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Limits of Protein Folding Inside GroE Complexes*

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The GroE chaperones of *Escherichia coli* promote the folding of other proteins under conditions where no spontaneous folding occurs. One requirement for this reaction is the trapping of the nonnative protein inside the chaperone complex. Encapsulation may be important to prevent unfavorable intermolecular interactions during folding. We show here that, especially for oligomeric proteins, the timing of encapsulation and release is of critical importance. If this cycle is decelerated, misfolding is observed inside functional chaperone complexes.

The GroE chaperone system from *Escherichia coli* prevents the aggregation and supports the folding of polypeptides (*cf.* 1–3). It consists of GroEL, a tetradecameric double-ring cylinder of identical 57-kDa subunits (4) and its cochaperone GroES, a heptameric dome-shaped ring structure of 10-kDa subunits (5). The GroEL double ring itself contains two binding sites for unfolded polypeptides on the inner rims of its two rings and an ATP binding site in each subunit (4). ATP binding induces large structural changes in GroEL, resulting in the out- and upward movement of the apical domains (6–8). As a consequence, hydrophobic residues, which are responsible for polypeptide binding, move away from the interior of the ring. This leads to the ejection of bound substrates into the central channel of the GroEL cylinder and the concomitant binding of the cochaperone GroES. Thus, substrate polypeptides are encapsulated in a hydrophilic folding cage (8–11).

Importantly, the ATP-induced domain movements in GroEL lead to the doubling in volume of the central channel (7, 8). Thus, substrates up to 60 kDa can fold inside the GroE cavity (12–14). ATP hydrolysis and the negative cooperativity for ATP binding between the two GroEL rings (15, 16) trigger the release of GroES and the substrate every 20 s (11, 17). Therefore, the bound substrate can fold only for a short period of time in the protected environment of the central cavity before it is ejected into the bulk solution independent of its folded state (11, 17–20). Binding of non-native polypeptides by GroEL decreases the concentration of aggregation-prone intermediates in solution, and sequestration of polypeptide chains inside the GroE cage allows folding in an unique folding environment without interaction with other folding intermediates (10). In addition to this passive role, the GroE machinery is able to accelerate protein folding (20, 21), possibly by active unfolding of kinetically trapped intermediates, which gives them a new chance to fold (22).

A key question remaining in this scenario is whether folding inside GroE always leads to committed intermediates or whether unfavorable folding reactions can occur inside GroE complexes. To address this question, we analyzed folding reactions occurring under stringent conditions. As a model substrate protein, we used dimeric citrate synthase (CS)[1] (23). We had shown previously that GroE binds monomeric unfolding intermediates of CS (24). A folding reaction inside the central cavity of the GroE complexes leads to an intermediate that is committed to associate to the native dimer, even under nonpermissive conditions. Thus, the GroE system shifts the kinetic partitioning between irreversible aggregation, which is the favored folding pathway in the absence of GroE, toward a productive folding reaction (25). Using this experimental system, we show here that CS can undergo folding reactions inside GroE complexes that lead to irreversibly misfolded proteins.

EXPERIMENTAL PROCEDURES

Proteins—GroEL and GroES were purified from the *E. coli* strain JM 109 TZ 136 bearing the multicopy plasmid DHαpOF 39 as described previously (26). Similary, the GroEL single ring mutant SR1 (9) was purified from the *E. coli* strain BL21 (DE3) pLysS bearing the plasmid pTrc99a. The concentrations of these proteins were determined spectrophotometrically using the following extinction coefficients: $E_{276}^{0.1\%}$ = 0.142 for GroES, $E_{280}^{0.1\%}$ = 0.173 for GroEL, and $E_{276}^{0.1\%}$ = 0.193 for SR1 (calculated according to Gill and von Hippel (27)). 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 (28). In addition, the GroEL and the 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 (EC 4.1.3.7), ATP, and ATPγS were obtained from Roche Molecular Biochemicals and treated as described (23). CS concentration refers to dimers, concentrations of GroEL, SR1, and GroES in the text refer to the 14-mer and 7-mer, respectively. Apyrase from potato (grade IV) was from Sigma.

Inactivation of CS—CS was diluted 1:100 to a final concentration of 0.075 μM into 50 mM Tris/HCl, pH 8.0 (25 °C), 10 mM KCl, 10 mM $MgCl_2$, 1 mM dithioerythritol in the presence of ATP (2 mM) or 0.3 μM GroEL, 0.6 μM GroES, and 2 mM ATP at 25 °C. Inactivation was initiated by a temperature shift to the indicated temperatures. To determine the inactivation kinetics, aliquots were withdrawn at the indicated time points, and CS activity was measured at 25 °C according to Srere (29).

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 (24). After shifting the temperature to 45 °C $SR1_7$·$GroES_7$·ATP_7 complexes were formed by the addition of GroES (0.3 μM) and ATP (2 mM). To dissociate these cis complexes, the samples were incubated on ice for 30 min (30). Reactivation was then measured at 25 °C. To determine the amount of intermediates that lack

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[1] The abbreviations used are: CS, citrate synthase; GdmCl, guanidinium chloride; GroE, complex composed of GroEL, GroES, and ATP or ADP; SR1, single ring mutant of GroEL; wtGroEL, wild type GroEL; ATPγS, adenosine 5′-*O*-(thiotriphosphate); Rubisco, ribulose-bisphosphate carboxylase/oxygenase; HPLC, high performance liquid chromatography.

any affinity to GroEL after folding inside of SR1·GroES complexes, 1 μM of SR1 was added upon reactivation. This excess of SR1 ensures that all intermediates that did not fold to the committed state are immediately trapped (25).

