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CHAPERONIN FUNCTION DEPENDS ON STRUCTURE AND DISORDER IN CO-CHAPERONIN MOBILE LOOPS

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Co-chaperonins from diverse organisms exhibit mobile loops which fold into a β hairpin conformation upon binding to the chaperonin. GroES, Gp31, and human Hsp10 mobile loops exhibit a preference for the β hairpin conformation in the free co-chaperonins, and the conformational dynamics of the human Hsp10 mobile loop appear to be restricted by nascent hairpin formation. Backbone conformational entropy must weigh against binding of co-chaperonins to chaperonins, and thus the conformational preferences of the loops may strongly influence chaperonin-binding affinity. Indeed, subtle mutations in the loops change GroEL-binding affinity and cause defects in chaperonin function, and these defects can be suppressed by mutations in GroEL which compensate for the changes in affinity. The fact that high-affinity co-chaperonin binding impairs chaperonin function has implications for the mechanism of chaperonin-assisted protein folding.

1. Structure and Dynamics in Co-chaperonin Mobile Loops

1.1 The GroEL/GroES Complex

The co-chaperonin mobile loop provides an extraordinary example of a disordered ligand-binding site in a protein. In the chaperonin complex, a ring of seven mobile loops displayed on one side of the co-chaperonin binds to a ring of seven apical domains on the ATP-bound chaperonin, thereby closing the protein-folding chamber. In the free co-chaperonin, the mobile loops are nearly as disordered as expected for a random coil polypeptide, yet substantial evidence points to a highly specific and sensitive interaction with the chaperonin.

NMR and crystallographic studies of the *Escherichia coli* co-chaperonin GroES and chaperonin GroEL show that the mobile loop binds to GroEL in a well defined hairpin conformation. The NMR structure was obtained by molecular dynamics and simulated annealing constrained by internuclear distances derived from transferred nuclear Overhauser effects (trNOEs) in a synthetic mobile loop peptide[1]. The crystallographic study revealed the complete GroES-GroEL-ADP_7 complex[2]. Although the conformations differ in detail, both studies show the GroEL-bound loop as forming a hairpin turn centered on a conserved glycine residue and bound to GroEL by three hydrophobic residues on one side of the hairpin.

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                        10          20        30        40
E. coli          MNIRPLHDRVIV    KRKEVETKSAGGIVLTGSAAA KSTRGEVLAVGNG...
M. leprae     MAKVKIKPLEDKILV    QAGEAETMTPSGLVIPENAKE KPQEGTVVAVGPG...

                   10        20        30        40        50
T4          MSEVQQLPIRAVGEYVILVSEPAQAGDEEVTESGLIIGKRVQGEVPELCVVHSVGPD...

                    10          20        30        40        50
human        AGQAFRKFLPLFDRVLV    ERSAAETVTKGGIMLPEKSQG KVLQATVVAVGSG...
yeast     MSTLLKSAKSIVPLMDRVLV    QRIKAQAKTASGLYLPEKNVE KLNQAEVVAVGPG...
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Figure 1: Alignment of an N-terminal Portion of Co-chaperonin Sequences. Residues identified as mobile by NMR are underlined. Three residues conserved as hydrophobic and implicated in GroEL binding are highlighted. A glycine residue immediately preceding the hydrophobic tripeptide also is conserved.

1.2 Conservation of Disorder in Co-chaperonin Mobile Loops

Mobility has been conserved in the chaperonin-binding loops of co-chaperonins from divergent species. The three-dimensional structure of *E. coli* GroES[3], bacteriophage T4 Gp31[4] and human Hsp10 (Hsp10) (J.F. Hunt, B.J. Scott, L. Henry, J. Guidry, S.J. Landry and J. Deisenhofer, unpublished) is conserved throughout their roughly 100-residue lengths. Nevertheless, only a few features of mobile loop primary structure are universally conserved (Fig. 1). The length of the mobile loop is exactly conserved in all known co-chaperonins except Gp31 in which the loop is five residues longer. Mobile loops are slightly hydrophilic overall, and they contain a central glycine residue followed by the three GroEL-binding hydrophobic amino acids. The H^{α} chemical shifts of almost all loop residues in each of the co-chaperonins are similar to values observed for the same amino acids in model random-coil peptides[1,5,6]. NMR relaxation measurements indicate that the mobile loop of the human co-chaperonin is highly dynamic, experiencing motion on timescales consistent with fluctuations of large amplitude[6]. In the co-chaperonin crystals, mobile loops generally are disordered unless trapped by lattice contacts.

