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Catalysis of protein folding by symmetric chaperone complexes

(GroE/maltose binding protein/molecular chaperones/chaperonins/heat shock)

HELMUT SPARRER, KERSTIN RUTKAT, AND JOHANNES BUCHNER*

Institut für Biophysik & Physikalische Biochemie, Universität Regensburg, 93040 Regensburg, Germany

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ABSTRACT **The GroE chaperones of *Escherichia coli* assist protein folding under physiological and heat shock conditions in an ATP-dependent way. Although a number of details of assisted folding have been elucidated, the molecular mechanism of the GroE cycle remains unresolved. Here we present an experimental system that allows the direct analysis of the GroE-mediated folding cycle under stringent conditions. We demonstrate that the GroE proteins efficiently catalyze the folding of kinetically trapped folding intermediates of a mutant of maltose-binding protein (MBP Y283D) in an ATP-dependent way. GroES plays a key role in this reaction cycle, accelerating the folding of the substrate protein MBP Y283D up to 50-fold. Interestingly, catalysis of the folding reaction requires the formation of symmetrical football-shaped GroEL·GroES$_2$ particles and the intermediate release of the nonnative protein from the chaperone complex. Our results show that, in the presence of GroES, the complex architecture of the GroEL toroids allows maintenance of two highly interregulated rings simultaneously active in protein folding.**

Chaperonins play an important role in maintaining protein integrity under physiological as well as under heat shock conditions (1–4). Forming tight complexes with folding intermediates, they prevent aggregation and assist the folding of polypeptides that would not reach the native state in a spontaneous reaction, due to the nonpermissive conditions of the cellular environment (5–7).

The GroE chaperone complex comprises two different proteins. GroEL, the protein binding component, consists of two stacked rings with seven identical subunits each (8). Each ring contains a central cavity in which nonnative protein can be accommodated (9–11). The co-chaperonin GroES is a seven-membered ring (12–14). Binding of either one or two GroES rings to GroEL leads to the formation of asymmetric bullet-shaped or symmetric football-shaped particles, as identified by electron microscopy (15–17). The significance of football-shaped particles as obligate or facultative intermediates in the ATPase cycle as well as in the coupled protein folding cycle are still unclear (18–25). However, it was shown that, in principle, folding can occur in the cavity of GroEL covered by GroES (cis-bullet) in a single turnover event (23, 24, 26).

Although GroE has been shown to be able to rescue aggregated protein, thereby enhancing the yield and the kinetics of refolding (27, 28), and to reshuffle trapped folding intermediates to a productive folding pathway via an unfolding event (29, 30), until now no quantitative experimental system, describing the way GroE influences folding, has been reported, and the underlying mechanisms remain to be elucidated.

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Here we used the temperature-sensitive folding mutant MBP Y283D, a 40-kDa periplasmic protein of *Escherichia coli*, as a substrate protein for GroE. This maltose-binding protein (MBP) variant provides the opportunity to directly study the effects of GroE on a slow folding species that does not aggregate by fluorescence spectroscopy. Spontaneous folding of wild-type and the mutant MBP, as well as their interaction with GroEL and SecB, have been characterized extensively (31–34).

MATERIALS AND METHODS

Purification. Wild-type MBP, the mutant MBP Y283D, and mutant RBP CP632 (AZ7T) were prepared according to the protocol given in ref. 32. Purification of GroEL and GroES was performed according to the protocol given in ref. 17. It should be noted that after purifying GroEL to homogeneity, as judged by silver-stained SDS gels, a number of additional steps are required to remove bound substrate proteins, which can be identified by tryptophan fluorescence, since GroEL itself lacks tryptophans.

Concentrations of pure proteins were determined by spectrophotometry and by a colorimetric assay as described.

Folding. Folding kinetics of guanidinium-denatured MBP were monitored in 20 mM Tris·HCl, pH 7.6/0.2 M KCl/5 mM $MgCl_2$/1 mM ATP by following changes in the fluorescence intensity as described (32). Tryptophans were excited with an excitation wavelength of 295 nm. Emission was detected at 344 nm. If not indicated otherwise, slits were set to a bandwidth of 4.25 nm for excitation and 8.5 nm for emission. Folding of MBP was recorded in a thermostated cuvette under constant stirring. GroEL, GroES and nucleotide were preincubated before adding denatured MBP Y283D.

For competition experiments, ribose-binding protein (RBP) was denatured for at least 4 h in 3.3 M urea. Denatured RBP was added 30 s after the addition of MBP in a 200-fold excess. The final concentration of urea (30 mM) did not influence the catalyzed folding reaction in the absence of RBP.

