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Minimal and optimal mechanisms for GroE-mediated protein folding

(heat-shock/minichaperone/malate dehydrogenase)

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ABSTRACT **We have analyzed the effects of different components of the GroE chaperonin system on protein folding by using a nonpermissive substrate (i.e., one that has very low spontaneous refolding yield) for which rate data can be acquired. In the absence of GroES and nucleotides, the rate of GroEL-mediated refolding of heat- and DTT-denatured mitochondrial malate dehydrogenase was extremely low, but some three times higher than the spontaneous rate. This GroEL-mediated rate was increased 17-fold by saturating concentrations of ATP, 11-fold by ADP and GroES, and 465-fold by ATP and GroES. Optimal refolding activity was observed when the dissociation of GroES from the chaperonin complex was dramatically reduced. Although GroEL minichaperones were able to bind denatured mitochondrial malate dehydrogenase, they were ineffective in enhancing the refolding rate. The spectrum of mechanisms for GroE-mediated protein folding depends on the nature of the substrate. The minimal mechanism for permissive substrates (i.e., having significant yields of spontaneous refolding), requires only binding to the apical domain of GroEL. Slow folding rates of nonpermissive substrates are limited by the transitions between high- and low-affinity states of GroEL alone. The optimal mechanism, which requires holoGroEL, physiological amounts of GroES, and ATP hydrolysis, is necessary for the chaperonin-mediated folding of nonpermissive substrates at physiologically relevant rates under conditions in which retention of bound GroES prevents the premature release of aggregation-prone folding intermediates from the chaperonin complex. The different mechanisms are described in terms of the structural features of mini- and holo-chaperones.**

Heat-shock proteins GroEL and GroES (the GroE chaperonin system) from *Escherichia coli* are involved in the folding and assembly of newly synthesized polypeptide chains released from the translation machinery and the refolding of stress-denatured proteins (1). *In vitro*, GroEL and GroES carry two complementary activities that prevent heat- and chemical-denatured polypeptides from irreversible aggregation and assist their refolding into native proteins (1–3).

GroEL is a 14 × 57.5-kDa oligomer that has two heptameric rings stacked back-to-back. GroES forms a heptameric ring of identical 10-kDa subunits that can associate on one or two ends of the $GroEL_{14}$ cylinder and form two differently active GroE chaperonin heterooligomers, $GroEL_{14}.GroES_7$ and $GroEL_{14}.(GroES_7)_2$ (4, 5). In the absence of bound nucleotides, the apical domains of GroEL subunits display hydrophobic binding sites with high affinity for the hydrophobic regions of the nonnative protein substrates. The binding sites face the central cavity of the $GroEL_{14}$ cylinder. When nucleotides bind to the $GroEL_{14}.GroES_7$ complex, the volume of the cavity is dramatically increased, and the binding sites become cryptic (6, 7). In the holochaperonin, refolding of protein substrates has been shown to occur underneath the $GroES_7$ cap inside a unique "cis" chamber of the $GroEL_{14}.GroES_7$ oligomer (8), or, more efficiently, inside two chambers of the highly active symmetrical $GroEL_{14}.(GroES_7)_2$ oligomer (5, 9, 10). Although GroEL functions as a double-ring complex (11), a single ring suffices for the mammalian mitochondrial homologue Hsp60 *in vivo* (12). In addition, minichaperones (fragments encompassing the apical domain of GroEL) are effective in refolding several protein substrates *in vitro*, calling into question the importance of the encapsulation within the active $GroEL_{14}.GroES_7$ complexes for all substrates (13). Moreover, the smallest minichaperones (residues 193–335 and 191–345) can, to varying extents, complement temperature-sensitive *E. coli groEL* alleles and supplement low levels of GroEL activity in transformants of *E. coli* in which the *groEL* gene has been deleted (14).

Strict GroEL-, GroES-, and ATP-dependent refolding of a nonnative protein *in vitro* was discovered for urea-denatured RubisCO (2). However, chaperonin-mediated refolding of proteins such as enolase, tryptophanase, rhodanese, and glutamine synthetase can take place in the presence of ADP and nonhydrolyzable ATP analogs (15–17). GroEL-mediated refolding of lactate dehydrogenase, enolase, tryptophanase, and dehydrofolate reductase can occur with ATP but without GroES (15, 16, 18, 19). GroEL minichaperones, despite being monomeric and non-nucleotide-binding, efficiently mediate the refolding of some proteins, such as rhodanese and cyclophilin A (13, 14). Noticeably, the different chaperonin requirements of the various protein substrates were addressed under *in vitro* conditions that often allowed significant levels of spontaneous refolding (15, 20). This has led to various, seemingly contradictory models for a minimal mechanism of GroEL- or minichaperone-mediated protein refolding, some including, or specifically excluding λ GroES interactions and/or nucleotide interactions and/or ATP hydrolysis (15–22). *In vivo*, the GroE-assisted refolding reaction also depends on the nature of the polypeptide substrates (23, 24). Therefore, conclusions about the mechanisms of action of the GroE chaperonin system must be made according to the GroE-dependent or GroE-independent nature of the folding of substrates studied.

