higher force portion of the force-velocity curve and quite likely would tend to maintain the muscles at a relatively high power and efficiency (*11*). Further evidence that gearing may enhance the contractile performance of the calf muscles comes from the observation that during rapid acceleration the gear ratio remained relatively low (Fig. 2B). Again, this is what would be expected if the system were to function so as to maintain the muscles at high power.

Morphologists and muscle physiologists have long recognized that synergistic muscles often have different mechanical advantages around joints, and they have therefore suggested that different muscles use different gears (*12*). Leverage around joints has also been shown to vary with body size (*13*) and during ontogeny (*14*). But these separate muscle systems and allometric patterns are not variable gearing mechanisms for individual muscles within an individual. Variable gearing has been suggested to result from the unusual organization of the ankles of artiodactyls and lagomorphs (*15*) and has been documented in the flight system of blowflies (*16*). Additionally, humans have been shown to shift mechanical advantage around the hip, knee, and ankle when they change gaits from a walk to a run (*17*), presumably using mechanisms similar to those proposed here.

The concept of variable musculoskeletal gearing has received relatively little attention from physiologists. Given the variety of species specialized for terrestrial locomotion, and the grace, skill, and speed with which many animals run and jump, it would be surprising if variable gearing had not evolved.

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7. The difference in gear ratio between running and acceleration was significant at the 0.0001 level, as measured by repeated multivariate analysis of variance (MANOVA), for the four people from whom we have recordings of both running and acceleration.
8. In contrast to these results from humans, preliminary data we have collected from a dog show a pattern of decreasing gear ratio during ankle extension. The center of force translates forward during foot support in this dog as it does in humans, but the more vertical orientation of the foot results in the ankle catching up to the ground reaction force during late foot support. This results in a decrease in the length of *R* and a decrease in the gear ratio.
9. Our kinematic technique is limited by the fact that an unknown portion of the measured change in length resulted from the stretch of the tendon in series with the muscles.
10. This is an appropriate value of gear ratio to use for comparison, because mechanical advantage at midstance is often used in calculations and modeling by biometricians.
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Characterization of a Functional $GroEL_{14}(GroES_7)_2$ Chaperonin Hetero-Oligomer

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Chaperonins GroEL and GroES form two types of hetero-oligomers in vitro that can mediate the folding of proteins. Chemical cross-linking and electron microscopy showed that in the presence of adenosine triphosphate (ATP), two $GroES_7$ rings can successively bind a single $GroEL_{14}$ core oligomer. The symmetric $GroEL_{14}(GroES_7)_2$ chaperonin, whose central cavity appears obstructed by two $GroES_7$ rings, can nonetheless stably bind and assist the ATP-dependent refolding of RuBisCO enzyme. Thus, unfolded proteins first bind and possibly fold on the external envelope of the chaperonin hetero-oligomer.

Chaperonins, also called cpn60 and cpn10, belong to a ubiquitous class of sequence-related chaperone molecules in mitochondria, chloroplasts, and bacteria. In the cell, they are implicated in the folding of proteins (*1*) and in the molecular response to cellular stress (*2*). In vitro, chaperonins assist in the correct refolding of proteins by preventing aggregations (*3, 4*). As determined by electron microscopy, cpn60 from bacteria (GroEL) is an oligomer of 14 identical 57.3-kD subunits, with a structure of two stacked heptameric rings (*5–7*) arranged around a twofold axis of symmetry. This oligomer, $GroEL_{14}$, appears as a hollow cylinder, with a cavity that spans the sevenfold axis of symmetry of the molecule (*7–9*). The cpn10 from bacteria (GroES) is a heptameric ring of identical 10.4-kD subunits (*10*).