Electron Microscopy and Image Analysis. Denatured MBP (80 nM) was incubated with GroEL (40 nM) for 2 min at 40°C in 50 mM Tris, pH 7.5/40 mM $MgCl_2$/10 mM KCl, and then GroES (120 nM) and 5′-adenylyl-imido-diphosphate (AMP-PNP; 2 mM) were added. The final guanidinium chloride concentration was 4 mM in all samples. The samples were applied to carbon-coated grids and negatively stained with 3% uranyl acetate, and electron micrographs were recorded at 120 kV and at a magnification of ×45,000 with a Philips CM12 (Philips, Eindhoven, the Netherlands). Digitized images (pixel size, 0.3 nm) were aligned, averaged, and subjected to a classification procedure based on eigenvector–eigenvalue analysis (17, 35).

Abbreviations: MBP, maltose-binding protein; RBP, ribose-binding protein.

*To whom reprint requests should be addressed. e-mail: Johannes.Buchner@biologie.uni-regensburg.de.

RESULTS AND DISCUSSION

Folding of MBP Y283D is Decelerated at High Temperature. To further analyze the structure formation of MBP, we studied the temperature dependence of folding for both wild-type and mutant MBP. The Arrhenius plot for the folding of wild-type MBP shows a strictly linear correlation from 15°C to 40°C for both the fast and slow folding phase (data not shown). This indicates that the general folding pathway for wild-type MBP does not change with temperature. However, the MBP folding mutant Y283D, which was used exclusively in our experiments, exhibits a strikingly different folding behavior. Due to the mutation at position 283, folding is slowed down considerably in comparison to wild-type MBP (31, 34). At temperatures up to 30°C, there is a linear slope in the Arrhenius plot for Y283D. In contrast, at temperatures above 35°C, the rate of spontaneous folding is decreasing strongly (Fig. 1). This kink in the Arrhenius plot indicates a dramatic change in the folding mechanism of MBP Y283D at high temperatures.

Importantly, aggregation does not occur as a side reaction under our experimental conditions, since the amplitudes of the spontaneous and GroE-mediated folding are identical. This demonstrates that all molecules eventually reach the native state. Furthermore, CD measurements of the thermal transition of wild-type MBP (T_m = 73°C) and MBP Y283D (T_m = 62°C) indicate that, at 40°C, for both proteins, the native state is strongly favored (data not shown). Thus, we conclude that misfolding is the reason for the observed decrease of the folding rate. Spontaneously, these misfolded intermediates are only slowly converted to fast folding intermediates, and therefore the apparent rate of folding decreases.

GroE Catalyzes Folding of MBP. Next, we studied the influence of GroE on the folding of this defective mutant at various temperatures. Surprisingly, when the temperature dependence of GroE-mediated folding of MBP Y283D was analyzed, the shape of the Arrhenius plot of MBP Y283D was changed significantly (Fig. 1). Active participation of GroE leads to an acceleration of MBP folding. This effect is most pronounced at 40°C, where the Arrhenius plots of spontaneous and GroE-assisted folding diverge most dramatically. At 45°C, the GroE system fails to support the folding reaction (Fig. 1). The catalytic effect of GroE is dependent on the presence of hydrolyzable ATP and also on the relative ratios of the chaperonins (see below). Addition of ADP instead of ATP did not result in catalysis of folding (Fig. 1).

To determine the precise requirements for catalyzed refolding, we tested various orders of addition of GroEL and GroES. In the presence of GroEL alone, MBP folding is completely suppressed, due to the formation of a tight complex (32). The spontaneous folding reaction was not influenced by the presence of ATP and GroES. However, as soon as GroEL was added to this mixture, again MBP Y283D folding was accelerated due to the completion of chaperonin system (compare Fig. 4 *Inset*). Importantly, this result excluded the possibility that GroE acts by preventing the initial fast formation of a slow folding intermediate U′ and circumventing this folding trap by populating an alternative folding pathway. Rather, GroE seems to bind U′ and to shift it back to a fast folding pathway.