To compare the relative importance of each cofactor and the scope of minichaperone activity and to distinguish between minimal and optimal chaperonin requirements, we evaluated here, under strictly nonpermissive conditions, the roles of minichaperones, GroES and nucleotide interaction, and ATP hydrolysis in the specific refolding mechanism of GroEL-

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Abbreviation: mtMDH, mitochondrial malate dehydrogenase.

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bound mitochondrial malate dehydrogenase (mtMDH). mtMDH shows a strict requirement for GroE chaperonin under conditions where spontaneous refolding is extremely low. The apparent rates, as opposed to the yields of mtMDH reactivation, served as a sensitive means of comparing various chaperonin subreactions activated by ATP, ADP, and/or GroES in a concentration-dependent manner. We found that neither GroES binding nor ATP hydrolysis are obligatory steps for the specific reactivation of mtMDH by GroEL. However, nucleotide and GroES binding to a holoGroEL, ATP hydrolysis, and slow GroES-release are essential for driving the GroEL-mediated refolding activity at optimal physiologically relevant rates.

MATERIALS AND METHODS

Proteins. $GroEL_{14}$ and $GroES_7$ from *E. coli* (25) and GroEL minichaperones (13, 14) were purified as described. Pig-heart mtMDH was from Boehringer Mannheim and hexokinase and pyruvate kinase were from Sigma. Protein concentrations were determined by the Bradford protein assay (Bio-Rad) as in ref. 5. In this study, protein concentrations refer to protomers and not to oligomers.

Chaperone Activity Assays. Native mtMDH (0.3 μM) in denaturing buffer [50 mM triethanolamine, pH 7.5/20 mM $MgAc_2$/150 mM KCl/5 mM DTT] was heat-denatured at 47°C as described (3) in the absence or presence of 4 μM GroEL. The rate of mtMDH inactivation was 0.17 nM/min and was independent of the presence of the chaperone. After 30 min at 47°C, less than 1% of the mtMDH remained active. The structure and function of GroEL was not affected by heat treatment (3). Subsequent to the heat shock, the rate of mtMDH reactivation was determined in the absence or presence, as indicated, of GroES and/or ATP, or ADP as follows. The enzymatic activity of mtMDH was measured as in ref. 26 at 25°C in folding buffer [150 mM potassium phosphate, pH 7.5/10 mM DTT/0.5 mM oxaloacetate/0.28 mM NADH (Sigma)]. In experiments containing ATP, 7 μg/ml pyruvate kinase and 3 mM phospho(enol)pyruvate (both from Sigma) were supplemented to regenerate the ATP. The time-dependent oxidation of NADH by mtMDH was monitored at 340 nm. Native mtMDH dimers remain stable and active in solutions above 6 nM (protomers; ref. 27). With the exception of the very low rate of spontaneous refolding, apparent rates of mtMDH reactivation were calculated from the time-dependent recovery of mtMDH activity in solutions containing at least 6 nM active mtMDH, at which the enzymatic activity is in good correlation with the amount of refolded enzyme. Rates of mtMDH reactivation were expressed in nM/min.

Addition of Mn^{2+} Ions to the Chaperonin Reaction. $MnCl_2$ (2 mM) was supplemented at 25°C after the mtMDH denaturation (Table 1; Fig. 4). Mn^{2+} ions had no effect on the activity of native mtMDH, the spontaneous reactivation of mtMDH without chaperonins, or the chaperonin-mediated reactivation of GroEL-bound mtMDH without nucleotides (data not shown).

Light Scattering Experiment. The aggregation of 0.3 μM mtMDH in denaturing buffer in the presence or absence of GroEL (4 μM) or minichaperones (10 μM) was followed at 550 ± 5 nm (Cary 500 Scan apparatus; Varian) at 47°C for 20 min. Under these conditions, no light-scattering was observed for GroEL alone or minichaperones alone (data not shown).

RESULTS

Chaperonin-Assisted Reactivation of Heat-Denatured mtMDH. When mtMDH was heat-denatured in the presence of DTT and GroEL (4 μM) and incubated at 25°C with saturating amounts of GroES (12 μM) and a saturing amount of ATP (1 mM), mtMDH was rapidly reactivated. About 75% of the mtMDH was recovered within 30 min at an apparent rate of 18.6 nM/min, which was 465-fold faster than without GroES and ATP and ≈1,700-fold faster than without GroEL (Fig. 1; Table 1). The background of spontaneously refolded mtMDH without chaperonins was less than 4% after 24 hr (Table 1).

