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GroES in the asymmetric $GroEL_{14}$–$GroES_7$ complex exchanges via an associative mechanism

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ABSTRACT **The interaction of the chaperonin $GroEL_{14}$ with its cochaperonin $GroES_7$ is dynamic, involving stable, asymmetric 1:1 complexes ($GroES_7$·$GroEL_7$–$GroEL_7$) and transient, metastable symmetric 2:1 complexes [$GroES_7$·$GroEL_7$–$GroEL_7$·$GroES_7$]. The transient formation of a 2:1 complex permits exchange of free $GroES_7$ for $GroES_7$ bound in the stable 1:1 complex. Electrophoresis in the presence of ADP was used to resolve free $GroEL_{14}$ from the $GroES_7$–$GroEL_{14}$ complex. Titration of $GroEL_{14}$ with radiolabeled $GroES_7$ to molar ratios of 32:1 demonstrated a 1:1 limiting stoichiometry in a stable complex. No stable 2:1 complex was detected. Preincubation of the asymmetric $GroES_7$·$GroEL_7$–$GroEL_7$ complex with excess unlabeled $GroES_7$ in the presence of ADP demonstrated $GroES_7$ exchange. The rates of $GroES_7$ exchange were proportional to the concentration of unlabeled free $GroES_7$. This concentration dependence points to an associative mechanism in which exchange of $GroES_7$ occurs by way of a transient 2:1 complex and excludes a dissociative mechanism in which exchange occurs by way of free $GroEL_{14}$. Exchange of radiolabeled ADP from 1:1 complexes was much slower than the exchange of $GroES_7$. In agreement with recent structural studies, this indicates that conformational changes in $GroEL_{14}$ following the dissociation of $GroES_7$ must precede ADP release. These results explain how the $GroEL_{14}$ cavity can become reversibly accessible to proteins under *in vivo* conditions that favor 2:1 complexes.**

Molecular chaperones are proteins that have evolved to assist efficient protein folding, trafficking, and assembly of proteins *in vivo*. The most widely studied molecular chaperones are the so-called chaperonin proteins from *Escherichia coli*, $GroEL_{14}$ and $GroES_7$. $GroEL_{14}$ is a tetradecamer of 14 identical 57-kDa subunits arranged in two seven-member, doughnut-shaped rings that are stacked back-to-back to yield a cylindrical structure with identical ends (1). $GroES_7$ is a single ring of seven identical 10-kDa subunits (2). $GroES_7$ uses a large mobile loop from each of its subunits to bind to and regulate the activity of $GroEL_{14}$ (3, 4). $GroES_7$ is required for the successful refolding of polypeptides by $GroEL_{14}$ under conditions where spontaneous folding does not occur (5). During this stringent folding, the partially folded target protein binds to one end of the $GroEL_{14}$ cylinder. $GroES_7$ can then bind to the same end and displace the target protein into the protected space that is formed by the $GroEL_{14}$ cylindrical chamber and the overlying $GroES_7$ dome. The release of the target protein from this complex requires the dissociation of the $GroES_7$. The interactions with $GroES_7$, the release of target proteins, and the required conformational changes in $GroEL_{14}$ depend on ATP binding and hydrolysis and the release of the ADP and inorganic phosphate that are formed.

One difference among mechanisms proposed to explain the details of chaperonin-assisted folding relates to the stoichiometry of the $GroES_7$–$GroEL_{14}$ complexes formed during the cycle of chaperonin-assisted refolding. Electron microscopy and chemical crosslinking have demonstrated the existence of both 1:1 $GroES_7$–$GroEL_{14}$, and 2:1 $(GroES_7)_2$–$GroEL_{14}$ complexes under various conditions (6–8). The 2:1 complexes were further shown to be active in the refolding of RuBisCO (9). Ultracentrifugation studies have also demonstrated that 1:1 and 2:1 complexes could be quantified under various equilibrium conditions in the presence of ADP or nonhydrolyzable ATP analogs such as adenosine 5′-[γ-[S]thio]triphosphate (ATPγS) or adenosine 5′-imido triphosphate (10). This conclusion is supported by direct binding studies using fluorescently labeled $GroES_7$, and additional conditions were determined for the formation of 2:1 complexes (11). The results indicated that at the concentrations in the *E. coli* cell (12) (2.6 μM $GroEL_{14}$, 8 mM ATP, and $GroES_7/GroEL_{14} = 2$), 2:1 $GroES_7$–$GroEL_{14}$ complexes likely predominate. However, others have questioned the significance of the symmetric 2:1 complexes (13, 14).

