The molecular chaperones GroEL and GroES facilitate protein folding in an ATP-dependent manner under conditions where no spontaneous folding occurs. It has remained unknown whether GroE achieves this by a passive sequestration of protein inside the GroE cavity or by changing the folding pathway of a protein. Here we used citrate synthase, a well studied model substrate, to discriminate between these possibilities. We demonstrate that GroE maintains unfolding intermediates in a state that allows productive folding under nonpermissive conditions. During encapsulation of non-native protein inside GroEL-GroES complexes, a folding reaction takes place, generating association-competent monomeric intermediates that are no longer recognized by GroEL. Thus, GroE shifts folding intermediates to a productive folding pathway under heat shock conditions where even the native protein unfolds in the absence of GroE.

Molecular chaperones are known to play a major role in protein folding in the cell. One of the best characterized chaperones is the GroEL/GroES system from Escherichia coli. In vivo, the GroE system is essential for viability (1). It seems to be involved in the folding of ~5–10% of the polypeptide chains to their native, three-dimensional structure (2, 3). Under stress conditions, the GroE chaperones maintain viability by stabilizing unfolding proteins or by keeping unfolding intermediates in a reactive state and preventing irreversible side reactions like aggregation (4–7). This allows refolding of the bound intermediates after restoration of permissive folding conditions.

GroEL is a tetradecameric molecule consisting of two heptameric rings of identical subunits stacked back to back (8, 9). The GroEL double-ring cylinder has two equivalent substrate-binding sites (10) on the inner top of its central channel and an ATP-binding site in each subunit (9, 11). Substrate binding takes place via hydrophobic (10, 12, 13) and electrostatic (14, 15) interactions. For productive binding and release cycles of GroEL-associated substrate proteins, ATP binding and hydrolysis are necessary (16). These events lead to conformational changes in the GroEL molecule (17–19), thus lowering the substrate affinity in the GroEL rings (10, 20, 21). The presence of GroES, the heptameric ring-shaped co-chaperone of GroEL, together with ATP is required for increasing the efficiency of substrate folding and for folding under nonpermissive conditions (22, 23). This seems to be due to the ability of GroE to partially unfold kinetically trapped folding intermediates, thus giving these species a new chance to fold (23–25). During the folding process, the GroE system transiently encapsulates a single polypeptide chain in the central cavity of GroEL-GroES complexes, allowing folding in a protected environment, isolated from other polypeptide chains (26, 27). After one cycle of ATP binding and hydrolysis, i.e. every 15–30 s, the bound substrate is ejected from these so-called "cis-complexes" (25, 26, 28) independent of their folding state (29). Folding in cis-complexes is restricted by the size of the central cavity of the GroEL-GroES complexes to polypeptides smaller than 60 kDa (3, 30).

Under thermal stress conditions, GroEL is able to stabilize rhodanese, resulting in a deceleration of the inactivation kinetics (31). Other substrates, however, are not stabilized, but they are bound by GroEL during heat inactivation and kept in a reactivable state (4, 5, 7). After shifting the conditions from nonpermissive to permissive, in all the cases investigated, the bound substrate can be efficiently refolded in the presence of ATP or GroES/ATP. Experiments in which proteins are bound to GroE and subsequently reactivated under permissive conditions are important to gain information about in vitro properties of GroE. However, how the complete GroE chaperone system is able to fold proteins under nonpermissive conditions such as heat shock is not yet understood. The question that remains is how proteins are folded by GroE in vivo under conditions where denaturation prevails in its absence.

Here, we used CS to investigate the activity of the GroE system under conditions where the substrate loses its activity rapidly, due to unfolding and subsequent aggregation. After we had shown that GroEL binds dimeric and monomeric unfolding intermediates of CS (32), we were interested in analyzing how the GroE machinery is able to shift the unfolding pathway of CS toward the native state, thus maintaining the native conformation of a substrate protein under unfolding conditions. Our first observation was that the complete GroE system changed the inactivation kinetics of CS dramatically. Of importance, folding inside cis-complexes is essential to produce monomeric intermediates of CS, which are committed to fold and associate to the native dimeric state even under nonpermissive conditions. Shifting of monomeric CS intermediates from the unfolding pathway to an alternative folding pathway ensures that active protein is formed in the cell even under unfavorable environmental conditions.

