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Interaction of GroEL with a highly structured folding intermediate: Iterative binding cycles do not involve unfolding

(Fab fragment/protein folding/protein association/chaperones/heat shock proteins)

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ABSTRACT **The GroE proteins are molecular chaperones involved in protein folding. The general mechanism by which they facilitate folding is still enigmatic. One of the central open questions is the conformation of the GroEL-bound nonnative protein. Several suggestions have been made concerning the folding stage at which a protein can interact with GroEL. Furthermore, the possibility exists that binding of the nonnative protein to GroEL results in its unfolding. We have addressed these issues that are basic for understanding the GroE-mediated folding cycle by using folding intermediates of an Fab antibody fragment as molecular probes to define the binding properties of GroEL. We show that, in addition to binding to an early folding intermediate, GroEL is able to recognize and interact with a late quaternary-structured folding intermediate (Dc) without measurably unfolding it. Thus, the prerequisite for binding is not a certain folding stage of a nonnative protein. In contrast, general surface properties of nonnative proteins seem to be crucial for binding. Furthermore, unfolding of a highly structured intermediate does not necessarily occur upon binding to GroEL. Folding of Dc in the presence of GroEL and ATP involves cycles of binding and release. Because in this system no off-pathway reactions or kinetic traps are involved, a quantitative analysis of the reactivation kinetics observed is possible. Our results indicate that the association reaction of Dc and GroEL in the presence of ATP is rather slow, whereas in the absence of ATP association is several orders of magnitude more efficient. Therefore, it seems that ATP functions by inhibiting reassociation rather than promoting release of the bound substrate.**

Molecular chaperones have the unique property of selectively recognizing and binding nonnative proteins. The best characterized chaperone so far is GroEL, for which the crystal structure has recently been determined (1). However, the molecular mechanism leading to the discrimination between native and nonnative protein structures is still enigmatic. Conflicting evidence and models exist for the interaction of the Hsp60/GroEL class of chaperones with nonnative substrates. It has been proposed that GroEL recognizes folding proteins that contain a certain amount of secondary structure but no fixed tertiary structure (2–4). These features are reminiscent of the molten globule intermediate of protein folding (5). Recently, the interaction of GroEL with the well-characterized molten globule state of α-lactalbumin has been demonstrated (4). While there is a consistent body of evidence for a model suggesting that GroEL selectively interacts with folding proteins at the stage of the molten globule intermediate, a number of observations argue against this idea. It was demonstrated that GroEL interacts with largely unfolded lactate dehydrogenase in kinetic experiments (6) and, by using two-dimensional NMR techniques, it was shown that cyclophilin bound to GroEL is devoid of secondary structure (7). Also, for α-lactalbumin, Okazaki *et al.* (8) concluded that the denatured state of the protein can be bound to GroEL. Recently, it has been proposed (9, 10) that the central element of the mechanism of GroE-mediated folding is unfolding of the substrate protein upon binding to GroEL, thus giving a protein that has not succeeded in rapidly folding to the native state or a kinetically trapped species the chance to start folding from a more unfolded conformation. Since chaperonin-mediated protein folding seems to involve cycles of binding and release, the nonnative protein may undergo concomitant cycles of folding and partial unfolding (9–11). This would imply that, depending on the folding characteristics of the given protein, a certain fraction of the nonnative protein will, upon each release cycle, reach the native state while the remaining slowly or wrongly folding portion will be rebound by GroEL (10). It should be noted that the term "unfolding" does not necessarily imply that the protein will become devoid of any noncovalent interactions. The idea is rather that part of the bonds may be broken in the partially folded intermediate upon binding to GroEL. However, if this "unfolding" event should affect folding kinetics upon release, it should be possible to detect it directly not by a structural but by a kinetic approach. To test this hypothesis, we investigated whether late highly structured folding intermediates interact with GroEL and whether these intermediates will be unfolded during their interaction with GroEL. This would unambiguously establish whether unfolding is an essential part in the GroE cycle and whether GroEL is only able to interact with largely unfolded structures or those that are in equilibrium with the denatured state. To address this question specifically, we studied the interaction of GroEL with Dc, a well-characterized folding intermediate of an Fab antibody fragment that has defined secondary, tertiary, and quartenary structure (12, 13). We show that Dc can be used as a substrate for GroEL. Most interestingly, the structure of Dc is not disturbed upon binding to GroEL. Addition of ATP leads to a reactivation of Dc from the GroEL–Dc complex. However, the reactivation kinetics are determined by the rebinding of Dc to GroEL, even in the presence of ATP. This allowed us to determine the rate constants of dissociation and association of the GroEL–Dc complex.