To determine the overall amount of reactivatable intermediates, GroEL (0.3 μM) and GroES (0.6 μM) were added to ensure a GroE-assisted folding. Additionally, 40 mM GdmCl were added immediately after the start of reactivation at 25 °C, because GdmCl destabilizes SR1·GroES-substrate complexes (data not shown). This ensures that all CS intermediates have been dissociated from SR1 and have the chance to fold in a reaction mediated by wtGroE.

Formation of wtGroEL·CS Complexes—To bind monomeric CS intermediates to wtGroEL, CS (0.075 μM) was incubated at 43 °C in the presence of GroEL (0.3 μM) and GroES (0.7 μM) for 90 min (24). After adjusting the temperature to 45 °C, ATP (2 mM) was added. ATP hydrolysis was stopped by the addition of 25 mM EDTA.

Formation of wtGroEL·GroES·CS·Complexes—To bind monomeric CS intermediates to wtGroEL, CS (0.15 μM) was incubated at 43 °C in the presence of GroEL (0.2 μM) and GroES (0.4 μM) for 90 min. After adjusting the temperature to 45 °C, ATP (200 μM) was added to allow binding of GroES. After a further 10 s, apyrase (10 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 reactivation were determined after 120 min of incubation at 25 °C. This procedure only led to the formation of stable $wtGroEL_{14}$·$GroES_7$·CS cis complexes (31). Due to this we used twice the amount of CS for this experiment, because statistically only 50% of the GroEL bound CS monomers are sequestered underneath GroES. The other portion of CS monomers are bound to the GroEL ring opposite to GroES and cannot fold during the experiment. We plotted the time in cis complex against activity gained from CS monomers folded in GroEL·GroES·CS cis complexes.

HPLC-Size exclusion chromatography Experiments—CS-SR1 experiments were performed as described above. After incubation of SR1·CS·GroES complexes at 45 °C, aliquots were withdrawn and injected onto a TosoHaas TSK 4000 PW gel filtration column (30 cm in length). The column was operated at 25 °C with a flow rate of 0.75 ml/min in 50 mM Tris/HCl, pH 8.0, 10 mM KCl, and 10 mM $MgCl_2$. Elution of the proteins was detected on-line with an Amersham Pharmacia Biotech Uvicord VW 2251 UV detector at 280 nm. The peak areas for SR1·CS·GroES complexes were calculated from the data points using the Peakfit software (Jandel Scientific).

ATPase Activity of GroE—ATP hydrolysis was measured using a coupled enzymatic assay (32). The measurements were carried out in 50 mM Tris/HCl, pH 8.0, 10 mM KCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 0.2 mM NADH, 2 mM phosphoenolpyruvate, 10 μg/ml pyruvate kinase, and 25 μg/ml lactate dehydrogenase. GroEL (75 nM) or SR1 (100 nM) were added and preincubated for 5 min. To measure the effect of GroES on the ATPase of GroEL, 150 nM GroES was added to GroEL or SR1. Then ATP (2 mM) was added, and after a further 2-min preincubation period, the change in absorbance at 340 nm was measured over 10 min in a thermostatted Ultrospec 3000 Amersham Pharmacia Biotech spectrophotometer. The rate of ATP hydrolysis was calculated as described (32). The rate of ATPγS hydrolysis, a very slowly hydrolyzable ATP analog, was determined at 45 °C in the presence of 1 μM GroEL and 2 μM GroES.

Electron Microscopy—CS (50 nM) was denatured in the presence of GroEL (40 nM) at 43 °C for 80 min in 50 mM Tris/HCl, pH 8.0, 10 mM KCl, 10 mM $MgCl_2$, and 1 mM dithioerythritol. After a shift to the indicated temperatures, GroES (120 nM) and ATP (2 mM) were added. After 5 min, the samples were applied to carbon-coated grids and negatively stained with 3% uranyl acetate. Electron micrographs were recorded at 120 kV and a magnification of 45,000 with a Philips CM12 electron microscope (Philips, Eindhoven, The Netherlands). Digitized images were aligned, averaged, and subjected to a classification procedure based on eigenvector-eigenvalue analysis (26, 33).

Data Analysis—Rate constants for the unfolding and refolding kinetics of CS were obtained from non-linear fits using Sigma plot 4.0 (Jandel Scientific). Rate constants and equilibrium constants for association or for association followed by an unimolecular folding reactions were determined with the corresponding models using the program Scientist (Micromath). Simulations of the determined folding steps were also performed with Scientist.

RESULTS

The Ability of GroE to Rescue CS from Inactivation Decreases at Higher Temperatures—Having analyzed previously how the GroE chaperone system stabilizes CS during thermal unfolding (25), we were now interested in determining the limitations of folding inside GroE complexes using this assay.

