Fluorescence Detection of Symmetric GroEL$_{14}$(GroES$_7$)$_2$
Heterooligomers Involved in Protein Release during the
Chaperonin Cycle*

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The GroEL$_{14}$ chaperonin from Escherichia coli was labeled with 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (I-AEDANS), a hydrophobic probe whose fluorescent emission is sensitive to structural changes within the protein. Increasing concentrations of ATP or adenylyl imidodiphosphate but not ADP caused two successive GroES$_7$-dependent changes in the fluorescence intensity of AEDANS-GroEL$_{14}$, corresponding to the sequential binding of two GroES$_7$ heptamers and the formation of two types of chaperonin heterooligomers, GroEL$_{14}$, GroES$_7$, and GroEL$_{14}$(GroES$_7$)$_2$. The binding of thermally denatured malate dehydrogenase (MDH) caused a specific increase in fluorescence intensity of AEDANS-GroEL$_{14}$ that allowed the direct measurement in solution at equilibrium of ATP- and GroES$_7$-dependent protein release from the chaperonin. Structure/function analysis during the generation of ATP from ADP indicated the following sequence of events: 1) ADP-stabilized MDH-GroEL$_{14}$,GroES$_7$ particles bind newly formed ATP. 2) MDH-GroEL$_{14}$,GroES$_7$ particles bind a second GroES$_7$. 3) MDH-GroEL$_{14}$,(GroES$_7$)$_2$ particles productively release MDH. 4) Released MDH completes folding. Therefore, the symmetrical GroEL$_{14}$,GroES$_7$ heterooligomer is an intermediate after the formation of which the protein substrate is productively released during the chaperonin-mediated protein folding cycle.

In Escherichia coli cells, chaperonins GroEL$_{14}$ and GroES$_7$ are implicated in the folding of proteins and in the molecular response to cellular stress (Goloubinoff et al., 1989a; Cheng et al., 1989; Frydman et al., 1994; Martin et al., 1992; Horwich et al., 1993). Under stringent conditions (Schmidt et al., 1994a), chaperonin-assisted protein-refolding requires GroEL$_{14}$, GroES$_7$, K$^+$, Mg$^{2+}$, or Mn$^{2+}$ ions and ATP hydrolysis (Goloubinoff et al., 1989b; Vitanen et al., 1990; Diamant et al., 1995b). The mechanism by which chaperonins prevent the irreversible aggregation of stress-denatured proteins and assist them to recover a functional three-dimensional structure remains largely unclear (Martin et al., 1991, 1993; Engel et al., 1995; Azem et al., 1994b, 1995). One approach to this question is to address the relationship between the chaperonin protein folding activity and the structure of the various GroEL-GroES heterooligomers. However, for lack of a direct method to assess the structure of chaperonin heterooligomers in active protein folding solutions, models for the chaperonin mechanism have been designed on the sole basis of indirect structural information, obtained under conditions that may not guarantee preservation of the biochemical equilibrium (Langer et al., 1992; Martin et al., 1993; Azem et al., 1994b; Chen et al., 1994; Azem et al., 1995).

Negative-stain electron microscopy (EM) indicated that in the presence of ADP or low ATP or AMP-PNP concentrations only one GroES$_7$-co-chaperonin can bind a GroEL$_{14}$ core particle and form an asymmetric GroEL$_{14}$GroES$_7$ complex (Langer et al., 1992; Ishii et al., 1992; Azem et al., 1994b). In contrast, in the presence of physiological pH, Mg$^{2+}$, and ATP concentrations or high concentrations of AMP-PNP but not of ADP, two GroES$_7$-co-chaperonins can bind the GroEL$_{14}$ cylinder and form a symmetric GroEL$_{14}$(GroES$_7$)$_2$ particle (Azem et al., 1994b). Variable percentages of symmetric GroEL$_{14}$(GroES$_7$)$_2$ particle were described under various conditions of pH, low protein, and high Mg$^{2+}$ concentrations (Schmidt et al., 1994b; Llorca et al., 1994; Harris et al., 1994; Engel et al., 1995; Llorca et al., 1996). Although the asymmetric GroEL$_{14}$GroES$_7$ particle has been considered to be the only heterooligomer of functional significance to the protein folding cycle (Langer et al., 1992; Martin et al., 1993; Engel et al., 1995; Hayer-Hartl et al., 1995), solutions populated by over 90% symmetrical GroEL$_{14}$(GroES$_7$)$_2$ particles were observed by EM and SDS gels of cross-linked chaperonins under conditions that also supported maximal rates of protein folding (Azem et al., 1994b). Moreover, the rate and increased efficiency of the protein refolding reaction was found proportional to the amount of GroEL$_{14}$(GroES$_7$)$_2$ particles in a chaperonin solution (Azem et al., 1995; Diamant et al., 1995b). Whereas the binding of a nonnative protein can possibly increase the amount of GroEL$_{14}$(GroES$_7$)$_2$ particles detected by EM (Llorca et al., 1996), kinetic analysis of thermophilic chaperonins indicated that the release of a bound protein from asymmetric GroEL$_{14}$GroES$_7$ particles depends upon the binding of a second GroES$_7$ heptamer (Todd et al., 1995).

