transition rate between energy minimum states. In the present study, we applied this method to a 28-residue all- β protein named the YJQ8 WW domain (PDB ID: 1EON). The simulations were performed under three different conditions: the main-chain ϕ and ψ dihedral angles of the residues that form the β -sheet in the native structure were restrained to the ranges of $\pm 60^\circ$, $\pm 90^\circ$, and $\pm 120^\circ$ centered on β -sheet's representative dihedral angles (ϕ , ψ) = $(-120^\circ, 130^\circ)$. With these secondary structure restraints, constant-temperature MD simulations were performed for 1 μ s at 400 K. We observed the folding into the native structure most frequently during the simulation with the restraints of $\pm 90^\circ$.

2P-096 Two transient intermediates appeared in the refolding process of Fyn SH3

Two transient intermediates appeared in the refolding process of Fyn SH3

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We have reported that an alpha-helix-rich intermediate appeared on the folding pathway of src SH3. This transient intermediate is rich in alpha-helix ($[\theta]_{222}$ is $-8,700 \text{ deg}^*\text{cm}^2\text{dmol}^{-1}$) and compact (Rg is 18.5 Å). We have also reported that, in contrast to src SH3, refolding of Fyn SH3 (W37F) at pH 6 was monophasic with the rate of 76 s^{-1} at 4C, but biphasic at -20C with the rates of 32 s^{-1} and 1.1 s^{-1} in the presence of 45% ethylene glycol. We have done the refolding of Fyn SH3 with another mutant (V11S, V15E, A39V, V55F). This mutant also showed biphasic with the rates of 19 s^{-1} and 1.4 s^{-1} at -28C in the presence of 45% ethylene glycol, whereas it showed nearly monophasic with the rate of 4.5 s^{-1} at -24C. The $[\theta]_{222}$ values of the burst, the amplitude of the fast phase and the amplitude of the slow phase at -28C were -8000, 7500 and 4400 in $\text{deg}^*\text{cm}^2\text{dmol}^{-1}$, whereas those of the burst and the amplitude of the kinetic phase at -24C were -5500 and 7300 in $\text{deg}^*\text{cm}^2\text{dmol}^{-1}$. Stopped-flow X-ray scattering experiments were also done. It shows monophasic at -28C. Rg values changed from 20.7 Å to 15.7 Å with the rate of 26 s^{-1} . These results can be summarized as follows; (1) Two mutants of Fyn SH3 (W37F and V11S/ V15E/ A39V/ V55F) showed two transient intermediates (TI1 and TI2) at subzero temperatures, though both of the intermediates are formed much more rapidly than in the case of src SH3. (2) TI1 is similar in Rg and CD amplitude of those of src SH3, whereas TI2 takes the same Rg with the native state, but the half CD amplitude.

2P-097 計算機による“理想的な”蛋白質のデノボ構造デザイン

De novo computational design of "ideal" protein structure

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Computational design of an arbitrary structure from scratch provides a stringent test of our knowledge of protein folding, and allows us to determine the essential factors that lead to foldable sequences. Kuhlman et al., succeeded in designing the novel protein structure of Top7 by using Rosetta. However, it was observed that Top7 folds differently from other similarly sized naturally occurring proteins: Top7 exhibits non-native intermediates upon folding. These intermediates may be present because Top7 was designed by optimizing its sequence to stabilize the native structure, rather than optimizing the sequence in the context of the global energy landscape. This optimization strategy is unlikely to work for designing larger and more complicated structures because non-native intermediates can induce aggregation and prevent proper folding. Here, we explored a rational method of de novo structure design of $\alpha+\beta$ proteins by explicitly designing sequences that have smooth funnel-like energy landscapes. Our results suggest that secondary structure (SS) length is an important parameter for de novo structure design. Optimal SS lengths eliminate non-native topologies by restricting the backbone structure, and producing smooth funnel-like energy landscapes. Moreover, we found a rule for selecting optimal SS lengths for creating $\alpha+\beta$ proteins. By using optimized SS lengths, we were able to design an ideal structure of a ferredoxin-like fold, which was experimentally validated using CD spectrum and NMR.

