Molecular chaperones: **Inside and outside the Anfinsen cage**

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The GroEL/GroES chaperonin system acts as a passive anti-aggregation cage for refolding rubisco and rhodanese, and not as an active unfolding device. Refolding aconitase is too large to enter the cage but reversible binding to GroEL reduces its aggregration. Unexpectedly, confinement in the cage increases the rate of refolding of rubisco, but not rhodanese.

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The bacterial chaperonin system GroEL/GroES assists the completion of folding of some partly folded polypeptide chains after their release from ribosomes. Since this property was discovered [1], unravelling its mechanism has generated much ingenious experimentation. Two different types of mechanism were discussed from the outset. One suggests that the central cage of each double-ring GroEL oligomer acts as a passive sequestration chamber, inside which one partly folded polypeptide chain continues to fold, protected from the hazard of forming a non-functional aggregate with one or more similar chains [2,3]. The other suggests that the GroEL/GroES system actively unfolds misfolded chains, and so allows them another chance to fold correctly [4].

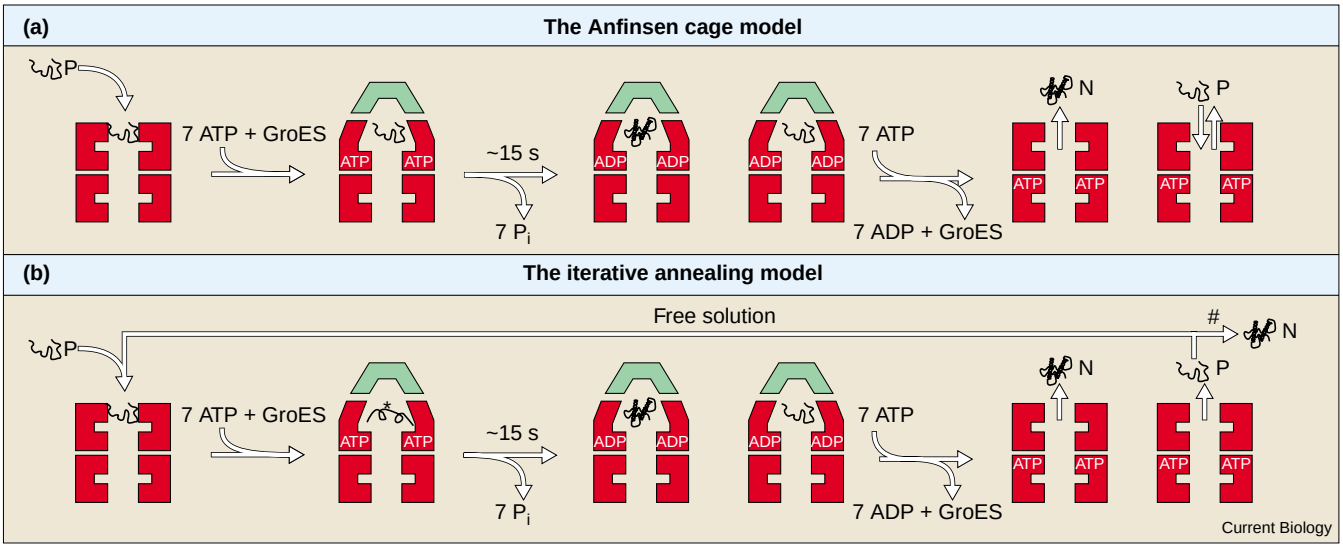
Neither of these two mechanisms conflicts with the principle of protein self-assembly, espoused originally by Caspar and Klug for viruses [5] and by Anfinsen for the refolding of denatured polypeptides [6]. Rather, they offer different, but not mutually exclusive, ways to explain how the GroEL/GroES system improves the efficiency with which the steric information encoded in the primary structure of a newly synthesized polypeptide creates a correctly folded protein. Recent work from the Hartl and Hayer-Hartl laboratory [7] favours the passive cage model over the active unfolding model for the two proteins tested, but also reports the unexpected finding that the rate of folding of one of these proteins is four-fold faster inside the cage than outside it. And a new paper from the Horwich laboratory [8] reports that the enzyme aconitase is too large to enter the cage, but that reversible binding to GroEL reduces its tendency to aggregate.

