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GroEL stimulates protein folding through forced unfolding

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Abstract

Many proteins cannot fold without the assistance of chaperonin machines like GroEL and GroES. The nature of this assistance, however, remains poorly understood. Here we demonstrate that unfolding of a substrate protein by GroEL enhances protein folding. We first show that capture of a protein on the open ring of a GroEL–ADP–GroES complex, GroEL’s physiological acceptor state for non-native proteins *in vivo*, leaves the substrate protein in an unexpectedly compact state. Subsequent binding of ATP to the same GroEL ring causes rapid, forced unfolding of the substrate protein. Notably, the fraction of the substrate protein that commits to the native state following GroES binding and protein release into the GroEL–GroES cavity is proportional to the extent of substrate-protein unfolding. Forced protein unfolding is thus a central component of the multilayered stimulatory mechanism used by GroEL to drive protein folding.

Many proteins cannot fold spontaneously in the concentrated environment of a cell^{1–3}. These proteins typically show slow and complex folding behavior that renders them susceptible to misfolding and aggregation^{4,5}. Aggregation of an indispensable cellular protein, and the loss of a key activity, is typically fatal. However, misfolding and aggregation of even nonessential proteins can result in cellular dysfunction and disease^{6–9}. Several large families of specialized proteins, known as molecular chaperones, have evolved to prevent protein misfolding and aggregation^{10–13}. In many cases, molecular chaperones seem to serve primarily as passive aggregation inhibitors, selectively binding aggregation-prone folding intermediates and blocking the self-association reactions that lead to aggregate formation^{10,11}. In other cases, it has been suggested that molecular chaperones actively facilitate protein folding by using the energy of ATP hydrolysis to alter the conformations accessible to a non-native protein^{14–16}.

Among the molecular chaperones, the chaperonins or Hsp60s use a distinct physical mechanism, capturing and confining non-native folding intermediates inside an isolated cavity^{17–21}. The chaperonin system of *Escherichia coli*, comprising GroEL and its smaller co-chaperonin GroES, is perhaps the best-studied example of this molecular chaperone family^{13,14}. GroEL is a large, homo-oligomer constructed from 14 subunits of 57 kDa arranged in a double ring complex, with 7 subunits in each ring²². Each GroEL ring

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AUTHOR CONTRIBUTIONS

Z.L. and D.M. contributed equally to the experimental design, execution of the experiments and the analysis and interpretation of the data; H.S.R. contributed to the experimental design, analysis and interpretation of the data and was primarily responsible for writing the manuscript.

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possesses a large central cavity that, in the absence of ATP and GroES, is lined with hydrophobic amino acids that capture poorly folded non-native substrate proteins^{22,23}. These hydrophobic binding sites are located on the inner surface of the GroEL apical domain at the open ends of each ring. The equatorial domain at the base of each GroEL subunit forms the interface between the two GroEL rings and contains the catalytic ATPase site that powers the GroEL reaction cycle^{24–26}. A GroEL-mediated folding reaction begins with the capture of a non-native substrate protein on an open GroEL ring^{27–30}. Subsequent binding of ATP to the same protein-loaded ring triggers the large-scale rearrangement of the GroEL apical domains, allowing GroES to bind to the GroEL ring^{18,31}. GroES binding results in the encapsulation and release of the non-native substrate protein into the isolated GroEL–GroES cavity (a *cis* complex)^{17,19–21}. Hydrolysis of ATP within the *cis* complex followed by ATP binding to the other GroEL ring (the *trans* ring) then causes GroES to be released and the substrate protein to be ejected into free solution, whether or not it has achieved a folded conformation that is committed to the native state^{17,26,27,30}. Substrate-protein molecules that fail to fold must then be recaptured for another passage through the GroEL reaction cycle.

Precisely how the capture and encapsulation of a non-native protein within the GroEL–GroES cavity leads to stimulated protein folding remains poorly understood. It was initially suggested that, as do many other molecular chaperones, GroEL functions as a passive aggregation inhibitor, with the GroEL–GroES cavity serving to simply sequester aggregation-prone folding intermediates^{32,33}. Because aggregation of a substrate protein would be prevented by encapsulation in the GroEL–GroES cavity, this model implied that GroEL's primary role is to 'infinitely' dilute substrate proteins and allow them to fold at their intrinsic spontaneous rates within the GroEL–GroES chamber³². Although simple binding of non-native proteins by GroEL does indeed inhibit aggregation, a considerable body of evidence suggests that, for many substrate proteins, GroEL is much more than a passive box and is actively involved in facilitating protein folding (for review, see refs. 13,14). On the basis of both experimental and theoretical studies, it has been suggested that confinement of a non-native protein within the GroEL–GroES cavity enhances folding by altering the substrate protein's folding landscape^{34–40}. In essence, this model suggests that the restricted volume, and perhaps the surface character, of the GroEL–GroES cavity inhibits formation of expanded and otherwise misfolded conformational states that slow or block folding of the protein in free solution. At the same time, collapsed conformations that are more likely to find the native state are favored, resulting in an overall smoothing of the substrate protein's folding landscape^{34–36,38,39}.

