Cell, Vol. 107, 223–233, October 19, 2001, Copyright ©2001 by Cell Press

Dual Function of Protein Confinement in Chaperonin-Assisted Protein Folding

Achim Brinker,[1] Guenther Pfeifer,[2] Michael J. Kerner,[1] Dean J. Naylor,[1] F. Ulrich Hartl,[1,3] and Manajit Hayer-Hartl[1,3]
[1]Department of Cellular Biochemistry
[2]Department of Molecular Structural Biology
Max-Planck-Institute of Biochemistry
Am Klopferspitz 18a
82152 Martinsried
Germany

Summary

The GroEL/GroES chaperonin system mediates the folding of a range of newly synthesized polypeptides in the bacterial cytosol. Using a rapid biotin-streptavidin-based inhibition of chaperonin function, we show that the cage formed by GroEL and its cofactor GroES can have a dual role in promoting folding. First, enclosure of nonnative protein in the GroEL:GroES complex is essential for folding to proceed unimpaired by aggregation. Second, folding inside the cage can be significantly faster than folding in free solution, independently of ATP-driven cycles of GroES binding and release. This suggests that confinement of unfolded protein in the narrow hydrophilic space of the chaperonin cage smoothes the energy landscape for the folding of some proteins, increasing the flux of folding intermediates toward the native state.

Introduction

The GroEL/GroES chaperonin system of *Escherichia coli* has an essential biological role in assisting the folding of a subset of cytosolic proteins (Fayet et al., 1989; Horwich et al., 1993; Ewalt et al., 1997; Houry et al., 1999). GroEL consists of two heptameric rings of 57 kDa subunits, which form a cylindrical structure with two cavities (Braig et al., 1994; Roseman et al., 1996). The apical domains of the subunits expose hydrophobic binding surfaces toward the ring center and engage in multiple contacts with a nonnative substrate protein (Fenton et al., 1994; Farr et al., 2000). The apical domain is connected via a hinge-like intermediate region with an equatorial ATPase domain that mediates most intersubunit contacts within and between GroEL rings (Figure 1A). GroES is a dome-shaped, heptameric ring of 10 kDa subunits that caps the ends of the GroEL cylinder (Hunt et al., 1996; Xu et al., 1997; Saibil, 2000).

The ATP-dependent interactions of GroEL with protein substrate and GroES have been studied extensively (reviewed in Hartl, 1996; Coyle et al., 1997; Richardson et al., 1998; Sigler et al., 1998; Ellis and Hartl, 1999; Saibil, 2000; Feltham and Gierasch, 2000). GroES cycles on and off GroEL in a manner dependent on the GroEL ATPase. Nonnative protein binds with highest affinity to the nucleotide-free state of GroEL. Subsequent binding of 7 ATP and of GroES induces the conversion of the inner GroEL surface from hydrophobic to hydrophilic and results in transient encapsulation of proteins up to 60 kDa in the so-called *cis* complex. The average enclosure time of 10–15 s at 25°C reflects the time required for the hydrolysis of the 7 ATP molecules in the *cis* ring of GroEL. Following hydrolysis, GroES is triggered to dissociate by ATP binding to the GroEL *trans* ring.

As a consequence of chaperonin action, productive protein folding is promoted and off-pathway aggregation is prevented. However, despite a detailed understanding of the enzymology of the system, the underlying principle of chaperonin-mediated protein folding is still a matter of debate (Ellis and Hartl, 1996; Coyle et al., 1997; Betancourt and Thirumalai, 1999; Wang and Weissman, 1999). Two competing models, the "Anfinsen cage" model and the "iterative annealing" model, have been proposed. Assuming that folding is generally efficient as long as off-pathway aggregation is prevented, the cage model holds that GroEL and GroES provide a passive box in which folding can proceed unimpaired by intermolecular interactions between nonnative polypeptides, comparable to folding at infinite dilution (Agard, 1993; Ellis, 1994; Wang and Weissman, 1999). Apparent acceleration of folding would only occur for proteins with a tendency to form reversible low-order aggregates. Indeed, proteins dependent on chaperonin for folding are typically aggregation prone and, for some substrates, production of the native state inside the cage-like structure of the GroEL:GroES complex is observed when the normal dissociation of substrate or GroES from GroEL is inhibited (Hayer-Hartl et al., 1996; Mayhew et al., 1996; Weissman et al., 1996; Rye et al., 1997). However, that folding occurs inside the cage during uninhibited GroES cycling has never been shown.

The iterative annealing model, on the other hand, supports the view that, for some proteins, formation of misfolded or kinetically trapped, but not yet aggregated, intermediates, precludes folding at a biologically relevant time scale. The proposed role of the chaperonin is to actively unfold these intermediates. Upon release into solution or into the GroEL cavity, the actively unfolded protein is then afforded a chance to partition between a fast pathway to the native state and a nonproductive pathway, again leading to trapped intermediate (Todd et al., 1994, 1996; Corrales and Fersht, 1996; Betancourt and Thirumalai, 1999; Shtilerman et al., 1999). Because spontaneous conversion of trapped intermediate to the native state is slow, iterative unfolding cycles would result in an overall acceleration of folding and, consequently, avoidance of aggregation. In support of this model, the time of protein encapsulation in the GroEL:GroES complex per reaction cycle is short (seconds) relative to the time required for the folding of many GroEL substrates (minutes). A protein typically undergoes multiple rounds of release from GroEL into solution in a nonnative state before reaching its native conformation (Todd et al., 1994; Weissman et al., 1994; Shtilerman et al., 1999), suggesting that, in the cycling system, folding may occur outside the

[3]Correspondence: uhartl@biochem.mpg.de [F.U.H]; mhartl@biochem.mpg.de [M.H.-H.]