Recent structural (4) and functional (15) studies have visualized the 1:1 $GroES_7$–$GroEL_{14}$ complex and demonstrated a mechanism requiring only 1:1 complexes in the functional cycle, and it was shown that ATP binding to the ring trans to the $GroES_7$ dome was sufficient to discharge the $GroES_7$. Although this particular model is depicted as involving an asymmetric 1:1 complex, considerations of symmetry have led to the suggestion that it would be possible to envision this mechanism as a special case of the more general situation involving a 2:1 complex (16, 17).

These studies have led to the view that the chaperonin cycle only involves the asymmetric complex $^{A}GroEL_7$–$^{B}GroEL_7$·ADP_7·$GroES_7$. Single-turnover experiments (18) showed that the bound $GroES_7$ underwent complete exchange with free $GroES_7$ on the time scale of ATP hydrolysis. Two exchange mechanisms, associative and dissociative, can be envisaged. In the associative mechanism, binding of ATP and $GroES_7$ to the trans ring ($^{A}GroEL_7$) of the asymmetric complex leads to the transient formation of an unstable, pseudosymmetrical complex $GroES_7$·ATP_7·$^{A}GroEL_7$–$^{B}GroEL_7$·ADP_7·$GroES_7$. Dissociation of the ligands from the $^{B}GroEL_7$ and ATP hydrolysis on the $^{A}GroEL_7$ ring leads to the regeneration of the asymmetric complex. In the dissociative mechanism, the departing $GroES_7$ is thought to dissociate before the association of the incoming $GroES_7$. The dissociative mechanism thus involves the transient formation of an unadorned $GroEL_{14}$ complex. Electron microscopy and cross-linking studies of mixtures of $GroEL_{14}$ and $GroEL_{14}$–$GroES_7$ in the presence of MgATP consistently show a high population of pseudosymmetrical, football-shaped particles, and the almost complete absence of unadorned $GroEL_7$–$GroEL_7$ particles, observations that are consistent with an associative mechanism (6–8).

These considerations make it interesting to consider the disparate views of the importance of complexes with different stoichiometries and to understand how the accessibility of the

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$GroEL_{14}$ cavity can be modulated under conditions that apparently favor the 2:1 complexes. It has recently been demonstrated by using direct binding measurements with fluorescently labeled $GroES_7$ that both 1:1 and 2:1 complexes could be formed with appropriate combinations of nucleotides (11). Thus, 2:1 complexes could be formed in the presence of ATP and high KCl, although only 1:1 complexes could be formed in the presence of ADP or adenosine 5′-imido triphosphate individually. Subsequent addition of the other nucleotide to preformed 1:1 complexes to give solutions with mixed nucleotides resulted in formation of 2:1 complexes, suggesting that an asymmetric distribution of nucleotides on the two rings favored a 2:1 complex. In the present study we have explored the stability of the asymmetric $^{A}GroEL_7$–$^{B}GroEL_7$·ADP_7·$GroES_7$ complex, imposing a symmetrical distribution of the nucleotide using only ADP. As before, no stable symmetric 2:1 $GroES_7$·ADP_7·$^{A}GroEL_7$–$^{B}GroEL_7$·ADP_7·$GroES_7$ could be detected. However, the existence of such a species can be deduced from the dependence of the rate of exchange of bound, isotopically labeled $GroES_7$ on the concentration of free $GroES_7$, a result which points to an associative exchange mechanism.

MATERIALS AND METHODS

Protein Purification. $GroES_7$ and $GroEL_{14}$ were purified as described previously (19, 20). Protein concentrations were determined by the method of Bradford (21).

Polyacrylamide Gel Electrophoresis. Some nondenaturing gel electrophoresis was performed by the method of Neuhoff *et al.* (22) by using 6% polyacrylamide gels at a constant 200 V for a 1-mm-thick gel. Where appropriate, gels were stained for protein with 0.05% Coomassie brilliant blue R250, 25% isopropyl alcohol, and 10% acetic acid.

Resolution of $GroEL_{14}$ from $GroES_7$–$GroEL_{14}$ complexes depended on the electrophoresis conditions, and the following conditions were found to give satisfactory resolution for our experiments. Electrophoresis was done under nondenaturing conditions with 4.5% polyacrylamide gels prepared in 0.5 M Tris borate (pH 8.5) supplemented with 5 mM magnesium acetate and 2 mM ADP. The running buffer contained 90 mM Tris borate, 1 mM magnesium acetate, and 0.2 mM ADP at pH 8.5. Before electrophoresis, samples were dissolved in a buffer consisting of 25 mM Tris borate (pH 8.5), 2% 2-mercaptoethanol, 10% glycerol, 2 mM ADP, 5 mM magnesium acetate, and 0.01% bromphenol blue as a tracking guide.