The term ‘Anfinsen cage’ was introduced to describe the idea that GroEL improves the efficiency of refolding of denatured proteins by binding each partly folded chain inside its oligomeric structure, thereby protecting it from aggregating with other similar chains [3]. ATP-triggered release of the bound chain into the cavity of the cage was suggested to allow it to fold in the same manner as it would in free solution in a classic Anfinsen refolding experiment. Subsequent *in vitro* experiments using reconstituted protein refolding systems in several laboratories have established that binding of GroES and ATP to the GroEL oligomer displaces the polypeptide inside the cage, where it continues to fold. GroES caps the cage to keep the sequestered chain from emerging into the medium for a time determined by the rate of ATP hydrolysis. If a chain does not fold sufficiently to internalise its GroEL-binding sites within about 15 seconds at 25°C, it is released into the buffer where it may either aggregate with similar chains or rebind to another GroEL oligomer (reviewed in [9,10]). Addition of crowding agents to the *in vitro* system reduces this release, suggesting that, *in vivo*, the crowded state of the cytoplasm ensures any released chain binds back rapidly to the same GroEL oligomer, and thus minimises the chance that it will aggregate with another partly folded chain ([11] and Figure 1a).

From this evidence, the chaperonins would appear to act as passive anti-aggregation devices or ‘mini-test tubes’, inside each of which a single chain can fold. Macromolecular crowding inside cells strongly favours the aggregation of some polypeptides as they fold [12], and genetic ablation of chaperonin activity in *Escherichia coli* has been shown to cause some proteins to aggregate [13]. Thus both *in vitro* and *in vivo* evidence supports the view that chaperonins function to reduce the probability that some newly synthesized polypeptides aggregate before they achieve their correctly folded conformations, and the Anfinsen cage model offers a plausible explanation for how they achieve this. But do chaperonins also actively unfold misfolded conformations? If they do, is this unfolding an essential aspect of chaperonin function or is it an epiphenomenon, an inevitable consequence of the cage mechanism of no functional significance?

Misfolding is conceptually distinct from aggregation, because it is usually taken to mean that a chain has reached a partly folded conformation that is stable enough not to be able to proceed to its functional conformation on a biologically relevant time scale. The energy landscape model of protein folding suggests that such misfolded chains form a subset of partly folded chains and, like them, vary in their susceptibility to aggregation [14]. To what extent misfolding is a real biological problem other than in disease is not clear, but the ability to unfold such misfolded conformations

Figure 1



(a) The Anfinsen cage model of GroEL/GroES action. A partly folded chain (P) binds to the apical domains of a GroEL oligomer (represented in red as a vertical slice through two stacked rings of seven subunits each). Binding of ATP and GroES – a single ring heptamer indicated in green – displaces this chain into the Anfinsen cage. After one round of ATP hydrolysis, the unbinding of GroES, triggered by binding of ATP to the opposite ring, allows the chain to diffuse into the free solution. If the chain has folded sufficiently (N) it will not rebind, but if it has not (P), it rapidly binds back under crowded conditions to the same GroEL oligomer, and so avoids aggregating with other partly folded chains in free solution. (b) The iterative annealing model of GroEL/GroES action. This is as in (a), but in this case displacement of the chain into the cage is accompanied by mechanical stretching that unfolds the chain further (*). Any chain that is still partly folded on unbinding of GroES enters the free solution, where it either completes its folding (#) or rebinds to another GroEL oligomer for a further round of unfolding.

before they are discharged into the Anfinsen cage might confer an added usefulness on the chaperonin system.

It has been observed for some proteins refolding *in vitro* in the presence of GroEL/GroES in uncrowded buffers that partly folded chains are released from the GroEL cage into the buffer after each ATPase cycle. Such chains bind to other GroEL oligomers and transit through the system repeatedly until they achieve the correctly folded state [15]. Such cycling may be an artefact of using uncrowded buffers [11,16], but these observations led to the iterative annealing model of unfolding, which suggests that large conformational changes in GroEL resulting from the binding of GroES exert a stretching force that actively unfolds bound misfolded chains. When released either into the cavity of the cage or into the buffer, these unfolded chains are suggested to have more opportunities to find routes to correct folding than if they remained misfolded, and thus they fold more rapidly ([17,18] and Figure 1b).

