Molecular Chaperones

174-Pos

Rocking Motion of a Protein-Folding Nano-Machine Revealed By Single-Particle Cryo-Em

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The protein folding machine Methanococcus maripaludis chaperonin (Mm-cpn) is a type II archael chaperonin that has a built-in lid. It is a 16-subunit homo-oligomer of ~1 MDa arranged in a two back-to-back rings that is structurally very similar to the mammalian chaperonin such as TRiC. The substrate folding is accompanied by a conformational change triggered by nucleotide binding and hydrolysis.

Using single particle cryo-EM and image reconstruction, we solve both the wild type and lidless mutant Mm-cpn in open and closed states respectively at resolutions between 10 and 4.3 Å. The open state is a nucleotide-free state while the closed state corresponds to the transition state of ATP hydrolysis. Cα backbone models of these four 3-D reconstructions have been hand traced or flexibly fitted depending on their resolutions. The models show clearly the subunits' equatorial domain rotation between the open and closed states, which is unique and different from the well-studied type I chaperonin (GroE) found in E.Coli.

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The Group II Chaperonin Mm-Cpn Binds and Refolds Human Gamma D Crystallin

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The aggregation of damaged or misfolded proteins is associated with a number of human diseases, including Alzheimer's disease, Huntington's disease, and cataract. In this study, we investigate the ability of the Group II chaperonin from Methanococcus marapaludis, Mm-Cpn, a homolog of the eukaryotic chaperonin TRiC, to bind and refold human γD crystallins. Crystallins are a family of structural proteins found in the lens of the human eye, and aggregation of these proteins is thought to be the cause of cataract. Mm-Cpn interactions with both wild type HγD-Crys, and damage and disease model mutant HγD-Crys were evaluated. Solution turbidity studies indicate that Mm-Cpn suppresses aggregation of both wild type and disease model mutant HγD-Crys, and exhibits a greater affinity for the destabilized mutant HγD-Crys. In addition, size exclusion chromatography and fluorescence spectroscopy show that Mm-Cpn can refold HγD to a native like state, as well as form a long-lived Mm-Cpn/HγD complex with both the wild type and mutant HγD crystallins. This long-lived complex may be ideal for imaging of the chaperonin/substrate complex by cryo-EM or x-ray crystallography. These data suggest that the Mm-Cpn/HγD interaction may reveal aspects of the mechanism of binding and refolding of beta-sheet domains by the Group II chaperonins.

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Chaperone Interactions of the Small Heat Shock Protein Human αb-Crystallin With Its Physiological Substrate γd-Crystallin and Its Isolated Domains

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α-crystallin, a small heat shock protein chaperone, is one of the ubiquitous crystallins in the vertebrate lens, along with the βγ-crystallins. It is a polydisperse complex of ~800 kD consisting of two subunits (~20 kD) αA- and αB-crystallin (αA- and αB-Crys). Its chaperone activity involves suppressing aggregation by binding aggregation-prone species. Aggregates isolated from mature-onset cataracts, the major cause of blindness worldwide, contain damaged and misfolded forms of βγ-crystallins. The γ-crystallins are structural, monomeric proteins that consist of four Greek-key motifs organized into two domains. Human γD-crystallin (HγD-Crys) is a stable and long-lived mammalian γ-crystallin localized in the lens nucleus. It can refold *in vitro* to its native state after unfolding in high concentrations of GdnHCl. However, at very low denaturant concentrations (< 1 M GdnHCl) aggregation of refolding HγD-Crys intermediates competes with productive refolding. We have previously determined that the conformation of the bound HγD-Crys substrate in γD-αB complexes resembles the partially folded intermediate populated during refolding/unfolding equilibrium experiments, which has its N-terminal domain unfolded and its C-terminal domain folded. We have utilized single domain constructs to further characterize the binding interactions of HαB-Crys to different regions of HγD-Crys. The HγD-Crys C-terminal domain construct (γD-Ctd) aggregates upon refolding, while the N-terminal domain construct (γD-Ntd) does not aggregate under similar conditions. However, when γD-Ctd and γD-Ntd are unfolded and refolded together, γD-Ctd recruits γD-Ntd into the aggregate. HαB-Crys can suppress the aggregation of the γD-Ctd and forms γD-Ctd-αB complexes. Using a no-Trp mutant of HαB-Crys (W9F/W60F), we have determined, through the fluorescence emission of γD-Ctd tryptophans, that the γD-Ctd in the γD-Ctd-αB complexes is partially folded. These results provide further insight into how α-crystallin interacts with aggregation-prone substrates *in vivo*.