MATERIALS AND METHODS

Reagents. The Fab fragment of the monoclonal antibody MAK33 (IgG1 with κ light chains), directed against human creatine kinase (EC 2.7.3.2.) (14), all ELISA reagents including streptavidin-coated tubes and biotinylated human creatine kinase, and ATP were obtained from Boehringer Mannheim. Trypsin was from Sigma. GroEL and GroES were purified as described (15). The concentration of Fab, GroEL, and GroES was determined by using extinction coefficients of $\varepsilon_{\text{Fab, 0.1\%}} = 1.6$, $\varepsilon_{\text{GroEL, 0.1\%}} = 0.2$, and $\varepsilon_{\text{GroES, 0.1\%}} = 0.142$, respectively, for

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Abbreviations: CS, citrate synthase; GdmCl, guanidinium chloride.
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a 1-cm path at 280 nm. Concentrations for GroEL and GroES given in text refer to the 14-mer and 7-mer, respectively.

Unfolding and Refolding of the Fab Fragment. To populate Uc (completely denatured Fab with all prolyl residues in the native conformation) and the late folding intermediate Dc, the Fab fragments were denatured in 6 M guanidinium chloride (GdmCl) at 4°C for 15–20 s and 3.5 M GdmCl at 10°C for 60 s, respectively. Under the latter conditions, Dc could be accumulated to ≈90% (13).

Renaturation of the Fab species was achieved by dilution into 0.1 M Tris·HCl (pH 7) at 10°C in the absence or presence of GroEL. If not indicated otherwise, the residual GdmCl concentration during renaturation was 60 mM. The protein concentrations used are indicated in the figures.

Release factors were added 25–30 min after initiation of complex formation. This time span was chosen to allow completion of refolding of the Fab fragment that was not trapped by GroEL. The amount of antigen binding activity regained upon renaturation was quantified by ELISA (16).

Fluorescence Kinetics. Denaturation kinetics of the GroEL-bound Fab fragment in 0.1 M Tris·HCl, pH 7/3 M GdmCl at 10°C (protein concentrations are given in the figures) were measured by using a SpexFluoromax fluorescence spectrometer. For excitation, a wavelength of 295 nm was used, and the emission wavelength was 350 nm. The slits for both excitation and emission were 4.25 nm. The kinetics were measured in a thermostated cell, equipped with a magnetic stirrer, by using a final Fab concentration of 1 μg/ml.

RESULTS

The Quaternary-Structured Folding Intermediate Dc Is a Substrate for GroEL. It has been shown, that the MAK33 Fab fragment from mouse interacts with GroEL during refolding (11, 17). However, no structural information of the GroEL-bound Fab fragment is available. We have established a model for the folding pathway of the oxidized Fab fragment (see model 1A in ref. 12) and characterized (13) one of the late folding intermediates, Dc. In this intermediate, all four domains of the Fab fragment have native-like structure. However, pairing of the domains occurs via nonnative interactions.

