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GroEL Traps Dimeric and Monomeric Unfolding Intermediates of Citrate Synthase*

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Holger Grallert, Kerstin Rutkat, and Johannes Buchner‡

From the Institut für Biophysik & Physikalische Biochemie, Universität Regensburg, 93040 Regensburg, Germany

The prokaryotic molecular chaperone GroE is increasingly expressed under heat shock conditions. GroE protects cells by preventing the irreversible aggregation of thermally unfolding proteins. Here, the interaction of GroE with thermally unfolding citrate synthase (CS) was dissected into several steps that occur before irreversible aggregation, and the conformational states of the unfolding protein recognized by GroEL were determined. The kinetic analysis of CS unfolding revealed the formation of inactive dimeric and monomeric intermediates. GroEL binds both intermediates without affecting the unfolding pathway. Furthermore, the dimeric intermediates are not protected against dissociation in the presence of GroEL. Monomeric CS is stably associated with GroEL, thus preventing further irreversible unfolding steps and subsequent aggregation. During refolding, monomeric CS is encapsulated inside the cavity of GroEL·GroES complexes. Taken together our results suggest that for protection of cells against heat stress both the ability of GroEL to interact with a large variety of nonnative conformations of proteins and the active, GroES-dependent refolding of highly unfolded species are important.

Chaperones are a functionally related group of proteins increasingly synthesized under heat shock conditions to prevent protein aggregation (1). In *Escherichia coli*, the most prominent chaperone is GroEL, a member of the Hsp60 family, and its co-chaperone GroES. This chaperone system has been studied extensively in the past (2). GroE, which is essential for viability under all environmental conditions (3) is a double-ring complex consisting of 14 identical subunits with a molecular mass of 57.2 kDa each (4). GroEL possesses a potassium-dependent ATPase, which is regulated by the co-chaperone GroES (5), a heptameric, ring-shaped molecule of 10-kDa subunits (6). *In vivo* as *in vitro*, the GroE-chaperone system increases the yield of correctly folded polypeptides by a highly dynamic, ATP-dependent binding and release mechanism (7, 8). GroEL binds unfolded or partially folded polypeptides via hydrophobic (9) and electrostatic (10, 11) interactions, thus preventing aggregation of folding intermediates (12). Under permissive folding conditions, the presence of ATP is sufficient for release and productive folding, whereas under nonpermissive conditions the co-chaperone GroES is essential for assisted folding (13). It is still not clear how GroE promotes the folding of polypeptides under conditions in which off-pathway reactions are preferred. During the chaperone cycle, GroE binds the nonnative proteins inside the central cavity of the GroEL·GroES complex, thus providing a folding environment in which no unspecific intermolecular interactions with other nonnative proteins occur (14, 15). During this so-called iterative annealing mechanism (16), the proteins are ejected from GroE into the bulk solution after ~15–30 s, because of the ATP binding and hydrolysis on the opposite ring of the GroEL molecule (17), independent of their folding state (17, 18). In addition, the GroE system is able to unfold kinetically trapped or misfolded nonnative polypeptides, which gives them a new possibility to fold correctly (17, 19, 20).

Because the experiments addressing the mechanism of GroE were mostly performed with "chemically" denatured proteins, little is known about the underlying mechanism of GroE under stress conditions (2). Under heat shock condition *in vitro*, GroEL has been shown to interact with several unfolding proteins. These tightly bound polypeptides are protected against irreversible aggregation (21). Reactivation is possible by changing the folding environment to permissive conditions in the presence of GroES and ATP (21–24).

Although these studies demonstrate the basic properties of GroE under heat shock conditions, little is known about the interaction of GroE with thermally unfolding, structured proteins. Here we set out to investigate the influence of GroEL on the thermal unfolding pathway of a model substrate protein in detail. For these studies we choose citrate synthase (CS)[1] as a substrate, because its thermal unfolding has been studied before (25, 26) and because CS was identified as an *in vivo* substrate of GroE in *E. coli* (27) and mitochondria (23). Furthermore, chemically denatured CS has been used previously to study GroE function (12). Mitochondrial CS from pig heart is a dimeric protein composed of two identical subunits with a molecular mass of 49 kDa each. It catalyzes the first reaction in the citric acid cycle, the condensation of oxaloacetic acid and acetyl-CoA to citric acid. The highly α-helical protein is nuclear-encoded and posttranslationally imported into mitochondria. Its three-dimensional structure was solved with a resolution of 2.7 Å (28). At elevated temperatures, CS loses its activity very rapidly with a midpoint of transition at 48 °C (29). The inactivation is accompanied by structural changes in the molecule. Addition of the substrates oxaloacetic acid and acetyl-CoA stabilize the enzyme (30, 31), shifting the midpoint of the thermal unfolding transition to 66.5 °C (29). This stabilization is due to huge conformational changes induced by substrate binding (28, 32, 33). During thermal inactivation, CS

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‡ To whom correspondence should be addressed: Institut für Biophysik & Physikalische Biochemie, Universität Regensburg, 93040 Regensburg, Germany. Tel.: 49-941-943-3039; Fax: 49-941-943-3039; E-mail: johannes.buchner@biologie.uni-regensburg.de.

