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The protein-folding activity of chaperonins correlates with the symmetric $GroEL_{14}(GroES_7)_2$ heterooligomer

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ABSTRACT **Chaperonins GroEL and GroES form, in the presence of ATP, two types of heterooligomers in solution: an asymmetric $GroEL_{14}GroES_7$ "bullet"-shaped particle and a symmetric $GroEL_{14}(GroES_7)_2$ "football"-shaped particle. Under limiting concentrations of ATP or GroES, excess ADP, or in the presence of 5′-adenylyl imidodiphosphate, a correlation is seen between protein folding and the amount of symmetric $GroEL_{14}(GroES_7)_2$ particles in a chaperonin solution, as detected by electron microscopy or by chemical crosslinking. Kinetic analysis suggests that protein folding is more efficient when carried out by a chaperonin solution populated with a majority of symmetric $GroEL_{14}(GroES_7)_2$ particles than by a majority of asymmetric $GroEL_{14}GroES_7$ particles. The symmetric heterooligomer behaves as a highly efficient intermediate of the chaperonin protein folding cycle *in vitro*.**

Chaperonins GroEL and GroES from *Escherichia coli* belong to a ubiquitous class of sequence-related chaperone molecules in bacteria, in organelles and in the cytoplasm of eukaryotes (for a review, see ref. 1). In the cell, chaperonins are implicated in the folding of proteins (2–4) and in the molecular response to cellular stress (5, 6). *In vitro*, chaperonins assist the correct refolding of proteins by preventing protein aggregation (7, 8). While the $GroEL_{14}$ core oligomer can spontaneously bind nonnative proteins (7), the hydrolysis of ATP in the presence of the cochaperonin $GroES_7$ is required for dissociation of a bound protein from the chaperonin complex (7, 9, 10). Central to understanding the mechanism by which chaperonins assist the folding of a large array of proteins is the relationship between the molecular structure and the specific functions of the various GroEL–GroES heterooligomers during protein folding. One model depicts the binding of a nonnative protein on the external envelope of a symmetric $GroEL_{14}(GroES_7)_2$ heterooligomer, from which it dissociates upon an ATP hydrolysis-dependent change in the affinity of the chaperonin surface for the folding protein (11). In contrast, another model depicts the binding of a nonnative protein within the central cavity of an asymmetric $GroEL_{14}GroES_7$ heterooligomer, from which it dissociates upon ATP hydrolysis, through the unobstructed end of the chaperonin (1, 9, 12–14).

Chemical crosslinking (15) and EM of chaperonin particles demonstrated that in the presence of ATP or the analog 5′-adenylyl imidodiphosphate (AMP-PNP), two $GroES_7$ rings can bind to a single $GroEL_{14}$ core oligomer and form a symmetric $GroEL_{14}(GroES_7)_2$ heterooligomer (15–18). In the presence of ADP, only asymmetric $GroEL_{14}GroES_7$ heterooligomers were observed (15–17, 19). Remarkably, a chaperonin solution populated with a majority of asymmetric $GroEL_{14}GroES_7$ particles appeared to assist the ATP-dependent refolding of the enzyme ribulose-bisphosphate carboxylase [Rubisco; 3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] equally as well as a chaperonin solution populated with a majority of $GroEL_{14}(GroES_7)_2$ particles, suggesting that both heterooligomers are functional species (15). This observation was substantiated by kinetic studies, indicating that one molecule of free $GroES_7$ can catalytically interact with many $GroEL_{14}GroES_7$ heterooligomers and thus form transient $GroEL_{14}(GroES_7)_2$ species during the ATPase cycle (20).

This study describes conditions *in vitro*, such as limiting concentrations of ATP or GroES, an excess of ADP or AMP-PNP, which specifically prevent or promote binding of the second $GroES_7$ to the asymmetric $GroEL_{14}GroES_7$ particle and correspondingly prevent or promote the protein folding activity of the chaperonin. The results strongly suggest that the symmetric $GroEL_{14}(GroES_7)_2$ chaperonin heterooligomer is an intermediate of the chaperonin protein folding cycle.

MATERIALS AND METHODS

Proteins. $GroES_7$ oligomers were purified to homogeneity as described (21), with small modifications. $GroEL_{14}$ oligomers were purified as described (22). Protein concentrations were determined by the Bio-Rad protein assay, with GroEL and GroES standard solutions, whose respective concentrations were determined by total amino acid analysis. Purified recombinant Rubisco from *Rhodospirillum rubrum* was kindly provided by G. H. Lorimer (DuPont). Pig heart mitochondrial malate dehydrogenase [mMDH; (*S*)-malate:NAD^+ oxidoreductase, EC 1.1.1.37] was from Boehringer Mannheim and hexokinase and pyruvate kinase were from Sigma.

