The 4th Open Workshop
"Chemistry of Biological Processes Created by Water and Biomolecules"

December 18(Mon)-19(Tue), 2006

Kyoto Terrsa
Kyoto city, Kyoto

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on Priority Areas from the MEXT of Japan
"Water and Biomolecules"

Organizer: Masahide Terazima (Kyoto univ.)

Dec.18

9:30-9:40 Opening remarks　Prof.K.Kuwajima

Chair person: Prof.Y.Okamoto
9:40-10:20
L001
Prof. Christopher M. Dobson, Cambridge University, UK
"Protein Folding and Misfolding: from Nanoscience to Neuroscience."
10:20-11:00
L002
Prof. Hideki Taguchi, The University of Tokyo, JAPAN
"Direct observation of yeast prion dynamics: from a single-molecule to a single-cell approach."
11:00-11:40
L003
Prof. José Nelson Onuchic, University of California at San Diego, USA
"Water Mediation in Protein Folding and Molecular Recognition."

11:40-13:00　Lunch

13:00-14:50 Poster session (Presentation of odd number posters)

Chair person: Prof.M.Ikeguchi
15:00-15:40
L004
Prof. Sheena E. Radford, The University of Leeds, UK
"Life on a Knife Edge: Tipping the Balance between Folding and Aggregation"
15:40-16:20
L005
Prof. Satoshi Takahashi, Institute for Protein Research, Osaka University, JAPAN
"Detection of Folding Dynamics of Freely Flowing Proteins in Solution at the Single Molecule Level"

16:20-17:00
L006
Prof. Shoji Takada, Kobe University, JAPAN
"Modeling and simulating biomolecular machines."
17:00-17:20
Prof. Jooyoung Lee, Korea Institute for Advanced Study, Korea
"High-accuracy protein structure prediction by global optimization"

18:00- Banquet

Dec.19

Chair person: Prof.N.Matsubayashi
9:00-9:40
L007
Prof. Ronald Levy, Rutgers University, USA
"Protein Folding, and Binding: Effective Potentials, Replica Exchange Simulations, and Network Models."
9:40-10:20
L008
Prof. Masahiro Kinoshita, International Innovation Center, Kyoto University, JAPAN
"Physical Basis for Characterizing Native Structures of Proteins."

10:20-10:30 break

Chair person: Prof.F.Hirata
10:30-11:10
L009
Prof. Montgomery Pettitt, University of Houston, USA
"Cellular Crowding: Modeling Activity in Highly Nonideal Solutions."
11:10-11:50
L010
Prof. Makoto Suzuki, Graduate School of Engineering, Department of Materials Processing,Tohoku University, JAPAN
"Increase of rotational mobility of water around actin filaments upon interaction with myosin and its physiological meanings."

11:50-13:00
Lunch

13:00-14:50
Poster session (Presentation of even number posters)

Chair person: Prof.M.Kataoka
15:00-15:40
L011
Prof. Keith Moffat, The University of Chicago, USA
"The role of internal hydrogen bonds and buried water in light-sensing and redox-sensing domains."
15:40-16:20
L012
Prof. Nobuo Niimura, Graduate School of Science & Engineering, Ibaraki University, JAPAN
"Neutron Protein Crystallography: Beyond the Folding Structure of Biological Macromolecules."

16:20-16:30 Closing remarks

PROTEIN FOLDING AND MISFOLDING: FROM NANOSCIENCE TO NEUROSCIENCE

Christopher M. Dobson, Department of Chemistry, University of Cambridge

Natural proteins are a very select group of molecules, and their properties have a number of very special characteristics when compared to random sequences of amino acids, one of which is the ability of specific sequences to fold to unique structures. This characteristic has enabled biological systems to generate a vast range of functions and an astonishing degree of specificity in their chemical processes. In order to help define the conceptual basis and fundamental principles that underlie the folding of natural proteins, we have exploited recently a number of approaches that combine experiment and theory. In particular, we have used a wide range of types of experimental data to restraint computer simulations so that they explore only those regions of conformational space that are consistent with the experimental measurements. Using this procedure it has been possible to define ensembles of protein structures that are accessible through dynamical fluctuations at different stages of the folding process of individual proteins. Such information is contributing to the development of a detailed understanding of the manner in which the unique folds encoded by specific sequences are achieved within a universal mechanism of protein folding.

In the light of the stochastic nature and complexity of the folding process, there is always a finite probability that any protein will fail to fold correctly or to remain in its correctly folded state. In some cases such failures give rise to serious cellular malfunctions and hence to diseases such as those associated with amyloid deposition [3]. We have shown that the *ability* to form amyloid structures is not restricted to the specific proteins whose deposition is associated with these diseases, but can be considered as a generic property of polypeptide chains. The *propensity* to form such a structure will, however, vary widely from one system to another. Our experiments also suggest that species associated with the formation of amyloid structures are often inherently toxic to cells, whether or not associated with known diseases. These findings indicate that a key feature of biological evolution has been the selection of protein sequences able to fold to unique structures but with an inherent resistance to aggregation, coupled with mechanisms that normally ensure effective control and regulation of the many possible conformational states that are in principle accessible to all proteins within the cell. In this talk I shall describe recent work from our laboratory that utilises a variety of experimental and simulational approaches to address the underlying origins of these phenomena, and which also begin to extend such studies from the test tube and computer into to living systems.

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L002

Direct observation of yeast prion dynamics: from a single-molecule to a single-cell approach.

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Prions are infectious proteins; an abnormal form of the prion protein causes an auto-catalytic conversion of a normal (soluble) form of prion protein to the abnormal amyloid form. This concept originated from the studies of mammalian neurodegenerative diseases, but spread to include other protein-based genetic elements from budding yeast *Saccharomyces cerevisiae*. In particular, the prion-inducing fragment of yeast Sup35, a determinant of the yeast prion-like phenotype [*PSI*$^{+}$], is comprised of a glutamine/asparagine-rich N-terminal domain, and is a valuable model protein to study the mechanism of prion.

Using the yeast prion Sup35 as a model, we have previously developed an imaging system using a fluorescence microscopy (ref. 1,2), and proposed a structural model based on an X-ray fiber diffraction analysis (ref. 3). As a first topic in this workshop, we will present our recent advances, in which we further improved the system to a single-molecule level using a total internal reflection fluorescent microscopy. We succeeded in monitoring live imaging of individual Sup35 fibrils elongation. Statistical analysis of rates of the fibril growths provides a novel insight into the mechanism of fibril growth.

Second, we directly monitored the dynamics of the prion aggregates in single living cells using an on-chip single-cell cultivation system as well as fluorescence correlation spectroscopy (FCS) (ref. 4). Single-cell imaging revealed that the visible foci of yeast prion Sup35 fused with GFP are dispersed throughout the cytoplasm during cell growth, but retain the prion phenotype. FCS revealed that [*PSI*$^{+}$] cells, irrespective of the presence of foci, contain diffuse oligomers, which are transmitted to their daughter cells.

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Water Mediation in Protein Folding and Molecular Recognition

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Water is essential for life in many ways, and without it biomolecules might no longer truly be biomolecules. In particular, water is important to the structure, stability, dynamics, and function of biological macromolecules. In protein folding, water mediates the collapse of the chain and the search for the native topology through a funneled energy landscape. Water actively participates in molecular recognition by mediating the interactions between binding partners and contributes to either enthalpic or entropic stabilization. Accordingly, water must be included in recognition and structure prediction codes to capture specificity. Thus water should not be treated as an inert environment, but rather as an integral and active component of biomolecular systems, where it has both dynamic and structural roles. Focusing on water sheds light on the physics and function of biological machinery and self-assembly and may advance our understanding of the natural design of proteins and nucleic acids.

L004

LIFE ON A KNIFE EDGE: TIPPING THE BALANCE BETWEEN FOLDING AND AGGREGATION

Professor Sheena E Radford

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Understanding how different proteins or protein fragments self-assemble into the ordered, insoluble aggregates associated with amyloid disease is an enormous challenge. Whilst it is generally accepted that protein unfolding is required for the formation of amyloid fibrils from natively folded proteins *in vitro* and, therefore, presumably also *in vivo*, the extent to which misfolding intermediates and partially folded species formed transiently during productive protein folding resemble each other, and the point at which the folding and aggregation free energy landscapes diverge remain obscure. Identifying rare aggregation-prone species under physiologically-relevant conditions and defining the structural properties that endow their amyloidogenic character is therefore an important challenge. We have attempted to address this question by determination of the folding mechanism of the naturally amyloidogenic protein, beta-2-microglobulin, and comparison of the concentration of different folding intermediates with the rates of aggregation. Using NMR the conformational properties of the amyloidogenic species has been identified. In addition, I will present our recent insights into the conformational properties of amyloid fibrils formed from beta-2-microglobulin *in vitro* using cryo-EM (in collaboration with Helen Saibil, Julie Hodgkinson and Sara Cohen-Krausz) and our attempts to pinpoint the role of individual residues in folding and assembly using mutagenesis approaches. In my presentation the results of our analysis will be discussed and the insights gained for the aggregation of this and other proteins will be described.

The Yin and Yang of protein folding. Jahn, T.R. & **Radford, S.E.** (2005) *FEBS J.* **272**, 5962-5970

Amyloid formation under physiological conditions proceeds *via* a native-like folding intermediate. Jahn, T.R., Parker, M.J., Homans, S.W.& **Radford, S.E.** (2006) *Nature Struct. and Molec. Bio*l. **13,** 195-201

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Detection of Folding Dynamics of Freely Flowing Proteins in Solution at the Single Molecule Level

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Events of protein folding involve complex and heterogeneous dynamics of polypeptides in a huge conformational space. Despite the importance to characterize the heterogeneity for the understanding of protein folding, the conventional methods relying on ensemble averaging can not resolve information of individual protein molecules. It is necessary to characterize protein folding dynamics at the single molecule level.

We constructed a new system for the detection of fluorescence signals from single molecules based on a capillary cell and a high sensitivity camera. A protein molecule is labeled by fluorescent dye so as the fluorescence intensity reflects the conformations of the protein. The labeled sample is introduced to the cell at concentrations that allow the flow of a single molecule at a time. The excitation laser is introduced into the capillary coaxially from the exit end of the sample stream. The camera is arranged to monitor the flow of molecules in the cell as traces of fluorescence image. Thus, the intensity profiles of the traces should reflect the time-dependent changes in the conformations of proteins freely flowing in solution.

Cytochrome *c* from *Saccharomyces Cervcisia* was labeled by Alexa 532 at the C-terminal cysteine (cyt *c*-Alexa). We confirmed that the native conformation of the labeled sample was retained. Based on ensemble titration measurements, the Alexa fluorescence of cyt *c*-Alxa was observed to be quenched and not quenched in the native and unfolded states, respectively. Furthermore, an equilibrium intermediate with moderate fluorescence intensity was observed. Therefore, the fluorescence intensity change of cyt *c*-Alexa corresponds to the conformational transitions of the sample.

We observed the fluorescence signals of cyt *c*-Alexa at the single molecule level by using the developed device. The sample was dissolved in solutions containing moderate concentrations of denaturants, where the native, intermediate and unfolded conformations exist simultaneously, and the transitions among the three states were observed. The obtained traces reproduced the three state transitions observed in the ensemble measurements. Furthermore, the traces gave various information on the dynamic property of each equilibrium state.

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L006

Modeling and simulating biomolecular machines

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Biomolecular machines in general are huge biomolecular complex that are designed to fulfill their functions through movement well controlled between atomic and 10 nanometer scale. Experimentally, X-ray crystallography and others are powerful to obtain structural insight on the machines, whereas laser-based experiments provide wealth of dynamic information. Yet, it is very difficult to directly get time-dependent & structural information of the whole biomolecular machines in any single experiment. In this sense, molecular dynamics simulations are potentially powerful to obtain time-dependent structural insight into biomolecules, but currently they can reach up to about microseconds, which are orders of magnitude too short to milliseconds that biomolecular machines function.

Recently, we initiated our effort to coarse-grain the representation of biomolecular complex performing simulations of dynamics that correspond to millisecond time scales: Using a Go-like model that has been used in protein folding study, we simulated rotary motion of *F_1-ATPase* addressing mechano-chemical coupling scheme and so on[1].

Along this line, in this talk, I will present our study on another biomolecular machine, *AAA+ ATPase* which unfolds and trans-locates the substrate proteins in degradation machines. Living things possess degradation machines for cellular quality control: The human being has *proteasome*, while bacteria possess analogous machines called *ATP-dependent protease*, which can serve as a model of proteasome. The ATP-dependent protease is a complex of two cylinders, a cylinder of AAA+ type ATPase domain and that of protease domain, which share a central pore. Substrate proteins are pulled into the pore of AAA+ ATPase cylinder and are trans-located to the protease cylinder where the substrates are cleaved. The pore is so narrow that the substrate proteins need to be unfolded for threading into the pore.

Here, through MD simulations, we address how the substrate proteins are unfolded and trans-located in the *HslU*, an example of AAA+ ATPase cylinder, using ATP hydrolysis energy. We first report atomic scale simulation of HslU showing non-trivial hydration in the pore. Based on this feature, we conducted simulation of power-stroke motion for translocation in a second-level coarse-graining model. Finally, even simpler modeling is used for elucidating overall unfolding and translocation of HslU.

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L007

PROTEIN FOLDING, and BINDING: EFFECTIVE POTENTIALS, REPLICA EXCHANGE SIMULATIONS, AND NETWORK MODELS

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Advances in computational biophysics depend on the development of accurate effective potentials and powerful sampling methods to traverse rugged energy landscapes. I will begin by reviewing features of the all atom effective potentials and replica exchange simulations we have designed for studies of protein folding, the binding of ligands to proteins, and for structure refinement [1,2].

We have developed an approach to the study of peptide and protein folding that makes use of the combined power of replica exchange simulations and a network model for the kinetics. We carry out replica exchange simulations to generate a very large (~10^6) set of states using an all-atom effective potential function and construct a kinetic model for the folding, using an ansatz that allows kinetic transitions between states based on structural similarity. Results are presented for the C-terminal peptide from the B1 domain of protein G [3,4]. We find that while the kinetics is two state following small temperature perturbations, the coil-to-hairpin folding is dominated by pathways that visit metastable helical conformations.

We are also using replica exchange simulations to study the binding of ligands to proteins; I will describe our work on the cytochrome P450s [5]. UV and NMR measurements of cytochrome P450 BM-3 in complex with N-palmitoylglycine (NPG) indicate that a conformational change occurs in the active site of the complex where the terminal atoms of the ligand move from a site distant from the heme iron, as seen in the low temperature crystal structure to a site proximal to the heme iron at room temperature. We have employed REMD to study this conformational change. The population of the proximal state is found to increase with temperature in agreement with the UV and NMR experiments. In addition our REMD study suggests that a new conformational state which is stabilized by conformational entropy is significantly populated at room temperature. A proposal for the entropic stabilization is provided on the basis of the structure of the newly identified state. We have gained additional insights into the conformational dynamics of substrate in the active site of cytochrome P450s through the analysis of NMR order parameters which I will discuss.

Despite the power of replica exchange sampling for traversing rugged biomolecular energy landscapes, a better understanding of the relationship between the physical kinetics of the systems being studied to their "kinetics" in the replica exchange ensemble is needed in order to use this new technology to maximum advantage. To illustrate some of the challenges we face, I will discuss our results using a network model to "simulate" replica exchange simulations of protein folding.

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L008

Physical Basis for Characterizing Native Structures of Proteins

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We have suggested that the translational motion of water molecules plays critical roles in the biological self-assembly [1-5]. Protein folding is no exception [6,7]. We argue that upon the structural change of a protein, the gain or loss of the intramolecular energy is largely compensated by the loss or gain of the hydration energy, when the folding is considered under the isochoric condition. We introduce the sum of the intramolecular energy and the hydration free energy as the key function. The change in this function is governed by the change in the hydration entropy. A protein is designed to fold into the structure that maximizes the entropy of water under the requirement that sufficiently many intramolecular hydrogen bonds be formed to compensate the dehydration penalty [8].

The major driving force in protein folding is a gain in the translational entropy of water. The formation of intramolecular hydrogen bonds is important just for reducing the dehydration penalty as much as possible during the folding process. Focusing the physical basis on these two factors, we construct a new, simple energy function which is free from the van der Waals and Coulombic force parameters. The energy function is calculated quite rapidly using our recently developed morphometric approach [9]. Seven different proteins are chosen, and the native fold and over 600 misfolded “decoy” structures are considered for each protein. It is shown for all the proteins that the energy function of the native structure is lower than that of any other possible structure [10].

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Cellular Crowding: Modeling Activity in Highly Nonideal Solutions

Nonideal solutions play a role in many aspects of biochemistry and therefore biology. The interior of cells is often less than 50% water. As concentrations increase, concentration itself becomes a less useful quantity to understand equilibria. Biological and medicinal chemistry often fail due to the difference between concentration and activity. An understanding of the impact of the crowded conditions in the cytoplasm on its biomolecules is of clear importance to biochemical, medical and pharmaceutical science. Work on the use of small biochemical compounds to crowd protein solutions indicates that a quantitative description of their non-ideal behavior is possible and straightforward. Here, we will show what the structural origin of this non-ideal solution behavior is from simulations and expressions derived from a semi-grand ensemble approach. We discuss the consequences of these findings regarding protein folding stability and solvation in crowded solutions through a structural analysis of the m-value or the change in free energy difference of a macromolecule in solution with respect to the concentration of a third component.

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L010

Increase of rotational mobility of water around actin filaments upon interaction with myosin and its physiological meanings

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Microwave dielectric spectroscopy can measure the rotational mobility of water molecules that hydrate proteins and the volume of the hydration shells. Using this technique, we have recently shown that apart from the water molecules with lowered rotational mobility that makes up a typical hydration shell, there are other water molecules around the actin filaments (F-actin) which have a much higher mobility than that of bulk water [1]. On the basis of our hypothesis that the negative charge-rich molecular surface of the double-helix structure of F-actin is responsible for induction of such hyper-mobile water (HMW), we have predicted that when F-actin interacts with some specific binding proteins, its dual hydration profile can be modulated. This is indeed the case. Thus, the volume of the hyper-mobile water markedly increases without significant change of that of the ordinary hydration shell when the myosin motor-domain (S1) binds to F-actin.(Fig.1 by the fixed ϕ analysis) No hyper-mobile component was found in the hydration shell of S1 itself.[2] Since the low viscosity of water is due to less hydrogen-bonds among water molecules, an important implication of the present result is that the solvent space around S1 bound to F-actin is asymmetric in the activity of water along the actin filaments, which may be able to drive myosin S1 due to the osmotic effect.(Fig.2)

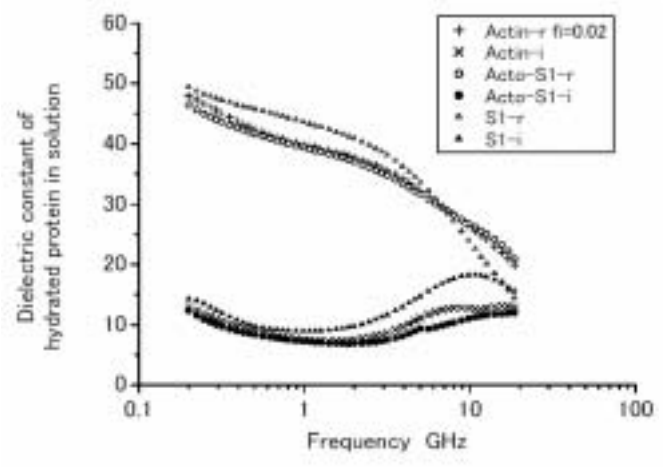

Fig.1 Partial dielectric spectra of F-actin, F-actin-S1 and S1 with water shells of constant thickness

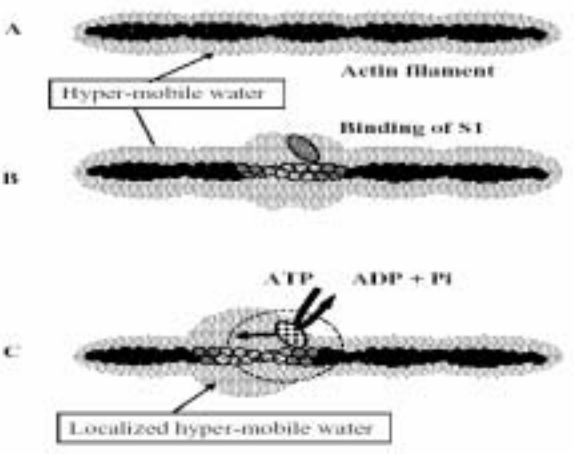

Fig.2 Possible motor model of actomyosin

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L011

THE ROLE OF INTERNAL HYDROGEN BONDS AND BURIED WATER IN LIGHT-SENSING AND REDOX-SENSING DOMAINS

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Light-sensing and redox-sensing signaling proteins contain a non-protein chromophore that is covalently or non-covalently embedded within the protein, and typically is not directly accessible to solvent. In at least one case, that of the redox sensor nifL, there are buried water molecules directly adjacent to and hydrogen-bonded to the chromophore; in all known cases, protein atoms are hydrogen-bonded to the chromophore. Based on static and time-resolved X-ray crystallography, it appears that a common theme is the rearrangement of these hydrogen bonds in response to a signal, such as absorption of a photon by the chromophore. Rearrangement can take the form of progressive lengthening, rupture or spatial reconfiguration, and may occur over a broad range of time scales. For example, water molecules may be displaced and adopt a different hydrogen bonding pattern, as in nifL; or a side chain such as that of glutamine may flip through 180^{o} and consequently remodel its hydrogen bonding pattern, as in the very different blue light sensor domains, AppA and LOV; or an extremely short hydrogen bond may lengthen and rupture, as in another blue light sensor, PYP.
The implications of these reconfigurations of internal water molecules and hydrogen bonds for signaling will be discussed.

L012

Neutron Protein Crystallography: Beyond the Folding Structure of Biological Macromolecules

Nobuo Niimura
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Hydrogen atoms and water molecules around proteins and nucleic acids could play a crucial role in many physiological functions. Neutron diffraction provides an experimental method of directly locating hydrogen atoms. [1,2,3] Several high-resolution neutron diffractometers, which exploit the neutron image plate, dedicated to biological macromolecules have been constructed to acquire high resolution neutron diffraction data for the location of hydrogen atoms and molecules of hydration in proteins and oligomeric nucleic acids.

(a) Since almost all the H atom positions can be identified experimentally, the geometrical details of certain types of H-bonds can be visualized;

(b) since the neutron scattering process distinguishes deuterium from hydrogen, information regarding the H/D exchange behavior of proteins can be obtained, and

(c) as far as mechanistic implications are concerned, the identification of protonation and deprotonation states of certain important amino acid residues can be carried out.

(d) The hydration structure around proteins and the hydration networks around DNA oligomers have been successfully characterized in several outstanding cases.

Theses will open the new field beyond the folding structure of bio-macromolecules such as:

1) Recognition of proteins and nucleic acids through the network structure of water molecules surrounding bio-macromolecules, and

2) The nature of chemical bond in proteins and nucleic acids elucidated by the accumulation of accurate structural information of hydrogen atoms.

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Morphometric approach to the solvation free energy of a protein

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The solvent has enormous effects on the structure and properties of a protein molecule immersed in it. Thus, the solvation free energy $F_{\mathrm{solvation}}$ is quite essential for understanding the structural nature of protein molecules. Despite the crucial importance of $F_{\mathrm{solvation}}$ of the protein in a given structure, relatively little is known about practical and effective ways of calculating this quantity. In this study we demonstrate the remarkable power of our new morphometric method [1] when applied to the calculation of $F_{\mathrm{solvation}}$ of a large, complex molecule such as a protein.

The idea of this approach is to predict the solvation free energy of the protein in a fixed structure based on four geometrical measures and corresponding thermodynamical coefficients:

$$F_{\mathrm{solvation}} = p\,V + \sigma A + \kappa C + \kappa' X,$$

where V, A, C, and X are the volume excluded by the protein, the surface area accessible to the solvent and the integrated mean and Gaussian curvature of the accessible surface, respectively. The corresponding thermodynamic coefficients are the pressure p, the surface tension σ of the solvent at a planar wall, and two bending rigidities κ and κ' which account for curvature effects. We determine these coefficients from calculations of the solvation free energy of spherical solutes with varying radius. In principle these coefficients can be determined using an arbitrary theoretical approach like integral equation, density-functional theory or computer simulations. The nature of the solvent-solvent and protein-solvent interaction is reflected in the values of those coefficients.

For a hard-sphere solvent, we calculate the $F_{\mathrm{solvation}}$ for the 600 structures of protein G that we studied within the 3D integral-equation approach. Although our two approaches are quite different in nature, the agreement between the 3D integral-equation theory and the morphometric approach is extremely good: the deviation is less than 0.7 % in all cases. This agreement is very encouraging, because the computing time for $F_{\mathrm{solvation}}$ is reduced by more than four orders of magnitude. Extension to a more realistic model of water is also possible and presented in detail.

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P002

DETECTING DOMAIN MOTIONS OF PROTEINS IN MOLECULAR DYNAMICS SIMULATIONS AND NORMAL MODE ANALYSES

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A method for detecting domain motions of proteins in molecular dynamics simulations and normal mode analyses is presented. Variance-covariance and correlation matrices are routinely used for analyzing correlated motions of proteins in molecular dynamics simulations and normal mode analyses. However, the matrices sometimes fail to detect correlated motions. For example, correlated motions in tangential directions are ignored in the matrices. In this study, we present a new variance-covariance and correlation matrices based on rigid-body motions of local segments. Using these matrices, we can detect dynamic domains that undergo such correlated motions. This method was applied to molecular dynamics simulations and normal mode analyses for some proteins, e.g. periplasmic binding proteins. Detected domain motions are in good agreement with domain motions observed in experiments.

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Comparison of solvated structure of water channels by using 3D-RISM method

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Aquaporins (AQP) are membrane proteins that selectively transport water or small molecule through the cell membrane. The comparisons of solvated structure of water channels of aquaporin family, AQP0, AQP1, AQP4 and AQPZ, were studied by method of statistical mechanics, 3D-RISM. The calculations of the three dimensional distribution functions suggested that in all AQP the distributions of water in ar/R region have low value, which is consistent with previous studies. AQP0, the lowest water permeability subtype, has a characteristic distribution of water. It has regions of excluded water, while the distributions of water are continuous or nearly continuous through the channels in the others types.

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P004

Theoretical Study on Thermodynamic Stability of Micelles

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Aggregation of amphiphilic molecules plays an important role in biosystem, e.g. bio-membrane. From the viewpoint of computational chemistry, one of the main objectives has been a free-energy-landscape of micellar system. For instance, a size distribution of micelles is one of the typical free energy landscapes. Recently, a MD simulation on the micelles composed of an ionic surfactant SDS (Sodium Dodecyl Sulfate) was performed by Yoshii et al., and the size distribution of the micelles has been reported [1]. One of the free-energy-landscapes of our interest other than size distributions is the free-energy-change along the "reaction coordinate", which is defined to include the process from a dispersed state to an aggregated state. In this study, the free-energy-landscape of the micelles composed of a nonionic surfactant C12E8 was calculated by using 3D-RISM theory.

In this study, the reaction coordinate is expressed as the radius of gyration R_g of a micellar aggregate, where for instance, smaller R_g corresponds to a more aggregated state. Free energy curve of the micelle of size (i.e. aggregation number) 40 is shown in Fig. 1. The existence of a local minimum in the free energy is observed in the vicinity of R_g = 20 angstrom. The free energy becomes higher values in the region R_g < 17 angstrom due to significant overlaps of atoms inside the micelle. The contributions of each component to the free energy are shown in Fig. 2.

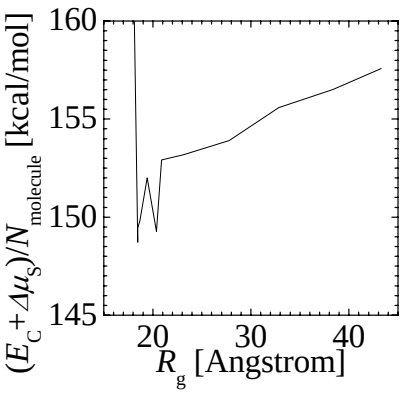

Fig. 1 Free energy curve along the reaction coordinate R_g.

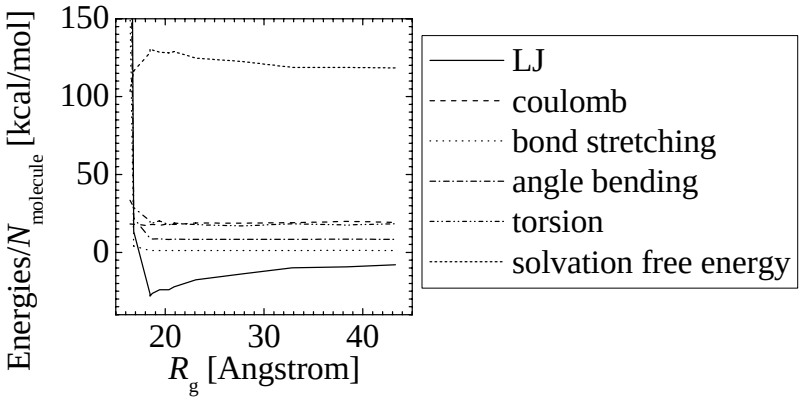

Fig. 2 Contributions of each component to the free energy.

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Hydration structures around and inside alpha-, beta- and gamma-cyclodextrins: 3D-RISM analyses

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Cyclodextrin is the name of a class of cyclic oligomers of glucose whose members are alpha-, beta-, gamma- cyclodextrin, etc. Cyclodextrin is a well-constructed miniature of an enzyme in the sense that it has a hydrophobic cavity of appropriate size, sites for introduction of catalytic groups at juxta positions, and satisfactory water solubility. The cavities normally full with water molecules held in place by hydrogen bonds in water (6, 12 and 17 water molecules for the alpha, beta and gamma forms, respectively), but the insides of the cones are less polar than the outsides, so that nonpolar organic molecules readily displace the water. Therefore it is interesting to investigate the solvation structure in the cavity of cyclodextrin. In the first step, we calculate the three dimensional (3D) distribution function of the oxygen and hydrogen of water molecules around and inside cyclodextrins. Fig 1 shows the temperature dependences of water molecule numbers in the cavities calculated from the 3D distribution function. The white (black) indicates the values calculated by water oxygen (hydrogen) distributions in each cyclodextrins. The numbers of the water molecules within the cavity of cyclodextrins calculated is 6.3, 11.7 and 15.3 for alpha-, beta- and gamma-cyclodextrins, respectively. Then it is good agreement with the experimental result.

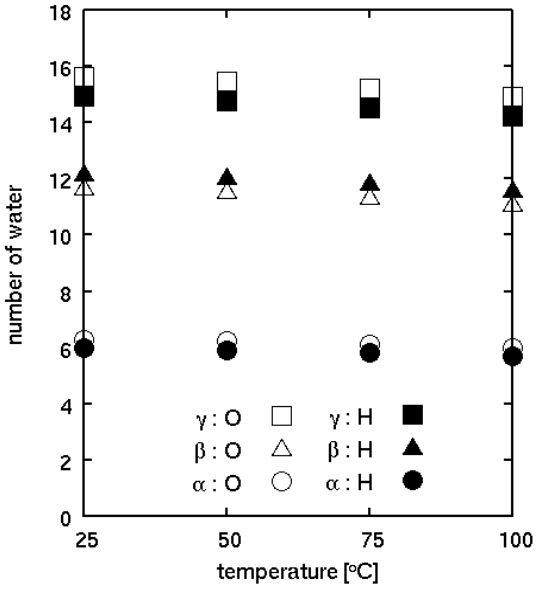

Fig. 1 The temperature dependency of number of water inside alpha-, beta- and gamma-cyclodextrins.

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P006

Theoretical Study of Ion Recognition by Human Lysozyme by the Integral Equation Theory

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Selective ion-binding by human lysozyme and its mutants is probed with the three-dimensional interaction site model theory, the statistical mechanical integral equation theory. Preliminary and partial results of the study have been already published [1]. The calculation was carried out for aqueous solutions of three different electrolytes $CaCl_2$, NaCl and KCl, and for four different mutants of the human lysozyme: wild type, Q86D, A92D, Q86D/A92D that have been studied experimentally. The discussion of this study focuses on the cleft, which consists of amino acid residues from Q86 to A92. For the wild type of protein in the aqueous solutions of all the electrolytes studied, there are no distributions observed for the ions inside the cleft. The Q86D mutant shows essentially the same behavior with that of the wild type. The A92D mutant shows strong binding ability to Na^+ in the recognition site, which is in accord with the experimental results. There are two isomers of the Q86D/A92D mutant, e. g. apo-Q86D/A92D and holo-Q86D/A92D. Although the both isomers exhibit the binding ability to the Na^+ and Ca^{2+} ions, the holo isomer shows much greater affinity compared to the apo isomer. Regarding the selective ion binding of the holo-Q86D/A92D mutant, it shows greater affinity to Ca^{2+} than to Na^+, which is also consistent with the experimental observation.[2]

We'll also report the rescent progress of our study.

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A theory of the triplet density correlations in molecular fluids

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We have developed a new theory to calculate the pair density correlation in molecular inhomogeneous fluids. The methods currently used for studies of inhomogeneous fluids are only applicable for the simple liquid consisting of spherical molecules. The new equation is derived from the inhomogeneous molecular Ornstein-Zernike equation with use of the RISM approximation[1]. The equation contains three unknown quantities, which are the direct and total correlation functions, and the singlet molecular correlation function of density. At first, we have modified HNC closure which relates the direct and total correlation functions suitably for inhomogeneous systems. Next, the single density correlation, which is included in the intramolecular susceptibility, is approximated at the crudest level for avoiding the calculation of the molecular Ornstein-Zernike equation. We have applied this method to the ion hydration phenomena and calculated the singlet and pair correlation functions of water molecules around an ion; Cl^-, Na^+, and K^+. In the vicinity of the ion, the pair correlation function calculated with the presented method exhibited dramatic change from that of pure water. The ionic size also gives a crucial effect on the hydration structure near the ion. In this paper, we also suggest a way how the unknown quantities are approximated at higher level. An extension of the theory to protein is expected to provide a powerful tool to study equilibrium as well as non-equilibrium hydration structure of the molecule.

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P008

Free-Energy Analysis of Binding into Self-Organizing, Nanoscale Structures in Solution

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Protein, membrane, and micelle are self-organizing and soft, nanoscale structures in solution. A key step of their functions is the binding of a molecule and is quantified with the (standard) free energy of binding. The free energy is a target of statistical thermodynamics, and an atomistic simulation with explicit solvent needs to be conducted to realistically incorporate the effect of specific interactions such as the hydrogen bonding and hydrophobicity. The binding activity reflects the cooperation and competition of the interactions among host (protein, membrane or micelle), guest (the molecule to be bound), and solvent (water and cosolvents), and the free-energy analysis at atomic resolution is the desirable route to the molecular understanding and control of the binding.

In the present work, we provide a unified, molecular approach to the binding in protein, membrane, and micelle. The basic idea of our approach is to view the aqueous solution with the host structure (protein, membrane or micelle) as a mixed solvent. The host is treated not as solute species, but as part of the mixed solvent system, and the guest molecule of interest is the only species regarded as the solute. In this view, the solvation free energy of the guest denotes the free-energy change for turning on the interactions of the solute (guest) with the solvent water, host, and cosolvents if present. The binding is then described by the difference in the solvation free energy between the host solution system and the neat solvent system. Typically, the host structure is dilute in solution and produces nanoscale inhomogeneity around it. The point of the free-energy analysis is to evaluate the solvation free energy in an inhomogeneous, mixed solvent system.

To approach the free energy, we adopt the method of energy representation developed in previous papers and combine it with a large-scale molecular simulation.[1,2] The method of energy representation is a theory of distribution functions in solution, and none of the system homogeneity, the thermodynamic limit, and the rigidity of molecule (and ion) is assumed in the formulation. A straightforward application is possible to an inhomogeneous system containing flexible species. The method was then applied to protein, membrane, and micellar solutions. As a protein system, the ligand-binding of benzamidine into α-thrombin and β-trypsin was analyzed and the explicit role of water is discussed. As membrane and micelle systems, the distribution of hydrophobic solutes within the host was determined for DMPC (dimyristoylglycerophosphocholine) bilayer and SDS (sodium dodecylsulfate) micelle and the validity of the pseudophase model is assessed.

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Interaction between Charged Colloidal Particles Immersed in Simple Electrolyte Solution Studied by the OZ-HNC Theory

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Recently, we have studied the interaction between macromolecules in crowding media and pointed out the importance of the entropic interaction arising from the translational motion of solvent molecules [1]. The cytoplasm in a living cell has not only water molecules and a variety of macromolecules but also electrolyte (e.g. NaCl). A macromolecule comprises groups with positive, negative, and almost zero partial charges. Moreover, the electrolyte concentration is over one order of magnitude higher than 0.01M and the charges are screened rather strongly. The macromolecules can approach each other up to a separation where the microstructure of the solvent near them comes into play.

In the present study, the interaction between charged colloidal particles immersed in electrolyte solution is analyzed using the OZ-HNC theory. They are highly charged in the same sign, and it is not definite if the translational motion of solvent molecules has essential effects on the particle interaction. We adopt an explicit model for the solvent molecules, ions, and colloidal particles. However, the solvent molecules do not possess partial charges. The Coulombic interaction is divided by the dielectric constant of water. Our model is more advanced than the conventional DLVO theory in the respect that the solvent granularity and the effects of the translational motion of solvent molecules are taken into consideration. It has been found that the dependence of the interaction on the electrolyte concentration is similar to that in the DLVO theory, although the strong van der Waals interaction between the colloidal particles is not considered in the present model. We will discuss the physical interpretation of the interaction, on the basis of comparing the present results with the previous results for more realistic model [2].

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P010

Molecular dynamics simulations of water molecules around solutes and in constant electric fields: Analysis of the dynamical properties

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Recent studies from both the simulations[1] and experiments[2] suggest accelerated motion of a part of water molecules around some types of solute molecules, although hydrating water molecules move much slowly compared with pure water. To investigate the dynamics of water molecules around solutes such as metal ions, rare gases, and small organic molecules, MD simulations were done. Dynamical properties (translational diffusion coefficient, rotational correlation time, etc.) were analyzed from the MD trajectory data, and the properties were compared with those of pure water. Especially, in case of ions, the dynamical properties of surrounding water molecules varied largely due to the radius and the electric charge of the ion. To investigate the effect of electric field, simulations under the constant electric field (10MV/m~1GV/m) were done for SPCE and TIP5P water models. In almost cases, the motion under the electric field seemed to be slowed down or unchanged. Especially, in case of rigid body approximation, almost no prominent effect on the dynamical properties was found although the water dipoles ordered according to the strong electric field. We also examined the calculation conditions such as system size, cutoff length, and water models and found that the effects were not ignorable in some cases. Rotational correlation time, translational diffusion coefficient, and hydrogen bond patterns of water molecules around the solutes were obtained as functions of the distance from the solute. Although a calcium ion with higher electric charge and a smaller radius had larger rotational correlation time of surrounding water (i.e., smaller motion), the value of bulk region was similar to that of iodide ion bulk region and pure water. The results of smaller systems with 12Å cut-off were always smaller time (i.e., faster dynamics), which suggested the small cut-off distance affected the dynamics.

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DRIVING FORCE FOR POLYPEPTIDE CHAIN TRANSLOCATION THROUGH PROTEIN CONDUCTING CHANNEL

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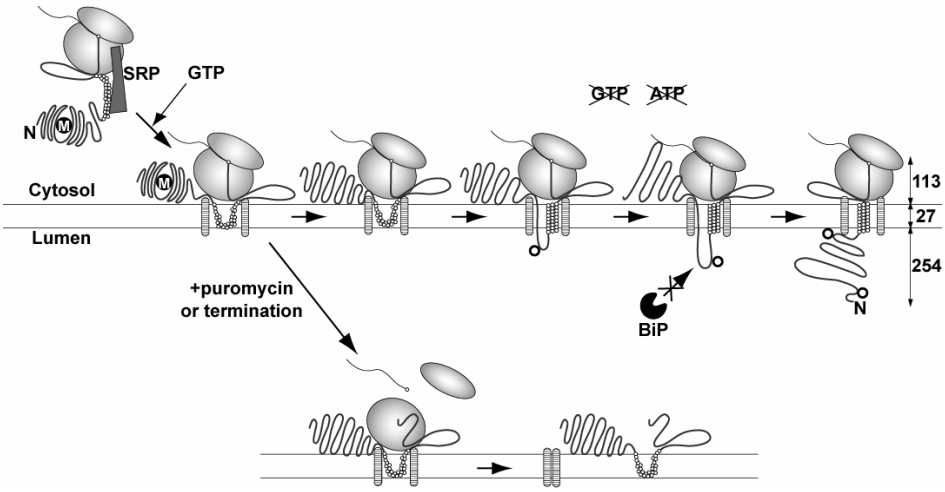
Nearly 70% of cell membrane proteins are synthesized and integrated into the membrane at the endoplasmic reticulum (ER). The membrane protein folding occurs cotranslationally on the ER. We investigated polypeptide chain translocation and folding of membrane proteins via a protein translocation channel (so-called translocon). Amino-terminal domain (N-domain) is translocated by the following type I signal-anchor sequence (SA-I) (Figure and reference [1]). The driving force was evaluated by streptavidin binding peptide (SBP) tag or titin domain, which had been fused at the N-terminus of SA-I. We demonstrated the follows: (i) the translocation of SBP-tagged N-domain was arrested depending on the affinity to the streptavidin. (ii) Translocation driving force balanced with the interaction between N-terminal SBP-tag and SAv with an affinity of Ca. 5 x 10^{-8} M. (iii) Folded titin domain is rarely translocated, while its mutant with less mechanical folding stability was translocated. (iv) Driving force for the initial phase of translocation is larger than that in the advanced stages. (v) The driving force was affected by the sequence character of the hydrophobic domain of signal sequence. These suggested that the driving force directly provided by the free energy reduction during transmembrane helix formation of signal sequence.