2P-098 新しいさや流セルを使用した蛋白質-分子の折り畳み軌跡の観測 Direct observation of single-molecule trajectories of protein folding using a new sheath flow cell

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A protein is able to find its native structure among a myriad of unfolded structures much faster than the time scale expected for a random search. However, the mechanism of the efficient search remains unclear because of limited experimental data on the direct observation of folding dynamics. Recently, we developed a technique for the detection of fluorescence signals from free single molecules for extended time periods. Here, we further developed the single-molecule system, in which a combination of microscopic lens and sheath flow cell was utilized. The improved system enables us to detect time-series data from single molecules more effectively. We applied this system to observe unfolded-state dynamics of iso1-cytochrome c labeled with a fluorescent dye under a mild-denaturing condition. Based on the intensity and dynamics of fluorescence traces from single molecules, we classified the traces into the 2 states, I and U. We further estimated the time scale of the effective equilibration time (EET) of the I state. EET was defined as the time that the cumulative intensity distribution of the time-series data from single molecules becomes

indistinguishable from that of the ensemble average. EET of the I state was estimated to be within 5 ms, suggesting that the conformational space of the I state is much smaller than the entire space. Thus, the formation of a folding intermediate might help to suppress the unnecessary conformational search during the folding.

2P-099 The transient and equilibrium intermediates of src SH3 refolding

The transient and equilibrium intermediates of src SH3 refolding

Xianju Jin, Jinsong Li, Masaji Shinjo, Yoshitaka Matsumura and Hiroshi Kihara (Dept of Physics, Kansai Medical University)

We have reported that an alpha-helix-rich transient intermediate (TI) appeared on src SH3 refolding pathway both at pH 3 and 6 at 4C (1). We have also reported that a mutant of src SH3 (A45G) formed an alpha-helix-rich equilibrium intermediate (EI) at pH3 (2). Rg values of TI were the same (20.2 ± 0.6 Å) regardless of pH and wt/A45G within experimental errors. In contrast, $[\theta]_{222}$ values are different at pHs 3 and 6 (wt type: $-8700 \text{ deg}^*\text{cm}^2\text{dmol}^{-1}$ at pH3 and $-6500 \text{ deg}^*\text{cm}^2\text{dmol}^{-1}$ at pH 6, A45G: $-6200 \text{ deg}^*\text{cm}^2\text{dmol}^{-1}$ at pH 6). Rg and $[\theta]_{222}$ value of EI of A45G at pH 3 were 19.1 Å and $-10300 \text{ deg}^*\text{cm}^2\text{dmol}^{-1}$, respectively. Thus, it is of interest to compare TI and EI. Recently, we have done refolding of A45G at pH 3 monitored by x-ray scattering, which showed no changes within a few seconds at 4C. This indicates EI and the TI of A45G at pH3 are the same in terms of Rg. We have also done pH-jump experiment of A45G from pH 3 to pH 6. It shows no burst phases. This suggests the conformational change of A45G from its EI to the native state at pH 6 does not pass through TI appeared at pH6 refolding process. These results suggest: (1) TIs at pH3 and pH 6 take the same Rg but different $[\theta]_{222}$ value. (2) TI at pH3 and EI are the same in Rg and $[\theta]_{222}$. (3) At pH-jump of A45G from pH 3 to 6 does not pass through TI (pH 6) on its isomerization pathway.1. Li et al. (2007) Biochemistry, 46, 5072-5082.2. Li et al. (2007) J. Mol. Biol., 372, 747-755.