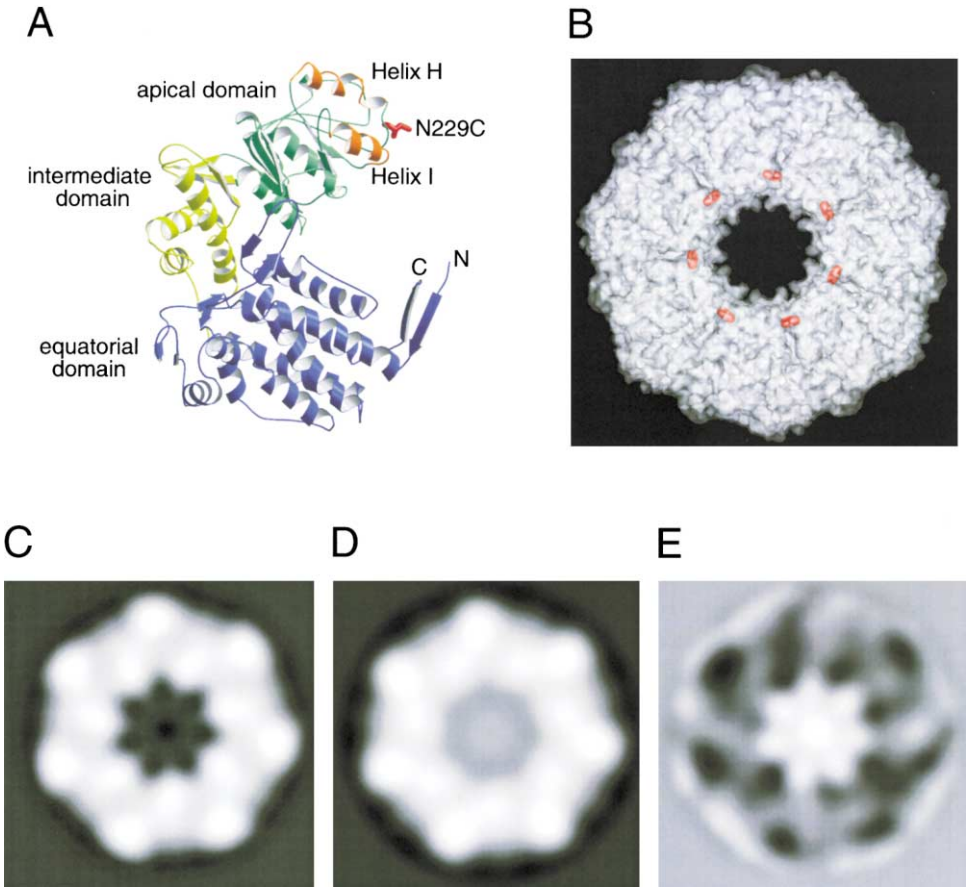

Figure 1. Occlusion of the Central GroEL Cavity upon Binding of Streptavidin to Biotinylated GroEL

(A) Ribbon diagram of the GroEL subunit (Xu and Sigler, 1998); pdb 1AON (MOLSCRIPT: Kraulis, 1991; Raster3D: Merrit and Bacon, 1997). Helices H and I (orange) expose hydrophobic amino acids toward the ring cavity for binding of nonnative protein. (B) Top-view of the GroEL *trans* ring in the crystal structure of the GroEL:GroES:ADP complex (Xu and Sigler, 1998); pdb 1AON. The position of Asn229 is indicated in red. (C-E) Electron micrographs of negatively stained EL229C-B (C), EL229C-B/SA (D) and a difference image of C and D (E). See Experimental Procedures for details.

chaperonin cage. GroES-mediated unfolding has recently been reported with bacterial ribulose-bisphosphate carboxylase (RuBisCo) as the substrate (Shtilerman et al., 1999). This "forced" unfolding may result from a rotation of the apical GroEL domains upon ATP and GroES binding (Xu et al., 1997; Saibil, 2000), possibly exerting a stretching force on the bound protein. However, the mechanistic significance of such unfolding has remained elusive. Although rate enhancement of folding by chaperonin has been observed with various model proteins (Ranson et al., 1997; Sparrer et al., 1997; Viitanen et al., 1990; Coyle et al., 1999), a contribution of GroES-mediated unfolding to accelerated folding has not yet been demonstrated.

The principal difference between the cage model and the iterative annealing model is that, in the former, the polypeptide must fold within the cage to avoid aggregation, while in the latter it does not. Moreover, acceleration of folding in the absence of aggregation is predicted for the annealing model but not for the Anfinsen cage model. In order to distinguish between these proposed mechanisms, we have analyzed the fate of nonnative proteins upon release from GroEL into solution with regard to the yield and rate of folding. Our results show that for two stringent chaperonin substrates, RuBisCo and rhodanese, fully efficient folding is independent of iterative, GroES-mediated unfolding cycles. Folding of these proteins must occur inside the GroEL:GroES cage in order for off-pathway aggregation to be prevented. Strikingly, we find for RuBisCo that confinement of unfolded protein in the narrow space of the chaperonin cage results in a significant acceleration of folding as compared to folding in free solution.

Results

Biotin-Streptavidin-Based Inactivation of GroEL

Whether folding occurs inside or outside the GroEL:GroES complex in a normally cycling chaperonin reaction is the focus of an active debate. This question has been difficult to resolve, because release of nonnative protein from GroEL into solution is followed by rapid GroEL rebinding. To definitively address this problem, a method to interrupt chaperonin cycling and to block the rebinding of nonnative protein by GroEL was developed, taking advantage of the rapid, high-affinity inter-

action of streptavidin (SA) with a biotinylated version of GroEL. Biotin binds free SA at 25°C at a rate of $\sim 10^8$ M^{-1} s^{-1} and a K_D of 10^{-14} M (Green, 1990). A mutant form of GroEL, EL229C, was constructed, in which the three endogenous cysteines of the wild-type protein were replaced by alanine and a new cysteine introduced at position Asn 229 (Figure 1A). This residue is located in the apical domain at the opening of the GroEL cylinder (Figure 1B), close to but not within the binding regions for GroES and substrate protein on helices H and I (Figure 1A). EL229C was quantitatively biotinylated to yield EL229C-B. Two to three molecules of SA, a tetramer of ~17 kDa subunits, bound to each ring of EL229C-B, as determined spectroscopically, taking advantage of the fact that SA contains 6 tryptophans per subunit and GroEL none (data not shown). On examination by negative stain electron microscopy, averaged end-on views of EL229C-B showed the typical 7-fold symmetry of the GroEL cylinder (Figure 1C). In contrast, end-on images of EL229C-B/SA (Figure 1D) deviated from 7-fold symmetry and the presence of a stain-excluding mass, occluding the central portion of the ring, was visualized by difference imaging (Figure 1E).

Both EL229C and EL229C-B were functionally equivalent to wild-type GroEL in mediating GroES and ATP-dependent protein folding (Figure 2A and results shown below). As expected, modification of EL229C-B with SA completely blocked the binding of nonnative protein as determined by gel filtration analysis of GroEL complexes (Figure 2B). Surface plasmon resonance measurements with immobilized GroES showed that EL229C-B bound and released GroES normally in an ATP-dependent manner, whereas modification of EL229C-B with SA blocked the interaction with GroES almost completely (Figure 2C). SA binding occurred at a fast rate ($t_{1/2}$ ~200 ms), as determined in a stopped-flow experiment by recording the decrease in tryptophan fluorescence of SA upon binding of biotin on EL229C-B (Figure 2D). This rate is equivalent to the reported rates of GroEL binding for nonnative RuBisCo or GroES ($\sim 10^7$ M^{-1} s^{-1}) under the experimental conditions (Hayer-Hartl et al., 1995; Rye et al., 1999). Based on these results, addition of SA is expected to interrupt GroEL/GroES function sharply, essentially within a single reaction cycle, by blocking the rebinding of a nonnative substrate protein to GroEL after release into solution.