Catalysis of the Folding Reaction Is Strongly Dependent on the Ratio of GroEL to GroES. The MBP folding assay enabled us to test the obligatory steps of the GroE cycle under stringent folding conditions. The importance of the co-chaperonin GroES for the ability of GroE to catalyze the folding of MBP Y283D at 40°C is shown in Fig. 2. At all GroE concentrations, from 25 nM to 200 nM, a strong dependency of the folding efficiency on the concentration of GroES was observed. At a concentration of 25 nM GroEL and MBP Y283D, the apparent folding reaction was inefficient up to an equimolar ratio of $GroES_7$ to $GroEL_{14}$. When a ratio of about one $GroES_7$ to one $GroEL_{14}$ was reached, an increase of the apparent folding rates to about the rates of spontaneous folding was observed. A further increase in the apparent refolding rate could be obtained by shifting the $GroES_7$ to $GroEL_{14}$ ratio to higher values. Highest efficiency was achieved at an at least 4-fold excess of GroES, with an apparent acceleration by a factor of four compared with unassisted folding. This result argues strongly for symmetric particles as active intermediates in the folding cycle.

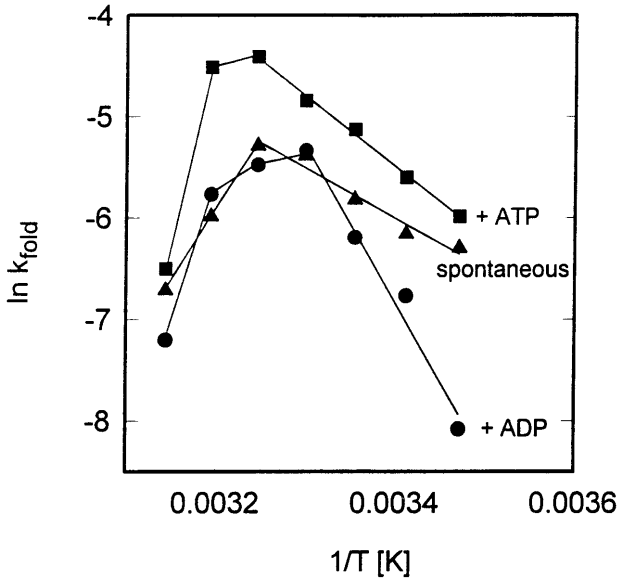

FIG. 1. Arrhenius plots for spontaneous and GroE-assisted folding of MBP Y283D. The temperature dependence of the spontaneous folding reactions of 25 nM MBP Y283D was measured from 15°C to 45°C. The logarithm of the obtained folding rate was plotted against $1/T$ [K]. The rates of the folding reactions in the presence of chaperonin (25 nM GroEL and 50 nM GroES) were determined in the presence 1 mM ATP and 1 mM ADP, respectively.

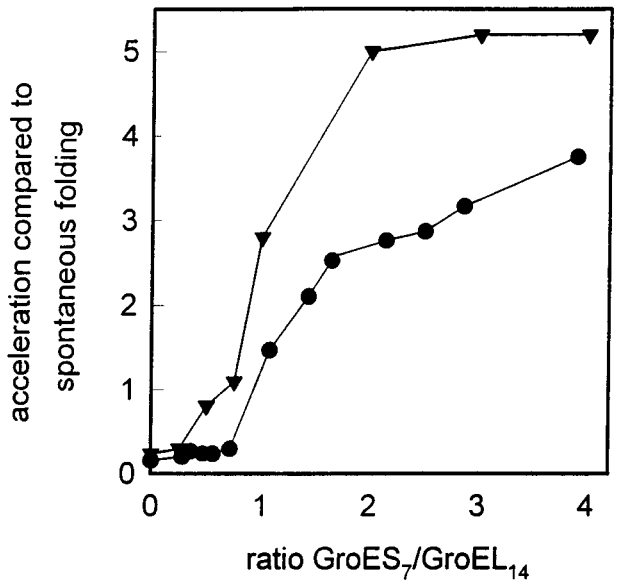

FIG. 2. Dependence of the efficiency of the GroE-mediated folding of MBP Y283D on the ratio of GroES to GroEL. To determine the folding efficiency of the GroE system at different ratios of GroES to GroEL, GroES was titrated from substoichiometric amounts to a fourfold excess relative to the two different MBP concentrations used. The rates for the respective folding kinetics were divided by the spontaneous folding rate. The results for concentrations of 25 nM (●) and 200 nM (▼) MBP Y283D are shown in the graph. Folding of MBP Y283D was recorded at 40°C.