Crosslinking of Chaperonin Heterooligomers. Crosslinking of chaperonin heterooligomers was as described (15, 22). Typically, $GroEL_{14}$ (3.5 μM monomer) was preincubated at 37°C in 50 mM triethanolamine (TEA) (pH 7.5) containing 20 mM $MgOAc_2$, 10 mM KCl, and variable amounts of $GroES_7$, ADP, ATP, or AMP-PNP as specified in the text. Crosslinking of the chaperonin heterooligomers was performed in the presence of 0.22% glutaraldehyde (GA) (Sigma) at 37°C for 7 min. The crosslinking reaction was stopped prior to SDS electrophoresis or native EM as described (15). SDS gel electrophoresis of crosslinked proteins was carried out as described (15, 22, 23). Gels were stained and scanned as described (22).

Fraction of $GroEL_{14}GroES_7$ and $GroEL_{14}(GroES_7)_2$ Particles. Due to the better resolution on SDS gels of the intermediate crosslinking species $GroEL_7$ and $GroEL_7GroES_7$, as compared to the poor resolution of the final crosslinking products of $GroEL_{14}GroES_7$ and $GroEL_{14}(GroES_7)_2$ (15), the fraction of $GroEL_{14}GroES_7$ and $GroEL_{14}(GroES_7)_2$ particles in a chaperonin solution was determined from the ratio of crosslinked species $GroEL_7$ and $GroEL_7GroES_7$. This is possible due to the fact that the crosslinking reactions within the $GroEL_7$ toroids and between the $GroEL_7$ and $GroES_7$ rings

Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; DTT, dithiothreitol; GA, glutaraldehyde; mMDH, mitochondrial malate dehydrogenase; TEA, triethanolamine; Rubisco, ribulose-bisphosphate carboxylase.
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(Fig. 1*A*) are much faster than across the two $GroEL_7$ toroids of the chaperonin particles (22). Accordingly, a native $GroEL_{14}GroES_7$ particle produces an equal amount of crosslinked $GroEL_7$ and $GroEL_7GroES_7$ species and, in contrast, a native $GroEL_{14}(GroES_7)_2$ particle produces only $GroEL_7GroES_7$, with no $GroEL_7$ crosslinked species (Table 1, Fig. 1*A*). The values obtained by a Gaussian fitting (24) of the two signals were first translated in terms of molar fraction to correct for the contribution of $GroES_7$ to the $GroEL_7GroES_7$ signal. Azem *et al.* (15) previously showed that the affinity of the second $GroES_7$ for the asymmetric $GroEL_{14}GroES_7$ particle is much lower than of the first $GroES_7$ for the $GroEL_{14}$ core oligomer. The existence of negative cooperativity between the two opposite sides of the chaperonin core oligomer with regard to the binding of $GroES_7$ thus prevents the coexistence of all three types of chaperonin oligomers—$GroEL_{14}$, $GroEL_{14}GroES_7$, and $GroEL_{14}(GroES_7)_2$—in the same solution. Therefore, three situations can be discriminated.