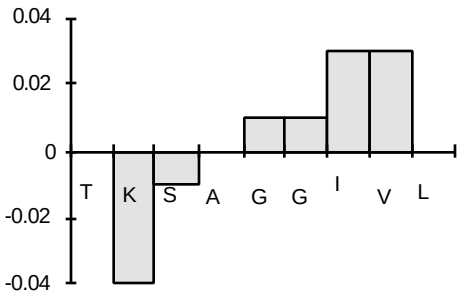

Figure 2: Change in Chemical Shift for H^{α} of the Central Nine Residues of the GroES Mobile Loop Upon Temperature Increase from 12 to 32 °C. Residues exhibiting the largest changes (K20, I25, and V26) participate in cross-strand interactions in the GroEL-bound hairpin conformation. NMR data were recorded as described.[5]

1.3 Nascent Structure in the Mobile Loop

The co-chaperonin mobile loop is poised for binding to the chaperonin by preferentially sampling the bound conformation. Residues forming the presumptive hairpin turn of GroES, Gp31, and Hsp10 exhibit prominent NH/NH nuclear Overhauser effects (NOEs), suggesting that the mobile loops preferentially sample a turn at this position, and these NOEs coincide with a strong trNOE in the chaperonin-bound GroES mobile loop peptide[1,6]. Although GroES mobile loop H^{α} chemical shifts are similar to the random-coil values, the temperature dependence of the H^{α} chemical shifts of K20, I25 and V26 indicates that structure is present (Fig. 2). These residues could be sensitive to formation of the GroEL-bound hairpin conformation because their sidechains are involved in cross-strand interactions (Fig. 3). Similar indications of nascent hairpin structure were observed for the Hsp10 mobile loop.[6] First, the H^{α} chemical shifts of V25, T26 and M31 deviate by more than 0.1 ppm from random-coil values toward that of an extended conformation, and these deviations are reduced at elevated temperature. Second, NH/H^{α} coupling constants for residues flanking the hairpin turn, E23-T26, I30 and M31, deviate toward values expected for extended structure. Third, the NMR relaxation profile indicates a local restriction of motion in the turn-forming sequence, and this restriction dissolves at high temperature (see below).

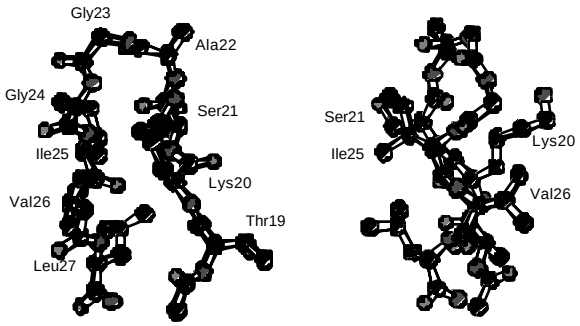

Figure 3: GroEL-bound Hairpin Conformation for the Central Nine Residues of the GroES Mobile Loop Determined by trNOE NMR Spectroscopy with a Synthetic Peptide.[1] Cross-strand interactions between S21 and I25 and between K20 and V26 could stabilize the hairpin conformation. The view at right is rotated 90° along the vertical axis relative to the view at left.

1.4 Simulating Mobile Loop Dynamics

To have some idea of the dynamics profile of the loop as a random coil, molecular dynamics of the Hsp10 mobile loop were simulated *in vacuo* using Discover (MSI) with the CVFF force field and an annealing protocol. The simulation started with the loop in the conformation trapped in the GroES crystal lattice[3]. All non-loop atoms of a single GroES subunit were included in the simulation but fixed. Ten structures were generated, and the average root-mean-square deviation (RMSD) from the average structure was calculated for the amide nitrogen of each residue in the

mobile loop. As expected, the RMSD profile resembles a parabola with a broad maximum centered over the middle of the loop (Fig. 4B, open squares).

