and F. U. Hartl, *Annu. Rev. Biochem.* **62**, 349 (1993).
20. Studies involving the cross-linking of large protein oligomers have indicated that non–cross-linked proteins are poor molecular weight standards for cross-linked species, and often the cross-linked molecule serves as its own standard [S. Daraweshe and E. Daniel, *Eur. J. Biochem.* **201**, 169 (1991)]. Azem *et al.* (*32*) identified the molecular weights of the cross-linked species [GroEL1-6], [GroEL7], and [GroEL14] according to their respective migration patterns in a gradient polyacrylamide gel, which aligned perfectly on a semi-logarithmic scale. The uniform tube gels used in Fig. 1 also showed a good alignment on a semi-logarithmic scale of the three cross-linked species [GroEL14], Form1, and Form2, which differed from one another by two successive shifts of approximately 70 kD (*33*).
21. It is noteworthy that nearly equimolar amounts of Mg-ATP and GroEL protomers suffice to support the formation of 50% of Form1, at the expense of the $GroEL_{14}$ cross-linked species (Table 1). Biochemically, the sensitivity of the first $GroES_7$-binding reaction to $GroEL_{14}$ cannot much exceed that revealed by the cross-linking reaction. Thus, cross-linking with GA is unlikely to have displaced the equilibrium toward dissociation of the chaperonin hetero-oligomer. Conversely, it is also unlikely that cross-linking displaced the equilibrium toward association of otherwise free $GroEL_{14}$ with $GroES_7$, during the 60-min cross-linking reaction, because, in a control experiment, we found that the application of GA only 10 s before the addition of ATP to the chaperonin solution (as in Fig. 1A, lane 3) prevented all associations between free $GroEL_{14}$ and $GroES_7$. A concomitant addition of GA and ATP resulted, however, in the complete formation of Form1 hetero-oligomers (*33*). This concurs with a previous observation (*32*) that the ATPase of $GroEL_{14}$ becomes fully inactivated by GA within seconds. Cross-linking with GA is concluded to be a faithful tool in the assessment of the oligomeric state of chaperonin hetero-oligomers in solution.
22. When traces of ^{35}S-labeled $GroES_7$ were included in this experiment, gel slices containing Form2 were found to have twice as many ^{35}S counts as gel slices containing Form1, whereas only background counts were recovered from gel slices containing $GroEL_{14}$ (*33*).
23. M. Schmidt *et al.*, *Science* **265**, 656 (1994).
24. The fact that similar football-shaped structures were observed with chaperonin solution that was not treated with GA demonstrated that the symmetric football-shaped structures in Fig. 2B are not cross-linking artifacts.
25. The cross-linked species in Fig. 1C, lanes 2 to 5 but not lane 1, immunoreacted on protein immunoblots with antibodies to RuBisCO (*33*). In the presence of ADP, unlike AMP-PNP, the slowest migrating complex RuBisCO-$GroEL_{14}(GroES_7)_2$, was not observed (*33*). This is expected because ADP does not support the binding of a second $GroES_7$ to $GroEL_{14}GroES_7$.
26. The RuBisCO-$GroEL_{14}GroES_7$ is a labile oligomer that tends to dissociate when GroEL and GroES are in submicromolar amounts. This was exemplified in the following experiment: 660 nM GroEL was reacted with a threefold excess of $GroES_7$, ADP, and unfolded RuBisCO. The resulting RuBisCO-$GroEL_{14}GroES_7$ complex was then separated from unbound $GroES_7$ and RuBisCO by gel filtration in the presence of ADP (13 to 16 min, Superose-6B, Pharmacia) and consequently diluted 1:10. The recovery of RuBisCO from the isolated RuBisCO-$GroEL_{14}GroES_7$ complex was 40% lower with a subsequent activation with ATP alone than with ATP plus $GroES_7$. This indicated that a significant loss of bound $GroES_7$ occurred during the gel filtration. However, when this experiment was performed with an initial concentration of 3.3 μM GroEL and a corresponding concentration of GroES, the loss of GroES-dependent recovery of RuBisCO was reduced to 15% (*33*). In the cell, 1 to 10% of the soluble protein is GroEL. The chaperonin concentrations thus range between 50 and 500 μM (*34*).
27. More than 80% of active RuBisCO could be recovered from AMP-PNP–preformed RuBisCO-$GroEL_{14}(GroES_7)_2$ complexes, as in Fig. 1C, that were incubated with 2 mM ATP for 2 hours instead of GA (*33*). Thus, once the initial interaction has occurred, an unfolded protein can remain stably bound to a $GroEL_{14}(GroES_7)_2$ complex in a form whose efficient refolding can later be successfully assisted by the chaperonin.
28. M. J. Todd, P. V. Viitanen, G. H. Lorimer, *Science* **265**, 659 (1994).
29. H. Christensen and H. R. Pain, *Eur. Biophys. J.* **19**, 221 (1991).
30. R. Grimm, G. K. Donaldson, S. M. van der Vies, E. Schäfer, A. A. Gatenby, *J. Biol. Chem.* **268**, 5220 (1993).
31. A. Ziemienowicz *et al.*, *ibid.*, p. 25425.
32. A. Azem, S. Diamant, P. Goloubinoff, *Biochemistry* **33**, 6671 (1994).
33. A. Azem, M. Kessel, P. Goloubinoff, data not shown.
34. S. M. Hemmingsen *et al.*, *Nature* **333**, 330 (1988).
35. We thank S. Diamant and C. Weiss for useful discussions. Supported in part by grant 00015/1 from the United States–Israel Binational Science Foundation, grant 1180 from the Joint German Israeli Research Program, grant 158-93 from the Israel Science Foundation to P.G., and a grant from the Golda Meir foundation to A.A.