[1] The abbreviations used are: CS, citrate synthase; HPLC, high performance liquid chromatography; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

unfolds via inactive, dimeric intermediates, which are in equilibrium with the native state (25, 26). Further unfolding of these intermediates leads to irreversible reactions and subsequent aggregation. We show here that GroEL interacts with structured dimeric unfolding intermediates of CS. These intermediates dissociate into monomers, which are stably associated with GroEL and held in a reactivatable state at elevated temperatures.

MATERIALS AND METHODS

Purification of Proteins—GroEL and GroES were purified from the *E. coli* strain JM 109 TZ 136, bearing the multicopy plasmid DHαpOF 39 as described previously (34). The concentrations of both proteins were determined spectrophotometrically using the following extinction coefficients: $E^{0.1\%}_{276\ nm} = 0.142$ for GroES (34, 35), and $E^{0.1\%}_{280\ nm} = 0.173$ for GroEL (calculated according to Ref. 36). The extinction coefficient used for the calculation of the GroEL concentration was corrected for minor tryptophan impurities present in the solution of the purified protein as determined by a titration of the tryptophan fluorescence (37). In addition, the absorbance spectrum of GroEL was corrected for intrinsic light scattering of the solution due to the large particle size of GroEL. Concentrations of GroEL and GroES in the text refer to the 14 or 7 mer, respectively. Dimeric mitochondrial CS from porcine heart (EC 4.1.3.7), with a molecular mass of 49 kDa per subunit, was obtained from Boehringer Mannheim. CS stock solutions were prepared as described by Buchner *et al.* (26). The concentration of CS was determined using an extinction coefficient of $E^{0.1\%}_{280\ nm} = 1.78$ (26).

Inactivation and Reactivation 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 $MgCl_2$, and 1 mM dithioerythrol in the presence or absence of GroEL (0.15 μM or as indicated in the figure legends) at 25 °C. Inactivation was then initiated by a temperature shift to 43 °C. To determine the inactivation kinetics, aliquots were withdrawn at the indicated time points, and CS activity was measured according to the method of Srere *et al.* (31). Activity measurements were carried out at 25 °C. Acetyl-CoA was obtained from Boehringer Mannheim; oxaloacetic acid and DTNB were from Sigma. Reactivation of CS was started by shifting the temperature to 25 °C and by addition of different components as indicated in the figure legends. The kinetics of reactivation were determined as described above.

Data Analysis—Rate constants for the unfolding and refolding kinetics of CS were obtained from nonlinear fits using Sigmaplot 4.0 (Jandel Scientific, Chicago, IL). Rate constants for association or bi-uni-molecular reactions were fitted or simulated with the corresponding models using Scientist (Micromath, Salt Lake City, UT).

HPLC-Size Exclusion Chromatography—CS (0.075 μM) was inactivated at 43 °C in the presence or absence of GroEL (0.15 μM) in 50 mM Tris/HCl, pH 8.0, 10 mM KCl, 10 mM $MgCl_2$, and 1 mM dithioerythrol. At the time points indicated, aliquots were withdrawn and immediately injected onto a TSK 4000 PW gel filtration column (TosoHaas, Stuttgart, Germany). The runs were performed 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 online with a Jasco FP-920 fluorescence detector. The excitation wavelength was 295 nm, and the emission wavelength was 326 nm; in both cases the slits were set to 10 nm. CS dimer peak areas and CS-GroEL peak areas were calculated from the data points with the Borwin software (Jasco, Groß-Umstadt, Germany). CS·GroEL complex peaks were corrected for fluorescence originating from GroEL.

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 dithioerythrol. After temperature shift to 40 °C, GroES (120 nM) and AMP-PNP (2 mM) were added. After a further 5 min at 43 °C 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 micrograph (Philips, Eindhoven, the Netherlands). Digitized images were aligned, averaged, and subjected to a classification procedure based on eigenvector-eigenvalue analysis (34, 38).