Profiles of the ^{1}H-^{15}N NOE for mobile loops of Hsp10 and GroES suggest that motion in the loop is constrained by local structure. At 25 ºC, two (negative) peaks are observed in the ^{1}H-^{15}N NOE profile for both Hsp10[6] and GroES (Fig. 4C). As the temperature increases from 25 to 45 ºC, the profile of ^{1}H-^{15}N NOE of Hsp10 becomes more negative and the dual peaks are overtaken by a single peak (Fig. 4A). Simplification of the NOE profile at increased temperature suggests that the dual peaks arise from a structural feature in the loop that can be melted out. However, even at 45 ºC, the shape of the ^{1}H-^{15}N NOE profile differs from the profile of RMSD. Most notably, the peak in the ^{1}H-^{15}N NOE is more sharp and occurs on the N-terminal side of the loop center. In order to probe the origin of the deviation from random coil behavior, the dynamics simulation was repeated with a single restraint corresponding to an H-bond between the CO of T26 and NH of I30, corresponding to a putative S21-I25 H-bond in the GroEL-bound conformation of the GroES peptide[1]. The resulting profile of RMSD is remarkably similar to the profile of ^{1}H-^{15}N NOE at 45 ºC (Compare Fig. 4B, filled triangles with Fig. 4A, open triangles). The presence of nascent structure in the mobile loop suggests that mutations in the loop could have large effects on its conformational behavior. For example, Flanagan and coworkers observed large effects of mutations on the radius of gyration of denatured staphylococcal nuclease[7].

The mobile loop of GroES exhibits much greater mobility than the mobile loop of Hsp10 at 25 ºC, and a key difference between GroES and Hsp10 mobile loops is the presence of P33 in Hsp10. The pyrrolidine ring of P33 could restrain backbone mobility in the carboxy-terminal portion of the mobile loop. Mutation of P33 to any other residue should substantially increase mobility and therefore reduce Hsp60 binding affinity. Two research groups independently isolated temperature-sensitive mutants in the gene for yeast Hsp10 (yHsp10) that specified substitutions for proline in the position homologous to P33[8,9]. Both mutants resulted in decreased affinity of yHsp10 for GroEL. In the study by Hohfeld *et al.*, the temperature-sensitive defect was recapitulated *in vitro*[8]. yHsp10 binding to GroEL was lost at the non-permissive temperature and recovered upon shift to the permissive temperature. These authors concluded that the defective protein could revert to the active form. This behavior may be explained by excessive disorder in the mutant mobile loop. If the dynamic flexibility is too great, then the entropy loss upon binding may be too unfavorable. It is possible that greater mobility in the loop of GroES explains its failure to function with eukaryotic Hsp60 proteins[17].

Dual peaks in the ^{1}H-^{15}N NOE profiles of both Hsp10 and GroES indicate structure in the middle of the loop (Fig. 4C). Structure stabilized by the hydrophobic tripeptide could constrain loop motion in this region. Alternatively