A third model of GroEL action, based on the idea that GroEL stimulates productive protein folding through unfolding, has also been proposed (for review see refs. 14,15). The most general form of this model posits that substrate proteins that strictly depend upon GroEL for their folding (so-called 'stringent' substrate proteins) are those most likely to fall into deep energetic traps that ambient thermal fluctuations cannot reverse⁴¹. These quasi-stable, misfolded states are highly prone to irreversible aggregation. In principle, a simple way to stimulate the productive folding of such a protein would be to partially disrupt the nascent structure of the trapped protein conformation, giving the substrate protein a fresh opportunity to fold from a higher point on its free energy landscape^{41,42}. Although a large body of evidence suggests that capture of a substrate protein by GroEL can indeed result in protein unfolding, the magnitude and impact of this effect has remained unclear¹⁴. In addition, unfolding by the full GroEL–GroES system has not been directly linked to enhanced folding of any stringent substrate protein to date. A variation on the unfolding model has also been proposed, in which it is suggested that GroEL delivers a mechanical unfolding force to a substrate protein in the course of GroES binding and substrate-protein encapsulation^{42–44}. Although an early tritium-amide exchange study was consistent with this

mechanism, termed ‘forced’ unfolding, subsequent and more detailed deuterium-amide exchange experiments were not^{44–46}. Thus, the existence and importance of protein unfolding by GroEL has remained controversial^{14,36,45}.

Here we demonstrate that unfolding of a stringent substrate protein by GroEL substantially enhances productive protein folding. We first examine the conformation of a non-native substrate protein bound to the open *trans* ring of a GroEL–ADP–GroES complex, GroEL’s physiological acceptor state for non-native proteins *in vivo*. Unexpectedly, we find that the non-native substrate protein is considerably more compact when bound to a *trans* ring than when bound to the GroEL tetradecamer alone. We then show that the captured folding intermediate is subjected to a rapid unfolding force when ATP binds to the loaded *trans* ring. Notably, we find that the fraction of the substrate protein that commits to the native state upon release into the GroEL–GroES cavity is directly proportional to the extent of substrate unfolding. Forced protein unfolding is thus a central component of the multilayered stimulatory mechanism employed by GroEL to drive protein folding.

RESULTS

Non-native Rubisco bound to a GroEL *trans* ring

The capture of a non-native substrate protein on an open GroEL ring is a necessary first step in GroEL-mediated protein folding. Because protein unfolding could, in principle, have a profound impact on productive folding, it remains crucially important to determine whether or not substrate-protein capture by a GroEL ring causes substantial protein unfolding. However, our current understanding of protein capture by GroEL is principally based on studies involving double-ring GroEL in the absence of nucleotide and GroES (referred to here as apoGroEL)^{11,14,47}. Under *in vivo* conditions, by contrast, the open *trans* ring of an asymmetric GroEL–ADP–GroES complex (an ‘ADP bullet’) is the acceptor state for non-native substrate proteins (Fig. 1a and refs. 21,27,48). Whether or not the conformation of a substrate protein bound to the *trans* ring of an ADP bullet is the same as that populated on an apoGroEL ring has remained unclear.

We therefore examined whether the conformation of a substrate protein bound to the *trans* ring of an ADP bullet is the same as that populated on an apoGroEL ring. We used Rubisco from *Rhodospirillum rubrum* as a model substrate protein for this analysis, because it is one of the most stringently dependent GroEL substrate proteins known²⁹. We also used a mixing protocol that yields a nearly homogeneous population of asymmetric ADP bullets (see Methods). The ADP bullets are stable for at least an hour when maintained in low concentrations of ADP (5–15 μ M; Supplementary Fig. 1 online). Binding of non-native Rubisco to the *trans* ring of an ADP bullet is comparable, in both rate and strength, to apoGroEL binding (ref. 27 and data not shown).

For these studies, we used an intramolecular fluorescence resonance energy transfer (FRET) assay that reports on changes in Rubisco conformation. In earlier work, we developed a mutagenesis and labeling protocol that allows the specific attachment of exogenous fluorescent probes to particular sites on the Rubisco monomer³⁵. The fluorescent dyes have no detectable effect on the stability, enzymatic activity or GroEL-mediated folding of this substrate protein. By coupling distinct probes to the amino- and carboxyterminal domains of the Rubisco monomer, we demonstrated that the average expansion of the non-native Rubisco monomer could be monitored by FRET³⁵. Binding of non-native Rubisco to an apoGroEL ring results in a near total loss of detectable FRET between the labeled regions of the Rubisco monomer (Fig. 1b,c and ref. 35). This observation suggests that, upon binding to an apoGroEL ring, the Rubisco monomer is subjected to a dramatic stretching or conformational expansion³⁵. By contrast, when the Rubisco monomer is bound to the *trans*

ring of an ADP bullet, we observe a much higher level of energy transfer, as evidenced by the considerable quenching of the donor fluorescence emission and decrease in the donor lifetime (Fig. 1b,c). These observations indicate that the Rubisco monomer populates a distinct conformational state or family of states when captured by a *trans* ring.

The large FRET signal of *trans* ring-bound Rubisco suggests that the non-native monomer is more compact when bound to the ADP-bullet *trans* ring than when bound to an apoGroEL ring. Quantitative analysis of both steady state and time-resolved FRET data support this conclusion, indicating that the labeled segments of the non-native Rubisco monomer are, on average, approximately 25 Å closer together when the protein is bound to an ADP-bullet *trans* ring (Fig. 1b, inset). Binding of Rubisco to an ADP-bullet *trans* ring thus results in an expansion of no more than 4–6 Å of the monomer relative to a kinetically trapped and misfolded state of the protein³⁵. By contrast, non-native Rubisco binding to an apoGroEL ring results in an expansion of 30 Å or more³⁵. Because these FRET observations probe only the dimensions of the Rubisco monomer along a single spatial axis, we examined the overall conformational properties of GroEL-bound Rubisco with two more methods. A more collapsed Rubisco monomer would be expected to show (i) reduced susceptibility to proteolytic fragmentation; and (ii) decreased reactivity of buried cysteine residues toward soluble thiol alkylating agents. Consistent with our FRET observations, non-native Rubisco bound to the *trans* ring of an ADP bullet is substantially less susceptible to protease digestion than when it is bound to apoGroEL (with chymotrypsin, Fig. 2a,b; with trypsin, Supplementary Fig. 2 online). In addition, *trans* ring-bound Rubisco shows digestion patterns with both proteases that are distinct from those observed with apoGroEL. We also observe changes in the chemical reactivity of several endogenous Rubisco cysteine residues in *trans* ring-bound Rubisco (Fig. 2c). These cysteine residues are fully buried and nonreactive to small fluorescent probes in native Rubisco, but they are exposed and highly reactive when non-native Rubisco binds to apoGroEL (Fig. 2c). The same cysteine residues, however, show intermediate reactivity when the non-native Rubisco monomer is bound to the *trans* ring of an ADP bullet, consistent with the internal structure of the protein being considerably less exposed to solvent. Overall, these observations strongly suggest that the non-native Rubisco monomer bound to an ADP-bullet *trans* ring is more compact and is present in a different conformation than when it is bound to an apoGroEL ring.