RESULTS

GroEL Binds Thermal Unfolding Intermediates of CS without Influencing the Inactivation Kinetics—Mitochondrial CS is a dimer consisting of two identical 49-kDa subunits. We chose this substrate to investigate the influence of GroE on protein unfolding under heat shock conditions because its thermal unfolding has been used before to analyze the function of other chaperones (25, 26, 39, 40). It was known that equimolar concentrations of the GroEL tetradecamer are sufficient to suppress the aggregation of chemically unfolded CS completely (12). Under the conditions used here, thermally unfolding CS loses its activity, with an apparent first order reaction within 50 min. The presence of stoichiometric or higher concentrations of GroEL exhibited no influence on the decrease in activity (Fig. 1*A* and data not shown). To test whether GroE affects the refolding of thermally denatured CS, reactivation of CS unfolding intermediates were induced by shifting the temperature to 25 °C (26). As shown in Fig. 1*B*, only a few percent of CS activity can be recovered after a 15-min incubation at 43 °C in the absence of GroEL. In contrast, after inactivation in the presence of GroEL, almost all of the inactive CS intermediates can be refolded. However, the co-chaperone GroES and ATP are required for efficient reactivation (Fig. 1*B*). Thus, GroEL binds CS unfolding intermediates stably and keeps them in a reactivatable state.

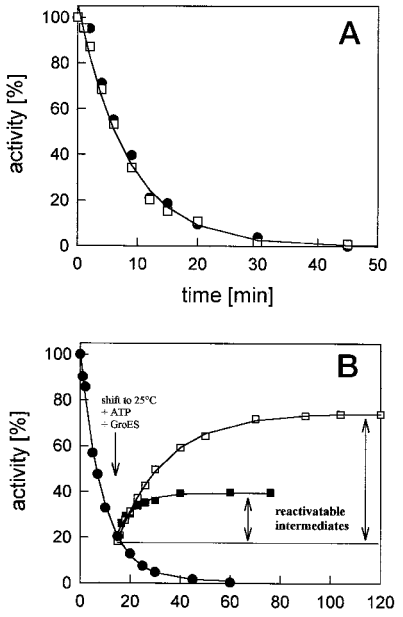

FIG. 1. **Influence of GroE on the thermal inactivation and reactivation of CS.** *A*, influence of GroEL on the thermal inactivation of CS. CS (0.075 μM) was incubated at 43 °C in the absence (●) and presence (□) of 0.15 μM $GroEL_{14}$. At the indicated time points, aliquots were withdrawn, and the activity was determined as described. The *solid line* represents a single exponential fit to the data. *B*, influence of GroE on the reactivation of CS at 25 °C. CS was inactivated at 43 °C in the presence (data not shown) or absence of GroEL (● and see above). Reactivation was started by a temperature shift to 25 °C and the addition of $GroES_7$ (0.3 μM) and ATP (2 mM). The reactivation kinetics after inactivation in the absence (■) or presence (□) of $GroEL_{14}$ (0.15 mM) is shown.

GroEL Binds Dimeric Unfolding Intermediates of CS—Next we addressed the question of which unfolding intermediates are bound to GroEL. Previous experiments had shown that inactivation involves the formation of at least two inactive unfolding intermediates, which are still dimeric (25). Here we determined the overall amount of reactivatable intermediates by monitoring the enzymatic activity and the amount of dimeric CS molecules during heat inactivation by HPLC-size exclusion chromatography. Fig. 2*A* shows the time course of formation of reactivatable CS intermediates during inactivation at 43 °C. In the absence of GroEL, the amount of intermediates increases during the first minutes, reaching a maximum at ~12 min. The subsequent decrease is due to the aggregation of CS after further inactivation. In the presence of GroEL, the amount of reactivatable intermediates increases over 30 min