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P012

Fluctuating Hydration Structure around Single Walled Carbon Nanotubes

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The nanometer-scale hydration and hydrophobic interactions are studied by simulating aqueous solutions of single-wall carbon nanotubes (SWCNs) as example systems. This is subsequent to the study of hydrophobic hydration around spherical solutes, C_{60} and $C_{60}H_{60}$ [1]. Molecular dynamics simulation of the solutions reveals that hydration around the nanometer-size solute is strongly dependent on the topology of the solute configuration: When a single SWCN is solved in water, water molecules are attracted to the surface of SWCN due to the dispersion interactions between carbons and water molecules. Water molecules in the first hydration layer around the SWCN move more slowly than in bulk. When a pair of SWCNs approach each other, water density in the interstitial region between SWCNs largely fluctuates from trajectory to trajectory. The water-mediated force between SWCNs also largely fluctuates and becomes attractive when the water density in the interstitial region is small in fluctuation. When a triplet of SWCNs come close to each other, water molecules are expelled from the interstitial region to leave a vacant space, which drives SWCNs to aggregate. Correlation between the fluctuating caging and the fluctuating drying suggests that the topology-dependent drying takes place when the water configuration becomes too much constrained to keep a consistent hydrogen bond network in the narrow region surrounded by the nanometer-size hydrophobic solutes.

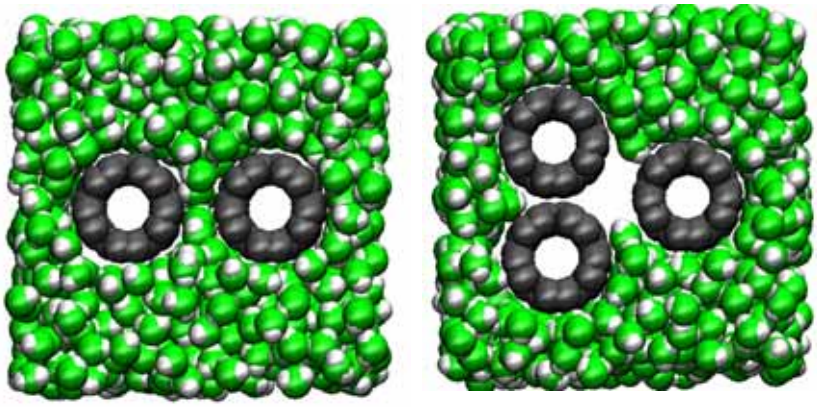

Fig. 1. Snap shots of MD simulation. (a) a pair of SWCNs with the center-of-mass distance = 12.80 Å. (b) a triplet of SWCNs with the center-of-mass distance = 12.85 Å.

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THE NATURE OF CHEMICAL BOND OF SOLVATED MOLECULES: USING THE NEW ANALYSIS OF MOLECULAR ORBITAL WAVE FUNCTIONS BASED ON THE RESONANCE THEORY

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It is essential to know the nature of chemical bond of the solvated molecule for solution chemistry. Very recently, we developed a novel method to calculate ionicity and covalency of a bond from a molecular orbital wave function.[1,2] A combination of this method and RISM-SCF theory enable us to discuss solvation effect on the nature of chemical bond. Figure 1 shows the weights of ionic and covalent structures of NaCl in the gas and aqueous solution phases. In both phases, the covalent bond becomes important when R decreases. This is consistent with our chemical intuition that a covalent bond is a short-range interaction and an ionic bond is a long-range one. In gas phase, the weight of ionic structure is only 73.1 % at equilibrium distance. On the other hand, in aqueous phase, it is 94.5 % and 98.7 % at CIP (contact ion pair) and SSIP (solvent separated ion pair) minimums. In aqueous solution, with increase of R, the weight of ionic bond increases rapidly compared to that in gas phase. This result clearly shows that the solvation effect enhances ionic character. This enhancement of ionic character plays crucial role on the dissolving process of NaCl.

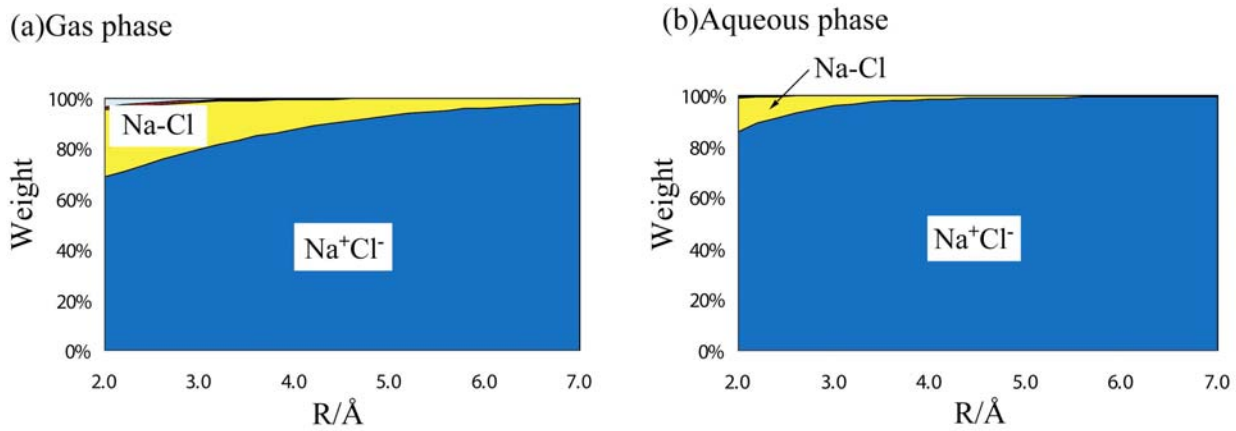

Figure 1. The changes of the weights of resonance structures of NaCl with respect to the distance between Na and Cl (R), in (a)gas and (b)aqueous phases.

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P014

A new procedure to evaluate 3D solvation structure based on integral equation theory

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Introduction Theoretical methods based on integral equation theory are very important for evaluation of microscopic and thermodynamic properties. In this work, we developed a new procedure to evaluate 3D solvation structure and evaluated hydration free energy of some organic compounds by this method.

Method To evaluate 3D correlation functions, real-solid harmonics are employed [1]. The coefficients of total correlation functions and direct correlation functions are evaluated from the following OZ- type equation and HNC-like closures:

$$h_{\eta\gamma,l'm'}(r_\eta) = \sum_{lm}\sum_{\alpha\beta}\left[\omega_{lm,l'm'}(\mathbf{R}_{\eta\alpha}) * c^{(\alpha)}_{\alpha\beta,lm} * (\omega_{\beta\gamma} + \rho h_{\beta\gamma})\right](r_\eta)$$

$$c_{\alpha\delta,l'm'}(r_\alpha) = \int \left[\exp(-\beta u_\delta(\mathbf{r}) + \tau_{\alpha\delta}(\mathbf{r})) - \tau_{\alpha\delta}(\mathbf{r}) - 1\right] S_{l'm'}(\hat{r}_\alpha)\mathrm{d}\mathbf{\Omega}_{r_\alpha}$$

$$c^{(\alpha)}_{\alpha\delta,l'm'}(r_\alpha) = \int w_\alpha(\mathbf{r})\left[\exp(-\beta u_\delta(\mathbf{r}) + \tau_{\alpha\delta}(\mathbf{r})) - \tau_{\alpha\delta}(\mathbf{r}) - 1\right] S_{l'm'}(\hat{r}_\alpha)\mathrm{d}\mathbf{\Omega}_{r_\alpha},$$

where $\tau_{\alpha\delta,lm}=h_{\alpha\delta,lm}-c_{\alpha\delta,lm}$ and $\omega_{lm,\,l'm'}$ is defined as follows:

$$\omega_{lm,l'm'}(k,\mathbf{R}) = (-1)^l i^{l'+l}\int \mathrm{e}^{i\mathbf{k}\cdot\mathbf{R}} S_{lm}(\hat{k}) S_{l'm'}(\hat{k})\mathrm{d}\mathbf{\Omega}_k \ .$$

Results and discussion The hydration free energies of some organic compounds calculated by RISM and the present method are shown in Fig. 1. Available experimental data are also shown in the figure. In the case of the present method, electrostatic potentials are evaluated from OPLS parameters (Model I) and multipoles calculated by quantum mechanics (Model II) [2]. By introducing 3D character for correlation functions and more realistic electrostatic potential (Model II), the calculated hydration free energies are better than RISM and Model I.

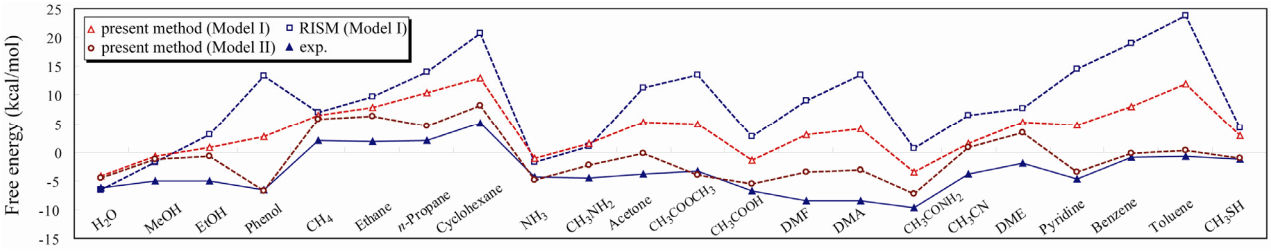

Fig. 1: Hydration free energies calculated by RISM and the present method. Electrostatic potentials are determined by OPLS and quantum mechanics. Available experimental data are also displayed.

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SOLVATION EFFECT IN THE REACTION OF $CO_2 + OH^- \rightarrow HCO_3^-$

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INTRODUCTION

The reaction of carbon dioxide with hydroxide anion plays important biological role such as blood pH regulation and the transportation of carbon dioxide in living systems. Davidson et al. theoretically show there is no barrier to this reaction in the gas phase, whereas there is barrier in solution [1]. However, detailed information on the solvation structure is not sufficiently known. RISM-SCF gives clear picture of the solvation which leads the detailed understanding of solvation effect.

RESULTS AND DISCUSSION

All calculations were carried out at the Hartree-Fock level. The distance between carbon and oxygen of hydroxide anion is chosen as the reaction coordinate (R). Fig.1 shows the energy profile along R, calculated by standard *ab initio* MO method and RISM-SCF. The barrier in aqueous solution (14.4 kcal/mol) is close to that of experimental value (12.1 kcal/mol) [1]. The site-site pair correlation functions (PCF) between the carbon in carbon dioxide and water hydrogen are shown in Fig. 2. In the reactant, there is a broad peak (2.8~4 Å) which is the mixture of two peaks: one is attributed to the hydrogen bonding between the oxygen in carbon dioxide and water hydrogen, and the other to the interaction between the carbon and water oxygen. In the product, there is sharp peak (2.5~3 Å) originated from the hydrogen bonding between the oxygen of carbon dioxide and water hydrogen. The change of the solvation structure along the pathway plays the central role in the reaction.

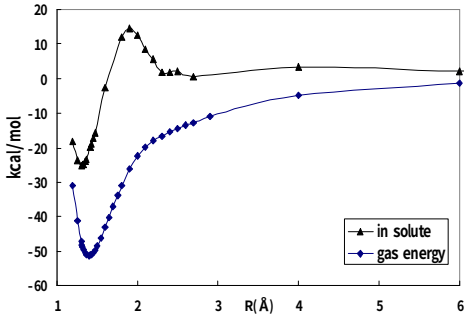

Figure 1. Energy profile for the reaction $OH^- + CO_2 \rightarrow HCO_3^-$ in gas phase and aqueous solution at 298.15 K using RISM-SCF.

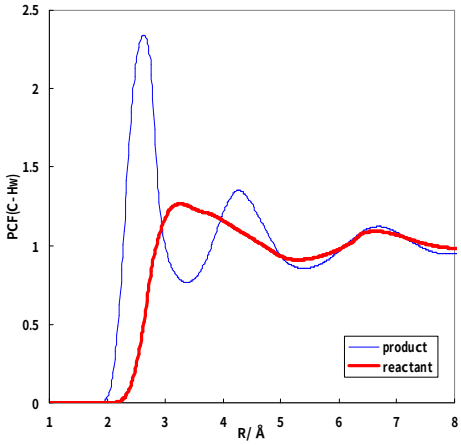

Figure 2. Computed pair correlation functions between the carbon and water hydrogen.

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P016

Theoretical study of solvation structure of glycine in HCl solution based on RISM theory

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【Introduction】 Amino acids become protonated form or zwitterions in acid aqueous solution. In order to analysis this phenomenon theoretically, it is essential to deal with multi component solution containing several kinds of ions and solvent. Reference Interaction Site Model (RISM) integral theory is one of the theories that enable us to treat multi component solution. Actually RISM is applied to peptides / NaCl aqueous solution [1]. In the present study, we applied RISM to glycine / HCl aqueous solution (HCl(aq)) system and investigated the radial distribution function near glycine and its solvation free energy (SFE) to changes in pH of the solvent.

【Method】 RISM equation is given by

$$h^{VV} = w^{V} c^{VV} w^{V} + w^{V} c^{VV} \rho^{V} h^{VV} \quad (1)$$

$$h^{UV} = w^{U} c^{UV} w^{V} + w^{U} c^{UV} \rho^{V} h^{VV} \quad (2)$$

where U and V represent solute and solvent, respectively. h is the matrix of total correlation functions and c is the matrix of direct correlation functions. ρ is the matrix of number density and w is the intramolecular correlation matrix. Eq.(1) is for balk properties. Using the h^{VV} matrix h^{UV} matrix can be calculated from Eq.(2). HCl(aq) is regarded as three components (H_2O, H_3O^+, and Cl^-) solution. The model of H_2O molecule is SPC/E. The temperature of the solution is at 298.15K.

【Result and Discussion】 Two different conformations of glycine are examined (Fig.1). In the range of 2.0 < pH < 6.0, both of conformation 1 (C1) and conformation 2 (C2) are stabilized than in pure water (pH = 7.0) (Fig2). In the range of pH = 0.0~2.0 the SFE of C1 and C2 drastically increased with addition of ions. In all range of pH the SFE of C2 is greater by about 1.0 kcal/mol in absolute value. This is opposite to the gas phase situation. In gas phase, C1 is more stable by 1.9 kcal/mol than C2 (HF/6-31G** level). It is considered that the cause is the large difference from the dipole moments between the two conformers, since the magnitude of dipole moments of C1 and C2 are 1.31 and 2.06 D, respectively.

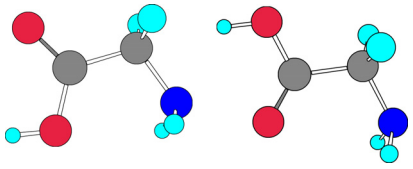

Fig.1 Conformations of the glycine. Conformation 1 (left) and conformation 2 (right).

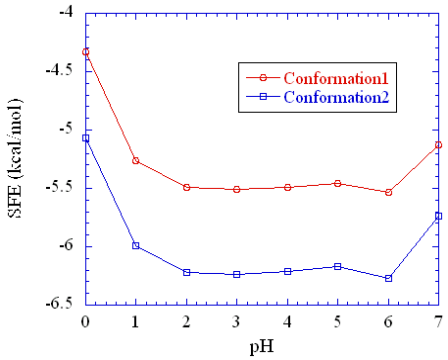

Fig.2 The change of SFE of glycine C1 and C2 in HCl(aq).

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ANALYSIS ON WATER CLUSTER IN TERMS OF ATOMIC AND DIATOMIC CONTRIBUTIONS: A MONTE CARLO STUDY

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Introduction

Recently, a new energy partitioning scheme has been suggested by Sato *et al.* [1] Their scheme reduces multi-center total energy in RHF to one- or two-center energy differently from Ichikawa's [2] and Mayer's schemes[3]. In this study, combining this partitioning scheme and Monte Carlo simulation, we investigated water dimer cluster and discussed the distribution of partitioned energy at different temperatures.

Computational Details

We performed MO calculation (RHF) using GAMESS modified for the energy partitioning and Monte Carlo simulation. 30,000 samples were produced at 50K, 100K, 150K, and 200K, respectively, by Monte Carlo method. The geometry of a water monomer was fixed at optimized one. 6-31G(d) and 6-31G basis sets were used for oxygen and hydrogen atoms, respectively.

Results and Discussion

We found the intermolecular energies were from -9 kcal/mol at 50K to -7.5 kcal/mol at 200K. These values are larger than the normal hydrogen bonding energy of -5.5 kcal/mol at the same geometry.

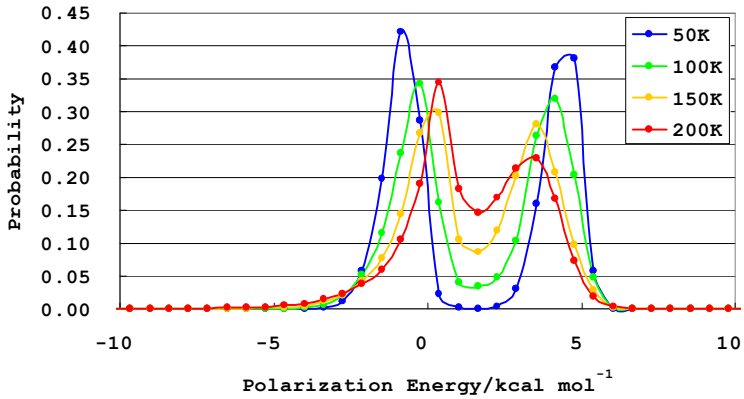

Figure. Probability Distribution of Polarization Energy

We also calculated the polarization energy (Figure). The peaks at 50K are located at -1 and 5 kcal/mol, and they don't overlap at all. It means that the "donor"(O-side) water molecule doesn't change into "acceptor"(H-side) one, and vice versa. The higher the temperature goes, the larger their overlap becomes: Donor easily changes into acceptor.

The center of the "valley" is located at about 2 kcal/mol, not at 0 kcal/mol. This is consistent with the fact that intermolecular interaction energy is larger than normal hydrogen bonding energy.

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P018

Effects of solvent on protein dynamics studied by molecular dynamics simulation of crystalline Staphylococcal nuclease

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The effects of solvent on protein dynamics are studied by molecular dynamics (MD) simulation of crystalline protein. We perform MD simulations in two hydration states, “dry” and “wet”, of crystalline Staphylococcal nuclease (SNase) at six temperatures ranging from 100 K to 300K. The “dry” and “wet” systems were hydrated to h=0.09 g D_2O per g protein and h=0.49 g D_2O per g protein, respectively.

The mean-square fluctuation averaged over hydrogen atoms, $<\Delta r^2>$, calculated by MD simulation in “wet” state is in good agreement with that in “dry” state at lower temperatures bellow 220 K, but is much larger at higher temperatures. As pointed out by the experiment [1], the magnitude of glass-like transition expected by simulation varies with hydration level.

Neutron scattering spectra are calculated using the results of MD simulations. As expected by our previous study [2], the peak frequency of the protein boson peak in “wet” state is higher than that in “dry” state at lower temperature bellow 220 K. This result is also consistent with our experiment [3]. At higher temperatures the protein boson peak becomes buried in the quasi-elastic contributions in “wet” state, but is still observed in “dry” state. It indicates that protein dynamics in “wet” state is more diffusive and more anharmonic than in “dry” state.

From the detailed analysis of simulation results, we will discuss the reason why protein dynamics varies with the hydration level as described above.

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NAD binding releases blocking loop for cooperative domain closing in liver alcohol dehydrogenase

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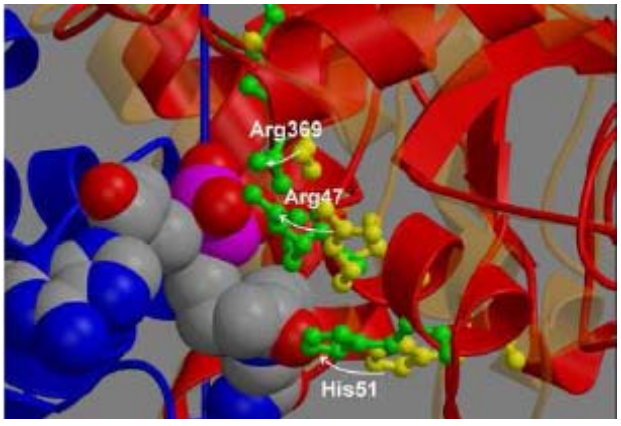
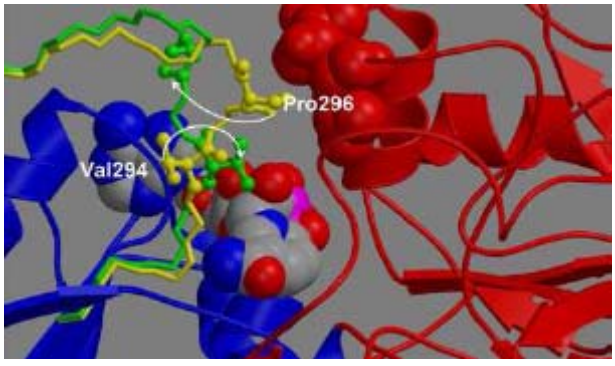
Horse liver alcohol dehydrogenase is a homodimer, the protomer having a coenzyme-binding domain and a catalytic domain. Using all available X-ray structures and 50 nanoseconds of molecular dynamics simulations, the mechanism of NAD+-induced domain closure was investigated [1]. When the well-known loop at the domain interface was modelled to its conformation in the closed structure, the NAD+-induced domain closure from the open structure could be simulated with remarkable accuracy. Native interactions in the closed structure between Arg369, Arg47, His51, Ala317, Phe319 and NAD+ were seen to form at different stages during domain closure. Removal of the Arg369 side-chain charge resulted in the loss of the tendency to close so verifying that specific interactions do help drive the domains closed. Further simulations and a careful analysis of X-ray structures suggest that the loop prevents domain closure in the absence of NAD+, and a cooperative mechanism operates between the subunits for domain closure. This cooperative mechanism explains the role of the loop as a block to closure, as in the absence of NAD+ it would prevent the occurrence of an unliganded closed subunit when the other subunit closes upon NAD+. Simulations that started with one subunit open and one closed supported this.





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ENERGY LANDSCAPE OF INTERMOLECULAR INTERACTION BETWEEN A MYOSIN MOLECULE AND AN ACTIN FILAMENT

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Recent single molecule experiments have shown that a myosin molecule slides along an actin filament directionally and stepwise. It is shown that the step size coincides with the actin monomer size, and the number of steps resulting from a single ATP hydrolysis varies stochastically, suggesting the mechano-chemical coupling is loose. In this study, to make an inquiry into a possible loose-coupling mechanism, we conduct a molecular dynamics simulation of the actomyosin system, consisting of a subfragment 1 of myosin II and a 55-nm long actin filament. It is observed that the myosin molecule slides along the actin filament directionally and stepwise, as has been observed in the single molecule experiments. It is shown that a key to understand the observed sliding of myosin lies on the energy landscape profile for the intermolecular interaction between the myosin molecule and the actin filament. The energy landscape exhibits two characteristics: i) successive depressions appearing at the interval of actin monomer, and ii) a slope inclined toward the barbed end spanning over several actin monomers. Key residues participating in the energy landscape profile are specified. Coulombic interactions between positively charged residues in the loop 2 of myosin and charged residues in actin are found to play an important role. Robustness of these energy landscape characteristics are examined by calculating energy landscapes for several intermolecular interaction models, including the Debye-Huckel model, the generalized Born model for Coulombic interactions, and for hydrophobic interactions, the solvent accessible surface area model and an enhanced pair-wise potential model for hydrophobic residues. We discuss a possible mechanism of the experimentally observed loose-coupling by referring to the energy landscape we calculated, and also discuss the relevance to other molecular motors such as myosin V, myosin VI and kinesin, some of which exhibit sliding motion similar to that observed in myosin II.

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P022

EFFECTIVENESS OF THE TRANSITION PATH SAMPLING TO STUDY RARE STRUCTURAL CHANGES OF A PROTEIN IN AQUEOUS SOLUTION

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To understand structural changes of a protein in aqueous solution, computer simulations are useful because it provides detailed information at the moment of transition. However, it takes large computation time to obtain statistically sufficient transition events (i.e., transition paths) by conventional molecular dynamics (MD) simulation. The transition path sampling (TPS) method [1] is a promising method to overcome this sampling problem. The TPS method effectively generates an ensemble of transition paths, with the path probabilities properly reproducing those obtained by conventional MD simulation.

In the TPS method, transition paths are sampled by generating a "new path" from an previous obtained "old path" one after another, just in the same way as the Monte Carlo structural sampling in which a new structure is generated from the previously obtained (accepted) old structure one after another. Therefore some correlation between the paths should remain, as is the case for the structural sampling, which prevents efficient path sampling in the "transition path space", raising a sampling problem again. Because this problem may completely spoil the potential of TPS, it is important to know the effectiveness of the TPS method under the various conditions.

To do this, we first studied a 2-dimensional double well potential system, because we can use the result of conventional MD simulation as the reference to evaluate the effectiveness of TPS. The effectiveness of TPS is examined by changing such important parameters of TPS as the path length, the strength of perturbation to be added to the old path, and the choice of the point at which a new path branches. After this basic examination, we apply TPS to the study of large structural changes in myosin, employing an extended Go-like model with superimposed structural bi-stability. Effect of water on the transition paths in myosin is also examined by using an implicit representation of water molecules [2].

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Theoretical study on the One-electron Reduction of a Cofactor in Aqueous Solution by the QM/MM-ER Approach

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Determination of the free energy change associated with a chemical process in a condensed phase is a matter of great significance in theoretical chemistry. However, it generally requires a substantial amount of ensemble averages for molecular configurations to attain convergence. It is, hence, desirable to develop a novel electronic structure calculation in combination with a statistical mechanics in order to explore the reaction path in biological system as well as in solution. Recently we proposed a method[1] on the basis of the QM/MM approach combined with the theory of energy representation. In the present paper, we apply the method to compute the reduction free energy $\Delta\mu_{\mathrm{red}}$ for the active site of the cofactor flavin adenine dinucleotide (FAD) which plays an essential role in many redox processes in biological systems. The $\Delta\mu_{\mathrm{red}}$ of the active site (isoalloxazine ring) of FAD is computed in the aqueous environment to elucidate the role of the apoprotein on the reduction process.

Within the theory of energy representation[2], as differed from the conventional theory of solution, the solvation free energy $\Delta\mu$ of a solute is expressed in terms of the distribution functions of the solute-solvent interaction energy, thus,

$$\Delta\mu = -k_{\mathrm{B}}T\int d\varepsilon\left[\rho(\varepsilon)-\rho_0(\varepsilon)+\beta\omega(\varepsilon)\rho(\varepsilon)-\beta\int_0^1 d\lambda\omega(\varepsilon,\lambda)\left(\rho(\varepsilon)-\rho_0(\varepsilon)\right)\right]. \qquad (1)$$

where $\rho(\varepsilon)$ and $\rho_0(\varepsilon)$ are the energy distribution function for the solution and the pure solvent systems, respectively, and β denotes the reciprocal of $k_{\mathrm{B}}T$. λ in Eq. (1) is the coupling parameter with respect to the solute insertion into the solvent and $\omega(\varepsilon;\lambda)$ is the indirect part of the solute-solvent potential of mean force. The energy distribution functions are to be constructed through the QM/MM simulations.

In the application of QM/MM-ER to the reduction of FAD, we have regarded the excess electron to be attached on the active site as a *solute* and the remaining molecules including the active site of the system have been treated as solvent. The interaction energy between the excess electron and the active site is defined by using the Hamiltonian H_0^{QM} for the solute at gas phase as

$$v_{\mathrm{QM}} = \left\langle \Psi_{N+1}\middle|H_0^{\mathrm{QM}}\middle|\Psi_{N+1}\right\rangle - \left\langle \Psi_N\middle|H_0^{\mathrm{QM}}\middle|\Psi_N\right\rangle$$

where $\left|\Psi_{N+1}\right\rangle$ is the electronic ground state in solution for the reduced active site and $\left|\Psi_N\right\rangle$ is the wavefunction in the gas phase for the solute before reduction. The computation is now in progress and the results will be presented at the conference.

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P024

Improving efficiency of conformation sampling in multicanonical molecular dynamics simulation

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The multicanonical molecular dynamics (MD) method has been widely used to sample conformation space of biomolecules. In this method, a multicanonical potential function is used in place of the potential energy function to produce a flat energy distribution. By analogy to the spectrum of white noise, it is often said that the multicanonical method realizes a “random” walk in energy space [1]. However, the simulation is conducted by solving deterministic equations of motion, and therefore the energy trajectory is, of course, not random, but is determined by the nature of the energy surface. The efficiency of the conformation sampling highly depends on the frequency of transitions from high to low energy state. Although the frequency can be optimized by improving the flatness of the energy distribution, it is limited by the nature of the energy surface: if the energy surface has a deep energy minimum state, the MD trajectory stays there for long time, resulting in a decrease in the transition frequency.

To solve this problem, we combined the conformational flooding method, which flattens the energy surface with umbrella potentials, with the multicanonical method. We applied this method to a system of a 10-residue mini-protein, chignolin. The umbrella potentials were calculated from free energy profiles plotted against C^{α} RMSDs from representative conformations that form deep free energy wells [4]. As a result, the transition frequency was increased from 48.9 ns^{-1} to 80.9 ns^{-1}. Our method is therefore useful to improve the efficiency of conformation sampling.

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VIBRATIONAL SPECTROSCOPIC FEATURES ARISING FROM NON-RANDOM STRUCTURES OF HYDRATED "RANDOM COIL" PEPTIDE CHAINS SIMULATED BY TIME-DOMAIN COMPUTATIONAL METHOD

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Recently, there has been much interest in the question of what are the real structures of peptide chains in the so-called "random coil" state in aqueous solution. This question is studied from the vibrational spectroscopic viewpoint by measuring the amide I bands of those peptide chains. It has been well known that the amide I bands of peptide chains are sensitive to their secondary structures and are useful in quantitative estimation of the content of secondary structure elements in proteins.

In many studies on the analysis of the amide I band profiles, the one-to-one correspondence between the features of the band and the (φ, ψ) angles tends to be discussed. However, peptide chains in aqueous solution are expected to be moving over a wide range on the Ramachandran map with some interconversions between different secondary structures. Therefore, to correctly analyze various features of the vibrational spectra of those peptide chains, it is important to elucidate the relation between those features and the dynamically changing structures of the peptide chains.

In the present study, a time-domain computational method of the vibrational spectra of coupled-oscillator systems (extended MD/TDC/WFP method) developed previously [1] is combined with Amber 8 [2] and is applied to the amide I band of $(Ala)_4$ in the so-called "random coil" state in a solvent cap of r = 21.7 Å. The ff03 force field [3] is used for the MD simulations. The vibrational couplings between neighboring peptide groups are determined by referring to the map [4] obtained from ab initio MO calculations. Considering that the amide I mode has a large dipole derivative, the vibrational frequency shifts of individual peptide groups are assumed to be induced by the effect of the electric field from the surrounding peptide groups and water molecules.

In agreement with the experimental result [5], the calculated spectrum has shown the negative noncoincidence effect, i.e., the frequency of the isotropic Raman band is higher than those of the IR and anisotropic Raman bands. This feature is consistent with the ppII-like structures of $(Ala)_4$ in aqueous solution, in that (1) the vibrational couplings between neighboring peptide groups are positive on average and (2) the dipole derivatives of those neighboring peptide groups make angles larger than 90°.

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P026

Molecular dynamics simulation of aspartate racemase from *Pyrococcus horikoshii* OT3

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X-ray crystallography has revealed two similar α/β domains of the aspartate racemase from the hyperthermophilic archaeum, *Pyrococcus horikoshii* OT3. The active site is located in the cleft between the two domains where two cysteine residues face each other. This arrangement allows the substrate to enter the cleft and enables the two cysteine residues to act synergistically. However the distance between their thiolates was estimated to be 9.6 Å, which is beyond the cooperative distance. We examined the molecular mechanism for the racemization reaction of this hyperthermophilic aspartate racemase using mutational analyses and molecular dynamics simulation. The mutational analyses revealed that Arg48 and Lys164 were essential for catalysis in addition to the putative catalytic cysteine residues. The molecular dynamics simulations revealed that the distance between the two active γ-sulfur atoms of cysteine residues oscillate to periodically become shorter than the predicted cooperative distance at high temperature. In addition, the conformation of Tyr160, which is located at the entrance of the cleft and inhibits the entry of a substrate, changes periodically to open the entrance at 375K. The MD simulations of mutants revealed that the opening of the gate is induced by the motion of the 164th residue, which locates next to Tyr160. The entrance of an aspartate molecule was observed by MD simulations driven by the force of the electrostatic interaction with Arg48, Lys164 and also Asp47. These results provide insights into the roles of amino acid residues at the catalytic site and also the activation mechanism of a hyperthermophilic aspartate racemase at high temperature.

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STRUCTURAL AND THERMODYNAMIC ANALYSIS OF HYDROPHOBIC SOLVATION BY MOLECULAR DYNAMICS SIMULATION

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The hydrophobic effect plays an important role in many chemical and biological processes. The unitary free energy change accompanying the transfer of a nonpolar molecule from nonpolar liquid phase to aqueous phase quantifies the magnitude of its hydrophobicity. The transfer process can be divided into two processes, (1) one from nonpolar liquid phase to gaseous phase, and (2) the other from gaseous phase to aqueous phase. Unique characteristics of transfer thermodynamic quantities appear in the latter process. Structural and thermodynamic aspects of hydrophobic hydration have so far been investigated by theoretical, experimental and computational methods. However, the physical mechanism underlying hydration thermodynamic properties and the structure of hydration water have not yet been fully clarified. The aim of this study is to elucidate molecular mechanism of the hydrophobic hydration of a nonpolar solute in water. Structural characteristics of hydration water and hydration thermodynamic quantities were examined with molecular dynamics (MD) simulation. A krypton atom and TIP5P water were taken for the solute and solvent respectively.

First, we analyzed the hydrogen bonds (H-bonds) formed between water molecules in the hydration shell. Compared with bulk phase, the first hydration shells have a significantly smaller mean number of H-bonds per water molecule. It indicates that hydration water molecules form weaker H-bonds to each other, which is opposed to the classical iceberg model. Second, the solvation thermodynamic quantities were examined by using two solvent models of TIP5P and ‘nonpolar water (NPW)’. The NPW model is a hypothetical spherical molecule having the same mass, size and density as those of TIP5P. While there is virtually no difference in solvation free energy between TIP5P water and NPW solvent, the solvation entropy and enthalpy in NPW solvent is large and positive unlike that in TIP5P water. Each of the expressions for solvation enthalpy and entropy could be separated into two contributions from the solute-solvent term and the solvent-solvent term or solvent reorganization. Contribution from the solute-solvent term in the solvation entropy and enthalpy has only small difference between TIP5P and NPW. The large difference in the solvation entropy and enthalpy originates from the component of solvent reorganization. These results suggest that the H-bonding characteristic of water has large effects on the negative hydration entropy and enthalpy.

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P028

Molecular Simulation of Polypeptides Using the SAAP Simulation Program

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A force field, which is an essential tool for the study of protein structures and functions at an atomic resolution, has been extensively refined to reproduce protein structures and the folding pathways. However it is still difficult to predict experimental results by molecular simulation using the force field with a limited computer resource. We are working on developing a new protein force field, called the single amino acid potential force field (SAAP)[1,2], for a high-speed simulation of polypeptides and proteins. The SAAP force field assumes that the total potential energy of a protein molecule comprises single amino acid potentials of individual amino acid residues and inter-amino acid interactions. In addition, solvent effects are incorporated in the force field parameters. Hence, with the SAAP force field molecular simulation proceeds in water as fast as in vacuo and can be carried out on a personal computer.

In this paper, we have developed the SAAP simulation program for arbitrary amino-acid sequences containing all 20 amino acids. The program was based on the core algorithms to connect amino acid residues together and calculate the total potential energy and was coded for the Monte Carlo simulation. The details will be presented along with demonstration of the software.

Accuracy of the developed SAAP simulation program was evaluated by using two model peptides; Met-enkephalin, a pentapeptide with an anodyne function (For-Tyr-Gly-Gly-Phe-Met-NH_2), and C-peptide, a peptide of 13 amino acid residues based on the N-terminal helix of ribonuclease A (For-Ala-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Leu-Arg-Ala-His-Ala-NH_2). The results obtained by the simulations will be compared with those having been obtained by experiments and simulations using the other force fields.

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Diffusion of large particles

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Purpose: Few authors have studied effects of the interaction of solvent particles on diffusion of large particles. Observing diffusion of protein, Nishida *et al.* have claimed the importance of hydrogen bonding [1]. In the present study, a theory is developed to calculate effects of attraction and repulsion from solvent particles.

Yamaguchi *et al.* have recently developed a theory to calculate diffusion coefficients [2]. They considered effects of a solute when calculating the dynamics of solvent particles. Since one cannot neglect this effect when a solute is large, their theory is useful for the present purpose.

Basic equations: The theory of Yamaguchi *et al.* [2] is given by

$$\nabla \cdot \mathbf{J}(\mathbf{r}) = 0, \tag{1}$$

$$\rho_{\rm eq}(\mathbf{r})\nabla\frac{\delta\rho(\mathbf{r})}{\rho_{\rm eq}(\mathbf{r})} - \int d\mathbf{r}'\rho_{\rm eq}(\mathbf{r})\nabla c(\mathbf{r}-\mathbf{r}')\delta\rho(\mathbf{r}') + \int d\mathbf{r}'\mathbf{M}(\mathbf{r}-\mathbf{r}')\frac{\mathbf{J}(\mathbf{r}')}{\rho_{\rm eq}(\mathbf{r}')} = 0. \tag{2}$$

Here, $\mathbf{J}(\mathbf{r})$ is the current density field at the steady state, satisfying the boundary condition: $r \to \infty, \mathbf{J}(\mathbf{r}) \to \rho_{\rm eq}(\mathbf{r})\mathbf{u}$, where r is the distance from the solute, $\rho_{\rm eq}(\mathbf{r})$ is the equilibrium density field when the solute does not move, and $\mathbf{u}$ is the velocity of the solute. In addition, $\delta\rho(\mathbf{r}) = \rho(\mathbf{r}) - \rho_{\rm eq}(\mathbf{r})$ where $\rho(\mathbf{r})$ is the density field at the steady state, and $c(\mathbf{r})$ is the direct correlation function of solvent particles without the solute, and $\mathbf{M}(\mathbf{r}-\mathbf{r}')$ is a tensor related with the time correlation function of a random force. The tensor depends only on the difference between the two positions $\mathbf{r}$ and $\mathbf{r}'$ because of their approximation. These equations enable one to calculate the diffusion coefficient from the values of $\delta\rho(\mathbf{r})$.

Large-solute limit: In the infinity limit of the solute size, the following slip addition was exactly obtained on the surface of the solute:

$$v_r(\mathbf{r}) = 0, \tag{3}$$

$$\frac{\partial v_\theta(\mathbf{r})}{\partial r} = 0, \tag{4}$$

$$\frac{\partial v_\phi(\mathbf{r})}{\partial r} = 0 \tag{5}$$

Here, $v_r(\mathbf{r}), v_\theta(\mathbf{r})$, and $v_\phi(\mathbf{r})$ are the components of a vector $\mathbf{v}(\mathbf{r})$ in the spherical coordinate at the center of the solute where $\mathbf{v}(\mathbf{r}) = \mathbf{J}(\mathbf{r})/\rho(\mathbf{r})$. From the limit, one can expand the diffusion coefficient in terms of $1/R$ where R is the radius of the solute.

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Molecular Dynamics Study on the Ligand to Metal Charge Transfer Dynamics in a Blue Copper Protein Plastocyanin

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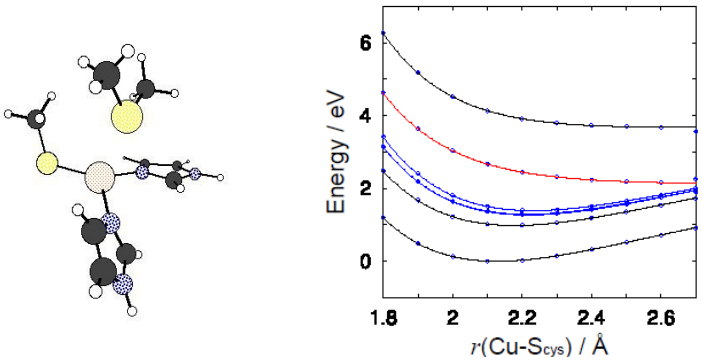
Plastocyanin contains a copper ion as the redox center that is surrounded by four amino acid ligands. It is believed that the ligand-to-metal charge transfer (LMCT) excitation is the origin of its blue color. Assuming that the dynamics induced by the LMCT excitation will be similar to those associated with the physiological redox reaction, a number of spectroscopic studies have been carried out. In order to offer complementary microscopic information on the CT mechanism, we carry out combined quantum chemical and molecular dynamics (MD) simulations. Of particular focus will be the interrelationship between the local structural change around the copper center and the large-scale protein motions, and its significance in the redox function of the protein. For example, calculations on the model complex suggested [1] that the vibrational breathing motion of the two histidine ligands may assist non-adiabatic transitions from the CT state to the low lying d-d ligand field excited states. We aim to clarify couplings of protein collective motions to this kind of local motions with the use of mixed quantum-classical MD simulations [2].