ATP binding causes forced protein unfolding

The more compact state of the Rubisco monomer indicates that the *trans* ring of an ADP bullet cannot induce the same level of binding-driven protein unfolding as an apoGroEL ring³⁵. We refer to this type of unfolding as ‘passive unfolding’ because it depends on only simple, physical contact between the non-native Rubisco monomer and the GroEL ring. Although passive unfolding has also been observed with other substrate proteins, a direct linkage between this effect and GroEL-stimulated folding has been difficult to establish¹⁴. Furthermore, although passive unfolding of a substrate protein on an apoGroEL ring can be extensive, it is also slow. With Rubisco, much of the passive unfolding seen with apoGroEL proceeds much more slowly ($t_{1/2} = \sim 5\text{--}8$ min at 25 °C) than GroEL’s functional reaction cycle ($t_{1/2} = \sim 15\text{--}20$ s at 25 °C)^{25,27,35,41}. In this context, our observation of limited passive unfolding of Rubisco by the ADP-bullet *trans* ring raises a key question: how much unfolding, if any, is necessary for productive folding? The modest level of passive unfolding on an ADP-bullet *trans* ring might represent a maximally effective level of substrate unfolding. Alternatively, passive unfolding on the ADP-bullet *trans* ring might represent only the first phase of substrate-protein unfolding. A bound Rubisco monomer might be subjected to further unfolding before encapsulation within the GroEL–GroES cavity. In particular, the dramatic rearrangements of the GroEL apical domains caused by ATP

binding, movements that precede and are required for GroES binding, might well induce further alterations in the bound substrate protein^{42,43}.

To address this question, we examined conformational changes in the *trans* ring-bound Rubisco by using a FRET-based rapid mixing assay (Fig. 3a and ref. 35). Notably, upon ATP and GroES binding to the Rubisco-occupied *trans* ring of a wild-type GroEL bullet, we observed two initial phases of conformational alteration in the non-native Rubisco monomer, based on the occurrence of distinct, time-dependent changes in intramolecular FRET: an initial rapid drop in FRET efficiency followed a slower increase in FRET efficiency (Fig. 3b). The same overall changes in FRET signal are also seen with a second pair of FRET dyes, as well as with a distinct set of dye-attachment sites, indicating that the conformational changes we observe occur across multiple spatial axes of the Rubisco monomer (Supplementary Fig. 3 online). The rising phase of the FRET signal is reminiscent of previous observations and probably reflects the compaction of the folding intermediate upon GroES binding³⁵. This conclusion is supported by the fact that the rate of the rising phase is dependent on the concentration of GroES in a classically bimolecular fashion (Fig. 3c). A third phase of FRET signal change is also detectable when experiments are conducted under conditions that allow extended observation of the stable complex (manual mixing and rapid enzymatic depletion of ambient ATP; Supplementary Fig. 4 online). Under these conditions, the initial drop and subsequent rise in FRET efficiency are followed by a much slower decrease in FRET efficiency, consistent with previous observations³⁵, and this corresponds to substrate-protein release and folding inside the GroEL–GroES *cis* complex.

It is important that the rising FRET signal indicative of GroES binding is preceded by a rapid decrease in FRET efficiency (Fig. 3b). This initial falling phase reflects a rapid increase in the distance between the labeled segments of the Rubisco monomer and, therefore, a conformational expansion or partial unfolding of the captured Rubisco intermediate. Because the observed rate of this expansion phase shows no dependence on the concentration of GroES (Fig. 3c), this unfolding event seems to occur independently of and before GroES binding. The observation of rapid, partial unfolding of the Rubisco intermediate before GroES binding suggests that ATP binding alone, and the attendant structural shifts of the GroEL ring, are sufficient to drive a forced conformational rearrangement of the bound substrate protein. We tested this idea by repeating a series of experiments similar to those shown in Fig. 3, but under conditions in which GroES was prevented from binding to the Rubisco-occupied *trans* ring (excess SR1, a single-ringed GroEL variant, added as a GroES trap²¹). Under these conditions, the consequences of ATP binding alone to the substrate-occupied *trans* ring can be observed (Fig. 4a). Binding of ATP to the *trans* ring of a wild-type GroEL bullet induces a rapid drop in the Rubisco FRET efficiency (Fig. 4b), similar to the rapid expansion that precedes GroES binding in Figure 3. The same overall changes were also observed with a second set of FRET probes (Supplementary Fig. 3). The partial unfolding event is well characterized by a single exponential rate law across a 20-fold range of ATP concentration, and the observed rate constant shows classical saturation behavior (Fig. 4c). The fitted rate constant of the fast phase in the absence of free GroES seems to be slower than the fast FRET change observed in the presence of GroES (Fig. 4c). However, this disparity is probably an artifact of the competing change in FRET efficiency, induced by the binding of GroES, artificially increasing the apparent rate of the initial falling phase in the presence of GroES.