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Identification of a Consensus Motif in Substrates Bound by a Type I Hsp40

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Protein aggregation is a hallmark of a large and diverse number of conformational diseases. Molecular chaperones of the Hsp40 family (Escherichia coli DnaJ homologs) recognize misfolded disease proteins and suppress the accumulation of toxic protein species. Type I Hsp40s are very potent at suppressing protein aggregation and facilitating the refolding of damaged proteins. Yet, the molecular mechanism for the recognition of nonnative polypeptides by Type I Hsp40s such as yeast Ydj1 is not clear. Here we computationally identify a unique motif that is selectively recognized by Ydj1p. The motif is characterized by the consensus sequence GX[LMQ]{P}X{P}{CIMPVW}, where [XY] denotes either X or Y and {XY} denotes neither X nor Y. We further verify the validity of the motif by site-directed mutagenesis and show that substrate binding by Ydj1 requires recognition of this motif. A yeast proteome screen revealed that many proteins contain more than one stretch of residues that contain the motif and are separated by varying numbers of amino acids. In light of our results, we propose a 2-site peptide-binding model and a plausible mechanism of peptide presentation by Ydj1p to the chaperones of the Hsp70 family. Based on our results, and given that Ydj1p and its human ortholog Hdj2 are functionally interchangeable, we hypothesize that our results can be extended to understanding human diseases.

178-Pos

Investigating Interactions Between the Hsp90 Molecular Chaperone and Unfolded Protein Substrates

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The ubiquitous protein chaperone Hsp90 plays an integral role in cellular homeostasis and protein folding by interacting with substrate proteins. Only recently have three-dimensional structures of the full-length Hsp90 been determined and a reaction cycle been proposed. Despite this achievement, remarkably little is known about the molecular basis for substrate interactions. To investigate this issue, I am utilizing a well-studied model of an unfolded protein, Δ131Δ, a fragment of staphylococcal nuclease. Using small angle x-ray scattering and structure-based fitting we have found that Hsp90 undergoes an open/closed conformational change in the presence of Δ131Δ. NMR measurements of Δ131Δ and domain fragments of Hsp90 have indicated the interaction location on both the chaperone and the substrate. We use this information to build a mechanistic model of how Hsp90 interacts with unfolded protein substrates and how different nucleotide states influence these interactions.

179-Pos

Insertion of Hsp70 Into Membranes Correlates With the Flipping of Phosphatidlyserine Across the Lipid Bilayer

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Expression of heat shock proteins is the primary event in the cellular response to stress. Indeed, these proteins are crucial in preventing cell death and recovery after different physiological and environmental stresses. Hsp70 (Hsp72), which is the major stress-inducible member of the heat shock protein family, is primarily located in the cytosol. However, recent evidence has shown that this protein can be detected on the cell surface of transformed cells inserted into the plasma membrane. Hsp70 does not contain any hydrophobic domains that could predict its insertion into membranes. Consequently, the possible mechanism for translocation into membranes is likely a non-classical process. Pure recombinant Hsp70 was incubated with phosphatidylserine (PS) liposomes, and a concentration-dependent incorporation of the protein into the bilayer was observed. On the contrary, Hsp70 did not get incorporated into phosphatidylcholine (PC) liposomes. Liposomes made of a PS:PC mixture

showed that insertion of Hsp70 into the bilayer was proportional to the PS concentration. In contrast, Hsp90 did not incorporate into PS liposomes. Hsp70 was found integrated into the lipid bilayer as demonstrated by lack of extraction by sodium carbonate or sonication treatment. Hsp70 inserted into PS liposomes could only be solubilized by non-ionic detergents. In cells, the presence of Hsp70 on the plasma membrane correlates with the flipping of PS to the outside of the membrane. These results demonstrate that Hsp70, which does not contain a predictable hydrophobic trans-membrane region, can spontaneously get inserted into a lipid environment by a process that may require the traslocation of PS across the lipid bilayer.