Protein Folding Occurs inside the GroEL:GroES Cage during Chaperonin Cycling

We first analyzed the effect of GroEL inhibition on protein folding under conditions in which the substrate protein fails to refold spontaneously but instead aggregates (nonpermissive conditions). Efficient acquisition of the native state is then stringently dependent on GroEL, GroES, and ATP. As depicted schematically in Figure 3A, the iterative annealing model would predict that, upon prevention of protein rebinding to GroEL, a fraction of nonnative molecules can partition to the native state in solution (U to N), whereas partitioning to kinetically trapped intermediate (U to I) ultimately results in aggregation. According to the cage model, preventing the reassociation of folding intermediate with GroEL should block folding due to rapid aggregation outside the cage (I to Agg) (Figure 3A).

Two stringent chaperonin substrates, bacterial RuBisCo, a homodimer of ~50 kDa subunits (Goloubinoff et al., 1989), and mitochondrial rhodanese, a monomer of ~33 kDa (Martin et al., 1991), were analyzed. At a final concentration of 0.5 μM, spontaneous refolding of these proteins to the native state is completely outcompeted by aggregation, but efficient refolding is achieved in the presence of EL229C-B, GroES, and ATP (Figures 3B and 3D). When SA was added at various times after initiating assisted folding with ATP (i.e., after multiple chaperonin cycles), further folding of both proteins was inhibited (Figures 3B–3E). However, folding continued uninterrupted when nonbiotinylated EL229C was added together with SA (Figures 3B and 3D). This result demonstrates that SA rapidly and specifically blocks rebinding of nonnative protein to EL229C-B but does not hinder its release from chaperonin into bulk solution. It also confirms that the substrate protein leaves GroEL completely in every reaction cycle and can move freely between GroEL molecules (Todd et al., 1994; Weissman et al., 1994). Strikingly, the block in RuBisCo folding upon addition of SA to cycling chaperonin was instantaneous and complete (Figures 3B and 3C). Assuming that folding occurs by iterative annealing in solution (Figure 3A), the fraction of molecules expected to reach native (assembly-competent) state upon release from GroEL would be at least 7%, i.e., the fraction completing folding per average chaperonin cycle. Such an increase in RuBisCo activity after SA addition is not observed (Figures 3B and 3C). Thus, kinetic partitioning to the native state must occur inside the GroEL:GroES cage, when the protein is displaced from the apical GroEL domains by GroES binding. As will become clear later, the block in folding upon preventing protein recapture by GroEL is due to rapid association of folding intermediates in the medium, consistent with the formation of pelletable aggregates (data not shown).

Similar results were obtained with rhodanese as the substrate, although with this protein, inhibition of GroEL function by SA did not inhibit folding instantaneously. Instead, a small fraction of molecules (5%–10% of total) continued to fold slowly to the native state before folding came to a halt (Figure 3D). This effect was independent of the time of addition of SA during the folding reaction (Figure 3E) and could be consistent with partitioning of unfolded protein in solution between a productive folding pathway and formation of a trapped intermediate. More likely, the rhodanese chains released from GroEL after partial folding in the cage aggregate less rapidly than those of RuBisCo (see below). This interpretation is also consistent with the observation of some rhodanese folding with GroEL/ATP in the absence of GroES (Figure 3D).

Fully Efficient Folding Is Independent of Chaperonin Cycling

We next investigated whether efficient folding is achieved simply by protein sequestration inside the GroEL:GroES complex, or whether repetitive cycles of GroEL/GroES-mediated unfolding are also required. Inactivation of GroEL by SA was employed to analyze the effect of shifting a substrate protein from normal, GroES-cycling GroEL, to a noncycling single-ring variant of GroEL, SR-

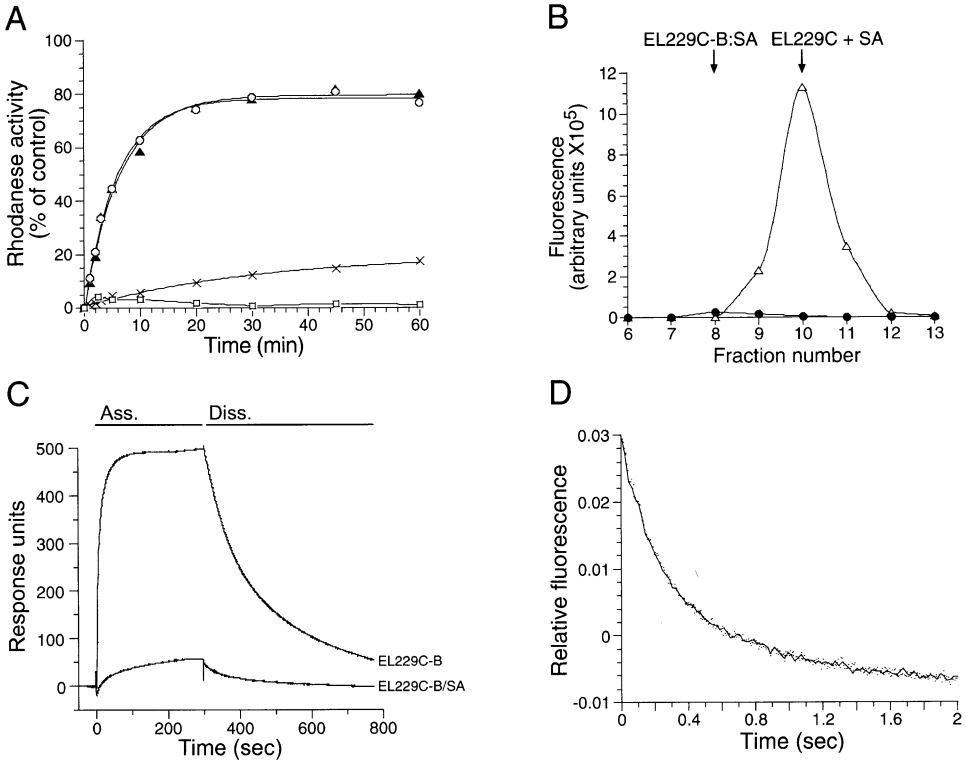

Figure 2. Functional Characterization of GroEL Mutant EL229C upon Binding of Biotin and Streptavidin

(A) Refolding of 0.5 μM rhodanese bound to either 0.5 μM EL229C (▲) or EL229C-B (○) in the presence of 1 μM GroES and 5 mM ATP. As a control, rhodanese bound to EL229C-B was incubated with ATP in the absence of GroES (×). Spontaneous reactivation in the absence of chaperonin (□). The activity of an equivalent amount of native rhodanese was set to 100%.

(B) Gel filtration of denatured fluorescent labeled rhodanese, added to EL229C (in the presence of SA) (△) or EL229C-B/SA (●). Peak positions of EL229C and of EL229C-B:SA elution are indicated.

(C) Binding of EL229C-B and EL229C-B:SA complex to GroES analyzed by surface plasmon resonance in the presence of ATP. GroES was immobilized as described in Experimental Procedures. Ass., association phase; Diss., dissociation phase.

(D) Stopped-flow kinetics of SA binding to EL229C-B. The decrease in tryptophan fluorescence of SA upon biotin binding is followed by fluorescence spectroscopy.