Preparation of ^{14}C-Labeled $GroEL_{14}$ and $GroES_7$. ^{14}C-labeled $GroEL_{14}$ and $GroES_7$ were prepared by reductive methylation using sodium cyanoborohydride essentially by the method of Jentoft and Dearborn (23, 24). For a typical labeling of $GroES_7$, 20 mM oligomer was treated with ^{14}C-labeled formaldehyde at a ratio of formaldehyde/lysine of 1.8 using a formaldehyde solution that was 59.9 μCi/ml (3 μCi/mM; 1 Ci = 37 GBq). The solution was 20 mM in cyanoborohydride and 0.1 M sodium phosphate, pH 7.6. The sample was incubated at room temperature for 2 hours. The 150-μl sample was freed of excess label and small molecules by two successive treatments with Sephadex G50 gel filtration spin columns of 1 ml each, equilibrated with 50 mM Tris·HCl (pH 7.8) containing 0.5 mM DTT. $GroEL_{14}$ was similarly labeled. The protein concentrations of $GroES_7$ and $GroEL_{14}$ were determined as indicated above. Each radiolabeled sample was tested and found to be active in refolding of denatured rhodanese by using the assay noted above.

Preparation of [α-^{32}P]ADP and Measurement of ADP Exchange. [α-^{32}P]ADP was prepared by treating 60 μl of 10.5 mM [α-^{32}P]ATP (48 μCi/μM) in a total volume of 160 μl of a solution containing hexokinase (375 units/ml), glucose (0.2 M), $MgCl_2$ (10 mM), and Tris·HCl (50 mM, pH 7.8). TLC on polyethyleneimine-cellulose using 1 M LiCl confirmed that the conversion to ADP was complete at 30 min. The sample was diluted 1:5 with 10 mM triethylammonium bicarbonate, pH 7.5, and loaded onto a DEAE-cellulose column (0.8 × 4.5 cm) previously equilibrated with the same buffer. ADP was eluted by using a gradient of 10–450 mM triethylammonium bicarbonate, pH 7.5. ADP-containing fractions were collected, their identity confirmed by chromatography, pooled, and vacuum dried.

$GroEL_{14}$·[α-^{32}P]ADP·$GroES_7$ complexes were formed by coincubating $GroEL_{14}$ (1 μM oligomer), 0.9 μM $GroES_7$, 50 μM [α-^{32}P]ADP (20 μCi/μM), 50 mM KCl, 5 mM Mg acetate, and 10 mM Mops·KOH, pH 7.2 in a final volume of 120 μl. The sample was incubated for 15 min at room temperature. The following additions were made to separate aliquots of the complex: (*i*) No addition; (*ii*) unlabeled ADP (2 mM final); (*iii*) $GroES_7$ (5-fold excess over $GroEL_{14}$); and (*iv*) unlabeled ADP (2 mM) + $GroES_7$ (5-fold excess over $GroEL_{14}$). Samples were incubated at room temperature for 2 hr, and aliquots were subjected to electrophoresis as described below. For the zero-time sample, electrophoresis was started within 5 min of mixing the samples.

Detection and Quantitation of Radiolabeled $GroEL_{14}$, $GroES_7$, and ADP. Gel electrophoresis was performed as indicated in the individual experiments in *Results and Discussion*. Resulting gels were dried under vacuum onto Whatman 3MM paper. Radiolabel was detected and quantified by using a storage phosphor screen and a PhosphorImager from Molecular Dynamics. Linearity of the response of the system was evaluated for each protein by determining standard curves using increasing quantities of radiolabeled samples containing known protein concentrations. These standard curves were used to relate the PhosphorImager responses to protein concentrations. Analogous procedures were applied to quantitation of [α-^{32}P]ADP.

Quantitation of $GroES_7$ Exchange Using Gel Permeation Chromatography. Labeled $GroEL_{14}$–$GroES_7$ complexes were formed by incubating 5 μM $GroEL_{14}$ with 5 μM ^{14}C[$GroES_7$] in 50 mM Tris·HCl (pH 7.8) containing 10 mM $MgCl_2$, 10 mM KCl, and 1 mM ADP. After 1 hour, unlabeled $GroES_7$ was added to the solutions to give the desired molar ratios of $GroES_7/GroEL_{14}$, which diluted the complexes to 3.2 μM. Labeled $GroES_7$ remaining in the complexes at a particular time was quantified by diluting an aliquot of the incubation mixture to 0.256 μM and injecting 100 μl onto a 7.8 × 300 mm Bio-Sep SEC 4000 HPLC gel permeation column (Phenomenex) that was developed at 0.5 ml/min with 50 mM Tris·HCl (pH 7.8) containing 10 mM $MgCl_2$, 0.5 mM KCl, 0.5 mM DTT, and 50 μM EDTA. Fractions of 500 μl were subjected to scintillation counting. This procedure resulted in baseline separation between the peaks containing $GroEL_{14}$ and free $GroES_7$. The initial incubation typically led to incorporation of 0.8–0.85 $GroES_7/GroEL_{14}$.