2P-100 固体 NMR と TEM によるヒトカルシトニンの線維形成過程の測定と形成機構解析

Fibril formation process and reaction mechanism of hCT as studied by high-resolution solid-state NMR and transmission electron microscope

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Human calcitonin (hCT) is peptide thyroid hormone, plays a central role in calcium-phosphorus metabolism and has been realized close in relation to medullary carcinoma of the thyroid. hCT is known to form amyloid-like fibril in concentrated aqueous solution. We have previously showed that a π - π stacking between benzene rings of side chains in hCT can stabilize fibrils. We, therefore, examined the effect of particular phenyl ring using hCT mutants (F19L-hCT and F16L-hCT). This allows to determine the role of specific F in fibrillation by comparing the fibrillation rate of hCT mutant with that of Wt-hCT. ^{13}C NMR and TEM experiments were performed for the fibrillations at pH3.3 and 7.4. The ^{13}C NMR signals showed that structural transition of hCT mutants at pH3.3 occurred from random coil to β -sheet and random coil near Ala26 during fibrillation. The kinetic behavior of the fibrillation of hCT mutants was analyzed, by considering the two-step autocatalytic reaction mechanism, in which the first step is a fibril nucleation (k_1) and the second step is a fibril elongation (k_2). Rate constant, k_1 s of hCT mutants are determined to be as fast as those of Wt-hCT, while k_2 s of hCTs were decreased. These results suggest that the hCT mutants pronouncedly inhibit the fibril elongation process. Moreover, TEM experiments visually showed the initial step of fibrillation in hCT and the nuclei were clearly seen in the initial step. This observation supports that hCT fibril takes the two-step autocatalytic reaction mechanism.

2P-101 固体 NMR と TEM によるグルカゴンのバイセル膜存在下でのアミロイド様線維形成機構の解明

Fibrillation mechanism of glucagon in the presence of phospholipid bicelles as studied by ^{13}C solid-state NMR and TEM

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Glucagon is a 29 amino acid peptide hormone secreted by α -cells of pancreatic islet and plays a central role in the maintenance of normal circulating glucose. Glucagon is known to form amyloidogenic fibril which is cytotoxic through the activation of apoptotic signaling pathways. To understand fibril structures and fibrillation mechanism of glucagon under aqueous acetic acid solution (pH 3.3) in the presence of phospholipid bicelles as a mimic of living cell, ^{13}C solid-state NMR and TEM measurements were performed. In the process of glucagon fibril formation, α -helix changed to β -sheet structures around N-terminal and C-terminal regions in the absence of bicells as observed in ^{13}C NMR spectra. However, in the presence of bicells, glucagon fibril showed α -helix structures around N-terminal, whereas β -sheet structures around C-terminal. ^{13}C NMR signals of fibril grew after a certain delay time, suggesting that the fibrillation could be explained by a two step autocatalytic reaction mechanism. The first step is a homogeneous nuclear formation (rate constant of k_1), and the second step is a fibril elongation (rate constant of k_2). In the presence of bicells, k_1 became larger and k_2 became smaller than the case in the presence of bicells. From the measurement of TEM, it turned out that glucagon fibril strongly interacted with

bicelles. In summary, we found that the fibril structures and fibrillation kinetics in the presence of bicells were different significantly from those in the absence of bicells.

2P-102 超分子複合体タンパク質の微小重力条件下での結晶化

Crystallization of supramacromolecular complexes of proteins under microgravity condition

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High-quality single-crystals are essential to obtain structures of proteins by means of X-ray crystallography. Crystallization of supramacromolecular protein complexes is more of a challenge than any other small protein. Crystallization in space is expected to have some significant advantages to obtain better crystals, because microgravity environment could reduce some unfavorable effects on crystallization caused by convection and sedimentation. We carried out crystallization of two supramacromolecular complexes as target proteins in space experiments. One is a kind of giant hemoglobin composed of 180 subunits with molecular mass of 3,600-kDa (complex I) and the other complex having ATPase activity consists of 8 subunits with 400-kDa (complex II). Crystallization in space was performed using the counter diffusion method and some crystals of both protein complexes were successfully obtained. As for the complex I, the maximum resolution of the crystals was improved and we succeeded in determining phases with the molecular replacement method. There was a remarkable change in the size of the lattice of the complex II. The unit-cell constants were approx. 2% shorter in all the axes than those of the crystals grown by the vapor diffusion method. These results indicate that crystallization under microgravity using the counter diffusion method is useful on the crystallization of supramacromolecular protein complexes. This work is performed in collaboration with the members of JAXA and Confocal Science, to whom the authors' thanks are due.