EL (Weissman et al., 1995). As shown by surface plasmon resonance, SR-EL binds GroES in an ATP-dependent manner, but is unable to dissociate it due to the absence of an allosteric signal from the GroEL *trans*-ring (Figure 4A). Thus, GroES may stably encapsulate protein substrate in SR-EL and allow folding to proceed inside the SR-EL:GroES complex without the possibility of active unfolding (Hayer-Hartl et al., 1996; Weissman et al., 1996). Essentially instantaneous dissociation of GroES from SR-EL is achieved by addition of the Mg^{2+} chelator CDTA (Figure 4A). This stops further folding of RuBisCo subunits due to stable GroEL binding and releases folded monomers for assembly.

Folding of RuBisCo bound to EL229C-B was initiated in the presence of GroES and ATP. After 45 s (~3 chaperonin cycles), SA was added either alone or together with SR-EL (Figure 4B). Again, modification of EL229C-B with SA blocked further RuBisCo folding. In contrast, efficient continuation of folding was observed upon simultaneous addition of SA and SR-EL, both with respect to yield and rate (Figure 4B). Essentially the same result was obtained with monomeric rhodanese as the substrate (data not shown). When CDTA was omitted, preserving the integrity of the SR-EL:GroES cage, ~70% of the enzymatically active rhodanese produced was recovered inside the cage on size exclusion chromatography (Figure 4D). The remaining ~30%, produced by cycling GroEL/GroES prior to addition of SA and SR-EL, fractionated as the free protein.

Based on the results described so far, assisted folding occurs in the GroEL:GroES cage, even though nonnative protein is repeatedly released into solution between chaperonin cycles. This explains how aggregation is avoided under nonpermissive conditions. Repeated cycles of protein binding and release, possibly mediating unfolding, are not required for efficient assisted folding.

GroEL:GroES Acts as an Infinite Dilution Cage for the Folding of Rhodanese

Whether and how the chaperonin system accelerates protein folding must be examined under permissive folding conditions where both reversible and irreversible off-pathway aggregation is avoided and spontaneous folding can proceed. The iterative annealing model then predicts that upon inhibiting rebinding of nonnative protein to GroEL, a fraction of the molecules will continue to fold in solution (U to N) at a fast rate k_{IA1}, followed by slower folding at a rate k_{IA2}, limited by the spontaneous

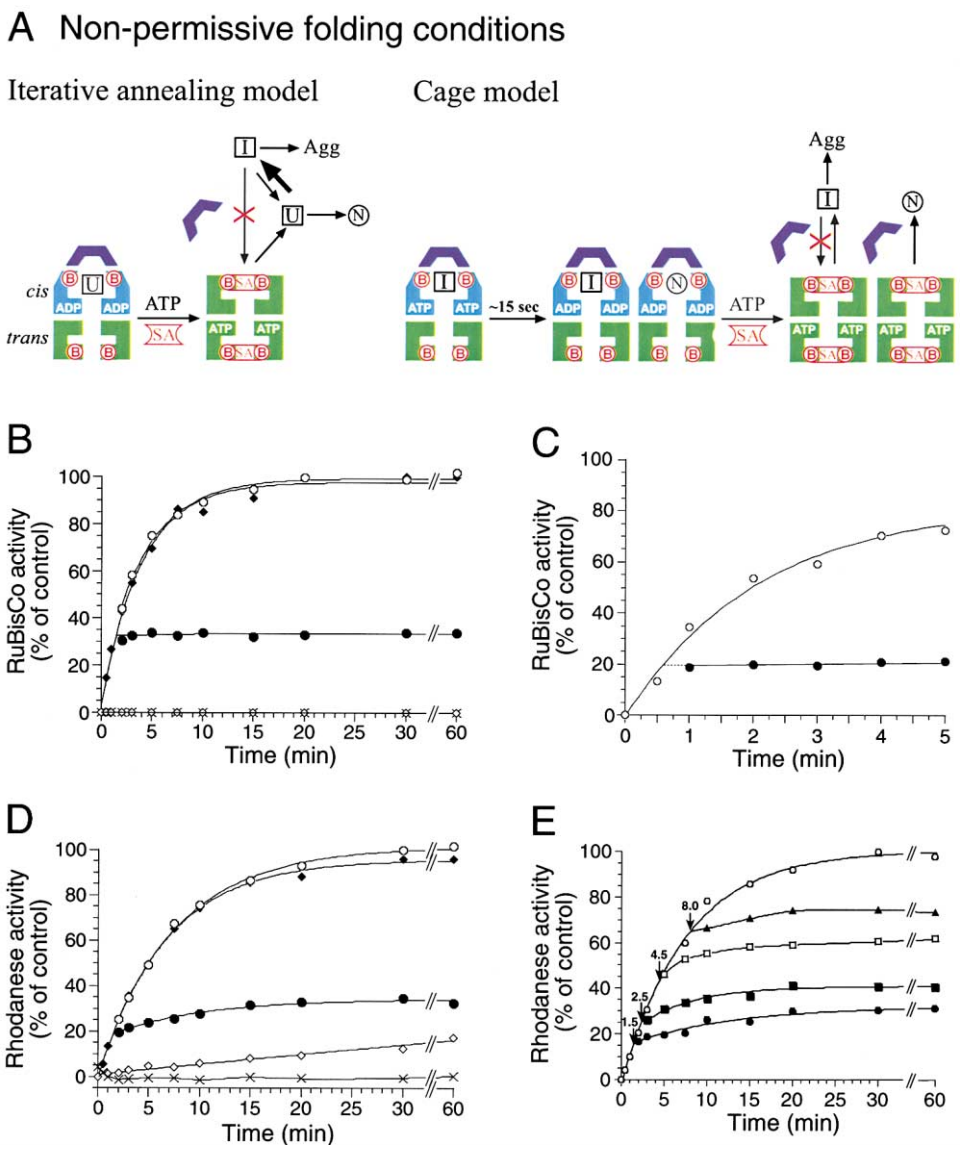

Figure 3. Effects of Interrupting Chaperonin Cycling by SA under Nonpermissive Folding Conditions

(A) Fate of nonnative protein upon release from GroEL and prevention of rebinding predicted by the iterative annealing model (left) and the cage model (right) under nonpermissive folding conditions. GroEL is shown schematically as a vertical cut through the cylinder. U, actively unfolded protein; I, aggregation-prone folding intermediate; N, native protein; Agg, aggregate; B, biotin; and SA, streptavidin. In the annealing model shown, partitioning to the native state is assumed to occur predominantly outside the cage. Note that in the cage model, only a fraction of folding intermediate (I) reaches native state in a single encapsulation cycle.

(B and C) Refolding of 0.5 μM RuBisCo and (D) of 0.5 μM rhodanese bound to 0.5 μM EL229C-B in the presence of 1 μM GroES and 5 mM ATP without further addition (◆) or with addition of 5 μM SA (●) or 1 μM EL229C/5 μM SA (○) at 1.5 min (B and D) or at 45 s (C) after initiating refolding with ATP. As controls, 0.5 μM denatured RuBisCo or rhodanese added to buffer B alone (x) or bound to EL229C-B in the presence of ATP (◇) were analyzed. Activities are expressed as a percentage of final activity reached in (○).