The high dependency of the mtMDH refolding reaction on the presence of GroEL during and after the heat shock permitted further addressing of the individual roles of ATP, ADP, and GroES under conditions of limiting but significant chaperonin activity. mtMDH refolding was ≈4 times faster in the presence of GroEL alone than without GroEL. The presence of saturating amounts of ADP with GroES or ATP without GroES accelerated 11- and 17-fold, respectively, the rate of GroEL-mediated refolding (Table 1). Hence, neither ATP hydrolysis nor GroES interaction are obligatory requirements for the specific refolding of mtMDH by $GroEL_{14}$.

Light scattering showed that minichaperones suppress the aggregation of mtMDH at 47°C (data not shown), indicating that minichaperones bind and prevent the aggregation of denatured mtMDH during heat stress. However, the rate of minichaperone-bound mtMDH reactivation after heat stress was equal to or lower than the spontaneous rate of mtMDH reactivation (data not shown).

Effect of ATP or ADP Concentrations on mtMDH Reactivation. We next examined the effect of increasing nucleotide concentrations on yields (Table 1) and the apparent refolding rates (Fig. 2) of the chaperonin reaction with and without GroES. In the presence of GroES, saturating amounts of ATP improved the yields (4.5-fold) and the rate (42-fold) compared

Table 1. Rate and yields of mtMDH and barnase reactivation

Present during denaturation	Supplemented after denaturation							
GroEL	ADP	ATP	Mn^{2+}	GroES	Rate, nM/min	Relative rate, mtMDH	Relative rate, barnase†	mtMDH yield, %
−	−	−	−	−	~0.011	~0.3		4
+*	−	−	−	−	0.040	1	1	6
+	+	−	−	−	0.090	2.2	3.2	6
+	+	−	+	−	0.235	6	—	—
+	+	−	−	+	0.445	11	8 (8)‡	17
+	−	+	−	−	0.674	17	15	25
+	−	+	−	+	18.6	465	100 (18)‡	78

Apparent rates of mtMDH reactivation (nM/min) were relative to the reference baseline rate, *. Conditions were as in Fig. 1–3. The following components were added during mtMDH denaturation at 47°C or renaturation at 25°C: 4 μM GroEL, 12 μM GroES, 1 mM ADP, 1 mM ATP, and 2 mM Mn^{2+}. Refolding yields for mtMDH were determined after 24 hr and expressed as % of the initial native mtMDH. When ATP was present, the ATP regeneration system was active for at least 5 hr without GroES and 10 hr with GroES.

†Relative rate constants at 25°C for the refolding of barnase (1 μM) when the denatured form is bound to GroEL (generally 2 μM $GroEL_{14}$) and the other reagents added in 100 mM Mes (pH 6.3), 2 mM KCl, and 2 mM $MgCl_2$ as described (34).

‡GroES/GroEL = 0.5, otherwise = 1.

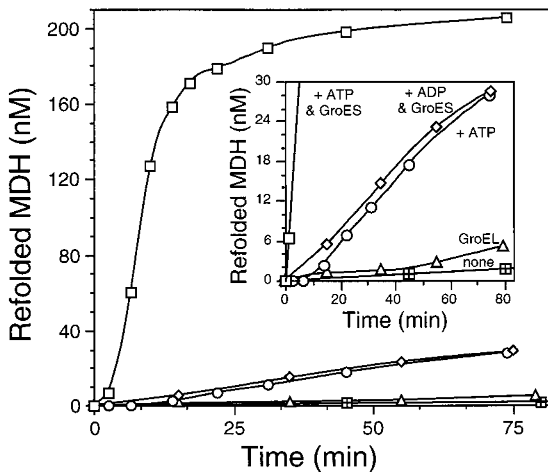

FIG. 1. Chaperonin-assisted reactivation of heat-denatured mtMDH. mtMDH (0.3 μM) was heat- and DTT-denatured in the absence (⊞), or in the presence of GroEL (4 μM). The time-dependent reactivation of the MDH was measured at 25°C without additional cofactors (△) or with supplemented ATP (1 mM; ○), GroES (12 μM) and ADP (1 mM) (◇), or GroES (12 μM) and ATP (1 mM; □). (*Inset*) Threefold enlargement of the slow kinetics.

with saturating amounts of ADP (Table 1). Without GroES and nucleotides, the refolding rate was low but significantly higher than the rate without chaperonins, and increasing concentrations of nucleotides consistently increased the refolding rates. The activating effects of nucleotides and GroES were synergistic. Half of the apparent V_{max} was reached without GroES at 117 μM ATP and 315 μM ADP and with saturating GroES at 14 μM ATP and 47 μM ADP (Fig. 2). Thus, GroES can reduce by about a factor of seven the nucleotide-requirement of the refolding reaction. Noticeably, a protomer ratio of GroES/GroEL = 0.5, as used in many *in vitro* chaperonin activity assays (21, 28), was clearly rate-limiting at all nucleotide concentrations. However, the yields of the ATP reaction were identical in the presence of limiting or saturating amounts of GroES (data not shown), indicating that the refolding rate is a more sensitive tool than yields for comparing limiting factors of the chaperonin reaction.