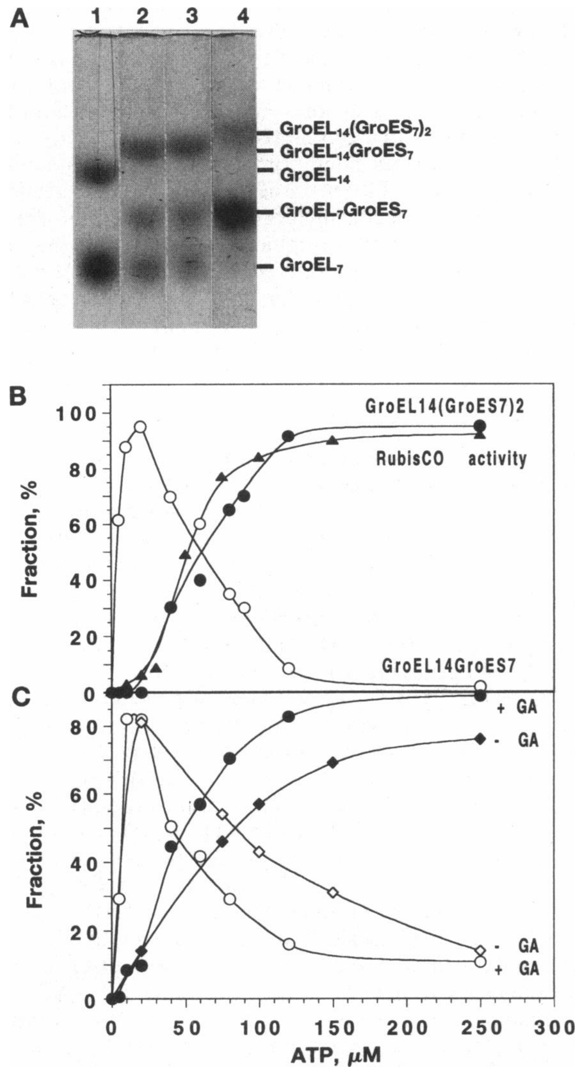

FIG. 1. ATP-dependent distribution of chaperonin heterooligomers and protein folding activity. $GroEL_{14}$ (3.5 μM monomer) and $GroES_7$ (9.6 μM monomer) were preincubated at 25°C for 2 min in the presence of 0, 5, 10, 20, 40, 60, 80, 120, and 250 μM ATP and an ATP regeneration system and then exposed to GA at 37°C as described. (*A*) SDS/polyacrylamide gel of representative samples with 0, 10, 20, and 250 μM ATP (lanes 1–4, respectively). (*B*) ATP-dependent stoichiometry of chaperonin heterooligomers and Rubisco refolding activity. Relative amounts (%) of $GroEL_{14}GroES_7$ (○) and $GroEL_{14}(GroES_7)_2$ (●) particles were obtained by computation of the data from the densitometer scan of $GroEL_7GroES_7$ and $GroEL_7$ crosslinked species on SDS gels as in *A*. The ATP-dependent Rubisco refolding activity (▲) was expressed as the fraction (%) of maximal chaperonin activity in the presence of 1.4 mM ATP, which was 92% of a nondenatured control. (*C*) Detection by EM of the ATP-dependent distribution of $GroEL_{14}GroES_7$ and $GroEL_{14}(GroES_7)_2$ chaperonin heterooligomers pretreated (○, ●) or not (◇, ◆) with GA.

Table 1. SDS species of crosslinked GroEL–GroES heterooligomers

Native species before crosslinking	SDS species after crosslinking				
	$GroEL_7$	$GroEL_7 \times GroES_7$	$GroEL_{14}$	$GroEL_{14} \times GroES_7$	$GroEL_{14} \times GroES_{14}$
$GroEL_{14}$	++	−	+	−	−
$GroEL_{14}GroES_7$	+	+	−	+	−
$GroEL_{14}(GroES_7)_2$	−	++	−	−	+

Distribution on SDS gels of crosslinked species from native oligomers $GroEL_{14}$, $GroEL_{14}GroES_7$, and $GroEL_{14}(GroES_7)_2$ after 7 min of crosslinking with GA. Due to the slower crosslinking reaction between $GroEL_7$ toroids (22), the transient species $GroEL_7$ and $GroEL_7GroES_7$ are found in larger amounts (++) than the fully crosslinked final products of the reaction (+) (Fig. 1*A*).

(*i*) The molar fraction of $GroEL_7 > GroEL_7GroES_7$. In this case, the solution cannot contain $GroEL_{14}(GroES_7)_2$ particles but only $GroEL_{14}$ and $GroEL_{14}GroES_7$ particles. In such a situation,

$$GroEL_{14}GroES_7 = \frac{2(GroEL_7GroES_7)}{(GroEL_7GroES_7 + GroEL_7)}$$

$$GroEL_{14} = \frac{(GroEL_7 - GroEL_7GroES_7)}{(GroEL_7GroES_7 + GroEL_7)}.$$

(*ii*) The molar fraction of $GroEL_7 = GroEL_7GroES_7$. In this case, the solution contains only asymmetric $GroEL_{14}GroES_7$ particles (see ref. 15).