The molecular mechanism by which chaperonins assist the folding of a large array of proteins (*11*) remains obscure (*12, 13*). Central to this issue is the molecular architecture of the GroEL-GroES hetero-oligomers and of the $GroEL_{14}$ core oligomer which can spontaneously bind unfolded proteins (*3*). The step leading to the dissociation of the protein-$GroEL_{14}$ complex and the subsequent correct refolding of the assisted protein is coordinated by the co-chaperonin $GroES_7$ and requires Mg-ATP (*3, 12*). Electron microscopy reveals that one $GroES_7$ ring can asymmetrically bind on either end of the $GroEL_{14}$ cylinder and thus obstruct one end of the central cavity (*7, 8*). Refolding proteins may compete with $GroES_7$ for the same binding sites on either end of the $GroEL_{14}$ cylinder (*14, 15*). However, electron micrographs of $GroEL_{14}$ molecules previously incubated with denatured proteins indicate that proteins bind directly within the central cavity (*9, 16*). Biochemical analysis suggested that the asymmetric $GroEL_{14}GroES_7$ complex is a functional chaperonin hetero-oligomer (*8, 17, 18*). Hence, a model for the chaperonin reaction cycle is an asymmetric $GroEL_{14}GroES_7$ hetero-oligomer that assists the folding and release of a protein from within the central cavity through the unobstructed end of the $GroEL_{14}$ cylinder (*13, 19*).

In contrast, we now show that the asymmetric $GroEL_{14}GroES_7$ molecule is only one of two active forms of chaperonin hetero-oligomers. In solution, there exists a second symmetric $GroEL_{14}(GroES_7)_2$ chaperonin that can stably bind and efficiently assist the refolding of the RuBisCO enzyme. Both the asymmetric $GroEL_{14}GroES_7$ and the symmetric $GroEL_{14}(GroES_7)_2$ hetero-

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oligomers may serve as intermediate species of the chaperonin cycle.

When $GroEL_{14}$ and a molar excess of $GroES_7$ oligomers were incubated with increasing amounts of Mg-ATP and then exposed to glutaraldehyde (GA), three cross-linked species successively appeared on SDS-polyacrylamide gels in a stepwise, ATP-dependent manner (Fig. 1A). In the absence of ATP, there was a single cross-linked species in the range of 800 ± 40 kD that comigrated with the cross-linked product of the $GroEL_{14}$ oligomer alone (*20*). Incubation with 5 μM ATP led to the appearance of a second cross-linked species in the range of 870 ± 40 kD, Form1, at the expense of the $GroEL_{14}$ species. When the ATP concentration was raised to 500 μM, a third cross-linked species, Form2, appeared in the range of 940 ± 50 kD, at the expense of Form1 (Fig. 1A) (*21*). Various concentrations of adenosine diphosphate (ADP) or adenylimidodiphosphate (AMP-PNP), but not of guanosine triphosphate (GTP), substituted for ATP in the reaction. However, ADP only supported the appearance of Form1 and not of Form2 (Table 1).

Form1 and Form2 also appeared in a $GroES_7$-dependent manner, in the presence of a saturating amount of ATP (Fig. 1B). When the molar ratio between GroES and GroEL protomers was 0.22, the protein signal distributed nearly equally between the $GroEL_{14}$ and Form1 cross-linked species. A GroES to GroEL ratio of 0.44 shifted the protein signal almost entirely into Form1, at the expense of $GroEL_{14}$. Form2 appeared when the molar ratio exceeded 0.5. It became the major species at the expense of Form1 when molar ratios exceeded 1.1 (*22*). From these results we deduce that in solution two $GroES_7$ rings can bind a single $GroEL_{14}$ core oligomer.

Electron microscopy confirmed that Form1 is the asymmetric $GroEL_{14}GroES_7$ hetero-oligomer and Form2 a symmetric $GroEL_{14}(GroES_7)_2$ chaperonin oligomer. Electron micrographs of a solution highly populated with Form1 chaperonins (Fig. 2A) revealed that it was enriched with asymmetric bullet-shaped structures (Fig. 2B), which were identical to $GroEL_{14}GroES_7$ hetero-oligomers (*8*, *9*). In contrast to Form1, where a single $GroES_7$ ring was seen asymmetrically bound to $GroEL_{14}$, Form2 molecules displayed a symmetric football-shaped structure (the shape of an American football). When a non–cross-linked chaperonin solution was incubated in the presence of ATP, similar symmetric structures were initially identified by Schmidt *et al.* (*23*) and further characterized as $GroEL_{14}(GroES_7)_2$ hetero-oligomers (*24*).