Effect of the GroES Concentration on mtMDH Reactivation. In the presence of ATP (1 mM) but not GroES, up to 25% of the GroEL-bound mtMDH was recovered. GroES tripled the yield and improved by 28-fold the rates of the reaction with GroEL alone (Table 1). To investigate further the GroES requirement of the GroEL chaperonin reaction, we examined the effect of increasing GroES concentrations on the apparent refolding rates of mtMDH reactivation (Fig. 3).

At saturating concentrations of ATP, increasing amounts of GroES activated the mtMDH refolding rates in an apparently cooperative manner, suggesting that optimal folding occurs under conditions in which more than one $GroES_7$ oligomer binds to the mtMDH–$GroEL_{14}$ (Fig. 3). Thus, when the GroES/GroEL ratio was 0.25, at which $GroEL_{14}.GroES_7$ and $GroEL_{14}$ complexes are about equimolar (5), the refolding rate was only 14% and not 50% of the V_{max}, as expected if the asymmetric $GroEL_{14}.GroES_7$ oligomer was the only optimally active protein-folding species (21, 28). Moreover, an inflection in the refolding rate was consistently observed around a GroES/GroEL ratio of 0.75, further suggesting the presence of more than one active chaperonin species (Fig. 3). V_{max} was nearly reached at GroES/GroEL = 1, showing that maximal refolding of mtMDH was achieved with equimolar GroES and GroEL, as in the cell (29). The first-order rate constants for the refolding of GroEL-bound barnase displayed a GroES-dependent behavior that paralleled the rates of mtMDH reactivation (Table 1). The rate constants for barnase refolding in the presence of ATP was also optimal for GroES/GroEL ratios of 1 or greater (30), when the chaperonin solution is populated by a majority of $GroEL_{14}.(GroES_7)_2$ and a minority of $GroEL_{14}.GroES_7$ particles (5, 9, 31).

At saturating concentrations of ADP, the rate of mtMDH refolding increased in a monophasic manner with the GroES concentration (Fig. 3). The V_{max} was reached at a GroES/GroEL ratio of 1. Together with previous evidence that

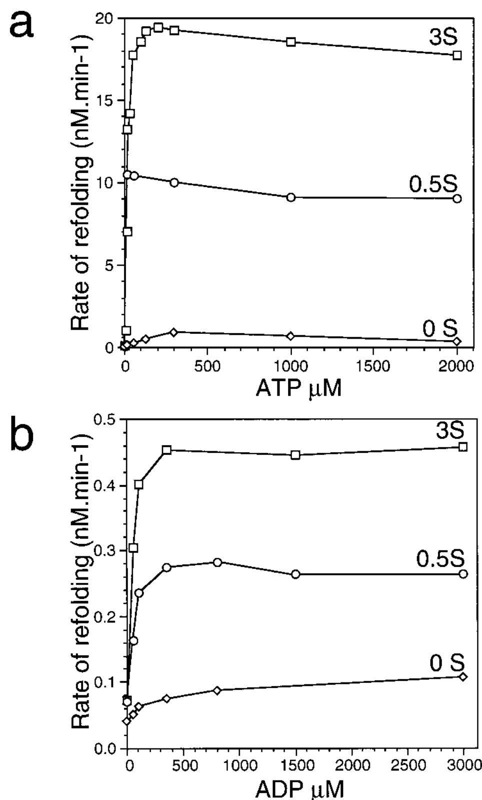

FIG. 2. Effect of ATP or ADP concentrations on the rates of mtMDH reactivation. The apparent rates of GroEL-mediated reactivation of mtMDH (4 μM GroEL, 0.3 μM mtMDH) was measured as in Fig. 1, in the presence of increasing concentrations of ATP (*a*) or ADP (*b*), without GroES (0 S), with limiting GroES (0.5 S; 2 μM), or with saturating amounts of GroES (3 S; 12 μM).