MATERIALS AND METHODS

Purification of Proteins—GroEL and GroES were purified from E. coli strain JM109 T7136 bearing the multicopy plasmid DHarOF39 as described previously (33). The GroEL single-ring mutant SRI (34) was purified from E. coli strain BL21(DE3) PlysS bearing the plasmid pTrc99a according to the protocol for wild-type GroEL (33). The concentrations of these proteins were determined spectrophotometrically in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: CS, citrate synthase; wtGroEL, wild-type GroEL; HPLC, high performance liquid chromatography; Rubisco, ribulose-bisphosphate carboxylase/oxygenase.
using the following extinction coefficients: $E_{276}^1$ = 0.142 for GroES (33) and $E_{276}^1$ = 0.173 for GroEL and $E_{276}^1$ = 0.193 for SR1 (calculated according to Ref. 35). The extinction coefficients used for the calculation of GroEL and SR1 concentrations were corrected for minor tryptophan impurities present in the solution of the purified proteins as determined by a titration of the tryptophan fluorescence (36). In addition, the GroEL and SR1 absorbance spectra were corrected for intrinsic light scattering of the solution due to the particle size of the protein complexes. Mitochondrial CS from porcine heart (citrate synthase, EC 4.1.3.7) was obtained from Roche Molecular Biochemicals (Mannheim, Germany) and treated as described (37). CS concentration refers to dimers, and GroEL concentration and SR1 and GroES concentrations refer to the 14-mer and 7-mers, respectively. Apyrase (grade VI) was obtained from Sigma.

Inactivation of CS—CS (7.5 μM) was diluted 1:100 into 50 mM Tris-HCl, pH 8.0 (25 °C), 10 mM KCl, 10 mM MgCl2, and 1 mM dithioerythritol in the presence of ATP (2 mM) or various concentrations of different GroE components (as indicated in the figure legends) at 25 °C. Inactivation was initiated by a temperature shift to 40 °C. To determine the inactivation kinetics, aliquots were withdrawn at the indicated time points, and CS activity was measured at 25 °C as described (38).

Formation of SR1-CS Complexes—To form SR1 complexes with bound monomeric CS, CS (0.075 μM) was incubated at 43 °C in the presence of SR1 (0.2 μM) for 90 min (32). After shifting the temperature to 25 °C (as indicated in the figure legends), SR1-GroES-ATP complexes were formed by addition of GroES (0.3 μM) and ATP (2 mM). To dissociate these cis-complexes, the samples were incubated on ice for 30 min (39). After a further temperature shift to 25 or 40 °C, the activity was determined as described above.

Formation of wtGroEL-GroES-CS Complexes—To bind monomeric CS intermediates to wtGroEL, CS (0.075 μM) was incubated at 43 °C in the presence of GroEL (0.1 μM) and GroES (0.2 μM) for 90 min (32). After adjusting the temperature to 25 °C, ATP (200 μM) was added to allow binding of GroES. After a further 20 s, apyrase (8 units) was added to hydrolyze the ATP to ADP and AMP. To dissociate the GroEL$_{14}$-GroES$_{7}$-CS complexes, the samples were incubated on ice for 30 min. The end points of reaction were determined after 120 min of incubation at 25 °C.

Data Analysis—Rate constants for the unfolding and refolding kinetics of CS were obtained from nonlinear fits using SigmaPlot 4.0 (Jandel Scientific). Rate constants and equilibrium constants for association or for association followed by unimolecular folding reactions were determined with the corresponding models using the program Scientist (MicroMath Science Software).