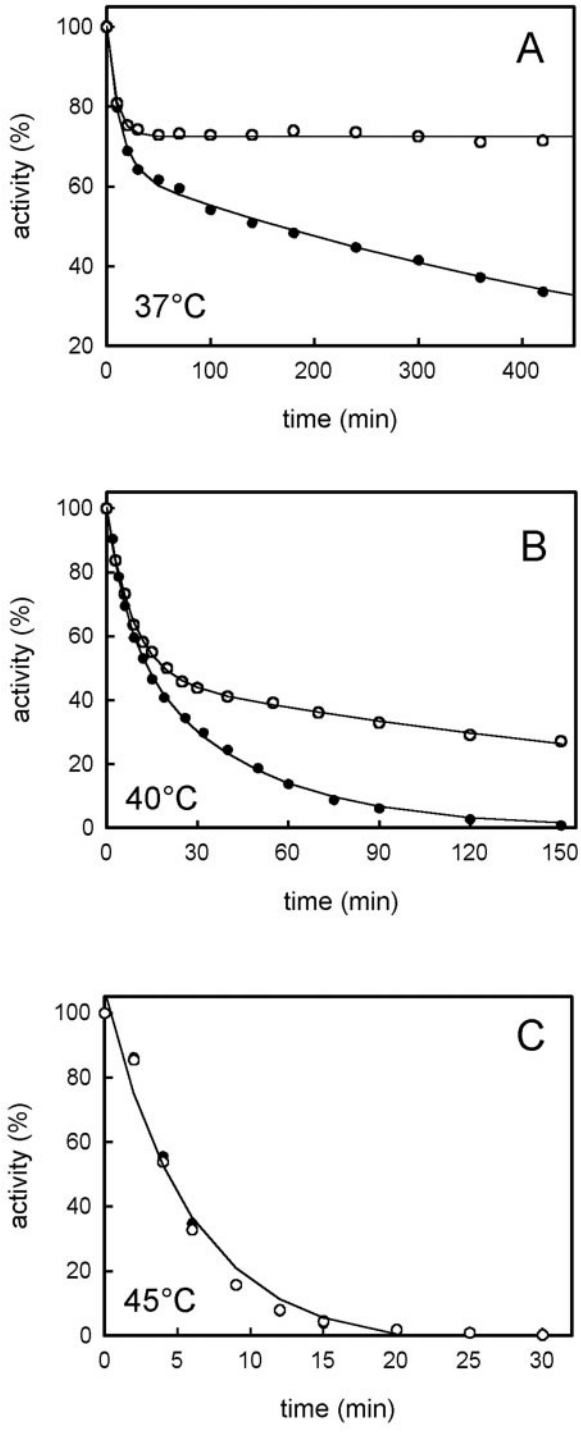


FIG. 1. **Temperature dependence of CS inactivation in the absence or presence of GroE.** CS (0.075 μM) was incubated in 50 mM Tris/HCl, pH 8.0, 10 mM KCl, 10 mM $MgCl_2$, and 2 mM ATP at 37 °C (*A*), 40 °C (*B*), and 45 °C (*C*) without additional components (●) or in the presence of 0.3 μM GroEL and 0.6 μM GroES (○).

To test the temperature range in which GroE is able to fold CS, we inactivated native CS in the presence or absence of GroEL, GroES, and ATP at different temperatures. As shown in Fig. 1, the ability of GroE to stabilize CS during heat denaturation decreased with increasing temperatures. Interestingly, even at physiological temperatures (37 °C), CS is inactivated slowly but completely in the absence of the chaperone