(E) Refolding of rhodanese as in (D) but with addition of SA at 1.5, 2.5, 4.5, and 8 min after initiating folding.

conversion of a kinetically trapped intermediate to the native state (I to N) (Figure 5A). In contrast, if GroEL/GroES functions solely as an aggregation prevention cage, acceleration of folding by chaperonin is not expected. Upon inhibiting rebinding of nonnative protein to GroEL, folding should proceed in solution at the rate k_C of assisted folding (Figure 5A).

Aggregation can usually be reduced by lowering the concentration of folding protein. Upon dilution from denaturant to a low concentration of 20 nM, rhodanese renatured spontaneously (Figure 5B). In contrast, in the presence of EL229C-B without ATP, renaturation was blocked (Figure 5B), indicating that binding of nonnative protein to GroEL is faster than spontaneous folding. Assisted folding occurred upon addition of ATP and GroES, at a rate indistinguishable from that of spontaneous folding (Figure 5B). Blocking the rebinding of nonnative rhodanese to EL229C-B by SA had no effect on the rate of folding and caused only a small reduction in yield. Thus, under permissive folding conditions, rhodanese

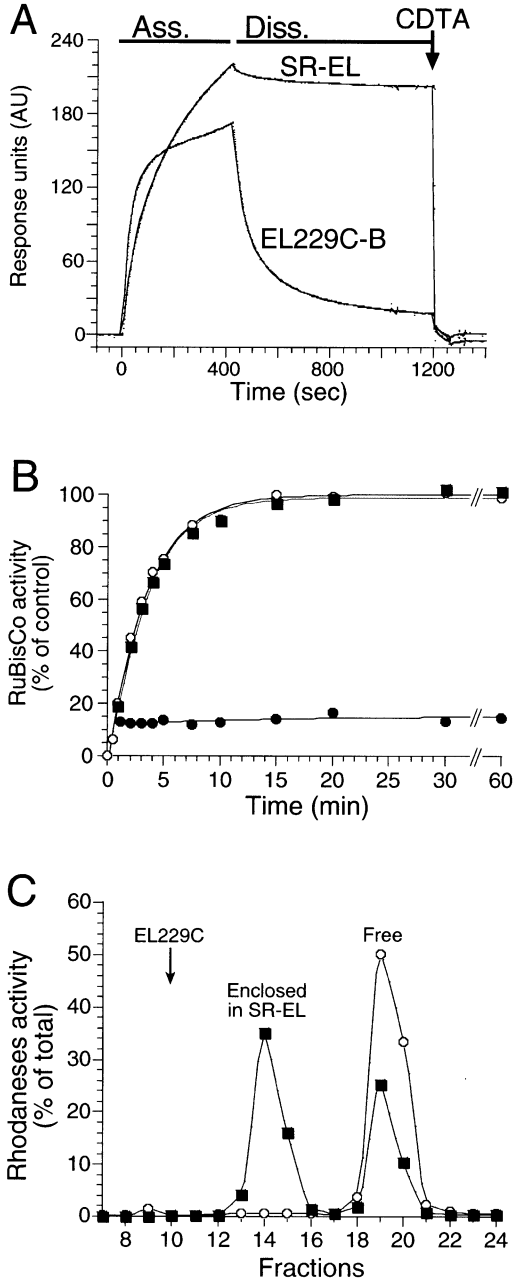

Figure 4. Shifting Nonnative Substrate Protein from Cycling GroEL/GroES to Noncycling SR-EL/GroES

(A) Binding of EL229C-B and of SR-EL to GroES analyzed by surface plasmon resonance in the presence of ATP. Ass., association phase; Diss., dissociation phase. Addition of CDTA, causing instantaneous dissociation of the SR-EL:GroES complex, is marked.

(B) Refolding of 0.5 μM RuBisCo bound to EL229C-B with the addition of either 5 μM SA (●) or 1 μM EL229C/5 μM SA (○) or 3 μM SR-EL/5 μM SA (■) at 45 s after initiating refolding, immediately followed by further addition of 4 μM GroES.

(C) Refolding of rhodanese bound to EL229C-B was initiated as in (B) followed by addition of EL229C/SA (○) or SR-EL/SA (■) at 1.5 min. After a total refolding time of 30 min, reactions were subjected to gel filtration on a Superdex200 PC 3.2/10 column and rhodanese activity determined in column fractions.

reaches native state at essentially the same rate in the presence or absence of a cycling chaperonin machinery (Figure 5B). We also conclude from these results that the block in folding observed upon inhibition of GroEL under nonpermissive conditions (Figure 3D) is due to aggregation of nonnative protein in free solution, not to formation of monomeric, kinetically trapped intermediate. Thus, with rhodanese as the substrate, the chaperonin system functions exclusively as an infinite dilution cage, preventing aggregation, and does not accelerate folding by unfolding kinetically trapped intermediates.

Confinement in the Chaperonin Cage Speeds the Folding of RuBisCo

RuBisCo has a much greater tendency to aggregate during folding than rhodanese, explaining the immediate stoppage of folding upon exclusion of rebinding of folding intermediate to GroEL under nonpermissive conditions (Figures 3B and 3C). The critical aggregation concentration of RuBisCo is strongly temperature dependent, increasing from ~10 nM at 25°C to ~900 nM at 4°C (van der Vies et al., 1992; Schmidt et al., 1994; Luo et al., 2001). Upon dilution from denaturant at low temperature, a folding intermediate (RuBisCo-I) forms that converts slowly to folded monomer, but aggregates rapidly at higher temperature (van der Vies et al., 1992; Luo et al., 2001). Conditions for efficient renaturation of RuBisCo were established at 10 to 15°C (Figure 5C). Both the rate and yield of folding at 10°C remained essentially constant at RuBisCo concentrations between 10 and 400 nM (Table 1). Moreover, RuBisCo-I behaved as a monomer on sizing chromatography (data not shown; see Luo et al., 2001), indicating that the folding rate was not limited by reversible aggregation. Interestingly, GroEL-assisted folding of RuBisCo was consistently approximately 4-fold faster than spontaneous folding (Table 1). This rate increase was dependent on GroES and ATP (Figure 5C). In the presence of GroEL and ATP alone, folding was slower than spontaneous renaturation, due to inefficient protein release from GroEL. Thus, rhodanese and RuBisCo have different folding properties. Although conditions permissive for spontaneous folding can be established for both proteins, the chaperonin accelerates the folding of RuBisCo but not of rhodanese.

The approximately 4-fold rate difference between assisted and spontaneous folding of RuBisCo is preserved at 25°C, as suggested by extrapolating the rates of spontaneous folding determined at lower temperatures (Table 1). This acceleration of folding by chaperonin could be consistent with iterative annealing (Betancourt and Thirumalai, 1999). However, a comparison of RuBisCo folding rates obtained with cycling GroEL/GroES and noncycling SR-EL/GroES at different temperatures showed that the rate of assisted folding is independent of iterative unfolding steps driven by chaperonin cycling (Figure 4B and Table 1).