When $GroEL_{14}$ and increasing amounts of $GroES_7$ were preincubated with AMP-PNP, mixed with urea-denatured RuBisCO, and then reacted with GA, three slow-migrating cross-linking species appeared above the $GroEL_{14}$ marker (Fig. 1C). The $GroES_7$-dependent, stepwise apparition of each species, at the expense of the other, identified them as the cross-linked species of RuBisCO-$GroEL_{14}$, RuBisCO-$GroEL_{14}GroES_7$, and RuBisCO-$GroEL_{14}(GroES_7)_2$ chaperonin complexes, respectively (*25*). It was previously suggested that the binding of unfolded citrate synthase, but not of unfolded rhodanese, can cause a transient dissociation of $GroES_7$ from ADP-preformed chaperonin hetero-oligomers (*13*), which we now know to be dilution-sensitive (*26*), asymmetric $GroEL_{14}GroES_7$ hetero-oligomers. In contrast, we showed here that within minutes of the initial binding of unfolded RuBisCO, $GroES_7$ is an integral part of RuBisCO-$GroEL_{14}GroES_7$ and of RuBisCO-$GroEL_{14}(GroES_7)_2$ complexes (Fig. 1C).

The binding of a single $GroES_7$ to a protein-$GroEL_{14}$ binary complex has been suggested to be sufficient to drive the correct release of the refolding protein from the chaperonin (*8*, *17*). The two orders of magnitude difference between the ATP concentrations necessary to form $GroEL_{14}GroES_7$ and $GroEL_{14}(GroES_7)_2$ oligomers (Table 1) suggests that $GroES_7$ has a higher affinity for $GroEL_{14}$ than for $GroEL_{14}GroES_7$. Consequently, $GroEL_{14}$ and $GroEL_{14}(GroES_7)_2$ tend not to coexist in the same solution (Fig. 2C); thus, protein refolding activity can be determined for chaperonin solutions that contain, at most, two identified species of hetero-oligomers. Solutions of $GroEL_{14}$ preincubated in the presence of ATP with various amounts of $GroES_7$ were assayed for RuBisCO refolding activity (Fig. 3). Maximal recovery of RuBisCO was already achieved when the molar ratio between GroES and GroEL approximated 0.5, and the chaperonin solution was accordingly popu- lated with a majority of asymmetric $GroEL_{14}GroES_7$ hetero-oligomers. Moreover, when the molar ratio was preset above 1.5 and the chaperonin solution was accordingly populated with a majority of symmetric

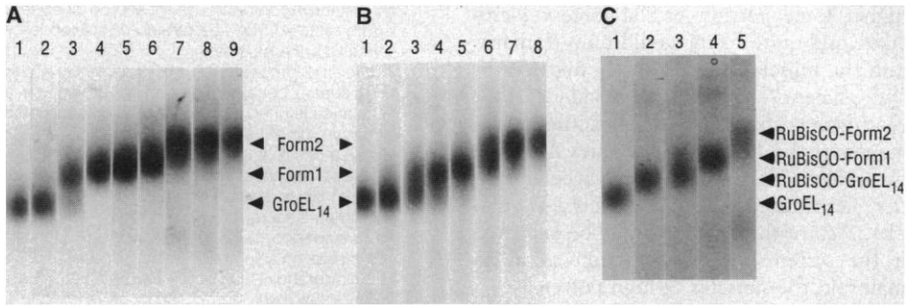
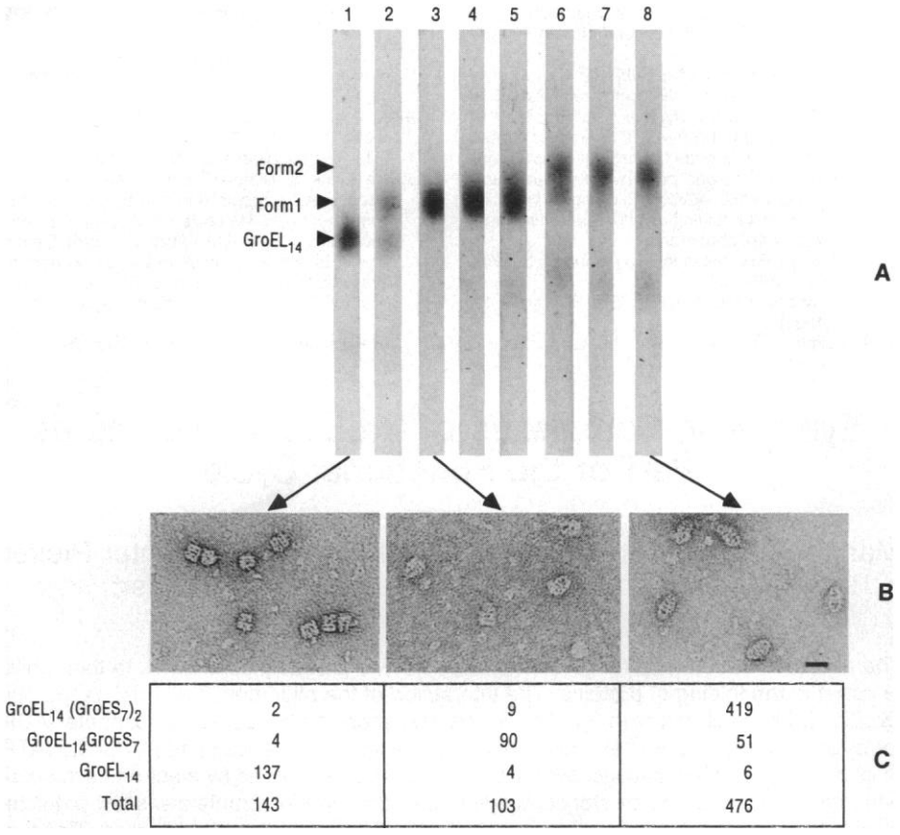