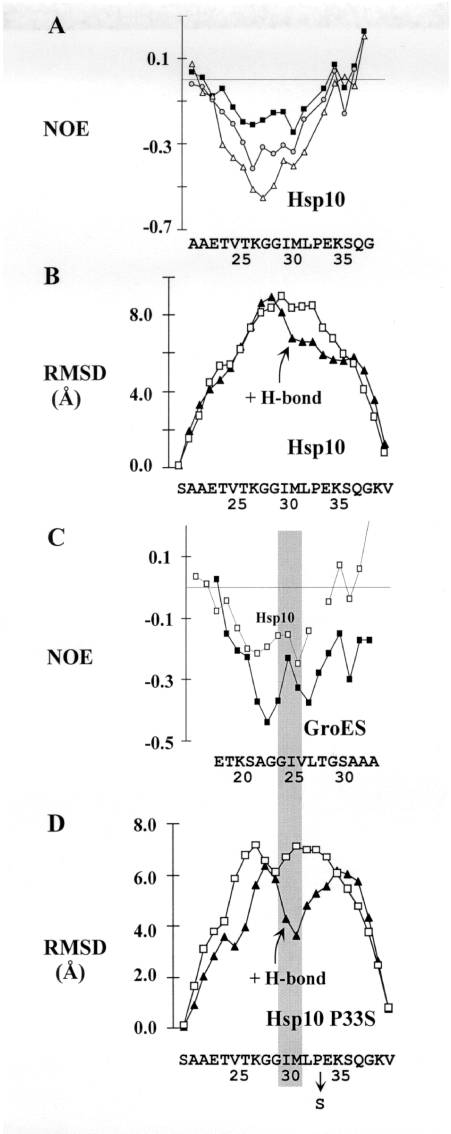

Figure 4. Experimental and simulated dynamics profiles for various (indicated) Hsp10 mobile loops. A. ^{1}H-^{15}N NOE of human Hsp10 at 25 (squares), 35 (circles), and 45 °C (triangles). B. RMSD from simulations +/- T26-I30 H-bond. C. ^{1}H-^{15}N NOE of GroES (filled) and human Hsp10 (open) at 25 °C. D. RMSD from simulations of P33S mutant +/- T26-I30 H-bond. NMR relaxation data for GroES at pH 4.0 were recorded as described for Hsp10.[6]

dual peaks in the ^{1}H-^{15}N NOE profiles could arise from a twisting mode of loop motion stabilized by an H-bond. The twisting motion may be described as a large vibration in which the loop rotates first in one direction and then the other, around an axis of rotation stretching from the base to the tip of the loop. The tip of the loop would be a node of relatively little motion.

The prominence of dual peaks in the profile of ^{1}H-^{15}N NOE is much more striking for GroES than for Hsp10. Since GroES lacks Pro at the position homologous to P33, annealing molecular dynamics simulations were carried out with the Hsp10 loop containing the P33S substitution. The RMSD profile exhibits dual peaks. When the T26-I30 H-bond is introduced, the node between the peaks of RMSD grows deeper and shifts to a position that coincides with the node between peaks in the ^{1}H-^{15}N NOE profile of GroES. Inspection of the ten structures generated by molecular dynamics suggests that they sample a segment of conformational space that could be traveled by a twisting motion of the loop.

2. Consequences of Mobile Loop Properties for Chaperonin Function

2.1 Affinity Governed by a Mobile Loop Folding Transition

Mutant co-chaperonins have been obtained by selection for resistance to bacteriophage λ, screens for suppression of a block on bacteriophage T4 by a mutation in *groEL*, and screens for temperature-sensitive growth of yeast *Saccharomyces cerevisiae* (Table 1). All mutants characterized to date contain an amino acid substitution in the mobile loop. In most cases, biochemical analysis revealed that the substitution reduced chaperonin-binding affinity, but "high-affinity" mutations also have been obtained by selection or design. The fact that mutations can disrupt chaperonin binding is not surprising, but the fact that mutations can increase binding affinity suggests that chaperonin binding is maintained at moderate levels by evolutionary selection. The entropic penalty of mobile loop ordering provides a mechanism for moderation of binding affinity. If such a mechanism is at work in this system, what are the consequences? In the interaction of well-ordered components, one often can understand the consequences of mutations by analyzing the structures of the binding interfaces. When binding is coupled to protein folding, the effects of a mutation also must be interpreted in terms of the equilibrium between folded and unfolded conformations.

Mutations in *groES* and gene *31* can be sorted into two classes on the basis of their genetic interaction with *groEL* alleles (Ref. 10; A. Richardson and C. Georgopoulos, unpublished). Since the GroEL alleles contain amino acid substitutions in a hinge region distant from the mobile loop binding site, we hypothesized that this genetic interaction results from an unconventional mechanism based on compensatory changes in affinity (A. Richardson, S. M. van der Vies, F. Keppel, C. Georgopoulos, A. Taher, and S. J. L., unpublished). Low-affinity mutants are suppressed by high-affinity mutants and *vice-versa*. Biochemical characterization of the purified proteins confirmed this hypothesis, and the changes in chaperonin-affinity of GroES mutants can be explained by predicted changes in the stability of the GroEL-bound hairpin conformation.