In support of the unfolding aspect of this model, hydrogen exchange measurements have detected partial unfolding of bound chains of bacterial ribulose bis-phosphate carboxylase (rubisco) during a single reaction cycle [19]. In contrast, no significant unfolding was found in a nuclear magnetic resonance (NMR) analysis of refolding malate dehydrogenase [20]. It is thus relevant to this debate that the recent work by Brinker *et al.* [7] employed bacterial rubisco as one of two protein substrates studied, the other being rhodanese. Both these substrates are prone to aggregation, and recycle repeatedly in the GroEL/GroES system in uncrowded buffers.

There are two testable differences between the cage and annealing models. In the cage model, the polypeptide must fold within the cage to avoid aggregation, while in the latter this is not the case. And the annealing model predicts that, under conditions where aggregation does not occur, the rate of correct folding will be faster once unfolding has taken place, while the cage model implies it will be the same. To distinguish experimentally between these possibilities, Brinker *et al.* [7] coupled biotin to cysteine residues located at the apical domain of GroEL. This modification does not prevent the normal functioning of the chaperonin system, but provides a method of preventing released chains from rebinding to GroEL, as biotin binds rapidly ($t_{1/2}$ about 200 ms) and irreversibly (K_D about 10^{-14} M) to streptavidin. Addition of streptavidin blocks entry to the cage and thus interrupts chain rebinding well within a single reaction cycle.

Brinker *et al.* [7] found that, under buffer conditions where partly folded chains of rubisco readily aggregate, the addition of streptavidin after several ATPase reaction cycles blocked rubisco folding completely and instantly. This

observation is inconsistent with the iterative annealing model, which predicts that some released chains would fold correctly in free solution, but it is in accord with the cage model, which predicts that correct folding occurs only within the cage. This result is not surprising, in view of the known high propensity of partly folded rubisco chains to aggregate. Partly folded chains of rhodanese do not aggregate so rapidly as those of rubisco, and addition of streptavidin to the rhodanese/GroEl/GroES system was found to allow some of the released chains to fold correctly. Experiments with a non-cycling single-ring variant of GroEL, which binds but does not release GroES, showed that correct folding of rubisco and rhodanese does not require the repeated cycles of binding and release implicit in the iterative annealing model.

Testing the second prediction required the use of buffer conditions where released partly folded chains do not aggregate. Addition of streptavidin under these circumstances does not affect the rate of rhodanese folding, which is the same as the rate of folding in free solution or in the presence of the uninhibited GroEL/GroES system. Thus any unfolding of misfolded rhodanese chains by the chaperonin system does not result in an acceleration of correct folding. Similar experiments with rubisco, however, revealed that the rate of folding of this protein is four-fold faster inside the cage than outside the cage. This effect is similar to the enhanced rate of folding of reduced lysozyme caused by the addition of crowding agents in the absence of the chaperonin system [21]. A possible explanation of both these enhancements is that compaction of unfolded chains is favoured by confinement in a narrow chamber (rubisco) and by crowding in free solution (lysozyme). Such effects are predicted by macromolecular crowding theory [12,22].

The Anfinsen cage is too small to accommodate chains larger than about 60 kDa. Nevertheless, there is *in vivo* evidence that some chains larger than 60 kDa bind transiently to GroEL shortly after synthesis, and mitochondrial aconitase (82 kDa) was found to aggregate *in vivo* in yeast [23] and *E. coli* [8] cells deficient in either GroEL or GroES. Chaudhuri *et al.* [8] found that the *in vitro* refolding of denatured aconitase is stimulated four to five-fold by addition of GroEL/GroES/ATP, but the role of GroES in this case is distinct from its role in the folding of smaller chains encapsulated inside the cage. Instead of binding to the same ring to which the aconitase is bound, GroES binds to the opposite ring, and this triggers the release of bound aconitase into the free solution where it folds. This entire cycle is repeated until the aconitase polypeptide has folded sufficiently not to rebind to GroEL [8].

These experiments with aconitase were carried out with uncrowded buffers, but the simplest interpretation is that repeated binding to and release from GroEL reduces the probability that partly folded chains of aconitase will aggregate with one another by transiently shielding exposed hydrophobic areas. In this respect the action of GroEL on aconitase resembles that of the Hsp 70/40 chaperones and minichaperones, which are too small to provide Anfinsen cages, but prevent aggregation by temporarily shielding hydrophobic areas on partly folded chains [10]. The chaperonins continue to spring surprises.

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