Left: Copper ion center of plastocyanin with truncated amino acid models of Cys, Met and two His ligands. Right: Ground and excited state potentials along the Cu-S(Cys) distance calculated by MCSCF-ORMAS method with 6-31G* basis set. [1]

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DNA Sequence Specific Hydration Responsible for Conformational Flexibility - molecular dynamics simulation on all possible tetramer sequences -

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DNA conformation and deformability depend on sequence composition. For example, it is known that a sequence containing contiguous AT steps is more deformable. The variability in DNA conformation and deformability has been believed to be one of important factors in specific recognition of DNA sequence by regulatory proteins. Several factors can be considered to yield the difference in DNA conformation and deformability among distinct DNA sequences. One is the mechanical stiffness inherent in the DNA itself, which is originated from base-pairing hydrogen interactions and base-stacking interactions. Another important factor affecting the DNA conformation and deformability is hydration. The hydration effect is inevitable when discussing the structural properties of DNA, since DNA is physiologically surrounded by water molecules, and the structure cannot be maintained without water.

A characteristic hydration pattern observed for DNA is the spine of water, which is composed of the highly ordered water molecules aligned along the floor of the minor groove. Such a hydration pattern as well as the conformation of DNA is dependent on the sequence. Consequently, the following question arises: How and to what extent does the hydration affect the sequence-dependent deformability of DNA ? To address this question, a comparative study of various DNA sequences is required. Recently, the sequence dependence of DNA conformation and deformability has been studied by MD simulations, where all possible 136 patterns for tetramer were examined [1]. In the present work, we report further analysis of these 136 MD trajectories focused on the hydration water. We compared the both properties of DNA hydration and deformation, and found a possible relationship between them [2].

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P032

A critical test of the electrostatic cutoff length for bimolecular simulations

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Coulomb interaction has been implemented in MD simulation in various ways such as cutoff, Ewald sum, multipole expansion, and continuum model. The cutoff is the simplest approach among them, which is available in any types of system such as periodic boundary cells or finite spheres. In this treatment, interactions between atom pairs only with a distance shorter than a given cutoff length are considered, and effects from more distant pairs are neglected. The cutoff treatment has been rigorously tested for various systems, and it has been revealed that the cutoff approximation can severely affect their properties.

One of the most problematic points with the cutoff calculation is to determine the cutoff length to be used. It is not an easy task to find a proper cutoff length that is able to suppress the artifact to a desired degree. It has been pointed out that the commonly used cutoff length ~12 Å is too short to maintain the folded structure, and a much longer length ~18 Å is required[1]. However, some contradictions have been reported. Three kinds of cutoff lengths 6, 10 and 14 Å were tested in the system of a hydrated helix-forming peptide by Schreiber and Steinhauser[2]. The native helix structure was maintained only in the simulation with the medium cutoff length of 10 Å. In the other cases of 6 and 14 Å, the helix broke down spontaneously. This result suggests that an increased cutoff length does not necessarily improve the simulation result. Recently, another example [3] showing the same tendency has been reported by the author, where bulk water systems were tested by using 9 and 18 Å cutoff lengths. Any critical error was not seen in the 9 Å cutoff simulation. On the other hand, in the 18 Å cutoff simulation, an artificial phase transition of water molecules was induced, and an extremely ordered structure was observed.

In the present work, in order to remove confusion of the cutoff lengths mentioned above and to obtain deeper understandings, we performed MD simulations of bulk water with various cutoff lengths. The bulk water system is particularly suitable compared with solution systems of peptides and proteins, because the result from such a homogeneous system is less influenced by the initial conditions used. We can thus obtain a more reliable conclusion from the calculation. In the present work, we made detailed investigations over the wide range of cutoff lengths 9-18 Å and successfully obtained a clear tendency of inducing the artifact. As the cutoff length increases, the artifact gradually increases [4], causing the phase transition reported in[3]. Our results clearly show that increasing cutoff length can make simulation results worse in terms of orientational behavior of water molecules.

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P033

Secondary structure formation studied by molecular dynamics simulations of short peptides

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Secondary-structure formation is a key step of protein folding. Two secondary-structure elements are usually connected by a turn or a loop structure that locates in the surface of the protein molecule. β-hairpin structure is such a secondary structure. We investigated the role of the turn region of a β-hairpin peptide ("G-peptide") for folding by molecular dynamics simulations. The amino-acid sequence of G-peptide is GEWTYDDATKTFTVTE.

We have recently observed folding events from unfolded conformations to the native conformation of G-peptide several times by multicanonical replica-exchange molecular dynamics simulation. The analysis of the simulation results has shown that (1) not only the hydrophobic inter-side-chain contacts between the aromatic residues (Y5 and F12) but also the native-like turn formation take place in an early stage of folding of G-peptide and that (2) the native-like turn formation prevents misfolding of G-peptide to non-native β-sheet conformations with the aromatic inter-side-chain contact formed.[1] These results are consistent with experimental studies on folding and stability of G-peptide.

The foldability of G-peptide, however, depends on the force field used in the simulation. The above results (with GROMOS96 force field) suggest that this dependence comes from the force-field dependence of the conformational preference of the turn sequence. We performed molecular dynamics simulations of a shorter peptide whose amino-acid sequence corresponds to the turn region. Though the turn peptide does not fold to the native structure, the similarity to the native structure has been shown being dependent on the force field.

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P034

THEORETICAL STUDIES FOR AMYLOIDOGENESIS OF Aβ(29-42)

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Amyloid β-peptide (Aβ) is comprised of 39 to 43 amino-acid residues and has tendency to form amyloid fibrils, which are associated with Alzheimer's disease. In these amyloid fibrils, Aβ is perceived to have an intermolecular β-sheet structure. Furthermore, Aβ(29-42), which is comprised of from the residues 29-42 of Aβ, has an intermolecular antiparallel β-sheet structure in aqueous solution.

The multicanonical-multioverlap algorithm has advantages of both the multicanonical algorithm and the multioverlap algorithm [1-3]. Namely, the multicanonical-multioverlap simulations can sample effectively the conformational space including specific configurations. Applying this method to protein systems, therefore, we can obtain effectively the accurate free-energy landscapes of these systems including specific configurations.

In this poster, we apply the multicanonical-multioverlap algorithm to Aβ(29-42) dimer with implicit water. By employing a β-sheet structure of Aβ(29-42) as a reference conformation in the multicanonical-multioverlap simulations, we study the secondary structures and aggregation properties of Aβ(29-42).

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Dynamical Properties of Polytheonamide B: A Normal Mode Analysis

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Recent progress in X-ray crystallography and NMR techniques has led to the determination of three-dimensional structures of many biological molecules. In the post-genome era, it is a challenging problem to identify the relationship between structures and functions of biomolecules.

Polytheonamide B is a highly cytotoxic polypeptide isolated from the marine sponge *Theonella swinhoei* in 1994. The NMR and amino-acid analysis indicated that polytheonamide B is a linear 48-residue peptide with alternating D- and L-amino acids and contains seven kinds of methylated and hydroxy variants of proteinogenic amino acids [1]. To investigate the dynamical properties of polytheonamide B we carried out energy minimization and normal mode analysis by using the program CHARMM. We calculated root-mean-square displacements of backbone atoms, root-mean-square fluctuations of the backbone dihedral angles (ϕ, ψ), and correlation factors for the dihedral angle fluctuations. The normal mode analysis revealed that polytheonamide B shows the elastic rod behavior in the very low frequency modes and that the libration motions of backbone peptide planes, which would associate with the function of polytheonamide B, have the modes with relatively low frequencies. We compare our results with those of another D, L-alternating polypeptide to discuss the structure and function.

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P036

Explicit symplectic molecular dynamics simulations for rigid-body molecules in the canonical ensemble

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We propose an explicit symplectic molecular dynamics (MD) algorithm for rigid-body molecules in the canonical ensemble [1]. Employing the symplectic MD algorithm, there is a conserved quantity which is close to Hamiltonian. Therefore, we can perform a MD simulation more stably than by conventional non-symplectic algorithms.

We applied this algorithm to TIP3P water at 300 K and compared the time evolution of the Hamiltonian with that by the non-symplectic algorithms. We found that the Hamiltonian was conserved well by the symplectic algorithm even for a time step of 4 fs. This time step is longer than typical values of 0.5 fs to 2 fs which are used by the conventional non-symplectic algorithms.

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MULTIPLE SEQUENCE ALIGNMENT USING CONFORMATIONAL SPACE ANNEALING

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Multiple sequence alignment is one of the most important problems in bioinformatics. However finding an optimal alignment of a given objective function is known as an NP-complete problem. Since the global minimum of a given function with a complex energy landscape is hard to be found effectively, practically all current methods use heuristic algorithms such as the progressive alignment. Moreover, even if we could find the global minimum, we do not know the perfect objective function to be optimized. In this paper we try to find diverse lower energy alignments possibly containing the global minimum of a consistency-based objective function by applying the conformational space annealing (CSA) method. The CSA is a powerful optimization tool especially suited for combinatorial optimization problems. By enforcing a systematic diverse conformational search, the CSA alleviates the problem arising from using an inaccurate objective function. Four benchmark tests along with discussions on the advantages and disadvantages of the method are presented.

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P038

TEMPLATE BASED MODELING BASED ON GLOBAL OPTIMIZATION

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For the prediction of the 3D structures of 100 CASP7 targets, we have developed a procedure which is based on global optimization of score functions in three levels. The whole procedure is composed of the following five steps:

1. Fold recognition: To collect fold candidates of a given target sequence, we considered top scoring templates from the meta-server provided by http://bioinfo.pl/~3djury, and another top scoring templates from an in-house method called FoldFinder. FoldFinder is a profile-profile alignment method utilizing predicted secondary structures. After collecting these templates, we performed a preliminary assortment of structural clustering often leading to 2 or 3 sets of template lists.

2. Multiple sequence/structure alignment by MSACSA: This is the most crucial step. We have performed multiple sequence/structure alignment for each template list obtained from the fold recognition step. We have applied a rigorous global optimization method to an in-house consistency-based scoring function by using the conformational space annealing [3] (CSA) method. The lowest scoring alignment among the 100 final ones from the CSA is used as the input to the following 3D modeling step.

3. Modeling of the 3D structure by ModellerCSA: The 3D structures of target proteins are constructed by optimizing the MODELLER energy function using the CSA method. For each multiple alignment (containing up to 25 templates), a total of 100 models are generated.

4. List selection and the clustering of models for final model selection: For most cases, we have more than one list of templates, and we have applied a neural network based in-house procedure to assess the quality of the models obtained for each list. We find the center model of the cluster.

5. Side-chain modeling for selected targets by ROTCSA: For each list, a rotamer library is constructed based on the consistency of the side chains in the final 100 models obtained in the step 3. To this library, we have added a backbone dependent and sequence specific rotamer library. Using the CSA, we have optimized an in-house scoring function which contains energy terms from SCWRL and DFIRE.

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Molecular dynamics simulations of chaperonin in water

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The chaperonin GroEL accelerates the protein folding by confining a protein in the large cage which is filled by water molecules. The purpose of our project is to investigate physico-chemical properties of water molecules confined in the cage by molecular dynamics (MD) simulations. In the first year of our project, we prepared the chaperonin in the water environment by immersing the GroEL-GroES complex (PDB ID: 1SX4) with crystal waters and counter ions in the water sphere of 140 Å. Since the GroEL-GroES complex has the large total charge of -273, we placed positive counter ions (Na^+) around the negative amino-acids to neutralize the entire system, where the electronic potentials around the amino acids were evaluated by performing the preliminary short MD simulations. The total number of amino acids, water molecules, and atoms were about 8000, 336000, and 1129000, respectively. All MD simulations were performed on a supercomputer (Fujitsu Primequest) at Research Center for Computational Science using a program COSMOS90 developed by one of the authors (M.S.). The performance speed of COSMOS90 was 3 sec/step for 64 processors of the Primequest. The entire system was heated up by performing 10-ps MD simulations at 1 °K, 50 °K, 150 °K, 200 °K, 250 °K, and 300 °K. After the equilibrating the entire sytem at 300 °K, we will calculate the physico-chemical properties such as the diffusion constant for water molecules and will clarify the difference in the properties between the confined water in the cage and bulk water.

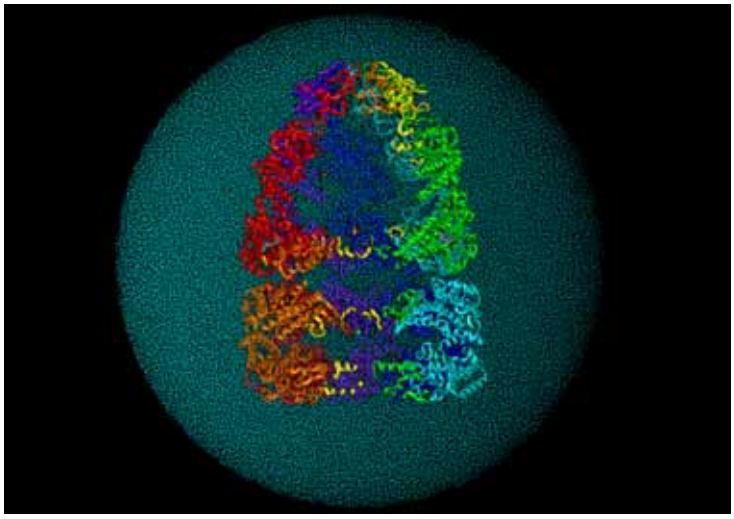

Figure: The GroEL-GroES complex in the water sphere of 140 Å. The total number of atoms is about 1129000.

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 P040

Molecular Dynamics Study on the Interaction between Trehalose and Phospholipid Bilayer

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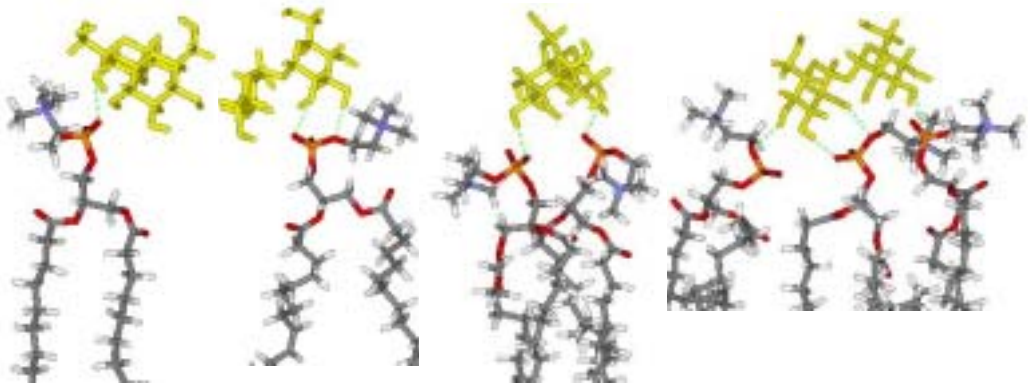
Trehalose acts as a good protectant against various water stresses such as desiccation and freezing. The water replacement hypothesis has been proposed as a possible mechanism by which trehalose protects biological components such as membrane and proteins in low water activity. Here, to investigate this hypothesis at atomic resolution, molecular dynamics simulations were carried out for trehalose/phospholpid bilayer systems, where the bilayer was constructed from 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC). Trehalose molecules were dissolved in the interbilayer medium at a concentration of 0.133 mol/kg or 1.22 mol/kg. After a 10 ns simulation for each system, we analyzed the hydrogen bond network formed among trehalose, water and the head group of POPC. In consequence, it was found that a part of the trehalose molecules directly hydrogen bond to the head group of POPC without being interrupted by any water molecules and that the stability of the lipid bilayer increases with an increase in trehalose concentration. These results support the validity of the water replacement hypothesis.



Direct interactions between trehalose and the head group of phospholipid

Reference

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Molecular design of functional peptide nanotubes

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It is known that protein nanotubes with hollow tubular fibrous structure perform diverse biological functions such as ion transport activity and antimicrobial activity. Here we try to design *de novo* cyclic peptides with amino acid side-chains sticking out inside the ring were designed to accommodate Cu^{2+} specifically. First, we designed two types of cyclic dodecylpeptide that had two regions of contiguous three L-amino acids in place of alternating L-, D-amino acids, and had His or Asp side-chains in the middle of three L-amino acids whose side-chain stayed inside the ring. Antiparallel ß-sheet structure is expected to be formed by mixing these two types of cyclic peptide with molar ratio 1:1. When Cu^{2+} was added to this peptide mixture at pH 7.5, CD spectra indicated transformation to ß-sheet, although long fibers were not observed in AFM images. On the other hand, CD spectra with Ni^{2+}, Zn^{2+}, Co^{2+} or Cd^{2+} showed no signal for ß-sheet formation. Moreover, NMR signals for His disappeared and the peaks of Asp became notably broader compared to other residues when the concentration of Cu^{2+} was raised. These results suggested that Cu^{2+} was situated in the position of His and Asp side-chains inside the ring. This metal-bound peptide was capable of being transformed into nano-fiber by adding methanol up to 55%. Since the nanotube can incorporate metal ions inside the ring specifically, the peptide nanotube is expected to be utilized as a functional molecular nanodevice.

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 P042

Molecular Dynamics Simulation on the Glassy States of Trehalose and Neotrehalose

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Trehalose (TH) is a good protectant against various environmental stresses in many organisms. For instance, a larva of *Polypedilum vanderplanki* accumulates a large amount of TH when it is desiccated and its metabolic activity becomes undetectable, a state called anhydrobiosis. Recently our group demonstrated that the anhydrobiosis is maintained as a result of vitrification of the accumulated TH [1]. In addition, from DSC measurements, it was found that TH has the highest glass transition temperature (Tg) and the largest activation energy in the glassy state among the naturally occurring gluco-disaccharides [2]. In this study, to understand why TH has such excellent properties as a desiccation protectant, we studied its glassy state at atomic level using molecular dynamics simulations and compared the results with those for neotrehalose (NTH). The simulations revealed the following points: 1) there is no apparent difference in the average number of intermolecular hydrogen bonds between TH and NTH, 2) the root-mean square displacement of the center of mass is smaller in TH than in NTH, 3) the free volume formed in the glassy matrix is relatively smaller in TH than in NTH, and 4) the glycosidic bond of TH takes a single conformation but that of NTH has more than two conformations. These results suggested that the higher Tg and higher stability of TH glass is due to the increasing packing density of the glassy matrix, which originates from the conformational simplicity of this sugar, a characteristic feature of α, α-1,1-linkage.

Molecular structures of A) trehalose (α-D-glucopyranosyl α-D-glucopyranoside and B) neotrehalose (α-D-glucopyranosyl β-D-glucopyranoside).

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Spectral tuning, photoisomerization, and energy transfer pathways of photoactive yellow protein

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We have studied the dynamics (1), the electronic state of the p-coumaric acid chromophore (2) of photoactive yellow protein (PYP). A new method, Multi-Layer Self-Consistent Molecular Orbital (MLSCMO) method (3,4) accurately calculated the optical absorption maxima for 12 mutants of PYP (Figure 1). The driving force for the photoisomerization reaction of PYP in atomic detail by using QM/MM calculations (5,6). We introduced a new quantity, microscopic site-site energy conductivity (7) and investigated the energy transfer pathways in PYP (Figure 2).

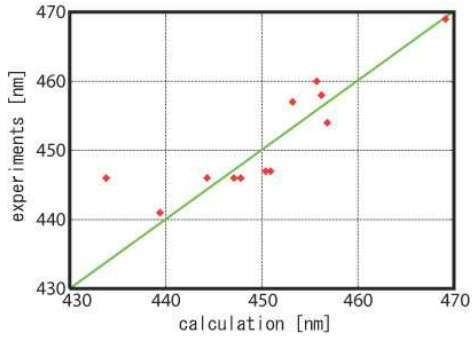

Figure 1.

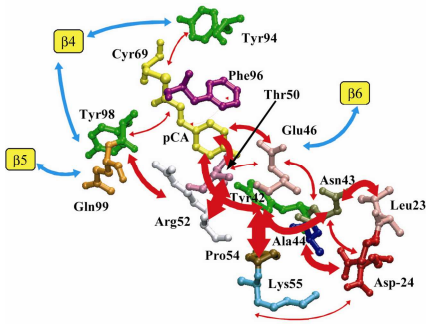

Figure 2.

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Free Energy Calculation on the Interaction of Trehalose with Hydrophobic Compounds

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In previous studies [1, 2], we reported that trehalose can bind to the cis type C=C double bond of unsaturated fatty acid (UFA) and diene through OH···π and CH···O interactions in aqueous solution and as a result of this it effectively depresses the oxidation of UFA. In addition, our recent NMR study demonstrated that trehalose also binds directly to benzene and its derivatives in aqueous solution [3]. Such intriguing complexation phenomena were not observed between other disaccharides (maltose, neotrehalose and sucrose) and the unsaturated compounds. Based on these results, it is expected that trehalose is able to specifically modify the hydrophobic interaction that plays an important role in stabilization of protein. In fact, we found that trehalose modify the aggregation process of β-amyloid (Aβ), especially for random-coil Aβ[4]. Therefore, it is of great interest to elucidate what kinds of driving force works in the complexation processe between trehalose and the unsaturated compounds. To address this problem, we performed explicit-solvent molecular dynamics simulations for a trehalose/benzene system. Using the umbrella sampling method combined with the weighted histogram analysis, we obtained the potential of mean force (PMF) for the complex formation process. The resultant PMF profile indicated that in aqueous solution trehalose and benzene forms a stable intermolecular complex at the distance of 5 Å between the centers of mass, with a stabilization energy of about 1 kcal/mol. At this energy minimum, the benzene molecule was most frequently located in the hydration pocket formed around the $–C_5$-O-C_1- bonds of the glucose rings of trehalose. In conclusion, benzene binds to trehalose with their relative orientations where dehydration penalty becomes as small as possible.

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The effect of simplified amino acid sequence on conformational stability and catalysis of Staphylococcal nuclease

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The Amino acid sequence of a protein basically contains almost all information required for structural formation and function of the protein. Therefore the elucidation of the information encoded in the amino acid sequence is essential for the understanding of the relationship between. The tertiary structure of a protein is tolerant to amino acid substitutions, suggesting that each amino acid residue is not equally important for the structure and function of a protein. This fact also suggests the possibility of the simplification of the sequence. We attempted to simplify the sequence of Staphylococcal nuclease (SNase). The existence of the residues whose side chains do not contribute to structural formation and functions makes the extraction of meaning information, we complicate intended to make the simplified protein to derive the essential information.

Our previous study on the systematic alanine insertion into SNase revealed the structural elements and the functional elements. We simplified the residues belonging to the other regions then there elements. The simplification was performed with a set of simple rules. (1) The hydrophilic residues (Ser, Thr, Asn and Glu) were changed to Ser. (2) The hydrophobic residues (Ala, Leu, Ile, and Met) were changed to Ala. (3) The charged residues (Asp, Glu, Lys, and Arg) were changed to Asn. (4) The aromatic residues (Phe, Tyr, and Trp) were changed to Phe. We could prepare the gene of the simplified SNase. The simplified protein could be overexpressed in E. coli and purified. The structure and the function of the simplified SNase will be discussed.

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P046

INVESTIGATION OF THE STRUCTURAL AND FUNCTIONAL REGION OF PHOTOACTIVE YELLOW PROTEIN BY USING SYSTEMATIC ALANINE INSERTION

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To establish the principle of protein folding and design, it is essential to extract information about structural formation and functional expression from the primary structure of a protein. We previously performed systematic alanine insertion analysis upon Staphylococcal nuclease (SNase). Where we created all possible single alanine insertion mutants and examined their foldability and enzymatic activity. Consequently, we can separate the regions bearing the structural and/or functional information on the sequence. We call them “structural element” and “functional element”, respectively. It is quite interesting that the structural elements correspond to the regions that form native like topology in the early stage of folding. It is also concluded that the systematic alanine insertion analysis is a useful technique for extracting structural and functional information on the amino acid sequences. In order to confirm the usefulness of the alanine insertion analysis, we have applied it to the other proteins such as Photoactive yellow protein (PYP) and Dihydrofolate reductase (DHFR). In the present report, we will show the results on PYP.

We constructed a series of alanine insertion mutants for the entire PYP sequence and reconstituted these mutants with *p*-coumaric acid chromophore. Because PYP turns to yellow upon binding of the chromophore and forming native structure, the color can be used as an indicator of the structural formation and functional expression. All of reconstitution assays were performed with *Escherichia coli* BL21 (DE3) containing tyrosine ammonia lyase (TAL) and *p*-coumaric acid ligase (pCL), where the chromophore of *p*-coumaric acid is bound to apo-protein in vivo. Thus, if a mutant forms the native like structure, the colored holo-protein can be obtained from the supernatant of the sonicated bacterial cells. The effects of alanine insertion were categorized into three groups: 1. holo-protein existing in supernatant, that is, no effect on the chromophore binding ability; 2. loss of the chromophore binding ability but the apo-protein existing in supernatant; 3. loss of the chromophore binding ability and apo-protein existing in inclusion body. The mutants that exhibit a common phenotype appeared as a continuum region, as seen in the analysis of SNase. Although the mutants of both group 2 and 3 lacked their chromophore binding ability, they can be distinguished by location of apo-proteins. The group2 mutants exist in supernatant, while the group 3 mutants are integrated into the inclusion body. This indicates that the solution structures of the apo-protein between the group 2 mutants and the group 3 mutants are different. We are now purifying these mutants to characterize their solution structures [1].

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Dynamics of protein and its hydration water studied by incoherent neutron inelastic scattering

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The protein dynamics have been expressed with the energy landscape. The realistic landscapes have the multiple substates, among which the protein conformation changes. Inelastic neutron scattering experiments of Staphylococcal nuclease in hydrated and dehydrated states at a wide range of temperatures from 10 K to 300 K were performed to study hydration effect on protein dynamics. It was found that the low frequency collective modes are highly coupled with the hydration, while the hydration hardly affects the local vibration with the higher frequency. A protein boson peak around 3 meV shifts to higher frequency upon hydration. This result indicates that the protein energy landscape is more rugged in the hydrated state, which was predicted by the molecular simulation[1]. Quasi-elastic scattering of hydrated state is more remarkable than that of dehydrated state at room temperature. These results allow us to give a comprehensive insight into the change of protein energy landscape upon hydration.

Another hydration related protein dynamical feature is a dynamical transition. Dynamics of protein and its hydration water at the transition was examined. The transition appears at around 240K above h～0.2 hydration level. Above the threshold hydration level, the hydration water dynamics drastically change. The anomalous dynamics of hydration water is coupled with the protein collective modes. Such a dynamical coupling should drive the hydration dependent protein dynamical transition. On the basis of the neutron spectrum observed, we showed that the origin of the boson peak and the dynamical transition is hydration-coupled low frequency modes, and then suggests the hydration-related energy landscape pictures.

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P048

ROOM-TEMPERATURE FTIR SPECTROSCOPY OF ACTIVE INTERNAL WATER MOLECULES IN THE BACTERIORHODOPSIN PHOTOCYCLE

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The important role of internal water molecules in the proton pump mechanism of bacteriorhodopsin (bR) has been largely documented in the literature, especially from low temperature Fourier-transform infrared (FTIR) spectroscopy [1, 2]. However, it is not known whether the same changes for the internal water bands also occur at room temperature. In this study, we conducted a series of time-resolved step-scan FTIR experiments of bR films hydrated either with $H_2{}^{16}O$ or $H_2{}^{18}O$, aiming to detect water O-H bands under weak hydrogen-bounding conditions (3750-3500 cm^{-1}). The covered time window (2.5 μs - 12 ms) and the experimental conditions allow us to obtain L-bR and M-bR spectra with minor contributions of other intermediates. The M-bR spectrum obtained at room temperature (293 K) was almost identical to the M-bR spectrum trapped at 230 K, also in the weakly hydrogen bounded water region (Fig. 1). In contrast, the L-bR spectrum obtained at room temperature differed significantly from the L-bR trapped at 170 K (Fig. 1). Besides some differences in the already studied 1800-900 cm^{-1} region, the room-temperature L-bR showed the lack of some positive bands corresponding to internal waters under weakly hydrogen-bounded conditions. This suggests that internal water molecules change differently during the bR photocycle at room and 170 K.

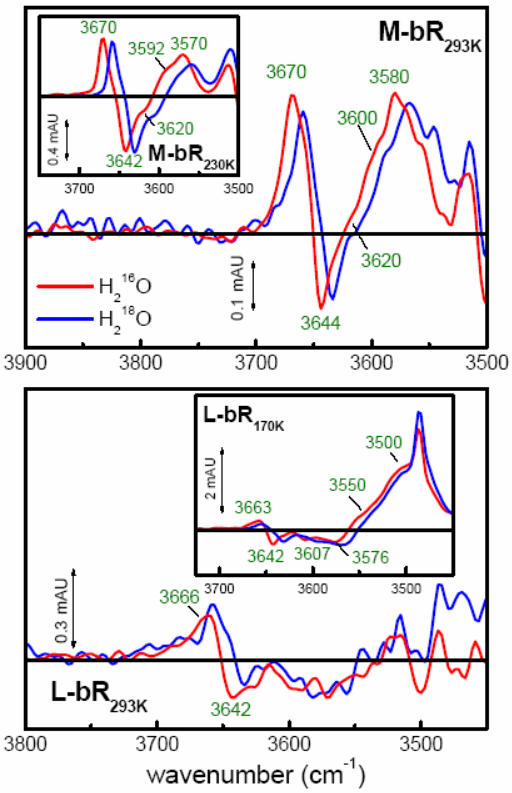

Fig.1. FTIR spectra. Bands shiffing in $H_2{}^{18}O$ are labeled in green.

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FTIR STUDIES OF HALIDE-BOUND D85S AND D212N BACTERIORHODOPSIN MUTANTS

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Bacteriorhodopsin (BR), a membrane protein found in *Halobacterium salinarum*, functions as a light-driven proton pump. The Schiff base region has a quadropolar structure with positive charges located at the protonated Schiff base and Arg82, and counterbalancing negative charges located at Asp85 and Asp212 (Figure). It is known that BR lacks a proton-pumping activity if Asp85 or Asp212 is neutralized by mutation. On the other hand, binding of Cl^- brings different effects for pumping functions in mutants at D85 and D212 position. While Cl^--bound D85T and D85S pump Cl^-, photovoltage measurements suggested that Cl^--bound D212N pumps protons at low pH.

In this study, we measured low-temperature FTIR spectra of D85S and D212N containing various halides to compare the halide binding site of both proteins. In the case of D85S, the N-D stretching vibrations of the Schiff base were halide-dependent [1]. This result suggests that the halide is a hydrogen-bond acceptor of the Schiff base, being consistent with the X-ray crystal structure. On the other hand, no halide dependence was observed for vibrational bands of the retinal skeleton and the Schiff base in the D212N mutant. This result suggests that the halide does not form a hydrogen bond with the Schiff base directly, unlike the mutation at D85 position. Halide-dependent water bands in the Schiff base region also differ between D85S and D212N. From these results, halide binding site of both proteins and role of two negative charges in BR will be discussed.

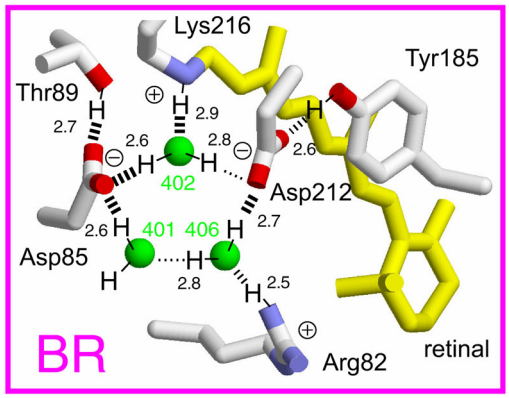

Figure X-ray crystal structure of the Schiff base region in bacteriorhodopsin. Dotted lines represent supposed hydrogen bonds. Hydrogen atoms and strength of hydrogen bond are revealed from our research [2], where strong hydrogen bonds are shown by thick dotted lines.

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P050

LONG-RANGE INTERACTION BETWEEN THE CYTOPLASMIC CARBOXYLIC ACID AND THE RETINAL IN A FUNGAL LIGHT-DRIVEN PROTON PUMP

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Fungal rhodopsins are eucaryotic structural homologs of the archaeal light-driven proton pump bacteriorhodopsin (BR) and have been discovered in the course of genome sequencing projects. Among them, two fungal rhodopsins were studied *in vitro* and exhibited very different photochemical behavior. *Neurospora* rhodopsin (NR) possesses a slow photocycle and has no ion transport activity, reminiscent of sensory rhodopsins, while *Leptosphaeria* rhodopsin (LR) has a fast bacteriorhodopsin-like photocycle and pumps protons light-dependently. Such a significant difference is surprising considering very high sequence homology of the two proteins. Our recent FTIR study has revealed that important structural difference between LR, NR and BR, especially in the structure of water molecule near the Schiff base [1]. In this paper, we investigate whether the chemical structure of a cytoplasmic carboxylic acid, the homolog of Asp96 of BR serving as a proton donor for the retinal Schiff base, can define the photochemical properties of fungal rhodopsins. We studied mutants of LR in which this aspartic acid was replaced by Glu or Asn using infrared and visible spectroscopy. We show that Glu at this position is lacking in ability for a proton donor similar to a non-protonatable Asn. Moreover, this replacement induces long-range structural perturbations of the retinal environment, as evidenced by changes in the vibrational bands of retinal (especially, hydrogen-out-of-plane modes) and neighboring aspartic acids and water molecules. The conformational coupling of the mutation site to the retinal may be mediated by helical rearrangements as suggested by the changes in amide and proline vibrational bands. We conclude that the difference in the photochemical behavior of fungal rhodopsins from *Leptosphaeria* and *Neurospora* may be attributed, to some extent, to the replacement of the cytoplasmic proton donor Asp by Glu [2].

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PHOTOCHROMIC BEHAVIOR OF *ANABAENA* SENSORY RHODOPSIN

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Anabaena Sensory Rhodopsin (ASR) is an archaeal-type rhodopsin found in eubacteria[1]. The gene encoding ASR forms a single operon with a soluble protein of 125 residues (14 kDa). *Anabaena* photosynthetic system has two accessory light-harvesting proteins, phycocyanin (absorbing red light) and phycoerythrocyanin (absorbing green light), and the expression levels of both phycobilisomes are controlled by the environmental light. ASR has two isomeric states of retinal, all-*trans* and 13-*cis* forms[2,3], and the isomeric ratio is altered depending on the wavelengths of illumination. Therefore, ASR may be a photochromic sensor that distinguishes between red and green light. For the photochromic sensory function, the photoproduct of the all-*trans* form should be the 13-*cis* form, and vice versa. However, it is well known that most archaeal-type rhodopsins have a photocycle, where the final photoproduct of the all-*trans* form is all-*trans*, like bacteriorhodopsin. What percentage is converted into the other isomeric form for ASR? In this study, we attempted to determine the branching ratio in the photoreactions of the all-*trans* and 13-*cis* forms by means of low-temperature UV/visible spectroscopy.

Surprisingly, the photo--chromic ratio (conversion of each isomeric state) was very high for both all-*trans* and 13-*cis* forms, suggesting that ASR is optimized for photo--chromism.

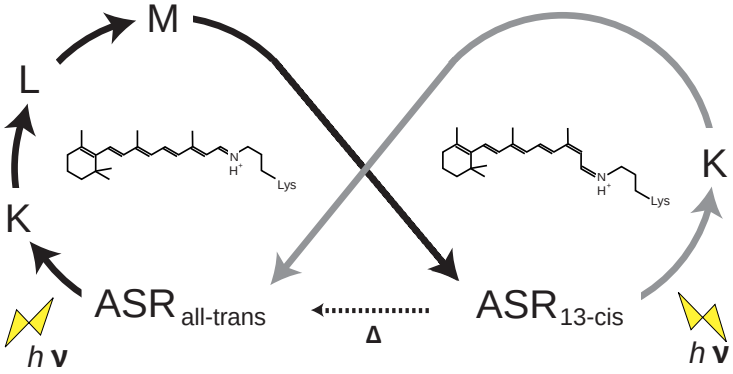

Figure 1 The photoreaction scheme of ASR

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P052

IDENTIFICATION OF THE N-H STRETCH OF ASN1008 BY FTIR SPECTROSCOPY IN THE LOV2 DOMAIN OF *ADIANTUM* PHYTOCHROME3

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Phototropins are blue-light receptors in plants. They have two photosensory domains named LOV which bind FMN as a chromophore. We have investigated the light-induced structural changes for the LOV2 domain of *Adiantum* phytochrome3 (phy3-LOV2) by use of low-temperature FTIR spectroscopy [1]. We observed the bands at 3490 (+)/3479 (-) cm^{-1} upon formation of the S390 intermediate, which was not deuterated upon hydration with D_2O (solid line in Figure 1a) [2]. The bands exhibited spectral downshifts by 10 cm^{-1} for the uniformly ^{15}N-labeled sample and also for the reconstituted sample of ^{14}N-FMN/^{15}N-apoprotein (dotted line in Figure 1a). This result clearly shows that the bands originate from apoprotein. Specific labeling of amino acids revealed that the bands originate from asparagine(s). Mutational study strongly suggested that the N-H group comes from Asn1008 (Figure 1b). The frequency shows that the N-H group is free from hydrogen-bonding. Since an N-H group of the Asn1008 side chain forms a hydrogen bond with the C(4)=O group of FMN, the observed stretching probably comes from the N-H group that is not involved in the hydrogen bond with FMN. The N-H group band showed a downshift in the Q1029L mutant (Figure 1c) which lacks the hydrogen bond with C(4)=O group of FMN, implying the rearrangement of the environment around Asn1008. The environment and the structural changes of Asn1008 will be discussed.

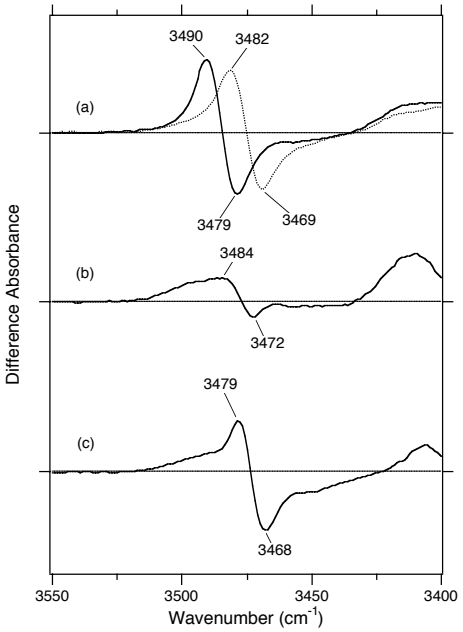

Figure 1. Light-induced difference FTIR spectra of unlabeled phy3-LOV2 (solid line in a), ^{14}N-FMN/^{15}N-apoprotein (dotted line in a), N1008V (b) and Q1029L (c) at 150 K in the 3550–3400 cm^{-1} region. One division of the y-axis corresponds to 0.0025 absorbance unit. Samples are hydrated with D_2O.

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ROLE OF PHE-1010 IN THE LIGHT-INDUCED STRUCTURAL CHANGES OF LOV2 DOMAIN OF *ADIANTUM* PHYTOCHROME3

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Phototropin, a blue-light sensor protein in plants, leads to various functions such as tropic responses, relocation of chloroplast, and stomata opening. Phototropin possesses two LOV domains. The LOV2 domain is more important for the light-induced kinase activation than the LOV1 domain, and we have shown that peptide backbone alterations are more prominent, particularly in the α-helical and β-sheet regions, in LOV2 than in LOV1 of phytochrome3 (phy3) [1, 2]. Nearly identical crystal structures of LOV1 and LOV2 domains suggest that some crucial amino acids are responsible for greater protein structural changes in the LOV2 domain.

In this study, we focused on Phe-1010 in phy3-LOV2, which sandwiches FMN with the reactive cysteine. Phenylalanine at this position is highly conserved for LOV2 domains, while the corresponding amino acid for LOV1 domains is Leucine. Unlike the wild-type LOV2, the FTIR spectra of F1010L exhibited no temperature dependence, which was similar to LOV1. In addition, spectral changes in amide-I were significantly smaller than in the wild-type LOV2, which also resembles LOV1. Thus, the replacement of Phe-1010 with Leu partially converts LOV2 into LOV1 in terms of protein structural changes. We will discuss the roles of Phe and Leu in LOV2 and LOV1 domains, respectively.

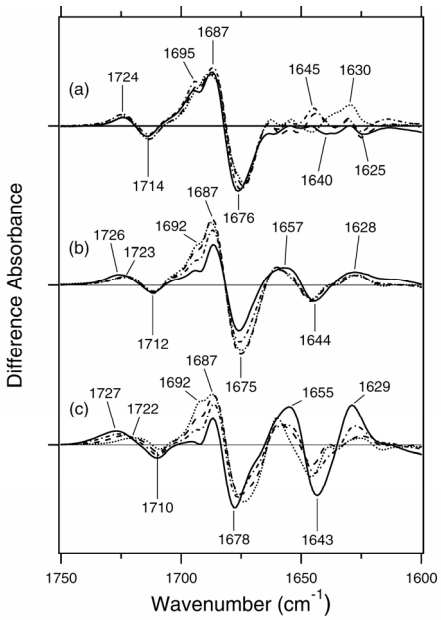

Figure 1. Light minus dark infrared spectra for phy3-LOV1 wild-type (a), phy3-LOV2 F1010L (b), and phy3-LOV2 wild-type (c) in the 1750-1600 cm^{-1} region. Spectra are measured at 150 K (dotted line), 200 K (broken line), 250 K (dash-dotted line), and 295 K (solid line). One division of y-axis corresponds to 0.009 absorbance units.