(*iii*) The molar fraction of $GroEL_7 < GroEL_7GroES_7$. In this case, the solution cannot contain $GroEL_{14}$ particles but only $GroEL_{14}GroES_7$ and $GroEL_{14}(GroES_7)_2$ particles. In such a situation,

$$GroEL_{14}GroES_7 = \frac{2(GroEL_7)}{(GroEL_7GroES_7 + GroEL_7)}$$

$$GroEL_{14}(GroES_7)_2 = \frac{(GroEL_7GroES_7 - GroEL_7)}{(GroEL_7GroES_7 + GroEL_7)}.$$

EM. Crosslinked or noncrosslinked chaperonin samples (Fig. 1*C*) were negatively stained with 1% aqueous uranyl acetate and detected by EM as described (15). Side views of the $GroEL_{14}$ (as in refs. 15 and 25), asymmetric $GroEL_{14}GroES_7$ particles (as in refs. 19, 26, and 31), and symmetric $GroEL_{14}(GroES_7)_2$ particles (as in refs. 15–18 and 31) were identified by negative-stain EM. The distribution of chaperonin species in Fig. 1*C* was counted from a sample of at least 500 identified side views for each ATP concentration. In the presence of ATP or ADP, but in the absence of a previous crosslinking treatment, the fraction of chaperonin side views ranged between 20% and 30% of the total amount of particles (see refs. 16 and 18).

Assays. Chaperonin protein refolding activity was assayed with Rubisco (7) or mMDH (27) as reporter enzymes as described, with small modifications. Rubisco (13 μM monomer) was incubated in 5 M urea and 10 mM dithiothreitol (DTT) for 1 hr at 25°C and then rapidly diluted 1:52 into a solution of $GroEL_{14}$ (3.5 μM monomer), $GroES_7$ (9.6 μM monomer), 50 mM TEA (pH 7.5), 20 mM $MgOAc_2$, 10 mM KCl, 20 mM glucose, 2 mM phospho*enol*pyruvate, pyruvate

kinase (20 μg/ml), and ATP as specified. The Rubisco refolding assay at 37°C was terminated after 12 min by addition of hexokinase (40 μg/ml) to deplete the pool of ATP. Under these conditions, 55% of the maximal Rubisco recovery was achieved. mMDH (18 μM monomer) was denatured at 25°C for 3–4 hr in 6 M urea containing 1 mM EDTA and 10 mM DTT. Renaturation was initiated by a 1:70 dilution into 50 mM TEA (pH 7.5), 20 mM $MgOAc_2$, 20 mM KCl, 10 mM DTT, 2 mM phospho*enol*pyruvate, pyruvate kinase (20 μg/ml), 0.75 mM ATP, $GroEL_{14}$ (1.75 μM monomer), and $GroES_7$ as indicated. The mMDH activity was assayed after 8 min of incubation at 37°C. Under these conditions, 46% of the maximal mMDH recovery was achieved (S.D. and P.G., data not shown).

Hydrolysis of ATP was measured as described (28, 29). The assay at 37°C for 10 min was carried out under the same conditions as described for Rubisco and mMDH refolding and for the crosslinking procedure. The inhibition constants (K_i) for ADP and AMP-PNP were calculated by the equation of Cheng and Prussoff (30): $K_i = IC_{50}/(1 + [S]/K_d)$, where IC_{50} is the concentration of the inhibitor that causes a 50% inhibition, [S] is the concentration of ATP, and K_d is the dissociation constant of ATP. Rubisco activity was assayed at 25°C for 10 min as described (7). The activity of mMDH was assayed at 25°C in 150 mM potassium phosphate buffer (pH 7.5), 10 mM DTT, 0.5 mM oxaloacetate, and 0.28 mM NADH (Sigma). The time-dependent oxidation of NADH by mMDH was monitored at 340 nm.

RESULTS

Quantitation of Chaperonin Species. Crosslinking with GA followed by analysis on SDS/polyacrylamide gels was used as described (15) to assess the fraction of asymmetric $GroEL_{14}GroES_7$ and symmetric $GroEL_{14}(GroES_7)_2$ heterooligomers in chaperonin solutions under conditions that also support the refolding of nonnative enzymes such as Rubisco or mMDH. When the fraction of chaperonin heterooligomers was measured in the presence of an ATP regeneration system and increasing amounts of ATP, as little as 20 μM ATP was sufficient to saturate the chaperonin solution with >90% asymmetric $GroEL_{14}GroES_7$ particles (Fig. 1 *A* and *B*). Above 20 μM ATP, symmetric $GroEL_{14}(GroES_7)_2$ particles appeared at the expense of asymmetric $GroEL_{14}GroES_7$ particles. In the presence of 62 μM ATP, the chaperonin solution was equally populated with asymmetric and symmetric particles. In the presence of 150 μM ATP, the solution contained >90% $GroEL_{14}(GroES_7)_2$ particles. Remarkably, the ATP-dependent distribution of chaperonin heterooligomers was nearly identical when inferred from SDS gels of crosslinked chaperonins or from the counting of negatively stained particles on electron micrographs (Fig. 1*C*). Moreover, the ATP-dependent distribution of chaperonin heterooligomers by EM was similar whether the chaperonin solutions were pretreated or not with GA prior to EM (Fig. 1*C*). This confirms that SDS gel analysis of crosslinked chaperonins is as valid a procedure as EM to assess the oligomeric states of chaperonins in solution, since crosslinking with GA does not appear to interfere with the equilibrium between the various heterooligomers.