It remained possible that under permissive conditions a single round of GroES-mediated unfolding is sufficient to prepare RuBisCo for rapid folding in solution. To explore this possibility, assisted folding was initiated with EL229C-B, GroES, and ATP at 10°C and then rebinding of nonnative RuBisCo to GroEL blocked by addition of

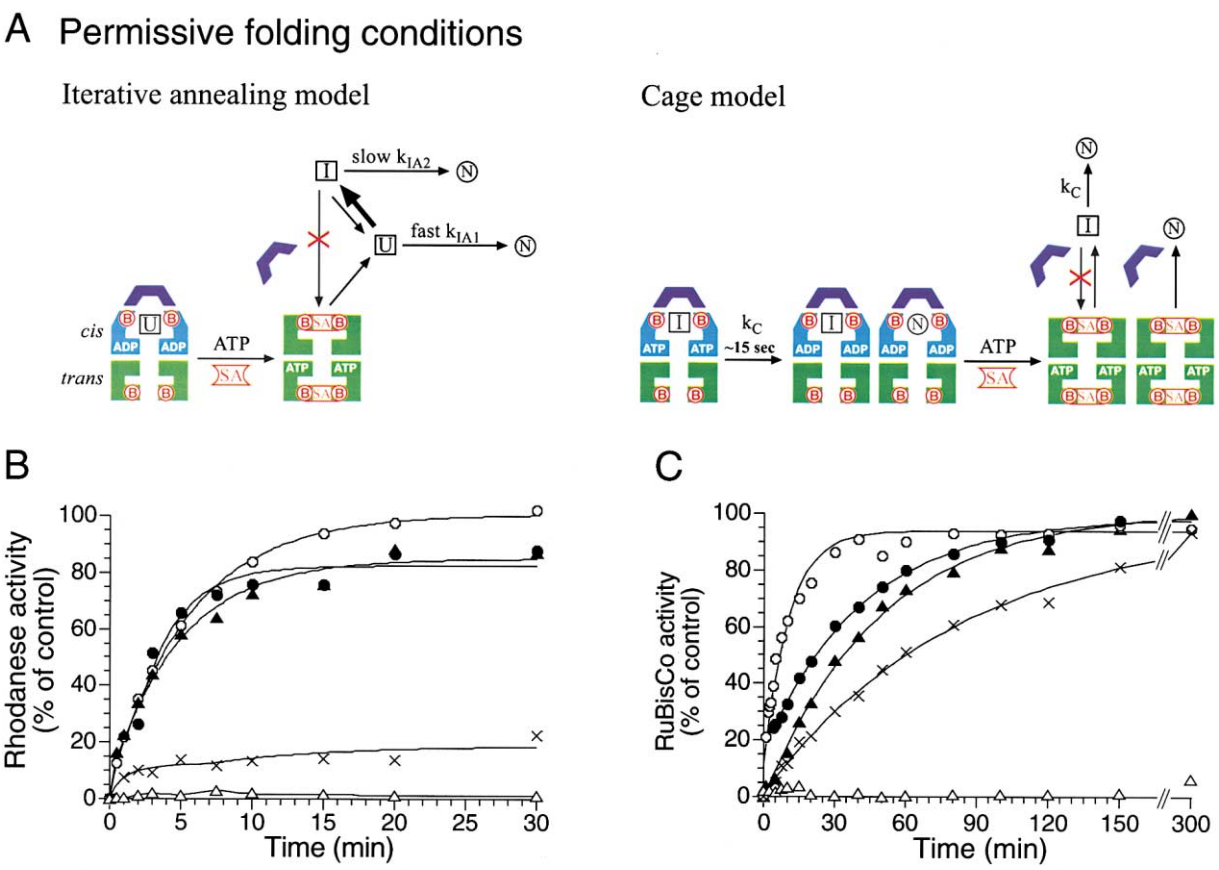

Figure 5. Spontaneous and Assisted Refolding of Rhodanese and RuBisCo under Permissive Folding Conditions

(A) Fate of nonnative protein upon release from GroEL and prevention of rebinding predicted by the iterative annealing model (left) and the cage model (right) under permissive conditions. k_{IA1} and k_{IA2}, rates for the partitioning of U to N and kinetically trapped intermediate I to N, respectively, in iterative annealing; k_C, rate for the conversion of nonnative intermediate I to N in the cage model.

(B) Refolding of rhodanese (final concentrations 20 nM) at 25°C in the absence of chaperonin (▲) or in the presence of 0.1 μM EL229C-B/0.4 μM GroES and ATP either without (○) or with addition of 1 μM SA (●) at 1.5 min after initiating refolding. GroES was omitted in rhodanese control reactions containing EL229C-B/ATP (×) or EL229C-B (△).

(C) RuBisCo refolding (10 nM final concentration) at 10°C in the absence of chaperonin (▲) or in the presence of 0.1 μM EL229C-B/0.4 μM GroES and ATP either without (○) or with addition of 1 μM SA (●) at 3.5 min after initiating refolding. GroES was omitted in control reactions containing EL229C-B/ATP (×) or EL229C-B (△).

SA. Strikingly, this resulted in an immediate shift of the fast assisted rate of folding to the slower spontaneous rate (Figure 5C). No fast folding component was detectable after SA addition. Thus, accelerated folding of RuBisCo critically depends on the confinement of the unfolded protein in the hydrophilic environment of the GroES-capped GroEL cavity.

Discussion

This study provides evidence for a dual role of protein sequestration in the GroEL:GroES cage. Folding in the cage not only serves to prevent rapid off-pathway aggregation, which precludes folding in solution, but surprisingly, mere confinement of protein in the cage can produce a significant rate enhancement of folding as compared to spontaneous folding. This effect is independent of multiple chaperonin cycles associated with iterative annealing. Instead, acceleration of folding is suggested to result from a smoothing of the energy surface of folding induced by the specific physical environment of the chaperonin cage (Figure 6).