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P054

Promiscuos binding of ligands by β-lactoglobulin involves hydrophoic interactions and plasticity

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Bovine β-lactoglobulin (βLG) binds a variety of hydrophobic ligands, though precisely how is not clear. To understand the structural basis of this promiscuous binding, we studied the interaction of βLG with palmitic acid (PA) using heteronuclear NMR spectroscopy. The titration monitored using tryptophan fluorescence and a HSQC spectrum confirmed a 1:1 stoichiometry for the PA-βLG complex. Upon the binding of PA, signal disappearances and large changes in chemical shifts were observed for the residues located at the entrance and bottom of the cavity, respectively. This observation indicates that the lower region makes a rigid connection with PA whereas the entrance is more flexible. The result is in contrast to the binding of ligands to intestinal fatty acid-binding protein, another member of the lipocalin family, in which structural consolidation occurs upon ligand binding. On the other hand, the ability of βLG to accommodate various hydrophobic ligands resembles that of GroEL, in which a huge hydrophobic cavity and flexible binding site confer the ability to bind various hydrophobic substrates. Considering these observations, it is suggested that, in addition to the presence of the hydrophobic cavity, the plasticity of the entrance region makes possible the binding of hydrophobic ligands of various shapes. Thus, in contrast to the specific binding seen for many enzymes, βLG provides an example of binding with low specificity but high affinity, which may play an important role in protein-ligand and protein-protein networks.

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Dynamics of Water Molecules in Protein Folding: Volume Profile Analysis by High Pressure Spectroscopy

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We determined the volume changes associated with protein folding of reduced cytochrome *c* from the unfolded state to the native state. The equilibrium constant between the unfolded state and native state (K_{UN}) was determined by a change in the absorption (420 nm) at various pressures between 0.1 and 200 MPa and at various concentrations of denaturant (guanidine hydrochloride) between 3.2 and 4.0 M. Dependence of K_{UN} on these factors revealed that the volume change at ambient pressure in the absence of denaturant is negative (ΔV_f = -13 $cm^3 \cdot mol^{-1}$) (Fig. 1). We also followed pressure dependence of the folding rate (k_{UN}) to determine the activation volume ($\Delta V_f^{0\ddagger}$) for the process from the collapsed state to the native state. By using the photo-induced protein folding reaction[1], $\Delta V_f^{0\ddagger}$ was estimated to be -15 $cm^3 \cdot mol^{-1}$ as shown in Fig. 1 Such negative volumes can be accounted for by a decrease in volume resulting from the dehydration of hydrophobic groups, primarily the heme group, and the dehydration is mainly induced in the process from the collapsed state to the native state. Dehydration, which increases the entropy of the protein system, compensates for a decrease in the entropy accompanying the formation of the more compact and ordered transition state as illustrated in Fig. 1. We, therefore, propose that the positive change in the activation entropy for the folding reaction is due to the dehydration of hydrophobic groups, and dehydration entropically promotes the protein folding reaction.

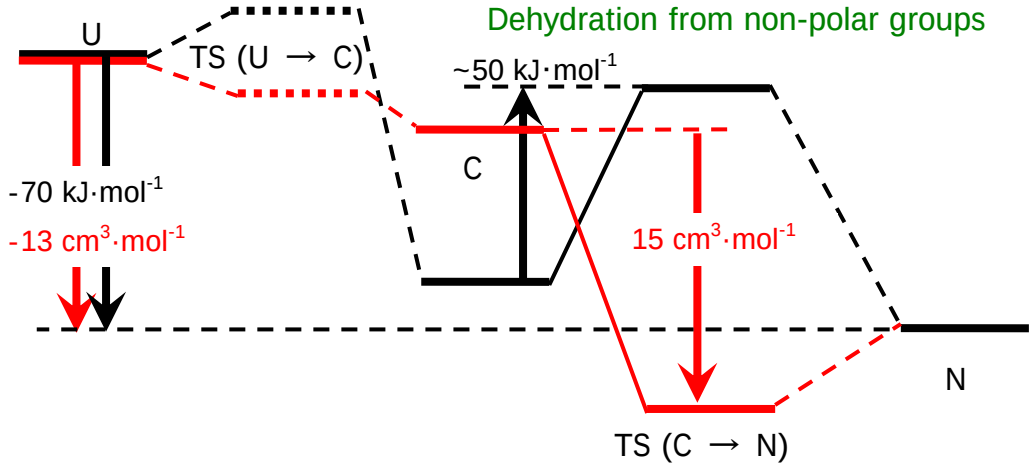

Fig. 1. Volume Profile for the Folding Reaction in Cytochrome *c*

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P056

PRESSURE EFFECT ON HELIX-COIL TRANSITION FOR AN ALANINE-BASED PEPTIDE IN WATER

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The α-helix is the most fundamental secondary structural element in proteins. It is important to understand the stability of α-helix in detail as a step toward understanding how proteins form stable native structures. An alanine-based peptides form a α-helix in the absence of tertiary interactions[1]. The past studies of these peptides have promoted our understanding of the stability of α-helix. However, while there has been the progress in understanding temperature, pH or salt effects on folding of α-helix, less known about the pressure effect. From pressure-tuning study, it is possible to investigate how large the partial molar volume of α-helix compared to coil is. The first study of pressure effect on a small 16-residue alanine-based peptide using infrared spectroscopy showed that α-helix is preferred rather than random coil under high pressure[2]. In addition, a molecular dynamics simulation study was carried out for a 20-residue alanine-based peptide[3]. Although this study was helpful to understand the microscopic structure of the peptide, it eventually concluded that the α-helix unfolds under high pressure. These experimental and theoretical results have been not in agreement with each other. In this study, we investigate pressure effect on the helix-coil transition for the 20-residue alaine-based peptide using infrared spectroscopy. Firstly, the thermally induced decrease of helical content was observed by circular dichroism. The amide I' infrared absorption at 1633 cm^{-1}, which is due to the α-helical structure, simultaneously decreased. Secondary, as increasing pressure, the infrared absorption at 1633 cm^{-1} increased. This indicates that the α-helix is stable under high pressure. Furthermore, the peak of amide I' band shifted to lower frequency with increasing pressure. This agrees with the simulation result. Hence, both results strongly support that the amide hydration is accelerated by pressure.

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P057

RAMAN STUDY OF CONFORMATIONAL EQUIBLIA OF THE DISULFIDE BRIDGES OF PROTEINS

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The disulfide bridge of proteins is one of the most important components for the structural stability of proteins. It is known that the conformational equilibra of the disulfide bridge can be observed by Raman spectroscopy [1]. However, there have been few physicochemical studies of the conformational equibrium of the disulfide bridge of protein due to the experimental difficulty. In this work, the effects of temperature on the conformational equibria of lysozyme and insulin in aqueous solution were studied by Raman spectroscopy. Lysozyme has four disulfide bridges. Insulin has three disulfide bridges. Stretching vibrational mode of –S-S- group formed by the cross-linking of two –SH groups is sensitive to conformation and occurs in the region 490-540 cm^{-1}. Raman bands observed at 510, 525, 540 cm^{-1} are assigned [1] to *gauche-gauche-gauche* (GGG), *trans-gauche-gauche* (TGG) and *trans-gauche-trans* (TGT) conformer of C-S-S-C. The enthalpy differences between TGG and GGG were determined from the temperature dependences of the intensities of the corresponding S-S stretching bands, which provide strong intensity and well-resolved peaks. The enthalpy difference of lysozyme and insulin in aqueous solution was 2.3 ± 1.3 kJ/mol and -6.1 ± 4.8 kJ/mol, respectively. On the other hand, the enthalpy difference of diethyl disulfide, which is a model compound of disulfide bridge, in neat liquid was previously reported to be -1.1 ± 0.3 kJ/mol [2]. Furthermore, we also report the conformational equibria of the disulfide bridges of crystallized proteins and denaturing agent added proteins.

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P058

Variable pressure NMR reveals conformational fluctuation around cavities in a protein: A cavity mutant of T4 lysozyme

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We have carried out $^{15}N/^{1}H$ TROSY-HSQC experiments on wild type (WT) and the cavity-creating mutant (L99A) of T4 lysozyme (pH6.0) at pressures between 3 and 300 MPa at 25℃. In L99A, the mutation replacing Ala with larger Leu creates a large cavity (atomic defect) containing one or more water molecules in the hydrophobic core part of T4 lysozyme. With increasing pressure, some cross-peak intensities of L99A are reduced preferentially for residues surrounding the cavity, while the rest of the cross-peaks remain intact except for the pressure-induced chemical shifts. Under the same condition, no cross peak intensities are lost for the wide type, either. These results indicate that L99A has excited-state conformers with disordered polypeptide chain surrounding the cavity under physiological condition, whose population increases with pressure because of their lower volume. The cavity-originated higher-energy conformers detected earlier indirectly in the ^{15}N spin relaxation dispersion experiment [1] [2] is very likely to be the same type of conformers detected directly in the present high pressure NMR experiment.

The fact that the preferential reduction of cross peaks in L99A is not accompanied by the appearance of “denatured” signals suggests that the disordered segments are multi-conformational and undergo slow fluctuations in millisecond ~ microsecond time range. A recent high-pressure crystallography study revealed that the number of water molecules within the cavity of L99A increases considerably with increasing pressure [3]. It is likely that the slow conformational fluctuations around the cavity of L99A detected in the high pressure NMR experiment is the phenomenon associated with the dynamics of water penetration into the cavity.

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The energy landscape of BTK SH3 domain revealed by variable pressure NMR

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The Src homology 3 (SH3) domain mediates protein–protein interactions in intracellular signal transduction. We have studied conformational fluctuations of bulton's tyrosine kinase (BTK) SH3 domain using variable-pressure NMR spectroscopy in the pressure range 0.1-300 MPa at 15 °C, 25 °C and 37 °C. $^{15}N/^{1}H$ HSQC spectral changes are fully reversible with pressure. The analysis of the spectra shows that the SH3 domain fluctuates among four conformers; two folded conformers (N_1 and N_2), a locally disordered conformer I and the unfolded conformer U. The structures of N_1 and N_2 differ mainly in the orientation of the Trp251 ring, although their overall folds are similar as previously reported [Hansson et al. *Biochemistry* **37**, 2912-2924 (1998)]. Conformer I is unique in that it is disordered preferentially around the large isolated cavity of the protein close to its ligand-binding site, suggesting that conformer I may be directly involved in the intracellular signal transduction. From the experiments, relative free energy levels as well as relative partial molar volumes have been determined for all these conformers, which reveal an approximate free energy landscape of the SH3 domain under a closely physiological condition.

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P060

Variable pressure NMR study on multiple conformational states of OspA, a vaccine candidate for Lyme disease

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Outer surface protein A (OspA, 31kDa) from Borrelia burgdorgeri has been shown to be involved in the transmission of Lyme disease from the host tick to uninfected mammal. A vaccine based on a recombinant form of OspA was shown to be partially effective but with unwanted side effects. The proposed mechanism of attachment to the tick gut involves a high energy conformation of OspA. To prepare a better vaccine, it is necessary to study the structure and dynamics of this high energy conformation. Variable pressure NMR allows one to investigate both structure and thermodynamics of the high energy conformer by increasing its equilibrium population at high pressure, but at neutral pH and ambient temperature. OspA is of particular interest in this respect, as it has a peculiar single-layer β-sheet structure. In the ^{1}H-NMR spectrum, the intensity of the upfield-shifted methyl signal of V199 exhibited a clearly bi-phasic but reversible transition with pressure, indicating the presence of the secondary native conformation and unfolding intermediate partially unfolded in the C-terminal side of the protein. The structure of this intermediate was examined in detail by carrying out two dimensional ^{15}N/^{1}H-TROSY experiments at varying pressure.

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Non-native structure of α-lactalbumin-fatty acid complex as an apoptosis induction factor

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Alpha-lactalbumin (α-LA), second major protein of milk whey, takes a part in the components of lactose synthase in the lactating mammary gland, while an interesting feature has been reported that a complex of human apo-α-LA and oleic acid induces apoptosis in tumor cells but spares mature cells [1]. The complex adopts a molten globule-like state at neutral pH. It was described that active complexes are only converted in an anion exchange column with ready-prepared oleic acid and recovered with high salt. Moreover, the other mammal α-LA becomes less efficient in conversion.

We have tried to produce the active complex in process of denaturation of α-LA by heat or pH treatment for the purpose of high efficient conversion from bovine α-LA (BLA). As the result, it was achieved by heat treatment over 50°C. The BLA-oleic acid complex induced tumor-cell death as well as that reported previously. Furthermore, it is implied that complexes showed different activity in treatment temperature, incubation time, and species between bovine and human. This difference is thought to come from α-LA's thermal stability in presence of oleic acid. Then, quantitative analyses were performed in correlation between structural stability and cell-death activity of those complexes for recombinant and authentic α-LA using CD spectroscopy and apoptosis assay. Recombinant α-LA has N-terminal methionine that destabilizes native state. Transition temperature of holo-BLA in phosphate-buffered saline was 47°C for RBLA and 57°C for WBLA (Fig. 1, upper). RBLA complex showed stronger apoptotic activity against L1210 cells than WBLA (Fig. 1, lower).

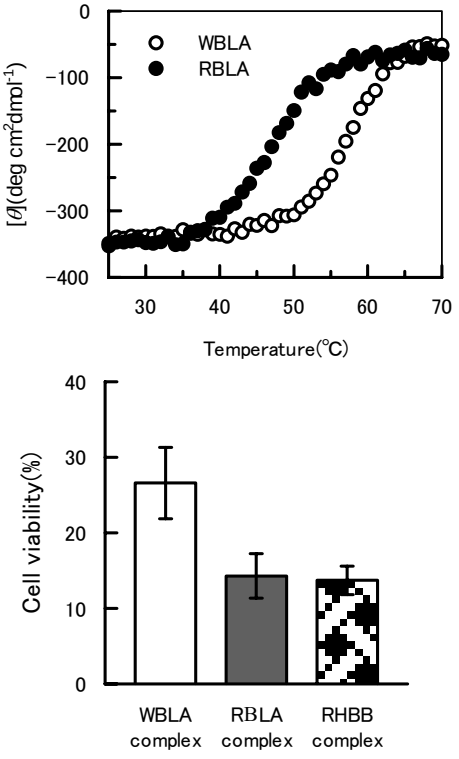

Fig. 1 Temperature dependency of near CD band of holo-BLA , authentic (WBLA) and recombinant (RBLA) (upper) and cell viability of BLA-oleic acid complexes (lower).

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P062

Construction of stabilized lysozyme against spontaneous deamidation

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Asparaginyl deamidation is one of the common non-enzymatic degradation of proteins and peptides, introducing a negative charge by transforming an asparaginyl residue into an aspartic or isoaspartic acid residue. It requires deprotonation of the backbone amide and hydrolysis of the intermediate. Since it undergoes spontaneously and irreversibly in neutral-basic solution, charge heterogeneity can be accumulated during purification, preservation and experiments. If the sample protein is labile to deamidation, control of experimental conditions or site-directed mutation are recommended to prevent deamidation.

Canine milk lysozyme (CML), a useful sample for protein folding study, has exhibited charge heterogeneity after sample preparation. We have found that four asparaginyl residues in CML deamidated under mild condition, pH 8.0 and 30°C, during 2-8 days incubation. The high reactivity of CML indicates that the deamidation can easily occur during experiments involving incubation. Substitution of these asparaginyl residues to glutaminyl residues prevented deamidation effectively. Although a glutaminyl residue can undergo deamidation as well as an asparaginyl residue, glutaminyl deamidation is much slower and must not be detected in the experiments here. Besides the four residues, one asparaginyl residue that did not deamidate in the native state was labile to deamidation in the unfolded state. Because preparation of lysozyme by *E. coli* expression requires denaturing processes, we constructed the quintuple mutant CML that has no deamidation site in the native and unfolded state. The structure formation of the mutant was confirmed and the thermostrability was slightly decreased. The overall conformation of this mutant was equivalent to that of wild-type in the native state. The most interesting character of the quintuple mutant CML, the molten globule formation, was observed as well as wild-type CML. Therefore, the mutant without labile asparaginyl residues will enable more precise analyses.

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EXCAVATION OF NOVEL PEPTIDYL-PROLYL ISOMEASES ON THE BASIS OF INFORMATION ACQUIRED BY COMPREHENSIVE MUTATIONAL ANALYSIS

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Peptidyl-prolyl isomease (PPIase) activity is a well-known function performed by a large number of proteins belonging to the PPIase family, e.g., cyclophilin, FKBP, and parvulin. The reaction mechanism of PPIase function, however, has not been fully elucidated. We focused on the common active site of the PPIase function, and then investigated a minimal condition required for the activity by comprehensive mutational analysis. As a result, we found that only the main-chain configuration of the amino acid residues constituting a small binding pocket was enough to show the activity[1]. This result implied that the traditional classification for the PPIase family, where a specific domain constituted of ~100 sequential residues was annotated as the functional unit of PPIase activity, was improper to comprehend all proteins possessing PPIase activity.

In the present study, we tentatively made up a new strategy on the basis of information acquired by the comprehensive mutational analysis in order to judge whether a protein has PPIase activity. Applying the method to all proteins registered in the Protein Data Bank, various non-PPIase proteins as well as known PPIases were picked up. Some of these non-PPIase proteins indeed showed the PPIase activity. This suggests that a protein, even if annotated as non-PPIase, may have PPIase activity and we have to reconsider what the PPIase activity is.

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P064

Counterion Condensation and Self-Condensation of Single Polyelectrolytes

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For a highly charged polyelectrolyte, counterions are known to condense onto the polyelectrolyte. Moreover, under certain conditions, the polyelectrolyte itself condenses. For example, a single DNA, which is a highly charged semiflexible polyelectrolyte, condenses into a compact structure such as a toroidal structure in the presence of multivalent cations.[1] From molecular dynamics simulations of semiflexible polyelectrolytes, it has been reported that single polyelectrolytes self-condense into toroidal or rod structures when the counterion valence is equal to or larger than three.[2] In contrast, the present authors have confirmed that the self-condensation can occur independent of the counterion valence if the electrostatic interaction is sufficiently strong.[3]

In this study, the relation between the the counterion condensation and the conditions for the self-condensation of single polyelectrolytes is studied by performing molecular dynamics simulations of a single flexible polyelectrolyte. We consider a system consisting of a polyelectrolyte with N_m monomers and N_c counterions. The polyelectrolyte is assumed to be flexible and represented by a bead-spring model. The charge of each monomer is $-e < 0$ and that of each counterion is $z_c e > 0$. We choose $N_m = z_c N_c$ so that the charge neutrality condition for the system holds. The nondimensional parameter $\lambda_M = e^2/(\varepsilon a k_B T)$, which is called the Manning ratio, describes the strength of the electrostatic interaction relative to the thermal energy. Here, ε, a, k_B and T are the dielectric constant of the solvent, the distance between two consecutive monomers, the Boltzmann constant and the temperature of the system, respectively. Note that $\lambda_M = 0$ corresponds to the case of the neutral polymer. The simulations are performed for various values of ε at a constant temperature T by using the Langevin-type equations of motion, where $N_m = 24, 48$ and 96 and $z_c = 1, 2, 3$ and 4.

As λ_M is increased from zero, the mean square average $\langle R_e^2 \rangle$ of the end-to-end distance of the polyelectrolyte first increases and then decreases after reaching a maximum value. The first increase is due to the increase of the strength of the repulsive electrostatic interaction between the monomers of a single polyelectrolyte. Screening of this repulsive interaction by the counterions condensed onto the polyelectrolyte causes the decrease in $\langle R_e^2 \rangle$. For large values of λ_M, $\langle R_e^2 \rangle$ is smaller than that for $\lambda_M = 0$, which signals the self-condensation of the polyelectrolyte. The self-condensation of the polyelectrolyte implies the existence of an effective attractive interaction among the monomers. If the repulsive interaction between the monomers is counterbalanced with the effective attractive interaction, the polyelectrolyte is expected to behave as an ideal chain, where the ratio of $\langle R_e^2 \rangle$ to the the mean square average $\langle R_g^2 \rangle$ of the radius of gyration of the polyelectrolyte becomes 6. By determining the onset of the self-condensation of the polyelectrolyte by the condition $\langle R_e^2 \rangle / \langle R_e^2 \rangle = 6$, it is found that the self-condensation occurs when about 90% of the charge of the polyelectrolyte is neutralized by the condensed counterions, which agrees with the experimental fact.[1] It is also found that $\langle R_e^2 \rangle$ takes its maximum value when about 13% of the charge of the polyelectrolyte is neutralized.

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OPTICAL CONTROL OF PROTEIN—PEPTIDE COMPLEX FORMATION USING A PHOTOCLEAVABLE CYCLIC PEPTIDE: RECOGNITION BY PHOSPHATIDYLINOSITOL 3-KINASE SH3 DOMAIN

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We have introduced photocleavable molecules into proteins to study the mechanism of protein folding, where the native protein structure was produced instantly by irradiation of light to the modified protein. In this research, we applied this technique to study protein molecular recognition, and a new photocleavable molecule was introduced into the peptide which interacts with a protein. A system to optically control the formation of the protein—peptide complex was constructed. We made a photocleavable cyclic peptide, which could not adjust its structure to form a sufficient complex with the protein. This peptide immediately converted to a linear flexible form from a cyclic stiffened form by light irradiation. After the light irradiation, the peptide started to form a tight complex with the protein.

Interaction between the SH3 domain of phosphatidylinositol 3-kinase (PI3K) and its proline-rich peptide ligand, (RKLPPRPSK, RLP1) is studied. PI3K is a signal transmission protein in a cell. A new peptide (CRKLPPRPSKC, C-RLP1-C), in which cysteins were introduced into both the N- and C-terminals of RLP1 was used. To make a cyclic peptide, the cysteins were bridged by a new photocleavable reagent, 2,5-bis(bromomethyl)nitrobenzene.

The modified peptide was purified by reversed phase HPLC. The molecular weight of the peptide was confirmed to be m/z = 1473.76 by the MALDI-TOF MS spectrum. It was in agreement with the calculated mass (m/z = 1473.75) of the cyclic peptide (modC-RLP1-C). In addition, no SH group was detected from the modified C-RLP1-C by the Ellman's test, which demonstrated that cysteins at both ends were modified. By these results, it was confirmed that a new photocleavable cyclic peptide was obtained. By UV-irradiation on the SH3 domain in the presence of modC-RLP1-C, the intensity of the CD band derived from the aromatic amino acids of the protein increased and that derived from α and 3_{10} helices decreased. These changes in the CD spectrum were similar to those obtained for the SH3 domain by the interaction with the RLP1. These results show that the present method would be a useful tool to optically control molecular interaction between a protein and a peptide.

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P066

Pressure effect on photoreaction dynamics of Photoactive Yellow Protein

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Thermodynamic properties, such as the partial molar volume, enthalpy, heat capacity and so on, are very important in researches of proteins to characterize the nature of states. Although it was impossible to measure these quantities for short lived species, we have developed a method to measure them in time domains by using transient grating (TG) method. Indeed, we have revealed ΔV, ΔH, $\Delta\alpha$, ΔC_p of reaction intermediates of various proteins so far. For example, this technique has been applied to the reaction of photoactive yellow protein (PYP), which is a water-soluble photo-response protein functioning the negative phototaxis in *Ectothiorhodospira halophila*. PYP has a chromophore, p-hydroxycinnamyl. Upon photoexcitation, the ground state species (pG) is converted into a red-shifted intermediate (pR_1) within less than 2 ns. Subsequently, pR_1 is converted into pR_2 without the spectrum change. The pR_2 species decays on the sub-millisecond time scale into blue shifted intermediate (pB'), which is converted to pB without spectrum change. This pB finally returns to pG in seconds time scale. Some of the thermodynamic properties of PYP intermediates were measured by Takeshita et al. [1, 2, 3]. However, some of them, in particular those related with the pressure could not be determined, because of experimental difficulty. Nevertheless, information of the pressure effect on the structure and dynamics should be essential for the biological function. To overcome this limitation, we are now trying to develop a TG system applicable to various pressures. We have already observed the TG signal of PYP at high pressures and found that the signal of PYP exhibited interesting pressure dependence. We also observed pressure dependent reaction rates in the process of pB' to pB. We are going to present and discuss these results in detail.

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Kinetic measurement of photoinduced dimerization process of BLUF protein YcgF

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Sensor proteins of blue light using Flavin Adenin Denucleotide (FAD) are members of a blue-light receptor family called BLUF protein. The photo-dynamic behavior of the BLUF proteins has been attracting considerable attention recently. The *Escherichia coli* YcgF is a fusion BLUF protein consisting of the N-terminal FAD-binding hold (BLUF domain) and the C-terminal glutamate-alanine-leucine (EAL) domain. The EAL domain of YcgF is predicted to have a cyclic-di-GMP phosphodiesterase activity. Although the BLUF domain absorbs blue light to start photo-reaction and then interacts with EAL domain to transmit the light signal to a lower stream, there was no evidence for demonstrating this mechanism so far. In order to understand the light sensing mechanism, it is necessary to detect the global conformational change of protein and/or the change of interaction between proteins in time-domain. However, it is generally difficult to trace reaction dynamics, which is spectrally silent. In this study, we studied photo-induced structural change of the YcgF from the view point of volume change as well as diffusion coefficient change in time-domain by using the pulsed laser-induced transient grating (TG) method.

After the photoexcittion of YcgF, the observed TG signal exhibits the thermal grating signal due to the heat released from the excited state and dynamics of two phases of the species grating with time constants of 13 μs and 2ms. These dynamics are assigned to the conformational change of BLUF region, and the global reaction of BLUF and EAL region, respectively. Interestingly, we observed a significant diffusion change associated with the 2ms reaction and the rate constant increased in proportion to the concentration of the protein. This concentration dependence indicates that the time dependent D is caused by the dimerization reaction of the YcgF. We suggested that the conformational change that causes the dimerization formation occurs with a time constant of 13 μs. We will discuss the mechanisms of signal transduction on the basis of these results. The dynamic detection from a view point of diffusion will give new insight of the photochemistry of this protein.

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P068

A NEW INSIGHT TO THE EXCITED STATE PHOTOCHEMISTRY OF BLUF DOMAIN OF AppA BY DIFFERENT PHOTOTHERMAL METHODS

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AppA is a novel blue-light receptor and function as a negative repressor of photosynthetic gene expression via interaction with its noncovalently bound partner protein, PpsR [1]. Although AppA mediated signal transduction is rather well understood, its initial photochemistry and signal transduction pathway still remain unexplored. Therefore, we have investigated AppA (BLUF domain of AppA, $AppA_{5\text{-}125}$) after the blue light activation by time-resolved transient grating (TG) method and transient lens (TrL) methods. The concentration dependency TG signals (Fig.1) indicate that in the excited state AppA form dimer in several millisecond time-scales depending upon concentration. As AppA already exist as a dimer in the ground state, this dimerization reaction indicates that AppA form tetramer in the signaling state.

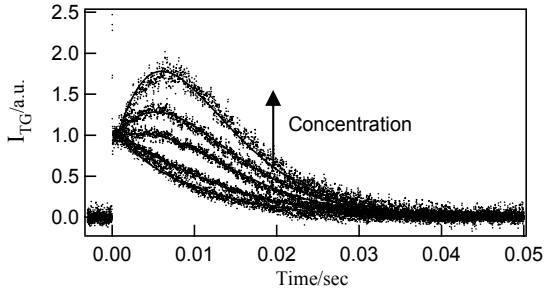

Fig.1: Concentration dependency TG signals of AppA at $q^2=1.3\times10^{12}$ m^{-2}.

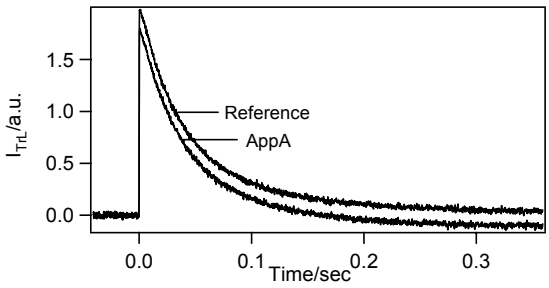

Fig.2: Typical TrL signals of reference sample and AppA at 298K.

We have also measured the enthalpy change (ΔH) associated with the formation of the first intermediate (formed in < 1 ns time-scale after excitation [2]) by the TG method. The ΔH value of this intermediate was ~40 (±10) kJ/mole at 25^0C. This ΔH value depends on temperature and this temperature dependence indicates positive heat capacity change (ΔC_p) during the formation of this intermediate. This positive ΔC_p value (1.4 (±0.28) kJ/mol/K) suggests that the hydrophobic surface area of this protein is exposed at this intermediate and this exposure of hydrophobic surface to the solvent may be the driving force for the subsequent tetramer formation. Moreover, we have also measured the ΔH associated with the tetramer formation reaction by the TrL method (Fig.2) and we have noticed that ΔH for this tetramer formation reaction decreases with increasing temperature. From the analysis we found that ΔC_p change for this tetramer formation reaction is negative ($\Phi\Delta C_p$= -1.5 (±0.25) kJ/mol/K). Generally, the negative ΔC_p indicates burial of hydrophobic surface from the solvent. Thus it indicates that hydrophobic surface, which is exposed to the solvent by photoexcitation of AppA, is prevented from solvent through protein-protein interaction by the tetramer formation.

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A method to detect conformational dynamics of Calmodulin induced by calcium ion

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Calmodulin is a well-known calcium binding protein. We investigated the conformational dynamics of Calmodulin by the transient grating (TG) method. For detecting the dynamics in time domain by the TG method, we use a caged calcium (Caged-Ca^{2+}) to trigger the reaction.

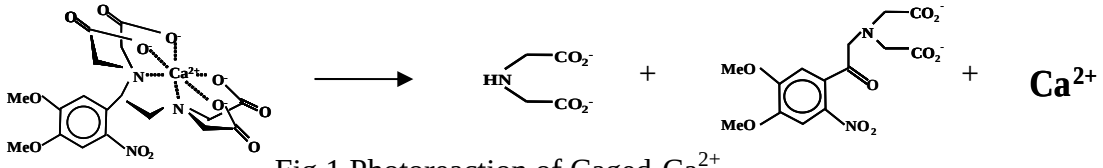

Fig.1 Photoreaction of Caged-Ca^{2+}

i) Photoreaction of Caged-Ca^{2+}

After photo-excitation of the Caged-Ca^{2+}, a TG signal that exhibits several phases was observed (Fig. 2(a)). The signal was analyzed in terms of the dissociation reaction (k_1= 20μs and k_2=200μs) of the Caged-Ca^{2+} and subsequent molecular diffusion ($D = 5\times10^{-10}m^2s^{-1}$).

ii) Detection of conformational dynamics of Calmodulin

The TG signal showed dramatic change, when Calmodulin was added to the solution (Fig. 2(b)). In particular, a remarkable change is appearance of rise and decay components in the last stage of the signal. These components reflect the molecular diffusion process after photoexcitation. From the rate constant, we determined the diffusion coefficient ($D = 1.0\times10^{-10}m^2s^{-1}$). This value clearly showed the diffusion of Calmodulin (Mw = 17kDa). Hence, we now know that the conformation of Calmodulin is changed by the Ca^{2+} release from the caged compound. We examined the q-dependence of the signal intensity and profile in detail and analyzed these signals. We concluded that a phase with a time constant of 40μs was observed in the signal. We consider that this phase reflects the interaction between Calmodulin and Ca^{2+}. We are now analyzing q-dependence of the signals to obtain information on the conformational change.

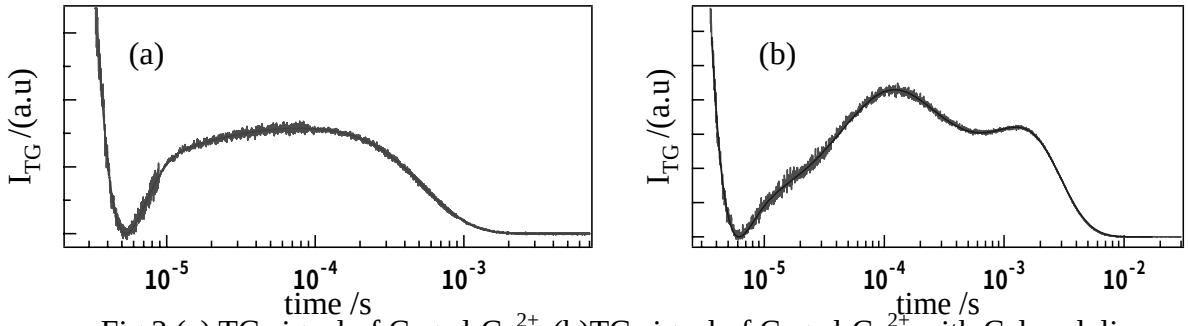

Fig.2 (a) TG signal of Caged-Ca^{2+}. (b)TG signal of Caged-Ca^{2+} with Calmodulin.

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P070

TIME-RESOLVED THERMODYNAMICS ON PHOTOCHEMICAL INTERMEDIATES OF PHOTOTROPIN ASSOCIATED WITH CONFORMATIONAL CHANGE

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Phototropin is a plant light sensor protein which controls phototropism, stomatal opening, and chloroplast relocation. When phototropin is illuminated with blue light, a chromophore FMN (flavin mononucleotide) absorbs the light and it activates a Ser/Thr kinase that is located at the C-terminal halves. At the ground state, FMN has no binding with the protein part. Upon blue light illumination, a covalent bond is formed between FMN and the protein within 4 μs. According to an X-ray crystallographic analysis, this photo-induced reaction is accompanied only by a change around the chromophore. However, a larger conformational change is expected for inducing the biological signal. Therefore, a study of conformational change in aqueous solution is necessary. Previously, by using the diffusion measurement in time-domain, we have observed a significant reduction of the diffusion coefficient for the LOV2 domain with the linker, but not for the LOV2 domain without the linker. The result was interpreted in terms of the unfolding of the α-helices in the linker region, which increases the intermolecular interaction between the protein and water.

In this study, we focused our attention on the thermodynamical properties of the short lived intermediates of the phototropin. In particular, we measured the enthalpy change, the heat capacity change, the volume change, and the thermal expansion coefficient change during the photo-cycle of LOV2 and LOV2-linker sample by using the transient lens method and the transient grating method. For the LOV2 sample, a spectrally silent dynamics with a lifetime of 10ms was found in the transient lens signal. This dynamics was attributed to the volume expansion and/or enthalpy relaxation processes. For the LOV2-Linker sample, another new dynamics was observed. Furthermore, it was found that the enthalpy change for the formation of this new species was temperature dependent, which suggests that the heat capacity of this species is larger than that of the ground state. This indicates that the hydrophobic area is exposed at the final step of the photo-cycle. The results will be discussed in detail.

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Study on the transducer activation dynamics by sensory rhodopsin II

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The *pharaonis* sensoryrhodopsin II (NpSRII, *pharaonis* phoborhodopsin) is a photoreceptor discovered in the cell membrane of *Natronomonas pharaonis*. This protein absorbs the blue-green light and initiates the signal transduction which eventually causes the movement of flagella motor to avoid blue-green visible and near-UV light. The conformational change of NpSRII triggered by absorbed photon affects the interaction between NpSRII and cognate transducer protein (NpHtrII) and activates NpHtrII to transmit the signal to the downstream proteins in the cytoplasm. The Schiff base of all-*trans* retinal which is the chromophore of NpSRII transfers proton to Asp75 within photocycle of NpSRII. This proton transfer was considered as the one of the main factors to change the protein conformation which activates NpHtrII. However, although the NpSRII D75N mutant lacks Asp75 and is incapable of proton transfer, the cell which possesses this rhodopsin showed the photophobic response upon light illumination. This result implies that the proton transfer is not a key event to activate NpHtrII and we need more molecular mechanical information to understand the mechanism of NpHtrII activation by NpSRII.

In this study, the interaction dynamics of D75N with NpHtrII was studied in time-domain by the transient grating (TG) method. As a model compound, we used the D75N-FP120 complex (fusion protein which consists of D75N and 120 residues of N-terminal region of NpHtrII connected to the C-terminal of D75N with linker peptide). We found that, D75N receptor has four intermediates (K_1-K_4) which have similar spectrum to each other on its photocycle and recovers to initial state in the same time scale of chromophore relaxation. On the other hand, the FP120-D75N complex has an additional intermediate Tr* after the decay of K_4. Based on the grating wavenumber q dependence of TG signal, we determined diffusion coefficients of K_4, Tr* and initial state of FP120-D75N. While D of Tr* and initial state was 9.5×10^{-11} m^2/s, D of K_4 was drastically smaller, 4.7×10^{-11} m^2/s. The origin of this D change, which is observed only in the case of fusion protein with NpHtrII, should be the structural change of the solvent accessible area of NpHtrII (HAMP domain). The HAMP domain is considered to play important role in signal transduction form transmembrane part to cytoplasmic domain and the observed structural change in this study should be related with this function.

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P072

Hydration Effects on Partial Molecular Volume of Proteins

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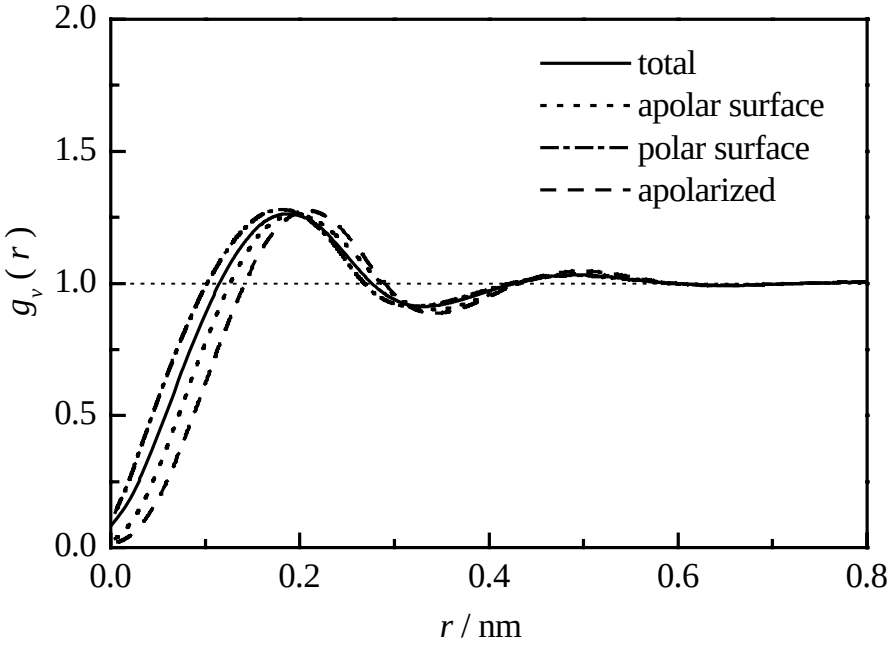
The partial molecular volume V_p of a protein is an observable thermodynamic quantity that provides information on its 3D structure and the spatial distribution of hydration water. To discuss protein structures in various conformational states based on the experimental value of V_p, it is necessary to estimate accurately the contribution of hydration water to V_p of proteins with arbitrary conformation. The purpose of this paper is to describe a method applicable to estimating V_p of proteins in either of the native and the nonnative states. We examined the spatial distribution of water around various solutes by MD simulation. The figure below shows the volume distribution function of lysozyme in aqueous solution. The result of simulation revealed that the space between nonpolar surface and water contributes significantly to V_p of solute and its thickness averaged over the molecular surface is about 0.06 nm. Especially, the contribution from this space is larger in the conformational state having the larger molecular surface such as the unfolded state. It was also found that the thickness of cavity is decreased by the electrostatic interaction between solute and solvent. In addition, the effect on V_p of electrostatic interactions between a polar group and water depends on the shape of the molecular surface where it is located. By taking into account these effects, it has become possible to estimate accurately V_p of molecules widely different in size and conformation. Based on the results of estimation, various structural models will be discussed for the nonnative states such as the fully unfolded and incompletely denatured intermediate states and so on.



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FT-IR STUDY ON MYOGLOBIN AGGREGATES

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Most of proteins are formed aggregates by physical conditions such as heat, high pressure, pH, and denaturants. The aggregates are well known to be the intermolecular β-sheet structure. Particularly some of them formed the amyloid fibrils in specific conditions. Amyloid fibrils are polypeptide aggregates which backbone is arranged in the specific twist type of β-sheet conformation with cross-β structure. They are associated with Alzheimer's disease, transmissible prion disorders, and so on. It is important to understand the molecular mechanism of the formation process of amyloid fibrils and what is reason to form them. The amyloid fibrils' formation is confirmed by the enhancement of fluorescence intensity of thioflavin T and direct observation by EM and AFM [1,2]. However the mechanism have not still solved at the present time.

In this study, we used holomyoglobin(HMB) and apomyoglobin(AMB) to explore the structure of the aggregates. These proteins have a similar three-dimensional structure with only α-helical content in the native state. AMB formed amyloid fibrils but HMB did not [2,3]. FT-IR spectroscopy is effective to study the secondary structure of protein aggregates. HMB formed the amorphous aggregates which have two strong absorption bands at 1618 cm^{-1} and 1685 cm^{-1} due to the antiparallel intermolecular β-sheet structure. However AMB formed amyloid fibrils which show the only one absorption band at 1685 cm^{-1}. Accompanying to increase the concentration of AMB, the new absorption at near 1618 cm^{-1} appears in addition to 1685 cm^{-1}, that is, the structure is the same as the HMB aggregates. We discuss about the structure of HMB and AMB aggregates.

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P074

The structure of insulin aggregates and the aggregation mechanism induced by the reduction of disulfide bonds

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Protein aggregation induces the major diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease, and Prion disease, which are neurodegenerative diseases in brain regions. Protein aggregation is relative to not only brain diseases but also cellular senescence. It is very important to clarify the structure and property of aggregation and the mechanism of protein aggregates. Many recent amyloid studies have revealed that various proteins form the amyloid fibrils under appropriate conditions [1]. Amyloid fibrils have the relatively rigid and the straight conformation consisting of characteristic cross β-sheet structures and grow up to highly twisted structure.