Fig. 1. Cross-linking of chaperonin hetero-oligomers. (**A**) Cross-linking of $GroEL_{14}$ and $GroES_7$, preincubated with increasing concentrations of ATP. We purified $GroES_7$ and $GroEL_{14}$ to homogeneity as described in (*18*) with small modifications, and as in (*32*), respectively. Oligomers of $GroEL_{14}$ (3.3 μM protomers) and $GroES_7$ (9.6 μM protomers) were preincubated for 20 min at 37°C in 50 mM triethanolamine, pH 7.5, containing 20 mM $MgCl_2$, 2 mM KCl, 1 mM dithiothreitol (DTT), and increasing amounts of ATP (0, 2, 5, 50, 100, 250, 500, 750, and 1000 μM, in lanes 1 to 9, respectively). (**B**) Cross-linking as in (A) of $GroEL_{14}$ preincubated with ATP (1 mM) and increasing amounts of $GroES_7$. The molar ratio between GroES and GroEL was 0, 0.11, 0.22, 0.33, 0.44, 0.67, 1.10, and 2.90 in lanes 1 to 8, respectively. (**C**) Cross-linking of chaperonin oligomers with unfolded RuBisCO. We preincubated $GroEL_{14}$ (3.3 μM protomers) with 2 mM AMP-PNP (Sigma) and increasing amounts of GroES as in (B). The molar ratio between GroES and GroEL protomers was 0, 0, 0.44, 1, and 2 in lanes 1 to 5, respectively. RuBisCO (26 μM) in 5 M urea was diluted 1:52 in the chaperonin solutions (except in lane 1, in which only 5 M urea was diluted) and then further incubated 30 min at 37°C. Chaperonin solutions in (A), (B), and (C) were exposed for 60 min to 0.22% GA at 37°C. The cross-linking reaction was stopped by the addition of a one-third volume of 1 M tris-glycine (pH 8.8), 4% SDS, and 10% 2-mercaptoethanol. Boiled samples (2 min) were submitted to a uniform SDS–polyacrylamide (2.8%) gel electrophoresis and stained with Coomassie Brilliant blue R-250 (Sigma) as in (*32*).

Table 1. The effective concentration of nucleotide that is necessary to drive the formation of 50% (EC_{50}) of the cross-linked species Form1, at the expense of $GroEL_{14}$, and of Form2, at the expense of Form1. Electrophoresis and Coomassie blue stain of the gels was as in Fig. 1A. Quantification of the cross-linked products was obtained by scanning the gels with an Ultrascan-XL (LKB) as in (*32*). Dashes indicate that no measurable reaction occurred.

Nucleotide	EC_{50} (μM)	
	$GroEL_{14}$:Form1	Form1:Form2
ATP	3.5	400
ADP*	30	—
AMP-PNP	100	500
GTP	—	—

*ADP was tested up to 6 mM.