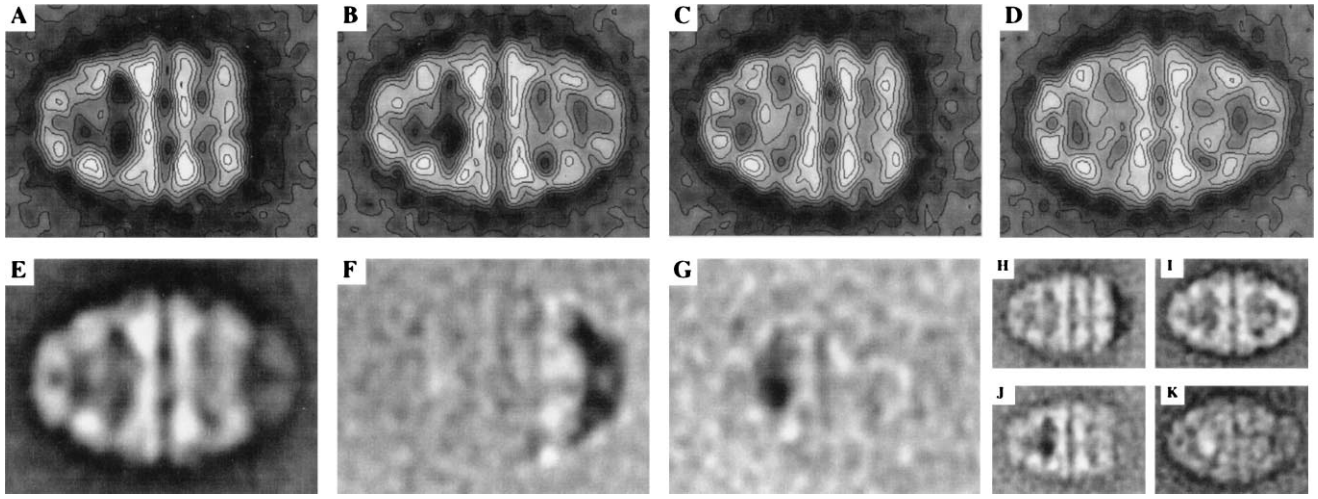

FIG. 3. Analysis of side views of GroEL·GroES complexes in the presence of denatured MBP by electron microscopy and image analysis. (*A–D*) The four major structural classes of GroE complexes in the sample (498 particles) based on the first two eigenvectors. (*A*) Empty bullet (24%). (*B*) Football with one MBP bound (23%). (*C*) Bullet with MBP bound inside (25%). (*D*) Footballs with two MBPs bound (28%). The eigenvector–eigenvalue analysis of the sample is documented in *E–K*. (*E*) Reference particle for the analysis (the average of all side views). (*F*) The most significant difference (eigenvector) between the particles. In *H* and *I*, this difference is depicted in terms of different classes of particles (bullets and footballs). (*G*) The second major difference obtained and (*J* and *K*) the two corresponding classes of averages differing in the presence of stain-excluding mass inside the GroEL·GroES complex.

Since at low GroE concentrations, the observed stoichiometry may be influenced by the affinity of GroEL for GroES, we repeated the titration of GroES with respect to GroEL and MBP Y283D at an 8-fold higher protein concentration. Under these conditions where affinity is no longer interfering with stoichiometry, again efficient acceleration of MBP folding required more than one GroES per GroEL. The increase in catalytic activity up to a factor of five was now already achieved at a 2-fold excess of GroES (Fig. 2). This is in agreement with the observation that the efficiency of GroE is limited by diffusion at lower concentrations (see also Fig. 4). Due to the the higher concentration of GroEL, GroES, and MBP Y283D, the probability of the formation of a productive complex is increased. It should be noted that saturation of catalysis at any stoichiometry that is above 1:1 is proof for the involvement of symmetric particles in the functional cycle. The result of the concentration variation stresses the importance of the ratio of the components and not their absolute concentrations for the efficiency of GroE-mediated folding. Whereas an absolute concentration of 50 nM GroES clearly supports catalyzed folding at a GroEL concentration of 25 nM, the same concentration of GroES results in a suppression folding at a concentration of 200 nM GroEL.

Catalysis can be achieved to some extent already at substoichiometric ratios of GroES to GroEL. Electron microscopy confirmed that under these conditions symmetric GroE particles are present in addition to asymmetric ones and empty GroEL (data not shown).