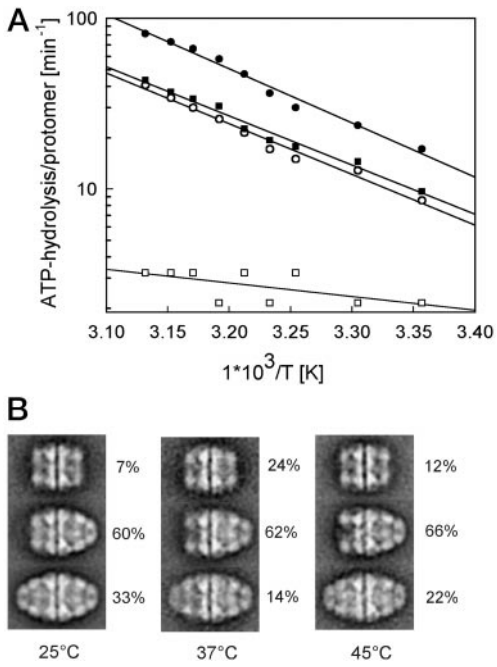


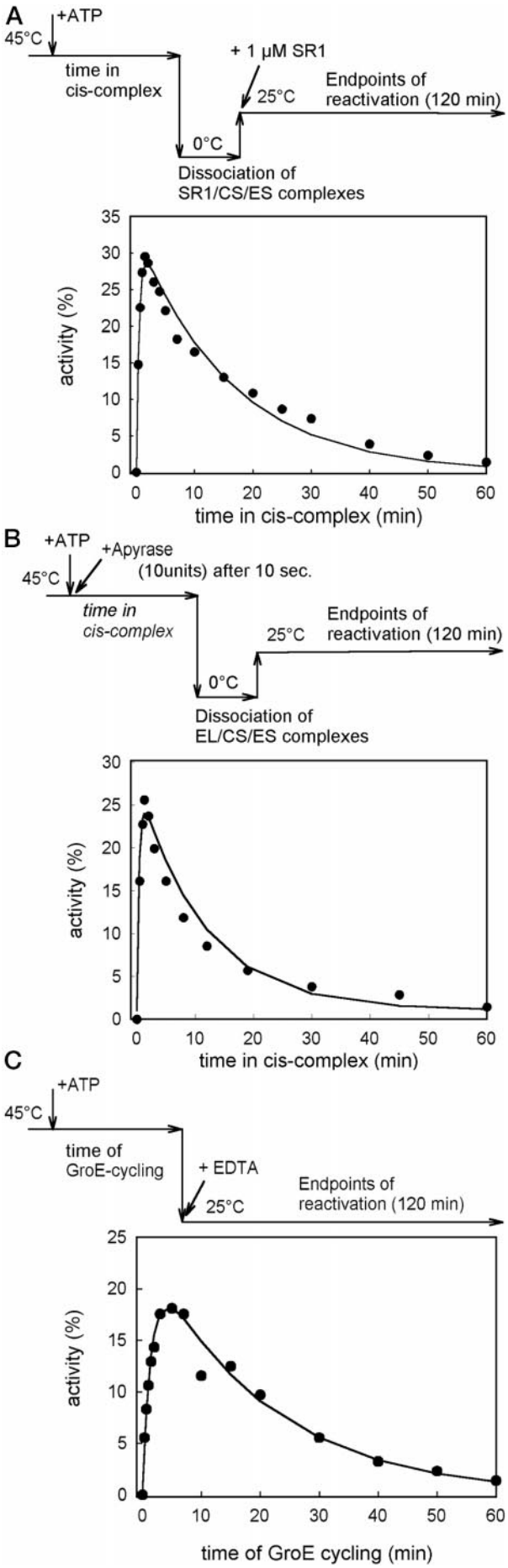
FIG. 2. **Temperature dependence of functional aspects of the GroE-system.** *A*, temperature dependence of the GroE ATPase. ATPase activity was determined between 25 °C and 45 °C using a coupled enzymatic assay (see "Experimental Procedures") for 75 nM GroEL (●), 75 nM GroEL and 150 nM GroES (○), 75 nM SR1 (■), 75 nM SR1and 150 nM GroES (□). *B*, electron microscopic distribution of asymmetrical (*bullets*) and symmetrical (*footballs*) GroEL·GroES complexes at 25 °C, 37 °C, and 45 °C. Samples were prepared at the indicated temperatures in the presence of ATP, and pictures were taken and analyzed by image processing (see "Experimental Procedures"). The distribution of GroE particles is indicated in percent.

system. The reaction can be described with two exponential kinetics with rate constants of $k_1 = 0.09$ min^{-1} and $k_2 = 0.0015$ min^{-1}, respectively. In the presence of GroE, the level of native CS can be held constant at approximately 75%, and the initial loss of activity follows a single exponential reaction ($k = 0.1$ min^{-1}). At 45 °C, a stabilizing effect of GroE is no longer detectable (Fig. 1*C*). The inactivation kinetics can be described by a single exponential function with a rate constant of 0.2 min^{-1}. The inability of GroE to stabilize CS at 45 °C could be either due to a change in the functional mechanism of GroE at higher temperatures or it could represent a folding defect of CS that GroE cannot rescue. To discriminate between these possibilities, we analyzed functional features of GroE and the GroE-assisted folding of CS at different temperatures.

Temperature Dependence of the GroE ATPase Activity—First, we determined the ATPase activity of GroEL and SR1, a single ring mutant of GroEL (9), in the absence or presence of GroES between 25 °C and 45 °C. Changes in the GroE ATPase mechanism would result in a kink in the temperature dependence. The Arrhenius plot of the GroEL-ATPase in Fig. 2*A* shows clearly that there is a linear relationship between ATPase activity and temperature in the investigated temperature range. As expected, in the presence of GroES the rate of the GroEL ATPase activity was approximately half that observed in the absence of GroES due to its inhibition of the ATPase. These results clearly demonstrate that the ATPase mechanism of the GroE chaperone machinery does not change at higher temperatures. Furthermore, the ATPase rates per subunit are very similar for SR1 and wtGroEL (Fig. 2*A*). This shows that SR1 has similar ATPase properties as wt GroEL under the conditions used. As expected, the rate of ATP hydrolysis for SR1 in the presence of GroES decreased to almost zero. The inhibition of ATPase function is due to the fact that SR1 binds GroES in the presence of ATP and hydrolyses the bound ATP but cannot release GroES and ADP (9). This result also demonstrates that the SR1·GroES complexes are stable in the investigated temperature range.

Distribution of Symmetric and Asymmetric GroE-Particles—Since there are indications that the association between GroEL and GroES is perturbed at elevated temperatures (34, 35), we investigated potential changes in structure of GroEL·GroES complexes formed at different temperatures. We used electron microscopy and image processing to visualize and classify asymmetric $GroEL_{14}$·$GroES_7$ complexes, so called "bullets," and symmetric $GroEL_{14}$·$GroES_{14}$ complexes, so called "footballs," at different temperatures. Furthermore, we prepared GroE complexes in the presence of CS to investigate potential substrate-induced changes in complex formation. At all temperatures investigated, GroEL bullets and footballs could be detected (Fig. 2*B*). However, no temperature-dependent differences in the distribution of the three species were observed. Also, the binding of CS had no influence on the complex formation at the investigated temperatures.