RESULTS AND DISCUSSION

The binding of $GroES_7$ to $GroEL_{14}$ produces a complex that has altered electrophoretic properties (25). The resolution of $GroEL_{14}$–$GroES_7$ complexes from $GroEL_{14}$ itself depends on the conditions used, and the method described in *Materials and Methods* using a Tris borate buffer was found to give clearer and more reproducible separations than earlier procedures. Fig. 1*A* shows the results of electrophoresis of $GroES_7$–$GroEL_{14}$ complexes formed and run in the presence of ADP. Lanes 2–8 show radiolabeled bands from $GroES_7$ as increasing amounts of [^{14}C]$GroES_7$ are added to a fixed concentration of $GroEL_{14}$. Lane 1 is a control that shows the position of [^{14}C]$GroEL_{14}$ (labeled L); all other lanes contain radioactivity only in $GroES_7$. The $GroES_7$, when added to $GroEL_{14}$, only migrates in positions corresponding to free $GroES_7$ (labeled S) and the complex marked C. No $GroES_7$ comigrates with uncomplexed $GroEL_{14}$. Lanes 2–8 represent increasing ratios of radiolabeled $GroES_7/GroEL_{14}$ from 0.5:1 to 32:1. Although

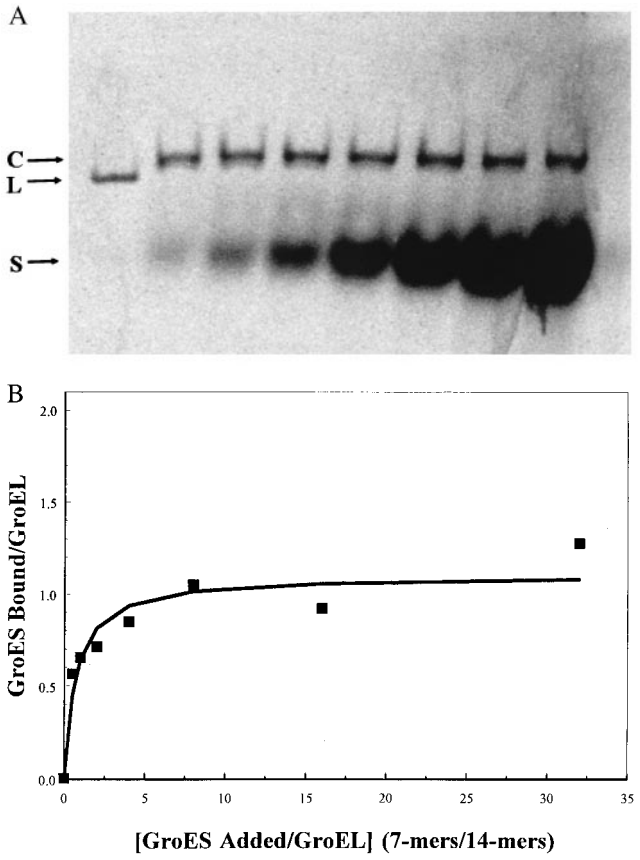

FIG. 1. Titration of GroEL with increasing concentrations of GroES. (*A*) Nondenaturing electrophoresis of GroEL–GroES complexes containing radiolabeled GroES. All bands in lanes 2–8 contain 2.88 pg of unlabeled GroEL and radiolabeled GroES at increasing molar ratios of GroES/GroEL (0.5, 1, 2, 4, 8, 16, and 32, respectively). Lane 1 shows 2.88 pg of radiolabeled GroEL and is included to mark the position of uncomplexed GroEL. L, position of GroEL; C, position of GroES/GroEL complex; S, position of uncomplexed GroES. Each incubation sample contained the following concentrations: 0.24uM $GroEL_{14}$, 2 mM ADP, 5 mM magnesium acetate, 50 mM KCl, and 10 mM Mops·KOH (pH 7.2). (*B*) The ratio of bound GroES/complex as a function of added $GroES_7/GroEL_{14}$. Line is a least-squares fit to a binding isotherm. The maximum binding corresponds to 1.1 ± 0.08 GroES/GroEL.