23 February 1994; accepted 1 June 1994

Symmetric Complexes of GroE Chaperonins as Part of the Functional Cycle

Marion Schmidt, Kerstin Rutkat, Reinhard Rachel, Günter Pfeifer, Rainer Jaenicke, Paul Viitanen, George Lorimer, Johannes Buchner*

The particular structural arrangement of chaperonins probably contributes to their ability to assist in the folding of proteins. The interaction of the oligomeric bacterial chaperonin GroEL and its cochaperonin, GroES, in the presence of adenosine diphosphate (ADP) forms an asymmetric complex. However, in the presence of adenosine triphosphate (ATP) or its nonhydrolyzable analogs, symmetric complexes were found by electron microscopy and image analysis. The existence of symmetric chaperonin complexes is not predicted by current models of the functional cycle for GroE-mediated protein folding. Because complete folding of a nonnative substrate protein in the presence of GroEL and GroES only occurs in the presence of ATP, but not with ADP, the symmetric chaperonin complexes formed during the GroE cycle are proposed to be functionally significant.

Chaperonins are abundant, indispensable proteins that participate in protein folding in vivo and in vitro (*1*, *2*). The *Escherichia coli* chaperonins comprise two proteins, GroEL and GroES. These proteins have a particular oligomeric structure, as detected by electron microscopy (*3*–*6*) and x-ray crystallography (*7*). Native GroEL (subunits of ~57 kD) is a cylindrical tetradecamer composed of two stacked rings with sevenfold symmetry, whereas GroES (subunits of ~10 kD) is a single heptameric ring (*8*). GroEL binds nonnative proteins with little or no specificity (*2*, *9*). Binding is thought to occur in the central cavity (*6*, *10*), although nonnative proteins can still be cross-linked to GroES, are prone to proteolysis, and can interact with antibodies (*11*). ATP induces changes in the quaternary structure of GroEL that lead to a rotation of the individual GroEL subunits (*5*). The K^+-dependent hydrolysis of ATP occurs cooperatively (*12*–*15*). GroEL also binds ADP and nonhydrolyzable analogs, although with lower affinity (*14*, *15*). As for GroES, it couples ATP hydrolysis with the ability of GroEL to refold nonnative proteins (*12*). Upon association of GroES with GroEL, cooperativity of ATP hydrolysis is enhanced (*13*–*16*), and the rate of ATP turnover is reduced to 50% (*17*). Under nonpermissive refolding conditions in vitro, GroES is essential for GroE-mediated protein folding (*18*).