Unfolding enhances productive Rubisco folding

We thus observe two phases of Rubisco unfolding by GroEL: (i) slow passive unfolding of the protein following capture by an open GroEL ring; and (ii) rapid, ATP-driven unfolding. Although these observations are highly suggestive, the ultimate significance of substrate-protein unfolding by GroEL rests on whether unfolding is linked to productive folding. In

principle, substrate-protein unfolding provides a simple means of repositioning a kinetically trapped folding intermediate to a higher point on its free energy landscape, potentially opening efficient and otherwise inaccessible routes to the substrate protein's native state^{14,15}. This model predicts that the fraction of a non-native substrate protein that commits to the native state should be correlated with the extent of GroEL-induced unfolding (Fig. 5). Unfortunately, testing this prediction on the *trans* ring of an ADP bullet is difficult, given the speed with which ATP-driven unfolding proceeds. A powerful alternative approach, however, is provided by the much slower rate of passive unfolding induced by the single-ringed GroEL variant SR1 (ref. 35).

Upon binding to an SR1 ring, Rubisco is subjected to a substantial level of passive unfolding³⁵ (Fig. 6a). SR1 also binds ATP and GroES and initiates a fully productive folding reaction inside a long-lived SR1–GroES complex^{17,19,20}. Importantly, however, SR1 supports only a minimal level of ATP-driven unfolding. In previous experiments, we detected a weak, ATP-driven expansion of the FRET-labeled Rubisco intermediate with SR1 (ref. 35). The magnitude of this apparent unfolding was small, and it was not detectable in the presence of GroES. However, these experiments were conducted under conditions (a 20-min preincubation of the SR1–Rubisco binary complex) in which passive unfolding on the SR1 ring is complete (Fig. 6a). SR1 could fail to support ATP-driven unfolding under these conditions because the long preincubation time allows maximal expansion of the substrate protein through passive unfolding. We therefore examined the ability of SR1 to cause ATP-driven Rubisco unfolding at different points along the SR1 passive-unfolding curve (Fig. 6b). Binary complexes between labeled Rubisco samples were formed and rapidly loaded into the syringe of a stopped flow apparatus. The preincubation time in this experiment takes into account the time (~15–20 s) required for complex formation and loading. When ATP is mixed with an SR1–Rubisco complex at preincubation times for which passive unfolding is less extensive, SR1 shows the same, low level of ATP-driven unfolding that is observed at longer times (Fig. 6b,c). Additionally, SR1 seems incapable of maintaining even this reduced level of ATP-driven expansion of the Rubisco monomer. Within 3–4 s, the SR1-bound Rubisco relaxes back to a conformational state similar to the state it populated before ATP addition (Fig. 6b). By contrast, the more extensive ATP-forced expansion of the Rubisco monomer on an ADP-bullet *trans* ring is stable for at least 7–10 s (Fig. 4b). SR1 thus shows a considerably reduced ability to support ATP-driven substrate unfolding. As a consequence, the slow passive unfolding of Rubisco on an SR1 ring provides a straightforward way to control the extent of substrate unfolding (Fig. 7a,b).

We therefore used SR1 to test whether unfolding stimulates productive Rubisco folding. We conducted a series of refolding experiments in which non-native Rubisco was bound to SR1 and incubated for various times before the addition of ATP and GroES (Fig. 7b). Productive folding was assessed by tracking the regain of enzymatic activity as a function of time (Fig. 7c). Whereas the overall refolding curves at each preincubation time are similar, the zero-time preincubation point (that is, the y-intercept in Fig. 7c) is directly proportional to the extent of SR1-mediated unfolding up to the point (~150 s) at which passive unfolding on the SR1 ring is complete (Fig. 7d). Thus, the fraction of non-native Rubisco that commits to the native state in the first few seconds following GroES encapsulation on SR1 increases in proportion to the amount of time that passive unfolding is allowed to proceed on the SR1 ring. Importantly, the final yield of native Rubisco following extended incubation within the SR1–GroES cavity is independent of the preincubation time (Fig. 7d, inset), demonstrating that the effect of a short preincubation time is not the result of incomplete Rubisco binding or encapsulation. Our results with SR1 thus strongly suggest that substrate unfolding by GroEL leads directly to improved partitioning of the substrate protein between more and less efficient folding routes.

DISCUSSION

Although several mechanisms have been proposed to explain how GroEL facilitates protein folding, the role of substrate-protein unfolding has remained controversial. Here we show that GroEL-induced unfolding can have a profound and direct influence on the productive folding of the stringent substrate protein Rubisco. Using GroEL's physiological acceptor state for non-native proteins, the ADP bullet, we show that non-native Rubisco is substantially more compact when bound to a *trans* ring than when bound to an apoGroEL ring. This observation suggests that the *trans* ring of an ADP bullet is much less capable of passive protein unfolding, although precisely why this is the case remains unclear. Passive unfolding is probably caused by the high density of hydrophobic surface on the inner apical face of an open GroEL ring, where non-native proteins like Rubisco make multiple, simultaneous binding contacts⁴⁹. The high local concentration of hydrophobic surface on the interior of an open GroEL ring probably catalyzes the rearrangement and partial unfolding of the protein, perhaps in a manner analogous to surface-mediated denaturation^{50,51}. The observation that the *trans* ring of an ADP bullet is less capable of passive unfolding suggests that either a *trans* ring cannot engage a substrate protein across multiple apical domains in the same way that an apoGroEL ring can, or that the *trans* ring is not capable of dynamically adjusting its structure in response to substrate-protein binding in the same manner as the less constrained apoGroEL ring⁵². Whatever the ultimate reason, our observations suggest that, at least for substrate proteins like Rubisco, the conformation of a substrate protein bound to the *trans* ring of an ADP bullet can be substantially different from that observed with an apoGroEL ring.