180-Pos

Single-Molecule Imaging of 1:2 Groel-Groes Complexes in Zero-Mode Waveguides

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GroEL is an *Escherichia coli* chaperonin which is composed of two heptameric rings. GroEL interacts with its cofactor GroES and assists protein folding in an ATP dependent manner. Because of negative cooperativity between two rings of GroEL in the binding of ATP, it has been generally believed that an asymmetrical 1:1 complex is only a functional form for over a decade. Contrary to the belief, we revealed that a symmetrical 1:2 GroEL-GroES complex can be formed in the presence of denatured protein using fluorescence resonance energy transfer (FRET) and fluorescence correlation spectroscopy (FCS). However, the dynamics of GroEL-GroES interaction including a 1:2 GroEL-GroES complex is still unclear. To clarify this issue, 1:2 GroEL-GroES complexes were observed using zero-mode waveguides (ZMWs) at a single molecule level. ZMWs are nanoholes array fabricated in a thin metal film. They can reduce excitation volume compared to total internal reflection illumination; therefore, they make it possible to observe individual 1:2 GroEL-GroES complexes at sub-μM concentrations that are required to form the complexes. Cy3-GroES and Cy5-GroEL binding to and dissociating from Alexa488-GroES immobilized on the bottoms of ZMWs were visualized. Cy3-GroES and Cy5-GroEL were co-localized in GroES-immobilized ZMWs for ~ 3 s. The duration time in ZMWs without immobilizing Alexa488-GroES, which reflected non-specific adsorption of GroEL-GroES complex to the bottoms of ZMWs, was ~ 1 s. These results showed that 1:2 GroEL-GroES complexes were successfully observed at a single-molecule level and their duration time was estimated to be ~3 s. Furthermore, the same experiment was carried out in the absence of denatured protein. The duration time of Cy3-GroES and Cy5-GroEL in Alexa488-GroES-immobilized ZMWs was ~1 s. This result indicated that 1:2 GroEL-GroES complexes disappeared in the absence of denatured protein, being consistent with FRET and FCS experiments.

181-Pos

Crystal Structure of *Drosophila* Unc-45, a Putative Myosin Chaperone

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UNC-45 is a chaperone that may aid in folding myosin's motor domain. Mutations in UNC-45 cause muscle defects and dysfunction, due to the importance of myosin in muscle structure and contraction. UNC-45 is composed of an N-terminal tetra-tricopeptide (TPR) domain, a C-terminal UCS (UNC-45/Cro1/She4 homology) domain, and a central region that links these. Here we present the crystal structure of *Drosophila* UNC-45 (dUNC-45), which should serve as a basis for attaining a detailed understanding of its mechanism of action. Bacterially expressed recombinant His-tagged dUNC-45 was purified sequentially using immobilized metal affinity chromatography and size exclusion chromatography. The protein eluted as a single peak, indicating a homogeneous population of protein suitable for crystallization. Crystals were prepared by hanging drop vapor diffusion and x-ray diffraction data were collected to a limit of 3.0 Å resolution. For phase determination, a seleno-methionine derivative dUNC-45 crystal was prepared for single wavelength anomalous dispersion (SAD) experiments. Synchrotron data were collected at the Berkeley National Laboratory Advanced Light Source. The diffraction data were processed in HKL2000. Selenium positions were determined and refined in Phenix. The resulting structure was refined against native data using Phaser and maximum-likelihood refinement with Refmac5. Model building was performed in COOT. Our current model has *R*-cryst and *R*-free values of 0.24 and 0.28 respectively. The TPR domain is not visible in the model, likely due to flexibility of the domain within the crystal. Overall, the UCS domain is composed of α-helices that form armadillo repeats which stack together to form a supercoiled structure. Based on our structure, it appears that the central and UCS domains make up a single structural unit. Currently, we are pursuing structure-based biochemical assays to pinpoint the protein surfaces that are involved in the binding and chaperone activities of dUNC-45.

