Conditions for Nucleotide-dependent GroES-GroEL Interactions

GroEL$_{14}$(GroES$_{7}$)$_{2}$ IS FAVORED BY AN ASYMMETRIC DISTRIBUTION OF NUCLEOTIDES*

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Boris M. Gorovits‡, Jesse Ybarra‡, Jeffrey W. Seale‡, and Paul M. Horowitz§

From the Department of Biochemistry, University of Texas Health Sciences Center at San Antonio, San Antonio, Texas 78240-7760

A still unresolved question regarding the mechanism of chaperonin-assisted protein folding involves the stoichiometry of the GroEL-GroES complex. This is important, because the activities of the *Escherichia coli* chaperonin GroES are modulated by the cochaperonin GroEL. In this report, the binding of GroES to highly purified GroEL in the presence of ATP, ADP, and the nonhydrolyzable ATP analogue, 5′-adenylyl βγ-imido-diphosphate (AMP-PNP), was investigated by using the fluorescence anisotropy of succinimidy1-1-pyrenebutyrate-labeled GroES. In the presence of Mg$^{2+}$-ATP and high [KCl] (10 mM), two GroES rings bind per one GroEL$_{14}$. In contrast, in the presence of ADP or AMP-PNP only one molecule of oligomeric GroES can be tightly bound by GroEL. With AMP-PNP, binding of a small amount (<20%) of a second GroES can be detected. In the presence of ADP alone, a second GroES ring can bind to GroEL weakly and with negative cooperativity. Strikingly, addition of AMP-PNP to the solution containing preformed GroEL$_{14}$(GroES$_{7}$) complexes formed in the presence of ADP results in an increase in the fluorescence anisotropy. Analysis of this effect indicates that 2 mol of GroES oligomer can be bound in the presence of mixed nucleotides. A similar conclusion follows from studies in which ADP is added to an GroEL$_{14}$(GroES$_{7}$) complex formed in the presence of AMP-PNP. This is the first demonstration of an asymmetric distribution of nucleotides bound on the 1:2 GroEL$_{14}$(GroES$_{7}$)$_{2}$ complex. The relation of the observed phenomena to the proposed mechanism of the GroEL function is discussed.

Although it is widely accepted that all the information required for proper folding of proteins is encoded entirely within the primary sequence (1, 2), it is also clear that molecular chaperones have evolved to assist in the efficient proper folding, trafficking, and assembly of proteins in vivo (3–5). The most widely studied molecular chaperones are the GroE chaperonin proteins of *Escherichia coli*, GroEL and GroES (3). GroEL is a tetradecamer of 14 identical 57-kDa subunits arranged in two heptameric rings that are stacked upon one another back-to-back (6). This results in a cylindrical oligomer whose two ends are identical.

GroEL possesses a weak, K$^{+}$-dependent ATPase activity that is partially inhibited by the presence of GroES (7–10). The quantized hydrolysis of ATP by GroEL has been proposed to provide the energy necessary for the release of polypeptide substrates (11, 12).

GroES is a heptamer of seven identical 10-kDa subunits arranged as a single ring (13). GroES regulates the activities of GroEL by the binding to GroEL of a large mobile loop from each GroES monomer (14, 15). It has been shown that GroES is required for successful refolding of polypeptides by GroEL under conditions where spontaneous refolding of the substrate does not occur (16).