The knowledge of the rate-limiting steps during Fab folding and unfolding and the possibility to accumulate the quaternary-structured intermediate Dc enabled us to ask at which stage during refolding GroEL is able to interact with the Fab fragment. For this purpose, we initiated the refolding process in the presence of GroEL starting from differently denatured Fab species. By using Dc, further reactivation to the native state could be blocked by GroEL (Fig. 1*A*), indicating formation of a complex of GroEL and the Fab folding intermediate. Titration experiments revealed that the amount of Fab trapped by substoichiometric concentrations of GroEL was identical to the respective GroEL concentration (data not shown). A simulation of these data showed that the formation of the complex between Dc and GroEL has to occur at least with a rate of $k_{ass} > 1 \times 10^8\ M^{-1}\cdot s^{-1}$. Otherwise a much higher percentage of Dc would escape GroEL binding and refold spontaneously to the native state. However, this interaction of Dc with GroEL does not prove that the structure of Dc is maintained upon GroEL binding.

From the GroEL–Dc complex, reactivation of Fab in the absence of any release factors is observed as a very slow reaction (data not shown). Addition of ATP to the GroEL–substrate complex results in a release of the bound Fab fragment, but the kinetics of reactivation are significantly slower than the spontaneous refolding of Dc ($k_{GroEL/ATP} = 5 \times 10^{-4}\ s^{-1}$ and $k_{spont} = 0.005\ s^{-1}$, respectively; Fig. 1*A*). In the presence of ATP and GroES, the kinetics of reactivation ($k_{GroEL/ES/ATP} = 0.002\ s^{-1}$) were faster but still slower than the spontaneous folding of Dc. The yield of reactivation is not influenced by the GroE system, because for Dc there is no competition between productive folding and off-pathway reactions such as aggregation.

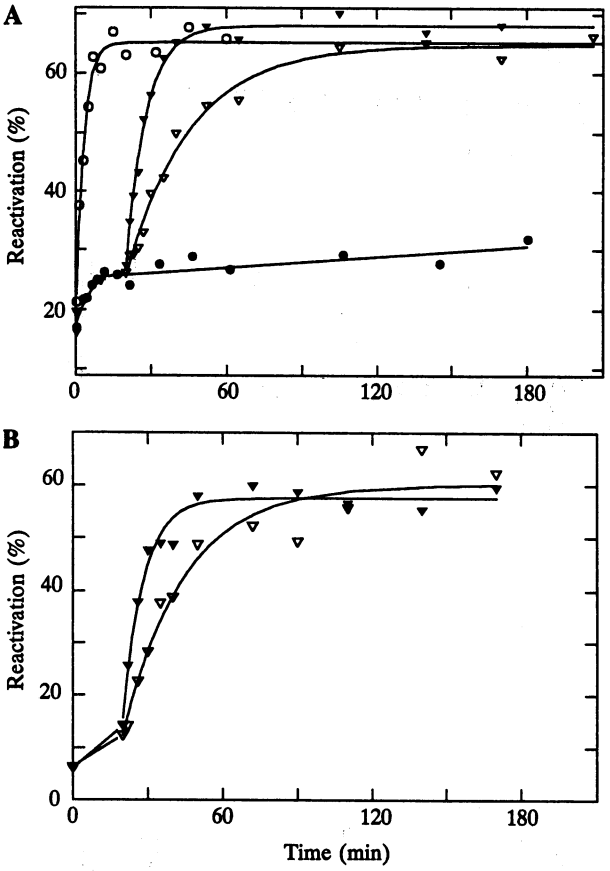

FIG. 1. Interaction of GroEL with the Fab folding intermediates Dc (*A*) or Uc (*B*) (10 μM) was diluted 1:50 into renaturation buffer at 10°C containing 0.2 μM GroEL. After 30 min, buffer (●), ATP (▽), or ATP and 0.5 μM GroES (▼) were added. For comparison, the reactivation of Dc in the absence of other factors (○) is shown. As indicated, aliquots were withdrawn, further reactivation was blocked by adding trypsin (400 μg/ml), and the amount of native Fab was quantified by ELISA. The initial fast phase in the presence of GroEL (●) might be due to a mixing artefact or a small error in the determination of the protein concentrations.