The validity of structure/function correlations depends on whether EM and SDS gels of cross-linked chaperonins reflect the true distribution between the various heterooligomers in active chaperonin solutions at equilibrium. The possible artificial recruitment of a second GroES$_7$ by GroEL$_{14}$GroES$_7$ during glutaraldehyde cross-linking (Azem et al., 1994b)}
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RESULTS

The Effect of Nucleotides—In the presence of an ATP regeneration system, increasing concentrations of ATP caused a decrease in the intensity of the steady-state fluorescence of AEDANS-GroEL$_{14}$. This effect was specific, because 1 mM ATP caused less than 1% change in the fluorescence intensity of free AEDANS or of the same amount of AEDANS-labeled malate dehydrogenase as controls (not shown). When AEDANS-GroEL$_{14}$ alone was exposed to increasing concentrations of ATP, the change in the fluorescence intensity was monophasic. A linear representation of the data in Fig. 1A clearly shows that the fluorescence signal saturates around 14.3% (Fig. 1A) with an EC$_{50}$ of 56 µM.

When AEDANS-GroEL$_{14}$ in the presence of a molar excess of GroES$_{7}$ was exposed to increasing concentrations of ATP, the decrease in the fluorescence intensity was sharper and biphasic with a first EC$_{50}$ of 5.5 µM and a second approximate EC$_{50}$ of 60 ± 20 µM (Fig. 1, A and B). When, in a control experiment, GroES$_{7}$ was first chemically modified by a pretreatment with glutaraldehyde (as in Fig. 4B), repurified, and then used as in Fig. 1A, the ATP-dependent decrease in the fluorescence intensity of GroEL$_{14}$ was monophasic, as if GroES$_{7}$ was absent from the solution (not shown). Thus, the two-step ATP-dependent transition in the fluorescence intensity of GroEL$_{14}$ is specific to the presence of unmodified GroES$_{7}$ in the solution.

Similarly to ATP, increasing concentrations of the ATP analog AMP-PNP caused a monophasic change in the fluorescence intensity of AEDANS-GroEL$_{14}$ alone, with an EC$_{50}$ value of about 100 µM. In the presence of GroES$_{7}$, increasing AMP-PNP concentrations above 160 µM caused a biphasic change in the GroEL$_{14}$ fluorescence with a first EC$_{50}$ of about 315 µM and a second EC$_{50}$ above 2 mM (Fig. 1, C and D).

In contrast to ATP and AMP-PNP, increasing concentrations of ADP caused a monophasic change in the fluorescence intensity of AEDANS-GroEL$_{14}$ both in the absence and the presence of GroES$_{7}$, with similar EC$_{50}$ values of 136 and 113 µM, respectively (Fig. 1E). Thus, in the presence of GroES$_{7}$, ATP, and AMP-PNP share the same ability to cause a biphasic change, as opposed to ADP, which causes a monophasic change in the fluorescence intensity of AEDANS-GroEL$_{14}$ (Fig. 1F).