Although much has been learned about the functions of GroEL and GroES, the detailed mechanism by which the chaperonins assist the refolding of proteins has not been definitively elucidated. One of the controversies surrounding the mechanism of GroEL-GroES action is the stoichiometry of the complex(es) involved. Electron microscopy has provided evidence for the existence of both 1:1 GroEL$_{14}$(GroES$_{7}$) and 1:2 GroEL$_{14}$(GroES$_{7}$)$_{2}$ complexes under various conditions (reviewed in Ref. 17). Schmidt et al. (18) used electron microscopy to show that 1:2 GroEL$_{14}$(GroES$_{7}$)$_{2}$ complexes could be formed in the presence of nonhydrolyzable ATP analogs, and using the kinetic data of Todd et al. (11), they suggested that the formation of 1:2 GroEL$_{14}$(GroES$_{7}$)$_{2}$ complexes preceded the rate-limiting step in the cycle. GroEL$_{14}$(GroES$_{7}$)$_{2}$ complexes were identified by Azem et al. (19) using electron microscopy and chemical cross-linking. Those experiments also suggested that these 1:2 complexes were active in the refolding of RuBisCO in agreement with the proposal that GroEL$_{14}$(GroES$_{7}$)$_{2}$ complexes served a role in the chaperonin ATPase cycle proposed by Todd et al. (11). In contrast, it has been reported that the formation of 1:2 GroEL$_{14}$(GroES$_{7}$)$_{2}$ complexes occurs at unphysiologically high Mg$^{2+}$ concentrations and increased pH (20). This latter work also showed that formation of GroEL$_{14}$(GroES$_{7}$)$_{2}$ complexes prevented the binding of unfolded polypeptide, calling into question the significance of 1:2 GroEL$_{14}$(GroES$_{7}$)$_{2}$ complexes. Using surface plasmon resonance, another study from that group showed that stable GroEL$_{14}$(GroES$_{7}$) complexes could be formed and dissociated with subsequent ATP hydrolysis with no intermediate formation of 1:2 GroEL$_{14}$(GroES$_{7}$)$_{2}$ complexes (21). Overall, it has been demonstrated that single site-directed mutations as well as nominal variations of solution conditions can have dramatic effects on the structure and function of GroEL. Therefore, the question of the role of the sometimes vigorous conditions required to demonstrate various complexes has not been directly addressed.

In this report, we show using direct binding measurements that GroEL$_{14}$(GroES$_{7}$)$_{2}$ complexes can be formed in the presence of ATP and high [KCl], although only one heptamer of GroES can be tightly bound to the oligomeric GroEL in the

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‡ These individuals contributed equally to this paper.

§ To whom correspondence should be addressed: Dept. of Biochemistry, University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78284-7760. Tel.: 210-567-3737; Fax: 210-567-6595; E-mail: Horowitz@bioc09.uthscsa.edu.

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presence of ADP or AMP-PNP, respectively. Subsequent addition of either AMP-PNP or ADP to the preformed 1:1 GroEL-GroES complex results in formation of GroEL14(GroES7)$_2$, suggesting that the 1:2 complex contains different nucleotides bound on the opposite rings. The relationship of the observed phenomenon to the proposed mechanism of the GroEL function is discussed.

MATERIALS AND METHODS

Reagents—Succinimidyl-1-pyrenebutyrate was purchased from Molecular Probes Inc. (Eugene, OR). All reagents were of analytical grade. Protein Purification—GroES and GroEL were purified as described previously (22, 23). Protein concentrations were determined by the method of Bradford (24).

The Refolding Activity of Rhodanese by GroEL-GroES—The refolding activity of rhodanese by GroEL-GroES was measured as described previously (25).

GroES Binding to GroEL—For labeling of GroES with pyrene, GroES (3.2 mg/ml) was incubated in 50 mM triethanolamine hydrochloride, pH 7.8, for 2 h at room temperature with a 1.5 molar excess over oligomer of succinimidyl-1-pyrenebutyrate added from a stock solution in N,N-dimethylformamide. The labeling solution contained 5% N,N-dimethylformamide. The sample was then dialyzed against 50 mM Tris-HCl, pH 7.8, 10 mM MgCl$_2$, overnight at 4°C to remove the free label. SDS-polyacrylamide gel electrophoresis confirmed that there was no detectable noncovalently bound pyrene. The product contained 0.8 pyrene moieties per each oligomer of GroES. The pyrene adduct was quantified using $e_3(340 \text{ nm}) = 43,000 \text{ M}^{-1} \text{cm}^{-1}$.