In the presence of adenine nucleotides, the physical interaction between GroEL and GroES is mediated through a flexible loop in the cochaperonin (*19*). Electron micrographs of such complexes (*5*, *6*) show a large structural rearrangement of GroEL when GroES binds at one end of the GroEL cylinder. The binding of one GroES has been proposed to confer a change in the structure at the distal end of the GroEL cylinder, thereby precluding the association of a second GroES (*6*). In addition, bio-

M. Schmidt, K. Rutkat, R. Jaenicke, J. Buchner, Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, Universitätsstrasse 31, D-93040 Regensburg, Germany.
R. Rachel, Institut für Mikrobiologie, Universität Regensburg, Universitätsstrasse 31, D-93040 Regensburg, Germany.
G. Pfeifer, Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, D-82153 Martinsried, Germany.
P. V. Viitanen and G. Lorimer, Molecular Biology Division, Central Research and Development Department, E. I. DuPont de Nemours and Company, Experimental Station, Wilmington, DE 19880, USA.
*To whom correspondence should be addressed.

chemical data point to a stoichiometry of one GroES heptamer per one GroEL tetradecamer (*6*, *14*, *15*, *20*). However, stoichiometry does not define the mechanism, it only sets mechanistic constraints. We now show that in the presence of ATP, but not ADP (that is, under conditions necessary for GroE-mediated protein folding), GroEL and GroES form a symmetric particle, which may represent an obligatory, although transient, intermediate in a functional cycle (*17*).

The asymmetric complex of both chaperonins formed in the presence of ADP is stable (*17*); however, it is not a fully functional complex, because ATP hydrolysis is a prerequisite for GroE-mediated protein folding (*20–22*). Therefore, the observed stoichiometry of GroES to GroEL cannot be used as a basis for a general mechanism of chaperonin function. To determine the structural basis for the functional cycle, we compared the association of the two chaperonins in the presence of ATP (Fig. 1A) versus ADP (Fig. 1B). With ATP, four different GroEL structures were detected after negative staining (Fig. 1A): (i) top views of GroEL with sevenfold rotational symmetry and an electron-dense center; (ii) brick-like structures with four stripes, representing typical side views of GroEL, consisting of two stacked heptameric rings of subunits, each having two distinct domains; (iii) asymmetric bullet-shaped particles corresponding to the association of one GroES ring with only one of the two GroEL rings; and (iv) symmetric football-like structures, formed upon binding of GroES rings to both GroEL rings. (The football shape refers to an American football.) Similar images can be obtained after cross-linking (*23*). The optimal ratio of GroES to GroEL, necessary to form symmetric particles, was achieved with a subunit molar equivalency. Higher concentrations of GroES did not increase the number of symmetric particles. These results were consistent with the stoichiometric association of two heptameric GroES rings with one tetradecameric GroEL particle. In contrast, only asymmetric complexes were observed in the presence of ADP (Fig. 1B) (*6*).

To obtain a clearer and more detailed structure of the symmetric complexes, we averaged 560 football particles after translational and rotational alignment (*24*). A representative structure is shown in Fig. 2A. The binding of two GroES heptamers to opposite ends of the GroEL cylinder conferred a pronounced alteration in GroEL side views. The four stripes, characteristic of GroEL alone, were reduced to two. Although the inner domains of each GroEL ring were unchanged upon the binding of GroES, the outer domains were altered by the interaction with the cochaperonin. Both end layers had a bulged appearance upon association with GroES. Most likely the tip of the bulged ends of bullets and footballs represents the side view of the additional GroES; thus, GroES probably binds to GroEL, creating a common sevenfold axis.