$GroEL_{14}(GroES_7)_2$ Particles Correlate with Protein Folding Activity. The chaperonin-assisted refolding activity of urea-denatured Rubisco (Fig. 1) or mMDH (data not shown) displayed a similar dependence on ATP concentration as the amount of symmetric $GroEL_{14}(GroES_7)_2$ particles in the solution, with half of the maximal rate of protein refolding activity achieved in the presence of ≈50 μM ATP and a maximal activity achieved in the presence of ≈150 μM ATP (Fig. 1*B*). Thus, limiting concentrations of ATP place a constraint on the formation of $GroEL_{14}(GroES_7)_2$ particles and thus on release of the folding protein.

The correlation between $GroEL_{14}(GroES_7)_2$ particles and protein folding activity was further exemplified in the presence of a saturating concentration of ATP and increasing concentration of GroES (Fig. 2). When the molar ratio of GroES to GroEL was <0.5, only $GroEL_{14}$ (data not shown) and asymmetric particles were detected in the chaperonin solution. Above a molar ratio of 0.5, $GroEL_{14}(GroES_7)_2$ particles appeared at the expense of the $GroEL_{14}GroES_7$ particles and were found in equal amounts at a molar ratio of 1.0 (Fig. 2). The chaperonin solution contained mostly $GroEL_{14}(GroES_7)_2$ particles when the GroES/GroEL ratio exceeded 1.5. Noticeably, chaperonin-assisted refolding activity of urea-denatured mMDH displayed a similar dependency on GroES concentration with half the maximal rate of protein refolding activity achieved at a GroES/GroEL ratio of 1.0 and a maximal activity achieved when the molar ratio exceeded 1.6 (Fig. 2).

$GroEL_{14}(GroES_7)_2$ Is a Highly Efficient Species. The efficiency of the folding reaction, in terms of ATP molecules hydrolyzed per refolded mMDH molecule, dramatically increased with the GroES concentration. Thus, 65 times more ATP was required for the successful folding of mMDH when the chaperonin solution was populated by 40% asymmetric $GroEL_{14}GroES_7$ and 60% $GroEL_{14}$ particles than when it contained 40% $GroEL_{14}GroES_7$ and 60% symmetric $GroEL_{14}(GroES_7)_2$ particles (Fig. 2). This result suggests that the two activities of ATP hydrolysis and protein folding are poorly coupled in a chaperonin solution containing a majority of asymmetric $GroEL_{14}GroES_7$ particles and better coupled in a chaperonin solution containing a majority of symmetrical $GroEL_{14}(GroES_7)_2$ particles.

The Uncoupling Effect of ADP. It has been previously shown that an excess of ADP over ATP inhibits the ATPase of chaperonins and does not induce the rapid and fruitful release of bound Rubisco from the chaperonin (20). The relationship between the symmetric $GroEL_{14}(GroES_7)_2$ particle and protein refolding activity was further confirmed in the presence of excess ATP and increasing amounts of ADP (Fig. 3*A*). As ADP concentrations increased, the Rubisco refolding activity decreased, despite the presence of a 10-fold excess of ATP (1.5 mM) and a 2-fold excess of GroES (Fig. 3*A*). Thus, when ADP