Although the extent of passive unfolding on the ADP-bullet *trans* ring is limited, it is only the first stage of substrate-protein unfolding by GroEL. Here we show that the binding of ATP to a Rubisco-loaded *trans* ring results in the rapid conformational expansion of the bound protein. This ATP-driven stretching of the GroEL-bound folding intermediate is highly consistent with forced protein unfolding^{42–44}. In the original forced-unfolding model, extensive destabilization of a substrate protein's internal structure was directly linked to substrate-protein encapsulation within the GroEL–GroES cavity, requiring the binding of both ATP and GroES. By contrast, the forced conformational change we observe seems to depend only on ATP binding, and it actually precedes GroES binding. Furthermore, detailed hydrogen-deuterium exchange experiments with malate dehydrogenase (MDH) and Rubisco failed to detect evidence for the extensive, global unfolding that was suggested by the original forced-unfolding model^{45,46}. These studies, however, left open the possibility that less extensive and longer-range structural alteration of a misfolded protein, including disruption of stabilizing tertiary contacts not readily observed by hydrogen-deuterium exchange, could be important for initiating productive folding. Our observations are consistent with the forced disruption of this kind of residual structure in a GroEL-bound folding intermediate and provide the best current evidence that the ATP-driven allosteric shift of a GroEL ring can cause partial, forced unfolding of a stringent substrate protein.

Our observations also provide direct experimental evidence that protein unfolding by GroEL is mechanistically linked to enhanced protein folding. By exploiting the slow rate of passive Rubisco unfolding by SR1, we show that unfolding directly enhances the efficiency with which Rubisco commits to the native state upon substrate-protein release into the SR1–GroES cavity. At the same time, however, encapsulation of Rubisco within the GroEL–GroES cavity also dramatically stimulates folding under conditions in which spontaneous folding is not possible^{17,19,20,35,36}. Several mechanisms have been proposed to explain how simple confinement of a non-native folding intermediate within the spatially confined GroEL–GroES cavity could lead to enhanced folding (for review, see ref. 14). Notably, even when unfolding by an SR1 ring is reduced, long-term confinement within the SR1–GroES

cavity results in fully productive folding^{34–36} (Fig. 7). This raises an important question: if confinement within a GroEL–GroES cavity can be fully efficient, to what extent can substrate-protein unfolding actually contribute to productive folding?

The answer to this question rests, at least in part, with the limited lifetime of the GroEL–GroES cavity during a physiologically relevant GroEL reaction cycle. Although long-term confinement within an isolated SR1–GroES cavity supports nearly complete Rubisco folding, Rubisco unfolding results in a maximal stimulation of ~10% for any given release event (Fig. 7d). However, in the case of Rubisco folding by SR1, the Rubisco monomer is bound and ejected only once into the productive SR1–GroES cavity, where the protein then spends the total remainder of the experimental observation time in an optimized folding environment. In the case of the cycling GroEL–GroES system, however, the relatively short lifetime (15–20 s) of the GroEL–GroES cavity during the course of a typical reaction cycle^{25,27} limits the capacity of *cis* cavity confinement to drive folding. During a typical GroEL reaction cycle, a non-native Rubisco monomer must spend a substantial fraction of its time in one of several states that have little or no capacity to fold. These states include: (i) kinetically trapped, non-productive states that are populated after the protein is ejected into free solution with each turn of the GroEL cycle and that must then be recaptured; (ii) bound to an open GroEL ring, waiting for ATP and GroES to bind; and (iii) stretched on a GroEL ring as GroES binds and encloses the protein. In other words, a protein transiting the cycling GroEL–GroES system has access to only a fraction of the in-cavity folding time of a protein confined within the artificially stable SR1–GroES cavity. A several-fold improvement in folding efficiency caused by substrate-protein unfolding during each turn of the GroEL cycle will, therefore, have a considerable impact.

The ability of GroEL to unfold kinetically trapped folding intermediates also provides a simple explanation for how GroEL and GroES drive folding under conditions in which encapsulation of a substrate protein is not possible. In several cases, large substrate proteins that cannot be accommodated within the GroEL–GroES cavity have been shown to gain a considerable folding benefit from binding to the *trans* ring of a GroEL–GroES complex, followed by direct ejection of the protein into free solution^{53,54}. Indeed, *trans*-only GroEL–GroES complexes that are incapable of protein encapsulation can substantially enhance the folding Rubisco and another classical GroEL substrate protein, MDH⁵⁵. Partial unfolding of these proteins on the open GroEL–GroES *trans* ring before their ejection into free solution provides a simple and elegant explanation for these observations.

The ATP-driven, forced expansion of a folding intermediate by GroEL can be viewed, then, as a means to rapidly complete the process of substrate unfolding that is initiated by the much slower step of passive, binding-driven unfolding. The combined steps of passive and forced protein unfolding serve to disrupt misfolded and inhibitory conformations of a substrate protein, providing the protein with a fresh opportunity at productive folding^{14,15}. Confinement of the non-native protein within the constricted space of the GroEL–GroES cavity then enhances and amplifies the probability that a protein finds a productive folding path^{34–36}. In folding Rubisco, GroEL thus makes use of a multilayered folding mechanism, involving both passive and forced partial unfolding, in combination with brief confinement within the GroEL–GroES cavity. GroEL's ability to use multiple physical methods to stimulate Rubisco folding may also explain how this chaperonin machine assists the folding of diverse substrate proteins that possess a range of physical and energetic constraints on their productive folding.