Insulin is the representative protein to study amyloidosis and/or the structure of amyloid fibrils. Amyloid fibrils of insulin are induced by the low pH and high temperature condition and/or in the presence of organic solvents and agitation [2]. On the other hand, it was found that the reduction of disulfide bonds in insulin induced aggregation [3]. The reduced aggregate of insulin is coursed by the accumulation of B-chain in insulin. Recently, we have focused the structure and functions of protein disulfide isomerase (PDI) which catalyzes the disulfide exchange reaction (oxidation, reduction, and isomerazation). In these results, we found PDI specifically accelerated to form reduced insulin aggregates. The structure of reduced insulin aggregates was based on the anti-parallel β-sheet by using FT-IR spectroscopy. We also tried to investigate the morphological image about the reduced insulin aggregates by AFM and the base structure of aggregation by using MALDI TOF-MASS spectrometer. We report here the mechanism and structure of reduced insulin aggregates promoted by PDI.

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Crystalization and dissolution of ubiquitin crystal under high pressure

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Although it has been generally accepted that conformational fluctuation plays a key role in protein function, actual changes in three dimensional structure associated with the putative motions have seldom been obtained experimentally at the atomic level. Recently, a large number of protein structures have been solved by solution and solid NMR spectroscopy and X-ray crystallography. They mainly revealed basic folded conformers, so called “native conformer”, of proteins, however, leaving the knowledge of high-energy conformers of proteins, such as alternative and locally disordered conformers. In order to collect such knowledge, we aim to crystallize high-energy conformers of proteins using pressure perturbation. Pressure causes a relative shift of populations of conformers with smaller volumes, which are usually high-energy conformers and hence rarely populated at atmospheric pressure (Akasaka, Chem. Rev. 2005). Here, we present our high pressure observation cell system and pressure effects on ubiquitin crystals. The observation cell having pressure resistive sapphire windows is connected to the pressure pump. The cell contains an inner cell and has resistance up to 200 MPa pressure.

Ubiquitin is a small (76 residues) and single domain protein. Recently, variable-pressure NMR revealed a presence of high-energy conformers of the protein under high pressure, namely alternative (N_2) and locally disordered conformers (I), which are fairly distinct structures from the basic folded conformer (N_1) (Kitahara et al., PNAS 2003, JMB 2005, 2006). These high-energy conformers are possible targets for crystallization. Our first approach is a pressurization of the crystal obtained at its native state condition, namely at pH 6.4, 20 °C and atmospheric pressure. The size of the crystal is ~ 500 μm, giving so far poor diffraction (~10 Å). The crystal was pressurized to 200 MPa, where in solution condition, N_2 is populated about 50 % and I less than 1% in equilibrium with N_1 (~50 %). Although it is not sure whether such thermodynamic equilibria are applicable for the protein in crystal or not, our observation showed that the crystal in its reservoir solution with 2 mM ubiquitin was intact from cracking and dissolution within 2 days. However, the crystal in 0.3mM ubiquitin solution mixed with the reservoir solution changed into less color at 200 MPa and few hours, indicating dissolution of the crystal. Then, the crystal again grew as pressure was decreased to 0.1 MPa. This shows that our observation system regulates crystallization and dissolution of the protein by pressure, enabling to study crystal growth of the single target crystal under different pressure.

High pressure macromolecular crystallography (HPMX) using diamond anvil cell is now a full-fledged technique (Fourme et al. 2001, 2006). The diamond anvil cell has in principle a higher pressure resistance than 1 GPa. We are going to make X-ray structural determination of the protein at different pressures in collaboration with Prof. Fourme.

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P076

BACTERIAL UNSATURATED GLUCURONYL HYDROLASE, GLYCOSAMINOGLYCAN-DEGRADING ENZYME, TRIGGERS HYDRATION OF VINYL ETHER GROUP BUT NOT OF GLYCOSIDIC BOND

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Unsaturated glucuronyl hydrolase (UGL) is a novel bacterial virulence factor responsible for degradation of glycosaminoglycan. The enzyme acts on unsaturated oligosaccharides produced by polysaccharide lyases and releases a 4-deoxy-L-threo-5-hexosulose-uronate derived from unsaturated glucuronic acid (ΔGlcA). Here, we show crystal structures of a mutant UGL and its complex with a substrate (Fig). Based on the structure of the enzyme-substrate complex and enzyme properties through site-directed mutagenesis together with the feature of the substrate, a novel reaction mechanism with a trigger of hydration of the vinyl ether group in ΔGlcA catalyzed by UGL is postulated. Asp^{149} acts as a general acid to protonate ΔGlcA C4 atom and also as a general base to deprotonate water molecule. The deprotonated water molecule attacks ΔGlcA C5 atom to yield unstable hemiketal, followed by spontaneous conversion of the aldehyde (4-deoxy-L-threo-5-hexosulose-uronate) and leaving saccharide through formation of hemiacetal and cleavage of glycosidic bond. This catalytic reaction mechanism quite differs from a generally accepted mechanism in glycosidases, suggesting that the design of specific inhibitors for UGL is promising for treating bacterial infectious diseases.

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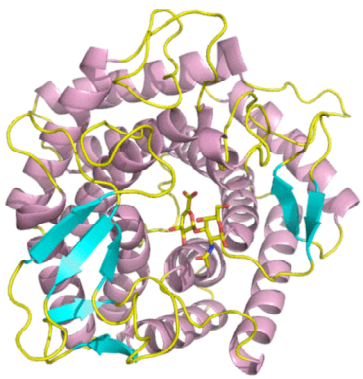

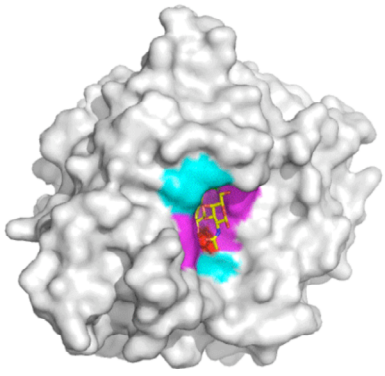

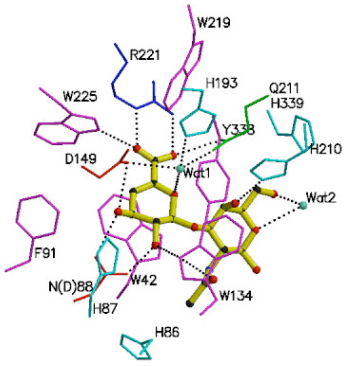

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PROMOTION OF COBALT-INCORPORAITON INTO THIOCYANATE HYDROLASE BY THE ACTIVATOR PROTEIN, P15K

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Thiocyanate hydrolase (SCNase) of *Thiobacillus thioparus* THI115 is the first enzyme in the bacterial degradation system of thiocyanate (SCN^-) and hydrolyzes SCN^- to carbonyl sulfide and ammonia. DNA sequences of the cloned genes revealed the close relation of SCNase to nitrile hydratase (NHase) including the NHase metal binding motif, C1-T(or S)-L-C2-S-C3. In NHases, C2 and C3 are post-translationally modified to cysteine sulfinic acid (Cys-SO_2H) and sulfenic acid (Cys-SOH), respectively. Recently, we have shown that SCNase contains one Co atom per $\alpha\beta\gamma$ hetero-trimer, and γCys131 (corresponding to C2 in the motif) is modified to Cys-SO_2H [1]. The results suggest that SCNase and NHases form a novel metalloprotein family having post-translationally modified cysteine ligands. In the present study, we developed the recombinant expression system of SCNase and found that the SCNase activator protein, which is likely to promote the incorporation of cobalt ions to SCNase protein [2].

When the genes for SCNase α, β and γ subunits were expressed in *Escherichia coli*, the subunits assembled to form a hetero-dodecamer, $(\alpha\beta\gamma)_4$, like native SCNase but exhibited no catalytic activity. Metal analysis indicated that the recombinant SCNase was expressed as an apo-form irrespective of the presence of cobalt in the medium. In general, NHase requires the co-expression of its specific activator protein encoded just downstream of its coding genes for the functional expression in recombinant systems. We searched the orfs located downstream of the SCNase genes, and the orf, *p15k*, was found as the candidate of the activator protein. The holo-form of SCNase was expressed when P15K was co-expressed in cobalt-enriched medium. P15K exhibited slight amino acid sequence homology with the SCNase β subunit, suggesting that P15K interact with α or γ subunits. Actually, P15K formed the complex with SCNase γ subunit tightly. We are characterizing the SCNaseγ-P15K complex. The latest results will be presented.

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P078

EFFECT OF pH ON ALLOSTERIC REGULATION OF HUMAN METHYLENETETRAHYDROFOLATE REDUCTASE

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Methylenetetrahydrofolate reducase (MTHFR) catalyzes the reduction of CH_2-H_4folate to form CH_3-H_4folate using NAD(P)H as a reducing agent. The methyl group of CH_3-H_4folate is used to form methionine by a methyl-transfer reaction catalyzed by methionine synthase. Mammalian MTHFR activity is inhibited by an allosteric regulator, *S*-adenosylmethionine (AdoMet). Thus, MTHFR is an important enzyme for regulating the flux of one-carbon units into methionine synthesis. Previous studies showed that mammalian MTHFR is a homodimer of ~70 kDa subunits, which can be divided into two domains, a catalytic domain with bound FAD at the N-terminus and a regulatory domain for AdoMet binding at the C-terminus. This enzyme could be a good model to understand how an allosteric factor regulates enzyme activity. A study of the *E. coli* MTHFR crystal structure revealed that the catalytic domain has an $\alpha_8\beta_8$ barrel structure and the isoalloxazine ring of the FAD cofactor sits in the center of the barrel. Based on a significant homology in the deduced amino acid sequences of *E. coli* and mammalian MTHFRs, the catalytic domain of mammalian MTHFR is probably also a barrel. However, some differences are known between prokaryotic and eukaryotic MTHFR. *E. coli* MTHFR is an NADH-dependent enzyme that is a homotetramer of smaller (33 kDa) subunits; therefore, it lacks sensitivity for AdoMet. At this moment, the topology of the regulatory domain is unknown. For allosteric regulation to occur, some signal deriving from the binding of AdoMet to the regulatory domain must be transmitted to the active site which must be located near the center of barrel. Despite the physiological importance and the enzymological interest of this allosteric regulation, there are few reports about the mechanism of allosteric regulation of mammalian MTHFR. We used recombinant human MTHFR to elucidate the mechanism. Human MTHFR, which contains 13 Trp resides in the regulatory domain, has ~10-fold higher protein fluorescence than *E. coli* enzyme. More than 20 % of the protein fluorescence is quenched by adding 20 μM AdoMet. We have also found that the quenching is slowed and accelerated at higher and lower pH, respectively. Furthermore, polypeptides obtained by limited proteolysis from human MTHFR in the presence of NADPH gave different fragment patterns than in the presence of AdoMet. These results suggest that major conformational changes happen upon AdoMet binding. These conformational changes may be needed for the allosteric regulation of human MTHFR by AdoMet.

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NEW APPROACHES TO DYNAMIC-FRACTAL WATER/PROTEIN STRUCTURES USING BROADBAND DIELECTRIC SPECTROSCOPY

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Broadband dielectric spectroscopy (BDS) was applied to complex systems consisting of water and biological molecules. Our original BDS measuring system offers physical pictures of structures and dynamics of the complex system in a wide time/frequency window from 5ps/30GHz up to 2Ms/1μHz. (Fig. 1)

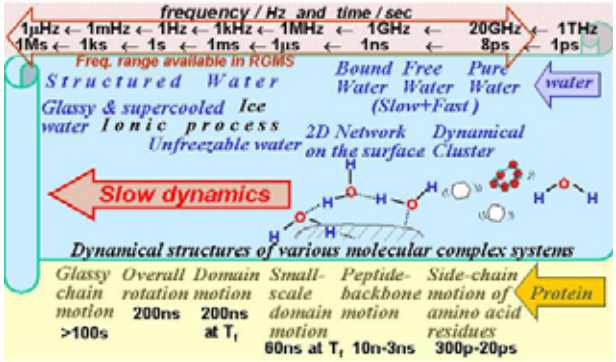

Fig.1. Slow dynamics of water and proteins observed by the BDS system.

Newer approaches to investigate dynamic behaviors of water/protein structures using BDS are suggested as follows:

1. Dynamic behaviors and the slow dynamics of dynamic clusters of water molecules are observed in wide time/frequency domain. Fractal behaviors [1] are obtained as a diagram of relaxation time vs. Cole-Cole parameter related to the distribution as shown in Fig. 2 for water structures and dynamics in aqueous systems. [2]
2. Dynamic behaviors in various time/length scales for proteins, such as overall, domain, and chain motions affected by water and ions in wide temperature range
3. Dynamic behaviors of unfreezable water, ice, and proteins in low temperature region including the glass transition temperature.

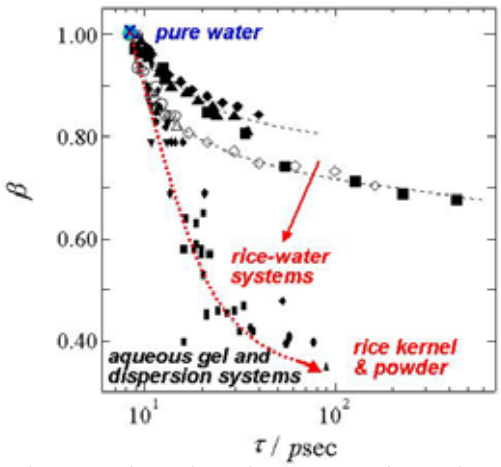

Fig.2. Relaxation time vs. Cole-Cole shape parameter diagram.

Typical results and explanations of dynamic behaviors with water structures are presented.

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P080

Hydration number of biomolecules revealed by dielectric constants in THz frequency region

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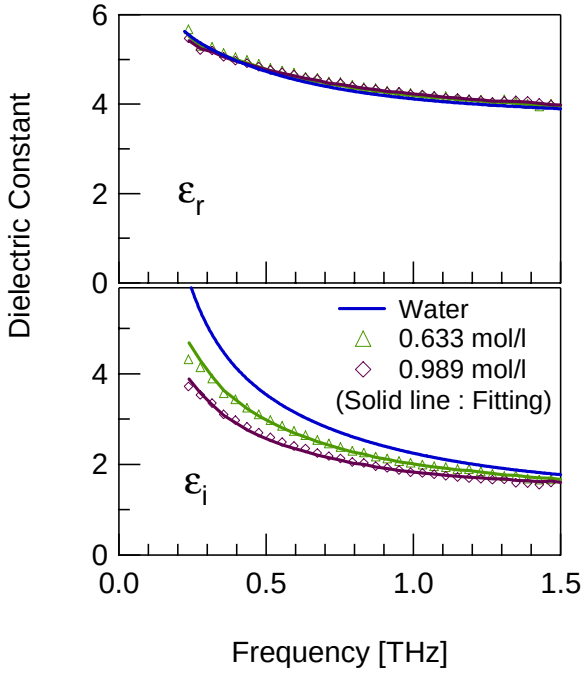
The hydrated water molecules bound to one biomolecule have been investigated intensively to recognize the role of water for macroscopic biomolecular motions. Here we preset a novel evaluation method of the hydration from dielectric properties in terahertz (THz) frequency region. Since water molecules with permanent dipole moment show dielectric relaxation with timescales of several picoseconds, the motion of water molecules is highly restricted when water molecules become strongly bound on the surface of biomolecule by hydrogen bonding. In THz frequency region where higher frequency tail of relaxational component appears in bulk water, hydrated water shows no relaxational polarizability. Therefore the decrement of relaxational polarizability can be considered to reflect the information of water molecules related to hydration.

We experimentally perform THz time-domain attenuated total reflection (TD-ATR) spectroscopy in several biomolecular solutions to determine dielectric constants exactly. Figure shows the dielectric constant of sucrose solutions with different concentrations. A solution with higher concentration has smaller imaginary part of dielectric constants because the number of hydrated water molecules which cannot respond to THz radiation is larger in solution with higher concentration. From this decrement of polarizability, we can evaluate the number of water molecules bound to one biomolecules using Onsager's local field correction. Thus we fitted real and imaginary part of dielectric constant of solution simultaneously changing unknown parameters of hydration number n_H, radius of hydrated water α_h and molecular polarizability of solute α_s. Best-fitted curves denoted by dashed curves are well reproduced by experimental results. Obtained parameters (n_H=17.4, $\alpha_h=0.81\alpha_w$, and $\alpha_s=12.1\times10^{-30}[m^3]$) are almost reasonable in comparing with other experimental results. This method will provide an easy way for the understanding of hydration of biological systems.



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Evaluation of the degree of water structure breaking effect by microwave dielectric spectroscopy

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[Background] Water molecules around proteins are classified into several categories such as bound water, non-freezing water, bulk water, etc. We have been developing an evaluation method of dielectric property of water around solute by microwave dielectric spectroscopy to extract dielectric property of hydration water, such as dielectric relaxation frequency :fc, dielectric relaxation amplitude :δ, and volume fraction of hydration water:φ based on Wagner mixture theory and Debye functions. [1] But this method had some problems. Since the boundary between the bulk water and the hydration water layer is not necessarily clear, fc and δ are determined on average depending on φ. In addition, the number of hydration water calculated from φ cannot be compared among different solutes, since fc and δ are different from one to the other. This time we improved the analysis method to solve these problems.

[Results] The dielectric relaxation study of NaI (water structure breaker) and NaF (water structure maker) aqueous solution were measured at 10-20C and calculated dielectric properties of molecules around these salts. Fig.1 is the imaginary dielectric spectra of NaI solution at various φ(10C). When φ is sufficiently large, peak top representing fc is close to that of bulk water (12.6GHz), because many bulk water molecules are in the boundary of large φ. With decreasing φ, fc increased. This means that with decreasing bulk water ratio, in other words with increasing hydration water ratio, average rotational mobility of water molecules in φ increases. Therefore, hydration water around NaI have high rotational mobility than that of bulk water. Assuming that NaI affect 10% of water molecules (φ=0.1), fc is 16.1GHz.

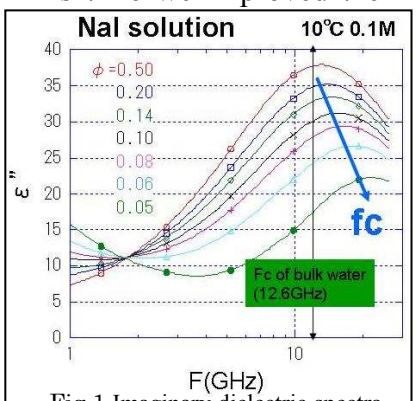

Fig.1 Imaginary dielectric spectra of NaI solution at various ϕ

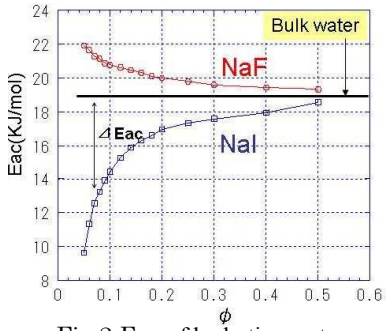

Fig.2 Eac of hydration water molecules at various ϕ

Fig.2 shows the relation between ln(fc)TR and φ of NaI and NaF solutions calculated by the Arrhenius plot analysis. Therefore these plots represent the average activation energy of hydration water molecules at φ (E_{ac}) with respect to the rotational mobility. When φ was large, E_{ac} was close to that of bulk water(19.1kJ/M). As decreasing φ, the property of hydration water is magnified. In the case of NaI solution (structure breakers) E_{ac} decreased with decreasing φ, while E_{ac} of NaF solution (structure maker) increased. We think this value may correspond to the hydrogen bond energy to be broken. Therefore the product of difference (ΔE_{ac}) between E_{ac}s of bulk water and salt solution and the moles number (N) of water molecules in φ should be the total change of hydrogen bond number by the solute. The product $\Delta E_{ac}N$ became constant -254±1.3 (S.E.) KJ/mol for NaI and 91±2.1 KJ/mol for NaF at all φ respectively. Thus, we can evaluate degree of water structure breaking or making around solute.

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P082

FLUORESCENT SPECTROSCOPY OF RIBOFLAVIN IN POLYELECTROLYTE SOLUTIONS

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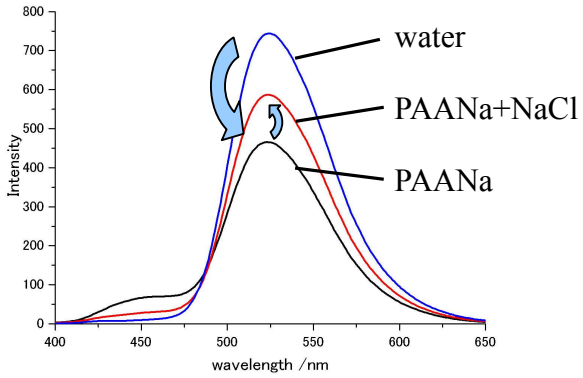
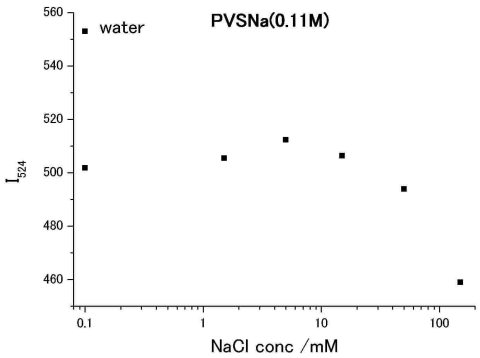
Water molecules around large halogen ions and highly charged polymers such as sodium polyacrylate(PAANa) were found to have higher dielectric relaxation frequency than that of bulk water. We call this hyper-mobile water(HMW).[1] However dielectric method requires a fair amount of sample and with low time resolution. Therefore we focused our attention on the fluorescence spectra of riboflavin(RF) as a fluorescent probe and examined the correlation between fluorescence intensity and the solute properties such as the electric field around those solutes, hydration properties including HMW.

We observed a suppression of RF-fluorescence in PAANa solution forming HMW. Since it is thought that HMW is formed in an electric field of sufficient strength around charged polymers, we measured the RF-fluorescence spectra for different NaCl concentrations expecting electrostatic screening effect. As a result the RF-fluorescence intensity increased by adding NaCl.

As in the case of PAANa solution, a decrease of the peak intensity of RF-fluorescence at 524nm (I_{524}) was observed in poly(vinylsulfonic acid) sodium salt(PVSNa) solution. When adding NaCl to the PVSNa solution, the I_{524} increased below 5mM NaCl, and above 5mM NaCl I_{524} decreased because of the suppression effect of NaCl on I_{524}.

These results show strong correlation between the RF-fluorescence spectra and the electric field around solutes.





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EFFECT OF POTASSIUM IODIDE AND CHONDROITIN SULFATE FORMING HYPER-MOBILE WATER ON THE VISCOSITY OF POLY(VINYL ALCOHOL) HYDROGELS

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Effect of salvation of polymers on the osmotic pressure is a general interest in polymer science and biological materials. To evaluate the polymer-solvent interaction to the osmotic pressure, we measured the swelling ratio of poly(vinyl alcohol) (PVA) hydrogels swollen in aqueous solutions of potassium halide at different temperatures. KI and KBr are known to break hydrogen bond networks among water molecules[1][2]. Fig.1 shows that swelling ratio of gels becomes larger for larger ions, such as I^-, in other words, caotropic ions, which form hyper-mobile water(HMW)[2] The amount of HMW also increases along with the order of Hofmeister series. For the estimation of strength of solute-solvent interaction, we use Flory's equation.

Second, we carried out viscoelasticity measurements (initial strain: 5% of gel thickness, vibration amplitude: 2.5%, frequency range 5~60Hz) for gels (1.PVA-chondroitin sulfate(CS); PVA:10wt%, CS:3,5wt%, 2.PVA-potassium polyvinyl sulfate(PVSK); PVA:10wt%, PVSK:0.15~0.25M, 3.PVA in potassium iodide(KI) solutions;PVA:10wt%, KI:0~1M). The value of viscosity and elasticity were calculated based on a Vogt model.

In the PVA-CS and -PVSK gels, we observed lower viscosity than that of gels including pullulan or poly(ethylene glycol) as references. In PVA gels in KI solutions, we found that the viscosity became lower with increasing of KI concentration. These results correlate with the property of hyper-mobile water.

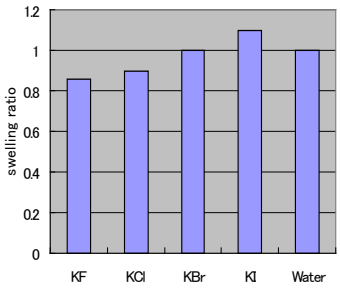

Fig.1 Swelling ratio of gels

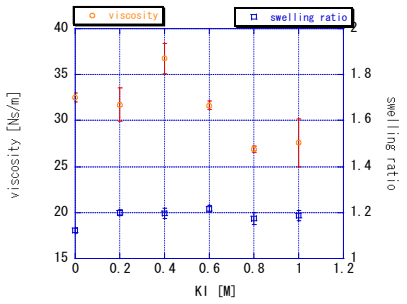

Fig.2 Viscosity and swelling ratio of PVA in KI solutions

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P084

Effect of Magnesium Ions On the Hydration Property of Poly(2-Acrylamide-2-Methyl Propane Sulfonic Acid

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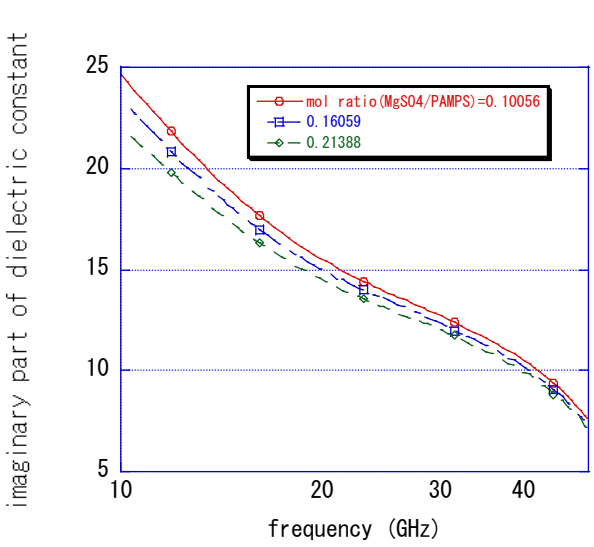
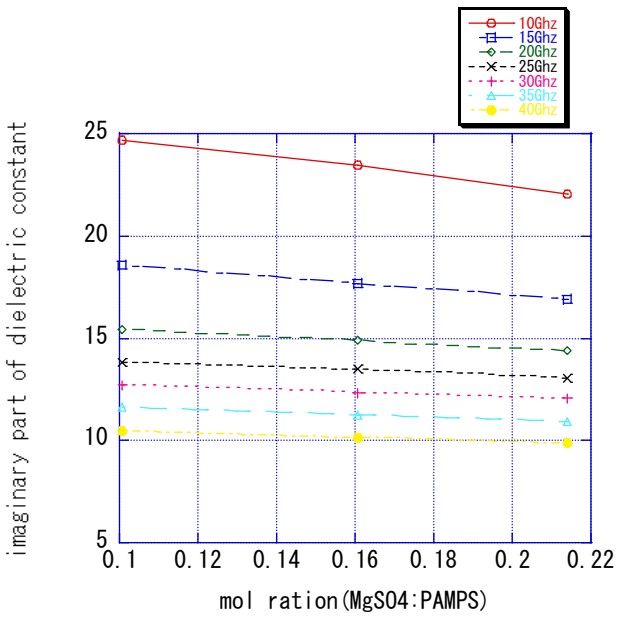
Recently we developed a microwave dielectric technique to measure the relaxation spectra of hydrated solutes to determine the relaxation frequency(fc) and dielectric loss. From our previous studies, In KI, KBr, poly-(acryl acid)Na (PAANa),and Poly-(2-acrylamide-2-metyl propane sulfonic acid) (PAMPS) solution, we found water named hyper mobile water (HMW) which has higher dielectric relaxation frequency than that of bulk water,17GHz. It suggested electric field of sufficient strength formed around ions or charged groups breaks the hydrogen bond network among water molecules, it is thought to be the origin of HMW. In this study, we measured dielectric relaxation of PAMPS solution as charged polymer at with different $MgSO_4$ concentrations. As a result, over 10 GHz, it was found imaginary dielectric constant decrease with increasing $MgSO_4$ concentration, indicating that the amount of HMW decrease with increasing $MgSO_4$. this result suggests that Mg^{2+} neutralized sulfonic groups' negative charge, while SO_4^{2-} ion don't generate HMW because of its strong hydration feature.





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PROTON DIFFUSION COEFFICIENTS AND DIELECTRIC RELAXATION FREQUENCY OF WATER AROUND ACTIN FILAMENTS

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In the previous studies, we showed a presence of hyper- mobile water(HM W) ,which has higher rotational mobility than that of bulk water around actin filaments. [1] Since it is thought that HMW is formed in an electric field of sufficient strength ,we expected that amount of HMW should decease by addition of salt due to the electro static screening. In this study we carry out two periments.

(1) By microwave dielectric spectroscopy(DS), we obtained the partial dielectric spectra of F-actin solutions in buffer A(Tris base 2mM,$CaCl_2$ 0.1mM,ATP 0.1mM,DTT 1mM,and KCl **50**mM) and buffer B(Tris base 2mM,$CaCl_2$ 0.1mM,ATP 0.1mM,DTT 1mM,and KCl **200**mM). shows fixed ϕ spectra for 0.05M KCl and 0.2M KCl.As 0.2M KCl condition the real part of dielectric constant was higher than that in 0.05M KCl condition in the 1 to 10GHz range, while the imaginary part increased at17GHz , indicating clear loss of HMW component , which has low dielectric constant than the bulk water.(Please see P081)

(2)The proton diffusion coefficient (D_H) in F-actin solution prepared by Spudich and Watt method was measured by Palused-field gradient spin echo (PFGSE) ^{1}H NMR at 25℃. Surprisingly, at low KCl concentration, D_H in F-actin was apparently higher than that in the buffer solution in spite of high polymer (actin) concentration. D_H decreased with KCl concentration below 150mM and then increased, while in the buffer solution, D_H increased monotonically with KCl concentration. The result can be explained by the reduction of hyper mobile water around F-actin by addition of KCl of 150mM. Thus, both DS and NMR results are consistent.

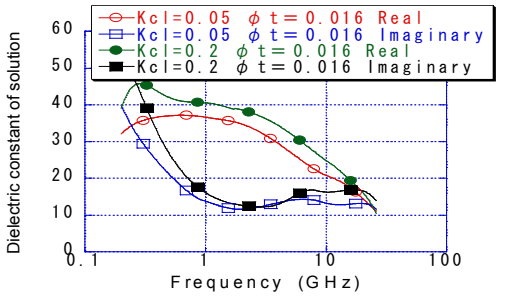
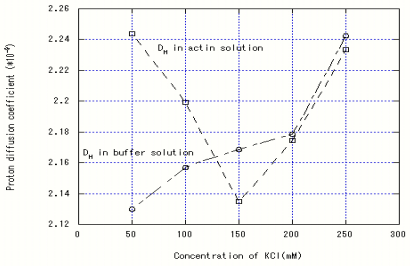


Fig.1 Dielectric constant vs Frequency



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P086

Glycerol induces lysozyme in non-native states to recover the native-like conformation – Effects of glycerol on kinetics of regeneration of disulfide bridges in disulfide-deficient variants of lysozyme

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We have studied the detailed structure of disulfide-deficient variants of lysozyme in order to elucidate the folding path of protein. Intact lysozyme has four disulfide bridges, and some of them were removed by amino acid substitution of Ala for Cys. Folded structures of 3SS-variants were demonstrated to be substantially the same as that of wild type. Among 2SS-variants, 2SS(1+2) with Cys6-Cys127 and Cys30-Cys115 had a native-like solid structure in the α-domain but a significantly flexible one in the β-domain, while 2SS(3+4) with Cys64-Cys80 and Cys-76-Cys94 was unstructured in whole. We are currently studying the conformation of 2SS(1+3) variant with Cys6-Cys127 and Cys64-Cys80, because it was found to recover near-UV CD spectra similar to those of wild type in the presence of 30 % glycerol. We have extensively studied amide H-D exchange reaction of 2SS(1+3) in the solution containing 30% glycerol at pH3.0 and 4° C by using NMR spectroscopy. As a result, L8-R14 in A-helix, W28-F34 in B-helix and G54-S60 in β3-strand were significantly protected against the H-D exchange reaction. In addition, some residues were partly protected in the region of C-helix and 3_{10}-helix in the β-domain. In order to demonstrate that these protected regions arise from the native-like conformation of lysozyme, we carried out disulfide regeneration experiments for 2SS(1+3) variant and showed effects of glycerol on them. Kinetics of the regeneration of disulfide bridges within 2SS(1+3) were followed under various experimental conditions at 0-3.0 mM GSH, and 0.1-1.0 mM GSSG, in the absence and presence of 30% glycerol. Fully reduced 2SS(1+3) disappeared with the formation of 1SS and/or 2SS intermediates of lysozyme, but its rate constant did not depend on the presence of glycerol. This means that glycerol does not influence the initial stage of disulfide regeneration. However, the yield of $2SS^N$ derivative with wild type disulfide bridges [Cys6-Cys127, Cys64-Cys80] significantly increased in the presence of glycerol under the condition of 1mM or 3mM GSH. On the other hand, under the condition of 0 mM GSH and 0.1 mM GSSG., $2SS^{non}$ derivatives with non-native disulfide bridges accumulated in both the absence and presence of glycerol. These data suggest that glycerol preferentially stabilize $2SS^N$ derivative more than intermediates with non-native disulfide bridges. Therefore, in the presence of GSH, the yield of $2SS^N$ increased due to its stabilization by glycerol.

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ON THE ROLE OF SOLVENT ENVIRONMENT IN ALCOHOL-INDUCED α-HELIX FORMATION OF MODEL PEPTIDES

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It has recently been found that alcohol-water mixtures undergo structure transition from tetrahedral-like water network to zigzag chain-like alcohol structure at specific alcohol concentrations with increasing concentration of amphiphilic alcohols [1-4]. It has also been found that a structural transition of peptides and proteins takes place from the random coil conformation to a helical state in alcohol-water mixtures [5]. Although alcohol-induced protein folding has been studied for a long time, the underlying mechanism has not yet been fully understood at the molecular level.

In the present study, the role of solvent environment in alcohol-induced α-helix formation of model peptides is discussed in view of the structure of aqueous solutions of alcohols: methanol (MeOH), ethanol (EtOH), 2,2,2-trifluoroethanol (TFE), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). The model peptides consist of 10 and 17 amino-acid residues (GLRDFIEKFK and KGRTFRNEKELRDFIEK) fragments of 28 amino-acid residues, Full Sequence Design-1 (PDB ID:1FSD). From CD results on the alcohol concentration dependence of molar ellipticity values at 222 nm in α-helix formation of the peptides, it has been found that the order of α-helix formation is consistent with that of solvent structural transition (HFIP>TFE>EtOH>MeOH); the result suggests that a structural transition of the peptides is strongly associated with a solvent clusters. Furthermore, we performed Replica Exchange MD (REMD) simulations and 2D-NMR measurements to reveal the details of solvent environment around the 10 residues fragment in the process of structure transition at the molecular level.

The REMD calculations have shown that the 10 residues fragment in ethanol-water mixtures at $\boldsymbol{x}$=0.1, 0.2, 0.3, 0.4 and 1.0 tends to have a helical state and that its structure in ethanol is more compact than those at other mole fractions of ethanol. We observed intermolecular interaction between Glu and terminal Lys from NOESY spectra in TFE-water mixture at $\boldsymbol{x}$=0.1, though, not in EtOH-water mixture at $\boldsymbol{x}$=0.4. Further analysis of the REMD and 2D-NMR data is now in progress.

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SINGLE MOLECULE IMAGING OF CHAPERONIN FUNCTIONS USING ZERO-MODE WAVEGUIDES

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In this study, we aim to investigate chaperonin functions. Chaperonin GroEL, which is composed of two rings, mediates protein folding while co-chaperonin GroES binds to each ring of GroEL alternatively. We have analyzed GroEL-GroES interactions at a single molecule level using a total internal reflection fluorescence microscopy (TIRFM), and revealed the important intermediate state in the chaperonin reaction cycle at 10 nM GroES concentration. However, it is still unclear whether there is other intermediate state such as GroES-GroEL-GroES complex because GroES exists at about 5 uM concentration in E.coli. In order to analyze chaperonin functions at the physiological condition, the illumination volume of 0.2 fL in TIRFM should be reduced to less than 1 aL. Arrays of zero-mode waveguides (ZMW), that is, nano-holes arrays lined up on a metal-clad quartz glass can reduce the illumination volume by three orders of magnitude than that by TIRFM.

After IC5- and biotin- labeled GroEL was immobilized via biotinylated BSA and streptavidin on ZMW, the solution containing 200 nM Cy3-GroES, 2 mM ATP, denatured protein as substrate was introduced on the quartz slide. On the position of the IC5-GroEL preassigned, Cy3-GroES appeared and disappeared again and again. The histogram of durations of on-time, the time from binding to release, indicated that GroES released from GroEL after two sequential steps, which agreed with previous studies. Next, we are analyzing about GroES-GroEL-GroES complex.

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P090

Direct observation of conformational dynamics of single biomolecules as sequential state transitions

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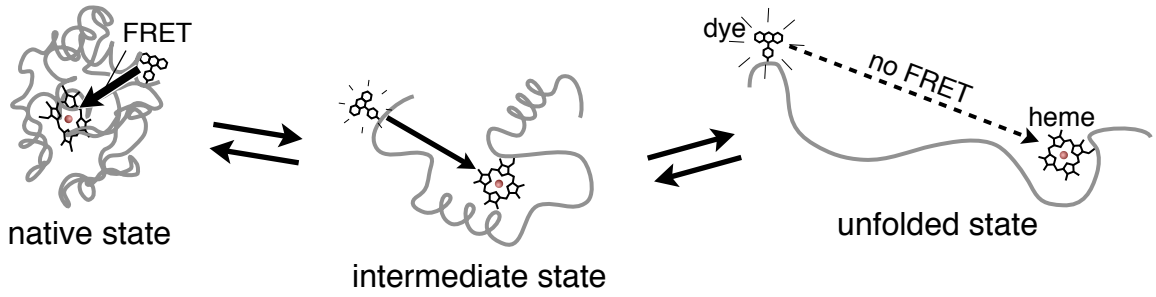
Since the biological molecules, such as proteins, lipids and nucleic acids, realize their functions by interactions with other molecules including solvent or other biomolecules, it is crucial to understand molecular dynamics for elucidating their functions and finally life itself. For example, folding process of cytochrome *c* (cyt *c*) protein has been investigated with a great deal of interests. The ensemble fluorescence measurements, which detects the conformational change by way of intramolecular FRET, revealed the existence of at least two intermediate states and the transition rates between them.

We investigate the conformational dynamics of cyt *c* using a single molecule technique, which can unveil information lost in ensemble averaging. Cyt *c* molecules can take one of several kinds of structure depending on the solvent condition. We investigated the conformational change of cyt *c* under the various solvent conditions. Especially under the equilibrium condition, molecules fluctuate between a compact (native or molten-globule) and a unfolded structure and intermediate states in unfolding-refolding process are expected to be observed. Structure change of dye-labeled cyt *c* can be detected through the fluorescence intensity change, since cyt *c* has a heme, which can quench fluorescence emission of a dye in its vicinity with fluorescence resonance energy transfer (FRET), and the FRET efficiency depends on the heme-dye distance.



We introduce a novel single molecule fluorescence detection system and statistical analysis, which is based on information theory. In order to analyze very weak signal from single molecules, we introduce an assumption that, at molecular level, dynamics can be regarded as sequential transitions between multiple molecular states. Each state is generally quite stable showing almost constant signal and jumps to another state instantaneously. Thus the information we have to obtain is which state the molecule stays in and the accurate time of transitions. We utilize a photon counting module and record the arrival time of every photon to the detector instead of the common photon counting scheme and applied the statistical analysis to obtained data. In our results, some intermediate states were resolved and the time resolution in the order of millisecond was achieved. Our technique realizes direct observation of transient intermediate states in fast conformational dynamics of single protein molecules.

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SINGLE MOLECULE FRET STUDY OF THE BACTERIAL IMMUNITY PROTEINS

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The bacterial immunity protein Im9 is a small 4-helical protein. Its thermodynamics and kinetics have been intensively investigated as a model for the folding of helical proteins by ensemble techniques. Im9 folds *via* two-state transition at neutral pH[1], but by reducing pH or introducing the mutations which stabilise non-native hydrophobic interactions, its folding mechanism can be switched to three state in which an on-pathway intermediate state is populated during folding[2,3].

Single molecule fluorescence detection has developed remarkably over the last decade and can now be applied to the detection of conformational changes of biomolecules such as proteins and nucleic acids[4,5].

We have characterised the urea-induced unfolding an Im9 variant (Im9S81C) using single molecule Förster resonance energy transfer (FRET) at neutral pH. The protein was labelled specifically with Alexa Fluor® 594 and 488 at Cys23 and Cys81. Urea titration of the doubly labelled protein showed that the population of the native species decreased and that of the denatured species increased with increasing urea concentration. No other species was detected at all urea concentrations, consistent with two-state unfolding. Interestingly, the mean proximity ratio, an indication of the relative distance between the dye pair, of the denatured species increased non-linearly as urea concentration decreased, suggesting the compaction of the denatured species at low urea concentrations. Similar results have obtained for other proteins using guanidium chloride[6-10]. The peak widths of the denatured species in mildly denaturing conditions were beyond those predicted from the shot noise effects, suggesting the presence of conformational heterogeneity within this ensemble or changes in the rate of chain reconfiguration. Temporal measurements on immobilised single molecules are now underway to directly investigate this heterogeneity.