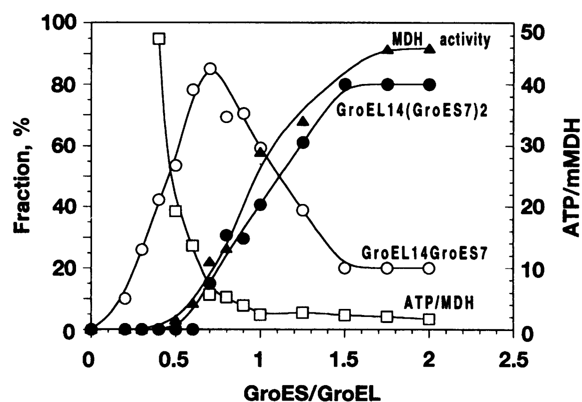

FIG. 2. GroES-dependent equilibrium of chaperonin heterooligomers and protein folding activity. GroEL (1.75 μM) was incubated in the presence of 0.75 mM ATP and increasing concentrations of GroES. After 8 min of incubation at 37°C, the samples were crosslinked with GA. $GroEL_{14}GroES_7$ (○) and $GroEL_{14}(GroES_7)_2$ (●) were identified and quantified as in Fig. 1*B*. Denatured mMDH was diluted in the chaperonin buffer containing GroEL, ATP, and increasing concentrations of GroES. mMDH activity was expressed as the fraction of the maximal activity in the presence of a saturating concentration of GroES. The efficiency of protein refolding (□) is expressed as the number of hydrolyzed ATP molecules (×1000) per refolded mMDH molecule.

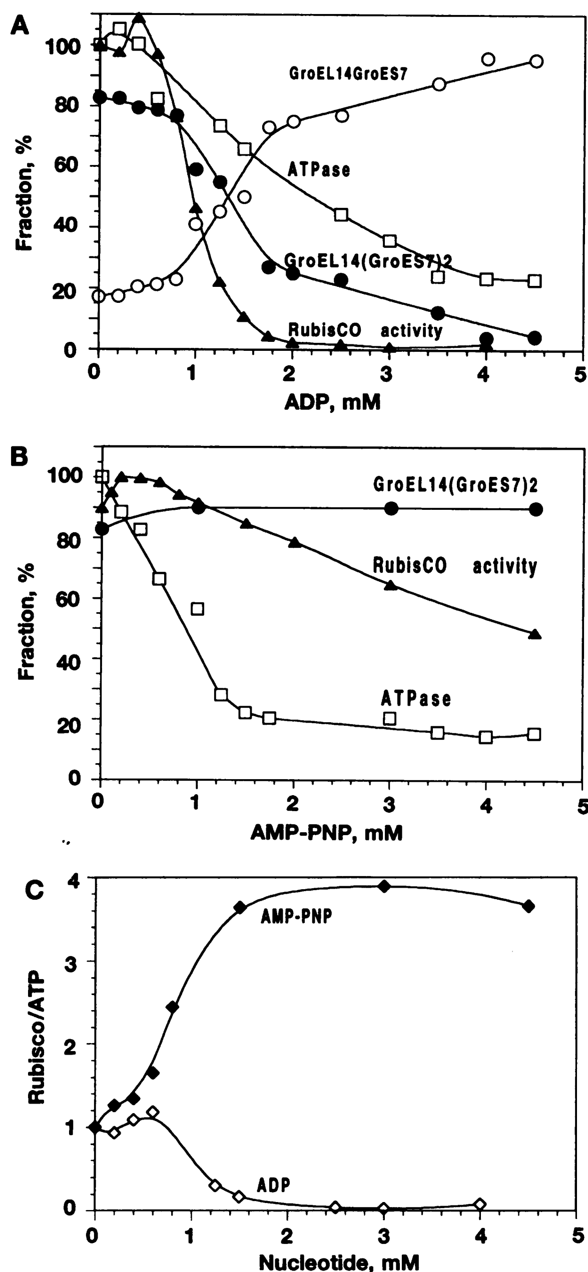

FIG. 3. Chaperonin stoichiometry and ATP-dependent activity in the presence of nonhydrolyzable ATP analogs. $GroEL_{14}$ and $GroES_7$ were preincubated as in Fig. 1*B* for 5 min at 25°C with 1.5 mM ATP and increasing concentrations of ADP (*A*) or AMP-PNP (*B*). Rubisco refolding activity (▲) was measured as in Fig. 1*B* but in the absence (*A*) or presence (*B*) of pyruvate kinase to regenerate the ATP. The fraction (%) of $GroEL_{14}(GroES_7)_2$ (●) and $GroEL_{14}GroES_7$ (○) particles in the chaperonin solution was measured as in Fig. 1*B*. ATPase activity (□) in the presence of ADP or AMP-PNP was expressed as the fraction (%) of the activity in the presence of ATP alone. (*C*) The yield of Rubisco refolding activity in the presence of 1.5 mM ATP and increasing amounts of ADP (◇) or AMP-PNP (◆) was expressed as the number of refolded Rubisco molecules per 100 hydrolyzed ATP molecules.