Table 1. Phenotype of Co-chaperonin Mutants

Co-chaperonin Allele	Chaperonin Affinity	Allele-specifically Suppressed by	Allele-specific Suppressor of
GroES(G24D)[a]	low	GroEL(V190I)[f]	
GroES(G24A)[b]	low		
GroES(G23D)[a]	high (predicted)	GroEL(V174F)[f]	
GroES(G23A)[b]	high		
Gp31(L35I)[c]	high		GroEL(E191G)[g]

Gp31(L35I,T31A)[c]	slightly high	GroEL(A383T)[c]
yHsp10(P33S)[d]	low	
yHsp10(P22H)[e]	low	

[a] Ref. 5.

[b] A. Taher, A. Richardson, F. Keppel, C. Georgopoulos, and S. J. Landry, unpublished.

[c] A. Richardson, S. van der Vies, F. Keppel, C. Georgopoulos, A. Taher and S. J. Landry, unpublished.

[d] Ref. 8.

[e] Ref. 9.

[f] Ref. 10.

[g] Ref. 11.

2.2 Chaperonin Dysfunction: Not Firing on All Cylinders

How does inappropriate co-chaperonin binding affinity result in poor chaperonin function? GroES has been called a "coupling factor" that couples ATP-dependent conformational changes in GroEL with substrate protein folding[12]. A key aspect of the chaperonin mechanism may be the enclosure of the substrate inside the "Anfinsen cage" where the protein is protected against aggregation[13]. Another important aspect may be the ability of GroES to commit all seven GroEL subunits to ATP hydrolysis before discharge of GroES and substrate[14]. Either aspect could explain the dysfunction of the chaperonin without co-chaperonin. In the absence of GroES, GroEL binds unfolded proteins and blocks their aggregation, and addition of ATP promotes discharge of the proteins[15]. However, in conditions that do not support unassisted folding, proteins released from GroEL without GroES fail to fold[16].

