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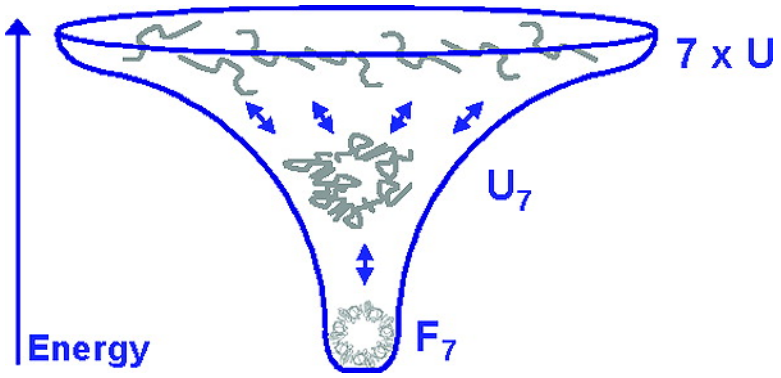

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Unfolding of Heptameric Co-chaperonin Protein Follows "Fly Casting" Mechanism: Observation of Transient Nonnative Heptamer

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Protein−protein interactions, resulting in transient or long-lived homo- or heterooligomers, play an essential role in many biological functions but sometimes also result in disease.[1−3] Thus, revealing the dynamic principles for protein−protein assembly, and how it may couple to protein-structural changes, is crucial for the understanding of protein signaling, function, malfunction, and drug design. Proposed mechanisms for formation of oligomeric proteins include "induced-fit" (i.e., flexible recognition), "lock-and-key" (i.e., rigid-body docking), and "conformational-selection" (i.e., recognition of preselected conformers) scenarios.[1,4−7] While these three mechanisms assume the presence of almost completely folded monomers before association, another possibility, the "fly casting", mechanism was recently proposed.[8] In this scenario, recognition between unfolded polypeptides results in enhanced speed of assembly since it provides a greater capture radius than that of a folded protein. However, subsequent simulations on a large set of dimers demonstrated that a strong "fly casting" effect was only detected for one protein,[4] suggesting this scenario to be rare.

The homoheptameric co-chaperonin protein 10 (cpn10)[9−12] is an attractive model for studies of the interplay between folding and assembly when the molecularity is high (i.e., seven). Cpn10 (GroES in *Escherichia coli*) normally functions as the cap to cpn60 (GroEL in *E. coli*) in which substrate folding is facilitated.[13] In addition, human mitochondrial cpn10 is identical to an immunosuppressive growth factor found in maternal serum,[14] and it is overexpressed during carcinogenesis[15] and in several protein-misfolding diseases.[16] The structure of cpn10 appears conserved in all organisms: in the heptamer, each cpn10 monomer adopts an irregular β-barrel topology. The dominant interaction between subunits is an antiparallel pairing of the first β-strand in one subunit and the final β-strand in the other subunit.[17]

The majority of the overall stability of the human mitochondrial cpn10 heptamer comes from interface interactions.[18] Cpn10 monomers can adopt folded structures in solution but exhibit marginal stability.[19] Equilibrium unfolding of cpn10, induced by guanidine hydrochloride (GuHCl) or heat, is a reversible, apparent two-state reaction that results in unfolded monomers.[18] In contrast, non-native heptamers become populated when urea is used as denaturant.[18,20] We here address the presence of such a non-native species on the kinetic free-energy landscape that connects folded heptamers with unfolded monomers. Since the GuHCl-induced equilibrium curves are strongly protein-concentration dependent,[18] complete Chevron plots are unfeasible; therefore, we have focused on the unfolding/dissociation side.

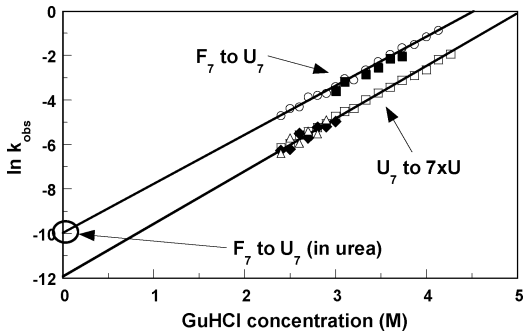

Figure 1. Semi-log plot of cpn10 unfolding/dissociation kinetics as a function of the GuHCl concentration.[21] (■) wild-type fluorescence, fast phase; (□) wild-type fluorescence, slow phase; (○) Phe90Trp fluorescence; (◆) wild-type $CD_{230\ nm}$; (△) wild-type $CD_{217\ nm}$, slow phase; fast $CD_{217\ nm}$ not shown. For comparison, extrapolated ln k_{obs} for $F_7 \rightarrow U_7$ in urea[20] is indicated.