RESULTS

Influence of the GroE System on the Thermal Unfolding of CS—In the presence of GroEL, the native CS dimer unfolds thermally via inactive dimeric intermediates, which dissociate into monomers. These monomers were stably associated with GroEL and kept in a reversible form at elevated temperatures. For efficient refolding under permissive conditions (i.e. lower temperature), the co-chaperone GroES and ATP are obligatory (32). However, under heat stress in vivo, it is not sufficient to only bind the denaturing polypeptides to GroEL; a stress period is also required. Therefore, we investigated how the GroE system is able to keep the substrate protein in its native state during heat stress. First, we inactivated CS at 40 °C in the presence of ATP and equimolar amounts of GroEL and titrated the GroES concentration from substoichiometric amounts to a 4-fold molar excess (Fig. 1A). The apparent time course of inactivation of native CS was slowed down with increasing concentrations of GroES. At a 2-fold molar excess of GroES$_{7}$ to

![FIG. 1. Stabilization of CS under nonpermissive conditions by the GroE chaperone system. A, dependence of the unfolding kinetics of CS at 40 °C on the GroES/GroEL ratio. CS (0.075 μM) was inactivated at 40 °C in the presence of 2 mM ATP ( ). GroES was titrated from substoichiometric amounts to a 4-fold molar excess compared with GroEL (0.075 μM): 0.0575 ( ), 0.075 ( ), 0.11 ( ), 0.15 ( ), and 0.3 ( ) μM GroES. Inset, dependence of the two apparent rate constants of the inactivation kinetics of CS at 40 °C on the GroES/GroEL ratio. B, dependence of the influence of GroE on the thermal inactivation of CS on the ratio of CS to GroE. CS (0.075 μM) was inactivated at 40 °C in the presence of increasing concentrations of GroE. The GroEL concentrations used were 0.025 ( ), 0.05 ( ), 0.075 ( ), 0.15 ( ), 0.2 ( ), and 0.3 ( ) μM. The stoichiometry between GroEL$_{14}$ and GroES$_{7}$ was held constant at 1:2. Inset, dependence of the two apparent rate constants of the inactivation kinetics of CS at 40 °C on the ratio of CS to GroEL.

GroEL$_{14}$, the apparent stabilization of CS reached a plateau (Fig. 1A, inset). Thus, the efficiency of the GroE system in stabilizing CS depends strongly on the GroES concentration. Interestingly, under the conditions used, the inactivation of CS can be described by two exponential functions, of which only one is influenced by the GroES concentration (see below). The GroES dependence indicates that GroEL-GroES complexes are involved in stabilization of CS. Furthermore, this points to the involvement of CS monomers in the stabilization of CS because dimeric CS molecules are too large to fit in the central cavity of GroEL-GroES complexes (cf. Ref. 32).

Influence of the Ratio of GroEL to CS on the Inactivation of CS—The above-described effects of GroE on CS inactivation can be explained by two models. First, GroE may shift the equilibrium between monomeric intermediates and the native enzyme toward the native state by populating monomeric intermediates and preventing the drain of protein by irreversible aggregation. Second, GroE could shift intermediates from the unfolding pathway to a completely different, productive folding pathway. To test these models, we investigated the influence of the GroEL concentration on CS inactivation. To this end, the GroEL$_{14}$/GroES$_{7}$ ratio was held constant (at a 2-fold molar excess of GroES to GroEL), and the GroEL concentration was increased up to an 8-fold molar excess compared with CS. Interestingly, the inactivation of CS was decelerated in the presence of increasing amounts of the GroE system (Fig. 1B). This is difficult to explain with a model for CS unfolding/folding in which the association of CS monomers is rate-limiting be-
cause with increasing GroE concentrations, the amount of CS intermediates free in solution should decrease, and association should thus be disfavored. However, the data can be well explained by the second model if one assumes that the intermediates released from GroE are in a conformation that can no longer be recognized by GroEL. This would allow them to associate to native dimers also in the presence of increasing concentrations of GroE.