Electron Microscopy Reveals the Presence of GroE Football Particles Complexed with MBP. This increase in efficiency at higher ratios of $GroES_7$ to $GroEL_{14}$ was unexpected, since current models of GroE-mediated folding suggest only asymmetric particles to be of functional importance (9, 24, 26). Symmetric particles were regarded to occur under nonphysiological conditions or as transient facultative intermediates (23). To obtain structural data on the GroE particles formed under our experimental conditions†, we performed electron microscopy and image analysis of GroE·MBP complexes. As shown in Fig. 3, classification of the particles obtained revealed that symmetric and asymmetric GroE particles are formed to equal amounts. Both species are able to bind nonnative MBP inside the cavity provided by GroEL and GroES, as demonstrated by the appearance of a stain-excluding mass in the MBP-containing sample. Interestingly, football particles containing one or two MBP molecules were formed to equal extent. The amount of footballs observed (≈50%) was not influenced by the presence of nonnative protein. These results argues for the possibility that substrate proteins as big as MBP can be bound within the cavity provided by GroEL and GroES and, importantly, that GroE is able to handle two proteins simultaneously. Sequestration of MBP Y283D inside GroE particles was confirmed independently by protease digestion as observed with ornithine transcarbanylase (OTC) medium or rhodanese (ref. 23; data not shown).

Catalysis of Folding Requires Multiple Binding Cycles of GroE and MBP. A prominent feature of current models of GroE function is that some, if not all, folding steps can occur while the protein is trapped inside the GroEL cavity in a cis-bullet complex (23, 24, 26). To explain the acceleration of folding, the release of MBP Y283D must occur in a form that has a structure different from that of the trapped intermediate when it was bound to GroEL. To test whether MBP folding and unfolding occurs completely shielded from bulk solution, we titrated the amount of GroE with a constant ratio of two $GroES_7$ per $GroEL_{14}$. The apparent folding rate in the presence of increasing amounts of chaperonin accelerated steadily up to a factor of eight compared with the spontaneous folding rate (Fig. 4). Thus, an increased number of encounters of GroE with released MBP intermediates is important for accelerated folding. Since GroEL does not interact with native MBP Y283D, the intermediates must be in a nonnative form. After dissociation from GroE, the released intermediate either partitions to the native state on a fast folding pathway or is trapped again as an unproductive intermediate and undergoes further GroE cycling.

The necessity for multiple interactions of free MBP with GroE was confirmed using RBP as a competitor (32). Since RBP does not contain tryptophans in its primary sequence, it does not interfere with the refolding assay, which is based on tryptophan fluorescence. When a refolding reaction of MBP Y283D in the presence of GroE was initiated and RBP was added in 200-fold excess after 30 s (i.e., after a few ATP

†The folding kinetics and the influence of GroE on MBP folding were identical under the buffer conditions used for electron microscopy and the kinetic analysis.

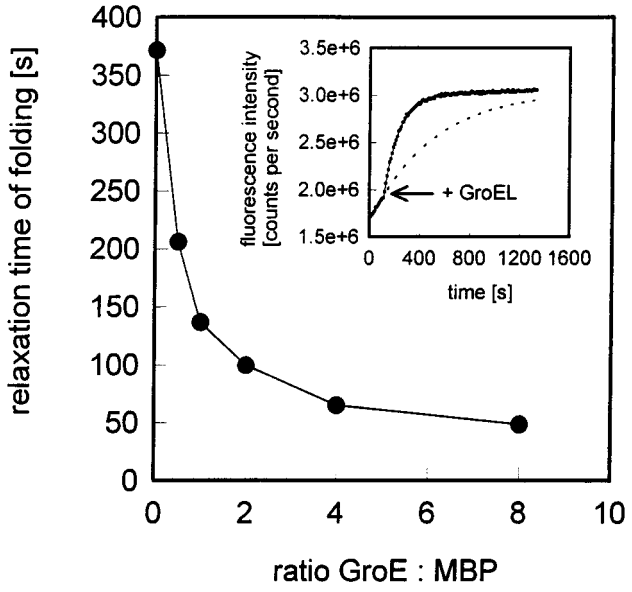

FIG. 4. Dependence of GroE-mediated folding of MBP Y283D on the amount of GroE. The apparent rate of folding of MBP Y283D in the presence of increasing amounts of GroE was recorded at 40°C. The concentration of MBP Y283D was 25 nM. The GroEL concentration was increased from substoichiometric amounts (12.5 nM) to an 8-fold excess (200 nM), where saturation of the catalytic effect of the GroE system on the folding of MBP Y283D was achieved. The concentration of GroES in all experiments was always twice the concentration of GroEL. (*Inset*) Unprocessed data of a catalyzed folding reaction. In the initial slow folding part of the kinetic, 1 mM ATP and 50 nM GroES were present. Neither GroES nor nucleotide influenced the folding rate compared with the spontaneous folding (dotted line). When the chaperonin system is completed with the addition of 25 nM GroEL after 200 s, catalyzed folding is induced (solid line).