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P092

EFFECT OF EXTERNAL TORQUE ON THE ROTATION OF F1-ATPASE MOTOR

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F_1-ATPase, a water-soluble portion of F_oF_1-ATP synthase, is a rotary motor in which the central γ-subunit rotates in the $\alpha_3\beta_3$ cylinder. The driving force of the rotation is the free energy of ATP hydrolysis and its entropy term corresponds to the absorption of heat from surroundings. Actually, under physiological conditions, half of the free energy change is of the entropic origin. It is interesting how such a motor behaves under external torque. Application of an external torque is important also to understand the energetics of the motor.

In the present study, we adopted a system of electrorotation (1) to exert constant torque on the rotating F_1-ATPase. In this system, rotating electric field induces dipole moment in the beads duplex attached to the γ subunit of F_1-ATPase and hence a torque is induced. The amplitude of the torque was estimated from the rotational Brownian motion of a control beads duplex and angular velocity induced by the rotating electric field.

Under ATP hydrolysis condition, external torque in the ATP hydrolysis direction accelerated the rotation proportionally to the applied torque. When external torque in the opposite direction was applied, stepping behavior became more evident and when external torque greater than some 30 pN nm was applied in the opposite direction, the rotation almost stopped on average but hardly rotated in the reverse direction. The observation suggests some rectifying mechanism in the F_1-ATPase (ref. 2). At that point of balance, we observed large fluctuation. The fluctuation may reflect the F_1-ATPase which takes over and back thermal energy from environment to try to achieve its function.

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PHOTO-CLEAVABLE MODIFICATION OF APOPLASTOCYANIN AND ITS PROTEIN FOLDING

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We developed a new method to investigate the protein folding based on a pulsed laser light triggering method and a unique detection method. For triggering the folding reaction, a denaturant is photodissociated from a protein by a pulsed-laser. For monitoring the folding process, the time developments of the molecular volume change as well as the diffusion coefficient, which are difficult to detect by conventional spectroscopy, are monitored by the transient grating (TG) method. The side chain of the cysteine residue of apoplastocyanin (apoPC) was site-specifically modified with a 4,5-dimethoxy-2-nitrobenzyl derivative, where the CD and 2D NMR spectra showed that the modified apoPC was unfolded. The substituent was cleaved with a rate of about 400 ns by photoirradiation, which was monitored by the disappearance of the absorption band at 355 nm and the increase in the transient grating signal. After a sufficient time from the photocleavage reaction, the CD and NMR spectra showed that the native β-sheet structure was recovered. Protein folding dynamics was monitored in the time-domain with the transient grating method from a viewpoint of the molecular volume change and the diffusion coefficient, both of which reflect the global structural change including the protein–water interaction. The observed volume decrease of apoPC with a time scale of 270 μs is ascribed to the initial hydrophobic collapse. The increase in the diffusion coefficient (23 ms) is considered to indicate a change from an intermolecular to an intramolecular hydrogen bonding network.

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P094

Direct time-resolved measurement of the enthalpy-, heat capacity- and volume-changes along apo-plastocyanin folding by transient grating method

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Proteins can form their unique native conformations from unfolded structures. Molecular mechanisms of this protein folding reaction have been one of central topics in a biophysical field. A number of previous researches suggested that hydrophobic collapse plays very important role in the protein folding, in particular for β-sheet rich proteins. However, studies on the folding process of all-β-sheet proteins have been very scarce. Furthermore, although time-resolved measurements are essential for understanding protein folding reactions, it has been rather difficult to trace time-dependences of a variety of properties associating with the protein folding with a fast time resolution. Recently, Hirota et al.[1] succeeded in preparing chemically-modified apoplastocyanin (apoPc), which dominantly consists of the β-sheets, to trigger the folding process by the photo-dissociation of 4,5-dimethoxy-2-nitrobenzyl group upon laser light irradiation. Using this protein, it was found that apoPc folds via two intermediates with reaction time constants of 270μs (volume contraction) and 25ms (diffusion coefficient change) at 298K. The first volume contraction reaction may suggest the hydrophobic collapse. However, detailed properties of the intermediates have not been clear.

In this study, we investigated the enthalpy change (ΔH) and heat capacity change (ΔCp) during the folding process to obtain thermodynamic evidence for the existence of the hydrophobic collapse by the transient grating (TG) method, which can detect enthalpy change of a short-lived intermediate. For the measurement of longer lived intermediates, we used the transient lens method, which has a capability to detect the thermal energy in a longer time region.

From the TG signals after photoexcitation of apoPC and a calorimetric standard sample, we determined ΔH of –64 and –40 kJ/mol at 298K, and ΔCp of –9.0 and –8.5 kJ/(mol K) for the 270μs- and 25ms-dynamics, respectively. The negative ΔH indicates that this protein folding is driven by the entropy change (ΔS>0). The negative ΔCp and positive ΔS is the general feature of the hydrophobic collapse. The principle of the measurement and the folding mechanism will be discussed.

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Development of sheath-flow system to observe protein folding events at a single-molecule level

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Information on the energy landscape of protein folding obtained by conventional ensemble measurements was limited, because signals from individual molecules are obscured by the averaging. The direct observation of folding trajectories of single molecules should enable us to clarify details of the landscape such as heterogeneity of folding pathway, ruggedness of the landscape, and the time scale for sampling all conformations within individual states.

Recently, we succeeded in developing a single-molecule detection system that can observe the dynamics of proteins in a flow cell. The system enables us to observe fluorescence signals from freely-diffusing molecules in solution without the artifacts caused by cell surfaces and other molecules. However, a signal to noise ratio of the data obtained by the system was limited, which was partially caused by the low efficiency of the light collection of the camera lens. Although it is desirable to use a microscopic lens for the efficient collections of photons, the confinement of single molecules within a narrow focal depth of the microscopic lens was extremely difficult.

Here, we developed a new sheath-flow system to spatially focus molecules in a narrow stream formed inside of the capillary cell. The performance of the flow cell was tested by using the absorbance of bromocresol purple (BCP). The stream of BCP was focused to a narrow cross section with radius of 4 μm in the capillary of 100 μm i.d.. Thus, the system enabled the confinement of freely-diffusing proteins in solution within a narrow space. Furthermore, we succeeded in observing fluorescence from single Alexa 532 dye using the developed device. We are currently investigating the dynamics of proteins at the single-molecule level.

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THE NATIVE AND MOLTEN GLOBULE STATES OF GOAT α-LACTALBUMIN CHARACTERIZED BY THE NMR HYDROGEN-EXCHANGE TECHNIQUE

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α-Lactalbumin (α-LA) is a small globular protein and has been extensively used as a model protein for studying the mechanisms of protein folding. This protein exhibits the molten globule (MG) state under mildly denaturing conditions, which has been shown to be identical to the transient intermediate observed during the kinetic folding. The MG state of bovine and human α-LA has been characterized in terms of the structure and stability. In addition to the studies on bovine and human α-LA, the structure of the transition state of the folding of goat α-LA was characterized by using the Φ-value analysis at the residue level [1]. To further investigate the folding mechanism of goat α-LA, we incorporated the hydrogen exchange technique to characterize the structural details of the native and the MG states of this protein by NMR spectroscopy. The HSQC spectrum of ^{15}N-labeled goat α-LA was assigned by three-dimensional ^{15}N-edited TOCSY and ^{15}N-edited NOESY experiments. Based on the resonance assignments of goat α-LA, the rates of hydrogen exchange were measured by NMR spectroscopy at 25 °C in the native state at pH* 6.3 and in the MG state at pH* 2.0. The protection against hydrogen exchange was much weaker in the MG state than in the native state. Significant protection was observed in β-sheets involved within the β-domain and the C-helix located at the interface between α- and β-domains (protection factor > 10^5), in the native state, whereas significant protection was observed in the C-helix (protection factor ~ 10-15) in the MG state. The pattern of the protection factor was similar between bovine and goat α-LA, reflecting the similarity of the amino acid sequence between these proteins. These results and the Φ-value analysis support that goat α-LA follows the hierarchical mechanism of protein folding.

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THE EFFECT OF ADP ON THE KINETICS OF THE ATP-INDUCED ALLOSTERIC TRANSITION OF GroEL

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The chaperonin GroEL from *Escherichia coli* is a tetradecamic protein of 14 identical 57 kDa subunits arranged in two heptameric rings stacked back-to-back with a central cavity. The ATP-dependent control of the chaperone functions of GroEL is underpinned by two levels of allostery, one within each ring and the other between the rings. To study this intricate system, we have used a single ring variant of GroEL (SR1) in addition to wild-type GroEL. We investigated the ATP-induced allosteric transitions of single-tryptophan variants of SR1 (Y485W-SR1) and wild-type GroEL (Y485W-GroEL) by stopped-flow fluorescence measurement. In each case, the ATP concentration dependence of the rate constant of allosteric transition exhibits a multiple sigmoidal behavior. To explain this behavior, we have assumed that GroEL has a second ATP binding site besides the well-known first ATP binding site. This assumption leads to the idea that blocking ATP binding to the second nucleotide binding site brings about changes in the ATP concentration dependence of the rate constant of the allosteric transition. We thus investigated the ATP-induced allosteric transition of Y485W-SR1 and Y485W-GroEL in the presence of excessive amount of ADP as an inhibitor of ATP. Based on these results, we will discuss the possibility of the existence of the second nucleotide binding sites and the effect of inter-ring interactions.

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EFFECT OF THE CHAPERONIN GroEL/ES ON THE REFOLDING KINETICS OF A STAPHYLOCOCCAL NUCLEASE MUTANT

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It is well known that protein folding *in vivo* is not a spontaneous process but requires a series of helper proteins such as molecular chaperones. Among a variety of molecular chaperones, the chaperonin GroEL/ES from *Escherichia coli* is one of the best studied chaperones.

We have analyzed the effect of the chaperonin GroEL/ES on the refolding kinetics of a staphylococcal nuclease mutant (SNase(Pro-)A69T) by stopped-flow fluorescence measurement. The refolding kinetics of SNase in the absence of GroEL are represented by a three-phase exponential process which arises from parallel folding pathways. On the other hand, the refolding kinetics of SNase in the presence of GroEL are represented by a double-phase exponential process, and slower than the refolding kinetics in the absence of GroEL. In addition, the amplitude of each phase of SNase refolding kinetics in the absence and presence of GroEL is different from each other. This result indicates that unfolded SNase binds to GroEL, forms intermediates in the GroEL-bound state and then folds into the native state, and that GroEL binding changes the refolding pathway of SNase.

We performed stopped-flow double-jump measurement where GroEL was added after the kinetic partitioning of SNase refolding was occurred. By this measurement, we related each phase of SNase refolding kinetics in the presence of GroEL to that in the absence of GroEL. The amplitudes of this refolding curve were the same as those of the SNase refolding kinetics in the absence of GroEL. This result demonstrated how the rate constants of SNase refolding were affected by the binding of GroEL, and we confirmed directly that GroEL binding retarded protein folding.

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FOLDING KINETICS OF THE A90S VARIANT OF PROLINE - FREE STAPHYLOCOCCAL NUCLEASE

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Proline-free variants of staphylococcal nuclease (SNase; 149 amino acid residue) have played a significant role in investigating the folding mechanisms of SNase. They show the simpler folding kinetics than those of the wild type protein due to the lack of the slow folding process rate-limited by proline isomerization. We have used a prolin-free variant where all the six proline residues found in the wild type protein were replaced by Ala, Thr or Gly (P11A/P31A/P42A/P47T/P56A/P117G; Pro-). To further elucidate the mechanisms of the folding of SNase, we previously investigated the folding kinetics of several variants of Pro- (SNasePro- V66L, G88V, V66L/G88V, A69T) by stopped-flow fluorescence and demonstrated that these proteins reach the native state through parallel pathways with accumulating on-pathway folding intermediates. In the present study, we investigated folding kinetics of another variant of SNasePro-, SNasePro-A90S, by stopped-flow fluorescence. We measured the refolding kinetics induced by a pH jump from pH 2.1 to pH 6.4 as well as formation of the native state as a function of time by a double-jump protocol. Together with the results on the folding kinetics of the other variants obtained previously, we will discuss the folding mechanisms of SNase.

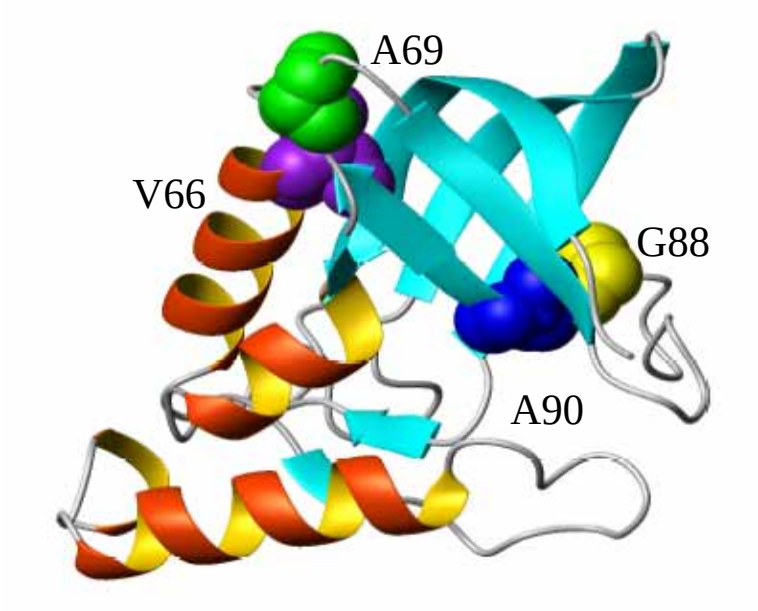

Figure. Staphylococcal nuclease and its mutation sites

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P100

Interactions responsible for early structures in the folding of equine β-lactoglobulin

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Equine β-lactoglobulin (ELG) forms a molten globule at an acidic pH (A state), which is known to be indistinguishable from a burst phase folding intermediate [1, 2]. ELG also forms an expanded and helical conformation at low temperatures (C state), which is a possible model of an earlier folding intermediate [3]. The structure of a single disulfide mutant C66A/C160A is similar to the A state in the presence of salts, while it is similar to the C state at low anion concentrations [4]. We have investigated the temperature-dependent change in the secondary structure using circular dichroism and proline scanning mutagenesis. At low anion concentrations, the helical content increased linearly as temperature decreased. In the presence of salts, the A state was cooperatively transformed into the C state at low temperatures. This suggests the importance of hydrophobic interactions for stabilizing the A state. Peptides encompassing native-like and nonnative α-helices were synthesized to investigate the interactions responsible for helix formation in the A and C states. These did not form stable helices, indicating that not only the helices in the A state but also the helices in the C state are stabilized by long-range interactions. A longer fragment, CHIBL, which encompasses the structured region in the A and C states, showed a helical structure. Proline-substituted mutants of CHIBL showed CD spectral changes similar to the corresponding mutants of the full-length protein in the C state. Therefore, CHIBL has a structure similar to the corresponding region of the full-length protein in the C state. This result indicates that interactions responsible for helix formation in the C state reside in the sequence of CHIBL, and that the sequences outside CHIBL are essential for secondary structure formation in the A state.

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Extraction of the Essential Regions of the Dihydrofolate Reductase sequence by Alanine Insertion Perturbation Analysis

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The extraction of the essential regions bearing information on structure formation is useful and effective to understand the principle of a protein architecture. Iwakura et al. succeeded to define such regions from dihydrofolate reductase (DHFR) with the circular permutation analysis. They called such regions as “folding elements” (FE), the circular permutation analysis was effective for DHFR, because the N- and C-termini are close to each other. However, in general, the two termini are not necessarily close to each other, and it is difficult to apply the method for various proteins. We demonstrated that the essential elements for the function and/or the foldability of staphylococcal nuclease can be extracted using the systematic alanine insertion analysis. In order to compare the two analytical methods, the alanine insertion analysis was performed upon DHFR.

We made the alanine insertion mutants in which an alanine is inserted into every interval on the sequence in the N-terminal region (1-65) of DHFR. The DHFR activity of each mutant was screened for resistance to TMP, a competitive inhibitor of DHFR. The mutants, 4A5, 27A28, 30A31 to 49A50, and 54A55 to 60A61 did not exhibit their DHFR activity, suggesting that these regions include essential information of the function and/or the foldability. It is interesting that these regions are closely related to the FEs. It can be proposed that the alanine insertion analysis provides similar information to circular permutation analysis, and is useful for the extraction of FEs without any restriction of the positions of the N- and C-termini. We will continue to examine particular information of the regions, by determining the enzyme activity and the secondary structure of the mutants.

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Elucidation of the unfolding-state and the folding process of the disulfide-bond introduced mutant of Staphylococcal nuclease

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Staphylococcal nuclease (SNase) is composed of two sub-domains, the N-terminal and the C-terminal sub-domain. Our previous studies demonstrate that the two sub-domains are connected by non-local interactions, and W140 is essential for the formation of the interaction to stabilize the native structure. We showed that the interaction is replaceable with the SS bond by I139C/Y54C. The folding kinetics of the SS mutant indicates that SNase is folded via parallel pathways and the non-local interaction is formed within the dead time of the stopped-flow CD (22ms).

In order to investigate the earlier folding event, the structures of the unfolding-states of the SS mutants and WT were examined by the solution X-ray scattering. The Guanidine Hydrochloride (GdHCl) concentration dependence of the folding kinetics for these proteins was also measured by the stopped-flow fluorescence (SF-FL) apparatus with the shorter dead time (2ms) than CD.

While the SS mutants and WT exhibited the same CD spectrum in the acidic condition, the scattering experiment showed that the tertiary structure of acid denatured state of the oxidized form was different from those of the reduced form and WT. From the SF-FL experiment, the rollover effects were observed under the lower GdHCl concentration for the 2nd phase and the 3rd phase of the oxidized form, as was seen in WT. The results suggest that, although the unfolding structure of the oxidized form forms the non-local interaction at the starting point due to the disulfide bond, the folding pathway observed in WT is also conserved in the SS mutant. Therefore, the non-local interaction of the intact SNase is formed at initial stage of the SNase folding.

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Relationship between pH-dependent conformational and stability changes of bovine β-lactoglobulin studied using NMR spectroscopy

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Bovine β-lactoglobulin (βLG) is a widely used model system in protein folding studies. Between pH 2 and pH 13, βLG exhibits a number of local and global structural transitions. βLG also shows pH-dependent stability change, which may be related to the structural change. Clarification of such relation is of biophysical importance and may have implications for protein folding.

To monitor the structural change of βLG at amino acid level, NMR measurements are performed. So far NMR measurements of βLG were limited to acidic pH because severe signal broadening caused by the monomer-dimer equilibrium occurs at neutral pH of the spectra. Thus, we prepared the βLG dimer mutant, A34C, which gave a spectrum of high quality compatible with a detailed NMR analysis.[1]

We performed pH titration and H/D exchange (H/D ex) experiments monitored by HSQC spectra. The principal component analysis (PCA) of pH titration data indicated that there are three transitions with pK_as of 3.0, 5.0, and 7.0, which is the same result as those reported previously. The result of PCA revealed which residues are responsible for each transition In addition, our H/D ex experiments suggested that the core regions of βLG molecule at different pH values are the same. Taking these results into consideration, the pathway of βLG folding does not change even pH changes. It is likely that the pH change causes slight change in ΔG between native and intermediate states. The relation between the stability change and the local conformational change will be discussed.

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Structural polymorphism of β_2-microglobulin amyloid fibrils induced by the addition of trifluoroethanol

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β_2-Microglobulin (β2-m), responsible for dialysis-related amyloidosis, forms amyloid fibrils *in vitro* under acidic conditions. They exhibit a highly organized cross-β core extended to almost 80% of the total sequence of the molecule, but little is known about the interactions achieved inside the core. To obtain insights into the architecture of fibrils, we have analyzed the effect of trifluoroethanol (TFE) on the structure of β2-m amyloid fibrils.

The formation of β2-m fibrils usually occurs only in the presence of seeds. However, it has recently been clarified that sonication induces fibril formation without seeds by promoting nucleation process [1]. When spontaneous fibrillation was induced by sonication in the presence of TFE at various concentrations, needle-like amyloid fibrils were formed up to 40% TFE. While all the formed fibrils exhibited similar far-UV CD spectra, they showed different patterns in tryptophan fluorescence spectrum, thioflavin T intensity and extension rate even under the same solvent condition, suggesting various fibril structures were produced depending on the concentration of TFE as a manifestation of polymorphism.

Interestingly, the fibrils formed at higher TFE concentrations exhibited significantly higher stability against guanidium hydrochloride. They also showed decreased sensitivity against pressurization. The exposure patterns of hydrophobic residues seemed to be perturbed depending on the concentration of TFE, as indicated by ANS binding, and it is thus suggested that the modulation of hydrophobic interactions is one of the major factor contributing to the production of polymorphic amyloid structures with different packing and stability.

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MICROSECOND HYDROPHOBIC COLLAPSE IN THE FOLDING OF *ESCHERICHIA COLI* DIHYDROFOLATE REDUCTASE, AN α/β-TYPE PROTEIN

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Using small-angle X-ray scattering combined with a continuous-flow mixing device, we monitored the microsecond compaction dynamics in the folding of *Escherichia coli* dihydrofolate reductase, an α/β-type protein. A significant collapse of the radius of gyration from 30 Å to 23.2 Å occurs within 300 μs after the initiation of refolding by a urea dilution jump. The subsequent folding after the major chain collapse occurs on a considerably longer time-scale. The protein folding trajectories constructed by comparing the development of the compactness and the secondary structure suggest that the specific hydrophobic collapse model rather than the framework model better explains the experimental observations. The folding trajectory of this α/β-type protein is located between those of α-helical and β-sheet proteins, suggesting that native topology determines the folding landscape.

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MICROSECOND FOLDING OF *ESCHERICHIA COLI* DIHYDROFOLATE REDUCTASE MONITORED BY TIME-RESOLVED FLUORESCENCE SPECTROSCOPY

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To monitor the kinetic refolding reaction of *Escherichia coli* dihydrofolate reductase (DHFR) with a microsecond time resolution, we used time-resolved fluorescence techniques combined with a continuous-flow mixing device. First, we measured the tryptophan excited-state decay kinetics along the folding reaction of DHFR by time-correlated single photon counting. The decay kinetics consists of two fluorescence lifetimes, indicating the presence of at least two classes of photophysical environments for the five tryptophans of DHFR. The photophysical environment corresponding to the longer lifetime, which may be attributable to Trp47 and Trp74, was in the unfolded state at 30 μs of the refolding, but the lifetime increased to 40% of the total change between the native and the unfolded state with a time constant of 540 μs. These results may suggest that the two tryptophans are susceptible to dynamic quenching by water molecules at 30 μs, but are partially buried inside the protein molecule in the subsequent folding phase.

In the second experiment, three mutants, each having one AEDANS label at Arg52, Asp87, or Gln146, were used for the FRET measurement from tryptophan donors to the AEDANS acceptor. The fluorescence decays of both tryptophans and AEDANS were measured along the folding reaction. Significant changes in the fluorescence properties were observed within 30 μs of the refolding, followed by a small increase in the FRET intensity with a time constant of 100 μs. These results support our previous SAXS studies showing the early compaction of DHFR within 300 μs of the refolding.

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ROLES OF CHAIN CONNECTIVITY IN THE ACQUISITION OF PROTEIN FOLDABILITY REVEALED BY SHUFFLING OF THE FOLDING ELEMENTS OF DHFR

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A "folding element (FE)" is a contiguous peptide segment crucial for a protein to be foldable and is a new concept that could assist in our understanding of the protein folding problem. To understand the roles of connectivity of the FEs in the acquisition of protein foldability, we performed block shuffling of the FEs of *Escherichia coli* dihydrofolate reductase (DHFR). The FEs were grouped into block A (FE1, FE2), block B (FE3 ~ FE5), and block C (FE6 ~ FE10), which were connected by Gly linkers with variable lengths from 3 to 13 residues. Foldable mutants were screened by the ability to bind an inhibitor, trimethoprim. We found that the mutants with the order of ABC, BCA, CAB, and BAC are foldable. The results indicate that the C-B connection makes the protein non-foldable, although a 13 Gly linker could compensate for the distance between blocks C and B. Thus, in addition to the presence of a complete set of the FEs, connectivity of the FEs is also an important factor in the acquisition of protein foldability.

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Polymerization and folding of neuroserpin: implications of the folding to the metastable state for serpinopathies

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Neuroserpin is a member of the serine protease inhibitor (serpin) super family that is predominantly secreted from neurons in the brain. The inhibitory function is expressed by a marked conformational transition "intra-molecular loop insertion", whereas this inherent molecular flexibility also results in polymerization of neuroserpin by the sequential linkage reaction "inter-molecular loop insertion". Neuroserpin polymers accumulate and form inclusion bodies within the endoplasmic reticulum of neurons, and they bring about a dementia Familial Encephalopathy with Neuroserpin Inclusion Bodies (FENIB). Four mutants of neuroserpin S49P, S52R, H338R and G392E are at present recognized to cause FENIB, and the peak ages of onset are 48, 24, 15 and 13 years, respectively. To investigate what is the key of the broadly different age of onset, we characterized wildtype neuroserpin (WT) and these four pathogenic mutants. In addition, a truncated mutant that lacks 10 amino acids at the C-terminus of WT (Δ_{10}WT) was also analyzed since C-terminally truncated mutants that lose polymerogenic properties have been found in other pathogenic serpins.

The rate of polymerization was directly related to the age of onset; the rate constants were G392E > H338R > S52R > S49P > WT ≈ Δ_{10}WT. In contrast, the thermostability in the native state was independent of the crisis; the melting temperatures were 56 ^{o}C for WT and Δ_{10}WT, 55 ^{o}C for S52R, 53 ^{o}C for G392E and 50 ^{o}C for S49P. The most remarkable result is that the refolding yield of the pathogenic mutants was extremely lower than that of WT. Moreover, the yield of Δ_{10}WT was over 50 % higher than that of WT. We therefore examined the refolding process of WT and the mutants. During the refolding of either WT or mutants, a compact intermediate that has about 60 % of secondary structure of the native protein was formed within 5 ms, and ANS binding to the intermediates was strong in order of G392E ≈ H338R > S52R > S49P > WT > Δ_{10}WT. Furthermore, in the refolding step from the intermediate to the native protein, the refolding rate of WT was faster than those of the pathogenic mutants but was slower than that of Δ_{10}WT. These results support the ideas: (1) Accumulation of neuroserpin in ER is likely to result from both polymerization of the native protein and aggregation of the folding intermediate. (2) The pathogenic point mutations result in an extended structure of the folding intermediate and slow folding, thereby misfolding was induced. (3) A structure near the C-terminus decelerates the folding after formation of the intermediate.

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P110

Cleaved ovalbumin mutant R339T refolds to a thermostable loop-inserted form *via* the metastable native structure

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Serine proteinase inhibitors (serpins) are believed to fold *in vivo* into a metastable native structure, and their inhibitory function is expressed by a transformation of the native protein to a thermostable loop-inserted form after receiving the P1-P1' cleavage by a target serine protease. To understand the unique folding and the remarkable conformational change, we investigated the refolding pathways of cleaved ovalbumin. Ovalbumin is a non-inhibitory serpin which lacks the ability to undergo the loop-insertion, hence the P1-P1' cleaved form stays a non-loop-inserted metastable structure. On the other hand, we have prepared and characterized a loop-insertion-competent ovalbumin mutant R339T that folds the metastable native structure but can transform to the thermostable loop-inserted form by the P1-P1' cleavage. We therefore examined the refolding process of the metastable cleaved form (cl-OVA) and the thermostable cleaved form (cl-R339T).

In the urea-denatured conditions, the cleaved proteins were completely dissociate to the heavy (Gly1-Ala351) and light (Ser352-Pro385) chains, and then the polypeptides were separated with a gel filtration column equilibrated with the denaturant. When the urea-denatured heavy chain from OVA or R339T was diluted with refolding buffer, immediately formed an initial burst refolding intermediate and stayed at the intermediate state during prolonged incubation. The intermediate produced from the heavy chain of OVA was indistinguishable from that of R339T by CD and fluorescence analyses. Adding the light chain, the intermediate from either OVA or R339T associated with the light chain and then refolded to metastable cl-OVA or thermostable cl-R339T, respectively. Furthermore, in the refolding pathways of cl-R339T, a pre-loop-inserted native like form was observed after reassociation of the heavy and light chains. These results support the idea that native serpins are in an intermediate state on the pathway of the folding to the thermostable loop-inserted structure. Serpins may be an instantial protein that is physiologically active in the intermediate state but inactive in the stable structure.

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Laser-induced temperature-jump study on the folding reaction of β-lactoglobulin

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A continuous-wave probed laser-induced temperature jump (cT-jump) system with advantages in simplicity and affordability has been constructed. This method was applied to monitor the changes in tryptophan fluorescence of the β-lactoglobulin during its folding; the kinetic phases were traced from 300 nsec to 10 msec after a temperature jump with a time resolution of 5 nsec. Notably, an early phase with typical squeezed-exponential characteristics, [$\exp\{-(kt)^{\beta}\}$, $\beta > 1.0$], was observed around several tens of microseconds after the temperature jump, which is actually the earliest phase ever observed for β-lactoglobulin. The squeezed-exponential phase has been detected only in the computer simulation. This process was theoretically analyzed according to a kinetic model suitable for sequential events occurring in one pathway, and it was demonstrated that this extremely early folding phase lacks distinct energy barriers and is considered to be the conformational shift comprised of approximately 20 sequential events occurring along one pathway, according to the proposed model. Therefore in the folding reaction of β-lactoglobulin, the conformational shift (U → U' transition) occurs in a successive manner prior to the non-native intermediate (I) formation.

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P112

The Role of C-terminal α-helix of pyrrolidone carboxyl peptidase from *P. furiosus* in folding and stability

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The refolding kinetics of cysteine-free pyrrolidone carboxyl peptidase (PCP-0SH) from a hyperthermophile, *Pyrococcus furiosus,* has been reported to be unusually slow in the acidic pH region [1]. The refolding process of PCP-0SH has been also observed using 2 D NMR in real time [2]. The denatured state of PCP-0SH can be trapped, which is the initial state (D_1 state) in the refolding process corresponding to heat denatured state but differs from the completely denatured state (D_2 state) in the concentrated denaturant. It is important to find the features of the denatured state for elucidating the mechanism of protein folding and stability. Therefore, to elucidate the structural basis of the denatured state of PCP-0SH under the physiological conditions, H/D exchange experiments in the D1 state were performed at pD 3.4 and 4 °C. HN protons in the C-terminal α6-helix region hardly exchanged to deuterium even after 24-hour incubation. The α6-helix (from S188 to E205) consists of 18 residues and the longest secondary structure region of PCP-0SH. In order to examine the role of α6-helix in folding and stability, Ala at position 199 in the α6-helix region was substituted by Pro that would be expected to affect the stability of α6-helix. H/D exchange experiments indicated that α6-helix region of A199P was partially unprotected in the D_1 state. On the other hand, some hydrophobic residues were protected from H/D exchange, which were not protected in the wild-type protein. Both of the stability and the refolding rate of A199P were also decreased by the mutation. This might be caused by that the D_1 state structure of A199P changed to temporally stable denatured state structure with non-native hydrophobic cluster, which might be induced by the partial destruction of α6-helix in the D_1 state. It is concluded that the C-terminal α6-helix of PCP-0SH is already constructed in the D_1 state and is necessary to correctly refold into the native structure and stabilize the native state.

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Stable α-helix-rich intermediate of single mutant A45G of src SH3

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We found that a single mutant of src SH3, A45G, took a stable α-helix-rich intermediate at acidic condition. This intermediate is identical to the intermediate that appeared on the folding pathway of wild-typed src SH3 as long as physicochemical properties are concerned. A45G takes fully native β-sheeted structure at pH6, but takes α-helix in 31 % at pH3.0. In contrast, wild-typed (WT) SH3 takes no α-helix at pH3 or pH6. The stable intermediate of A45G thus obtained was also investigated by x-ray solution scattering. Radius of gyrations were 18.39 Å, 17.37 Å and 15.38 Å at pH3.0, 4.0 and 6.0 respectively. Kratky plots of A45G show peaks at pH3.0, 4.0 and 6.0, though the peak was slightly shifted to the smaller h value at acidic pH.

Refolding experiment of A45G shows a α-helix-rich intermediate at pH6, as is the case with WT SH3. The amplitudes of circular dichroism of the kinetic intermediates were almost the same between A45G and WT SH3, while the folding rate became 9 times slower in A45G than that in WT. pH-jump experiment of A45G was performed from pH3.0 to pH6.0. This showed no bursts and the rate of refolding was exactly the same with the refolding rate of A45G at pH6.0.

It is concluded with these findings that the intermediate of A45G at pH3 is identical to the intermediate kinetically observed on the folding pathway of WT src SH3 as well as that of A45G.

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P114

Alpha-helix-rich intermediate in the early stage of protein folding

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We have studied folding process of proteins including α-helix-rich proteins (lambda repressor, apo-myoglobin, RNase A, lysozyme), β-structure-rich proteins (bovine and equine β-lactoglobulins, ubiquitin and FHA domain), and fully β-sheeted proteins (src SH3 and Fyn SH3). In all cases, we found that folding goes though at least two steps; appearance of a α-helix-rich burst followed by a resolvable folding process to the native state. This α-helical burst amplitudes of protein show good proportionality to the helical fraction calculated by helix2 program. This suggests that the initial event of protein folding is to form short unstable α-helix due to a rapid helix-coil transition, and then some α-helices are collapsed and stabilized by the interaction with each other. Such interaction leads to a compact state of the protein, which can further reorganize to the native structure.

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Early events of folding of lambda repressor investigated by cryo-stopped-flow method

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We did two kinds of kinetic refolding experiments of lambda repressor fragment (pseudo-wildtype (Y22W) and the mutant (Y22W, Q33Y, A37G, A49G)) in a stabilizing solvent, 45% by weight aqueous ethylene glycol at –28 ℃ by cryo-stopped-flow method. One kind experiment is circular dichroism at 222 nm (sensitive to helix content), and the other is small angle X-ray scattering (measuring the radius of gyration). The results show that refolding from guanidine hydrochloride denatured conditions exhibits very different time scales for collapse and secondary structure formation: the two processes become decoupled. Collapse remains a low-barrier activated process, while the fastest of several secondary structure formation time scales approaches the downhill folding limit.

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P116

Contribution of Disulfide Bond to the Stability and Redox Function of *Shewanella violacea* Cytochrome c_5

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Two cysteine residues forming disulfide bond in *Shewanella violacea* cytochrome c_5 (SV cytc_5) contributed to the overall protein stability but not to the redox poteintial. Cys-59 and Cys-62 were replaced with Ala in SV cytc_5, and the resulting C59A/C62A variant was characterized by means of UV-VIS optical absorption, circular dichroism (CD), and paramagnetic NMR. The spectral features of C59A/C62A variant resembled those of the wild-type SV cytc_5, indicating that the double mutations did not affect the formation of the overall protein structure. The protein unfolding of the C59A/C62A variant has been followed by differential scanning calorimetry (DSC) and CD at 222 nm. The results showed that the mutations caused a dramatic reduction of the stability: thermal transition temperature was down-shifted by 23 °C with respect to the wild-type SV cytc_5 in both DSC and CD analyses, and difference in Gibbs free energy change between the wild-type and variant was 11.7 kJ mol^{-1}. In contrast, the redox potentials of wild-type and variant were significantly the same, +309 and +303 mV, respectively. These results together suggest that the disulfide bond is a structural requirement significantly contributing to the overall stability of SV cytc_5, but the mutations did not affect the electronic structure in the vicinity of heme that could have influenced redox function. Therefore, protein stability can be controlled without changing redox function in SV cytc_5.

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Thermodynamics and Mutational Studies on Association of Natively Partially Folded EspB with α-Catenin Tail Domain

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Binding of natively partially folded EspB from *E. coli* O157 [1] to tail domain of human α-catenin (α-catT) can trigger actin rearrangement of host cells by interfering interactions between α-catT and vinculin and/or between α-catT and F-actin. Here, we analysed properties of the interaction between recombinant α-catT and EspB in vitro. Dissociation constants (K_d) at different temperature were estimated from titration curves monitored by fluorescence anisotropy. The positive entropy for the dissociation (80 J mol^{-1} K^{-1} at 20 °C) was estimated from the dependence of K_d on temperature, suggesting the structural ordering of EspB upon binding to α-catT. This was consistent with the decreased fluorescence of ANS upon binding of EspB to α-catT. The interaction of vinculin head domain (vH) with α-catT are affected by the presence of EspB. Furthermore, EspB was found to directly interact with vH. The mutational analysis on α-catT indicated that the site for vH binding are located around K704, Q781 and N827. However, the affinity of EspB to α-catT were unaffected by these mutations. These data suggest that EspB prevent the interaction between α-catT and vH by binding to both proteins either to shield the binding sites or to sterically conceal them.

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P118

THE INTERMEDIATE STRUCTURE OF PYRROLIDONE CARBOXYL PEPTIDASE STUDIED BY SITE-DIRECTED SPIN LABELING AND NMR

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Understanding how a polypeptide chain folds to its biologically active conformation is one of the major issues in molecular structural biology. For most proteins, the intermediate conformational state accumulates transiently at an early stage of refolding prior to the formation of the native state.

Previous study has shown that the refolding of the cysteine-free mutant of pyrrolidone carboxyl peptidase (PCP-0SH) from a hyperthermophile was unusually slow [1]. When the refolding reaction from the fully unfolded state was initiated at 5^{o}C and pH 2.2, the kinetic intermediate was populated and the refolding reaction was unusually slow, enabling us to investigate the kinetic intermediate by multidimensional nuclear magnetic resonance (NMR). Therefore, PCP-0SH offers unique advantages for the NMR studies of the kinetic intermediate at an early stage of refolding.

The ^{1}H-^{15}N HSQC spectrum of the PCP-0SH intermediate exhibited the line broadening and the limited ^{1}H resonance dispersion characteristic of unfolded conformations. We used the paramagnetic spin labeling and NMR to characterize the intermediate of PCP-0SH. Paramagnetic nitroxide spin labels cause substantial broadening of the resonances of spins within ca 15 Å of the labeling site. Single-cysteine mutants of PCP-0SH were created by site-directed mutagenesis technique and were subsequently modified with (1-oxy-2,2,5,5-tetramethylpyrrolidin-3-yl)methyl methanethiosulfonate. The six residues were replaced with cysteine for this study: R21, K54, K122, H152, E172, and E194, which are exposed to solvent in the native structure of PCP-0SH. Nitroxide spin labels can be specifically attached to PCP-0SH through disulfide bond formation with the side chain of cysteine residue. No significant interactions were observed between the N-terminal half and the C-terminal half, indicating the independence of these two regions in the intermediate.

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Stabilization mechanism of CutA1 protein from *P. horikoshii,* with unusually high denaturation temperatures

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Recently, we have found that the CutA1 (*Ph*CutA1) from *Pyrococcus horikoshii*, a hyperthermophile, has an unbelievably high denaturation temperature of nearly 150 ^{o}C [1]. To elucidate the mechanism of this unusually high stability, we compared profiles of the unfolding/refolding for three CutA1s from *P. horikoshii*, *Thermus thermophilus* (*Tt*CutA1) and *Oryza sativa* (*Os*CutA1) with different growth temperatures, which are 98, 75, and 28 ^{o}C, respectively. The molecular weights of a monomer are 11.6K to 12.5K. All three kinds of CutA1s were confirmed to be a trimer form in solution at pH 7.0 judged from an ultracentrifugation analysis. At pH 7.0 denaturation temperatures of *Ph*CutA1, *Tt*CutA1, and *Os*CutA1, were found to be 148.5, 113.9, and 98.9 ^{o}C, respectively, from DSC experiments, indicating that even *Os*CutA1 from a plant has an unusually high denaturation temperature. The guanidine hydrochloride (GuHCl)-induced unfolding and refolding of the proteins were monitored by the CD value at 220 nm. *Ph*CutA1 did not unfold even after heating for 30 min at 120 ^{o}C in 7.9 M GuHCl at pH 8, but completely unfolded after one hour incubation at pH 2.5 and 80 ^{o}C. The unfolded *Ph*CutA1 could be recovered by diluting concentration of GuHCl at pH 8.0 and 37 ^{o}C. The refolding curve of *Ph*CutA1 after 28 day-incubation in the same condition showed the mid-point of 5.8 M GuHCl, indicating that the transition points of unfolding and refolding are different. This kind of hysteresis was also observed in unfolding and refolding curves of *Tt*CutA1 and *Os*CutA1: the transition points of unfolding and refolding were 5.4 and 3.4 M for *Tt*CutA1, respectively, and 3.8 and 2.2 M for *Os*CutA1 at pH 8.0 and 37 ^{o}C. However, at pH 2.5 the mid-points of unfolding and refolding curves of *Ph*CutA1 were almost same to be around 3.6 M after one day at 37 ^{o}C, respectively, although those of *Tt*CutA1 were slightly different to be 4.1 and 3.8 M after one day at 37 ^{o}C, respectively, indicating that *Tt*CutA1 is slightly more stable than *Ph*CutA1 at pH 2.5. These results indicate that (1) *Ph*CutA1 in the acidic region might be remarkably destabilized due to protonation of a lot of ionizable residues that exist overwhelmingly in *Ph*CutA1 as compared with the other two proteins and (2) in the neutral pH there is hysteresis on the unfolding and refolding curves due to unusually slow unfolding rate of the three CutA1 proteins. These results support the thermo-stabilization mechanism due to ionic interactions, proposed on the structural basis of *Ph*CutA1 [1].