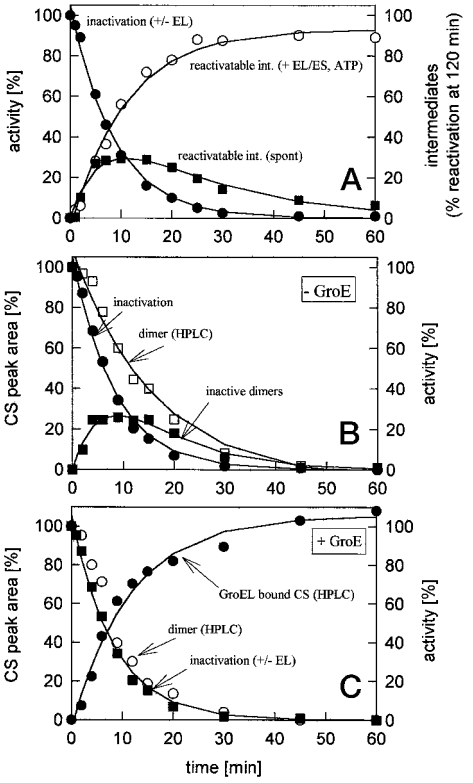

FIG. 2. **GroEL keeps thermal unfolding intermediates of CS reactivatable.** *A*, analysis of CS unfolding intermediates in the absence or presence of GroEL. CS (0.075 μM) was inactivated at 43 °C without (●) or with $GroEL_{14}$ (0.15 μM) (data not shown). At the indicated time points, activity was measured, or reactivation of CS intermediates was started by a temperature shift to 25 °C and the addition of $GroES_7$ (0.3 μM) and ATP (2 mM). After 120 min the end points of reactivation at 25 °C were determined. The amount of reactivatable intermediates (*int.*; see Fig. 1*B*) is plotted *versus* the inactivation time in the absence (■) or presence (○) of GroEL. *spont*, spontaneous. *B*, dissection of inactive and dimeric intermediates during the thermal unfolding of CS. CS (0.075 μM) was incubated at 43 °C; at the indicated times aliquots were withdrawn and immediately injected on an HPLC-size exclusion chromatography column. The peak areas of the CS dimer peaks were determined using Borwin software, and the percentages of intermediates were calculated (□). The difference between the amount of dimers (□) and active CS (●) represents the concentration of dimeric unfolding intermediates of CS (■). *C*, GroEL binds dimeric unfolding intermediates. CS (0.075 μM) was inactivated at 43 °C in the presence of $GroEL_{14}$ (0.15 μM); at the times points indicated aliquots were withdrawn and injected immediately onto the HPLC column, and the peak areas of free CS dimers (○) and the increase in the GroEL peaks (●), due to the bound CS intermediates, were determined. The inactivation kinetics of CS in the presence of GroEL was determined as described (■).

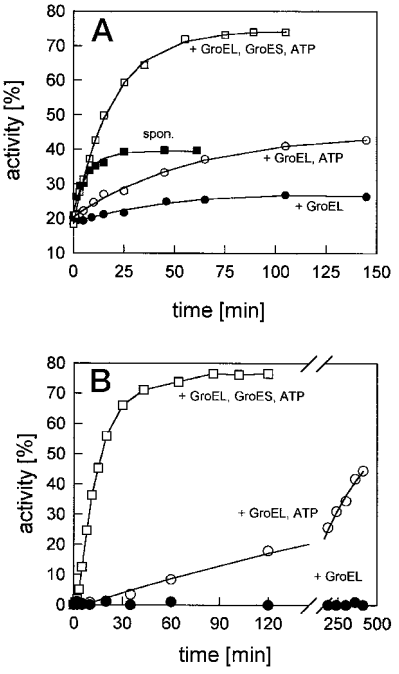

FIG. 3. **Different intermediates are populated during the thermal unfolding of CS.** CS (0.075 μM) was inactivated at 43 °C in the presence or absence of GroEL (0.15 μM) for 15 min (*A*) or 60 min (*B*). Reactivation of CS intermediates in the absence of GroEL was started immediately by a temperature shift to 25 °C (■). Reactivation of the GroEL-bound intermediates was initiated by a temperature shift to 25 °C (●) or after a 2-min preincubation at 25 °C by addition of 2 mM ATP (○) or addition of GroES (0.3 μM) and 2 mM ATP (□). *spon.*, spontaneous.

to ~90%. HPLC-size exclusion chromatography analysis of thermally unfolding CS shows that the disappearance of the CS dimer peak is much slower than inactivation of CS (Fig. 2*B*). Thus, in agreement with earlier suggestions (25) that inactivation clearly precedes dissociation. Comparing the inactivation and the HPLC data, the amount of inactive dimeric unfolding intermediates of CS can be calculated by subtracting the amount of active CS at the different time points from the overall amount of dimeric molecules. The time course of appearance and disappearance of these dimeric but inactive intermediates corresponded very well with the distribution of intermediates measured in the activity assay (Fig. 2, *A* and *B*), leading to the conclusion that during thermal inactivation all reactivatable CS intermediates are in the dimeric state. Monomeric intermediates were not detectable, probably because of the fast aggregation of these species. To determine whether GroEL binds inactive dimeric CS intermediates, we performed HPLC-size exclusion chromatography experiments in the presence of GroEL. Fig. 2*C* shows that the decrease in the dimer peak area in the presence of GroEL followed the same kinetics as inactivation in the absence of GroEL. Thus, GroEL binds dimeric unfolding intermediates of CS stably and holds them in a state that allows reactivation under permissive conditions.