Folding inside the GroEL:GroES Cage

Our results demonstrate that under normal conditions of chaperonin cycling, protein folding occurs inside the GroEL:GroES cage. A distinction between the cage model and the iterative annealing model of chaperonin action was achieved by following the fate of nonnative substrate protein upon its release from GroEL into solution. Under nonpermissive folding conditions, rapid streptavidin-biotin-mediated exclusion of nonnative protein from GroEL inhibits further folding. This effect is most dramatic for RuBisCo, which generates a folding intermediate that aggregates in vitro even at low nanomolar concentrations (at 25°C and above) (van der Vies et al., 1992), i.e., at concentrations much below those in vivo. Indeed, RuBisCo is the most abundant protein of the photosynthetic bacterium *R. rubrum* (10%–40%

Table 1. Single Exponential Rate Constants for Subunit Folding of RuBisCo

	Rate of RuBisCo refolding (k $min^{-1} \times 10^{-2}$)		
T°C	Spontaneous	Assisted GroEL-GroES	Assisted SR1-GroES
4	0.9 ± 0.05	—	—
10	2.2 ± 0.2	7.0 ± 2	—
10	2.1 ± 0.2; 2.0 ± 0.1[a]	—	—
15	3.8 ± 0.2	12.5 ± 1	14.6 ± 1
20	6.0 ± 0.2	22.5 ± 5	37.5 ± 5
25	11[b]	35 ± 5	38 ± 5
30	—	51 ± 5	44 ± 5

Except when stated otherwise, refolding was performed at a concentration of 10 nM RuBisCo. Averages +/− SD from 3 to 5 independent experiments are shown. The rate of spontaneous folding at 25°C was estimated by fitting the rates determined at 4 to 20°C to an exponential equation. Note that the SR-EL:GroES complex becomes less stable below 15°C.
[a] Rates at 200 and 400 nM RuBisCo, respectively.
[b] Extrapolated.

of soluble protein) (Schloss et al., 1979), with nascent, nonnative chains reaching concentrations of ~3 μM (10% of the concentration of ribosomes). Thus, although the chaperonin releases in every round of ATP-dependent action both native and nonnative protein into solution, nonnative molecules are recaptured and continue to fold in the GroEL:GroES cage, thereby avoiding aggregation. For this mechanism to be effective, GroEL rebinding of folding intermediate is faster than protein aggregation (and slow folding) in solution. Indeed, binding of nonnative polypeptide by chaperonin is a very rapid bimolecular process (k ~10^7 $M^{-1}s^{-1}$) (Corrales and Fersht, 1995; Rye et al., 1999). We estimate that under the experimental conditions at 25°C, nonnative RuBisCo spends less than 200 ms in solution between encapsulation cycles. Based on a rate of folding of ~0.1 min^{-1} (Table 1) and assuming on average 8 chaperonin cycles, less than 2% of total RuBisCo would reach native state outside GroEL, even under permissive folding conditions.

Chaperonin cycling may have been optimized to make folded subunits of oligomeric proteins available for assembly without delay and to discharge rapidly folding, chaperonin-independent proteins that may have inadvertently been enclosed. We note that the strong excluded volume effect of the bacterial cytosol resulting from macromolecular crowding is expected to increase the tendency of nonnative proteins to aggregate over that in dilute solution (van den Berg et al., 1999), but also to favor rapid rebinding of aggregation-sensitive folding intermediate to GroEL (Martin and Hartl, 1997).

Acceleration of Folding by Confinement in the Chaperonin Cage

Our results provide a new view on how chaperonin action can accelerate folding for some proteins. RuBisCo folding is complicated by the preferred formation of a kinetically trapped intermediate whose spontaneous conversion to the native state is slow (shown schematically in Figure 6A). Chaperonin-assisted folding of RuBisCo is approximately 4-fold accelerated over spontaneous folding. However, contrary to predictions from theoretical calculations with single-bead lattice chains (Betancourt and Thirumalai, 1999), accelerated folding of RuBis-

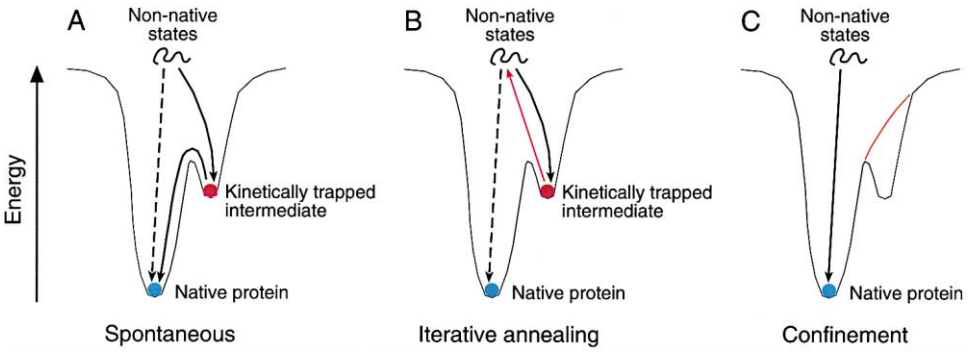

Figure 6. Schematic Representation of Accelerated Protein Folding by Chaperonin Using Simple Energy Diagrams

Folding of a protein such as RuBisCo, populating a kinetically trapped intermediate, is considered.

(A) Spontaneous folding. Unfolded protein partitions between a fast pathway to the native state and preferred formation of a trapped intermediate whose spontaneous conversion to the native state is slow.

(B) Model for accelerated chaperonin-assisted folding as a result of iterative annealing. Trapped intermediate formed either in the chaperonin cage or in solution is thought to be actively unfolded in an ATP and GroES-dependent reaction (red arrow), thereby having another chance to partition into a fast folding pathway.

(C) Accelerated chaperonin-assisted folding as a result of confinement. Formation (or accumulation) of trapped intermediate is thought to be avoided in the confined environment of the chaperonin cage. This is reflected in a smoothing of the energy surface (red line). In this model, the energy of ATP is utilized to discharge unfolded protein into the narrow hydrophilic space of the chaperonin cage.

Co is not achieved through repeated, ATP-dependent cycles of forced unfolding associated with iterative annealing (Figure 6B). As shown by blocking rebinding of nonnative protein to GroEL under permissive folding conditions, a single event of GroES-mediated unfolding, if it occurs, is not sufficient to achieve accelerated folding. Indeed, no significant unfolding was found in a recent structural analysis by NMR with malate dehydrogenase (MDH) as a chaperonin substrate (Chen et al., 2001). Moreover, as for MDH, the GroEL-bound forms of a number of other model substrates have been shown to contain varying amounts of fluctuating secondary structure but little, if any, native or nonnative tertiary structure (reviewed in Coyle et al., 1997). Thus, considering the energy landscapes of folding reactions (Dobson et al., 1998), further GroES-mediated unfolding of these intermediates prior to release would not increase their probability to partition rapidly to the native state (Figure 6B). Our data with RuBisCo rather suggest that, in the environment of the chaperonin cage, formation of certain kinetically trapped intermediates is either avoided or their progression toward the native state is facilitated. This effectively amounts to a smoothing of the energy landscape for folding inside the cage (Figure 6C). In this mechanism, the chaperonin does not utilize the energy of ATP for active unfolding but for forcing the nonnative polypeptide from a hydrophobic binding surface into a confined, hydrophilic space. Effects on the energy surface of folding are not anticipated by the original Anfinsen cage model, where the protein is thought to fold in the cage as it does at infinite dilution in solution.

Theoretical analysis of the effect of confinement of macromolecules inside narrow pores predicts that compact structures will be favored over less compact structures (Minton, 1992). Furthermore, confinement in the GroEL cage is expected to be strongly dependent on the size of the enclosed protein, consistent with the finding that folding of rhodanese (33 kDa) is not accelerated by chaperonin. So, considering that RuBisCo (50 kDa) closely approaches the size limit of the cage (~60 kDa), confinement may favor the adoption of compact chain conformations predominantly for proteins in a similar size range. It is noteworthy that a number of newly synthesized proteins that interact with GroEL are around 50 to 60 kDa in size (Ewalt et al., 1997; Houry et al., 1999). It will be interesting to compare the rates of folding of these proteins inside the chaperonin cage and in free solution.