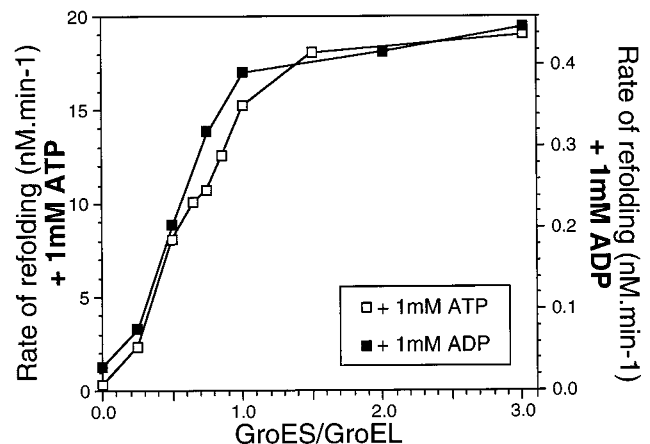

FIG. 3. Effect of the GroES concentration on the rate of mtMDH reactivation. The apparent rates of GroEL-mediated reactivation of mtMDH (4 μM GroEL, 0.3 μM mtMDH), was measured as in Fig. 1 in the presence of 1 mM ATP (□), or 1 mM ADP (■) and increasing concentrations of GroES.

asymmetric $GroEL_{14}.GroES_7$ particles are the predominant species in an ADP-containing chaperonin solution (5, 9), this confirms that ADP-formed $GroEL_{14}.GroES_7$ particles refold proteins such as mtMDH and barnase but do so at rates that are 40 and 13 times lower, respectively, as with ATP (Table 1).

Effect of Mn^{2+} Ions on GroE-Mediated Refolding of mtMDH. It was then of interest to test the effect of Mn^{2+} ions on GroE-mediated refolding of mtMDH because Mn^{2+} ions significantly increase the affinity of ATP and ADP for $GroEL_{14}$ (26). The rate of (GroEL + ADP)-mediated refolding of mtMDH was nearly tripled by the addition of 2 mM Mn^{2+} (Table 1; Fig. 4). Mn^{2+} ions (as GroES; Fig. 2) enhanced the rate of mtMDH refolding by increasing the apparent affinity of nucleotides for the chaperonin complex.

Moreover, at saturating amounts of ATP, Mn^{2+} ions strongly increase the affinity of $GroES_7$ for mtMDH–$GroEL_{14}$ while concomitantly increasing the rate of mtMDH refolding and decreasing the rate of ATP hydrolysis (26). This observation was exploited to address the role of GroES release during active GroEL-assisted refolding of mtMDH by using a large excess of free $GroEL_{14}$ as a trap for $GroES_7$ and for mtMDH intermediates that may be released from the chaperone during the reaction (Fig. 4). The GroEL-mediated refolding rate of mtMDH in the presence of near-limiting Mg^{2+} (6 mM; ref. 9) and equimolar amount of GroES (Fig. 3) was expectedly lower (5.1 nM/min) than optimal (Table 1). The addition of a 6.25-fold excess of free GroEL rapidly reduced the rate of mtMDH refolding to 2 nM/min (Fig. 4). This inhibition implies that the trap of free $GroEL_{14}$ can rapidly bind the free $GroES_7$ and possibly the mtMDH intermediates released from the initial complex created during the folding reaction. In contrast, when 2 mM Mn^{2+} ions were added together with 1 mM ATP before the addition of the GroEL trap, the refolding rate was expectedly higher (7.6 nM/min) than without Mn^{2+} (Fig. 4; see ref. 26). Remarkably, this rate remained unchanged by the GroEL trap. Hence, for up to 12 min after the addition of the GroEL trap, a significant fraction (23%) of mtMDH continued to be reactivated at the same rate (7.6 nM/min) as mtMDH without the GroEL trap (Fig. 4).

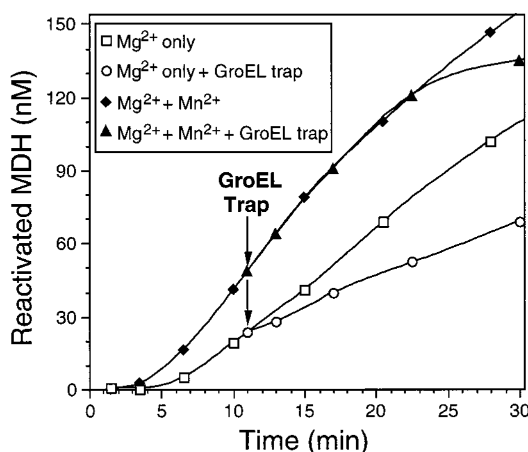

FIG. 4. Effect of Mn^{2+} ions on GroEL/GroES-mediated refolding of mtMDH. The time-dependent reactivation of GroEL-bound mtMDH (4 μM GroEL, 0.3 μM mtMDH) was measured in the presence of 1 mM ATP and an ATP-regeneration system [equimolar (4 μM) GroES] in folding buffer as in Figs. 1–3 but with a near-limiting 6 mM concentration of divalent ions instead of 20 mM Mg^{2+}. Open symbols: 6 mM Mg^{2+}. Filled symbols: 4 mM Mg^{2+} and 2 mM Mn^{2+}. A 6.25-fold excess of free GroEL (22 μM) was added 11 min after initiation of reactivation at 25°C with (○, ▲) or without (□, ◆) ATP added to the reaction.