The Distribution of GroEL-bound Intermediates Changes with Inactivation Time—To investigate whether the GroEL-bound intermediates remain dimeric or dissociate, we followed the time courses of reactivation after a 15- or 60-min incubation at 43 °C, respectively (Fig. 3). If there were changes in the distribution of different unfolding intermediates of CS in the presence of GroEL, these differences should be visible in the reactivation kinetics because of differences in their refolding behavior. After a 15-min inactivation period, both the spontaneous and the GroE-assisted reactivation of CS intermediates followed a single exponential kinetic, with rate constants of 0.135 and 0.05 min^{-1}, respectively. After inactivating CS for 15 min at 43 °C in the presence of GroEL, reactivation was initiated by shifting the temperature to 25 °C. Addition of ATP only, during reactivation, led to a much slower reactivation of CS than reactivation in the presence of the complete GroE system (GroEL·GroES·ATP) or the spontaneous folding reaction. This indicates that an additional reaction becomes rate-limiting, which should be the dissociation of the GroEL-bound intermediates from GroEL. So the detectable rate of folding corresponds to the apparent off rate of the CS intermediates from GroEL. Interestingly, this kinetic trace can only be described by two parallel first order kinetics. This indicates that at least two different intermediates were bound to GroEL. Because the off-rate of an intermediate from GroEL correlates with its affinity to GroEL, one of the intermediates has a high affinity for GroEL, resulting in an apparent rate constant for folding of 2.1×10^{-3} min^{-1}. The second intermediate has a

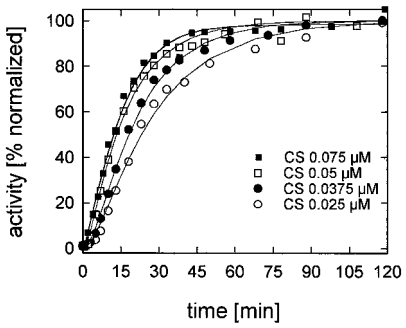

FIG. 4. **The reactivation of GroEL-bound CS unfolding intermediates after long time inactivation is concentration-dependent.** CS was incubated at 43 °C in the presence of GroEL for 60 min and then shifted to 25 °C. After a 2-min preincubation at 25 °C, GroES and ATP (2 mM) were added to initiate the reactivation of the GroEL-bound intermediates of CS. The CS concentration was varied among 0.075 μM (■), 0.05 μM (□), 0.0375 μM (●), and 0.025 μM (○). The stoichiometry among CS, $GroEL_{14}$, and $GroES_7$ was kept constant (1:1:2).

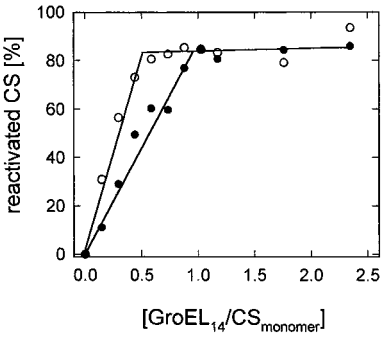

FIG. 5. **GroEL binds two monomeric CS unfolding intermediates after long term inactivation.** CS was thermally unfolded at 43 °C for 90 min in the presence of different $GroEL_{14}$ concentrations and then shifted to 25 °C. After 2 min the reactivation of the GroEL-bound intermediates was initiated by addition of a 2-fold molar excess of $GroES_7$ and 2 mM ATP. After 120 min at 25 °C, CS activity was determined and plotted against the $GroEL_{14}$/CS ratio (○). To generate a GroEL tetradecamer with only one accessible substrate binding site, $GroEL_{14}$ was incubated for 30 min at 25 °C with a 2-fold molar excess of $GroES_7$ and 1 mM ADP, which produces stable, bullet-shaped particles (20). CS was inactivated in the presence of $GroEL_{14}$·$GroES_7$·ADP complexes, as described above. Reactivation was started by the addition of 2 mM ATP (●).

lower affinity and therefore a faster apparent rate constant of folding (k = 0.015 min^{-1}).