182-Pos

Clpxp Degradation of Proteins Probed By Single-Molecule Fluorescence

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ClpXP is an AAA+ protease that unfolds and degrades target proteins. ClpX, a hexameric ring-shaped ATPase, recognizes specific proteins and then powers their mechanical denaturation and translocation into the degradation chamber of ClpP where polypeptide bond cleavage occurs. Although ClpXP degradation activities have been widely studied at the bulk solution level, the operating principles and detailed mechanisms of this complex macromolecular machinery remain unanswered. Here, we probe the kinetics of substrate unfolding and degradation by ClpXP using a single-molecule fluorescence assay. These assays employ a covalently crosslinked ClpX hexamer, immobilized on PEG coated surface illuminated by total internal reflection fluorescence. A series of substrates are engineered to contain fusion of an N-terminal Cy3 and a C-terminal GFP-titin-ssrA module. In the presence of ATPγS, ClpX stalls at GFP after degradation of titin-ssrA domains. These stalled pre-engaged substrates are stably bound to ClpXP even in the absence of Mg^{++}, but are released quickly upon the introduction of nucleotide-free solution. Exchange into ATP for pre-engaged substrate-ClpXP complexes allows synchronous resumption of unfolding and degradation of GFP and any following domains. The time required for complete degradation is measured by loss of the N-terminal Cy3 from the protease complex. GFP unfolding can also be monitored directly with quenching of intrinsic fluorescence by denaturation. Global fitting of single-molecule data for a set of related substrates yields time constants for ClpX unfolding, translocation, and a terminal step which may involve product release, and shows strong agreement with bulk solution measurements. It should be possible to extend these methods to allow single-molecule studies such as FRET for real-time assays of ATP-fueled conformational changes that drive the mechanical operations of the ClpXP protease. Support from the NSF Career Award (0643745) is gratefully acknowledged.

Protein-Ligand Interactions I

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Efficacy As An Intrinsic Property of the M2 Muscarinic Receptor in Its Oligomeric State

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G protein-coupled receptors exhibit a characteristic dispersion of affinities for agonists, and the breadth of that dispersion is correlated with efficacy. The implied heterogeneity is commonly attributed to a ligand-regulated transient complex between the receptor and the G protein; that is, agonists bind with higher affinity to the complex than to the receptor alone. Such an arrangement is at odds with observations that the receptor-G protein complex is less transient than required by such models, and that GPCRs exist as oligomers. The present investigation has been directed toward the alternative notion that heterogeneity emerges as a property of the oligomer and is independent of G proteins. M_2 muscarinic receptors were purified as monomers from *Sf*9 cells and reconstituted as tetramers devoid of G proteins in phospholipid vesicles (POPC/POPS/cholesterol). The antagonists *N*-methylscopolamine and quinuclidinylbenzilate recognized a single class of sites in assays with *N*-[^{3}H]methylscopolamine; in contrast, seven agonists recognized at least two classes (log K_H and log K_L). The magnitude of that dispersion was quantified empirically as the product of the difference in affinity for the two classes ($\Delta\log K$) and the fraction exhibiting higher affinity (F_H). The value of $F_H\Delta\log K$ equals the area between the observed curve and a curve of equal amplitude and a single affinity corresponding to log K_L. It therefore prefigures the shift that could be effected by GTP acting via a G protein, were it present. The values of $F_H\Delta\log K$ generally were higher for full agonists than partial agonists. This distinction among agonists and antagonists recalls the propensity of those ligands to elicit a response. It follows that the heterogeneity revealed by agonists, which is predictive of efficacy, is a property intrinsic to the receptor in its oligomeric state.