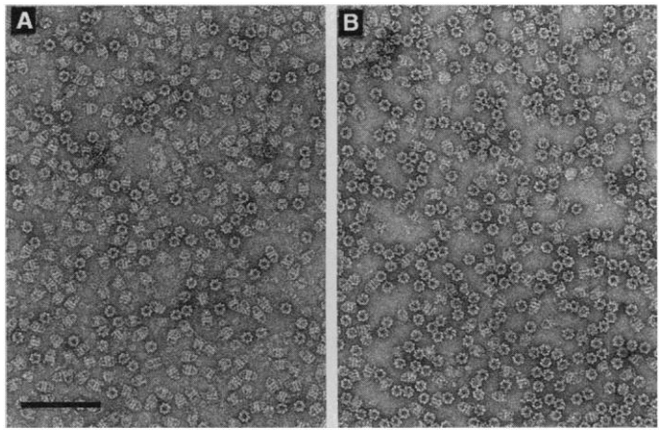

Fig. 1. Electron micrographs of negatively stained GroEL-GroES complexes in the presence of ATP or ADP. GroEL and GroES were purified to apparent homogeneity (*28*) from the *E. coli* strain JM 109 harboring the plasmid pOF39 (*29*), as judged by silver stained SDS gels (*30*). Subsequently, the pH of GroEL- and GroES-containing samples was rapidly shifted to pH 4.5. After application to a cation-exchange column (Mono S, Pharmacia), GroES showed no tryptophan-containing contaminations as judged by fluorescence spectroscopy. GroEL was further incubated with 10 mM ATP, 10 mM $MgCl_2$, and 10 mM KCl for 30 min at room temperature before the protein was applied to a gel filtration column (Superdex 200, prep grade, Pharmacia) for separation of aggregated peptides from the GroEL-containing peak. Concentrations were determined as described (*30*). For electron microscopy, 0.77 μM GroEL (monomers) and 1.48 μM GroES were incubated in the presence of 50 mM $MgCl_2$, 50 mM KCl, and 2.5 mM ATP (**A**) or ADP (**B**) in a buffer containing 50 mM tris (pH 8) for 30 min at room temperature. In control reactions in the absence of nucleotides, GroEL and GroES were not able to form hetero-oligomeric complexes. GroEL and complexes of GroEL-GroES in the presence of various nucleotides were negatively stained with 3% uranyl acetate. Electron micrographs were recorded with a Philips CM 12 at nominal magnifications of ×45,000 and ×60,000. The scale bar represents 100 nm.

An unbiased statistical distribution of the different complexes was obtained with eigenvector-eigenvalue analysis (*25*) after alignment. This mathematical procedure separates the images of particles into subclasses, according to structural similarities. The dominant features (dominant eigenvectors) were size and mass distribution of the complexes. With no additional information or restraints to create subclasses, we obtained six structurally distinguishable classes of GroE side views as a result of this analysis (Fig. 2, B to G). Partitioning of the complexes into more than six subclasses did not convey additional structural information. Two distinct classes, both of bullets and footballs, were observed on the basis of

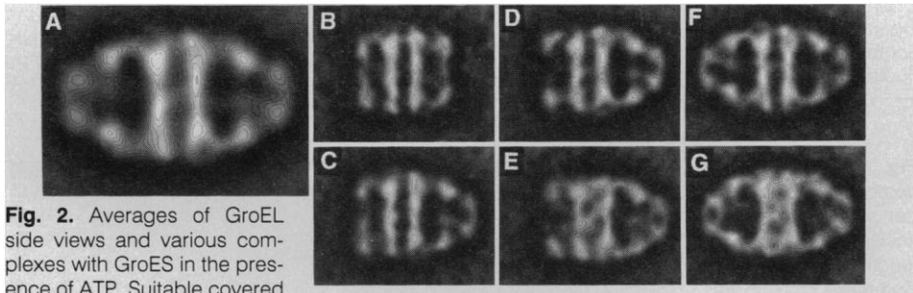

Fig. 2. Averages of GroEL side views and various complexes with GroES in the presence of ATP. Suitable covered areas of electron micrographs described in Fig. 1A were digitized with an EIKONIX CCD camera with a step size of 15 μm. Individual side views, selected interactively on a display system, were aligned iteratively with respect to translation and orientation by using standard correlation techniques (*24*). To detect interimage structural variations, the aligned images were subjected to a classification procedure based on eigenvector-eigenvalue data analysis (*25*). (**A**) Representative averages of 580 football particles and (**B** to **G**) averages of side views representing the six classes obtained by the described analysis: (B) unloaded GroEL side views, (C) bullets, (D) open bullets with defined masses in the center of the molecule, (E) the same structure as in (D) with diffuse mass distribution, (F) footballs with defined mass distribution, and (G) footballs with diffuse masses in the center of the GroEL particles. The dimensions of the particles were calculated to be (A) 15 nm × 14 nm, (C to E) 19.5 nm × 14 nm, and (F and G) 24 nm × 14 nm. The longitudinal diameter of the additional GroES in the complexes was ~7.2 nm. The number of particles averaged in the subclasses were (B) 48, (C) 81, (D) 106, (E) 68, (F) 104, and (G) 50.