2P-103 分子シミュレーションによる分子モーター RuvB の分岐点移動メカニズム解析

Analysis of mechanism of branch migration by RuvB using molecular dynamics simulation

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Homologous recombination occurs in all organisms, allowing for genetic exchange, the reassortment of genes along chromosomes, and the repair of broken DNA strands. A four-stranded DNA structure called a Holliday junction (HJ) is a central intermediate state in homologous recombination, and one of the crucial steps in the process of recombination is branch migration of the HJ. In *Escherichia coli*, branch migration of the HJ is promoted by proteins RuvA and RuvB. The RuvA tetramer specifically recognizes and binds to the HJ. The RuvB hexamer works as a molecular motor to drive branch migration at the expense of the energy of ATP hydrolysis. It is thought that the constituent RuvB monomers undergo conformational changes, according to their state: ATP-bound, ADP-bound, and nucleotide-free states. To understand the relationship between the conformational changes in RuvB and the translocation of the HJ DNA, molecular dynamics (MD) simulations of the RuvA/RuvB/HJ DNA complex were carried out at a constant pressure of one bar and a temperature of 350 K. Principal component analysis was performed to analyze what kinds of concerted motions in RuvB contribute to the translocation of the HJ DNA. It was found that several low frequency modes showed the motions of the RuvB hexamer were coupled with the translational motion of DNA. These motions implied that the C-terminal domain of RuvB is important for the translation motion of DNA. The results will be discussed in detail at the conference.

2P-104 網膜前駆細胞で働く bHLH 型転写因子の in vitro 解析

In vitro analyses of bHLH transcription factors expressed in retinal progenitor cells

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The helix-loop-helix (HLH) family of transcriptional regulatory proteins play key roles in a wide array of developmental processes. Basic HLH (bHLH) transcription factors form a homo-dimer or a hetero-dimer with other bHLH factors, and control the transcription of genes by binding to a specific DNA sequence, E-box. Inhibitor of differentiation (Id) that lacks the basic domain is also important as a negative regulator of bHLH factors. We explored bHLH factors and their negative regulators expressed in retinal progenitor cells of newts, and isolated three kinds of genes encoding, HEB (E-protein), neuroD4 (ND4 or Ath3), and ID2. We prepared recombinant ID2 protein, and bHLH domains of HEB and ND4 proteins. It was suggested from gel-shift assays that recombinant HEB protein selectively form a complex with an oligonucleotides containing an E-box sequence (E-oligo). ND4 protein binds to HEB protein and forms HEB-ND4/E-oligo complex, but ND4-ND4/E-oligo complex was not observed in our experiments. At low temperature, HEB-HEB/E-oligo complex was stable, but its dissociation was observed at high temperature. Our results suggested that dynamic behaviors of bHLH-bHLH/E-box complexes were important to elucidate the construction of neural tissues.

2P-105 電子顕微鏡法による出芽酵母 Rap1 とテロメア DNA 複合体の構造解析

Structure analysis of *S. cerevisiae* Rap1-telomeric DNA complex by Electron microscopy