The anisotropy of the pyrene-labeled GroES was recorded using a PC1 photon counting spectrofluorometer (ISS, Champaign, IL). For the anisotropy measurements, various concentrations of GroES were incubated with or without 160–200 nM GroEL in 50 mM Tris-HCl, pH 7.8, 10 mM MgCl$_2$, and either 0.5 or 10 mM KCl. The anisotropy was recorded within 1–3 min after the addition of specified nucleotide (ATP, ADP, or AMP-PNP). For ATP, this time was within the steady state phase of ATP hydrolysis, and the measurements were complete before ~5% of the ATP had been consumed. In case of binding in the presence of mixed nucleotides, GroEL and GroES were incubated in the presence of ADP for 5 min, and the anisotropy was recorded 1 min after the addition of AMP-PNP. In all cases the excitation wavelength and the emission wavelength were set at 345 and 395 nm, respectively.

The fraction of bound GroES$_7$ was calculated from the following relationships,

$$r_{\text{obs}} = \frac{(f_{\text{bound}})(r_{\text{bound}})}{(f_{\text{free}})(r_{\text{free}})} = \frac{(f_{\text{bound}})}{(f_{\text{free}})}$$

$$f_{\text{bound}} = 1 - f_{\text{free}}$$

Where $r_{\text{obs}}$ is the measured anisotropy for a given condition, $f_{\text{bound}}$ and $f_{\text{free}}$ are the fraction of GroES$_7$ bound, and $f_{\text{free}}$ is the fraction of free GroES$_7$. $r_{\text{bound}}$ is the anisotropy of bound GroES$_7$, and $r_{\text{free}}$ is the anisotropy of free GroES$_7$. The anisotropy of bound GroES$_7$ is the value measured in an excess of GroEL$_{14}$ and it gave a value of $r = 0.029 \pm 0.001$ independent of the presence of nucleotide. There was no significant difference in the fluorescence intensity between the free and bound GroES$_7$, indicating that no significant change in quantum yield was associated with binding.

Anisotropy data obtained in the presence of ADP were fit to the Scatchard (Equation 3) and Hill (Equation 4) equations using nonlinear least squares methods implemented in PSI plot,

$$B \frac{1}{F} = \frac{1}{2} K_f(q_1 - B) + K_d(q_1 - B)$$

$$+ \sqrt{[K_f(q_1 - B) + K_d(q_1 - B)]^2 + 4 K_d q_1^2 q_2}$$

$$\log[Y(1 - Y)] = h \log[S] - h K$$

1 The abbreviations used are: AMP-PNP, 5′-adenyllyl $\beta,\gamma$-imido-diphosphate; pyrene, succinimidyl-1-pyrenebutyrate; pyrene-GroES, GroES covalently labeled with pyrene.

where $Y$ is the degree of saturation of the acceptor, $h$ is the Hill coefficient, and $K$ is the dissociation constant (27).

The ATPase Activity of GroEL—The ATPase activity of GroEL was measured by the method of Viitanen et al. (28) with the modifications of Horovitz et al. (29).