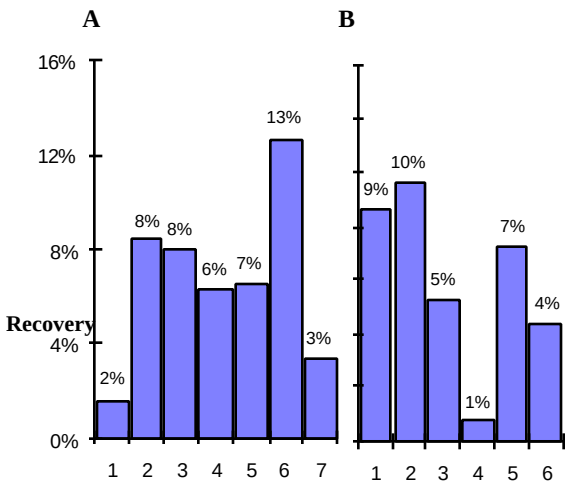

Figure 5: Citrate Synthase Transferred Between Chaperonins Can Refold but Is Rapidly Lost in the Absence of Appropriate Co-chaperonin. Citrate synthase was denatured in 6M guanidine-HCl, 50 mM K-PO_4 buffer (pH 7.4), 3 mM DTT, 2 mM EDTA and then diluted 180-fold into a renaturation mix composed of 4.2 µM GroEL (monomer), 1 mM oxaloacetate, 10 mM $MgCl_2$, 20 mM K-PO_4 buffer (pH 7.4). The final citrate synthase concentration was 0.2 µM. The "transfer" incubation was initiated by addition of ATP (2 mM) and other components where indicated below. [Hsp60] (1x) = 4.2 µM. [GroES] = 4.2 µM. [Hsp10] = 8.4 µM. After 8 min, co-chaperonin was added to promote refolding of chaperonin-bound citrate synthase, and citrate synthase activity was determined after another 8 min. (A) No co-chaperonin was present during the "transfer" incubation. (B) GroES was present during the "transfer" incubation. Transfer of non-native citrate synthase from GroEL to Hsp60 is indicated by increased recovery upon subsequent addition of Hsp10 (A6, B5, B6). Higher (2x) Hsp60 concentration in the "transfer" incubation with GroES present results in poorer recovery because citrate synthase is lost upon release from Hsp60 in the absence of Hsp10 (B4, B6).
A:
1, "spontaneous" renaturation
2, GroEL —5'— ATP —8'— Hsp10
3, GroEL —5'— ATP —8'— GroES
4, GroEL —5'— Hsp60 (1x), ATP —8'— Hsp10
5, GroEL —5'— Hsp60 (1x), ATP —8'— GroES
6, GroEL —5'— Hsp60 (2x), ATP —8'— Hsp10
7, GroEL —5'— Hsp60 (2x), ATP —8'— GroES
B:
1, GroEL —5'— GroES, ATP
2, GroEL —5'— GroES, ATP —8'— Hsp10
3, GroEL —5'— GroES, Hsp60 (1x), ATP
4, GroEL —5'— GroES, Hsp60 (2x), ATP
5, GroEL —5'— GroES, Hsp60 (1x), ATP —8'— Hsp10
6, GroEL —5'— GroES, Hsp60 (2x), ATP —8'— Hsp10

Preliminary experiments showed that refolding of citrate synthase becomes less efficient when addition of GroES is delayed after unfolded citrate synthase is bound to GroEL and ATP added (data not shown). The loss of efficiency could be due to misfolding or aggregation of citrate synthase while bound to GroEL or after unproductive discharge from GroEL. Thus, we wished to determine whether citrate synthase could be transferred from one chaperonin to another and whether transferred citrate synthase remains competent for folding.

Subsequently, we would examine whether co-chaperonins modify these processes.

Substrate protein that has been transferred from GroEL to hamster Hsp60 (Hsp60) can be detected by taking advantage of the specific requirement of Hsp60 for mammalian Hsp10[17]. Since

GroEL works equally well with GroES and Hsp10 (data not shown), the difference in recovery obtained with Hsp10 *versus* GroES from a mixture of GroEL and Hsp60 reflects refolding assisted by Hsp60/Hsp10. Citrate synthase was chemically denatured and then diluted into a solution containing GroEL. ATP and in some cases Hsp60 were added, and the mixture was incubated at 37 °C to allow citrate synthase to transfer between chaperonins. This incubation period must be kept short (here, 8 min) because, in the presence of ATP but absence of co-chaperonin, citrate synthase is rapidly lost to aggregation, resulting in low yields. Separate experiments show that when GroES is added at the same time as ATP, greater than 60% of the input citrate synthase is recovered; and similar yields are obtained with Hsp60 and Hsp10 (data not shown).

Very little recovery was observed if co-chaperonin was left out altogether, but substantial recovery was observed when either GroES or Hsp10 was added in the final incubation (Fig. 5A, compare reactions 2 and 3 with 1). Inclusion of one equivalent of Hsp60 during the "transfer" incubation slightly reduced the yield, and the same yield was obtained regardless whether Hsp10 or GroES was added in the final incubation (Fig. 5A, reactions 4 and 5). Inclusion of two equivalents of Hsp60 during the "transfer" incubation resulted in enhancement of yield after addition of Hsp10 and reduction of yield after addition of GroES (Fig. 5A, reactions 6 and 7). As expected, citrate synthase molecules that become associated with Hsp60 refold upon addition of Hsp10 but not GroES. The enhanced yield obtained with two equivalents of Hsp60 results from the increased efficiency of refolding at higher chaperonin concentration. However, one equivalent of Hsp60 does not enhance the yield. Apparently, some citrate synthase is lost to aggregation during the "transfer" incubation, and one equivalent of Hsp60 is insufficient to overcome the loss. These results not only indicate that citrate synthase which transfers to Hsp60 is viable for refolding but also demonstrate that the Hsp60-associated molecules are lost to aggregation faster than GroEL-associated molecules in the presence of ATP but absence of co-chaperonin.