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Telomeres are specialized nucleoprotein complexes that cap the ends of eukaryotic linear chromosomes, stabilizing them and protecting their integrity. Telomeric DNA of most eukaryotes is composed of tandemly repeated sequence units whose number is tightly regulated. Telomeres in budding yeast *Saccharomyces cerevisiae* cells are maintained between 250 to 350 bp through a dynamic balance of lengthening and shortening activities. Normal telomere length regulation requires telomerase as well as a telomeric protein-DNA complex. Rap1 is the major telomere binding protein and binds duplex telomeric DNA. Rap1 also recruits the Rif1 and Rif2 proteins, which are negative regulators of telomere length. To understand telomere length regulation, we have observed complexes of Rap1 with a DNA array of 10 or 37 consensus Rap1 binding sites by electron microscopy, and have revealed unique helical structures. The length of these structures varied and we interpret this as varying Rap1 occupancy on a given DNA molecule. Hence the heterogeneity of complexes, we carried out electron tomography and three-dimensional reconstructions were calculated to better visualize these Rap1-DNA complexes. When we visualized Rap1-DNA complexes using DNA with 37 Rap1 binding sites, only partial occupancy of Rap1 binding sites was observed. In some images, we observed bent or coiled Rap1-DNA structures. Taking all of the structural data together, we propose a model of Rap1 binding to DNA as a clamp.

2P-106 計算機シミュレーションによる RNA 結合タンパク質の塩基配列特異的認識機構の解明

Simulation study on sequence recognition mechanism of RNA-binding protein

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The quality and quantity of RNAs in the cells are managed by RNA-binding proteins (RBPs). The RBPs can recognize and bind their target RNAs specifically and express the functions, such as stabilization and destabilization of RNA, translational control, splicing and so forth. Most of the RBPs specifically recognize RNA sequences and/or 3D structures to bind RNAs. The binding sequence and binding constant of each RBP have been determined by binding assays and RBP-RNA complex structures have been solved by X-ray crystallographic analysis and NMR. Some NMR studies reported that an RNA changed its dynamics when it bound to RBP. As RBP expresses its function when it binds to RNA, the relationship between the RNA dynamics and the mechanisms of specific recognition is of interest. However, only a few research results on the relationship have been reported. In this study, we employed molecular dynamics simulation to characterize the mechanism of specific recognition from the dynamical aspect. We attempted a systematic and global identification of the change of the complex stability and dynamics by mutations depending on the sites, the types and the number of mutation sites. We used Pumilio as a model RBP, which specifically recognizes and binds UGU triplet. It is expected that the crystal structure reflects the mechanism of recognition, because Pumilio utilizes mostly hydrogen bonds to recognize RNA bases. The details will be reported at the meeting.

2P-107 Musashi-1 が有する二つの RNA 結合ドメインによる RNA 認識機構の常磁性効果 PRE、PCS、RDC を利用した解析

A simultaneous RNA-binding mode of two RNA-binding domains of Musashi-1 analyzed by paramagnetic effects, PRE, PCS, and RDC

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Mammalian Musashi-1 (Msi1) is an RNA-binding protein that regulates differentiation of central nervous system via translational repression of its target, m-numb RNA. Msi1 consists of two tandem RNA-binding domains (RBD1 and RBD2), both of which bind to a target sequence within m-numb RNA in a highly cooperative manner. In order to gain structural insight into how RBD1 and RBD2 simultaneously recognize the target RNA, we have performed an NMR analysis based on paramagnetic effects. Three Msi1 mutants, each of which possesses a single Cys residue, were prepared. The Cys residue of each Msi1 mutant was decorated with MTSL, which carries a stable paramagnetic nitroxide radical. The long-range distance restraints (20-40 Å) between two RBDs were derived from paramagnetic relaxation enhancement (PRE) caused by the paramagnetic center. Comparison of the PRE data obtained for Msi1 in free and complexed forms showed that the two RBDs approaches to each other upon binding with RNA. Moreover, the relative location of two RBDs in the complex was successfully determined on the basis of these long-range distance restraints. In addition, rigid lanthanide binding tag was attached to the Cys residue of some of the Msi1 mutants (in collaborating with Prof. Otting), then pseudo contact shifts (PCSs) and residual dipolar couplings (RDCs) caused by lanthanide ions were recorded.