the Coomassie stain of this gel shows some protein at the position of $GroEL_{14}$ at the lower concentrations of $GroES_7$ (data not shown), no radiolabel is detected in a position corresponding to uncomplexed $GroEL_{14}$. Thus, any binding of radiolabled $GroES_7$ in the presence of ADP leads to a shift in $GroEL_{14}$ to the position designated C. Fig. 1*B* shows the results of quantitation of the radiolabel in the complex, which demonstrate that even at very high levels of $GroES_7$, only 1:1 complexes are stable during electrophoresis in the presence of ADP. This conclusion is supported by the observation of insignificant amounts of radiolabel between the complex and free $GroES_7$ in each lane of Fig. 1, suggesting that no radiolabel was dissociating from the complexes during electrophoresis. Furthermore, although a comparable decrease in electrophoretic mobility was to be expected on formation of a 2:1 complex, no stable 2:1 complexes were detected after electrophoresis. Previous fluorescence anisotropy studies indicated that there was some amount of 2:1 complex in solution at steady state under comparable conditions. Together, these results indicate that the 2:1 complexes are present, but they are in dynamic equilibrium with 1:1 complexes, and under these dilute conditions the 1:1 complexes are favored. For example, at [ADP] = 2 mM used here, and at a ratio of 5:1 $GroES_7/GroEL_{14}$, the previous anisotropy results suggested that there were 1.3 $GroES_7$ per $GroEL_{14}$ (11). At a ratio of 32:1 $GroES_7/GroEL_{14}$, 2:1 complexes are expected to predominate. However, least squares fitting of the electrophoretic binding data shown here gives a maximum binding of 1.1 ± 0.08 $GroES_7/GroEL_{14}$. Therefore, the results in Fig. 1 *A* and *B* demonstrate that the only stable complexes observed in electrophoresis are 1:1. These complexes do not significantly dissociate during electrophoresis. This latter conclusion was confirmed by noting that increasing the duration of electrophoresis did not change the results described here, i.e., not much dissociation occurs, even if more time is allowed (the electrophoresis in Fig. 1*A* was for 5 hours). Thus, it is clear that in the presence of the levels of ADP used here, 2:1 complexes are not stable.

Fig. 2 shows the results of preincubation of radiolabeled 1:1 complexes with increasing ratios of unlabeled $GroES_7$. The samples for this experiment were incubated at room temperature for 60 minutes before electrophoresis. The bands corresponding to 'C' in Fig. 1*A* were quantified, and the results clearly indicate that the 1:1 complexes can exchange their radiolabeled $GroES_7$ when incubated in this fashion. It should be noted that $GroES_7$ migrates considerably faster than either the complex or the free $GroEL_{14}$. Therefore, the unlabeled, unbound $GroES_7$ rapidly separates from the $GroEL_{14}$ species so that the complex largely electrophoreses in the absence of unlabeled $GroES_7$. These results show that ≈50% of the radiolabeled $GroES_7$ can be displaced by this procedure at a molar ratio of 1:1 unlabeled to radiolabeled $GroES_7$, with the original $GroES_7$ being present at a molar ratio of 5:1 over $GroEL_{14}$. Therefore, based on Fig. 1, the stable complexes that were initially present contained approximately 0.8:1 $GroES_7/GroEL_{14}$. The counts remaining in the complex after incubation with increasing amounts of added unlabeled $GroES_7$ (Fig. 2) closely correspond to that expected for isotope dilution, indicating that equilibriation is complete after 60 min. Overall, the results confirm that the 1:1 complexes are stable and demonstrate that exchange occurs with excess $GroES_7$.

Fig. 3 demonstrates the exchange of $GroES_7$ from stable 1:1 complexes in a different way. These results demonstrate what