Having analyzed the effect of the GroE system on reactivation after 15 min of inactivation, we asked whether there were any differences in the refolding of CS after a long time inactivation in the presence of GroEL. After 60 min of incubation at 43 °C, the ATP-induced refolding of the GroEL-bound intermediates is very slow (Fig. 3*B*). The kinetic trace followed one single first order kinetic, with an apparent rate constant of 2.35×10^{-3} min^{-1}. This rate constant is very similar to the apparent slow folding phase of the ATP-induced renaturation reaction observed after 15 min of inactivation. Thus, the low affinity intermediate bound to GroEL is not protected against a conversion to a second high affinity intermediate during heat shock. The GroES·ATP-induced reactivation kinetics of GroEL-bound CS were very similar after inactivation for 15 or 60 min (Fig. 3*B*). However, the kinetic traces after 60 min inactivation were found to be sigmoidal (also see Fig. 4), so that they could not be described with a single exponential function. After 15 min of inactivation in the presence of GroEL, reactivation at 25 °C in the absence of GroES and ATP showed a slow increase in activity, following a single exponential function. In contrast, after 60 min of inactivation there is no reactivation detectable in the absence of GroES and ATP, because of the higher affinity of the intermediates formed during long-term incubation at 43 °C. Unfolding in the absence of GroEL for 60 min led to the completely irreversible denaturation of CS (data not shown). From the results presented so far, we conclude that GroEL interacts with two different intermediates of CS during thermal unfolding, which exhibit different affinity to GroEL and of which at least one is dimeric (see also discussion).

Refolding of the GroEL-bound Intermediates of CS after Long Time Inactivation Is Concentration-dependent—Having shown that GroEL binds dimeric unfolding intermediates of CS and that the GroEL-bound intermediates convert to a second, kinetically distinct intermediate with long term incubation at 43 °C, we asked whether these intermediates were still dimeric. To this end, we analyzed the concentration dependence of CS refolding in the presence of GroE after 60 min of inactivation in the presence of GroEL. As shown in Fig. 4, reactivation of these intermediates is strongly dependent on CS concentration, which implies that these intermediates are monomeric. The sigmoidal time courses further showed that the reactivation reaction comprises at least two consecutive reactions. At higher CS concentrations the kinetic traces converged, which indicates that a concentration-independent folding step becomes rate-limiting. It seems most likely that this step represents the folding of the inactive dimeric intermediate to the active enzyme, as previously suggested (25). This is supported by the disappearance of the lag phase of the refolding reaction.

GroEL Has Two Binding Sites for Monomeric Unfolding Intermediates of CS—To test the binding stoichiometry of GroEL for monomeric CS intermediates we performed a 90-min inactivation of CS in the presence of increasing amounts of GroEL. As shown in Fig. 5, at a ratio of 0.5 $GroEL_{14}/CS_{monomer}$ the maximum yield of reactivatable intermediates was recovered. Because the CS unfolding intermediates are all in a monomeric conformation after a 90-min heat treatment, this shows clearly that GroEL has two independent binding sites for CS. As a control, we preformed $GroEL_{14}$·$GroES_7$·ADP complexes in which one substrate binding site is stably associated with GroES, so that only one substrate binding site is available per GroEL. In this case, after a 90-min inactivation of CS in the presence of the GroEL·GroES complexes, the maximum yield of recovered intermediates was obtained at a $GroEL_{14}/CS_{monomer}$ ratio of 1. If the GroEL-bound intermediates were still dimeric, a ratio of 0.5 should have been sufficient to achieve the maximal yield of reactivation.

Electron Microscopy of GroE·CS Complexes—Electron microscopic studies have shown that substrates can be bound inside the central cavity of GroEL·GroES complexes (20). Due to the size of the cavity, only polypeptides smaller than 60 kDa can be sequestered in the GroEL cavity underneath a GroES molecule (41). Because refolding of long term thermally denatured CS by GroEL is GroES- and ATP-dependent, we asked whether monomeric CS species with a mass of 49 kDa are encapsulated into the internal GroE cavity during the chaperone cycle. To test this, CS was denatured in the presence of GroEL for 80 min at 43 °C. Then, after a temperature shift to 40 °C, GroES and the nonhydrolyzable ATP analogue AMP-PNP were added to form GroEL·GroES·CS complexes. Electron micrographs of these complexes show a stain-excluding mass inside the cavity of $GroEL_{14}$·$GroES_{14}$ complexes (Fig. 6*A*) and $GroEL_{14}$·$GroES_7$ complexes (Fig. 6*B*). As a control, GroEL was incubated at 43 °C for 80 min in the absence of CS, and then GroES and AMP-PNP were added. In this case, no stain-excluding mass inside the GroEL·GroES complexes was detectable (Fig. 6, *C* and *D*). This result proves directly that monomeric CS intermediates generated during heat inactivation are encapsulated