the defined or diffuse mass distribution in the center of the GroEL side view. The different classes probably reflect some variability of the particles in their orientation relative to the carbon support. However, we cannot rule out the possibility that they represent structurally different subclasses. Calculation of the relative proportion of the individual species showed that about 55% of all side views and complexes were symmetric (Table 1). Because complex formation in the presence of ATP is highly dynamic, as a result of the repetitive release and rebinding of GroES that accompanies ATP hydrolysis (*17, 20*), we expected a heterogenous distribution of symmetric and asymmetric complexes. The proportion of symmetric particles may depend on the ADP-ATP ratio at the time of the preparation for electron microscopy (*26*).

Our results indicate that there are two potential GroES binding sites at opposite ends of the GroEL cylinder, which can be simultaneously occupied in the presence of ATP. The association of two GroES molecules with one GroEL particle is supported by the observation that the chaperonin proteins of *Thermus thermophilus* have also been shown to form symmetric particles in the presence of ATP (*4, 27*).

Next we explored complex formation under conditions known to influence the adenosine triphosphatase (ATPase) activity of GroE proteins (*15*) to further characterize the functional significance of symmetric particles. The ATPase activity of GroEL-GroES complexes can be completely arrested by excess ADP (*15, 17*). If the observed symmetric particle in the presence of ATP is an intermediate in the catalytic cycle, it might be expected to disappear on addition of excess ADP, in accordance with the kinetic model (*17*). When ADP was added to a solution known to contain symmetric particles, only asymmetric particles were subsequently observed (Fig. 3A). Furthermore, the chaperonin ATPase is K^+-dependent, and at low K^+ concentrations, ATP hydrolysis by the GroEL-GroES complex comes to a halt after very few turnovers (*12, 15*). Therefore, we investigated complex formation with 1 mM K^+ instead of 50 mM K^+. As expected, only asymmetric particles were observed under this condition (*21*). Consistent with the kinetic model, these results support the idea that the symmetric football-shaped particles are present only under conditions where the GroE system is able to facilitate the refolding of nonnative proteins, that is, when it is actively hydrolyzing ATP.

We then determined whether the formation of the symmetric particle was induced by ATP binding or ATP hydrolysis. For this purpose the nonhydrolyzable analogs 5′-adenylyl-imido-diphosphate (AMP-PNP) and ATP-γ-S were used. Symmetric football-shaped particles predominated in the presence of AMP-PNP (Fig. 3B); only a few asymmetric, bullet-like particles were visible. Similar results were obtained with ATP-γ-S (*21*). We conclude that the binding of ATP was sufficient to create symmetric GroEL-GroES complexes. In agreement with the single turnover experiments (*17*), we suggest that ATP hydrolysis by this symmetric particle leads to the dissociation of one of the two GroES rings from the complex, so as to create the asymmetric particles. The distribution of asymmetric bullets and symmetric footballs might therefore reflect the rate of ATP hydrolysis and the relative concentrations of ATP and ADP. That footballs accumulate during the catalytic cycle suggests that they precede the rate-limiting step in the cycle.

It has been proposed that the functional state of GroEL-GroES complexes is the asymmetric bullet structure with an altered surface at the distal end of the GroEL particle (that is, the ring opposite GroES), which prevents the binding of a second GroES and preserves structural and functional asymmetry (6). In contrast, under conditions where protein folding is facilitated (that is, in the presence of ATP), we have shown here that a second GroES can bind and that the symmetric football and the asymmetric bullet are probably both intermediates in the catalytic cycle. The functional significance of the open-bullet–like particles (Fig. 2, D and E) is unclear. Perhaps they represent another intermediate species in the cycle. It is also possible that the open surface at the distal end of the GroEL cylinder is caused by stain-induced disrupture of football-shaped complexes during the preparation of the proteins on the grids.