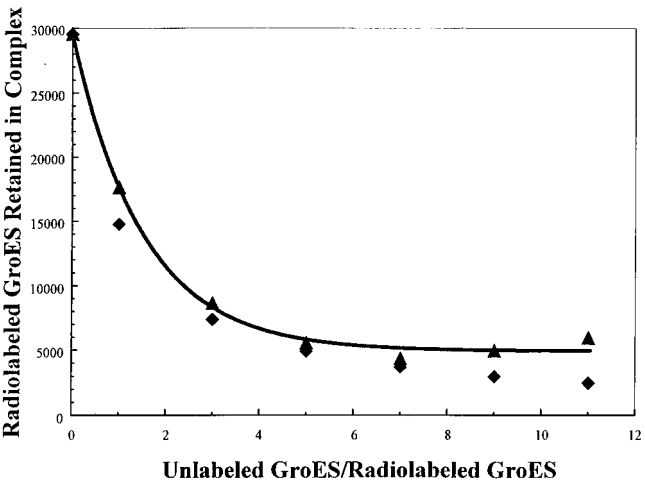

FIG. 2. Exchange of bound radiolabeled $GroES_7$ as a function of added unlabeled GroES. Plot shows radiolabeled $GroES_7$ retained in $GroES_7$–$GroEL_{14}$ complex vs. the ratio of added unlabeled GroES to radiolabeled $GroES_7$. The initial sample contained 0.34 μM $GroEL_{14}$ and 1.7 μM radiolabeled $GroES_7$ in the same buffer as in Fig. 1. The abscissa gives the ratio of added unlabeled $GroES_7$ over the initial radiolabeled $GroES_7$. Units of the ordinate are counts from the PhosphorImager output. Electrophoresis was performed as in Fig. 1, and the radiolabel was quantified as described in *Materials and Methods*. Actual data are represented by ▲; theoretical expectations for isotopic equilibration are represented by ◆. The line is an exponential fit to the data.

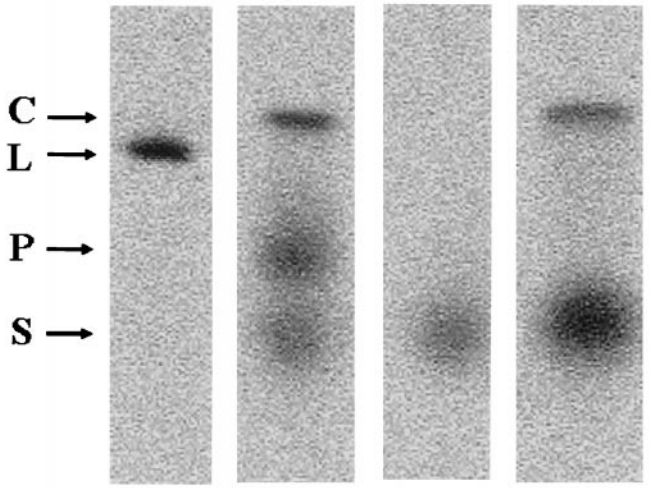

FIG. 3. Displacement *en passant* of labeled $GroES_7$ by unlabeled $GroES_7$ added after initiation of electrophoresis. Lane 1, labeled $GroEL_{14}$ (L) to mark the position of uncomplexed $GroEL_{14}$. Lanes 2–4 contain radiolabel only in $GroES_7$: lane2, $GroEL_{14}$–labeled $GroES_7$ complex prepared as in Fig. 1 by using equimolar labeled $GroES_7$ and incubated for 15 min at room temperature. Complex (3.6 pM) was electrophoresed for 60 min. Electrophoresis was briefly interrupted, and 100× unlabeled $GroES_7$ in 2 mM ADP was added and the run was continued for 4 more hours. Lane 3, radiolabeled $GroES_7$ alone to mark position of $GroES_7$. Lane 4, $GroEL_{14}$–$GroES_7$ prepared as for lane 2 but preincubated with 100× unlabeled $GroES_7$ before electrophoresis.

might be termed "exchange in passing," because the experiment takes advantage of the fact that $GroES_7$ migrates more rapidly on native gel electrophoresis than either the complex or $GroEL_{14}$, thus allowing $GroES_7$ that is added after initiating electrophoresis to pass the complex. While passing, the unlabeled $GroES_7$ has an opportunity to exchange with the radiolabeled $GroES_7$ in complex. Lanes 2, 3, and 4 contain only radiolabeled $GroES_7$, whereas lane 1 shows the position of radiolabeled $GroEL_{14}$ for comparison. Lane 3 shows the position of labeled $GroES_7$ electrophoresed in the absence of $GroEL_{14}$. Lane 2 shows the behavior of the $GroEL_{14}$–$GroES_7$ complex prepared with radiolabeled $GroES_7$ in which electrophoresis was stopped after 1 hour, at which time the tracking dye was approximately 1.7 cm into the gel. At that time, unlabeled $GroES_7$ was added to the sample well and electrophoresis was continued for an additional 4 hours. The added $GroES_7$ migrates rapidly, and it overtakes and passes the stable complex. These results demonstrate that there is displacement of radiolabeled $GroES_7$ as the unlabeled $GroES_7$ passes the complex. The displaced $GroEL_{14}$ migrates at the position labeled P, whereas the excess $GroES_7$ that was present with the original complex is at the position labeled S. Lane 4 shows that if the complex between $GroEL_{14}$ and radiolabeled $GroES_7$ is preincubated with excess unlabeled $GroES_7$ before electrophoresis, the only uncomplexed $GroES_7$ migrates at the position S, demonstrating that the exchange was complete before electrophoresis.