DISCUSSION

A fundamental problem in protein folding is the generation of metastable states that are "sticky" and have a tendency to aggregate and then precipitate. These states can be misfolded states in biosynthesis that must unfold to refold productively, folding intermediates that are kinetically trapped, or denatured or partly denatured states that arise from stresses such as heat shock. It is thus reasonable to speculate that the basic and minimal requirement for the mechanism of GroEL-assisted protein folding is to provide a hydrophobic surface that can bind the sticky folding intermediates or misfolded states (13, 32), eventually causing the unfolding of compact states (33) and sequestering them to prevent their aggregation, giving them an additional chance to fold successfully (30, 34). The complex structure of GroEL and the precise nature of its active site are refinements to optimize this function by enabling it to modulate the tightness of binding (Fig. 5).

Minimal Mechanism of GroEL-Mediated Protein Folding. Here we confirm that neither ATP hydrolysis nor GroES

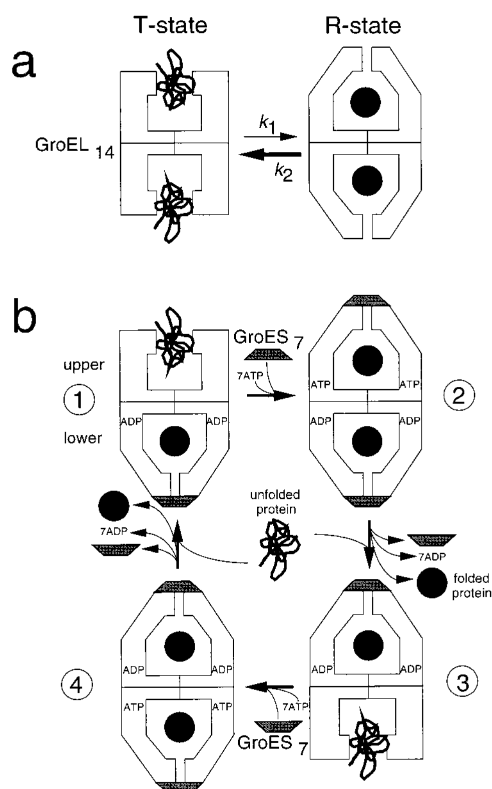

FIG. 5. Models for specific GroE-assisted refolding of proteins. (*a*) Minimal mechanism for the chaperonin activity without GroES and nucleotides. A single $GroEL_{14}$ oligomer is preferentially in a low-energy T state, in which it can bind up to two nonnative proteins at the apical domain of the two cavities. The stable T state, which can rarely convert into a high energy R state ($k_1 \ll k_2$) with lifted apical domains that release the proteins within the enlarged central cavities and initiate refolding. Factors (such as nucleotide exchange and GroES exchange, urea, or temperature) can accelerate the transition rate between the T and R states and thus favor multiple cumulative steps of protein folding to take place in the cavities of the R state. (*b*) Optimal mechanism for the chaperonin activity with GroES and ATP. An asymmetrical protein–$GroEL_{14}.GroES_7$ oligomer can bind an unfolded protein in the trans ring that is in the T state. Binding of seven ATPs and $GroES_7$ to the trans ring forms an all-R state $(protein)_2$–$GroEL_{14}.(GroES_7)_2$ oligomer. Hydrolysis of seven ATPs in the upper ring causes the release of one $GroES_7$, seven ADPs and the refolded protein in the lower ring, thus reforming a protein–$GroEL_{14}.GroES_7$ oligomer with the lower trans ring in the T state ready to bind a new unfolded protein.

interactions are essential to the minimal mechanism of GroEL, as described (4, 21). The activity of the minichaperones resulting from their exposed hydrophobic surface is reflected in their specificity of action *in vitro* (13). Minichaperones enhance the refolding of proteins that have a significant background of spontaneous refolding, such as rhodanese, cyclophilin A, and glyceraldehyde-3-phosphate dehydrogenase (unpublished data). Although minichaperones interact with the nonpermissive substrate (heat-denatured mtMDH) they do not assist in its reactivation after the stress (data not shown), in contrast to holo$GroEL_{14}$ (Fig. 1; Table 1). Therefore, in absence of any cofactor, our model for GroEL-assisted refolding of nonpermissive substrates (Fig. 5*a*) involves two states of the $GroEL_{14}$ molecule: a stable T state, in which exposed hydrophobic surfaces on the apical domains can cooperatively and strongly bind denatured proteins, and an unstable R state, in which the apical domains are lifted to form two enlarged central chambers (30, 35). The lifting of the apical domains masks the hydrophobic binding surfaces (7), thereby releasing the bound protein into the enlarged central chambers of the chaperonin, where refolding initiates. Because intra-GroEL protein release and protein folding takes place in the R state (8) and tight binding takes place only in the T state, successful refolding requires transitions between the two states. Monomeric minichaperones are not capable of similar transitions.