The SR1-GroES Complex Promotes cis-Folding of Monomeric CS Intermediates—To analyze the influence of GroE on CS folding in more detail, we used the GroEL single-ring mutant SR1 (34). SR1 hydrolyzes ATP and binds substrate and GroES, but does not release GroES and non-native proteins because GroES binding results in the complete inhibition of the SR1 ATPase activity (34). It is therefore possible to examine the folding of monomeric intermediates of CS triggered by a single round of ATP hydrolysis in the central cavity of a SR1-GroES-ATP complex. For these experiments, we inactivated CS for 90 min at 43 °C in the presence of SR1 to form complexes between CS monomers and SR1. At 25 °C, these SR1-substrate complexes were stable for at least 3 h (data not shown). After addition of ATP or GroES/ATP to SR1-CS complexes, only 3% of CS activity was detectable after 3 h without dissociating the SR1 complexes (data not shown). Thus, CS monomers are stably trapped inside SR1-GroES complexes. To investigate the influence of cis-complex incubation on CS folding, we dissociated the SR1-GroES/ATP complexes by a 30-min ice incubation (39) and recorded the reactivation kinetics of CS monomers after dissociation from SR1 at 25 °C (Fig. 2). During ice incubation, no increase in CS activity was detectable (data not shown), and the ice treatment seems to have had no influence on the folding behavior of CS (see below). After a 15-min incubation of the CS monomers inside a SR1-GroES/ATP complex at 25 °C, the yield of native protein reached up to 70% in a fast folding reaction (Fig. 2). In contrast, after incubation in the presence or absence of ATP alone, reactiva-

Fig. 2. GroES promotes cis-folding of monomeric CS intermediates in the presence of SR1. As shown in the scheme, CS (0.075 μM) was inactivated for 90 min at 43 °C in the presence of the GroEL single-ring mutant SR1 (0.2 μM). After a period of 2 min allowing temperature adjustment to 25 °C, either ATP (2 μM) or ATP and GroES (0.3 μM) were added. Directly after a 15-min incubation, the samples were shifted to 9 °C for 30 min to dissociate the SR1-GroES-substrate complexes. After a further temperature shift to 25 °C, the reactivation kinetics of CS were measured. As shown in the graph, CS was reactivated after a 15-min incubation in a SR1-GroES-ATP complex (●); after 15 min in the presence of SR1 and ATP and addition of GroES during the ice incubation (○); after 15 min in the presence of ATP (□); or after incubation in the absence of additional components (■).

Fig. 3. cis-Folding of CS monomers generates an association-competent intermediate that cannot be recognized by GroEL anymore. The experiment was performed as described in the legend to Fig. 2. CS was incubated in SR1-GroES/ATP, for either 0 or 15 min at 25 °C. Reactivation was started after a 15-min cis-folding incubation in the absence (●), or presence (○) of 1 μM SR1 after complex dissociation on ice. SR1 was added to trap all the intermediates, which still can be recognized by GroEL. Also shown are the refolding kinetics of CS after a 0-min cis-complex incubation in the absence of the SR1 trap (■) or in the presence of 1 μM SR1 (○).
elution profile of CS intermediates after a 60-min incubation in a 25 °C, samples were injected on the HPLC gel-filtration column. After a temperature shift and a 2-min preincubation at 43 °C. After a temperature shift and a 2-min preincubation at SR1 (0.3 mM) was performed as described for SR1 complexes, indicating stable shows that almost all CS molecules coeluted with the SR1 60-min incubation of CS in such complexes, the elution profile were formed by addition of GroES and ATP. After an additional monomers takes place in association with GroE complexes, we performed HPLC/size exclusion chromatography experiments on the column after 90 min of refolding.

Temperature-dependent—Having shown that monomeric CS folds inside of GroE cis-complexes, we asked whether this could explain the “apparent stabilization” of CS by GroE during inactivation at 40 °C. To test this, we performed experiments as described above, with the difference that the temperature during cis-incubation was varied between 25 and 40 °C. The yields of reactivation for the cis-folding kinetics of the Arrhenius plot were, in all cases, ~80%. The resulting plot shows clearly that folding inside the GroE complexes is strongly temperature-dependent, with a resulting activation energy of 92 kJ mol⁻¹ (Fig. 6). At 40 °C, the rate constant for the folding of CS to the association-competent monomers is 0.6 min⁻¹, which is much faster than the inactivation reaction at the same temperature (see Fig. 1). This fast folding step inside GroE allows CS to fold to the native state under nonpermissive conditions, if the resulting monomers would be association-competent at 40 °C.