To support the electrophoretic results, the kinetics of $GroES_7$ exchange were investigated by using gel permeation chromatography. An associative mechanism involving a 2:1 complex would be expected to show increasing rates of exchange with increasing concentrations of unlabeled $GroES_7$, whereas the normalized exchange rates for a mechanism that proceeds by dissociation of the 1:1 complex would be independent of the concentration of added $GroES_7$. The results shown in Table 1 are clearly in accord with the associative process. The rates strongly depend on the added $GroES_7$ concentration, and the exchange rates increase by a factor of

Table 1. Quantitation of $GroES_7$ exchange by gel permeation chromatography

Ratio [$GroES_7$/([^{14}C]$GroES_7$/$GroEL_{14}$)]	Apparent normalized rate constants for $GroES_7$ exchange $\times 10^4$ min^{-1}
Complex alone (no additions)	1.6
10	25
20	40
30	60

$GroEL_{14}$–$GroES_7$ complexes were prepared using [^{14}C]$GroES_7$ as described in *Materials and Methods*. After 1 hour at 25°C, the samples were brought to the indicated ratio of $GroES_7/GroEL_{14}$ by the addition of unlabeled $GroES_7$. The $GroES_7$ remaining with the complexes was quantified using gel permeation chromatography as described in *Materials and Methods.* The percent remaining labeled $GroES_7$ in complexes was quantified using gel permeation chromatography as a function of time between 0 and 235 min. The data were fit to first-order kinetics, and the derived pseudo-first-order rate constants are presented.

40 at a 30-fold excess of unlabeled $GroES_7$. The half-time for exchange with no added $GroES_7$ is almost 75 hr, whereas the half-time falls to 1.9 hr in the presence of a 30-fold molar excess of unlabeled $GroES_7$.§ As expected for an associative process in which the apparent rate constant includes the unlabeled $GroES_7$ concentration as a factor, the observed rates increase linearly with increasing unlabeled $GroES_7$.

Under comparable conditions, the exchange of ADP is 7–10 times slower than the exchange of $GroES_7$ from the $^{A}GroEL_7$–$^{B}GroEL_7$·ADP_7·$GroES_7$ complex (data not shown). In the structure of this complex (4), the ADP is locked into its binding site by the conformational change that accompanies $GroES_7$ binding. This suggests an obligatory order for association and dissociation of these ligands—first on, last off—in which nucleotide binds before $GroES_7$, whereas GroES release precedes nucleotide dissociation. The present results indicate that the events following the dissociation of $GroES_7$ that cause the release of ADP must be slow relative to the rate of rebinding of $GroES_7$. Otherwise the rates of ADP and $GroES_7$ exchange would be the same, which is not what is observed.

The present results demonstrate that stable 1:1 $GroES_7$–$GroEL_{14}$ complexes form in the presence of ADP, and they do not significantly dissociate during electrophoresis or gel permeation chromatography. Furthermore, 2:1 complexes detected by fluorescence anisotropy measurements (11) are not detected as stable species under the conditions used here. However, the $GroES_7$ in these stable 1:1 complexes can be exchanged with $GroES_7$ in solution by an associative mechanism involving the transient formation of unstable 2:1 complexes.

A previous study (27) detected little exchange of $GroES_7$ from 1:1 complexes at levels of $GroES_7$ at which the present work clearly demonstrates exchange. This difference can be rationalized, because it has been shown that, in addition to excess $GroES_7$, the formation of 2:1 complexes depends on the level of ADP present in solution (11). At the low ADP levels used in a previous studies (27), no 2:1 complexes could be detected by fluorecence anisotropy, whereas at the ADP levels used here, a significant population of 2:1 complexes (≈20%) could be observed (11). Thus, the present results are consistent with the model suggested previously indicating that $GroES_7$–$GroEL_{14}$ complexes are not stable if they are truly symmetric (i.e., ADP and $GroES_7$ on both rings).