was in 3-fold excess over ATP, the chaperonin solution was found to be populated with nearly 95% asymmetric $GroEL_{14}GroES_7$ particles, which did not drive the refolding of Rubisco (or mMDH; data not shown) but were, nevertheless, able to hydrolyze ATP at 20% of the rate without ADP. However, when the excess ADP was converted into ATP by subsequent addition of an ATP regeneration system, all the Rubisco refolding activity was recovered and a majority of $GroEL_{14}(GroES_7)_2$ particles was detected in the solution (data not shown). Thus, despite the presence of a saturating amount of ATP, excess ADP can destabilize the symmetric $GroEL_{14}(GroES_7)_2$ particle and cause its dissociation into an asymmetric $GroEL_{14}GroES_7$ particle, which, although able to hydrolyze ATP, does not release bound proteins.

The Coupling Effect of AMP-PNP. In contrast to the negative effect of ADP on the stability of the $GroEL_{14}(GroES_7)_2$ particle and, correspondingly, on the efficiency of the folding reaction, an excess of AMP-PNP over ATP promoted the stability of the $GroEL_{14}(GroES_7)_2$ particle and, correspondingly, the protein folding efficiency (Fig. 3*B*). When measured under the conditions described in Fig. 3*A*, the inhibition constants of the GroEL ATPase in the presence of GroES were 12 μM for AMP-PNP and 50 μM for ADP (30) and the dissociation constant of ATP was 23 μM. Thus, as expected from using a competitive inhibitor with a binding constant similar to that of ATP, the chaperonin ATPase activity was inhibited by AMP-PNP somewhat more efficiently than by ADP. However, Rubisco refolding activity was much less inhibited than expected from the degree of inhibition of the ATPase. Moreover, the efficiency of the reaction was four times higher in the presence of an equimolar amount of AMP-PNP (1.5 mM) and ATP than in the presence of ATP alone (Fig. 3*C*). In comparison, the efficiency of Rubisco refolding was 6.5-fold lower in the presence of an equimolar amount of ADP and ATP than in the presence of ATP alone (Fig. 3*C*). Hence, despite the fact that AMP-PNP and ADP are both nonhydrolyzable analogs that compete with ATP for the same sites on GroEL, AMP-PNP and ADP exert opposite effects on the stability of the $GroEL_{14}(GroES_7)_2$ particle, which translate into opposite effects on the efficiency of the chaperonin-assisted protein folding reaction. This confirms our results with limiting GroES that the two reactions of ATP hydrolysis and protein folding are best coupled in a chaperonin solution populated with a majority of $GroEL_{14}(GroES_7)_2$ particles and are least efficiently coupled in a solution of $GroEL_{14}GroES_7$ particles.

DISCUSSION

We have presented evidence suggesting that the symmetric $GroEL_{14}(GroES_7)_2$ chaperonin heterooligomer is an intermediate, possibly an obligatory intermediate, of the protein folding cycle *in vitro*.

Quantitation of Oligomeric Species in Solution. In the presence of an ATP regeneration system, the distribution of crosslinked chaperonin species is identical whether inferred from SDS gels or from electron micrographs. Thus, the use of SDS gels of crosslinked chaperonin species is as faithful a means to assess the fraction of $GroEL_{14}$, $GroEL_{14}GroES_7$, and $GroEL_{14}(GroES_7)_2$ particles in a chaperonin solution as EM. Furthermore, in the presence of an ATP regeneration system, the ATP-dependent distribution of chaperonin species is similar whether or not the sample was subjected to crosslinking with GA prior to EM (Fig. 1*C*). Thus, crosslinking does not introduce a significant change in the ATP-dependent distribution of the various chaperonin heterooligomers in solution. Moreover, the relatively low affinity of the second $GroES_7$, compared to the first $GroES_7$, for the chaperonin (Fig. 2) and the high sensitivity of the $GroEL_{14}(GroES_7)_2$ particle to ADP (Fig. 3*A*) suggest that the bond of the second $GroES_7$ is highly labile (15). Accordingly, unless the chaperonin is subjected to a prior crosslinking treatment with GA, the bound second $GroES_7$ is likely to be particularly sensitive to changes in the absolute and relative concentrations of ATP, ADP, GroEL, and GroES during gel filtration or preparation of the sample for EM (19, 31).