Long Term Incubation of Monomeric CS Intermediates in Stable Cis Complexes Leads to Misfolding—Having shown that the inability of GroE to stabilize native CS at higher temperatures is not due to mechanistic changes in the ATPase or the association of GroEL and GroES itself, we now focused on the folding of CS inside GroE at higher temperatures. As described previously (25), monomeric CS intermediates (M_1) fold inside GroE cis complexes to a state (M_2), which is committed to associate to native dimers. This reaction is responsible for the apparent stabilization of CS at 40 °C (see Fig. 1*B*). Now we tested whether this folding step occurs also at 45 °C. To this end, complexes between monomeric CS unfolding intermediates, SR1, and GroES were formed in the presence of ATP. HPLC size exclusion chromatography confirmed that the SR1·GroES·CS complexes were stable for at least 90 min at 45 °C (data not shown). After different incubation times at 45 °C, aliquots were withdrawn and dissociated on ice (30), and the number of CS molecules that reached the committed state was determined. Additional SR1 was added to trap all CS folding intermediates that could still be recognized by the chaperone (*cf.* the scheme in Fig. 3*A*). We found that after short term incubation in the cis complexes at 45 °C, about 30% of the CS molecules fold to the committed state (Fig. 3*A*). Upon further incubation, these committed intermediates disappear rapidly. The kinetic trace can be described by two consecutive reactions with rate constants of 1.9 min^{-1} and 0.06 min^{-1}, respectively. Taken together, these results show that monomeric CS intermediates (M_1) fold inside of SR1·GroES complexes to an association-competent state (M_2) also at 45 °C. Prolonged incubation leads to a misfolding reaction, resulting in the formation of the CS intermediate (M_3), which in contrast to M_2, is not association-competent anymore: $M_1 \leftrightarrow M_2 \leftrightarrow M_3$. The "overfolding" reaction ($M_2 \rightarrow M_3$) could reflect a specific property of SR1. In this case, M_3 would not be formed in wtGroE. To test this possibility, we inactivated CS in the presence of GroEL and GroES as described for SR1. Then ATP was added to form GroEL·GroES·CS complexes, and 10 s later, the ATP cycle was quenched by apyrase. Under the conditions used, apyrase hydrolyzed 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. 3*B*). The analysis clearly showed that monomeric CS intermediates fold with approximately the same rate constants to an association-competent monomer (M_2) as observed for SR1·ES complexes ($k_1 = 2.1$ min^{-1}). After pro-



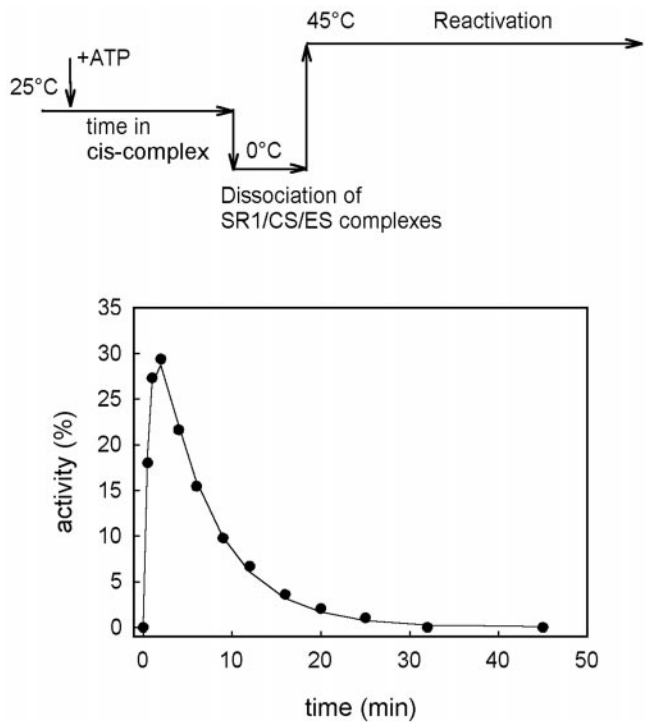


FIG. 4. **The association-competent monomer of CS is able to associate to the native dimer at 45 °C.** The association-competent monomer (M_2) was populated inside of SR1·GroES complexes at 25 °C as described previously, then the complexes were dissociated by ice incubation, and the reactivation time course was measured at 45 °C.

longed incubation, M_2 disappeared in a subsequent slower folding reaction (k_2 = 0.08 min^{-1}), as observed in SR1. Thus, folding inside SR1·GroES complexes is a valid model for GroE cis-folding even at higher temperatures.

Due to the apyrase treatment, CS became artificially locked inside wtGroE complexes. To test whether the disappearance of the committed intermediate (M_2) also occurs in the presence of a completely active, ATP-hydrolyzing GroE system, we analyzed the time course of the appearance and disappearance of