Similar results were obtained, when not Dc but the fully denatured state Uc [with all prolyl residues in their native conformation (12)] was used (Fig. 1*B*). This is due to the fact that in both cases the rate limiting step of folding is determined by the folding reaction Dc → N (12, 13). It should be noted that not Uc itself but, most likely, an early folding intermediate is bound in this case, because the structural information for the native conformation of the prolyl residues is maintained while the Fab fragment is bound to GroEL (data not shown). However, this intermediate is clearly different from Dc (see below).

Complex Formation Determines the Rate of Fab Reactivation even in the Presence of ATP. There are several possibilities to explain the differences in the reactivation kinetics observed under the conditions shown in Fig. 1. (*i*) The release of the GroEL-bound Fab fragment might be rate limiting, even in the presence of release factors, leading to slower reactivation kinetics compared to the spontaneous reaction. (*ii*) Rebinding of the released protein could occur after adding release factors. (*iii*) Structural changes of Dc upon binding to GroEL may lead to a change in the rate limiting step of refolding after release from the complex.

To differentiate between these possible explanations, we used three experimental approaches: (*i*) identification of the rebinding reaction by competition with unfolded citrate synthase (CS), (*ii*) variation of GroEL concentrations, and (*iii*) measuring denaturation kinetics of the substrate bound to GroEL.

If reactivation of Dc from the GroEL–Dc complex occurs via multiple rounds of release and rebinding, a protein that is known to bind to GroEL during its refolding process should be able to compete for rebinding of Dc and thus should accelerate the apparent reactivation kinetics. As shown in Fig. 2, reactivation kinetics, starting from the Fab–GroEL complex in the presence of ATP, were accelerated with increasing concentrations of unfolded CS. Unfolded CS itself had no effect on refolding of Fab, either starting from the fully denatured state or from Dc (data not shown). Since unfolded CS itself can either refold or aggregate, the exact concentration of the competitor cannot be determined and, therefore, a quantitative analysis of the kinetics is not possible. Nevertheless, these results imply that nonnative Fab dissociates from GroEL in a form that can be recognized by GroEL again. Rebinding has a marked influence on the overall reactivation kinetics. A quantification of the association constant should be possible by analyzing the concentration dependence of the reactivation process. Experiments starting from a defined concentration of the GroEL–Dc complex and various concentrations of additional free GroEL showed that the renaturation kinetics of Dc, initiated by adding ATP, is slowed down with increasing GroEL concentrations. The data shown in Fig. 3 can be quantified by the following model:

$$EL\text{–}Dc \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL + Dc \xrightarrow{k_2} N, \qquad [1]$$

where EL–Dc represents the complex of GroEL and Fab, EL and Dc are the nonassociated species, N is the native Fab fragment, and k_1, k_{-1}, and k_2 are the respective microscopic rate constants. By assuming that the folding intermediate Dc is released from the complex (see below), the rate k_2 of the reaction Dc → N is 0.005 s^{-1}. The kinetic simulation of the overall reactivation using the program KINSIM gave rate constants $k_1 = 8.3 \times 10^{-4}$ to 2.7×10^{-3} s^{-1} and $k_{-1} = 1.0 \times 10^4$ to 1×10^5 $M^{-1}{\cdot}s^{-1}$. The dissociation constant was determined as $K_d = 50$ nM, calculated from the microscopic rate constants given in Fig. 3. No great difference of the rate constants within the given range of error were obtained, when two binding sites on GroEL were used for the simulation. It should be noted that the given rates and dissociation constant are the sum of all

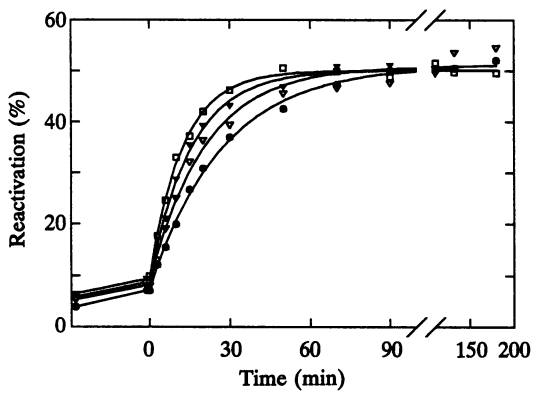

FIG. 2. Competition of Fab rebinding by denatured CS. The complex formation of Dc and GroEL was performed as described in Fig. 1. After 30 min, ATP (2 mM) was added either alone (●) or with 0.15 μM (▽), 0.2 μM (▼), or 0.3 μM (□) denatured CS. In all cases, the residual GdmCl concentration was 0.16 M. The kinetics of renaturation were measured as in Fig. 1.