In conclusion, our results suggest a functional role for symmetric complexes. A redefinition of the cycle of GroE-facilitated protein folding, incorporating this species, would therefore appear to be necessary. A unifying model consistent with these structural and stoichiometric considerations has been developed (*17*). In support of this model and in agreement with cross-linking studies (*23*), we show that symmetric complexes can be observed, but only under conditions where the system is able to promote the refolding of nonnative substrate proteins. Formation of symmetric complexes follows the binding of ATP, whereas the dissociation of one ring of GroES follows ATP hydrolysis. The dynamics of the system during ATP hydrolysis are reflected in the distribution of footballs and bullets. Both the asymmetric and the symmetric complexes are necessary to sustain the operation of the cycle.

Table 1. Distribution of chaperonin complexes in the presence of ATP. The statistical analysis is based on the averaging procedure presented in Fig. 2 (B to G). We excluded top views from the analysis because we cannot distinguish whether such particles contain one, two, or no molecules of GroES. DeM, defined mass in the center of the GroEL particle; DiM, diffused mass distribution in the center of the GroEL particle; 610 particles would be equivalent to 100%. Of these particles, 27 (4.4%) could not be attributed unambiguously to one of the structural classes obtained by the analysis.

Representative classes	Percent of side views	
Unloaded side views	3	(Fig. 2B)
Closed bullets	12.4	(Fig. 2C)
Open bullets (DeM)	14.6	(Fig. 2D)
Open bullets (DiM)	11.5	(Fig. 2E)
Footballs (DeM)	36.2	(Fig. 2F)
Footballs (DiM)	17.9	(Fig. 2G)

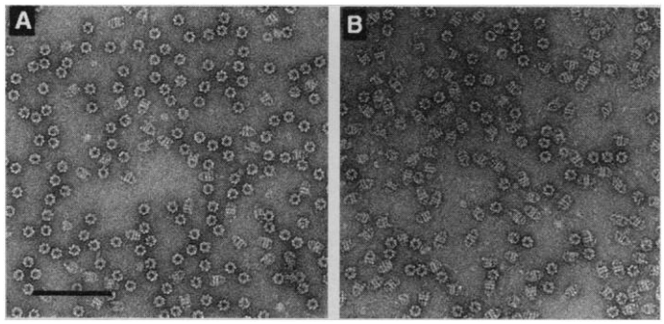

Fig. 3. GroEL-GroES complex formation under various conditions known to influence the functional cycle. (**A**) Complex formation was done as in Fig. 1A, with 0.77 μM GroEL and 0.74 μM GroES in the presence of 2.5 mM ATP. As a control, one sample was prepared for electron microscopy after 30 min of incubation at room temperature to make sure that football formation had occurred as expected. To a second sample 10 mM ADP was added. After another 30 min of incubation, this sample was applied to grids and negatively stained. (**B**) GroEL and GroES were allowed to form complexes as in (A), but with 2.5 mM AMP-PNP instead of ATP. The electron micrographs were recorded as in Fig. 1. The scale bar represents 100 nm.

REFERENCES AND NOTES

1. M.-J. Gething and J. Sambrook, *Nature* **355**, 33 (1992); J. Zeilstra-Ryalls, O. Fayet, C. Georgopoulos, *Annu. Rev. Microbiol.* **45**, 301 (1991).
2. R. Jaenicke, *Curr. Opin. Struct. Biol.* **3**, 104 (1993).
3. R. W. Hendrix, *J. Mol. Biol.* **129**, 375 (1979); P. Zwickl *et al.*, *J. Struct. Biol.* **103**, 197 (1990).