FIG. 3. **Cis folding of monomeric CS intermediates at 45 °C.** *A*, complexes between monomeric CS intermediates (0.15 μM) and the GroEL single ring mutant SR1 (0.2 μM) were incubated for 2 min at 45 °C. Cis folding was initiated by addition of GroES (0.3 μM) and ATP (2 mM). After different incubation times at 45 °C, encapsulated CS was released by a 30-min incubation on ice. Then the yield of CS intermediates lacking any affinity for GroEL was determined after 120 min of reactivation at 25 °C in the presence of a high excess of SR1 (1 μM). Excess SR1 trapped all CS molecules that did not fold to the association-competent state during incubation in cis complexes. *B*, distribution of CS intermediates with no apparent affinity to GroEL (M_2) during folding in cis bullets at 45 °C. CS (0.15 μM) was inactivated for 90 min at 43 °C in the presence of wtGroEL (0.3 μM) and GroES (0.7 μM). After a temperature adjustment to 45 °C, complex formation was started with ATP (200 μM). After a further 10 s, apyrase (10 units) was added to rapidly remove the ATP free in solution. The incubation time of unfolded CS intermediates in $wtGroEL_{14}$·$GroES_7$ complexes at 45 °C was varied. After a 30-min ice incubation to dissociate the complexes, the yield of reactivation was determined at 25 °C. In this case, the amount of intermediates in cis bullets was plotted against the time at 45 °C. *C*, distribution of CS intermediates with no apparent affinity to GroEL (M_2) during folding at 45 °C. Complexes between monomeric CS intermediates (0.15 μM) and the wtGroEL (0.3 μM) were incubated for 2 min at 45 °C. Folding was initiated by the addition of GroES (0.7 μM) and ATP 2 mM. After different incubation times of CS intermediates in the presence of GroE at 45 °C, the amount of CS intermediates lacking affinity for GroEL were determined in the presence of an excess of EDTA (25 mM) after 120 min at 25 °C. EDTA stops the GroE machinery by chelating the Mg^{2+} ions, which are essential for ATP binding to GroEL.

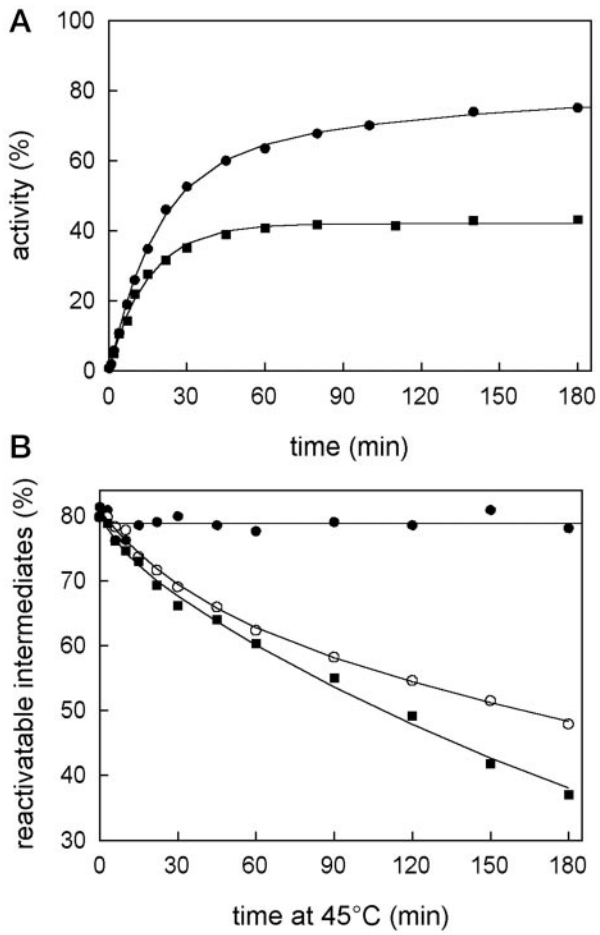


FIG. 5. **Influence of incubation time at 45 °C in SR1·GroES complexes or in the presence of wtGroEL·GroES on the amount of reactivatable intermediates.** *A*, reactivation kinetics of CS folding after 150 min at 45 °C in SR1·GroES cis complexes (■) or in the presence of wt GroEL·GroES (●). CS was treated at 45 °C in the presence of 0.2 μM SR1, 0.3 mM GroES, 2 mM ATP or in the presence of 0.6 μM GroEL, 1.2 μM GroES, and 2 mM ATP as described in Fig. 3. In the case of SR1·GroES complexes, reactivation was started at 25 °C after ice incubation and the addition of wtGroEL (0.15 μM), GroES (0.3 μM), and 40 mM GdmCl. GdmCl was added to destabilize the SR1·GroES complexes, which reassociate after the ice incubation. In the case of wtGroEL·GroES reactivation was started by a temperature shift to 25 °C. *B*, distribution of the reactive CS intermediates after incubation in SR1·GroES complexes, in the presence of wtGroEL·GroES or in the presence of wtGroEL·GroES with a 100-fold slower ATPase activity at 45 °C. The experiment was performed as described in *A*, and the amount of reactivatable intermediates were determined after different incubation times at 45 °C in the presence of SR1·GroES·ATP (■), GroEL·GroES·ATP (●), or GroEL·GroES·ATPγS (○). The GroE ATPase was slowed down by using 1 mM ATPγS during incubation at 45 °C. For reactivation, in all cases 2 mM ATP were added.

M_2 in the presence of wtGroE and ATP at 45 °C (Fig. 3*C*). At different time points, aliquots were withdrawn and incubated at 25 °C with EDTA to stop the GroE ATPase cycle rapidly and to dissociate the GroEL·GroES complexes. Interestingly, the formation and disappearance of association-competent CS intermediates is comparable with that observed in SR1·GroES complexes and can be well described by two consecutive reactions with rate constants of 0.65 min^{-1} and 0.05 min^{-1}, respectively. This implicates a kinetic partitioning between the productive association of M_2 to native dimers and the unproductive conversion of M_2 to M_3.