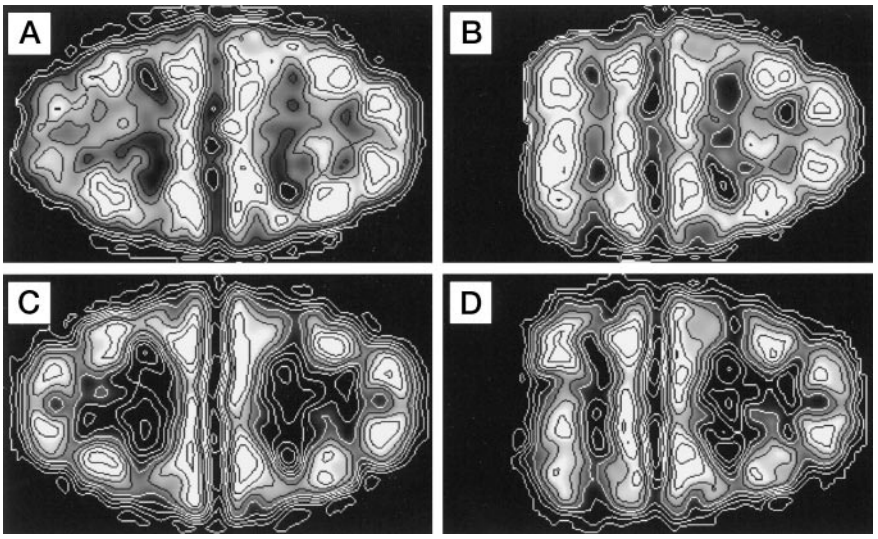

FIG. 6. **Analysis of side views of GroEL·GroES complexes in the presence or absence of CS by electron microscopy and image analysis.** GroEL was incubated in the presence or absence of CS for 80 min at 43 °C. After adjusting the temperature to 40 °C, GroES and AMP-PNP were added and incubated for 5 min before preparing the electron microscopy grids. *A*, symmetrical $GroEL_{14}$·$GroES_{14}$ complex with a stain-excluding mass inside the GroE cavities on both sides. *B*, asymmetrical $GroEL_{14}$·$GroES_7$ complex with stain-excluding mass inside the GroE cavity. *C* and *D*, symmetrical and asymmetrical GroEL·GroES complexes without a stain-excluding mass inside the cavities after preparation in the absence of substrate.

in the GroE cavity. After 15 min of inactivation and preparation as described above, we also found stain-excluding masses of CS inside the central cavity of GroEL·GroES complexes. These masses correspond to the fraction of monomeric intermediates of CS at this time point (data not shown). However, if a substrate is not sequestered underneath GroES, it is not possible by negative staining to determine whether a molecule is bound to GroEL. As in the absence of GroES, GroEL adopts open and closed conformations; it is difficult to distinguish unambiguously between a closed GroEL molecule without and an open one with bound substrate.

DISCUSSION

Under heat shock or other stress conditions many proteins lose their native conformation and denature rapidly, resulting in the population of irreversibly unfolded polypeptides and aggregates. To prevent such irreversible reactions and to maintain viability, the production of heat shock proteins is induced rapidly. In agreement with this view, elimination of the heat shock response in *E. coli* by disrupting the stress-sensitive transcription factor σ^{32} leads to protein aggregation and inclusion body formation with subsequent cell death after stress (42). It is known that the GroE chaperones promote protein folding under conditions under which no spontaneous folding occurs and prevent aggregation of many substrates under heat stress. However, because little is known about the thermal unfolding of substrate proteins, it remained unclear how the GroE system performs this task under unfolding conditions.

We show here that the interaction of GroE with thermally unfolding CS can be dissected into several steps that occur before irreversible aggregation. Generally, GroEL has no influence on the inactivation kinetics of CS at high temperatures but suppresses the heat-induced aggregation of CS completely (26). Addition of GroES and ATP to the GroEL-bound CS intermediates allows refolding under permissive conditions. This is in agreement with previous results on the effects of GroEL on thermally denaturing proteins (21–24).