Reactivation Efficiency of CS Monomers Depends Strongly on the Incubation Time in SR1-GroES-ATP cis-Complexes—Having demonstrated that monomeric intermediates of CS can fold inside SR1-GroES-ATP complexes to association-competent monomers, we examined the folding of CS in the SR1 complex in more detail. To analyze the time course of folding, we formed SR1-CS-GroES complexes as described above (see scheme in Fig. 2), now varying the incubation time of CS in the cis-complex at 25 °C. After dissociating the complexes by incubation on ice, a high molar excess of SR1 was added as a trap for CS molecules that did not fold to intermediates with low affin-

**FIG. 4. Monomeric CS intermediates are stably bound in SR1-GroES-ATP complexes.** A, elution profile of native CS (0.075 μM) and SR1, (0.2 μM) at 25 °C. B, elution profile of SR1-bound CS unfolding intermediates. CS was inactivated in the presence of SR1 for 90 min at 43 °C. After a temperature shift and a 2-min preincubation at 25 °C, samples were injected on the HPLC gel-filtration column. C, elution profile of CS intermediates after a 60-min incubation in a SR1-GroES-ATP complex. Inactivation of CS in the presence of SR1 was performed as described for B. After a precooling period at 25 °C, the SR1-GroES-ATP-substrate complex was formed by addition of GroES (0.3 μM) and ATP (2 mM). D, elution profile of refolded CS after a 60-min cis-complex incubation. Substrate cis-complexes were formed as described above. Dissociation of the SR1-GroES-ATP-substrate complexes was initiated by a 30-min ice incubation. Reactivation of CS was started by a temperature shift back to 25 °C. The sample was injected on the column after 90 min of refolding.

**FIG. 5. Kinetics of CS folding in the cis-complex.** The experiment was performed as described in the legend to Fig. 3. The incubation time of unfolded CS intermediates in SR1-GroES-ATP complexes at 25 °C was varied. At the start of the reactivation, 1 μM SR1 was added as a trap. The activity was measured after 120 min of reactivation.

**cis-Folding of CS Intermediates in SR1 Is Strongly Temperature-dependent**—Having shown that monomeric CS folds inside of GroE cis-complexes, we asked whether this could explain the “apparent stabilization” of CS by GroE during inactivation at 40 °C. To test this, we performed experiments as described above, with the difference that the temperature during cis-incubation was varied between 25 and 40 °C. The yields of reactivation for the cis-folding kinetics of the Arrhenius plot were, in all cases, ~80%. The resulting plot shows clearly that folding inside the GroE complexes is strongly temperature-dependent, with a resulting activation energy of 92 kJ mol⁻¹ (Fig. 6). At 40 °C, the rate constant for the folding of CS to the association-competent monomers is 0.6 min⁻¹, which is much faster than the inactivation reaction at the same temperature (see Fig. 1). This fast folding step inside GroE allows CS to fold to the native state under nonpermissive conditions, if the resulting monomers would be association-competent at 40 °C.