RESULTS

GroEL Is Able to Form 1:2 Complexes with GroES in the Presence of ATP—To directly evaluate the GroEL-GroES interaction in solution, the cochaperonin GroES was covalently labeled with succinimidyl-1-pyrenebutyrate, an amino group directed derivative of pyrene. Succinimidyl-1-pyrenebutyrate possesses a very low average fluorescence lifetime (>100 ns) that allows one to examine protein-protein interactions. GroES that was labeled in this manner was functional as assessed by its ability to support rhodanese refolding by GroEL (data not shown). Under conditions where the GroEL concentration was fixed and exceeded the $K_a$ of the GroEL-GroES interaction, GroES was in the presence of ADP or AMP-PNP, 1 and 2 additional increase in GroES resulted in the progressive decrease of the fluorescence anisotropy values ($r_{\text{obs}}$). By using Equations 1 and 2 (see “Materials and Methods”) $r_{\text{obs}}$ was transformed to the concentrations of free and bound GroES (Fig. 1A). The ratios of [bound GroES$_7$]/[total GroES$_{14}$] are given by the values on the ordinate. In this experiment the GroEL$_{14}$ concentration was 135 nM. In the presence of a high level of [KCl] (10 mM), the bound fraction of GroES sharply increased up to a value of approximately 270 nM, which corresponded to a 2:1 complex between GroES and GroEL (Fig. 1A, closed circles). These results are consistent with reports that 1:2 GroEL-GroES complexes can be detected under similar conditions by electron microscopy (18) or chemical cross-linking (19). It is interesting that at a low concentration of KCl (0.5 mM) only partial saturation of two binding sites on GroEL can be observed (Fig. 1A, open circles). The line shown represents a nonlinear least squares fit to the acquired data. The observed stoichiometry of about 1.5 suggests that under such conditions the system contains both 2:1 and 1:1 complexes. The data were fit to an equation describing a sum of 1:1 and 1:2 binding isomers (where the final concentration of the GroEL-GroES complex is a sum of the [GroEL$_{14}$(GroES$_7$)$_2$] and [GroEL$_{14}$(GroES$_7$)$_1$]). It was found that under such conditions the solution contains approximately 46 and 54% of the [GroEL$_{14}$(GroES$_7$)$_1$] and [GroEL$_{14}$(GroES$_7$)$_2$] complexes, respectively. This result is consistent with previous studies that suggested that the ability of GroEL to inhibit GroEL ATPase activity at low [KCl] was due to a significantly decreased apparent affinity of the 1:1 binary GroEL-GroES complex for MgATP versus MgADP under these conditions (10).

In the presence of AMP-PNP, a nonhydrolyzable analogue of ATP, mainly 1:1 complexes were formed (Fig. 1A, closed circles). Analysis of these data in terms of 1:1 and 1:2 binding isomers, as above, led to the conclusion that approximately 18% of the complexes were 1:2 in the presence of AMP-PNP. These conclusions are consistent with the previously reported electron microscopic studies showing that although 1:1 complexes were favored under these conditions, 1:2 complexes could be observed (31).

Similar data obtained in the presence of ADP did not give a clear plateau at high GroES concentrations, and they were analyzed by the Scatchard equation (Equation 3; see “Materials and Methods”). The resulting plot is presented in Fig. 1B. Here, the solid line represents the theoretical fit of Equation 3 to the experimental data using a nonlinear least squares procedure (“Materials and Methods”). By utilizing the Scatchard equation one can determine the total concentration of the ligand binding sites and apparent binding constant. Here the equation describing a protein with two binding sites was used. The two component fit gave, for the tighter binding site, an apparent
The corresponding apparent stoichiometry of the GroEL-GroES complex ([bound GroES]/[total GroEL]) of 0.61, because 122 nM GroES$_7$ were found to be bound to 200 nM GroEL$_{14}$. The corresponding apparent binding constant of the GroEL-GroES interaction was calculated as 20 nM. This is consistent with the previously reported ability of GroEL to form 1:1 GroEL$_{14}$/GroES$_7$ complexes in the presence of ADP (11). The fitting procedure yielded parameters for the weaker binding site. Thus, for the the 200 nM solution of GroEL$_{14}$, the oligomer is able to bind, additionally, 231 nM GroES$_7$ ([bound GroES$_7$ to the second site]/[total GroEL$_{14}$] = 1.155) with corresponding binding constant of 0.555 μM.

Therefore, the total number of binding sites is 353 nM, which is approximately 1.7 times larger than initial [GroEL$_{14}$], demonstrating the ability of the system to form 1:2 GroEL$_{14}$/GroES$_{7}$ complexes even in the presence of ADP. The curvature in the Scatchard plot also supports the idea of the existence of two different binding sites on GroEL for GroES, because the system starts with equivalent sites as demonstrated in the x-ray structure (6).

The low value of the second binding constant is a result of a strong negative cooperativity between two binding sites of GroEL, which was demonstrated by fitting the same data to the Hill equation (Equation 4; see “Materials and Methods”). The Hill coefficient was found to be 0.58. For this system that starts with two equivalent binding sites at the ends of the cylindrical oligomer of GroEL (27), the fact that the value of the Hill coefficient is <1 is consistent with strong negative cooperativity.