In addition, the kinetic analysis presented here suggests the following model for CS unfolding in the presence of GroEL at elevated temperatures (Fig. 7). The native CS dimer unfolds during heat inactivation to an inactive dimer, which exhibits native-like structure (25). These intermediates are bound weakly by GroEL. Later during the unfolding process, the inactive dimers dissociate into monomeric intermediates, which possess a high affinity for GroEL. Surprisingly, also in the presence of GroEL the dimeric intermediates dissociate

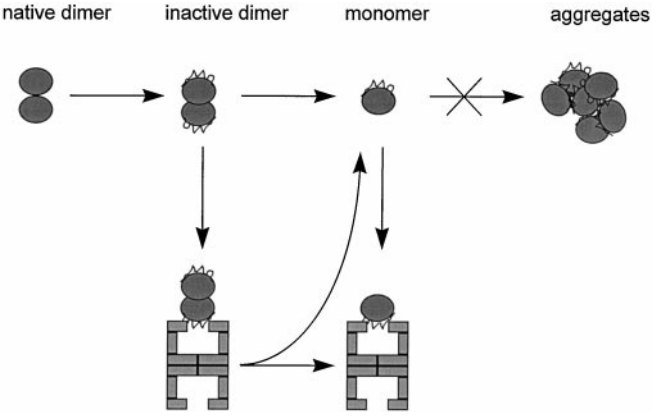

FIG. 7. **Scheme for the CS unfolding pathway in the presence of GroEL.** Native CS dimer unfolds at elevated temperatures (43 °C) to a partially unfolded, inactive dimeric intermediate. This intermediate can be bound by GroEL. Further inactivation results in the dissociation of the dimeric intermediate to a monomeric intermediate, which aggregates rapidly. Interestingly, the GroEL-bound dimeric intermediate cannot be stabilized by GroEL and dissociates into its monomeric form. The monomeric intermediates are stably bound to GroEL, which leads to a complete suppression of aggregation.

into monomers. This may be due to fast binding and release, so that dissociation occurs free in solution. The other possibility is that the dimeric intermediates are bound only via one CS subunit, so that one subunit may dissociate, whereas the other one is stably associated with GroEL. The possibility that GroEL binds only monomeric intermediates of CS, thus shifting the equilibrium between inactive dimers and monomers to the monomeric intermediate, can be excluded. In the presence of GroEL, such a shift in equilibrium would also affect the equilibrium between the native enzyme and inactive dimers, thus leading to accelerated inactivation. Such an effect was not detected in Fig. 1. Furthermore, in this case, inactivation should be dominated by monomeric species at any time point during inactivation. As a consequence, one would not expect to see any differences in the reactivation kinetics after 15 or 60 min of thermal unfolding, as was shown in Fig. 3.

Because of the size limits of the cavity, only monomeric CS intermediates can be encapsulated in GroEL·GroES complexes, leading to asymmetrical and symmetrical particles, the so-called "bullets" and "footballs" (20), with substrate inside. Sequestration of CS inside GroE complexes is a prerequisite for efficient refolding under nonpermissive conditions.

It is rewarding to compare the interaction of GroEL with thermally unfolding CS with that of two other major classes of chaperones. At least in the case of thermal unfolding of CS, these three classes of chaperones share the ability to suppress unspecific aggregation. The kinetic analyses, however, revealed differences in the interaction with nonnative conformations of CS. Hsp90 was found to interact only transiently with early dimeric unfolding intermediates, thus leading to an apparent stabilization of CS during thermal unfolding (25). In contrast, Hsp25, a member of the small heat shock protein family, was shown to bind all unfolding intermediates of CS stably during thermal unfolding. Similar to GroEL, it has no influence on the inactivation kinetics. So far, reactivation is only possible in cooperation with other chaperones under permissive conditions (40). A common theme that emerges from this and other studies is that chaperones seem to interact already with early, native-like unfolding intermediates, thus preventing the formation of aggregation-prone, unfolded conformations.

GroE is set apart from other chaperones both by the range of conformations it interacts with productively and, importantly, by its ability to support folding under nonpermissive conditions. Although the later has attracted quite some attention, the mechanistic importance of the recognition of different conformations of a protein has not been incorporated into the current models of GroE action. Binding of a protein at different stages of folding by GroEL, as shown here for CS, may be the rule rather than the exception, because similar phenomena have also been described for the interaction of GroE with chemically denatured proteins. In this context, GroEL has been shown to interact both with the highly structured, native-like dimeric folding intermediate of an antibody Fab fragment and with its unfolded, monomeric intermediate (43). Furthermore, GroEL binds two different nonnative states of β-lactamase (44). The interaction of GroEL with different nonnative conformations of a protein allows the chaperone to interfere early on with unfolding proteins by recognizing native-like species and preventing further irreversible species. At the same time, GroE is able to actively support the folding of largely unfolded proteins in a strictly GroES-dependent manner. The interplay of these reactions seems to be important for GroE to support survival under heat shock conditions.

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