Although apparently symmetric 2:1 complexes can form and appear to persist in the presence of ATP, this steady-state snapshot of the population of molecules obscures highly dynamic

§The rate of $GroES_7$ exchange observed by using chromatography to separate the $GroES_7$–$GroEL_{14}$ complex from free $GroES_7$ is slower than that observed by using PAGE. We attribute this discrepancy to excluded volume effects in the polyacrylamide gel matrix (26).

behavior. Pre-steady state studies of the catalytic cycle (15, 18, 28–30) indicate that the cycle proceeds unidirectionally, with the ligands $GroES_7$ and ADP dissociating from alternate $GroEL_7$ rings in the manner of a two stroke engine.; i.e., $^{A}GroEL_7$–$^{B}GroEL_7{\cdot}ADP_7{\cdot}GroES_7 \rightarrow GroES_7{\cdot}ATP_7{\cdot}^{A}GroEL_7$–$^{B}GroEL_7{\cdot}ADP_7{\cdot}GroES_7 \rightarrow GroES_7{\cdot}ATP_7{\cdot}^{A}GroEL_7$–$^{B}GroEL_7 \rightarrow GroES_7{\cdot}ADP_7{\cdot}^{A}GroEL_7$–$^{B}GroEL_7 \rightarrow GroES_7{\cdot}ADP_7{\cdot}^{A}GroEL_7$–$^{B}GroEL_7{\cdot}ATP_7{\cdot}GroES_7 \rightarrow {}^{A}GroEL_7$–$^{B}GroEL_7{\cdot}ATP_7{\cdot}GroES_7 \rightarrow {}^{A}GroEL_7$–$^{B}GroEL_7{\cdot}ADP_7{\cdot}GroES_7$. Although the binding of a second $GroES_7$ is not obligatory to operation of the cycle, at the cellular concentrations of $GroEL_{14}$, $GroES_7$, and ATP, there is every reason to expect its participation in the cycle *in vivo*.

The truly symmetric 2:1 complex $GroES_7{\cdot}ADP_7{\cdot}^{A}GroEL_7$–$^{B}GroEL_7{\cdot}ADP_7{\cdot}GroES_7$ invoked in the present study cannot be considered an intermediate in the chaperonin cycle for two reasons. If it were involved, a single round of ATP hydrolysis would cause the loss of only half of the radiolabled ligand (ADP or $GroES_7$) present in the starting asymmetric complex. In reality, 100% of the radiolabeled ligand is lost (18). Second, the asymmetry of the system is maintained by the fact that ATP hydrolysis by one $GroEL_7$ ring is inhibited so long as there is ADP present in the other $GroEL_7$ ring (30). Alternatively, asymmetry could be maintained by the cooperative hydrolysis of ATP on one ring of $GroEL_{14}$ provided that *both* products, ADP and P_i, remained trapped within that ring until ADP had dissociated from the other ring. However, it has not proven possible to trap $^{32}P_i$ from [γ-^{32}P]ATP in the stable asymmetric complex, although [α-^{32}P]ADP from [α-^{32}P]ATP can readily be trapped, as such a model would predict. Furthermore, unpublished results (cited in ref. 30) indicate that the release of P_i from the complex is coincident with ATP cleavage.

The recent x-ray structure (4) of the 1:1 complex with ADP and $GroES_7$ bound on the same ring of $GroEL_{14}$ suggests a common origin for the instability of the truly symmetric 2:1 $GroES_7{\cdot}ADP_7{\cdot}^{A}GroEL_7$–$^{B}GroEL_7{\cdot}ADP_7{\cdot}GroES_7$ complex observed here as well as for the pseudosymmetric 2:1 $GroES_7{\cdot}ATP_7{\cdot}^{A}GroEL_7$–$^{B}GroEL_7{\cdot}ADP_7{\cdot}GroES_7$ complex that is thought to form transiently during the normal catalytic cycle. Three things are coupled in the occupied $GroEL_{14}$ ring: (*i*) binding of $GroES_7$, (*ii*) binding of seven nucleotides, and (*iii*) changes in the orientations of the equatorial domains at the ring–ring interface. Because this interface is maintained by a tight coupling of the equatorial domains between the two rings, changes in one ring induce complementary changes in the opposite ring, with the result of reducing the symmetry that is present in unliganded $GroEL_{14}$. This symmetry-breaking at the interface can explain the observed anticooperativity in binding of ligands to the two rings of $GroEL_{14}$. Binding of ATP alone to the trans ring is sufficient to induce the dissociation of the ligands in the cis ring (15). However, binding of ADP to the trans ring does not induce the dissociation of the ligands from the cis ring. But, as shown here, binding of *both* ADP and $GroES_7$ to the trans ring induces the departure of $GroES_7$ from the cis ring. Thus, regardless of whether the 2:1 complexes are symmetric or merely pseudosymmetric, they are intrinsically unstable and tend to revert to asymmetric resting states.

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