The kinetics of cpn10 unfolding/disassembly induced by GuHCl was probed by manual and stopped-flow mixing experiments.[21] Progress of the reaction was monitored by far-UV CD and aromatic fluorescence, tools previously applied in equilibrium experiments.[18] The kinetics is biexponential when measured by tyrosine fluorescence (30% of amplitude, fast phase; rest in slow phase) and $CD_{217\ nm}$ (>55% amplitude in fast phase; rest in slow phase), but single-exponential (only slow phase) when probed by $CD_{230\ nm}$ (Figure 1). While $CD_{217\ nm}$ probes secondary structure, both tyrosine fluorescence and $CD_{230\ nm}$ report on tertiary interactions near cpn10's three tyrosines; since these are situated on, or near, the interfaces, interprotein interactions dominate the latter signals.[18] To directly isolate the unfolding step, we used a cpn10 variant with a tryptophan engineered into the core (Phe90Trp) that specifically senses polypeptide unfolding.[19] For this variant, which has wild-type stability,[19] there is only a fast fluorescence phase. Taken together, the data demonstrate that the first phase is an unfolding step (i.e., $F_7 \rightarrow U_7$), whereas the second step involves disassembly coupled to unfolding of the interface β-strands (i.e., $U_7 \rightarrow 7 \times U$). In accord with mechanistic reversibility, kinetic refolding/assembly experiments at a few accessible GuHCl concentrations are also biexponential processes.

The unfolding/disassembly reaction for human cpn10 was also investigated by high-temperature molecular dynamics (MD) simulations.[22] To induce denaturation, the temperature was raised 50 K at the beginning of every ns, for 10 ns, starting at 298 K. In all monomers, the reaction begins with unfolding of core β-strands while most of the interface β-strands remain paired. Not until the

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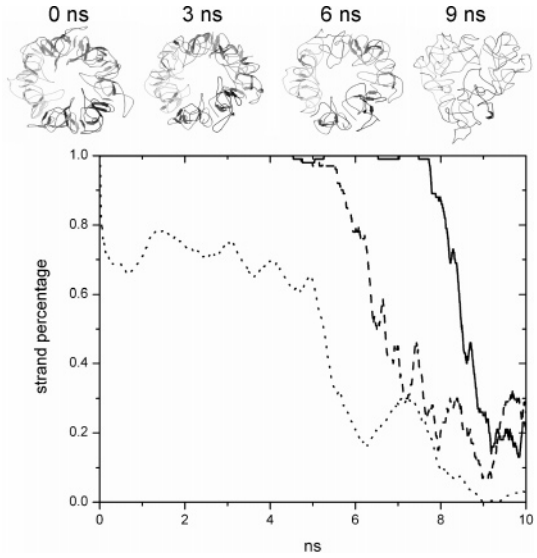

Figure 2. (Top) MD snapshots of cpn10 heptamer heating.[22] (Bottom) Secondary structure analysis of interfaces (dashed and solid lines; N- and C-terminal interfaces, respectively) and interior (dotted line) in a representative monomer as a function of simulation time (temperature). Curves were smoothed by taking averages of 100 1-ps frames. Same trends are observed for all monomers. The initial drop (in dotted line) is due to the dynamic equilibration at 298 K.

end of the 10-ns simulation, do the interfaces break apart, resulting in complete denaturation (Figure 2 and Supporting Information (SI)).

The qualitative agreement between MD simulations and time-resolved spectroscopy, together with apparent reversibility of the process, strongly suggests that cpn10's folding/binding free-energy landscape involves a transient on-path intermediate that has the characteristics of an unfolded heptamer. In addition, the rate constant extrapolated to water for the fast phase matches the unfolding rate constant reported for cpn10 unfolding in urea (extrapolated to water)[20] which quantitatively supports that the fast step is unfolding without disassembly. Since the interfaces provide almost 90% of the overall thermodynamic stability of the cpn10 heptamer,[18] it appears that the thermodynamic properties of the system define the shape of the kinetic folding/binding free-energy landscape.

The ability of unstructured cpn10 monomers to recognize each other before folding may be rationalized by the high plasticity of the interfaces: all interfaces in the GroES crystal structure differ slightly from each other in terms of side-chain and backbone positions,[17] and a cpn10 variant was recently shown to accommodate a large structural change at the interface without loss of either heptamer specificity or affinity.[23]

In summary, we here demonstrate that a non-native cpn10 heptamer, previously only observed as a dead-end species in equilibrium urea experiments, is an obligatory intermediate on the kinetic path between folded heptamers and unfolded monomers. This work has several fundamental as well as biological implications: (i) It suggests a minimally frustrated funneled-shape folding/binding free-energy landscape that is wide at the top but abruptly narrows (upon assembly) before approaching the folded heptamer at the bottom of the funnel. (ii) It demonstrates for the first time the applicability of the "fly casting" folding/binding mechanism to a homoheptamer. Earlier simulation work searched for this behavior, which has both functional and kinetic advantages, only among oligomers with molecularity of two.[4] (iii) Our understanding of the importance of unfolded proteins in the cell is constantly growing.[24,25] In the case of cpn10, interfaces that are in principle "glued together" may be a requirement to ensure efficient cycling on and off the cpn60 oligomer. (iv) Finally, coupling between folding and binding could allow cpn10 to interact with many targets. Such "fly casting" interactions may facilitate cpn10's roles in pregnancy and disease, for which little is known.

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Supporting Information Available: Technical details of simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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