The ATP-dependent two-step effect of GroES$_{7}$ on the fluorescence of AEDANS-GroEL$_{14}$ was compared with the ATP-dependent formation of GroEL$_{14}$GroES$_{7}$ and GroEL$_{14}$GroES$_{7}$ particles in chaperonin solutions, as previously measured under the same conditions by EM and SDS gels of cross-linked chaperonins (Azem et al. 1995; see Fig. 6A). In Fig. 1B, a clear correlation was observed between the ATP-dependent formation of asymmetric GroEL$_{14}$GroES$_{7}$ particles and the first ATP-

Experimental Procedures

Proteins—GroEL$_{14}$ was purified to homogeneity as in Azem et al. (1994a). GroES$_{7}$ was purified as in Todd et al. (1993) with minor modifications. Protein concentrations were determined by the Bradford protein assay (Bio-Rad) with GroEL and GroES standard solutions, whose respective concentrations were determined by total amino acid analysis. For the sake of clarity, in this work all chaperonin concentrations were expressed in terms of the individual GroES$_{7}$ and GroEL$_{14}$ oligomers. The concentration column (Pharmacia Biotech Inc.) in 50 mM triethanolamine, pH 7.5, and 20 mM MgOAc was incubated with 3 mM I-AEDANS (Molecular Probes Europe BV) in the dark at 37°C for 60 min. AEDANS-labeled GroEL$_{14}$ was then separated on a Superose 6 gel filtration column (Pharmacia Biotech Inc.) in 50 mM triethanolamine, pH 7.5. Under these conditions, about five AEDANS molecules were found covalently bound to each GroEL$_{14}$ monomer, possibly to nonessential cysteine and lysine residues (Hudson and Weber, 1973). Regardless of their identity, the target residues are not essential to the chaperonin function, because after a 22-min incubation at 47°C, AEDANS-GroEL$_{14}$ was found equally as well as unlabeled GroEL$_{14}$ to prevent the thermal aggregation and promote the subsequent reactivation at 25°C of thermally denatured MDH in a strict GroES$_{7}$- and ATP-dependent manner (not shown). AEDANS labeling did not impair the ability of GroEL$_{14}$ to form both asymmetric GroEL$_{14}$GroES$_{7}$ and symmetric GroEL$_{14}$GroES$_{7}$ particles, as detected by glutaraldehyde cross-linking (see Figs. 4B and 6).

The spectral properties of AEDANS-GroEL$_{14}$ were as described by Hansen and Gafni (1994). Because changes in the fluorescence intensity were more sensitive than shifts in the emission spectra, fluorescence intensity was used to monitor the structural changes in GroEL$_{14}$ in the presence of nucleotides, divalent ions, GroES$_{7}$, native, and nonnative MDH. In storage, the chaperonin activity and the fluorescence emission of AEDANS-GroEL$_{14}$ remained unchanged after 2 weeks of incubation in the dark at 4°C as a 20–30 µM solution in the presence of 50 mM triethanolamine, pH 7.5, and 2 mM MgOAc.
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**Fig. 1.** Nucleotide-dependent fluorescence of GroEL$_{14}$. The steady-state fluorescence of AEDANS-GroEL$_{14}$ (3.5 μM, protomers) in the presence of 50 mM triethanolamine, pH 7.5, 20 mM MgOAc, 20 mM KCl, and increasing concentrations of ATP (A and B), AMP-PNP (C and D), or ADP (E and F) in the absence (○) or the presence (●) of GroES$_{7}$ (10 μM, protomers). In A, 3 mM phosphoenolpyruvate and 20 μM pyruvate kinase were added to regenerate the ATP. B, D, and F, represent the net difference effect (●) caused by GroES$_{7}$ on the GroEL$_{14}$ fluorescence in A, C, and E, respectively. For comparison, the ATP-dependent distribution of GroEL$_{14}$GroES$_{7}$ (○) and GroEL$_{14}$GroES$_{7}$ (●) particles was reproduced in B, as initially measured under the same conditions by chemical cross-linking and by negative stain EM in Azem et al. (1995) and also as observed in Fig. 6.

**Fig. 2.** GroES$_{7}$-dependent fluorescence of GroEL$_{14}$. GroEL$_{14}$ fluorescence was measured under conditions as described in the legend to Fig. 1 in the presence of 20 mM Mg$^{2+}$ and 1 mM ATP (●) or ADP (○) and increasing concentrations of GroES$_{7}$ without (empty symbols) or with (●) 2 mM MnOAc.