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P120

Study on Structure Folding of Ribonuclease A Coupled with SS Rearrangement

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In the oxidative refolding pathways of proteins that have several SS linkages, SS formation and SS rearrangement reactions progress simultaneously. When sulfur reagents, such as glutathione and DTT^{ox}, are used in the oxidative refolding experiments of proteins, the structure formation coupled with the SS rearrangement is difficult to be observed because the SS formation reaction is slow by use of the sulfur reagents. However, we found that the use of a water-soluble selenium reagent, called DHS^{ox}, allows separation of the SS formation and SS rearrangement reactions: the structure formation coupled with the SS rearrangement reaction was clearly observed on the oxidative refolding pathways of ribonuclease A by using DHS^{ox} as an oxidizing reagent.

The SS formation reaction (oxidation) ended up in 1 minute after addition of three molar equivalents of DHS^{ox} to the solution containing the reduced form of ribonuclease A (R) at 25℃ and pH 8.0, and the SS rearrangement reaction proceeded subsequently. HPLC analysis revealed that after one minute the reaction solution contained R, 1S, 2S, 3S, and 4S intermediates but they gradually transformed to two specific intermediates (des[40-95] and des[65-72] intermediates having three native SS bonds) and N (the native state) in 5h. According to UV and CD spectral analysis, the two 3S intermediates were shown to have a similar folded structure to N. Structure formation accompanied with the formation of the des[40-95] and des[65-72] intermediates will be discussed.

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Molten globule-like intermediate state of cytochrome *c* observed in the thermal transition under weak acidic condition

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Molten globule (MG) structure has been frequently reported as the typical transient structure in the process of protein folding. The MG structure has been also observed as the stable structure of a thermodynamic state under several specific solution conditions. The mechanism that stabilizes the structure is inevitably important for understanding the folding mechanism and thermodynamic stability of protein molecules. In the case of horse cytochrome *c*, two distinct MG states, MG1 and MG2, were observed by spectroscopic methods under high salt concentration at around pH 2. Recently we have found that a stable intermediate state is detected by precise calorimetry and circular dichroism (CD) measurement even under low salt concentration at pH 4 (50 mM acetate buffer). In this study, the structural characteristics of the intermediate state observed under the weak acidic condition were further studied by CD and solution X-ray scattering (SXS), and the volumetric ones were elucidated by pressure perturbation calorimetry (PPC). The mole fractions of the thermodynamic states of horse cytochrome *c* under several solution conditions were evaluated as temperature functions by differential scanning calorimetry (DSC) with VP-DSC (MicroCal). The CD spectra of far- and near-ultra violet (UV) and visible region of the protein solution were monitored in the temperature range from 5°C to 95°C with J-720 (Jasco), and all the spectra were analyzed with three-state transition model using the mole fractions determined by the calorimetric measurement in order to determine the individual CD spectra of each thermodynamic states. In the analysis, the linear temperature dependence of the decomposed CD spectra was taken into account. The SXS measurement was performed at the BL-10c in Photon Factory (Tsukuba) and PPC was done using VP-DSC with pressure control unit (MicroCal), and the decomposed SXS profiles and PPC profiles were evaluated using the same procedure as the CD analysis mentioned above. The decomposed CD spectra of the intermediate (I) state observed in the thermal transition from the native (N) to the denatured (D) state of horse cytochrome *c* at pH 4.1 under low salt condition, 50 mM acetate buffer, agreed well with that of the MG1 state. Also the decomposed SXS profile of the I state and that of the MG1 state were found to be identical. In the case of SXS, these profiles also agree well with that of the N state indicating that these structures have almost the same shape and compactness. These results indicate that the structure of the I state has the same characteristics as that of the MG1. On the other hand, the partial molar volume of the I state showed the middle value between those of the N and the D states, suggesting the existing partially hydrated hydrophobic core in the structure of the I state.

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P122

Molecular mechanism of temperature dependent dissociation and interaction with unfolded proteins of small heat shock proteins from the fission yeast, *Schizosaccharomyces pombe*

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Small heat shock protein (sHsp) is one of the most ubiquitous molecular chaperones. They are grouped together based on a conserved domain, α-crystallin domain. In the genome of the fission yeast *Schizosaccharomyces pombe*, there are two sHsp genes, SPBC3E7.02c and SPCC338.06c which encode SpHsp16.0 and SpHsp15.8. Transcriptional analysis revealed that they are induced by heat shock. We expressed and analyzed molecular properties of SpHsp16.0 and SpHsp15.8. They exist as spherical complexes at room temperature. The complexes dissociated into smaller oligomers at elevated temperature, which reassembled into the parent structures immediately after temperature shift to the room temperature. Thermodynamics study of dissociation of sHsp complexes was performed by differential scanning calorimetry (DSC). They protected citrate synthase (CS) from thermal aggregation at 45 ºC and large sHsp-substrate complexes were observed by electron microscopy and HPLC. However, such large complexes were observed only at lowered temperature, but could not be detected at 45 ºC. To examine the nature of the interaction between SpHsp16.0 and denatured CS, fluorescence cross-correlation spectrometry was performed using fluorescent labeled SpHsp16.0 and CS. As a result, the interaction between denatured CS and SpHsp16.0 was transient at 42 ºC. Based on these results, we proposed that sHsps dissociate at elevated temperature, and the dissociated small oligomers function to suppress aggregation of denatured proteins by the transient interaction [1]. We will also present the results of the study of the interaction between SpHsp60 and CS at higher temperature by fluorescence anisotropy.

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Thermal-induced conversion of globular proteins into amyloid fibrils: Cases of β_2-microglobulin and hen egg-white lysozyme

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The thermodynamic parameters characterizing protein stability can be obtained for a fully reversible folding/unfolding system directly using differential scanning calorimetry (DSC). However, it is also clear that the reversible DSC profile can be altered by an irreversible step causing protein aggregation. In this study, the heat-induced structural conversion into orderly aggregated amyloid fibrils was studied with β_2-microglobulin and hen egg-white lysozyme by means of the combined use of agitation and heating by DSC. First, aggregates were formed by mildly agitating the soluble protein solution in the presence of NaCl. Then, the aggregated protein solution was heated in the cell of DSC in order to produce amyloid fibrils and estimate the thermal effects concomitant with the fibrillation. For β_2-microglobulin, as the increase in NaCl concentration at neutral pH, DSC thermogram began to show a clear transition accompanied by a large decrease in the heat capacity of the protein solution, followed by a kinetically controlled thermal response that is highly heating rate-dependent. The aggregated lysozyme solution at high NaCl concentration also revealed a clear transition reflected in the heat capacity and the similar kinetic thermal response after the transition over the wide pH range. The electron microscopy demonstrated the conformational conversion from the agitation-treated state into orderly aggregated amyloid fibrils as a result of the distinct transition. These results offer a glimpse at the molecular events leading to the conversion into amyloid fibrils in the heat-induced aggregation pathway.

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P124

Surface interaction effects on fibril morphology in the self-assembly of amyloid β (1-40) into amyloid

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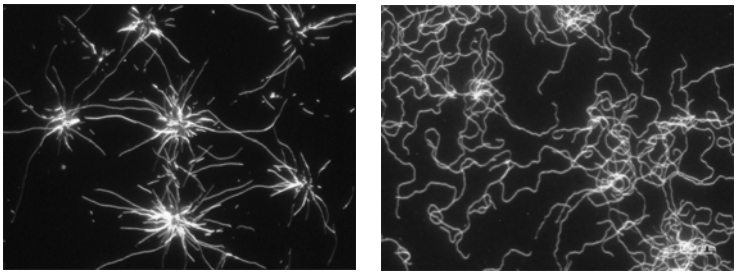
Amyloid fibrils form via a nucleation process, followed by a growth process. Thus, while spontaneous fibril formation is often difficult to occur showing a lag phase, seed-dependent growth, in which fragmented fibrils are added to the solution of monomers, occurs rapidly without a nucleation process. Direct observation of both the nucleation and growth process is important to clarify the mechanism of fibril formation and moreover to develop new therapies of amyloidoses. We have established that total internal reflection fluorescence microscopy (TIRFM) combined thioflavin T, an amyloid specific fluorescence dye, is a useful method to study the mechanism of amyloid fibril formation. Among various amyloidogenic peptides, we focus on amyloid β (Aβ) peptide associated with Alzheimer's disease. In our previous study [1], the seed-dependent amyloid fibril growth of Aβ (1-40) was visualized in real time at a single fibril level revealing the formation of spherulitic structure under the chemically modified quartz surface. On the other hand, nucleation process in the spontaneous fibril formation is not clear. Here to clarify the nucleation process, we studied the fibril formation of Aβ (1-40) in the absence of seeds.

Self-assembly of Aβ (1-40) on the quartz slide glass in the absence of seeds produced several types of amyloid fibrils with various and remarkable morphologies. In contrast, seed-dependent growth on the same quartz slide glass produced the amyloid fibrils with the same straight and rigid morphology as the template. These results suggest that, in the spontaneous fibril formation, nucleation step is critically affected by environmental factors, leading to the formation of several types of fibrils with distinct morphologies. We proposed that, in contact with surfaces, Aβ (1-40) exhibits a high potential forming various types of nuclei, and hence amyloid fibrils of distinct morphologies. The results help to elucidate the mechanism of amyloid fibril formation.



Direct observation of Aβ(1-40) amyloid fibrils growth by TIRFM

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Orientation of amyloid fibrils formed by β_2-microglobulin and its peptide fragment in a flow

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Dialysis-related amyloidosis is caused by the deposition of β2-microglobulin (β2-m) amyloid fibrils in patients of long-term hemodialysis. Amyloid fibrils have unbranched structure with lengths up to several μm. To investigate the orientation of fibrils in a flow, we used the β2-m fibrils and two kinds of protofilaments formed by its peptide fragment K3, which are called f210 and f218 fibrils, respectively.

Using circular dichroism (CD) and linear dichroism (LD) spectroscopy, we observed the orientation of the f210 fibrils in a flow. The f210 fibrils in a flow showed larger CD signals than those under nonflow conditions. As it is assumed that the contribution of LD signals is included in CD signals, we performed LD measurement. The LD spectra revealed that the f210 fibrils oriented along with the flow and net $\pi\pi^*$ transition moment of carbonyl groups of backbone and L_b transition moment of Tyr26 in K3 peptide oriented almost parallel to the fibril axis.

We compared the orientation of the β2-m and f218 fibrils in a flow with that of the f210 fibril. It is found that the orientation of fibrils depends on their morphology. Furthermore, we examined the effects of length of fibrils. We prepared the fragments of the f210 fibrils with various lengths by ultrasonication and measured their LD spectra. The result showed that the orientation of fibrils depends on their length as rod-like polymers. In this study, combining the LD measurement with a flow system, we could reveal the orientation in a flow and the internal structure of fibrils.

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P126

Folding and Unfolding Kinetics of β2-Microglobulin as Probed by Tryptophan Fluorescence

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β2-Microglobulin (β2-m), which has a typical immunoglobulin-fold, forms amyloid fibrils responsible for dialysis-related amyloidosis. The population of folding intermediates is predicted to be involved in the initiation of fibril formation, and it is important to examine the detailed mechanism of folding and unfolding of β2-m.

Here, we have investigated the kinetics of folding and unfolding of β2-m by the denaturant (Gdn-HCl)-dilution experiments with stopped-flow and manual mixing methods using Trp fluorescence as a probe. We adopted a mutant, W39 [W60F/W95F/L39W] in addition to wild type (wt), in which single Trp was introduced at the position corresponding to the conserved Trp common to other immunoglobulin domains. The introduced Trp39 exhibited a remarkable quenching in the native state, resulting in a dramatic change in fluorescence intensity during the reactions.

As a result of systematic analysis, we found that the kinetic pattern of W39 mutant was quite similar to that of the constant fragment of the immunoglobulin light chain which was previously analyzed.[1] We could interpret the results of wt based on the scheme for the constant fragment with some modifications, as well as W39. Furthermore, by analyzing P32V, we could discuss the cis-trans isomerization of Pro32 in more detail.

In the poster presentation, we will report the reaction mechanism of β2-m proposed from the present analysis.

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Replication mechanism of infectious prion protein (PrP^{Sc}): A nonamer disruption simulation using Go model

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Transmission mechanism in prion diseases still remains enigmatic. To understand the replication mechanism of scrapie-type prion (PrP^{Sc}) based on the tertiary structure of the prion, here, we conducted a simulation of the disruption process of PrP^{Sc} nonamer using Go model and a structure of PrP^{Sc} proposed by C. Govaerts et al. [1] at a relatively high temperature. The results showed that the nonamer finally disrupted into nine monomers in the following three steps; a nonamer → (i) a nonamer in which three molecules partially unfolded→ (ii) a hexamer +3 monomers → (iii) 9 monomers. These steps progressed in a cooperative manner, especially, disruption of a hexamer exhibited very strong cooperativity. This result indicates that the hexamers of the PrP^{Sc} are much more stable than the dimers or the trimers, implying that the hexamer or the oligomer larger than the hexamer works as a seed. To confirm the statistical mechanical significance of this observed phenomena, we conducted the disruption simulations using three different native contact sets. In summary, the cooperative, stepwise disruption behaviors were common to all simulations but the combination of three molecules which unfolded and dissociated from the nonamers in the early stage was entirely dependent on native contact set. According to the locations of causative mutations, here we propose a PrP^{Sc} propagation mechanism, in which the chain topology was incorporated. An oligomer ($\geqq 6$) can be a seed of prion's replication, and the monomer associates with the seed at the α-helices of the C-terminal region, and subsequently β-helices of the N-terminal region are formed. For comparison, the differences of the amyloid formation mechanisms between prion and short peptides (N-terminal of Sup35) are discussed.

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P128

PREFERENTIAL AND REVERSIBLE BINDING OF FLAVONOIDS TO THE AMYLOID FIBRIL STRUCTURE EXERTS ANTI-AMYLOIDOGENIC EFFECT AGAINST ALZHEIMER'S β-AMYLOID FIBRILS IN VITRO

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How various anti-amyloidogenic compounds inhibit the formation of Alzheimer's β-amyloid fibrils (fAβ) from amyloid β-peptide (Aβ) and destabilize fAβ remains poorly understood. Using spectrophotometry, spectrofluorometry, atomic force microscopy, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and surface plasmon resonance (SPR), we investigated the anti-amyloidogenic effects of five flavonoids on fAβ in vitro. Oxidized flavonoids generally inhibited fAβ(1-40) formation significantly more potently than fresh compounds. Characterization of the novel fluorescence of myricetin (Myr) emitted at 586 nm with excitation maximum at 430 nm in the presence of fAβ(1-40) revealed the specific binding of Myr to fAβ(1-40). By SPR analysis, distinct association and dissociation reactions of Myr to fAβ(1-40) were observed, in contrast to the very weak binding to the Aβ monomer. A significant decrease in the rate of fibril extension was observed when > 0.5 μM of Myr was injected into the SPR experimental system. These findings suggest that flavonoids, especially Myr exert an anti-amyloidogenic effect in vitro by preferentially and reversibly binding to the amyloid fibril structure of fAβ, rather than to Aβ monomers.

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β-SHEET CONFORMATIONAL TRANSITION AND AMYLOID-LIKE FIBRIL FORMATION OF THE POLYGLUTAMINE PROTEIN, AND SCREENING FOR THEIR INHIBITORS.

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Conformational alterations leading to aggregation and deposition of misfolded proteins have been recognized as a common molecular pathogenesis of neurodegenerative diseases including Alzheimer, Parkinson, and the polyglutamine (polyQ) diseases, which are classified as the conformational diseases. The polyQ diseases are a group of inherited neurodegenerative diseases which are caused by abnormal expansions of the polyQ stretch within the disease-causing proteins. Expansion of the polyQ stretch is thought to confer toxic properties on the disease-causing proteins through alterations in their conformation, leading to their assembly into insoluble amyloid-like fibrils. To elucidate conformational alterations of the expanded polyQ protein and its cytotoxic conformers formed during amyloid-like fibril formation, we performed structural analyses of thioredoxin-polyQ (thio-polyQ) fusion proteins. Circular dichroism analyses revealed that thio-polyQ proteins with an expanded polyQ stretch undergo a conformational transition to a β-sheet dominant structure, resulting in formation of insoluble β-sheet-rich amyloid-like fibrils. Analytical ultracentrifugation and size exclusion chromatography demonstrated that this β-sheet conformational transition occurs in the monomeric thio-polyQ protein, preceding its assembly into amyloid-like fibrils. Microinjection of thio-polyQ proteins with various structures into cultured cells revealed that the soluble β-sheet monomer as well as amyloid-like fibrils causes cytotoxicity. We further show that the polyQ binding peptide QBP1 prevents the β-sheet conformational transition of the thio-polyQ protein monomer, resulting in inhibition of amyloid-like fibril formation and neurodegeneration *in vivo*. We conclude that the toxic β-sheet conformational transition of the expanded polyQ protein monomer is a promising therapeutic target for the currently untreatable polyQ diseases, and possibly for the other conformational diseases.

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P130

Thermodynamic Analysis on the Amyloid Fibril Formation by β-Lactoglobulin

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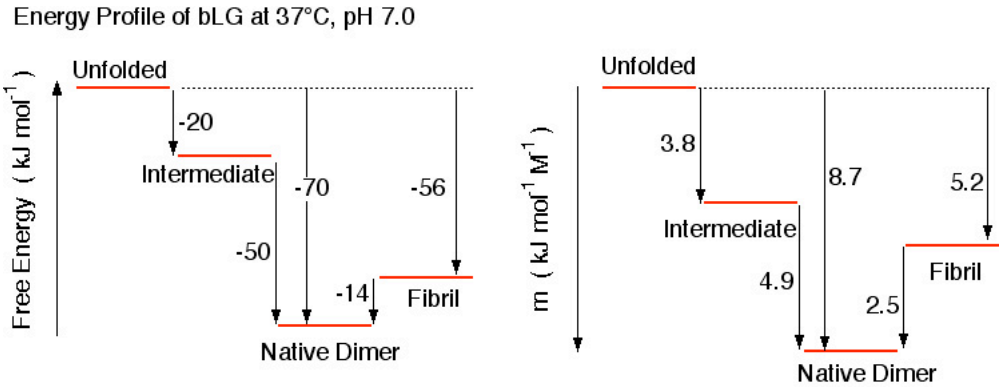
Protein aggregation into well-ordered fibrillar structure such as amyloid fibrils is one of the fundamental topics in protein science as well as in pathology. Historically, thermodynamic analysis on the stability of the native structures of proteins -- another well-ordered, functional structures formed by self-organisation -- has provided valuable information on the mechanism of protein folding. Thus, the similar strategy should be also of great benefit for understand the mechanism of protein aggregations. However, no general methodology to elucidate such information has not been established so far.

In this study, I used bovine β-lactoglobulin as a model system to assess this problem. The analysis is based on the following approximations. 1) The reaction fibril formation is a reversible process. and 2) Under equilibrium conditions, the number of amyloid fibrils in solution is unchanged. According to these, the dissociation constant or critical concentration for the reaction should be equal to the concentration of precursor proteins present in solution after the reaction is completed. These approximations are actually derived from the nucleation-dependent mechanism as usually suggested for this reaction, and already applied to amyloid Aβ peptide [1,2]. By applying the same approach to urea-induced fibril formation by β-lactoglobulin which also assumes the well-ordered native state unlike the above peptide system, I obtained complete energy profile for β-lactoglobulin including the native, intermediate, unfolded and fibrillar states. Application of this simple strategy to various protein or peptide systems will further provide the fundamental aspect of amyloid fibril formation by polypeptide chains.



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Polyglutamine fibrillization affected by non-pathogenic proteins – roles of cross-seeding in neurodegenerative diseases

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Amyloid is one of the protein fibrillar aggregates and is an important pathology found in several neurodegenerative diseases. Fibrillar aggregates form from a soluble folded protein in two steps: nucleation and elongation. In a nucleation step, a protein molecule is supposed to undergo conformational changes and form a nucleus for the following fibril elongation. Interestingly, the nucleus of a protein can sometimes induce fibrillization of a different protein, which is called a "cross-seeding" reaction [1]. Aggregation of the disease-causing proteins could thus be triggered by nucleation of different protein(s) [2] and *vice versa*; in fact, the amyloid-like aggregates in the patients contain various kinds of proteins [3]. Despite this, the protein fibrillization by cross-seeding reactions in physiological conditions remains elusive.

To test the relevance of cross-seeding reactions in protein aggregation, we have focused on the fibrillization of polyglutamine, which is involved in the neurodegenerative diseases such as Huntington's disease. The polyglutamine aggregation has been shown to accelerate upon cellular stress such as heat and UV [4], but we also note that the cellular stress leads to the formation of mRNA-containing granules, which is regulated by TIA-1. TIA-1 is thus a candidate protein that cross-seeds the polyglutamine aggregation, leading us to investigate the fibrillogenicity of TIA-1. Incubation of a granule-forming domain of TIA-1 at C-terminal region (TIA-1^C) increases the intensity of Thioflavin-T fluorescence and red-shifts the absorption peak of Congo red, both of which are typical changes upon formation of amyloid-like aggregates. While the soluble form of TIA-1^C slows the polyglutamine fibrillization, TIA-1^C fibrillar aggregates slightly shorten the time for nucleation of polyglutamine, which is consistent with the cross-seeding mechanism. In a cultured cell model, TIA-1^C co-localizes with polyglutamine aggregates, suggesting the intracellular interaction between TIA-1^C and polyglutamine. In order to reveal the intracellular cross-seeding between TIA-1 and polyglutamine, we are now examining effects of TIA-1 aggregation on intracellular kinetics of polyglutamine aggregation.

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P132

Construction of Green Fluorescent Protein (GFP) Variants with Amyloid β-Peptide Sequence

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Amyloid fibril formation of protein is related to the fatal diseases. Alzheimer's disease is a progressive neurodegenerative disease that is characterized by the abnormal accumulation of amyloid β-peptide (Aβ) in senile plaques. Aβ self-assembles into soluble oligomers, protofibrils, and amyloid fibrils. The recent studies implicated that the oligomeric species of Aβ have a potential for killing neurons in cultured hippocampal brain slices. Although the structures of amyloid fibrils and soluble oligomers have not been realized, the solid-state NMR analysis of fibrils composed of Aβ1-40 was reported. The NMR results indicated that 1-10 residues of Aβ1-40 was approximately disordered, and residues 12-24 and 30-40 formed parallel β-sheet structure in the fibrils. Aβ11-25, which is located in a central region of Aβ and known to be important for fibril formation, has been found to form amyloid fibrils as a similar morphology of full-length Aβ1-40 and 1-42 fibrils. On the other hand, the green fluorescent protein (GFP) isolated from the jellyfish *Aequorea Victoria* is a 238 amino acids protein that generates a fluorophore autocatalytically. GFP folds into β-barrel structure composed of eleven β-strands and the fluorophore on an α-helix buried in the center of barrel. Both parallel and anti-parallel β-sheets were included in the GFP structure. In the present study, we designed and synthesized the GFP variants, in which the Aβ sequence was embedded as the Aβ structural mimic. Since the Aβ can self-assemble by recognizing itself, it was expected that the GFP variants could bind to Aβ and inhibit the oligomerization of Aβ.

To insert the Aβ sequence into GFP, the central region of Aβ14-23 was selected due to the importance for aggregation of Aβ. To prevent destabilization of the GFP structure, some amino acids in Aβ14-23 (HQKLVFFAED or HQKLVFFAED; selected amino acids were underlined) were substituted for the surface residues upon GFP. GFP contains eleven β-strands aligned by ten anti-parallel and one parallel β-sheet topology. GFP variants, P13H and AP13Q, in which two β-strands were replaced by the Aβ amino acids, were designed, respectively, as a parallel and an anti-parallel β-sheet model. GFP and GFP variants bearing a 6-histidine tag at a C-terminal were purified by Ni-NTA resin with high purity. The GFP variants showed circular dichroism spectra and fluorescent spectra similar to those of GFP.

We examined the inhibition abilities of GFP and the variants for oligomerization of Aβ1-42. Oligomerizartion of Aβ1-42 was evaluated by an ELISA assay using anti-Aβ-antibody. During the incubation of Aβ1-42 in the presence of GFP, Aβ1-42 self-assembled into oligomers was scarcely inhibited as compared with that in the absence of GFP. In contrast, in the presence of the P13H or AP13Q variant, the amount of Aβ1-42 oligomers were obviously decreased as compared to that without the variant. These results suggested that P13H and AP13Q could inhibit the oligomerization of Aβ1-42 probably by binding to the Aβ1-42 molecule. Especially, P13H showed the higher inhibition activity. It seems that the parallel orientation of Aβ sequence on the GFP surface is more favorable to interact with the Aβ1-42 molecule than the anti-parallel orientation.

In conclusion, design of the molecules capable of binding to Aβ by using the GFP β-barrel scaffold and embedding the Aβ sequence with parallel orientation was successfully demonstrated. This strategy might be useful to construct the molecules that bind to the other amyloidogenic species.

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P133

On The Role of Solvent Cluster in Dissolution of Amyloid-like Fibrils in DMSO-Water Mixtures

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Amyloid fibril formation is now recognized as a phenomenon common to many proteins and peptides. Amyloid fibrils are associated with fatal diseases such as Alzheimer's, which is caused by misfolding of proteins. Thus, it is important to investigate the underlying mechanism of Amyloid formation for understanding the properties of Amyloid fibrils and preventing the formation of the fibrils.

It was reported that fluoroalcohols (TFE and HFIP) and dimethylsulfoxide (DMSO) completely dissolved β_2-microglobulin amyloid fibrils although a high concentration [*i.e.*, 80%(v/v)] was required for DMSO(Fig.1)[1]. The dissolution mechanism is not known, however. It is very likely that the dissolution of amyloid fibril is related to solvent environment in these mixtures.

In the present study, the microscopic and mesoscopic structure of dimethylsulfoxide (DMSO)-water mixtures has been investigated at 298 K as a function of DMSO mole fraction (x_{DMSO}) by X-ray diffraction and small-angle neutron scattering (SANS), respectively. From the analysis of the X-ray radial distribution functions of DMSO-water mixtures, it was found that the structure of water cluster similar to ice was gradually broken as DMSO mole fraction increases, followed by a structural transition to DMSO cluster at $x_{DMSO} \approx 0.5$. The maximum in the correlation length ξ obtained from the analysis of SANS data was found at $x_{DMSO} \approx 0.5$. As the correlation length ξ is a measure of concentration fluctuation, the SANS data also indicate the structural transition of solvent cluster at this composition. The role of solvent cluster in dissolution of β_2-microglobulin amyloid-like fibrils is discussed from the structure of DMSO-water mixtures obtained.

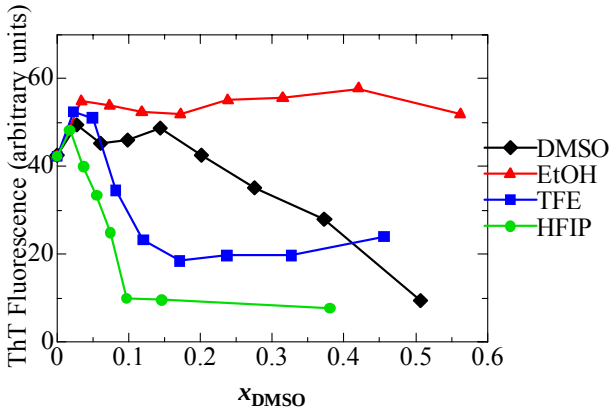

Fig. 1. Dissolution of β_2-microglobulin amyloid fibril in various alcohols and DMSO [2].

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P134

Design and Synthesis of an Oxidation-Responsible Amyloid Fibril Formation Protein, AG-Met, and Its Expression in Cell

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Protein aggregation occurring from protein misfolding results in amyloid disease (AD), such as Alzheimer's and Parkinson's disease. We have previously designed and synthesized a small globular protein, amyloidgenesin (AG) (Fig.1). AG undergoes to form amyloid protofilaments and then fibril clusters. There are many indications that oxidative damage plays an important role in AD. In this study, to investigate the relationship between fibril formation associated to oxidative stress and cytotoxity, we have designed and synthesized amyloidgenesin-Met (AG-Met) (Fig.1). When sulfur atom of Met is oxidized by chemical oxidative stress, amphiphilic sequences can take an amphiphilic β-structure.

AG: Ac-KKLKLLLKLL-GGGG-KKLKLLLKLL-GGGG-***LLLLWLLL***-GGGG-KKLKLLLKLL-GGGG-KKLKLLLKLL-OH

AG-Met: Ac-KKLKLMLKLL-GGGG-KKLKLMLKLL-GGGG-***LLLLWLLL***-GGGG-KKLKLMLKLL-GGGG-KKLKLMLKLL-OH

Fig.1. Primary structure of amyloidgenesin (AG) and AG-Met. Underlines show the sequences of amphiphilic α-or-β structure.

When sulfur atoms of Met were oxidized by chemical oxidative reagent, chloramine T of 2 equivalents for 4 Met(s), AG-Met underwent irreversible α-to-β conformational transition rapidly within 20 min with increasing ThT fluorescence (Fig. 2). Electron microscopic studies showed that amyloid-like fibril deposits were observed within 2 hrs after the treatment of chloramine T. These results show that AG-Met is a useful model protein on an oxidative-responsible amyloid fibril formation in vivo. We have also been studying the relationship between the expression of AG-Met, oxidative stress, and cytotoxicity in cells.

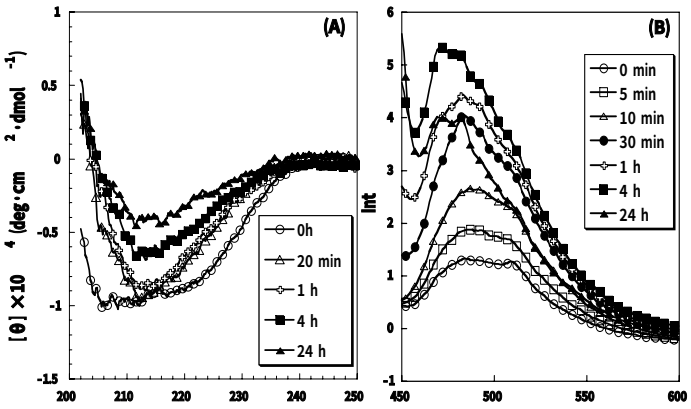

Fig. 2. Time dependent CD(A) and ThT fluorescence(B) spectra of AG- Met in the presence of chloramine T

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P135

Pressure dissociation kinetics of amyloid protofibrils

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We present here the first detailed kinetic study of the dissociation reaction of amyloid protofibrils utilizing pressure-jump fluorescence techniques. The experiments were carried out on typical protofibrils formed from the intrinsically denatured disulfide-deficient variant of hen lysozyme in the pressure range 3 MPa up to 400 MPa. Prviously, we followed the dissociation and association reactions repeatedly by NMR upon pressure-jump up and down, respectively [1,2]. In the present study, the pressure-dependence of the dissociation rate was studied in detail with Trp fluorescence as monitor under excessive dilution where reassociation reaction can be neglected. The dissociation reaction is strongly accelerated by pressure up to ~300 MPa with a negative activation volume ($\Delta V^{o\ddagger}$ = -50.5 ± 1.60 ml mol^{-1} at 0.1 MPa). However, the acceleration becomes less at higher pressure and the rate reaches a plateau at ~400 MPa, giving a negative activation compressibility ($\Delta\kappa^{\ddagger}$ = -0.013±0.001 ml mol^{-1} bar^{-1} or -0.9 x 10^{-6} ml g^{-1} bar^{-1}). These results indicate that the protofibril is a high-volume state, but the volume decreases in the transition state due probably to partial hydration. From the exponential time decay of the fluorescence and the exponential size distribution of the protofibrils studied by AFM, we conclude that the protofibril grows or decays by a linear polymerization mechanism, i.e., by detachment of a monomer at one end of the protofibril. The pressure-dependent rate of detachment of a monomer from the protofbril could be estimated experimentally. This rate is ~3.1 min^{-1} at 200MPa.

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Intrinsic rate of dissociation of monomeric protein from amyloid protofibrils

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A genetically engineered disulfide-deficient variant of hen lysozyme (0SS) spontaneously forms an amyloid protofibril. We have recently studied the dissociation kinetics of *matured* protofibrils of 0SS (prepared by incubating the 0SS solution for more than 6 months) as a function of pressure with Trp fluorescence as monitor and identified the mechanism of dissociation [1].

In the present work, we have studied the dissociation kinetics of *young* protofibrils of 0SS to compare their dissociation rates with that of the matured protofibril. Young protofibrils are formed from 0SS by incubating its solution in 30 mM NaCl, 20 mM sodium acetate, pH 4.0 for shorter periods of time starting from 40 min, 100 min and so forth. The population distribution of the young protofibril over its length, measured on atomic force microscopy image, is close to exponential, consistent with the linear polymerization mechanism that protofibrils grow and shrink by addition and detachment of monomer to its growing end, respectively [1].

The decrease of Trp fluorescence intensity upon pressure- jump from 3 MPa to a range of pressure up to 200 MPa, following an excessive dilution of the protofibril solution incubated for 40 min, 100 min and so forth, is considered to represents selectively the dissociation process of the young protofibril. The fluorescence intensity decreases exponentially with time, accompanied by a red shift of maximum emission from 338 nm to ~348 nm, indicating that the protofibrils are dissociated into monomers in which Trp residues are well exposed to the solvent. The intrinsic rates of dissociation of the monomeric species, extrapolated to 0.1 MPa, from protofibrils produced for shorter periods of incubation such as 40min, 100 min, 24 hr are in the range of 0.1-0.4 min^{-1}, comparable to 0.15 min^{-1} for 6 months. The activation volume for dissociation is negative, showing a tendency for decreasing with the incubation period; $\Delta V^{o\ddagger}$ = -77.65±9.71ml/mol, -66.93± 6.15ml/mol,-58.97±9.35ml/mol, and -50.5 ± 1.60 ml mol^{-1} , respectively, for 40min, 100 min, 24 hr and 6 months of incubation, while the activation compressibility $\Delta\kappa^{\ddagger}$ is also negative throughout the incubation period from 40 min to 6 months.

From these observations, we conclude that the linear polymerization mechanism applies for the protofibril formation and dissociation of 0SS for all incubation periods from 40 min to 6 months. although the activation process for dissociation, determining the intrinsic dissociation rate of the monomer, is affected slightly when the average length of the protoribril becomes shorter.

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Amyloid protofibril is highly voluminous and compressible.

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This study provides a first comprehensive analysis of equilibrium volumetric parameters on monomer-to-polymer transition in the amyloid protofibril formation. We have measured the partial specific volume and compressibility changes associated with the amyloid protofibril formation from the intrinsically denatured disulfide-deficient variant of hen lysozyme (0SS) on a high precision density meter and a ultrasound velocity meter at 25°C by simultaneously monitoring the progress of the reaction with circular dichroism (CD). The partial specific volume (ν) and the partial specific adiabatic compressibility (κ_s) of 0SS increase dramatically, as the self-association reaction proceeds with time as monitored by CD, giving $\Delta\nu = 0.040$ ml·g^{-1} (which corresponds to 571 ml mol monomer^{-1}) and $\Delta\kappa_s = 6.10\times10^{-12}$ ml·g^{-1}·cm^2·dyn^{-1}. The volume change is much larger than those for folding of globular proteins of similar size [1]. The results clearly demonstrate that the protofibril formation is accompanied by a dramatic increase in partial molar volume and indicate that the resultant protofibril becomes highly compressible, due probably to a loss of hydration with concomitant formation of a considerable amount of intra- and inter-molecular cavities. The result explains why the amyloid protofibril is easily dissociable by pressure.

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Morphology of Amyloid protofibrils from various disulfide-deficient mutants of hen lysozyme

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We have investigated here the effect of disulfide bonds on the morphology of amyloid fibrils using various disulfide variants of hen lysozyme. The disulfide variants include the disulfide-deficient mutants of hen lysozyme (0SS), four kinds of one-disulfide variants (1SS-1; 6-127), (1SS-2; 30-115), (1SS-3; 64-80), (1SS-4; 76-94), and two kinds of two-disulfide variants (2SS1+2; 6-127, 30-115 both in the α-domain), (2SS3+4, 64-80, 76-94 both in the β-domain), in which appropriate Cys residues have been replaced with Ser or Ala by genetic engineering [1]. The reaction for fibril formation has been carried out in aqueous solutions containing 20 mM sodium acetate, 50 mM sodium chloride (pH 4.0) at 25ºC. The fibril formation reaction has also been carried out for wild type hen lysozyme (4SS), but in a slightly different solution condition (80 mM NaCl, pH 2) at 57ºC.

All the variants except 2SS3+4 and 4SS are found to form long winding protofibrils of 1.5~2.5 nm in height, of which 1SS-2 and 2SS1+2 also form ring-shaped fibrils. They remain protofibrils and do not proceed to matured amyloid fibrils consisting of bundles of protofibrils even for prolonged incubation. In contrast, the variant 2SS3+4 and 4SS, which form straight protofibrils, form matured amyloid fibers after prolonged incubation at appropriate conditions. The result suggests that, for protofibrils to grow into matured amyloid fibers, certain restriction on the conformational freedom of the polypeptide backbone is required.

In all, the present work shows the intimate relationship between the location and the number of disulfide bonds and the morphology of the resultant amyloid fibrils.

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Characteristic features of amyloid fibril formation from wild-type hen lysozyme

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The wild-type hen egg white lysozyme (HEWL) forms amyloid-like fibrils at low pH simply by keeping the solution at elevated temperatures. We have followed the process of amyloid-like fibril formation on atomic force microscopy (AFM) images for HEWL (1 mg/ml) in 80 mM NaCl, pH 2.2 at 57℃, close to the transition temperature. The fibril formation started after a time lag of ~4 days, and a clear formation of typical protofibrils began after ~5 days, which consist of long straight fibers of 2.5～3.5 nm in height. Finally, after ~11 days, part of the protofibrils turned into amyloid fibers of 8~12 nm in height, apparently consisting of several bundles of protofibrils.

We have examined the length distribution of the protofibrils obtained from ~5 days incubation, which obeys nearly an exponential function of the fibril length. The result is consistent with the linear polymerization mechanism of the protofibril formation such that a monomer is attached successively to the growing end of the preformed protofibril [1]. This mechanism has recently been established for the amyloid protofibril formation for the first time from intrinsically denatured disulfide-deficient hen lysozyme (0SS) in the monomeric state [2]. Our present result indicating that the same mechanism applies to the protofibril formation from wild-type hen lysozyme is a step forward to understanding the molecular process of amyloidosis *in vivo*.

The long time-lag before starting the protofibril formation suggests that a seed formation is the rate-limiting step for the protofibril formation, as shown earlier for human lysozyme [3]. Indeed, when the matured amyloid firbils after ~11 days are made into pieces by sonication and added as seeds to the incubating solution of HEWL at 57℃, protofibrils start to form immediately without a time lag. During the reaction, the circular dichroism spectra depict a transition from closely native HEWL into β-rich protofibrils in a two-state manner. The result indicates that native HEWL can be transformed into β-rich protofibrils by contacting the seed, a process of conformational infection.

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Pressure effect on oligomric human prion

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We report here the first NMR investigation of oligomeric prion. Oligomeric human PrP^C ($HuPrP^C$) (23-231) has been prepared according to Jackson *et al* [1] and subjected to AMF analysis, fluorescence and ^{1}H-NMR observations. At ambient pressure, the 1D ^{1}H-NMR spectrum of oligomeric $HuPrP^C$ (23-231) shows broad signals in the high-field shifted CH_3 region (0 –1.0 ppm) in contrast to the well resolved NMR spectrum from monomeric $HuPrP^C$ (23-231) [2], indicating that the high molecular oligomeric $HuPrP^C$ (23-231) have a certain core structure. In contrast, the relatively sharp signals at around 0.9 ppm show the presence of unordered polypeptide chain in the oligomer. A plausible case is that the C-terminal core part of $HuPrP^C$ (23-231) is involved in the oligomerization phenomenon, while the N-terminal flexible region is left out in the oligomerization.

Next, pressure jump experiments are performed on the oligomeric $HuPrP^C$ (23-231) from 0.3 M Pa to 200 MPa, which are monitored by 1D ^{1}H-NMR and fluorescence spectroscopy. With pressure jump to 200 MPa, the ^{1}H-NMR signal at 0.3 ppm decreases along with a shift to lower magnetic field, while the relatively sharp signal at 0.9 ppm increases. The resultant spectrum at 200 MPa is similar to that of monomeric $HuPrP^C$ (23-231) at 200 MPa [2]. These results indicate that the oligomeric $HuPrP^C$ (23-231) is sensitive to pressure and dissociates into monomeric form at 200 MPa. The dissociation reaction finishes within 30 min of NMR signal accumulation. With pressure release to 0.3 M Pa, the original spectrum at ambient pressure recovers at least partially, showing that the dissociation reaction is basically reversible with pressure.

In contrast, the NMR signals from six Trp residues distributed mainly in the N-terminal segment are not affected by the pressure jump, indicating that they are not directly involved in the association, suggesting the view that only the C-terminal core part is involved in the oligomerization. The present result provides the first structural information for oligomeric prion.

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Neutron Diffraction Study of Cubic Insulin at Two Different pDs

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It is known that the change in protonation status of amino acid side chains can strongly affect the biological function and stability of a protein. It is often hard to estimate the protonation status of some amino acids because it not only depends on the pH of the solution and but also on the local environment of the amino acid residue. Therefore, monitoring the protonation status of a protein at different pHs is an effective method to determine protonation status. Neutron diffraction is a powerful tool to observe hydrogen atoms because of its strong interaction with hydrogen. Thus, a neutron diffraction study of cubic insulin, a 5700 Da protein, composed of two peptide chains; A-chain (21 a.a.) and B-chain (30 a.a.), has been carried out using the BIX-4 single crystal diffractometer at the JRR-3 reactor of the Japan Atomic Energy Agency and the result was compared with the previous neutron diffraction data at pD 9 [1] and by X-ray diffraction at pH 6 [2].