4. H. Tagouchi, J. Konishi, N. Ishii, M. Yoshida, *J. Biol. Chem.* **266**, 22411 (1991).
5. H. R. Saibil *et al.*, *Curr. Biol.* **3**, 265 (1993).
6. T. Langer, G. Pfeifer, J. Martin, W. Baumeister, F.-U. Hartl, *EMBO J.* **11**, 4757 (1992).
7. L. A. Svensson, B. P. Surin, N. E. Dixon, M. D. Spangfort, *J. Mol. Biol.* **235**, 47 (1994).
8. G. N. Chandrasekhar, K. Tilly, C. Woolford, R. W. Hendrix, C. Georgopoulos, *J. Biol. Chem.* **261**, 12414 (1986).
9. P. V. Viitanen, A. A. Gatenby, G. H. Lorimer, *Protein Sci.* **1**, 363 (1992).
10. K. Braig, M. Simon, F. Furuya, J. F. Hainfeld, A. L. Horwich, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3978 (1993).
11. E. S. Bochkareva and A. S. Girshovich, *J. Biol. Chem.* **267**, 25672 (1992); J. Martin *et al.*, *Nature* **352**, 36 (1991); N. Ishii, H. Tagouchi, H. Sasabe, M. Yoshida, *J. Mol. Biol.* **236**, 691 (1994).
12. P. V. Viitanen *et al.*, *Biochemistry* **29**, 5665 (1990).
13. T. E. Gray and A. R. Fersht, *FEBS Lett.* **292**, 254 (1991).
14. G. S. Jackson *et al.*, *Biochemistry* **32**, 2554 (1993).
15. M. J. Todd, P. V. Viitanen, G. H. Lorimer, *ibid.*, p. 8560.
16. E. S. Bochkareva, N. M. Lissin, G. C. Flynn, J. E. Rothman, A. S. Girshovich, *J. Biol. Chem.* **267**, 6796 (1992).
17. M. J. Todd, P. V. Viitanen, G. H. Lorimer, *Science* **265**, 659 (1994).
18. M. Schmidt, M. J. Todd, G. H. Lorimer, P. V. Viitanen, *J. Biol. Chem.* **269**, 10304 (1994).
19. S. J. Landry, J. Zeilstra-Ryalls, O. Fayet, C. Georgopoulos, L. M. Gierasch, *Nature* **364**, 255 (1993).
20. J. Martin, M. Mayhew, T. Langer, F.-U. Hartl, *ibid.* **366**, 228 (1993).
21. M. Schmidt and J. Buchner, unpublished results.
22. In a previous attempt to investigate complex formation in the presence of ATP, the complex was formed in the presence of ATP and subsequently applied to a size-exclusion column with ATP in the running buffer (*5*). However, this treatment changes the equilibrium of free compared with bound GroES, because released GroES is immediately removed on the column. This change in equilibrium might be why only asymmetric complexes were detected in electron micrographs.
23. A. Azem and P. Goloubinoff, *Science* **265**, 653 (1994).
24. J. Frank, A. Verschoor, M. Boublik, *ibid.* **214**, 1353 (1981).
25. M. Van Heel and J. Frank, *Ultramicroscopy* **6**, 187 (1981). All computations were performed with either the SEMPER [W. O. Saxton, T. J. Pitt, M. Horne, *ibid.* **9**, 109 (1979)] or EM (R. Hegerl and A. Altbauer, *ibid.*, p. 109) software systems.
26. An important preparative detail would be the time of incubation of the two proteins in the presence of ATP, before the sample is applied to the grid, because the ADP-ATP ratio is continuously changing. We routinely waited 30 min before preparing the samples for electron microscopy. Under the conditions used, we were sure that an excess of ATP was still present.
27. In a later report the observed stoichiometry of two GroES rings associated with one GroEL oligomer has been questioned by the authors [N. Ishii, H. Tagouchi, M. Sumi, M. Yoshida, *FEBS Lett.* **299**, 169 (1992)].
28. To be sure that no damage of the oligomeric proteins due to freezing procedures was occurring, we rapidly purified the protein without freezing it between individual purification steps (Fig. 1). However, when the so purified proteins were frozen and then again allowed to form complexes in the presence of ATP, we observed no differences on negatively stained electron micrographs. Thus, freezing did not change the structural and functional properties of GroE particles.
29. O. Fayet, J.-M. Louarn, C. P. Georgopoulos, *Mol. Gen. Genet.* **202**, 435 (1986).
30. J. Buchner *et al.*, *Biochemistry* **30**, 1586 (1991).
31. We thank W. Baumeister for equipment for image analysis and discussions, S. Volker-Mürkl for technical assistance, and the University of Regensburg, the Deutsche Forschungsgemeinschaft, and the Fonds der Chemischen Industrie for financial support to M.S., R.R., R.J., and J.B.