**GroES Does Not Completely Inhibit the ATPase Activity of GroEL**—Fig. 2 shows the inhibition of the GroEL ATPase by GroES. As has been previously shown, the hydrolysis of ATP by GroEL is K$^+$-dependent (10, 28). In the presence of 10 mM KCl, the maximum inhibition by GroES is approximately 50% (Fig. 2, closed squares). In the presence of low K$^+$, the ATPase activity is inhibited approximately 90% (Fig. 2, closed circles). These values are consistent with those previously reported (9, 10).

Under conditions of high [GroEL] and low [KCl], the ATPase activity of GroEL is sharply decreased as a function of [GroES] (Fig. 2, closed circles), reaching a minimum close to [GroES]/[GroEL] = 1. At this point, the GroEL activity is inhibited by approximately 80%. It is interesting that the GroEL ATPase activity could not be inhibited 100% at the low [KCl] and [GroES]/[GroEL] = 1, demonstrating a low intrinsic ability to hydrolyze ATP by the trans GroEL toroid. The ATPase activity of GroEL does not change significantly at higher GroES/GroEL ratios. This is consistent with the previous suggestion that there is an increased relative affinity of GroEL for ADP compared with ATP (10). Therefore, the rate of ADP exchange for ATP is the rate-limiting step for the formation of the 1:2 GroEL$_{14}$/GroES$_{7}$ complex and for ATP hydrolysis. Under these conditions, the exchange does not depend on the initial concentration of GroES. As shown above, under initial rate conditions for the hydrolysis of ATP, where [ATP] is greater than [ADP] and at low [KCl], GroEL is able to form a 1:2 complex with GroES (Fig. 1A). The ability of the chaperonin to slowly hydrolyze ATP at low [KCl] and [GroES]/[GroEL] > 1 would facilitate GroES exchange under such conditions.
GroES added shows that after AMP-PNP addition, two mixture containing 85 nM GroEL and 500 nM GroES. To the formed 1:1 complex AMP-PNP was titrated up to 4.7 mM. The anisotropy of pyrene-GroES was measured as described under “Material and Methods.” Concentration of the bound GroES was calculated using Equations 1 and 2 (see “Materials and Methods”). Measurements were made in buffer containing 10 mM MgCl₂ and 0.5 mM KCl. GroEL₁₄(GroES₇)₂ Complex Can Be Formed in the Presence of ADP and AMP-PNP Together—Because low [AMP-PNP] alone cannot facilitate formation of the GroEL₁₄(GroES₇)₂ complex (31), the hypothesis that the 1:2 GroEL-GroES complex contains an asymmetric distribution of nucleotides was tested. In this experiment, AMP-PNP was titrated into a solution containing 1:1 GroEL₁₄(GroES₇) complex preformed in the presence of ADP and 5-fold excess of GroES. In the presence of GroES, ADP was shown to have high affinity for GroEL (Kᵣ < 70 nM (30)). Therefore, in the 160 µM solution of the nucleotide all ADP binding sites are saturated. The KCl concentration was 0.5 mM to maintain ADP bound to the GroEL-GroES complex, because it was shown that under such conditions ADP remains bound to GroEL even in the presence of 2 mM of AMP-PNP (11). As a result, there was an additional increase of the r_obs indicating an increased concentration of the bound GroES (Fig. 3). A similar result was obtained when pyrene-labeled GroES was titrated into a fixed concentration of GroEL in the presence of ADP (0.05 mM), after which each sample was supplied with 1 mM AMP-PNP (Fig. 4). The resulting anisotropy value increased, indicating the appearance of the additional binding sites for the GroEL-GroES interaction. Data replotted as a function of the concentration of bound GroES versus total concentration of GroES added shows that after AMP-PNP addition, two GroES₂ molecules were able to interact with one oligomer of GroEL (Fig. 4). A similar conclusion follows from studies in which ADP was added to an 1:1 GroEL₁₄(GroES₇) complex in the presence of AMP-PNP (data not shown).