To rule out the possibility that the committed and association-competent intermediate (M_2) can no longer associate to native dimers at higher temperatures, we populated M_2 inside SR1·GroES complexes at 25 °C, released them, and followed

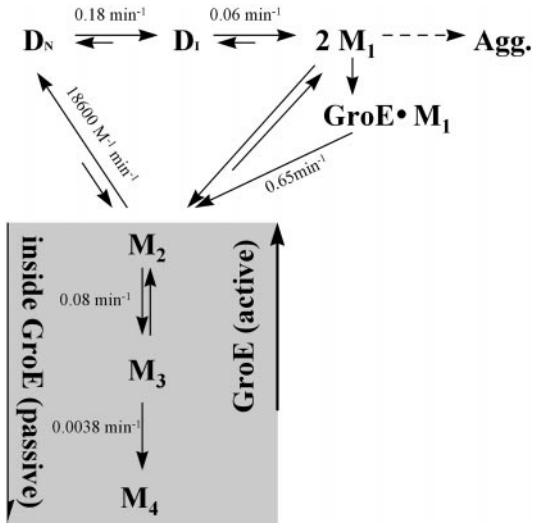


FIG. 6. **Model for the GroE-assisted folding at elevated temperatures.** Native CS dimers (D_N) unfold under nonpermissive conditions to inactive dimers (D_I). These dimers interact with the GroE chaperone system but are not stabilized (*cf.* Ref. 24). For reasons of simplicity, this interaction is not included in the scheme. Subsequently, dissociation of the inactive dimer leads to monomeric intermediates (M_1). These monomers preferably undergo irreversible reactions with subsequent aggregation (*Agg.*), but a small part can fold to association-competent monomers (M_2). However, GroE shifts the equilibrium between these reactions toward the intermediate M_2 by decreasing the concentration of M_1 in solution and allowing folding inside of cis complexes. Thus, association-competent monomers (M_2) are populated. After release from GroE complexes these monomers are able to associate to native dimers (D_N) even under nonpermissive conditions. At temperatures above 37 °C, the association-competent intermediates can also "overfold" inside of GroE complexes toward an intermediate M_3, which is still reactivatable at lower temperatures, and subsequently to an irreversibly "overfolded" intermediate M_4. The slow conversion of the intermediate M_3 to M_4 is only relevant at long term incubation inside of cis complexes. However, in the presence of an ATP-hydrolyzing GroE system, this reaction is prevented. Rate constants for the single reactions at 45 °C are indicated on the *reaction arrows*. The rate constant for the conversion from D_I to M_1 was determined at 43 °C (24).

the dimerization at 45 °C. As shown in Fig. 4, the activity of CS increased rapidly and decreased in a subsequent reaction. The first reaction represents the association of the monomeric intermediate (M_2) to the active dimer ($k = 18\,600\ M^{-1}min^{-1}$), and the second subsequent reaction corresponds to the unfolding of the native dimer (D_N) to the inactive dimer (D_I, $k = 0.18\ min^{-1}$) (*cf.* Ref. 24). This experiment clearly shows that the association-competent intermediate (M_2) can still associate to the native dimer at 45 °C. This together with kinetic simulations confirms that the conversion of M_2 to M_3 is essential to explain the complete loss of CS activity in the presence of GroE at temperatures higher than 37 °C (see Figs. 1 and 6).

The Amount of Reactivatable CS Intermediates Differs Strongly on Incubation in Different GroE Complexes at 45 °C—Having shown that in GroE complexes a misfolding reaction of monomeric CS intermediates occurs, we were now interested in determining whether the resulting CS species (M_3) can be reactivated by GroE. We incubated monomeric CS in stable SR1·GroES complexes or in the presence of wtGroEL·GroES at 45 °C as described above. Reactivation was started in the case of wtGroEL·GroES by a temperature shift to 25 °C and the addition of ATP. In the case of SR1·GroES complexes, we first dissociated the complexes on ice, then wtGroEL, GroES, ATP, and a small amount of GdmCl were added for reactivation. Fig. 5*A* shows the reactivation kinetics of CS intermediates after a 150-min incubation at 45 °C either with wtGroEL·GroES or in SR1·GroES. Interestingly, only 42% of CS activity can be recovered after incubation in SR1·GroES

TABLE I

Rate constants of CS folding in the presence of GroE complexes

The CS folding reactions were measured at 45 °C in stable SR1 · GroES complexes, in stable wtGroEL · GroES bullets, or in the presence of an ATP-cycling GroE system.

	SR1 · GroES complex	Stable GroEL · ES bullets[a]	GroEL · ES, ATP
	min^{-1}	min^{-1}	min^{-1}
$M_1 \rightarrow M_2$	1.9	2.1	0.65
$M_2 \rightarrow M_3$	0.06	0.08	0.05
$M_3 \rightarrow M_4$	0.0038	Not determined	0.002[b]

[a] wtGroEL/GroES cis bullets were created by an apyrase treatment (see "Experimental Procedures" and Fig. 3).

[b] Rate constant for the conversion of M_3 to M_4 in the presence of a wtGroE system with an 100-fold decelerated ATPase (see "Experimental Procedures" and Fig. 5).

complexes. In contrast, after incubation of CS in the presence of the wtGroE system, up to 80% of CS activity can be recovered. This leads to the conclusion that CS monomers misfold irreversibly inside stable SR1·GroES complexes to a monomeric intermediate M_4: $M_1 \leftrightarrow M_2 \leftrightarrow M_3 \rightarrow M_4$. To determine the kinetics of this folding reaction, we investigated the change in yield of reactivatable CS intermediates with increasing incubation times in SR1·GroES complexes at 45 °C (Fig. 5*B*). We found that the number of reactivatable intermediates decreased constantly with a rate constant of 0.0038 min^{-1}. After 180 min at 45 °C, less than 40% activity could be recovered. However, in the presence of an ATP cycling GroE system, even after prolonged incubation at 45 °C, no irreversible loss of CS activity could be detected. This result clearly demonstrates that long time sequestration in stable cis complexes can lead to irreversible folding reactions.