Experimental Procedures

Proteins

EL229C (Cys138, 458, and 519 to Ala; Asn229 to Cys) was generated by site-directed mutagenesis using the Quick Change Site Directed Mutagenesis Kit from Stratagene. GroEL, EL229C, SR-EL, and GroES were purified as described (Hayer-Hartl et al., 1994, 1996; Weissman et al., 1995; Rye et al., 1997) and concentrations determined spectrophotometrically at 280 nm. Bovine rhodanese (Sigma) was further purified (Weber et al., 1998). Rho488 was prepared by labeling rhodanese with the fluorescent dye Alexa488. Labeled rhodanese retained full enzymatic activity. The coding region of the RuBisCo gene from *R. rubrum* (GenBank accession number X00286) was amplified by PCR from pRR2119 (a kind gift from C.R. Somerville [Somerville and Somerville, 1984]) and inserted into pET-11a (Novagen). Wild-type dimeric RuBisCo and the monomeric mutant K168E (Mural et al., 1990) were purified as described (van der Vies et al., 1992). Affinity-purified streptavidin (SA) was from Sigma.

Modification of EL229C with Biotin and SA

EL229C was incubated in buffer A (20 mM MOPS/KOH [pH 7.5], 100 mM KCl, and 5 mM $Mg(OAc)_2$) with a 5-fold molar excess of EZ-Link maleinimide activated biotin (linker length 29.1 Å) (Pierce) over cysteines for 10 min at 25°C. Reactions were stopped by addition of 50 mM β-mercaptoethanol (β-ME) and excess reagents removed by gel filtration on a Superdex200 HR10/30 (Pharmacia). EL229C-B was concentrated on centricon100 (Amicon), stored at 4°C, and used within a few days. Modification was >90% by quantifying free cysteines with the fluorescent probe 4-maleinimidylsalicylicacid (Molecular Probes). SA modification of EL229C-B was performed in the presence of a 10-fold molar excess of SA over EL229C-B.

Assays

Rhodanese Refolding

Rhodanese was denatured in 6 M guanidinium-HCl (GuHCl) (Langer et al., 1992) and diluted 100-fold into buffer A in the absence or presence of GroEL at 25°C, as specified in the legends. A 2-fold molar excess of GroES over GroEL was added and refolding initiated with 5 mM ATP. At the times indicated, GroEL action was stopped with CDTA (50 mM) and apyrase (10 U) and rhodanese activity measured (Martin et al., 1991; Hayer-Hartl et al., 1996). Spontaneous and assisted refolding (20 nM rhodanese) was analyzed in buffer A/50 mM $Na_2S_2O_3$. Refolding reactions were stopped by adding a 5-fold molar excess of ELD87K (GroEL-trap) (Farr et al., 1997), which binds nonnative protein but does not release it.

RuBisCo Refolding

RuBisCo was denatured in 6 M GuHCl, 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, and 10 mM DTT for 1 hr at 25°C and diluted 100-fold into buffer B (50 mM Tris-HCl [pH 7.8], 250 mM KCl, 5 mM $Mg(OAc)_2$, 1 mg/ml BSA, and 200 mM β-ME [or 5 mM DTT]) in the absence or presence of GroEL, as specified in the legends. Assisted refolding was initiated by adding a 2-fold molar excess of GroES over GroEL and 5 mM ATP. At the times indicated, aliquots (60 μl) were withdrawn and rapidly mixed with solution containing 7.5 mM CDTA and apyrase (10 U) to stop GroEL action and RuBisCo folding. RuBisCo enzyme activity was determined after incubation at 25°C for 1 hr as described by Goloubinoff et al. (1989) with modifications. $Mg(OAc)_2$ (50 mM) was added in the enzyme assay to compensate for the presence of CDTA. At low RuBisCo concentrations (2–50 nM), dimer formation becomes rate limiting. To accelerate assembly, RuBisCo mutant K168E (van der Vies et al., 1992) was added in 50× molar excess over wild-type RuBisCo during refolding. RuBisCo(K168E) forms an enzymatically inactive, folded monomer that does not interact with GroEL. Catalytic activity is produced on formation of a heterodimer with wild-type RuBisCo.

Gel Filtration

One hundred micromolar fluorescent-labeled Rho488 was diluted 200-fold from 6 M GuHCl into buffer A at 25°C containing either 0.5 μM EL229C/5 μM SA or EL229C-B/5 μM SA. After 5 min incubation, 50 μl of the reaction was applied on a Superdex200 PC 3.2/10 column. Fractions were analyzed by fluorescence spectroscopy. Denatured rhodanese not bound to GroEL aggregates and is not recovered from the column.

Kinetic Analysis of Streptavidin Binding to EL229C-B

The kinetics of SA binding to EL229C-B was analyzed in a stopped-flow apparatus (Applied Photophysics). One syringe contained 1 μM EL229C-B and the other 10 μM SA in buffer A. The two solutions were mixed rapidly (1:1) and the decrease in tryptophan fluorescence of SA was followed. Ten rapid mixing experiments were performed and the last five averaged.

Surface Plasmon Resonance

GroES mutant 98C (Rye et al., 1999) was immobilized (50–100 RU) via a thioether linkage on a CM5 biosensor chip of a BIAcore 2000 instrument and analysis performed as described (Hayer-Hartl et al., 1995). Buffer A plus 2 mM ATP was used as the running buffer

for binding experiments at a flow rate of 20 μl/min at 25°C. The concentration of GroEL was 250 nM.

Electron Microscopy

EL229C-B:SA was concentrated to 20 μg/ml and negatively stained with 2% (w/v) uranyl acetate. Images of EL229C-B and EL229C-B:SA were recorded with a CM 20 FEG Philips electron microscope equipped with a 2k by 2k CCD-camera at a magnification of 47,000×. In order to apply standard correlation averaging methods, 790 (EL229C-B) and 930 (EL229C-B:SA) single molecules were extracted from band pass filtered images. The average of EL229C-B molecules was 7-fold symmetrized (Figure 1C) and used as a first reference for translation and orientation alignments of the EL229C-B/SA complexes. The resulting average was then employed as a new reference in a refinement pass. To detect interimage structural variations, the aligned molecules were subjected to a classification procedure based on eigenvector-eigenvalue data analysis (Engel et al., 1995). The result, a class average of 700 complexes, is shown in Figure 1D.

Acknowledgments

We thank Rosemarie Schiebel, Dirk Wischnewski, and Carola Griffel for excellent technical assistance; Holger Sondermann for Figure 1A; Frank Weber for establishing the spontaneous folding condition for rhodanese; and John Ellis for discussion. A.B. was supported by a fellowship from the Boehringer Ingelheim Foundation; D.J.N. was supported by a fellowship from EMBO.

Received July 9, 2001; revised August 22, 2001.

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