Elucidation of the Molecular Mechanisms of Protein Folding

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Kuwajima group is studying mechanisms of *in vitro* protein folding and mechanisms of molecular chaperone function. Our goals are to elucidate the physical principles by which a protein organizes its specific native structure from the amino acid sequence. In this year, they studied molecular mechanisms of the cytotoxicity of human α-lactalbumin made lethal to tumor cells (HAMLET) and other protein-oleic acid complexes, in which a protein folding intermediate forms a complex with oleic acid, and this complex has a unique apoptotic activity for the selective killing of tumor cells.

1. Molecular Mechanisms of the Cytotoxicity of Human α-Lactalbumin Made Lethal to Tumor Cells (HAMLET) and Other Protein-Oleic Acid Complexes

Although HAMLET (human α-lactalbumin made lethal to tumor cells), a complex formed by human α-lactalbumin and oleic acid, has a unique apoptotic activity for the selective killing of tumor cells, the molecular mechanisms of expression of the HAMLET activity are not well understood. Therefore, we studied the molecular properties of HAMLET and its goat counterpart, GAMLET (goat α-lactalbumin made lethal to tumor cells), by pulse field gradient NMR and 920-MHz two-dimensional NMR techniques. We also examined the expression of HAMLET-like activities of complexes between oleic acid and other proteins that form a stable molten globule state. We observed that both HAMLET and GAMLET at pH7.5 were heterogeneous, composed of the native protein, the monomeric molten globule-like state, and the oligomeric species. At pH 2.0 and 50 °C, HAMLET and GAMLET appeared in the monomeric state, and we identified the oleic acid-binding site in the complexes by two- dimensional NMR. Rather surprisingly, the binding site thus identified was markedly different between HAMLET and GAMLET. Furthermore, canine milk lysozyme, apo- myoglobin, and β_2-microglobulin all formed the HAMLET- like complex with the anti-tumor activity, when the protein was treated with oleic acid under conditions in which their molten globule states were stable. From these results, we conclude that the protein portion of HAMLET, GAMLET, and the other HAMLET-like protein-oleic acid complexes is not the origin of their cytotoxicity to tumor cells and that the protein portion of these complexes plays a role in the delivery of cytotoxic oleic acid molecules into tumor cells across the cell membrane.

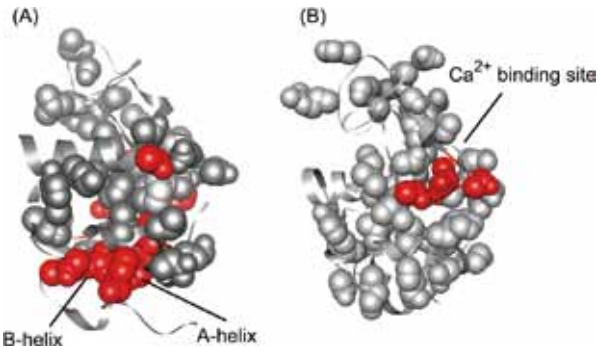

Figure 1. The oleic acid-binding sites of human α-lactalbumin in HAMLET (A) and of goat α-lactalbumin in GAMLET (B) as determined by differences in cross-peaks between the free molten globule state and the α-lactalbumin-olecic acid complex (HAMLET or GAMLET). The amino acid residues represented by the space-filling model are those whose cross-peaks in the ^{1}H-^{15}N HSQC spectra are assigned. The red residues indicate the oleic acid-binding site of each protein.

2. Native-State Heterogeneity of β_2-Microglobulin as Revealed by Kinetic Folding and Real-Time NMR Experiments

The kinetic folding of β_2-microglobulin from the acid-denatured state was investigated by interrupted-unfolding and interrupted-refolding experiments using stopped-flow double-jump techniques. In the interrupted unfolding, we first unfolded the protein by a pH jump from pH 7.5 to pH 2.0, and the kinetic refolding assay was carried out by the reverse pH jump by monitoring tryptophan fluorescence. Similarly, in the interrupted refolding, we first refolded the protein by a pH jump from pH 2.0 to pH 7.5 and used a guanidine hydrochloride (GdnHCl) concentration jump as well as the reverse pH jump as unfolding assays. Based on these experiments, the folding is represented by a parallel-pathway model, in which the molecule with the correct Pro32 *cis* isomer refolds rapidly with a rate constant of 5–6 s^{-1}, while the molecule with the Pro32 *trans* isomer refolds more slowly (pH 7.5 and 25 °C). At the last step of folding, the native-like *trans* conformer produced on the latter pathway isomerizes very slowly (0.001–0.002 s^{-1}) into the native *cis* conformer. In the GdnHCl-induced unfolding assays in the interrupted refolding, the native-like *trans* conformer unfolded remarkably faster than the native *cis* conformer, and the direct GdnHCl-induced unfolding was also biphasic, indicating that the native-like *trans* conformer is populated at a significant level under the native condition. The one-dimensional NMR and the real-time NMR experiments of refolding further indicated that the population of the *trans* conformer increases up to 7–9% under a more physiological condition (pH 7.5 and 37 °C).

3. Structural Insights into the Stability Perturbations Induced by N-Terminal Variation in Human and Goat α-Lactalbumin

Addition of an extra methionine at the N-terminus by recombinant expression of α-lactalbumin in *Escherichia coli* significantly destabilizes the protein, and this destabilization has hampered mutational analyses such as the mutational phi-value analysis of the protein. Deletion of residue 1 from the recombinant form recovers the stability in human and goat α-lactalbumin. Here, we thus determined the crystal structures of the residue 1-deletion variants of recombinant human and goat α-lactalbumin, and compared the structures with those of the authentic and recombinant forms. The results demonstrate the importance of the N-terminal backbone structure and hydrogen- bonding pattern for the stability of α-lactalbumin.

4. The H/D-Exchange Kinetics of the *Escherichia coli* Co-Chaperonin GroES Studied by 2D NMR and DMSO-Quenched Exchange Methods

We studied hydrogen/deuterium-exchange reactions of peptide amide protons of GroES using two different techniques: (1) two-dimensional ^{1}H–^{15}N transverse-optimized NMR spectroscopy and (2) the dimethylsulfoxide-quenched hydrogen-exchange method combined with conventional ^{1}H–^{15}N heteronuclear single quantum coherence spectroscopy. By using these techniques together with direct heteronuclear single quantum coherence experiments, we quantitatively evaluated the exchange rates for 33 out of the 94 peptide amide protons of GroES and their protection factors, and for the remaining 61 residues, we obtained the lower limits of the exchange rates. The protection factors of the most highly protected amide protons were on the order of 10^6–10^7, and the values were comparable in magnitude to those observed in typical small globular proteins, but the number of the highly protected amide protons with a protection factor larger than 10^6 was only 10, significantly smaller than the numbers reported for the small globular proteins, indicating that significant portions of free heptameric GroES are flexible and natively unfolded. The highly protected amino acid residues with a protection factor larger than 10^5 were mainly located in three β-strands that form the hydrophobic core of GroES, while the residues in a mobile loop (residues 17–34) were not highly protected. The protection factors of the most highly protected amide protons were orders of magnitude larger than the value expected from the equilibrium unfolding parameters previously reported, strongly suggesting that the equilibrium unfolding of GroES is more complicated than a simple two-state or three-state mechanism and may involve more than a single intermediate.

References

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