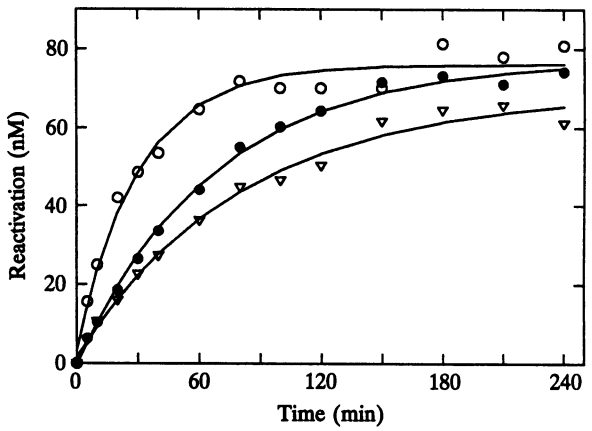

FIG. 3. Effect of the GroEL concentration on Fab reactivation in the presence of ATP. Dc (0.15 μM) was added to various concentrations of GroEL. The concentration of the complex formed was assumed to be the amount of Fab that could be renatured by addition of ATP after a 30-min incubation (0.075 μM). The kinetics start with addition of ATP. The concentration of native Fab at this time (0.05 μM) was subtracted. The GroEL concentrations used were 0.15 μM (○), 0.4 μM (●), and 1.2 μM (▽). The data were simulated according to model 1 with rate constants $k_1 = 0.06$ min^{-1}, $k_{-1} = 2 \times 10^4$ $M^{-1}{\cdot}s^{-1}$, and $k_2 = 0.3$ min^{-1}.

partial reactions occurring during the GroEL folding cycle. These values have to be taken as operational constants. The data do not allow a discrimination of partial reactions.

The Structural Integrity of Dc Is Not Affected by Binding to GroEL. Because the structure of the Fab fragment bound to GroEL cannot be directly examined by using spectroscopic methods, we used a kinetic approach for analyzing the conformation. The intermediate Dc is characterized kinetically by a fast folding reaction to the native state (Dc → N, $k = 0.005$ s^{-1}) and by slow denaturation kinetics (13). These re- and denaturation kinetics can be utilized to identify Dc unambiguously within the folding/unfolding pathway of Fab. This allowed us to unfold GroEL-bound Dc by adding GdmCl and monitor the unfolding kinetics by fluorescence. At the GdmCl concentration used (3 M), GroEL itself fully denatures within the mixing time (data not shown). Comparison of the unfolding kinetics of Dc in the absence of GroEL to the unfolding of Dc bound to GroEL allowed us to determine whether Dc retains its structural characteristics after binding to GroEL. If Dc became substantially unfolded upon binding to GroEL, i.e., by disruption of the nonnative domain contacts, the denaturation kinetics of the GroEL-bound species would be expected to be significantly faster.

A denaturation kinetic of the native Fab fragment in 3 M GdmCl, measured by fluorescence, is shown in Fig. 4. This denaturation is a biphasic process, in which first Dc is accumulated in a fast reaction, and then Dc is denatured with slow kinetics without a population of any other intermediate (13). When the GroEL–Dc complex is denatured in 3 M GdmCl, the kinetics and amplitudes of the denaturation process are identical to the denaturation of Dc free in solution (Fig. 4). Thus, we could show that Dc is bound by GroEL without disturbing its main structural features—i.e., the noncovalent domain pairing of the already native-like domains. In contrast, a complex formed of Uc and GroEL shows clearly different denaturation behavior (Fig. 4*B*). In this case, the denaturation kinetics is too fast to be detectable, clearly indicating that the GroEL-bound species has less intrinsic stability than Dc. This implies that GroEL can bind at least two structurally different intermediates of the productive folding pathway of Fab, one of them being the quaternary-structured intermediate Dc. This is confirmed by the renaturation kinetics measured after denaturation of the GroEL–Dc complex in 3 M GdmCl for 30 s,