For nonpermissive substrates, the ability to undergo recurrent transitions between high- and low-affinity states (such as the T and R states of $GroEL_{14}$) is necessary for the minimal mechanism of GroEL-mediated protein folding. Therefore, conditions that increase the stability of one state over the other may limit the rate of protein refolding. Hence, in the absence of nucleotides and GroES, the T state is very stable, and conversions to the R state are very rare ($k_1 \ll k_2$). But, mild destabilization of the T state by nondenaturing chemicals (36) or by temperature (22) promotes transitions between the two states and, consequently, efficient protein refolding. Hence, conditions that decrease the stability of the T state or increase up to a certain point the stability of the R state can reach an optimum at which the stability of the two species becomes similar ($k_1 = k_2$), resulting in optimal rates of transition between the two states and therefore of protein refolding. The binding of $GroES_7$ (Fig. 3) or of Mn^{2+}-nucleotides (Fig. 4) to the chaperonin complex, which is known to stabilize the R state (37), indeed does promote refolding. However, high concentrations of GroES (above the physiological equimolar ratio with GroEL) inhibit refolding rates of lactate dehydrogenase (3), suggesting that overstabilization of the R state by excessive binding/rebinding of $GroES_7$ ($k_1 > k_2$) also can limit the rate of transitions between two states.

In contrast to the nonphysiological conditions of a minimal reaction without GroES and nucleotides, intact GroEL is in equimolar concentration with GroES in the cell, and an equilibrium of 2–10 mM ATP and 0.5–1 mM ADP is maintained by an ATP regeneration system (29). In addition, minichaperones cannot complement the complete loss of GroEL *in vivo* (14), consistent with the known essential role of GroEL (1). Thus, a more elaborate and efficient GroEL-mediated refolding mechanism is likely to take place in the cell.

Optimal Mechanism of GroEL-Mediated Protein Folding. In current models of chaperonin action, GroES binding and release is driven by successive rounds of ATP hydrolysis in the two rings of the $GroEL_{14}$ oligomer (9, 30, 38, 39). Different proteins may require various numbers of folding steps and therefore, of transitions between the T and R states of $GroEL_{14}$, to finally reach a native conformation. Fig. 5*b* shows a model for the optimal mechanism of GroEL-mediated refolding. An asymmetric protein-$GroEL_{14}$.$GroES_7$ particle, containing one folding protein in the lower "cis" chamber from a previous cycle (Fig. 5*b*, species 1), binds a second unfolded protein in the upper ring in the T state. Binding of seven ATPs and $GroES_7$ to the upper ring discharges the bound protein from the hydrophobic regions of the apical domains and initiates folding in the enlarged upper cavity in the R state (37). The intermediate formed is a highly active near-symmetrical $protein_2$–$GroEL_{14}$.$(GroES_7)_2$ oligomer containing seven ADPs in the lower ring and seven ATPs in the upper ring (10), which can concomitantly assist two protein molecules, possibly at different stages of folding, in the two enclosed R chambers (Fig. 5*b*, species 2; refs. 5, 10, and 40). ATP hydrolysis in the upper ring releases from the lower ring seven ADPs, one $GroES_7$, and the first refolded protein. A stable asymmetric protein–$GroEL_{14}$.$GroES_7$ complex is thus reformed, which may enclose the second folding protein in the upper chamber (Fig. 5*b*, species 3). This complex can then bind a third unfolded protein in the lower ring or rebind incompletely folded protein intermediates and repeat the cycle, while reforming a new transient, highly active symmetric $protein_2$–$GroEL_{14}$.$(GroES_7)_2$ intermediate (Fig. 5*b*, species 4).

By assuming that a protein like mtMDH requires a given amount of time to reach a folded state within the chaperonin chamber, our results suggest that this is achieved in two nonmutually exclusive manners: (*i*) in multiple, cumulative steps under suboptimal conditions (e.g., with GroES but without Mn^{2+}), when the slower rates of folding become dependent on optimal T-R transition rates ($k_1 = k_2$) or (*ii*) in as little as a single step under optimal conditions, when the number of transitions between T and R states may be as low as one (to allow initial binding, then final release), as also described for the efficient refolding of RubisCO (11).