GroEL$_{14}$ (Azem et al., 1994b; Schmidt et al., 1994b), increasing concentrations of GroES$_{7}$ decreased the fluorescence intensity of AEDANS-GroEL$_{14}$ with an EC$_{50}$ of 0.91 (GroES/GroEL molar ratio) and a maximal fluorescence change of 7.1% from the GroES-less control (Fig. 2). In contrast, in the presence of saturating amounts of ATP and Mg$^{2+}$, where two GroES$_{7}$ can symmetrically bind GroEL$_{14}$ (Azem et al., 1994b, 1995); see Fig. 6A), increasing concentrations of GroES$_{7}$ decreased the fluorescence intensity of AEDANS-GroEL$_{14}$ with an EC$_{50}$ of 0.77 and a maximal fluorescence change of 14.1% from the GroES-less control (Fig. 2). Thus, in the presence of ATP, the net effect of GroES$_{7}$ on the fluorescence intensity of AEDANS-GroEL$_{14}$ was twice as high as that in the presence of ADP, confirming that fluorescence can distinguish between the binding of a single GroES$_{7}$ to GroEL$_{14}$ and the binding of a second GroES$_{7}$ to GroEL$_{14}$GroES$_{7}$.

Mn$^{2+}$ ions increase the affinity of GroES$_{7}$ for GroEL (Diamant et al., 1995b). The presence of 2 mM Mn$^{2+}$, in addition to the preexisting 20 mM Mg$^{2+}$ and ATP, increased the effect of GroES$_{7}$ on the GroEL$_{14}$ fluorescence and reduced the EC$_{50}$ from 0.77 to 0.51 (Fig. 2). Thus, when the GroES/GroEL molar ratio was 0.51 and the chaperonin solution was accordingly saturated with asymmetric GroEL$_{14}$GroES$_{7}$ particles (Diamant et al., 1995b), the GroES$_{7}$-dependent fluorescence signal was half-saturated. This demonstrates that GroES$_{7}$ does not only interact with GroEL$_{14}$ to form GroEL$_{14}$GroES$_{7}$ particles but also interacts to the same extent with GroEL$_{14}$GroES$_{7}$ to form symmetrical GroEL$_{14}$GroES$_{7}$ particles.

The Effect of Mg$^{2+}$ Ions—In the presence of saturating amounts of GroES$_{7}$ and ATP, increasing concentrations of Mg$^{2+}$ decreased the fluorescence intensity of GroEL$_{14}$ (Fig. 3A), as expected from the cumulative effects of free and ATP-bound Mg$^{2+}$ ions on the structure and activity of GroEL$_{14}$, as well as on the affinity of GroES$_{7}$ for both GroEL$_{14}$ and GroEL$_{14}$GroES$_{7}$ particles (Azem et al., 1994a; Diamant et al., 1995a, 1995b). However, at variance with previous evidence from EM (Engel et al., 1995), estimated physiological amounts of 2.5 mM of free Mg$^{2+}$ ions (Alatossava et al., 1985), sufficed to produce the fluorescence signal of a chaperonin solution saturated with GroEL$_{14}$GroES$_{7}$ particles (Fig. 3B).