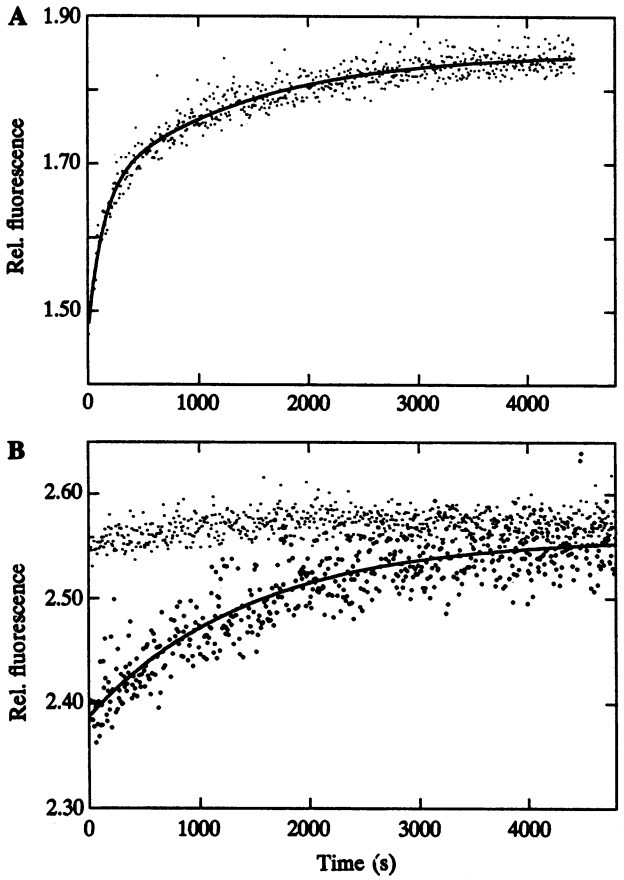

FIG. 4. Denaturation kinetics of Dc bound to GroEL. The EL–Dc complex was formed as described in Fig. 1, except that a 3.5-fold molar excess of GroEL was used to ensure that essentially no Fab molecules escaped from binding to GroEL. Similarly, the complex, starting from Uc, was formed. After 30 min, the protein was diluted into 0.1 M Tris·HCl, pH 8/3 M GdmCl at 10°C. The final Fab concentration was 1 μg/ml. The denaturation kinetics were followed by tryptophan fluorescence. For comparison the denaturation kinetics of native Fab under identical conditions are displayed (*A*). The data were fitted to two first-order reactions ($k_1 = 7.6 \times 10^{-3}\ s^{-1}, A_1 = 0.195$; $k_2 = 7.0 \times 10^{-4}\ s^{-1}, A_2 = 0.189$), with the second rate constant and amplitude characterizing the spontaneous denaturation of Dc. (*B*) The denaturation of EL–Dc (•) was fitted to a single first-order reaction with a rate constant of $k = 6.8 \times 10^{-4}\ s^{-1}$ and an amplitude $A = 0.171$. The denaturation kinetic of EL–Uc (·) was not resolvable.

which is very similar to that of the spontaneous reactivation of Dc (data not shown).

The structure of Dc is maintained not only upon binding to GroEL in the absence of any release factors but also if cycles of binding and release were performed in the presence of ATP: When the GroEL–Dc complex was incubated in the presence of ATP for 30 min with a high excess of free GroEL in solution, ≈20% of the Fab fragment could reach the native state. However, ≈85% of Dc was released from GroEL at least once within this period. Denaturation of the protein in 3 M GdmCl after a 30-min incubation with ATP resulted in the detection of 16% native protein (i.e., a fast phase of denaturation with only a small amplitude); the remaining Fab, although it had passed through at least one cycle of release and rebinding still maintained the structural features of Dc (data not shown).