DISCUSSION

It is tempting to suggest, based on the highly symmetrical structure of GroEL, that the oligomer can form a GroEL₁₄(GroES₇)₂ complex where the cochaperonin GroES is bound symmetrically on the opposite rings of the GroEL molecule. Indeed, using electron microscopy, cross-linking, and other methods it has been shown that under certain conditions GroEL is able to bind two GroES oligomers forming the 1:2 GroEL₁₄(GroES₇)₂ complex (17, 32, 33). To clarify the mechanism of the chaperone activity, it is important to understand the nature of nucleotides bound on the GroEL₁₄(GroES₇)₂ complex.

FIG. 3. Titration of AMP-PNP in the presence of fixed concentration of GroES and GroEL. ADP (0–161 mM) was titrated to the mixture containing 85 nM GroEL and 500 nM GroES. To the formed 1:1 GroEL-GroES complex AMP-PNP was titrated up to 4.7 mM. The anisotropy of pyrene-GroES was measured as described under “Material and Methods.” Concentration of the bound GroES was calculated using Equations 1 and 2 (see “Materials and Methods”). Measurements were made in buffer containing 10 mM MgCl₂ and 0.5 mM KCl.

FIG. 4. Addition of AMP-PNP to the solution of 1:1 GroEL₁₄(GroES₇) complex results in the increase of the concentration of bound GroES. GroES was titrated up to 1600 mM (total final concentration) in the samples containing 160 nM GroEL₁₄ and 0.1 mM ADP. The anisotropy of pyrene-GroES was measured as described under “Material and Methods” (shown as closed squares). AMP-PNP was added to maintain a final concentration of 1 mM. After careful mixing anisotropy was detected again (shown as open squares). Concentration of the bound GroES was calculated using Equations 1 and 2 (see “Materials and Methods”). Measurements were made in buffer containing 10 mM MgCl₂ and 0.5 mM KCl. The lines are drawn to guide the eye.

In this report, we have shown by directly measuring the binding of GroES to GroEL that GroEL₁₄(GroES₇)₂ complexes are formed under conditions of productive chaperonin-assisted protein folding, i.e., in the presence of ATP and high [KCl], supporting the conclusions of Azem et al. (32), and Torok et al. (33). The present method does not require cross-linking or fixation, and the state of GroES binding can be assessed in real time. GroEL₁₄(GroES₇)₂ complexes can be formed also in the presence of ATP and low [KCl] (Fig. 1A). But because the affinity of GroEL for GroES relative to the affinity for ATP under such conditions is increased, concentrations of the 1:2 GroEL₁₄(GroES₇)₂ complex are reduced (Fig. 1A).

Our results suggest that the 1:2 complex is not favored when only one type of nucleotide is bound to the GroEL double toroid. In fact, they suggest that only when ADP and ATP (or the ATP analog, AMP-PNP) are bound on the opposite rings of GroEL can significant amounts of 1:2 GroEL₁₄(GroES₇)₂ complex be formed under the physiological ratio of GroEL and GroES (1:2) (Figs. 3 and 4). These considerations only refer to stable complexes, and they do not preclude the possibility of transient 1:2 complexes forming in the presence of a single type of nucleotide. For example, binding affinities of the two binding sites of GroEL in the presence of ADP are different by approximately 27-fold (Fig. 1B). Because the affinity of the 1:1 GroEL-GroES complex for the second GroES oligomer under such conditions is highly reduced due to the strong negative cooperativity between two binding sites, the 1:2 GroEL₁₄(GroES₇)₂ complex, containing the same type of nucleotide bound on the opposite rings of GroEL, is highly unstable. Therefore, one of the bound GroES molecules can be exchanged with GroES in bulk solution. This exchange is slow, and it can be facilitated in the presence of the excess of [GroES] over [GroEL] (34).