To test this further, we analyzed the folding of the association-competent monomers resulting from a cis-complex incubation at elevated temperatures. For this we incubated monomeric CS intermediates at 40 °C for 15 min in SR1 cis-complexes. Then we dissociated the GroE-substrate complexes by a 30-min ice incubation and measured the folding kinetics of the resulting association-competent monomers at 40 °C. Fig. 7 shows that these intermediates fold to the native dimeric enzyme very fast, followed by a slower inactivation reaction. Fitting this reaction to a mechanism of association to active dimers followed by an unfolding reaction resulted in an apparent association rate constant of ~7000 M⁻¹ min⁻¹ and a rate...
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constant for the subsequent inactivation of \( \approx 0.1 \) min\(^{-1} \). The rate constant for inactivation determined in this experiment corresponds very well to the first, GroE-independent rate constant for the inactivation of the native enzyme (Fig. 1). Using these rate constants, we simulated the association of the monomers generated in cis-complexes to the native enzyme and the subsequent inactivation reaction. The simulations showed that under these conditions, CS association is possible even at very low protein concentrations (data not shown). This is a prerequisite for the stabilization of CS in the presence of GroE at elevated temperatures because only a fraction of CS molecules is in its association-competent state during inactivation. In summary, these experiments show that at elevated temperatures, monomeric intermediates of CS fold inside of GroE cis-complexes faster to association-competent monomers than native CS loses its activity. Therefore, these activated monomers are able to associate to the native state even under unfolding conditions.

\textbf{cis-Folding in Wild-type GroEL—}To test whether SR1 is a valid model to investigate the folding in cis-complexes, we performed cis-folding experiments with wild-type GroEL. In this case, we inactivated CS in the presence of GroEL and GroES as described for SR1. After adjusting the temperature to 25 °C, we added ATP (200 \( \mu \)M) to form GroEL-GroES-CS complexes. A further 20 s later, we added apyrase to stop the ATP cycle of GroE by hydrolyzing the ATP quickly to ADP and AMP. Under the conditions used, apyrase hydrolyses the ATP free in solution within 3–4 s to ADP and within 10 s to AMP (data not shown). Following the apyrase quench, we investigated the folding of monomeric intermediates of CS inside the GroEL-GroES complexes as described for SR1 (Fig. 8, scheme). Analysis of CS folding in wtGroE cis-complexes clearly showed that monomeric CS intermediates folded with exactly the same rate constant to an association-competent monomer as observed for SR1-GroES complexes (\( k_{\text{wtGroEL}} = 0.1 \) min\(^{-1} \); cf. Fig. 5). All the intermediates that did not fold to a state lacking any affinity for GroEL were rebound back to GroEL after the ice incubation. In comparison to the SR1 experiments, we were able to fold half the number of the CS intermediates inside of cis-complexes (Fig. 8), suggesting that after apyrase treatment, only asymmetrical GroEL-GroES complexes are present. This was confirmed by electron microscopy and image processing (data not shown).

\textbf{DISCUSSION}

Folding of proteins under nonpermissive conditions is necessary to preserve the viability of cells in stress situations. Recent \textit{in vitro} studies have demonstrated that the GroE chaperone system is able to promote folding of polypeptides under conditions where no spontaneous folding occurs. Furthermore, GroE is known to bind unfolding intermediates stably during thermal unfolding and to keep them in a refoldable state so that these intermediates can be recovered under permissive conditions after stress (cf. Ref. 32). For bacterial luciferase, GroE was shown to increase the number of intermediates that proceed along the productive folding pathway by disfavoring irreversible reactions (40). Under nonpermissive conditions, GroE was not able to shift the pathway to the native state. For
D
\longrightarrow D_\text{c}
\longrightarrow 2 \text{ M} \longrightarrow \text{Agg.}

GroE-M

2 M
\longrightarrow \text{GroE} \cdot \text{M}^* 

\text{cis-folding}

\text{GroE-assisted Folding under Nonpermissive Conditions}

Fig. 9. Model for the GroE-assisted folding of CS under nonpermissive conditions. Native CS dimers (D_S) unfold under nonpermissive conditions to inactive dimers (D_\text{c}). These intermediates interact with the GroE chaperone system, but are not stabilized (cf. Fig. 7 in Ref. 32). For reasons of simplicity, this interaction is not included in the scheme. Subsequently, dissociation of the inactive dimer leads to monomeric intermediates (M). GroE interacts with the monomeric intermediates via an ATP-dependent binding and release mechanism. The concentration of aggregation-prone intermediates in solution is thus decreased, resulting in the suppression of irreversible aggregation steps (Agg.). GroE changes the folding of the monomeric intermediate (M) in a reaction taking place in the cis-complex. Thus, association-competent monomers (M^*) are created, which are able to associate to native dimers (D_N) even under nonpermissive conditions. In the absence of GroE, the monomeric intermediates (M) may, to a small, experimentally not detectable extent, convert spontaneously to the association-competent form (M^*; dashed arrow).