A large single crystal of cubic insulin with a volume of 2.7 mm^3 (2.0 **x** 1.7 **x** 0.8 mm) was prepared based on the crystallization phase diagram determined by dialysis crystallization. The crystal diffracted to 2.5 Å resolution at pD 6. A total of 2,864 independent reflections was obtained with an overall R_{merge} of 15.9% from 6,975 observed reflections. The completeness of this data set was 95.2% in the range of 80-2.5Å resolution and was 93.7% ($I/\sigma>3$: 64.4%) for the outermost (2.59-2.50Å) resolution shell. The structure was refined to R-factor of 25.2% and free R of 29.9% using the diffraction data to 2.5Å resolution. Protonation status of ionizable amino acid residues at pD 6 are observed and are compared with the results obtained at pD 9. Since the pK of histidine side chains is just around 6.0, the charged statuses of .His5 and His10 in insulin at pD6 and PD9 are very interesting. It was found that only N_π of His5 in the B-chain is protonated whereas both N_π and $N\tau$ of His10 in the B-chain is protonated at pD6 and the results at pD10 are also the same. These indicate that neutron diffraction study is useful to determine the protonation status of a protein.

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NEUTRON DIFFRACTION AND HIGH RESOLUTION X-RAY CRYSTAL STRUCTURE ANALYSIS OF PHOTOACTIVE YELLOW PROTEIHN

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Hydrogen atoms are involved in many biochemical reactions during protein function. In the case of photoactive yellow protein (PYP), the protonation/deprotonation of certain amino acid residues and rearrangements in the hydrogen bond network are involved in the conformational changes of the PYP. Therefore, to understand the molecular mechanism, it is essential to determine the exact positions of hydrogen atoms. Neutron crystallography is one of the most effective methods to determine the hydrogen positions. However, it requires a large single crystal more than 1mm in each direction. We surveyed conditions for the crystallization to obtain a large crystal. It was found that a fairly large crystal of PYP ($1.5\times0.7\times0.7mm^3$) can be obtained using ammonium sulfate with sodium chloride as precipitants [1]. X-ray diffraction study was performed with the obtained large crystal at SPring-8. The crystal gave X-ray diffraction spots up to 0.84Å. Although some of the hydrogen atoms could be observed in the high resolution X-ray crystal structure, the hydrogen atoms responsible for hydrogen bonds were hardly observed. For the neutron diffraction experiment, it is necessary to replace the solvent from light water to heavy water, to reduce the background due to incoherent scattering of hydrogen. We, therefore, crystallized PYP from a buffer prepared with heavy water, but obtained only crystals with dendrite shapes even in the optimum concentrations of ammonium sulfate, sodium chloride, and PYP. However, larger crystals can be grown by optimization of pD of the solution (pD 6.9). Using BIX-4 at the JRR-3M reactor, we succeeded to obtain neutron diffraction pattern up to 2.1Å with the deuterated crystal. However it took 6hours to record the diffraction pattern for one frame. This means that 6 months are required to complete the entire set of diffraction data, which is not practical. In order to reduce the exposure time, we attempted to increase the crystal size. As a result, we succeeded to obtain crystals with larger size ($2.83\times0.83\times0.78mm^3$) under high pD (pD 9.0) condition.

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Structural Analysis of GGA (VHS-GAT) –ARF Complex Using SAXS Measurement

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GGAs (GGA1-3) are a family of clathrin adaptors involved in the protein sorting, and are recruited to the *trans*-Golgi network by a small GTPase, ARF. GGA consists of three domains, namely VHS, GAT and GAE. Each of these three domains has various functions independently in the protein sorting and transport processes, interacting with the other proteins. Although each domain structure has been determined by crystallographic studies, the domain assembly of GGA is still unclear. In order to understand the molecular mechanism of GGA, it is important to clarify the structure of GGA in the multi-domain state because the domain rearrangement upon an association with a binding partner would affect on the interaction with the other proteins.

We focused on the interactions of GGA with ARF, which is one of the initial processes of protein sorting and transport. To study the characteristics of the GGA-ARF complex in solution, we chose VHS-GAT domain construct as a model for the multi-domain state. The titration experiments of ARF with the constructs from GGA1 and GGA3 were performed using the SAXS measurement. The intensity profiles were subjected to SVD analysis and the intensity profiles of VHS-GAT-ARF complexes were obtained. Using the profiles, we then estimated the solution structure. It is indicated that GGA3-ARF exists mainly as a dimer, while GGA1-ARF is a monomer. The previous study showed that the apparent molecular weight of VHS-GAT3 is equivalent to ~1.4mer, and that of VHS-GAT1 is equivalent to monomer. The result suggests that the equilibrium of GGA3 between monomer and dimer shifts to the dimer state upon interacting with ARF. Therefore it is proposed that the most of GGA3 form a dimer even on the membrane surface while GGA1 remains to be a monomer. The different stoichiometrical manners between GGA1 and GGA3 imply the distinct roles of GGAs in the sorting and transport. We will discuss the molecular mechanism of the protein sorting and transport based on the solution structures of the complexes.

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REDOX-DEPENDENT DOMAIN REARRANGEMENT OF THERMOPHILIC FUNGAL PROTEIN DISULFIDE ISOMERASE STUDIED BY SOLUTION X-RAY SCATTERING

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Protein disulfide isomerase (PDI) is an essential catalyst of disulfide formation and isomerization in eukaryotic endoplasmic reticulum. PDI, comprising four thioredoxin-fold domains a, b, b', a' and a short segment c, has two active sites at domains a and a'. We studied redox-dependent conformational changes of a thermophilic fungal PDI by small-angle X-ray scattering. Scattering profiles of PDI show redox-dependent differences, and the apparent molecular weight of PDI estimated from the profiles in the oxidized state is about 1.1 times larger than that in the reduced state. But the radius of gyration and the maximum dimension of PDI in the oxidized states are slightly larger than that in the reduced. These results are interpreted as a redox-dependent conformational alternation accompanying a change of hydration of the PDI molecule in retaining the overall molecular shape. A low-resolution model restored from the profiles in the reduced state comprised discrete four globular domains arranged in a twisted "U" shape similar to that of the yeast PDI in the crystal (1). The a domain probably contacted with the b' domain in contrast to the crystal structure. A model in the oxidized state still had the twisted "U" shape, but the size become slightly larger than that in the reduced state and the domains separate unclearly. Thus, conformational alternation comes from the changes of interaction between the b' domain and domains with active sites.

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Structure-function relationship of scytalone dehydratase studied by X-ray crystallography of mutated enzymes

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Scytalone dehydratase (SD) of the rice blast fungus, damaging rice production, catalyses two key dehydration reactions in the melanin biosynthesis indispensable for the pathogenicity of the fungus. SD comprising 172 residues functions as a trimer, and the structure of a subunit is divided into two portions: the main body from 9 to 154 forming the active-site pocket and the C-terminal region from 155 to 172. Because the C-terminal region covers completely the active-site pocket in any SD-inhibitor complex analyzed so far, the region is expected to work as a lid. Several hydrophobic residues located around the marginal region of the active site pocket have van der Waals contacts with the C-terminal region and inhibitor molecules in the inhibited state of SD. Though the hydrophobic residues are not involved in the dehydration reaction, they are thought to be of importance for capturing and stabilizing substrate in the pocket. In the present study, we made point-mutated enzymes to exchange hydrophobic residues to alanines to understand the role of the residues. The mutated enzymes are studied by enzymatic kinetics, circular dichroic measurements of the ultraviolet region and crystal structure analyses. The mutations affect predominantly the affinity of the substrate to SD rather than the turn over rate, suggesting that the interactions are profoundly necessary to prevent the escape of substrate from the pocket before dehydration reaction. The crystal structures displayed conformational varieties of the C-terminal region at around Gly154 and the sheet region around the reaction center. Based on the results, we discuss the roles of the residues and protein dynamics in the dehydration reaction of SD.

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X-RAY SOLUTION SCATTERING STUDY OF ASSOCIATED PROTEIN, GRANCALCIN

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Grancalcin, a leukocyte-specific protein, is a member of a protein family named penta-EF-hand (PEF), which contains five repetitive EF-hand motifs [1]. Proteins in the family are characterized by their unique biological significance. X-ray scattering was performed to investigate their conformation and their associated states in solution. This protein is easily aggregated and actually scattering data showed aggregation. To analyze data, we used two-component or three-component analysis in Guinier plot as below.

$$I(h) = I_1 \exp(-\frac{R_{g1}^2 h^2}{3}) + I_2 \exp(-\frac{R_{g2}^2 h^2}{3}) \quad \text{(a)}$$

$$I(h) = I_1 \exp(-\frac{R_{g1}^2 h^2}{3}) + I_2 \exp(-\frac{R_{g2}^2 h^2}{3}) + I_3 \exp(-\frac{R_{g3}^2 h^2}{3}) \quad \text{(b)}$$

where I is intensity, Rg is radius of gyration and h is the scattering vector.

. In case of mixed solution of monomer and oligomers, the smallest Rg gives the Rg of the monomer, while the larger Rg's give the average Rg of oligomers, if they are well separated. The smallest Rg values show good coincidence between (a) and (b). Then we can estimate monomer component. By subtracting oligomers part from scattering intensity, we can get monomer intensity. Molecular shape of monomer calculated by DAMMIN program [2] is shown in Fig.1.

Fig.1. Calculated structure by DAMMIN

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Kinetic crystallography of Escherichia coli MutT: an activation mechanism of a nucleophilic water molecule

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Escherichia coli MutT specifically hydrolyzes a potently mutagenic and DNA replicational substrate, 8-oxo-dGTP to 8-oxo-dGMT in the presence of Mg^{2+} (Mn^{2+}). This event can prevent the misincorporation of 8-oxoguanine opposite adenine in DNA. Recently we have determined the crystal structures of MutT in the presence and in the absent of the reaction product 8-oxo-dGMP and shown that MutT specifically recognizes 8-oxo-dGMP through a number of hydrogen bonds to the protein and waters in the binding pocket with the large ligand-induced conformational change. The catalytic mechanism of MutT still remains unclear. In this paper we report the mechanism for 8-oxo-dGTP hydrolysis in the MutT crystal by kinetic protein crystallography.

In order to elucidate the reaction mechanism of MutT, the MutT-8-oxo-dGTP crystals were soaked in $MnCl_2$ under a variety of conditions and freeze-trapped in a nitrogen stream at 100K. The X-ray diffraction data were collected at SPring-8 and PF. When the crystals were soaked in 20mM $MnCl_2$ for 4 hours, 8-oxo-dGTP was perfectly hydrolyzed to 8-oxo-dGMP. Then many trials to catch the different reaction states of catalysis with soaking in 5mM $MnCl_2$ for various hours were performed. Thus we have succeeded in the visualization of the process for the activation of the nucleophilic water molecule synchronized with the binding of manganese ions. This reaction mechanism agrees very well with the previous mutational experiments and gives us the implications of the catalytic mechanism for the other nudix hydrolases with the MutT motif. Now in order to investigate whether the manganese cluster observed in the crystal is detected on the reaction process in the solution, we are planning to measure low temperature EPR spectra of a variety of MutT reaction states. Up to date, the time-resolved EPR spectra of MutT with Mn^{2+} ions were recorded in the presence of 8-oxo-dGTP at 277K and 288K. When 8-oxo-dGTP added to a solution including MutT and Mn^{2+}, the typical EPR spectra of mononuclear Mn^{2+} largely changed and decreased in intensity. The intensity increased as time went on, i.e, as the hydrolysis occurred. This fact suggests the existence of the manganese cluster on the hydrolysis process.

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Tetrameric Structure of Thermostable Direct Hemolysin *Vibrio parahaemolyticus*

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The thermostable direct hemolysin (TDH) is a major virulence factor of *Vibrio parahaemolyticus*. We have characterised the conformational properties of TDH by small angle X-ray scattering (SAXS), ultracentrifugation and transmission electron microscopy. Sedimentation equilibrium and velocity studies revealed that the protein is tetrameric in aqueous solvents. The Guinier plot derived from SAXS data provided a radius of gyration of 29.0 Å. The elongated pattern with a shoulder of a pair distance distribution function derived from SAXS data suggested the presence of molecules with an anisotropic shape having a maximum diameter of 98 Å. Electron microscopic image analysis of the negatively stained TDH oligomer showed the presence of C_4 symmetric particles with edge and diagonal lengths of 65 and 80 Å, respectively. Shape reconstruction was carried out by *ab initio* calculations using the SAXS data with a C_4-symmetric approximation (Fig.1). These results suggested that the tetrameric TDH assumes an oblate structure. The hydrodynamic parameters predicted from the *ab initio* model differed slightly from the experimental ones, suggesting the presence of flexible segments.

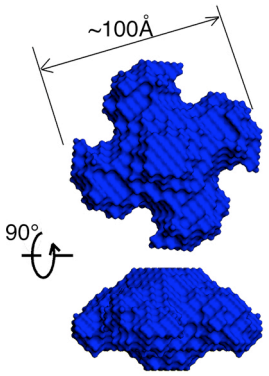

Fig. 1. Low resolution beads model of tetrameric TDH. The model has been constructed using the *ab initio* modelling program GASBOR to analyse SAXS data assuming C_4-symmetry.

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OBSERVATION OF THE SURFACE STRUCTURES OF PROTEIN CRYSTALS BY ATOMIC FORCE MICROSCOPY

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Structural changes and fluctuations of proteins are essential for their functions and are the subject of much discussion to understand structure-function relationship of proteins. Of experimental techniques to investigate structural changes and fluctuations of proteins, we are trying to detect those phenomena by atomic force microscopy (AFM). AFM scans surface structures of materials at a nano-meter resolution by using a sharp tip attached to a soft cantilever. Now, AFM equipments have been developed to measure materials bathed in aqueous solution such as biological samples.

Now, we are trying to use AFM as a probe to measure the surface structures of protein crystals in aqueous solution. This is a preliminary step to detect the structural changes and fluctuations of protein molecules located at the surface of protein crystals. When probing structural changes and fluctuations of protein molecules by AFM, protein crystals provide benefits to measure the surface structure: one can observe the same lattice plane at the crystal surface and the same orientation of the protein in every measurement. In addition, the effect as to the convolution of the probe-tip shape on the surface structure is minimized. We measured the surface structures of lysozyme crystals, as a standard sample, and glutamate dehydrogenase (GDH) crystals to examine the possibilities to detect domain movement of the enzyme at the surface of the crystals. X-ray crystallography of GDH revealed that the domain of the enzyme even in crystalline state exhibited conformational fluctuations of domains about 1nm [1,2]. Thus, the fluctuation of the domain might be detectable by an AFM measurement.

We currently measure the surface structures of the crystals by the contact mode. This measurement mode has an advantage to obtain surface images at a high resolution, the probe tip in closely contacting with sample surface, however, frequently degrade surface structures by chipping molecules away. Through a number of trials and various tuning of measurement conditions, we are now able to measure the surface structures of the two protein crystals. With a support to determine the miller indices of the lattice planes with X-ray diffraction experiments, we now resolve the orientation of individual lysozyme and GDH molecules at the surface of their crystals.

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SINGLE MOLECULE DYNAMICS OF BIOMOLECULES STUDIED BY ATOMIC FORCE MICROSCOPY

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Under physiological conditions biomolecules are dynamic in both their local and global conformations and these fluctuations play an important role in biological function. The dynamical nature of these systems implies that an understanding of the viscoelastic behaviour will help us to understand the function of these biological molecules. To address this issue, we have developed a new method for measuring viscoelastic response of single molecules based on analysis of thermally[1] or magnetically[2] driven oscillations of an atomic force microscope (AFM) cantilever. Using a simple harmonic oscillator model, the effective damping and elastic constant of a single molecule are obtained. The method was applied to the stretching of polysaccharides,[1,2] synthetic polymer[3] and protein concatamers[4] and their viscoelasticity were determined. Based on the force dependence of their viscoelasticity, the shape of the energy landscape of these biomolecules will be discussed.[5]

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UNUSUAL THERMODIFFUSION BEHAVIOR OF AQUEOUS SOLUTIONS OF POLYMERS UNDER A TEMPERATURE GRADIENT (I): DEXTRAN IN THE PRESENCE AND THE ABSENCE OF UREA

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The Ludwig-Soret effect, also called thermal diffusion or thermodiffusion, concerns the mass flows of fluid mixtures, which are induced by a temperature gradient. In a binary mixture the flux $\mathbf{J}_1$ of the component 1 is described in response to a temperature and concentration gradient [1]. The flux is given by

$$\mathrm{J}_1 = -\rho D \nabla w_1 - \rho w_1 (1 - w_1) D_T \nabla \mathrm{T}. \qquad (1)$$

Here, D is the translational mass diffusion coefficient, D_T the thermal diffusion coefficient, w the weight fraction, ρ the total mass density, and T the temperature. In a steady state with the presence of a temperature gradient, $\mathbf{J}_1$ vanishes ($\mathbf{J}_1 = 0$) and which yields

$$\frac{D_T}{D} = -\frac{1}{w_1(1-w_1)} \frac{\nabla w_1}{\nabla T}. \qquad (2)$$

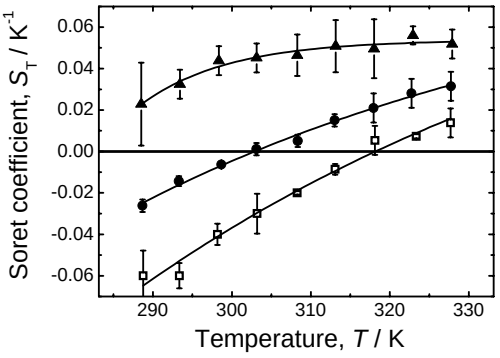

Fig. 1. Soret coefficient of 5.0 g/L dextran in urea/water. Symbols indicate 0M (□), 2M (●) and 5M (▲) urea.

The Soret coefficient S_T of component 1 is defined as the ratio of thermal and translational diffusion coefficients i.e., $S_T = D_T / D$. The value of S_T characterizes the magnitude of concentration gradient. The sign of S_T indicates the direction of flux. In the presentation, we will report the experimental results of thermal diffusion forced Rayleigh scattering (TDFRS) for the aqueous solutions of dextran in the presence and the absence of urea [2]. Figure 1 shows the Soret coefficient of dextran as a function of temperature. For 5M urea solution the S_T has positive values in the experimental temperature range, which is typical observation for polymer solutions. Whereas for 0 M and 2 M urea solutions the sign change of S_T is observed at 45.0 and 29.7 ^{o}C, respectively. The mechanisms of the negative sign of S_T and the sign change behavior of S_T will be discussed with taken into account molecular interactions.

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UNUSUAL THERMODIFFUSION BEHAVIOR OF AQUEOUS SOLUTIONS OF POLYMERS UNDER A TEMPERATURE GRADIENT (II): PNIPAM IN WATER AND IN ALCOHOLS

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Poly(*N*-isopropylacrylamide) (PNiPAM) in water has the Θ-temperature at 30.6 °C, where a coil-globule transition of the single chain takes place as a function of temperature. Since the key feature such as segment-segment or segment-solvent interactions can be varied experimentally according to the consequence of coil-globule transition of PNiPAM, it is expected that deeper understandings for contributions of molecular interactions on thermal diffusion behavior might be achieved. The experimental results of the Soret coefficient in the vicinity of coil-globule transition of PNiPAM are shown in Fig. 1 (upper). The Soret coefficient of PNiPAM shows an apparent peak at the Θ-temperature. The result indicates that the thermal diffusion behavior is associated with the interactions among segments and solvents [1]. The temperature dependence study is also carried out with the variation of alcohols (MeOH, EtOH, 1-PrOH, 2-PrOH, 1-BuOH and *tert*-BuOH) as the solvent [2]. The obtained S_T values are shown in Fig. 1 (lower). For the methanol solution (■) the sign of S_T is always positive in the experimental temperature range, which corresponds to that PNiPAM migrates to the cold side of the fluid (typical for polymers), while the sign is negative for larger alcohols. The results correspond to that the PNiPAM molecules move to the warm side of the fluid due to the temperature gradient. For ethanol solution (●), the sign of S_T changes from positive to negative at 35 °C by heating. The effect of solvent quality on the thermal diffusion behavior will be discussed in terms of Hildebrand solubility parameters.

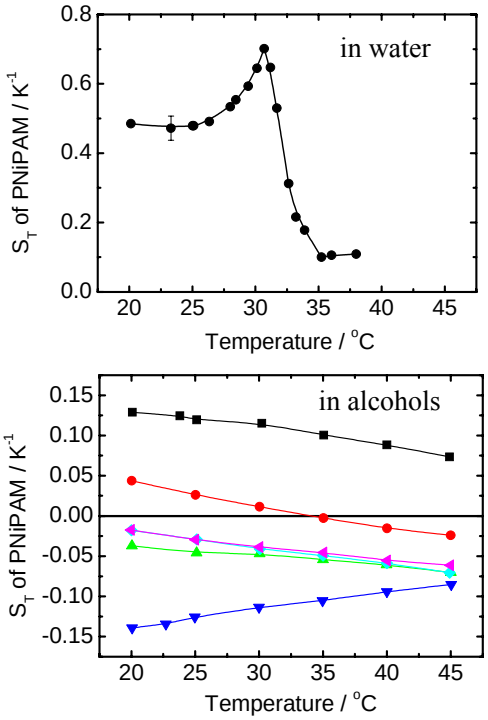

Fig. 1. Soret coefficient of PNiPAM in water (upper) and in alcohols (lower). Alcohols used in this study are MeOH, EtOH, 1-PrOH, 2-PrOH, 1-BuOH, and *tert*-BuOH from top to bottom.

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Chemotherapy with Drug-Free Hybrid Liposomes Composed of Dimyristoylphosphatidylcholine and Polyoxyethyledodecyl Ether

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We have produced hybrid liposomes (HL) which can be prepared by ultrasonicating a mixture of vesicular and micellar molecules in a buffer solution [1]. The physical properties of these liposomes such as shape, size, membrane fluidity, and the temperature of their phase transition can be controlled by changing the constituents and compositional ratios. Hybrid liposomes without drugs were also demonstrated to inhibit the proliferation of various tumor cells in vitro and in vivo [2-3]. In this study, we examined inhibitory effects of hybrid liposomes composed of phosphatidylcholine and polyoxyethylenealkyl ether on the growth of lymphoma cells *in vitro*, *in vivo* and clinical applications.

We examined morphology of HL composed of DMPC and $C_{12}(EO)_{23}$ on the basis of electron microscopy. A clear solution of HL having hydrodynamic diameter of 80-100 nm could be kept over one month on the basis of dynamic light scattering measurements. It is noteworthy that HL having diameter of 100nm could avoid the clearance by reticular endothelial system (RES) *in vivo*. We examined the fifty percent inhibitory concentration (IC_{50}) of HL on the growth of human B lymphoma cells (RAJI) in vitro. The IC_{50} values were 0.16mM for HL and 0.34mM for DMPC liposomes on the basis of the DMPC concentration, respectively. These results indicate that the inhibitory effects of HL should be advantage as compared with single-component DMPC liposomes. How do HL suppress the growth of tumor cells? Then, we tried to examine a plausible mechanism for inhibition of HL on the growth of RAJI cells. Firstly, the DNA content of RAJI cells treated with HL was measured using flow cytometer. A marked increase in the apoptotic DNA was observed in RAJI cells treated with HL. Next, we examined the nuclear DNA fragmentation with HL using agarose gel electrophoresis. It is noteworthy that exposure of RAJI cells to the hybrid liposomes caused DNA fragmentation characteristic of apoptosis. In addition, we examined induction of apoptosis by HL on the basis of confocal microscopy using staining method. The green color was also observed in fluorescence micrograph of RAJI cells treated with hybrid liposomes on the basis of TUNEL method. These results indicate that HL should induce apoptosis for RAJI cells. Furthermore, we examined therapeutic effects of HL on mice model of carcinoma in vivo. The prolonged survival of mice was obtained by the treatment with HL. It is of interest that significantly prolonged survival rate (150%) was obtained after the treatment with HL. Assessment of chronic toxicity of HL was carried out using normal rats after the injections of HL for six months. The number of red and white blood cells of rats treated with hybrid liposomes were within normal limits. In addition, all of the other biochemical data, such as ALP, GOT and GPT activities, as well as levels of albumin, urea nitrogen, creatinin, glucose, total protein, calcium, inorganic phosphorus, sodium, potassium and chloride, were the same as those observed in the controls. Furthermore, no weight loss was observed in the rats. These results indicate that HL should have no side effect *in vivo*. Clinical applications of HL without any drug for patients with lymphoma were examined after passing the committee of bioethics. The clinical diagnosis of one severe-stage patient at that time was as follows : (a) No effects of all chemotherapeutics were observed. (b) It was suggested that the future lifetime would be a few months. The prolonged survival more than one year was attained in the severe-stage patient with lymphoma after the intravenous injection of HL without any side effect. It is also worthy to note that the remarkable reduction of lymph node neoplasm (solid tumor) was obtained after the local administration of HL [4]

It is very important that chemotherapy with drug-free HL could be established without any side effects. Such successful clinical applications of HL without drugs were for the first time.

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Membrane Targeted Chemotherapy with Hybrid Liposomes for Colon Tumor Cells Leading to Apoptosis

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Molecular targeted therapeutics has attracted much attention in recent years as an efficient therapy for cancer on the basis of molecular level studies on human cells. Targeted molecules included protease, growth factor, receptor, oncogene and so on. On the other hand, it is already known that the fluidity of tumor cell membranes as molecular aggregate is generally larger than that of normal cells. However, there are few reports on therapy from the viewpoint of membrane fluidity of tumor cells.

We have produced hybrid liposomes (HL-n) which can be prepared by sonication of vesicular and micellar molecules in a buffer solution[1]. The physical properties of HL-n such as shape, size, membrane fluidity, and the temperature of phase transition can be controlled by changing the constituents and compositional ratios. In addition, HL-n inhibited the proliferation of various tumor cells along with apoptosis in vitro and in vivo[2,3]. Recently, lipid-mediated apoptosis for tumor cells has been observed[4]. However, as far as we know, there is no report on antitumor effects in relation to membrane fluidity of liposomes. In this study, we report the first successful experiment resulting in a good correlation between antitumor activity on the growth of human colon tumor cells and membrane fluidity of HL-n composed of dimyristoylphosphatidylcholine (DMPC) and 10 mol% polyoxyethylene(n)dodecyl ether ($C_{12}(EO)_n$; n = 4, 8, 10, 21, 23, 25) [5].

We examined the effect of HL-n composed of DMPC and 10 mol% $C_{12}(EO)_n$ on the growth of human colon tumor (WiDr) cells on the basis of WST-1 assay. Fifty percent inhibitory concentration (IC_{50}) of HL were 0.29 mM DMPC for HL-8, 0.25 mM DMPC for HL-10, 0.23 mM DMPC for HL-21, 0.26 mM DMPC for HL-23, and 0.30 mM DMPC for HL-25. On the other hand, IC_{50} of DMPC liposomes was greater than 1.2 mM. These results indicate that HL-n (n = 8, 10, 21, 23, 25) should be effective for inhibiting the growth of WiDr cells.

How do HL suppress the growth of tumor cells? We evaluated the fluidity of HL from fluorescence polarization (P) of 1, 6-diphenyl-1, 3, 5-hexatriene (DPH). It is worthy to note that a good correlation between the P values and IC_{50} was obtained. This indicates that HL having larger fluidity could suppress greater the growth of tumor cells. We examined further mechanisms for suppressing the growth of WiDr cells by HL. Apoptotic DNA rates for WiDr cells treated with HL using flow cytometer. HL-23 and -25 were effective for increasing apoptotic DNA rates among all the liposomes employed in this study.

Here, to get evidence about the fusion and accumulation of HL-n into WiDr cell membrane, total internal reflection fluorescence (TIRF) microscopy was examined using fluorescently labeled lipid with a IX71 Olympus fluorescence microscope. TIRF micrographs of WiDr and normal colon (CCD33Co) cells after the treatment with HL-23 including fluorescently labeled lipid are observed. Interestingly, the fluorescence intensity of fluorescently labeled lipid incorporated into HL-23 in WiDr cell membrane drastically increased after 10 min though that in CCD33Co cell was almost constant. The specific high accumulation of HL-23 in WiDr cell membrane was observed using TIRF microscopy for the first time.

In conclusion, this study demonstrated that growth inhibition and apoptosis for tumor cells by HL provides the possibility of therapy from a viewpoint of biophysical characteristics of tumor cell membranes and that we should consider both IC_{50} and apoptotic DNA rates for clinical application.

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Effect of high-frequency ultrasound on liposome solutions

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Phospholipids in water are known to form aggregates of closed sphere composed of bimolecular membrane, which is called *liposome*. Owing to its resemblance with biomembrane, it is often used as a model of biomembranes and also as the carrier of the drug delivery system. The application of ultrasound is one of the representative methods to prepare liposome solutions. The frequency of ultrasound is one of the important parameters to control various effects of ultrasounds. In previous works, however, the frequency of ultrasound used in the preparation of liposome is limited to several tens of kHz. In this work, we apply high-frequency ultrasound (480 kHz) to liposome solutions in order to seek a possibility to control the structure of liposome by frequency tuning.

The lipid used in experiment is dilauroylphosphatidylcholine (DLPC) whose concentration is 0.5 mmol / dm^3. The liposome solutions of relatively homogeneous size distribution are prepared through the extrusion method before sonication. A home-made bath-type ultrasonic reactor is used. The temperature of the solution is controlled to be 25 ^oC by flowing the thermostated water through the reactor. The frequency of the ultrasound is 480kHz, and the power consumed in the sample solution is estimated to be 13 W according to the calorimetric method. The size distributions before and after sonication is determined by the dynamic light scattering (DLS) measurement and Marquadt analysis. The solutions after sonication are also analyzed with the high-pressure liquid chromatography (HPLC) in order to examine the sonolysis of lipid molecules.

Figure 1 shows the change of the size distribution of liposome during sonication. The initially prepared liposome of 400 nm is broken to those of 100 nm by the sonication of 3 hours, and the formation of large aggregates is observed afterward. The dependences on the power and frequency of the ultrasound are under investigation, which will be presented and discussed in the workshop.

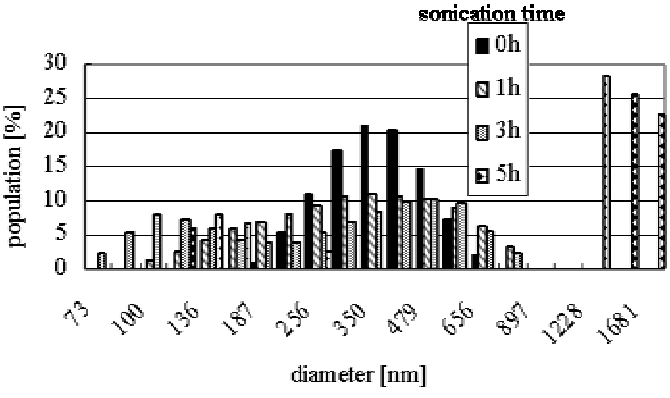

Figure 1. Size distribution of liposomes prepared from 5×10^{-4} M DLPC after filtration by 400 nm polycarbonate membrane filters

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Diffusion of Oxygen Molecules in Saccharide Solutions Measured by Fluorescence Correlation Spectroscopy.

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Saccharides such as trehalose, glucose, and sucrose are utilized as protectants by many organisms against severe dehydration and freezing. Some organisms even undergo a state of suspended animation called “cryptobiosis” to survive such hazardous conditions. Some small invertebrates can revive from years of dehydration. A group of frogs and turtles living in cold area can revive from complete freezing. In the case of freezing, ice is formed outside of the cell and the cell gets dehydrated because of the osmotic pressure. Therefore, cryptobiosis under dehydration and freezing are similar and saccharides are considered to play an important role. The saccharides work as a substituent of water and at low water concentration or at low temperature they undergo glass transition and isolate the biological substances form the outer world.

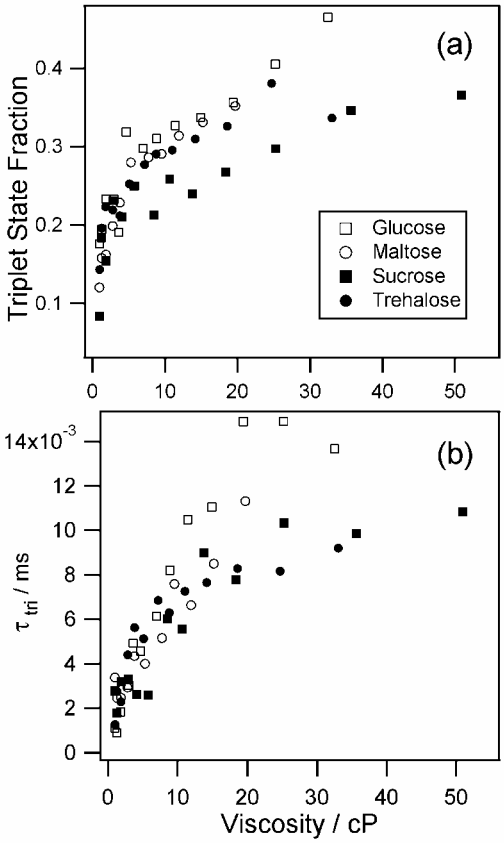

Figure 1. (a) The fraction and (b) the lifetime of the excited triplet state of rhodamine 6G in four types of saccharide aqueous solutions plotted against the solvent viscosity. The temperature was ~22 °C.

We are interested in molecular dynamics under the influence of saccharides and have been carrying out experiments of fluorescence correlation spectroscopy (FCS) in aqueous solutions of saccharides. The translational diffusion of rhodamine 6G (R6G) in saccharide aqueous solutions had a linear correlation with the viscosity as expected from the Stokes-Einstein equation. More interestingly, autocorrelation function obtained by FCS measurement exhibited a decaying component faster than the translational diffusion which was ascribed to the decay of an excited triplet state of R6G. The fraction and the lifetime, τ_{tri}, of the triplet state became larger and longer at higher saccharide concentrations (higher viscosity). These results indicate that oxygen diffusion becomes slower at higher saccharide concentrations. The triplet fraction of R6G in viscous linear alcohols was minor indicating that this effect is unique to aqueous solution of saccharides.

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LIQUID DYNAMICS AND INTERMOLECULAR INTERACTIONS IN AQUEOUS SOLUTIONS AND BIOLOGICAL SYSTEMS STUDIED BY FEMTOSECOND LASER SPECTROSCOPY

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Femtosecond laser spectroscopy has shown to be useful to investigate liquid dynamics and intermolecular interactions in condensed phases. In this poster we report our recent studies on ultrafast laser experiments such as third-order nonlinear infrared (IR) spectroscopy and time-domain terahertz spectroscopy in aqueous solutions and biological systems.

IR photon echo technique, one of the nonlinear IR spectroscopy, provides detailed information on the time scale and the magnitude of the time correlation functions (TCF) of the vibrational frequency fluctuations. The TCF for the vibration transition is considered to be sensitive to local fluctuations of the surroundings around the chromophores. We have employed IR photon echo technique to study the vibrational dephasing dynamics of ions in aqueous solutions. We found that the TCF decays biexponentially with time constants of several tens of femtoseconds and 1.5 ps. The results show that the time scale of the TCF depends only on the solvent and independent of the solute, whereas the coupling strength of the solute-solvent interaction is dependent on both the solvent and solute.

We have also performed IR pump-probe spectroscopy to study dynamics of the C=O double bonding group of acetic acid, which forms hydrogen bond with water molecules. To understand the chemical reaction in water, it is essential to obtain information on the interaction between solute and the surrounding water molecules. The interaction between the solute and the surrounding water molecules is mostly dominated by intermolecular hydrogen bonding. We measured the time-resolved difference spectra of CH_3COOD in D_2O after the C=O stretch excitation. The bleaching of the ground-state absorption band ($v=1\leftarrow0$, 1710 cm^{-1}) and the rise of the transient absorption band ($v=2\leftarrow1$, 1660 cm^{-1}) are observed. The time dependence of the absorbance change is well fitted by a double-exponential function with the identical time constants of 150 fs and 1.0 ps at all the wavenumbers for the measurements from 1610 to 1760 cm^{-1}. We obtained the spectra of the 150-fs and 1.0-ps components by global fitting. This result indicates that there are two components with different decay time and spectrum. The different spectral and dynamical properties of the C=O stretch vibration are possibly due to the presence of different solvation structures around acetic acid.

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EFFECT OF K+ AND NA+ COUNTERIONS ON DNA COMPACTION BY MULTICATIONS, BY CATIONIC NANOPARTICLES AND IN CONCENTRATED SOLUTIONS OF NEUTRAL POLYMER

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Monocations such as K^+ and Na^+ and the concentrational balance between them play a significant role in regulation of various cell processes. They are also supposed to be involved into regulation of the DNA compaction in cell. We have investigated the influence of monocation chemical nature on the efficiency of the long DNA compaction at a single-molecule level in different systems: (1) the DNA compaction in a crowding environment of neutral polymer (PEG), (2) the DNA compaction by multication (spermine), and (3) the DNA compaction by cationic nanoparticles with the sizes 15 and 100 nm. It was found that in the first system the DNA compaction is more efficient in the presence of Na^+ than K^+ salt. [1] In the second system, decompaction of DNA-spermine complex is more efficient in the case of Na^+, indicating that K^+ is more favorable for the effective DNA compaction in this system. [2] Finally, the DNA compaction by cationic nanoparticles is more effective in solutions with potassium salt with the more pronounced effect observed when smaller nanoparticles are used. These results are discussed in terms of monocation size and hydration effects in relation to electrostatic interaction with the DNA.

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PROBING BIOPOLYMER CONFORMATION BY METALLIZATION WITH NOBLE METALS

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We demonstrate different ways of silver deposition on DNA template and report the influence of silver nanostructures formation on DNA conformational state [1]. We Propose a novel method for the simple visual (colorimetric) and spectroscopic monitoring of the conformational state of a biopolymer. We present an experimental example of the detection of the change in the conformation of a giant DNA molecule [2]. This methodology is based on the difference in the manner of metallization with noble metals on a polymer scaffold depending on its conformation. Spectroscopic analysis of the metallization of DNA by metallic sliver or gold provides information on the critical concentration of DNA binder, at which the folding transition from the elongated into the compact state occurs, together with the dimension and morphology of a compact DNA condensate. This method may be suitable for use in a rapid screening procedure for the high-throughput analysis of large chemical libraries to evaluate their ability to induce DNA compaction, protein folding and similar important processes.

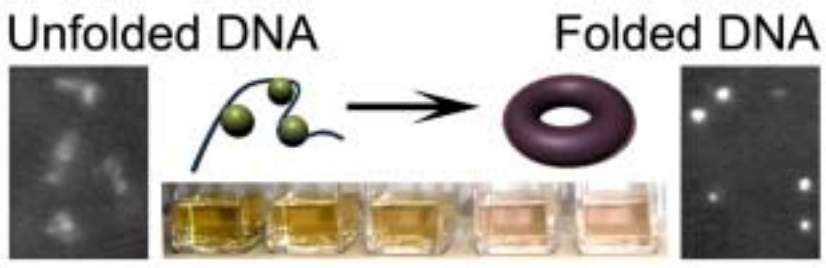

Figure. DNA high-order structure can be detected through metallization by noble metal (picture shows the example of silver metallization) at a very low concentration (10^{-9}g/L).

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Dynamics and thermodynamics of the dimerization of G-quadruplex DNA with various cations.

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A single repeat sequence of the human telomere, d(TTAGGG), has been shown to form a all-parallel G-quadruplex DNA in the presence of low K^+ concentrations ($[K^+]$), which spontaneously assembles to form a higher order structure in the presence of high $[K^+]$.[1] On the other hand, a parallel G-quadruplex DNA formed from d(TTAGGG**T**) does not form a higher order structure even if $[K^+]$ is increased, because the extra 3'-terminal thymine prevents the assembly of the G-quadruplex DNA. Our previous study using ^{1}H NMR and a size exclusion chromatography/ multi angle laser light scattering demonstrated that a series of oligonucleotide sequences, d(TTAG$_n$), where n = 3–5, formed a dimer through end-to-end stacking of the 3'-terminal G-quartets of parallel G-quadruplexes formed from these sequences (Fig. 1).[1] We report herein the effects of divalent cations such as Mg^{2+} and Ca^{2+} on the dynamics and thermodynamics of the dimerization of the G-quadruplex DNA, $(d(TTAGGG))_4$.

The present study demonstrated that Mg^{2+} promotes not only the dimerization of G-quadruplex DNA, but also the formation of G-quadruplex DNA. Mg^{2+} is likely to contribute to partial neutralization of the negative charges of DNA, which results in the promotion of the dimerization. The electrostatic interaction between Mg^{2+} and DNA backbone phosphate ions were manifested in ^{31}P NMR spectra. In addition, similarly to the case of Mg^{2+}, Ca^{2+} was also found to promote both the formation and dimerization of G-quadruplex DNA, due to the suppression of the electrostatic repulsion between monomers. These findings are useful for the molecular design of the "heme-DNA" complex.[2-5]

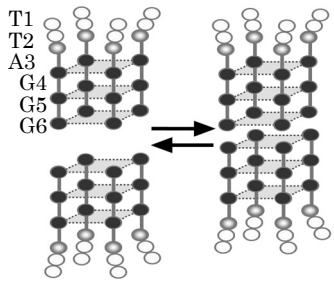

Fig. 1 Schematic representation of the dimerization of G-quadruplex DNA, $(d(TTAGGG))_4$.

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