Many models feature the folding of proteins inside the GroEL cavity, underneath a GroES “cap,” the so-called “Anfinsen cage” (35–37). One of the questions that arises from such models concerns the fate of complexes where unfolded polypeptide is bound trans from the GroES. There have been models...
proposed that feature multiple cycles of binding and release of substrate protein, such as the iterative annealing models (38, 39). It is conceivable that conformational changes involved in the GroEL ring containing bound GroES could cause dissociation of the polypeptide on the trans ring. However, if the 1:2 GroEL_{14}(GroES)_{7} complex is the functional chaperonin machine as suggested by these results along with others (32, 33, 40), then productive folding would naturally proceed from a cis complex, i.e. substrate protein and GroES bound to the same GroEL ring (41). This hypothesis does not imply that 1:1 GroEL-GroES complexes are insignificant in the chaperonin cycle. In fact, it can be easily imagined that the 1:1 complex is the acceptor state for unfolded polypeptide. Some productive folding could then be accomplished by competition between binding of the polypeptide and binding of the second GroES. This latter possibility would be appropriate, for example, with large proteins that could not be accommodated under the GroES cap (see below).

The following general mechanism for chaperonin-assisted folding can be suggested (Scheme 1). For simplicity, the scheme does not depict conformational changes in GroEL that occur in response to ligand binding and ATP hydrolysis. The acceptor state (1), the 1:1 GroEL-GroES complex, can interact with unfolded polypeptide. As a result, protein-substrate is bound on GroEL trans to the GroES oligomer ring (2). The succeeding binding of a second GroES molecule results in formation of 1:2 GroEL_{14}(GroES)_{7} complex (3) that contains bound unfolded polypeptide under a cap formed by the GroES. The following step of ATP hydrolysis results in liberation of the unfolded polypeptide, allowing it to fold inside of the cavity of GroEL. As a result of hydrolysis, a metastable, transient 1:2 GroEL_{14}(GroES)_{7} complex containing ADP bound on both rings of GroEL is formed (shown in brackets) (4). The GroES that is bound on the ring opposite to the side of ATP hydrolysis can dissociate under these conditions (10). Dissociation of the GroES oligomer produces a complex with substrate protein under a GroES cap (5). The opposite side is in an acceptor state that, under the appropriate conditions (e.g. high concentrations of a slow folding protein), might bind a second molecule of substrate protein. After the opposite side rebinds ATP, it can also interact with a second GroES oligomer to form a state that is isomorphic with respect to the stoichiometry of bound proteins but that is asymmetric with respect to bound nucleotides and detailed protein conformation (6). Repetition of the steps described above would then result in formation of a similar unstable complex (7). Because at this time ATP is hydrolyzed on the GroEL ring opposite to the position of the originally unfolded polypeptide, dissociation of the GroES molecule from the GroEL oligomer can result in complete liberation of the protein-substrate, if it was able to successfully fold during previous steps of the chaperonin action (8). Protein substrate will rebind to the GroEL oligomer, and it will be able to enter the next cycle if its folding was not completed.

This proposed mechanism can explain why some proteins can be refolded in the presence of ADP and GroES only, because the unstable 1:2 GroEL_{14}(GroES)_{7} complex (4 or 7) can be formed in the presence of this nucleotide. In the presence of ATP, cycling through the chaperonin cycle would be greatly facilitated (34). In this general scheme all interactions with ligands and proteins are actually equilibria, and this full scheme is most appropriate for small proteins that can fit under the GroES cap and that are folded under nonpermissive conditions. When these conditions are not met, the scheme can be appropriately modified. Thus, for example, a large, fast folding protein may be assisted by a process involving the equilibrium between species 1 and 2. The nucleotide hydrolysis provides the directionality and sequencing of interactions (12). The conclusions presented here in terms of nucleotide-dependent binding of GroES are compatible with studies of nucleotide binding to GroEL, in which it was suggested that negative cooperativity with respect to nucleotide binding favored asymmetric GroEL nucleotide complexes in which different nucleotides were bound on the two rings (42).

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