The Effect of ADP Conversion into ATP—Chemical cross-linking has shown that even in the presence of a saturating concentration of ATP, ADP can affect the stability of GroEL$_{14}$GroES$_{7}$ particles and displace the equilibrium in favor of asymmetric GroEL$_{14}$GroES$_{7}$ particles (Azem et al., 1995). In the absence of GroES$_{7}$, the slow conversion of ADP...
A transient protein-bound GroEL14(GroES7)2 ternary complex formation as for protein release suggests that the formation of the transient protein-bound GroEL14(GroES7)2 ternary complex is rate-determining for the protein folding cycle. Also after the initial release of GroEL14 particles (Fig. 4A), as expected from the higher affinity of the newly formed ADP, the decrease in GroEL14 fluorescence was measured as in Fig. 1A in the presence of 1 mM ATP and increasing concentrations of MgOAc (not shown), which is the signal from a solution saturated with ATP-stabilized GroEL14(GroES7)2 particles (see Fig. 1A). In contrast, when GroES7 was present, the same rate of ATP synthesis did not cause the same immediate decrease in fluorescence intensity of GroEL14. Only after a delay of about 3 min did the fluorescence signal shift from that of a majority of ADP-GroEL14GroES7 particles into that of a majority of ATP-GroEL14(GroES7)2 particles (Fig. 4A). The higher affinity of ATP for GroEL14(GroES7)2 particles, as compared with GroEL14 particles (Todd et al., 1994) and the consequent delay in the binding of ATP and of the second GroES7 can explain the observed delay in the fluorescence change (Fig. 4A). It should be noted that because after 10 min the maximal decrease in fluorescence was less than 25%, a minority of GroEL14GroES7 particles were likely to be also present in the chaperonin solution. This was confirmed by limited glutaraldehyde cross-linking (Fig. 4B). In the absence of nucleotide, GroES7 did not bind GroEL14, as revealed by the presence of the final GroEL14(GroES7)2, species and of a single transient GroEL7 species, without a transient GroEL7GroES7 species (Fig. 4B, lane 1). In contrast, 40 s after the addition of ADP, the chaperonin solution was populated by a mixture of asymmetric GroEL14GroES7 and GroEL14 particles (Fig. 4B, lane 2). The asymmetric GroEL14GroES7 particle steadily remained the major species in the solution during at least 3 min after the initiation of ATP synthesis, as demonstrated by the equal amounts of GroEL14(GroES7)2 and GroEL7 transient species (Fig. 4B, lanes 3-6). Beyond 3 min, symmetric GroEL14(GroES7)2 particles started to accumulate at the expense of asymmetric GroEL14GroES7 particles, as reflected by the formation of GroEL14GroES7 species in excess and at the expense of the GroEL7 transient GroEL7 species (Fig. 4B, lanes 7-9). Thus, the two successive fluorescence changes, first slow and then rapid, during ATP synthesis in the presence of ADP and GroES7, (Fig. 4A) coincided with a maintained maximal level of asymmetric GroEL14GroES7 particles, followed by the formation of symmetric GroEL14(GroES7)2 particles at the expense of the GroEL14GroES7 particles in the solution (Fig. 4B, lanes 7-9).

The Effect of a Nonnative Protein—The fluorescence intensity of GroEL14 after exposure to 47 °C for 22 min and then to 25 °C was the same as that of GroEL14 incubated for 22 min at 25 °C (not shown), indicating that the heat treatment did not irreversibly affect the structure of the chaperonin. However, when native MDH was added to GroEL14 at 47 °C, the steady-state fluorescence intensity of GroEL14 was increased within 5 min by 4.6% (Fig. 5A, inset). After 22 min at 47 °C and cooling to 25 °C, the steady-state fluorescence intensity of GroEL14 was increased by 7%, as compared with a corresponding treatment at a constant 25 °C (Fig. 5A) or with the same treatment at 47 °C in absence of MDH (not shown). The residual MDH activity, which was less than 1.5%, did not increase during more than 60 min after the heat treatment, as long as both GroES7 and ATP were not supplied (not shown), indicating that unbound MDH was irreversibly aggregated. The addition of a saturating concentration of ADP did not reduce the net fluorescence signal for bound MDH. The subsequent addition of GroES7 did reduce by 18% the signal for bound MDH; however, no MDH reactivation was observed in the solution. Only after the initiation of ATP synthesis by pyruvate kinase and a delay of about 2–3 min did the net fluorescence signal of bound MDH start to subside with a time constant of 2.5 min (Fig. 5B).

The fact that the same delay was necessary for GroEL14(GroES7)2 formation as for protein release suggests that the formation of a transient protein-bound GroEL14(GroES7)2 ternary complex is rate-determining for the protein folding cycle. Also after the

![Figure 3](image-url) Fluorescence Detection of GroEL14(GroES7)2 Chaperonins

**FIG. 3.** Mg2+-dependent fluorescence of GroEL14. GroEL14 fluorescence was measured as in Fig. 1A in the presence of 1 mM ATP and increasing concentrations of MgOAc (A) without (○) or with (●) GroES7 (5.25 μM protomers). B, net GroES7 effect (%) on the GroEL14 fluorescence (●) from A.