$GroEL_{14}(GroES_7)_2$ Particle as an Intermediate of the Folding Cycle. As previously shown (15), 20 μM ATP can fully saturate a chaperonin solution with $GroEL_{14}GroES_7$ particles, which, nevertheless, supports only a slow rate of protein folding activity. In contrast, all the Rubisco activity was recovered if additional ATP (150 μM) was subsequently provided (data not shown). Nonnative Rubisco has been shown previously to bind and remain stably bound to ADP-stabilized $GroEL_{14}GroES_7$ particles (20). Since the folding conditions in our experiments were such that nonnative Rubisco aggregates and cannot refold in the absence of chaperonins (7) and since the ATP-regeneration system was fully active even in the presence of as little as 20 μM ATP (data not shown), this suggests that the nonnative Rubisco can bind and remain stably bound to asymmetric $GroEL_{14}GroES_7$ particles even while the chaperonin complex is hydrolyzing ATP. This is confirmed in the presence of a large excess of ATP and an even larger excess of ADP (Fig. 3*A*). Therefore, ATP hydrolysis by asymmetric $GroEL_{14}GroES_7$ particles does necessarily result in the release and subsequent folding of a bound protein. Only when the concentration of ATP was increased, or that of ADP was decreased, did the protein folding activity increase in direct correlation with the amount of $GroEL_{14}(GroES_7)_2$ particles in the solution. Thus, a limiting amount of ATP, or an excess of ADP, can impose a constraint on the stoichiometry of chaperonin heterooligomer, which in turn correlates with a constraint on the ability of the chaperonin to assist protein folding.

While 400 μM ATP was previously reported (15) to be necessary to equally populate a chaperonin solution with $GroEL_{14}GroES_7$ and $GroEL_{14}(GroES_7)_2$ particles, we find here (Fig. 1) that only 50–75 μM ATP is necessary for this effect. This difference is due to the use of an ATP regeneration system, which minimizes the effect of ADP on the destabilization of $GroEL_{14}(GroES_7)_2$ particles.

The GroES-dependent correlation between the refolding of mMDH and the amount of symmetric heterooligomers detected provides additional evidence that $GroEL_{14}(GroES_7)_2$ can be an intermediate of the GroE protein folding cycle. In contrast to mMDH, Rubisco refolding activity was reported to be already saturated at a GroES/GroEL ratio of 0.5 (15). We attribute this difference to the observation that the correct chaperonin-mediated refolding of a mMDH molecule requires between 20 and 100 times more ATP hydrolyzed than a Rubisco molecule (Figs. 2 and 3*C*). Therefore, the refolding of mMDH is expected to be at least 20 times more sensitive than the refolding of Rubisco to substoichiometric amounts of $GroES_7$ and thus to suboptimal amounts of $GroEL_{14}(GroES_7)_2$ particles in the reaction.

We have shown that in terms of ATP hydrolysis the protein folding reaction is most efficient when the symmetric $GroEL_{14}(GroES_7)_2$ heterooligomer is the major species in the chaperonin solution and is dramatically less efficient when the asymmetric $GroEL_{14}GroES_7$ heteroöligomer is the major species. This suggests that, as in the case of the chaperonin ATPase cycle (20, 21), models of the chaperonin folding cycle (12) should include the symmetric $GroEL_{14}(GroES_7)_2$ particle as an intermediate. Moreover, $GroEL_{14}(GroES_7)_2$ particles have been shown by gel-filtration analysis to have a significantly lower affinity for nonnative protein than $GroEL_{14}$ (31), as would be expected from an intermediate involved in the particular step of protein release in the chaperonin cycle.

While evidence from x-ray crystallography of $GroEL_{14}$ suggested that the size of the central cavity can harbor a protein only half the size of a compact Rubisco monomer (32, 33), EM has shown that $GroES_7$ binding significantly increases the size of the cavity (26). In addition, mutant and EM analyses suggested that the protein substrate can bind the inner surface of the central cavity (26, 34) near the unoccupied end of a $GroEL_{14}GroES_7$ particle (26). In contrast, crosslinking experiments have suggested that $GroES_7$ and the protein substrate can bind to the same side of $GroEL_{14}$ (35). Thus, the existence of a transient or stable $GroEL_{14}(GroES_7)_2$ species in the central cavity of which a protein is bound and capped by $GroES_7$ is structurally possible. Our study does not address the dynamics of $GroES_7$ binding during the folding reaction. Therefore, the rates of $GroES_7$ exchange with the chaperonin during protein folding could be such that unfolded proteins bind and are released from the central cavity, undisturbed by the fact that chaperonins spend most of their time as symmetric $GroEL_{14}(GroES_7)_2$ particles.

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