5 April 1994; accepted 27 June 1994

Dynamics of the Chaperonin ATPase Cycle: Implications for Facilitated Protein Folding

Matthew J. Todd, Paul V. Viitanen, George H. Lorimer*

The *Escherichia coli* chaperonins GroEL and GroES facilitate protein folding in an adenosine triphosphate (ATP)–dependent manner. After a single cycle of ATP hydrolysis by the adenosine triphosphatase (ATPase) activity of GroEL, the bi-toroidal GroEL formed a stable asymmetric ternary complex with GroES and nucleotide (bulletlike structures). With each subsequent turnover, ATP was hydrolyzed by one ring of GroEL in a quantized manner, completely releasing the adenosine diphosphate and GroES that were tightly bound to the other ring as a result of the previous turnover. The catalytic cycle involved formation of a symmetric complex (football-like structures) as an intermediate that accumulated before the rate-determining hydrolytic step. After one to two cycles, most of the substrate protein dissociated still in a nonnative state, which is consistent with intermolecular transfer of the substrate protein between toroids of high and low affinity. A unifying model for chaperonin-facilitated protein folding based on successive rounds of binding and release, and partitioning between committed and kinetically trapped intermediates, is proposed.

Chaperonins are ubiquitous, indispensable proteins that facilitate protein folding in an ATP-dependent manner (*1*), enhancing the yield (but rarely the rate of formation) of properly folded substrate protein under conditions where spontaneous folding does not occur (*2*). Chaperonins are typified by the two *E. coli* heat shock proteins GroEL (chaperonin 60) and GroES (chaperonin 10) (*1*). GroEL forms a binary complex with many unfolded proteins (*3*) and thus is promiscuous rather than selective. To achieve the native state under nonpermissive conditions, a substrate protein must be discharged from the binary complex in a state that allows commitment to the native state, which usually requires the complete chaperonin system of GroEL, GroES, and the hydrolysis of ATP (*4*). Here, we describe how these three components interact with each other to assist protein folding. We propose that, in contrast to other models, the unfolded protein substrate plays a passive role in this process.

GroEL (*5*) consists of two stacked heptameric rings of seven identical 57-kD subunits each (*6*). GroES is a single homoheptameric toroid of 10-kD subunits (*7*). GroEL has a K^+-dependent adenosine triphosphatase (ATPase) activity (one turnover every 10 to 12 s) that is inhibited by GroES (*6–12*). The binding of one ring of GroES to one of the two rings of GroEL, which occurs only in the presence of adenine nucleotide, creates an asymmetric complex of $GroES_7 \cdot ADP_7 \cdot GroEL_7$–$GroEL_7$ (where ADP is adenosine diphosphate) (*5, 9*). The stoichiometry of this species has been inferred from kinetic measurements of GroEL ATPase activity (*8–12*) and observed directly by electron microscopy (*13, 14*). GroES binding inhibits GroEL ATPase from 50 to 100%, depending on the concentrations of ATP, ADP, and K^+ (*9*). At high K^+ concentrations, half of the sites remain fully active, resulting in 50% inhibition. At low K^+ concentrations, the ATPase activity of GroEL can be completely inhibited by a single ring of GroES after as little as one-half of the GroEL protomers (seven sites) have turned over (*9*). This complete inhibition can be reversed by raising the concentration of either ATP or K^+ or by removing the inhibitory ADP. Under such circumstances, ATP hydrolysis resumes at ~50% of the uninhibited rate. The GroES inhibition reflects a decreased affinity of unoccupied sites of the asymmetric complex for ATP (relative to ADP) (*9*).

Steady-state kinetics, however, do not provide an adequate description of the chaperonin ATPase cycle. We therefore exploited the slow hydrolysis of ATP and the stability of the asymmetric complex to analyze the dynamics of the chaperonin system. To examine the consequences of a single round of ATP hydrolysis (all seven sites on one ring of GroEL turning over once), we manipulated the concentrations of K^+, ATP, and ADP to turn on or off the hydrolysis of ATP at will, while the integrity of the chaperonin proteins was maintained.

E. I. DuPont de Nemours and Company, Central Research and Development Department, Experimental Station, Wilmington, DE 19880, USA.

*To whom correspondence should be addressed.