![Figure 4](image-url) Fluorescence transitions during ATP generation. A, the decrease in AEDANS-GroEL14 fluorescence was measured in the presence of 3 mM phosphoenolpyruvate under conditions as described in the legend to Fig. 1 for 200 s after the addition of 1 mM ATP in the absence (○) or the presence of 14 μM GroES7 (●). Then, to convert the ADP into ATP, a limiting amount of pyruvate kinase (PK, 2.5 ng/ml) was added, and the change in fluorescence was measured for 10 min. B, time-dependent analysis of the formation of ATP-GroEL14(GroES7)2 particles from ADP-GroEL14GroES7 particles as in A. Chaperonins were cross-linked with glutaraldehyde, before (lane 1) or 40 s after addition of ADP (lane 2) or 40, 80, 120, 180, 260, 420, or 600 s after the addition of pyruvate kinase (lanes 3-9, respectively). Cross-linked samples were separated by SDS gel electrophoresis as in Azem et al. (1995).
was saturating (0.25 mM). Thus, the transient protein-associated to the chaperonin (not shown), even when ATP that within that time, all the nonnative MDH still remained A core particle (Fig. 6, rate, MDH activity was recovered (t 1⁄2 of 9 min) (Fig. 5 B). Thus, consistent with similar observations using RubisCO and Rho-danese (Todd et al., 1994; Weissman et al., 1994), the difference between the rate of protein release and that of protein folding indicate that the final steps of protein release and relative activity of recovered MDH ( ) expressed as a percentage of a native MDH control.

FIG. 5. Fluorescence signal of bound protein. A, AEDANS-GroEL14 (3.5 μM) was preincubated with 0.25 μM MDH for 22 min at 25°C ( ) or at 47°C ( ). Then, at t = 0, both solutions were incubated at 25°C with 1 mM ADP for 200 s. At t = 200 s, GroES7 (7 μM) was added. At t = 300 s, 2.5 ng/ml PK was added, and the change in the fluorescence was measured as a function of time. Inset, time-dependent increase in the fluorescence of GroEL14 at 47°C upon the addition of native MDH. B, net time-dependent effect of nonnative MDH on the GroEL14 fluorescence ( ) from A and relative activity of recovered MDH ( ) expressed as a percentage of a native MDH control.

same delay, in parallel to the protein release, albeit at a slower rate, MDH activity was recovered (t50 of 9 min) (Fig. 5B). Thus, consistent with similar observations using RubisCO and Rho-danese (Todd et al., 1994; Weissman et al., 1994), the difference between the rate of protein release and that of protein folding indicate that the final steps of folding and assembly of a protein such as MDH, occur after it has been completely released from the chaperonin.

Cross-linking and fluorescence confirmed that transient MDH-GroEL14(GroES7)2 complexes form prior to the productive release of the protein. Hence, after 1 min of incubation at 37°C with increasing concentrations of ATP, both GroES7 bind the MDH-GroEL14 complex equally well as the free GroEL14 core particle (Fig. 6, A and B). In parallel, fluorescence revealed that within that time, all the nonnative MDH still remained associated to the chaperonin (not shown), even when ATP was saturating (0.25 mM). Thus, the transient protein-GroEL14(GroES7)2 complex can accumulate and become the major species in the solution, prior to the initiation of productive protein release.

DISCUSSION

We have shown that the interaction of Mg2+ ions, nucleo-tides, GroES7, and a nonnative protein caused distinct and specific changes in the quantum yield of the steady-state fluorescence of AEDANS-GroEL14. Thus, in the presence of increasing concentrations of ATP or AMP-PNP, the interaction of GroES7 caused a biphasic change, whereas in the presence of ADP, GroES7 caused a monophasic change in the AEDANS-GroEL14 fluorescence. Similarly, SDS gels and EM of cross-linked chaperonins have previously shown that in the presence of ATP or AMP-PNP, two GroES7 can bind both ends of GroEL14 whereas in the presence of ADP, GroES7 can bind only one end of the GroEL14 core chaperonin (Azem et al., 1994b; Schmidt et al., 1994b; Azem et al., 1995). Data from fluorescence experiments not only concurred qualitatively but also quantitatively with the cross-linking and EM data. Hence, the two fluorescence changes occurred at the same ATP concentrations previously reported for the successive formation of asymmetrical GroEL14(GroES7) as of symmetrical GroEL14(GroES7)2 particles in the solution (Figs. 1C and 6A; Azem et al., 1995). We conclude from this precise match that the three methods faithfully reflect the steady-state equilibrium between GroEL14GroES7 and GroEL14(GroES7)2 particles in the functional chaperonin solutions. Moreover, all three methods describe chaperonin solutions populated by a majority of symmetrical GroEL14(GroES7)2 particles under conditions that also support maximal rates of protein folding (Azem et al., 1994, 1995; Diamant et al., 1995b). Remarkably, these conditions of 2.5 mM Mg2+ and pH 7.5 contradict a previous suggestion that symmetrical GroEL14(GroES7)2 particles can form in significant amounts only under nonphysiological pH 8.0 and high (50 mM) Mg2+ concentrations (Engel et al., 1995).