METHODS

Proteins

Wild-type GroEL, cysteine-free GroEL and SR1 were expressed and purified using a combination of previously described methods^{17,27,56} (Supplementary Methods). Wild-type GroES, GroES98C, wild-type Rubisco and the various Rubisco cysteine mutants were expressed and purified as previously described^{17,27,35}.

Labeling of Rubisco with fluorescent dyes

Labeling of Rubisco variants was carried out as previously described^{27,35}. The thiol-reactive dyes used in this study were: 5-iodoacetamidofluorescein (F), 5-(2-acetamidoethyl) aminonaphthalene-1-sulfonate (AEDANS), tetramethylrhodamine-5-iodoacetamide (R) and 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM). All reactive dyes were obtained from Invitrogen (Molecular Probes) and were prepared fresh from dry powder in anhydrous dimethyl formamide (DMF) immediately before use. The extent and specificity of dye conjugation was confirmed by (i) determination of the protein:dye ratio using a combination of dye absorption and protein quantitation (Bradford assay; BioRad); (ii) denaturing ion-exchange chromatography of labeled proteins using anion-exchange chromatography in 6 M urea⁵⁷; (iii) quantitation of the residual free thiol content of proteins following dye conjugation using a thiol-detection assay (Invitrogen, Molecular Probes); and (iiii) analysis of dye-labeled peptides following trypsin digestion, reverse-phase (C18) chromatography and either in-line fluorescence detection⁵⁷ or MS. Unless otherwise stated, the extent of dye conjugation to specific cysteine residues was between 95% and 100%. The labeling efficiency of ES98F was ~15% (~1 dye per heptamer). Exposure of Rubisco cysteine residues was examined with a short pulse (10 min) of the coumarin-derived, maleimide-reactive dye CPM. Dye incorporation was examined by reverse-phase HPLC analysis. See Supplementary Methods for further details.

Refolding and enzymatic assays

The refolding of Rubisco with SR1 was assayed essentially as previously described^{17,35}. Rubisco was denatured in acid-urea and mixed with excess SR1 for the indicated times at 25 °C before the addition of GroES and ATP.

Asymmetric GroEL–GroES complex formation and protease protection

The binding of Rubisco to the *trans* ring of a GroEL–GroES complex was conducted by first creating an asymmetric GroEL–ADP–GroES complex. Samples of GroEL (7 μM), GroES (24.5 μM) and ATP (250 μM) were mixed in 50 mM HEPES (pH 7.6), 5 mM potassium acetate, 10 mM magnesium acetate and 2 mM DTT (buffer A) and incubated for 10 min at 25 °C. The asymmetric complex was diluted to the desired concentration and supplemented with acid-urea denatured Rubisco, and the sample was then incubated for 10 min at 25 °C to allow *trans* ring binding. For protease protection experiments, the asymmetric GroEL–ADP–GroES complex was mixed (120 nM GroEL, 350 nM GroES and 14 μM ADP final) with acid-urea denatured, fluorescein-labeled Rubisco (58-F, 100 nM). Binary complexes between apoGroEL and denatured, fluorescent Rubisco were created by mixing 100 nM denatured, fluorescent Rubisco with apoGroEL (120 nM). GroES (350 nM) and ADP (14 μM) were then added in order to match the total protein content of each reaction. Following a 30 min incubation at 25 °C, samples were supplemented with either trypsin (0.2 μg ml⁻¹; Sigma) or chymotrypsin (0.3 μg ml⁻¹; Sigma) and incubated at 25 °C. Aliquots were removed at various times, treated with 0.2 mg ml⁻¹ PMSF and then subjected to SDS-PAGE. The level of undigested Rubisco was determined by quantifying the amount of intact, fluorescent Rubisco using a Molecular Dynamics Storm 860 system.

Steady-state and time-resolved FRET

Steady-state fluorescence emission spectra were acquired with a PTI photon-counting spectrofluorometer equipped with a temperature-jacketed cuvette holder. Donor excited state lifetimes were measured in the time domain using a Photon Technologies International TimeMaster Fluorescence Lifetime Spectrometer. The lifetimes of donor-only and donor-plus-acceptor samples were measured using a pulsed, N₂ laser-pumped dye laser coupled to an optical boxcar detector⁵⁸. The average distance between donor and acceptor probes ($\langle r \rangle$) was calculated from the average FRET efficiency $\langle E \rangle$ as⁵⁹:

$$\langle r \rangle = R_0 \left(\frac{1 - \langle E \rangle}{\langle E \rangle} \right)^{1/6}$$

A value of R_0 was calculated for each donor-acceptor pair in each state examined. The value of κ^2 was assumed to be 2/3 for all distance measurements. See ref. 35 for more detailed descriptions of instrument settings and energy transfer calculations. In all cases, steady-state and time-resolved measurements of the GroEL–Rubisco complexes were taken only after a 10–20 min incubation of the complex at 25 °C to allow the conformational rearrangements of the Rubisco monomer to come to completion.