Interestingly, we observed optimal refolding of mtMDH when $GroES_7$ does not rapidly exchange with excess of the free $GroEL_{14}$ trap (Fig. 4), suggesting that complete release of all of the bound $GroES_7$ is not obligatory for efficient refolding inside the chaperonin chamber. This result corroborates with previous reports of active refolding *in vitro* within the cis chamber of $GroEL_{14}$.$GroES_7$ oligomers (8) and within single ring SR1 mutants with no $GroES_7$ dissociation (11, 21). Thus, under physiological conditions, effective refolding, when intermediates overcome kinetic barriers by repeated cycles of "iterative annealing" (39), may not necessarily have to take place in an uncapped $GroEL_7$ toroid, but also within the closed chamber of $GroEL_7$ strongly capped by $GroES_7$.

In the GroEL quench experiment (Fig. 4), efficient mtMDH reactivation despite excess GroEL implies that a minimal amount of capping $GroES_7$ oligomers was released to allow the dissociation of folded mtMDH from the chaperonin, but that this released GroES was not needed subsequently to assist in the refolding of the remaining GroEL-bound mtMDH. The ATP hydrolysis is considered to act as the timer (≈20 sec) that initiates GroES release and subsequently the delivery of folded substrate (11). Our findings suggest that the folding chamber can detect the folded state of a protein therein and signal for $GroES_7$ release once the protein is fully folded. The presence of such a signal is supported by the observation that without $GroES_7$, the ATP-driven reactivation of GroEL-bound MDH produced 75% of kinetically trapped MDH species but only 22% in the presence of capping $GroES_7$ (Table 1). Hence, under conditions *in vitro* comparable to those encountered *in vivo*, $GroES_7$ carries the complementary roles of controlling the formation and the stabilization of folding chambers in the R state and preventing premature dissociation and aggregation of early folding intermediates from the chaperonin complex.

Structural and Mechanistic Considerations. We can rationalize minimal and optimal mechanisms for GroEL action by using the structural model of (32) as a framework. The crystal structure of the minichaperone sht-GroEL(191–376) revealed a hydrophobic and flexible binding site for substrates. There is a tension at the active site of GroEL and of minichaperones to stretch a bound denatured hydrophobic patch into an extended

β-strand conformation, as detected in $^1H/^2H$-exchange experiments on barnase (33).

The minichaperone was built into the intact GroEL structure, where it could be seen that the binding sites formed a ring around the inside top rim of the GroEL cylinder (32). A ring of subsites would have a great avidity for substrates that occupied multiple subsites. Indeed, a single molecule of denatured barnase binds to GroEL with high affinity (34), presumably because multiple subsites are occupied. Conversely, further molecules of denatured barnase bind weakly because they compete for the subsites, and so each barnase molecule binds to fewer. The structures of GroEL (the T state) and its complexes, determined by electron microscopy, show that the ring of contiguous binding sites in GroEL is greatly expanded into a crown-like structure on the binding of ATP or ATP and GroES in the R states (6). The forcing apart of subsites would weaken the binding of substrates, as found experimentally for barnase, for example (30). Thus, the multimeric and allosteric nature of GroEL provides an exquisite system for regulating its avidity for denatured states. Conversely, the monomeric structure of the minichaperone gives a weaker avidity for substrates that cannot be regulated.

CONCLUSION

Different substrates have different requirements for being chaperoned *in vitro* (15, 20) and *in vivo* (23, 24). There is a spectrum of mechanisms for GroE-mediated protein folding, with minichaperones at one end and the full GroES + GroEL + ATP chaperonin machine at the other. Permissive substrates, which require just a temporary parking spot that prevents their aggregation and allows them the luxury of more time to refold, are chaperoned by minichaperones or even by GroEL (13). Conversely, nonpermissive substrates, such as heat-denatured mtMDH, require tight binding to multiple subsites, followed by their release on binding of cofactors. As such, the nonpermissive substrates will follow the mechanism of Corrales and Fersht (30, 41) or its analogous counterparts (39, 42), which have multiple rounds of weak and tight binding as ATP is hydrolyzed and GroEL switches between its R and T states. Intact GroEL operates by the optimal mechanism, whereas minichaperones operate by the minimal mechanism.

The yield of any refolded protein substrates critically depend on the precise folding pathways, which are greatly influenced by the experimental conditions. For example, detergents and hydrophobic proteins other than GroEL (e.g., bovine and human serum albumin, calmodulin), capable of interacting with aggregation prone folding intermediates, should have varying degrees of chaperoning capacity by exemplifying the minimal mechanism of GroEL.

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