 Symposium GroEL14(GroES7)2 particles often remained undetected by EM and even by fluorescence analysis. Thus, in the presence of low Mg2+ concentrations, extensive chaperonin dilutions, the absence of an ATP regeneration system, or without performing a prior thorough cross-linking of GroES to GroEL, GroEL14(GroES7)2 particles were not reported (Langer et al., 1992; Martin et al., 1993; Ishii et al., 1992, 1994). The lack of a specific fluorescence signal for GroEL14(GroES7)2 particles in a similar fluorescence analysis of pyrenyl-GroEL14 can now be attributed to suboptimal conditions, such as low ATP.
concentrations (10–40 μM), the absence of an ATP regeneration system, and nonsaturating amounts of GroES (Burston et al., 1995). More recently, varying minor amounts of symmetrical GroEL$_{14}$(GroES$_{7}$)$_{2}$ particles in the solution (Azem et al., 1994b; Weissman et al., 1995b; Diamant et al., 1995). Moreover, fully corroborating previous evidence using cross-linking, EM, gel filtration, and kinetic analysis (Azem et al., 1994b, 1995; Diamant et al., 1995b; Llorca et al., 1995; Todd et al., 1995), fluorescence, and cross-linking (Fig. 6) showed here that transient MDH-GroEL$_{14}$(GroES$_{7}$)$_{2}$ complexes can form, accumulate, and even become the major species of an optimally active protein folding solution of chaperonins.

How Can Proteins Be Released from GroEL$_{14}$(GroES$_{7}$)$_{2}$ Particles?—Protein release has been previously described from ADP-stabilized asymmetric GroEL$_{7}$(GroES$_{7}$) particles upon activation with ATP (Langer et al., 1992; Martin et al., 1993; Todd et al., 1994; Weissman et al., 1995). Remarkably, kinetic studies showed that productive protein release preferentially occurs from GroEL$_{14}$(GroES$_{7}$) particles in which the protein is bound in a cis conformation, i.e., on the same GroEL$_{14}$ toroid as the bound GroES$_{7}$ (Weissman et al., 1995). It should be noted that structurally speaking, a GroEL$_{14}$(GroES$_{7}$)$_{2}$ particle may contain as many as two protein binding sites in a cis configuration (Todd et al., 1995). Although it is not clear if the cis-bound protein is released directly from the asymmetric GroEL$_{14}$(GroES$_{7}$)$_{2}$ particle or from a transiently forming GroEL$_{14}$(GroES$_{7}$)$_{2}$ particle, this nevertheless demonstrates that during the folding cycle, a bound protein must reside on the same side as GroES$_{7}$ before it can be productively released (Bochkareva and Girshovich, 1992; Weissman et al., 1995; Todd et al., 1995).

Similar to the cis MDH-GroEL$_{14}$(GroES$_{7}$) complex (Weissman et al., 1995, 1996), we show here that MDH-GroEL$_{14}$(GroES$_{7}$)$_{2}$ complex can serve as an efficient species from which productive protein folding and release can occur. Although the sharp decrease in the chaperonin affinity for a bound protein appears to be a consequence of the binding of the second GroES$_{7}$, it is likely that the minority of MDH-GroEL$_{14}$(GroES$_{7}$)$_{2}$ molecules that release MDH at a given time do so after of the dissociation of the capping GroES$_{7}$ from the complex (Weissman et al., 1996).

A model for the protein folding cycle of chaperonins is proposed in Fig. 7. Due to the high affinity of GroES$_{7}$ for the GroEL$_{14}$ core oligomer (Azem et al., 1994b) and the consequent stability of the GroEL$_{14}$(GroES$_{7}$)$_{2}$ hetero-oligomer, active exchange of GroES$_{7}$ during protein folding is more likely to occur between the labile GroEL$_{14}$(GroES$_{7}$)$_{2}$ and stable GroEL$_{14}$ GroES$_{7}$ particles than between the stable GroEL$_{14}$(GroES$_{7}$)$_{2}$ and GroEL$_{14}$(GroES$_{7}$)$_{2}$ particle.
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