Stopped-flow fluorescence and data analysis

Stopped-flow experiments were carried out essentially as described previously³⁵, using an SFM-400 rapid mixing unit (BioLogic) equipped with a custom-designed, two-channel fluorescence detection system. The time-dependent change in donor-side FRET efficiency of the labeled Rubisco monomer was extracted from matched sets of donor-only and donor-acceptor stopped-flow experiments as described previously³⁵. Fitting of experimental data was accomplished with either Igor Pro (Wavemetrics) or Origin (OriginLab).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Non-native Rubisco is more compact when bound to the *trans* ring of an ADP bullet than when bound to apoGroEL. **(a)** The conformation of a substrate protein bound to either apoGroEL or the *trans* ring of an ADP bullet can be investigated with intramolecular FRET, where the distance between two exogenous fluorescent probes attached to different parts of the substrate protein (indicated schematically as colored circles labeled 'A' and 'D') is examined. **(b)** Steady-state FRET measurement of the distance between the N- and C-terminal domains of a non-native Rubisco monomer (100 nM) bound to an ADP-bullet *trans* ring and apoGroEL (120 nM in each case). The C-terminal domain of a previously described Rubisco variant³⁵ was labeled with a donor fluorophore and the N-terminal domain was labeled with an acceptor fluorophore. For these measurements, the donor probe was 5-(2-acetamidoethyl) aminonaphthalene-1-sulfonate (AEDANS; 454-ED) and the acceptor was fluorescein (58-F). The donor-emission spectra of donor-only (labeled apo-D and *trans*-D, respectively) and donor-acceptor (labeled apo and *trans*, respectively) samples are shown. **(c)** Time-resolved donor-intensity decays of the samples described in **b**. The donor-emission decay of donor-only and donor-acceptor samples, labeled as above, and the instrument response function (IRF) are shown. The average intraprobe distance was quantified from both steady-state and time-resolved FRET measurements (**b**, inset).

Figure 2.

Non-native Rubisco bound to the *trans* ring of an ADP bullet is less susceptible to protease digestion and shows reduced chemical modification of internal cysteine residues. (a) Fluorescently labeled Rubisco (58-F; 100 nM) was denatured and bound to either ADP bullets or apoGroEL (labeled *trans* and apo, respectively; 120 nM in each case), treated with chymotrypsin for the indicated times and then analyzed by SDS-PAGE and laser-excited, fluorescence gel scanning. Partial digestion fragments that appear uniquely well represented in either the apo or *trans* digestion experiments are indicated by a square bracket and asterisks. (b) The amount of full-length, fluorescent Rubisco was quantified for three independent protease experiments, and the average of these replicates is plotted as a function of digestion time (error bars show one s.d.). The $t_{1/2}$ for the disappearance of full-length Rubisco bound to apoGroEL and an ADP-bullet *trans* ring are 0.6 min and 1.3 min, respectively. (c) The internal structure of Rubisco monomers bound to apoGroEL and the ADP-bullet *trans* ring was probed with a small, highly reactive coumarin maleimide dye (CPM) that selectively modifies exposed cysteine residues⁶⁰. Only a single surface-exposed cysteine residue at position 58 (out of a total of five cysteine residues in wild-type Rubisco) is modified in native Rubisco (labeled Native (58C); 500 nM). The graph shows the total level of CPM fluorescence detected in the Rubisco sample following analysis by reverse-phase HPLC. When non-native Rubisco (500 nM) is bound to either apoGroEL (apo; 600 nM) or an ADP-bullet *trans* ring (*trans*; 600 nM), the internal Rubisco cysteine residues become highly reactive toward CPM. However, the reactivity of internal cysteine residues in Rubisco bound to apoGroEL is significantly greater than the *trans* ring-bound sample ($P < 0.003$ based on a paired t -test with $n = 3$ replicates; error bars show one s.d.). The maximum level of internal CPM incorporation expected under the solution conditions used is also shown (indicated by a dashed line, labeled five-fold 58C) and was estimated by assuming that the internal Rubisco cysteine residues in unfolded Rubisco would be as exposed and reactive as the surface-exposed 58C site.

Figure 3.

GroES and ATP binding to a Rubisco-occupied *trans* ring results in forced conformational expansion, followed by compaction, of the non-native protein. (a) Schematic of a FRET experiment designed to examine conformational changes in a non-native substrate protein upon encapsulation beneath GroES on the *trans* ring of an ADP bullet. (b) GroES binding and substrate encapsulation results in an initial rapid expansion of the non-native Rubisco intermediate, followed by a compaction. Substrate-bound complexes in the presence of excess GroES were rapidly mixed in a stopped-flow apparatus (60 nM) with ATP (1 mM). For these measurements, the donor probe was fluorescein and the acceptor was rhodamine. Each FRET trace is the average of $n = 10$ replicates of matched experimental pairs, calculated from donor-only (Rubisco 454-F) and donor-acceptor (Rubisco 454-F/58-R) samples. The inset shows the FRET change over the first second of data. The rising phase in the observed FRET signal reflects GroES binding and substrate-protein encapsulation and compaction³⁵. (c) The observed rate constants for the initial rapid drop in FRET efficiency (k_{obs}) and the subsequent rise upon GroES binding (k_{ES}) for a series of experiments at different concentrations of GroES are shown. In each case, a fixed concentration of the Rubisco-bound GroEL ADP bullets (60 nM) and ATP (1 mM) was used, whereas the total concentration of GroES was varied.

Figure 4.

ATP binding alone to a Rubisco-occupied *trans* ring drives a forced conformational expansion of the non-native protein. **(a)** Schematic of a FRET experiment designed to follow conformational changes in a non-native substrate protein upon ATP binding alone to the substrate-loaded *trans* ring of an ADP bullet. In this case, a large excess of a single-ringed GroEL variant SR1 (ref. 21) was added as a GroES trap. SR1 contains a set of equatorial mutations that prevent assembly of a double-ringed complex (indicated by red crosses). SR1 binds ATP and GroES, executes a single round of ATP hydrolysis and then stalls, failing to release GroES and ADP²¹. **(b)** The allosteric transition triggered by ATP alone results in the forced conformational expansion of the non-native protein. Stopped-flow FRET experiments were conducted in essentially the same manner as in Figure 3, except that SR1 was present in a 20-fold excess (1.2 μ M) to prevent GroES binding to the substrate-loaded GroEL ring. **(c)** The observed rate of Rubisco expansion on the *trans* ring of an ADP bullet at different concentrations of ATP is shown. Each data point in the plot represents a single exponential fit to the observed change in FRET efficiency of the labeled Rubisco calculated from the average of $n = 10$ experimental replicates. The solid line shows a fit of the data to the Hill equation with $k_{obs} = 2.49 \pm 0.06 \text{ s}^{-1}$, $K_{1/2} = 82.6 \pm 3.7 \text{ } \mu\text{M}$ and $n_H = 1.9 \pm 0.2$.