If co-chaperonin prevents citrate synthase aggregation by protecting the molecule until folding is complete, then inclusion of GroES during the "transfer" reaction should eliminate reduction in yield caused by addition of Hsp60. This is similar to experiments by others in which inactive GroEL "trap" molecules were included in order to capture unfolded substrate proteins released during chaperonin-assisted folding reactions[18,19]. As shown previously[18], some of the citrate synthase is released from GroEL in a form that is not committed to fold because it is captured by Hsp60. When GroES is the only co-chaperonin present, these molecules are lost (Fig. 5B, reactions 3 and 4); but when Hsp10 is added, some of these molecules are recovered (Fig. 5B, reactions 5 and 6). Less citrate synthase is recovered when two equivalents of Hsp60 are included (Fig. 5B, reaction 6), presumably because more citrate synthase is lost after transfer but before addition of Hsp10. This experiment

illustrates that "trapped" molecules can refold, but they are lost over time when the appropriate co-chaperonin is not present. Thus, the co-chaperonin cannot protect the chaperonin-bound substrate against transfer to another chaperonin molecule; and when the appropriate co-chaperonin is unavailable, the transferred substrate is rapidly lost to aggregation (Fig. 6).

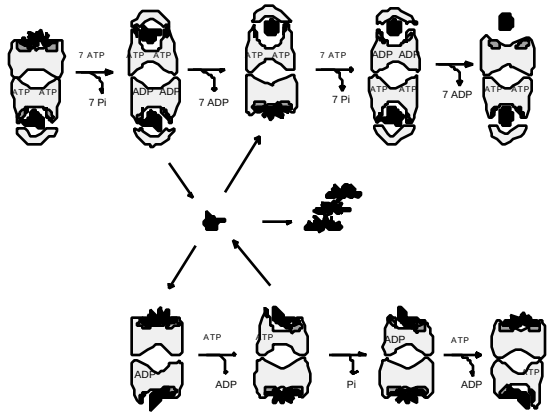

Figure 6: Diagram Showing Transfer of Substrate between Chaperonin Molecules and Loss of Substrate to Aggregation. Non-native substrate is released from all nucleotide-bound forms of the chaperonin (although release from only a few forms is shown for simplicity). Substrate molecules released without co-chaperonin either transfer or aggregate. In contrast, substrate molecules which are released with co-chaperonin have the opportunity to refold. Co-chaperonin binding that is either too weak or too strong could reduce formation of productive chaperonin-substrate-co-chaperonin complexes.

Co-chaperonin must bind to the chaperonin immediately after substrate binding. Although substrates can complete folding while sequestered beneath the co-chaperonin[20], if the chaperonin cycle time is shorter than the substrate folding time, then incompletely folded molecules will be ejected from the chaperonin, and a new round of coordinated release must be initiated. Herein lies the dysfunction of deviant co-chaperonin binding. Since substrate molecules are lost to aggregation while associated with an uncoordinated chaperonin, the co-chaperonin must join the substrate-chaperonin complex immediately after it forms. Weak co-chaperonin binding results in a lower steady-state level of productive complex. Strong co-chaperonin binding diminishes the pool of free co-chaperonin.

2.3 Conclusions and Prospects

The elaborate architecture of the chaperonin complex may require a minimum amount of specific contact interface at each of the seven mobile loop binding sites; and therefore, the interface cannot be reduced to obtain lower affinity. Mobile loop disorder in the unbound state provides a mechanism to moderate affinity. The disorder is tempered by a conformational bias that predisposes the loop toward the bound conformation. Nature must strike a balance of structure and disorder.

Disorder in macromolecular binding sites may provide a mechanism to uncouple affinity from specificity in many association reactions. Examples are

likely to include protein-nucleic acid and protein-protein complexes such as in the regulation of transcription and cell-cell contact, respectively.

New questions arising are foreshadowed by issues of protein folding in general. How much is the entropy of the unbound state affected by a conformational bias toward the bound conformation? Does a requirement for folding introduce a potential for kinetic traps that impede binding? How do important disordered regions avoid proteolytic degradation?

Acknowledgments

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