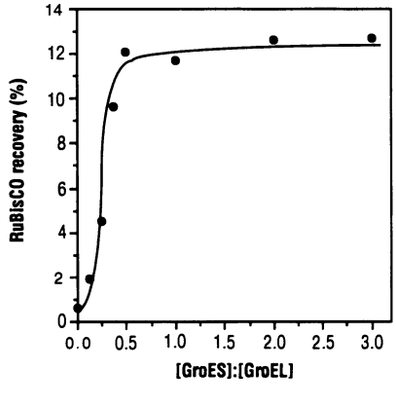


$GroEL_{14}(GroES_7)_2$	2	9	419
$GroEL_{14}GroES_7$	4	90	51
$GroEL_{14}$	137	4	6
Total	143	103	476

C

Fig. 2. Identification of cross-linked chaperonin species by electron microscopy. (**A**) SDS gels of cross-linked chaperonin hetero-oligomers. The $GroEL_{14}$ and $GroES_7$ oligomers were preincubated as in Fig. 1A except that ATP was replaced by 0, 0.1, 0.2, 0.4, 0.5, 0.6, 1.0, and 1.2 mM AMP-PNP (for lanes 1 to 8, respectively). (**B**) Cross-linked chaperonin hetero-oligomers viewed by electron microscopy. The left, central, and right panels show cross-linked chaperonin molecules from (A), lanes 1, 3, and 8, respectively. The cross-linking reaction was stopped by the addition of an equal volume of 1 M glycine, pH 7.5. Samples were applied to a glow-discharged, carbon-coated, collodion-covered 300 mesh copper grid and negatively stained with 1% aqueous uranyl acetate. Specimens were viewed in a Philips CM12 electron microscope operating at 100 kV. Micrographs were recorded on Kodak emulsion SO-163 at a nominal magnification of ×75,000. The bar equals 20 nm. (**C**) Distribution of identified side views of $GroEL_{14}$, $GroEL_{14}GroES_7$, and $GroEL_{14}(GroES_7)_2$ molecules in a larger sample of fields as in (B).

Fig. 3. $GroES_7$-dependent refolding of RuBisCO by $GroEL_{14}$ and ATP. The oligomer $GroEL_{14}$ (3.3 μM) in 50 mM triethanolamine (pH 7.5), 10 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1% glucose, and 1 mM Mg-ATP and increasing amounts of $GroES_7$ (GroES to GroEL molar ratio is indicated) were incubated with a 1:52 dilution of RuBisCO (26 μM) from *Rhodospirillum rubrum* (*3*) in 5 M urea and 10 mM DTT. The refolding of RuBisCO was interrupted after a 15-min reaction at 37°C by addition of hexokinase (100 μg/ml, Sigma) (*3*). Maximal recovery of RuBisCO after a 1-hour reaction was 37% of a nondenatured control.



$GroEL_{14}(GroES_7)_2$ hetero-oligomers, the recovery of RuBisCO was equally as efficient. Thus, the efficiency of protein refolding is the same whether an unfolded protein first interacts with an asymmetric or with a symmetric chaperonin hetero-oligomer (*27*).

Todd *et al.* (*28*) have proposed a model in which both asymmetric and symmetric hetero-oligomers are intermediates of the chaperonin adenosine triphosphatase (ATPase) cycle. They suggest that a single $GroES_7$ molecule binds repetitively to many asymmetric $GroEL_{14}GroES_7$ hetero-oligomers, thus converting them into transient $GroEL_{14}(GroES_7)_2$ complexes (*28*). This model, which assigns a catalytic role to the second $GroES_7$, can explain our unexpected observation that full RuBisCO recovery is equally achieved by chaperonin solutions populated either with asymmetric or with symmetric chaperonin hetero-oligomers.

In view of our results, the role of the central cavity remains unclear. Electron micrographs of $GroEL_{14}(GroES_7)_2$ show that both access paths to the central cavity are obstructed by the two $GroES_7$ rings [see (*23*)]. Nevertheless, $GroEL_{14}(GroES_7)_2$ is a fully potent chaperonin, which implies that the initial interaction and binding between unfolded RuBisCO and $GroEL_{14}(GroES_7)_2$ occurs on the outside surfaces of the chaperonin. If the folding of the protein was also to take place on the external envelope of the chaperonin, the limitation imposed by the size of the central cavity would be lifted (*29*), accounting for reports of chaperonin-assisted folding of large polypeptides such as phytochrome (*30*) and of *Escherichia coli* RNA polymerase (*31*). The central cavity may serve as an inexpensive backbone for the globular shape of the complex and thus provide optimal exposure of the chaperonin protein-binding surfaces to the surrounding protein-folding intermediates.

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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*).
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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.
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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.

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Symmetric Complexes of GroE Chaperonins as Part of the Functional Cycle

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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-

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