Figure 5.

A functional model for substrate-protein unfolding by GroEL. Partial substrate unfolding by GroEL occurs in two phases: (i) passive unfolding upon protein capture by a GroEL ring; and (ii) forced unfolding upon ATP binding to a substrate-loaded *trans* ring. Although substrate confinement within the GroEL–GroES cavity enhances folding, the GroEL–GroES cavity exists for only a short period of time (~15–20 s at 25 °C) during a functional GroEL cycle^{17,19,20,25,27,34–36}. Substrate unfolding provides, in principle, a method for driving a non-native substrate protein to a higher level of its folding free energy landscape, opening efficient folding paths (committed) that are not readily accessible to more compact states (uncommitted)^{14,15}. Substrate-protein unfolding before encapsulation might substantially enrich the fraction of protein that commits to a productive folding path with each round of the GroEL cycle. ATP binding to the *trans* ring of the asymmetric complex, once hydrolysis of ATP within the GroEL–GroES cavity is complete (Pi), induces disassembly of the GroEL–ADP–GroES complex and ejection of the substrate protein, folded or not^{17,26,27,30}. Uncommitted substrate must be recaptured on the *trans* ring of an ADP bullet for another round of unfolding and encapsulation (lower arrow). Two full rounds of ATP hydrolysis, one in each ring, followed by a round of substrate capture on the *trans* ring, returns the asymmetric ATP-containing complex to the starting ADP-bullet state.

Figure 6.

ATP binding to a Rubisco-loaded SR1 ring does not cause extensive forced unfolding. **(a)** SR1 and the *trans* ring of an ADP bullet drive passive unfolding of Rubisco at a similar rate. The passive unfolding of Rubisco by SR1 and the ADP-bullet *trans* ring was monitored by FRET using fluorescein as the donor and rhodamine as the acceptor. The time-dependent drops in FRET efficiency for each complex are shown. Matched samples of donor-only (454-F) and donor-acceptor (454-F/58-R) Rubisco were denatured in acid-urea and rapidly diluted 50-fold (100 nM final) into buffer containing either SR1 (400 nM final) or ADP bullets (120 nM final) at 25 °C. Under the conditions used for this experiment, binding is complete within 5 s²⁷. In both cases, mixing was conducted by rapid manual mixing into a thermally jacketed stirring cuvette in a standard fluorimeter to allow stable long-time observation of the sample. **(b)** SR1 does not support extensive forced unfolding of non-native Rubisco. Acid-urea denatured Rubisco was diluted to a final concentration of 100 nM into buffer containing 400 nM SR1. At different times over the course of a passive unfolding curve (main plot), aliquots of the SR1–Rubisco complex were rapidly mixed with ATP in a stopped-flow apparatus, and changes in the FRET efficiency of the labeled Rubisco sample were monitored for ~10 s. The insets show representative stopped-flow traces (red) taken at early (left) and late (right) points in the passive-unfolding process. For reference, the progress of passive unfolding over the same time window is also shown in each inset. The stopped-flow FRET traces are the average of $n = 3$ replicates of matched experimental pairs, calculated from donor-only (Rubisco 454-F) and donor-acceptor (Rubisco 454-F/58-R) samples. **(c)** The maximum amplitude of forced Rubisco expansion by SR1 upon ATP binding was measured for a series of stopped-flow experiments over the full passive-unfolding curve and is plotted as a function of time. The level of preexisting passive unfolding does not affect the weak forced unfolding supported by SR1 upon ATP binding.

Figure 7.

The fraction of non-native Rubisco that commits to the native state in a single round of GroES encapsulation increases in proportion to the extent of unfolding. **(a)** The slow, passive unfolding of Rubisco on SR1 can be used to examine the linkage between productive folding and substrate-protein unfolding. The progress curve of passive Rubisco unfolding by SR1, monitored by FRET at 25 °C, is shown schematically. For comparison, a schematic of the progress curve of Rubisco unfolding on the *trans* ring of an ADP bullet, and the extent of forced unfolding driven by ATP binding to the *trans* ring, are also shown. **(b)** Schematic of the experiment. Denatured Rubisco bound to excess SR1 was incubated for different preincubation times at 25 °C (indicated by red circles and letters a–d) to allow passive unfolding before the addition of GroES and ATP. Because SR1 shows a dramatically reduced capacity to support forced unfolding upon ATP binding (Fig. 6), the preincubation time primarily sets the level of unfolding imposed on the bound Rubisco monomer. Following addition of ATP and GroES, the encapsulated Rubisco is allowed to fold within the enclosed SR1–GroES cavity. **(c)** Rubisco folding is monitored as a function of time following GroES and ATP addition for several different preincubation times. Although the overall refolding kinetics are similar, the fraction of Rubisco that commits to the native state in the first few seconds following ATP and GroES binding increases considerably at longer preincubation times. **(d)** The extent to which unfolding on SR1 enhances the initial commitment of Rubisco to the native state is quantified by extrapolating refolding curves like those in **c** to zero preincubation time for a series of identical experiments ($n = 3$; error bars show one s.d.). The inset in **d** shows the final native Rubisco yield (following 30 min of folding within the SR1–GroES complex) observed at each preincubation point.