DISCUSSION

We have shown herein that GroEL is able to recognize two differently structured folding intermediates of an antibody Fab fragment. One of these intermediates, Dc, is already highly structured containing secondary, tertiary, and quaternary interactions and the other one is an earlier folding intermediate that is different from the completely denatured state (Uc). Recently, it has been proposed that several different conformational states are recognized by GroEL: (*i*) the denatured state (7, 8, 18), (*ii*) the molten globule state (2–4, 19), and (*iii*) "structured" folding intermediates (3, 20). While the binding of an early Fab folding intermediate is in good agreement with data in the literature, the complex formation of GroEL with a late folding intermediate is without precedent. Although Dc is still nonnative, it resembles the native protein in several aspects: The β-sheets are formed and packed together building up native-like hydrophobic cores in all four domains. Furthermore, domain–domain interactions occur although in a nonnative form. Binding of this intermediate indicates that secondary structure by itself is not the prerequisite for interaction with GroEL but rather that the GroEL-accessible surface properties determine recognition by GroEL. This view is supported by the fact that casein, a native protein, can interact with GroEL. The most important characteristic of such a surface seems to be its hydrophobic properties (21, 22). Based on the ability of GroEL to suppress aggregation as an unproductive side reaction in protein folding (23), it has been assumed that GroE functions by keeping the amount of free aggregation-sensitive folding intermediates low (24). Recently, a different model has been proposed in which GroEL has a more active role in the folding process (9, 10). In this scheme of events, GroEL binds nonnative proteins, releases them in a less-stable partially unfolded form, and rebinds the fraction that does not fold rapidly to a native (-like) state. It is assumed that the rebound species contains slow folding or kinetically trapped molecules that upon binding to GroEL are partially unfolded, thus allowing the individual molecules to start the folding process from scratch. For Dc, we could not observe any unfolding events induced by interactions with GroEL by comparing the denaturation kinetics of Dc free in solution and bound to GroEL. This holds not only for the rate constants of the denaturation reaction but also for the amplitudes measured by fluorescence, indicating that not only a minor fraction of GroEL-bound Dc was monitored. Using denaturation kinetics for the analyses of the conformation allows us to monitor changes that influence the cooperative stabilizing network of interactions. Interactions that do not contribute to this network and, therefore, have only extremely small or no effects on the unfolding kinetics cannot be detected by this method. For Dc, the intrinsic characteristics of the intermediate do not seem to be affected by the binding process. Thus, we could narrow down the proposed destabilizing effect of GroEL to Fab structures occurring earlier during protein folding than Dc in the case of the Fab fragment. This suggests that GroEL functionally distinguishes between native-like or native and misfolded proteins by the intrinsic stability of the respective molecules (22), but in both cases, GroEL has the ability to bind appropriate surfaces.

We do, as proposed in the "unfolding model," observe complete release and rebinding of Dc during the folding process by competition with unfolded CS and the apparent deceleration of the folding kinetics in the presence of excess GroEL. The apparent dissociation constant of Dc, calculated from the "operational" microscopic rate constants, is ≈50 nM in the presence of ATP. The dissociation constant of peptides in the absence of any release factors was previously determined to be in the range of 10^{-3}–10^{-5} M (25). This great difference clearly shows the importance of cooperative binding of a substrate protein to GroEL. For Dc, the competition between folding and rebinding determines the kinetics observed. Complex formation of Dc and GroEL (rebinding) in the presence of ATP is a slow process, but in the absence of ATP the association process is significantly faster. For barnase, it has been demonstrated that complex formation in the absence of

ATP is diffusion-controlled (26). Thus, ATP seems to control complex formation by strongly decreasing the association rate. If the rate constant of dissociation is not changed significantly by ATP, this would imply that ATP, rather than promoting release, functions by inhibiting reassociation of the nonnative protein with GroEL.

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