turnovers), saturation of the GroEL binding sites for denatured protein with the competitor prevented the interaction of MBP folding intermediates with the chaperonin. Consequently, the apparent folding rate was shifted back from the catalyzed rate to the spontaneous rate. Since protein is released in a coordinated manner with ATP hydrolysis of the trans-ring, this result implies that only a small fraction of MBP Y283D may have reached the native state before it is expelled from GroE. Therefore, as already concluded from the GroE titration experiment, MBP Y283D folding intermediates released in a nonnative form could not be converted quantitatively to the native state in a single ATP turnover.

Model for the GroE Reaction Cycle. Taken together, our experimental data argue for the following model of GroE-mediated folding (Fig. 5). In the presence of nucleotide and GroES, the asymmetric bullet is one of the prevailing species present in solution (36). Since the binding site at one ring is now occupied by GroES, association of nonnative protein can only occur at the ring distal to GroES. This leads to the formation of a trans-bullet. Free GroES readily associates to the trans ring, thereby forming a football-shaped intermediate with MBP Y283D sequestered inside the chaperonin. Hydrolysis of ATP, coordinated by GroES, leads to the effective release of the substrate protein still caged in the central cavity, allowing it to fold to a intermediate or to reach the native state (23–26). At the same time, GroES and ADP are released from the opposite ring. This ring can now be loaded with ATP and, in a further turnover, discharge the substrate protein. In the case of the MBP mutant, the dissociated protein partitions between a species that reaches the native state in a fast reaction and a kinetically trapped species, which will undergo further cycling.

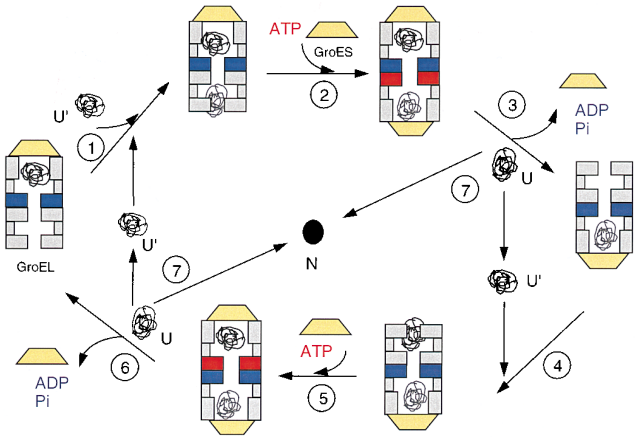

FIG. 5. Model for the reaction cycle of GroE. A cis-bullet particle with GroES, ADP, and nonnative protein (shown in black) attached to one GroEL ring binds a second folding intermediate (shown in gray) to the high-affinity binding site of the trans-ring (step 1). In a next step, ATP and a second GroES associate to form a football-shaped particle (step 2) encapsulating two folding intermediates. Hydrolysis of ATP in lower GroEL ring releases GroES, ADP, and protein from the upper ring (step 3), thus completing the flip-flop and restoring a new high-affinity acceptor state; however, it is in a rotated state. The released nonnative protein can either fold to the native state (N; step 7) or rebind to the chaperonin (step 1). To achieve catalysis, the conformations of the intermediate dissociated from GroEL and bound to GroEL have to differ with U representing an early folding intermediate and U′ a kinetically trapped species. The protein released from GroEL (U) will either fold to the native state (N) in a fast reaction or convert again to (U′) and undergo another unfolding cycle. A set of reactions (steps 4–6) equivalent to steps 1–3 completes the cycle and leads back to the original orientation. The GroEL domain complexed with ATP is shown in red, and the GroEL domain with ADP bound is shown in blue.

Alternatively, as shown in Fig. 5, a second substrate protein can associate to the unoccupied high-affinity binding site of the cis-bullet already loaded with one substrate protein in cis. In principle, GroE-mediated folding occurs now via the same set of reactions as described above. However, binding of two substrate proteins establishes an efficient flip-flop mechanism of binding, release in the cavity and ejection into solution, with the two rings of GroEL simultaneously active in protein folding. Loading GroEL with two proteins allows reduction of the energy cost for folding by a factor of two and, at the same time, makes full use of the complex architecture of the two highly regulated toroids. We suggest that under stress conditions, when the cell has to cope with large amounts of nonnative protein, this is the prevailing mechanism of GroE.

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