Decelerating the ATPase of GroE Leads to Misfolding in an Active GroE System —The above-mentioned results implicate that the time of encapsulation inside cis complexes is critical and that even in functional GroE complexes, the kinetic competition between productive folding steps and misfolding exists. Based on the preceding results we predicted that increasing the time of encapsulation in wtGroE complexes by slowing down the GroE ATPase should lead to irreversible misfolding even in the presence of an ATP-hydrolyzing GroE system. To test this hypothesis we aimed to artificially decrease the rate of ATP hydrolysis by GroEL. To this end we made use of the ATP analog ATPγS, which is hydrolyzed 100-fold more slowly than ATP by GroEL at 45 °C (data not shown). This allowed monitoring of the influence of a drastically decelerated GroE system on the folding of CS under nonpermissive conditions. We incubated CS in the presence of wtGroEL·GroES with ATPγS at 45 °C, as described above. Reactivation was started by a temperature shift to 25 °C and the addition of ATP. As shown in Fig. 5*B*, the amount of reactivatable intermediates decreased (k = 0.002 min^{-1}), with a slightly slower kinetic as in the case of SR1. In this experiment only 50% of the CS activity could be regained after 180 min at 45 °C. This experiment clearly demonstrates the importance of the GroE timer and folding kinetics for productive folding.

DISCUSSION

We show here that an irreversible misfolding reaction can occur in GroE complexes. This gives insight into the general mechanism of folding inside GroE, since no specific temperature-induced changes in GroE function were detected. Because the encapsulated CS intermediates are monomeric (24), we suggest the following model for their GroE-assisted folding (Fig. 6 and Table I). The unfolding intermediate, M_1, folds inside GroE complexes to the association-competent intermediate, M_2. One round of ATP hydrolysis is sufficient for this reaction to occur. This intermediate, which can be populated up to 80% in cis complexes, lacks any affinity for GroEL and is committed to associate to the native dimer even under nonpermissive conditions. Under nonpermissive conditions, M_2 undergoes a further folding reaction to a state M_3, which does not fold directly to the native state and can be trapped again by GroEL. In the absence of a binding and release cycle, the intermediate M_3 misfolds irreversibly inside GroE to the conformation M_4 in a subsequent reaction. Even after restoration of ATP cycling conditions, M_4 cannot be refolded, and CS activity cannot be restored. The conversion of M_3 to the irreversibly misfolded intermediate M_4 inside GroE is slow and, thus, highlights the critical importance of the timer function of the ATPase for productive folding.

Kinetic simulations using the reaction scheme presented in Fig. 6 showed that the conversion of intermediate M_2 to intermediate M_3 is sufficient to explain the complete loss of CS activity in the presence of GroE at temperatures higher than 37 °C. At physiological temperature (37 °C), CS inactivates slowly but steadily in the absence of the GroE chaperone system. In the presence of GroE, CS is stabilized at a constant level of activity. The fast decrease in activity at the beginning of inactivation reflects the fact that only monomeric and not dimeric CS intermediates fold productively in GroE cis complexes. This folding event leads to association-competent monomers (M_2) that associate again to the native dimer. The overfolding reaction ($M_2 \rightarrow M_3$) is not significant at this temperature. Thus, in this case, GroE modulates the kinetic partitioning between productive and unproductive folding steps as described for bacterial luciferase (36).

Since the irreversible conversion of M_3 to the intermediate M_4 occurred only in stable cis complexes, the ATP-hydrolyzing GroE system is able to actively shift the intermediate M_3 back toward the productive folding pathway, most likely by disrupting incorrect intramolecular interactions in CS. Such an unfolding activity of GroE was directly demonstrated for Rubisco by tritium exchange experiments (22). The three-dimensional structure of CS (37) gives a hint on the potential structural basis of the overfolding reaction. It could well be that the loop/helix extension of the monomer, which is normally intertwined with the other monomer, folds back on the dimer interface, thus blocking the correct dimer formation. Irreversible misfolding occurs when the protein is encapsulated for extended periods of time in GroE. This argues strongly against a passive role of GroE in which sequestration is the key element for folding under nonpermissive conditions. Analysis of a strongly decelerated GroE system confirmed this. Here, one round of ATPγS hydrolysis took approximately 10 to 15 min. During this time, the CS intermediates were sequestered in GroE, and in agreement with our hypothesis, the amount of reactivatable CS intermediates decreased as in SR1. At longer incubation times the loss of folding competence is slower compared with SR1, due to the hydrolysis of ATPγS.

Under nonpermissive conditions, the continuous binding, encapsulation, and release of nonnative proteins is required for GroE to allow folding (*cf.* Refs. 1, 2, and 38). GroEL is a slow

ATPase that is able to direct the energy from substrate binding and ATP hydrolysis to the promotion of the folding process (22, 39). What determines the rate of hydrolysis remained elusive. Based on our results we like to propose that the decisive factor for the evolution of the rate of ATP hydrolysis by GroEL is the competition between productive and aberrant folding inside the GroE complex.

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