productive folding, sequestration of polypeptide inside GroEL-GroES complexes is necessary (27, 34). However, it is not clear how the GroE machinery allows folding of a substrate protein to its native state under conditions where unfolding is rapid and accompanied by aggregation. Two alternative explanations exist: either GroE could influence the kinetic partitioning between irreversible side reactions and productive folding, or GroE could actually change the folding pathway of a polypeptide.

We show here that in the presence of GroES and ATP, the GroE machinery allows the folding of a monomeric unfolding intermediate inside cis-complexes to a state that associates to the native dimer under unfolding conditions. Taken together, our results suggest that the model of GroE-assisted folding under nonpermissive conditions (Fig. 9): Native dimeric CS (D_N) unfolds at elevated temperatures via dimeric intermediates (D_d). These intermediates interact with GroE, but are not protected against dissociation (cf. Ref. 32).

The monomeric intermediates (Fig. 9, M) are sequestered in the central cavity of GroEL underneath GroES (32). The interaction with GroE suppresses irreversible side reactions of the intermediate (M) and its subsequent aggregation. In addition, inside the GroE complex, a folding reaction occurs that transforms the initially bound intermediate (M) to the monomeric intermediate (M^*), which has no detectable affinity for GroEL anymore. Of importance, the intermediate (M^*) is association-competent under nonpermissive conditions. In the absence of the GroE machinery, this unfolding reaction may also occur spontaneously. This reaction was not detectable (dashed arrow) possibly because most of the intermediates (M) aggregate rapidly. For achieving the apparent “stabilization” of CS under unfolding conditions, association-competent monomers have to be constantly regenerated via GroE-mediated folding steps. This is reflected in the unfolding kinetics. The first phase of the biphasic CS inactivation kinetics (Fig. 1) represents the fast inactivation of the native dimer (D_N) to the inactive dimer (D_\text{c}). The second, slower, and GroE-dependent phase is the net rate of the unfolding and refolding reactions summarized in Fig. 9. This kinetic phase depends on the population of the monomers (M^*) and their association to the native dimer.

Encapsulation in GroE cis-complexes and exposure to the hydrophilic environment of the cavity seem to be the key elements for allowing the protein to fold more effectively than in solution. An explanation for the change of the CS folding pathway could be the GroE-induced unfolding of partially folded intermediates as shown directly for Rubisco (41). Unfolding may be mechanically driven by the ATP-induced domain movements of GroEL subunits (42, 43). In this scenario, the ATP-dependent interaction of GroEL with the CS intermediate (Fig. 9, M) could lead to significant unfolding, giving the protein a new chance to fold. The change in the folding pathway of CS could be directly demonstrated in the presence of SR1, ATP, and GroES, suggesting that one round of ATP hydrolysis is, in principle, sufficient to generate and stabilize the intermediate (M^*). Folding of CS inside the SR1-GroES cis-complexes is much slower than one round of ATP hydrolysis in wild-type GroE (25). Therefore, as in the case of rhodanese and Rubisco (26, 44), multiple rounds of binding and release are required to reach the native state in the presence of wild-type GroE. In this context, it has been proposed previously that GroE functions by “iterative annealing,” which implies that in each round of interaction with GroE, only a certain percentage of substrate proteins becomes committed to fold to the native state (25). In agreement with this suggestion, we found that increasing amounts of GroE decelerate the unfolding kinetics of CS most likely by increasing the chance of unfolding intermediates to refold in cis-complexes. Thus, GroE is not just a passive container that allows proteins to fold one at a time in infinite dilution. More important, while contacting the non-native protein, GroE may shift the unfolded protein to a different trajectory on the energy landscape of folding.

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