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Two Conformational States of β-Lactamase Bound to GroEL: A Biophysical Characterization

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Escherichia coli RTEM β-lactamase, in which both cysteine residues which form the single disulfide bond have been mutated to alanine residues, can form stable reversible complexes with GroEL under two different sets of conditions. Starting with the GdmCl-denatured enzyme, it is bound to GroEL in a state where no protons are protected against exchange with ^2H_2O, as determined by electrospray ionization mass spectrometry (ESI-MS). In contrast, when native protein is destabilized at high temperature and added to GroEL, a conformation is bound with 18 protected protons after two hours of exchange. While the high-temperature complex can form both with the wild-type enzyme (with intact disulfide bond) and the Cys-Ala double mutant, only the latter protein can form a complex starting from GdmCl denatured states. Thus, two different sets of conformations of the same protein can be bound, depending both on the conditions used to form the complex and on the intrinsic stability of the intermediate recognized by GroEL, and we have characterized the properties of both complexes.

Keywords: molecular chaperones; protein folding; β-lactamase; hydrogen/deuterium exchange; mass spectrometry

Introduction

The *Escherichia coli* chaperonin GroEL can bind various substrate proteins, and efficiently assists their folding in the cell and prevents their aggregation under heat-shock conditions (Hartl, 1996; Lorimer, 1996; Fenton & Horwich, 1997). GroEL is a cylindrical protein composed of two heptameric rings of 57 kDa subunits stacked back to back. The 2.8 Å X-ray crystal structure of GroEL (Braig *et al.*, 1994, 1995) reveals that each subunit consists of three distinct domains: equatorial, intermediate and apical. The equatorial domain, connected to the apical domain by a hinge-like intermediate domain, contains the ATP binding site and provides most of the contacts between the subunits within one heptameric ring and all contacts between the two rings. The flexible apical domain forms the opening of the cylinder and exposes hydrophobic residues to the inside of the toroid. This is the location where the co-chaperonin GroES (Xu *et al.*, 1997) and the substrate protein are bound (Braig *et al.*, 1993; Chen *et al.*, 1994; Buckle *et al.*, 1997). Indeed, mutations of these residues abolish polypeptide binding (Fenton *et al.*, 1994). Many experiments have been performed in order to understand the structural properties of the substrate bound to GroEL; however, experiments with a variety of substrate proteins have led to very different proposals about the nature of the bound state, ranging from fully unfolded to native-like conformations.

One powerful technique for determining the conformation of the bound substrate is H/^{2}H exchange (Hvidt & Nielsen, 1966; Roder & Wüthrich, 1986; Englander *et al.*, 1996) in combination with either two-dimensional NMR (Zahn *et al.*, 1994a, 1996a,b; Goldberg *et al.*, 1997; Nieba-Axmann *et al.*, 1997) or ESI-MS (Robinson *et al.*, 1994; Miranker *et al.*, 1996; Gervasoni *et al.*, 1996; Gross *et al.*, 1996). H/^{2}H exchange experiments performed with cyclophilin (Zahn *et al.*, 1994a; Nieba-Axmann *et al.*, 1997) and barnase (Zahn *et al.*, 1996a,b), monitored by NMR, have shown that GroEL is able to catalyze the exchange of those protons that are buried in the core structure of the native substrate proteins. These results have lead to the conclusion that cyclophilin and barnase

Abbreviations used: H/^{2}H, hydrogen/deuterium; Cys-Ala β-lase, Cys-Ala double mutant β-lactamase; CD, circular dichroism; ESI-MS, electrospray ionization mass spectrometry; GdmCl, guanidinium hydrochloride; DHFR, dihydrofolate reductase; AMP-PNP, 5′-adenylylimido-diphosphate; BSA, bovine serum albumin.

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undergo transient global unfolding while bound to GroEL.

In contrast, core protons of DHFR bound to GroEL did not exchange (Goldberg *et al.*, 1997). In $H/^2H$ exchange experiments monitored by ESI-MS the protection of GroEL-bound DHFR against $H/^2H$ exchange was maintained during rounds of binding and release, driven by ATP hydrolysis, suggesting that the bound DHFR is partially folded and that the protein contains stable structure that does not unfold further during iterative binding cycles (Gross *et al.*, 1996). Analogous $H/^2H$ experiments show properties similar to the native state for the thermally destabilized β-lactamase (β-lase) (Gervasoni *et al.*, 1996) and reduced α-lactalbumin (Okazaki *et al.*, 1997), but conformations similar to the molten globule for a scrambled three-disulfide form of α-lactalbumin (Robinson *et al.*, 1994) have been proposed. In the case of lactate dehydrogenase (Badcoe *et al.*, 1991) and α-lactalbumin (Okazaki *et al.*, 1994), using other biochemical and biophysical techniques, an unfolded bound state has been proposed, with the latter protein also having been suggested to be bound in a molten globule state (Martin *et al.*, 1991; Hayer-Hartl *et al.*, 1994). Furthermore, a late folding intermediate of an antibody Fab fragment with native-like domains was found to bind to GroEL (Lilie & Buchner, 1995), but also α-helices with amphipathic properties (Landry & Gierasch, 1991; Brazil *et al.*, 1997) and β-sheet secondary structural elements (Schmidt & Buchner, 1992) were shown to bind to GroEL.

Consequently, a full picture of the structural features of the substrate proteins recognized by GroEL still has to emerge. These structural features probably depend not only on differences between different proteins, but also on the experimental conditions used for forming the substrate protein-GroEL complex (Gorovits & Horowitz, 1997). Until now the only common feature of all substrate proteins of GroEL seems to be the presence of hydrophobic patches, which are normally buried in the native state.

Here we use RTEM β-lase as a model system to study the interaction with GroEL under different conditions for complex formation. This model system offers unique insight because the thermodynamics and kinetics of various forms of β-lase (Mitchinson & Pain, 1985; Schultz *et al.*, 1987; Goto *et al.*, 1990; Laminet & Plückthun, 1989; Ptitsyn *et al.*, 1990; Uversky, 1993; Uversky & Ptitsyn, 1994; Walker & Gilbert, 1995; Frech *et al.*, 1996; Gervasoni & Plückthun, 1997; Vanhove *et al.*, 1995, 1997) and their interaction with GroEL (Laminet *et al.*, 1990; Zahn & Plückthun, 1992, 1994; Gervasoni *et al.*, 1996; Gervasoni & Plückthun, 1997) have been extensively characterized. At physiological temperatures only the precursor of β-lase can interact reversibly *in vivo* (Bochkareva *et al.*, 1988; Kusukawa *et al.*, 1989) and *in vitro* (Laminet *et al.*, 1990; Zahn & Plückthun, 1992) with GroEL, whereas binding of mature β-lase to GroEL has only been detected at 48°C. At room temperature a stable complex between chemically denatured mature β-lase and GroEL could not be detected (Zahn & Plückthun, 1994).

We have previously shown that a mutant β-lase, from which the disulfide bond was removed by two Cys-Ala mutations (abbreviated as Cys-Ala β-lase), was able to form a stable complex with GroEL, when the enzyme was first denatured in GdmCl. Under these conditions, GroEL forms a stable complex with the enzyme only at early stages of folding, but it does not bind late folding intermediates, which are populated before the slow phases of folding (Gervasoni & Plückthun, 1997). Here we show that a stable complex with GroEL can also be formed when the Cys-Ala β-lase is thermally destabilized by heating to 42°C (just below the thermal transition midpoint of the mutant enzyme). To study the conformational features required for binding to GroEL under these different conditions we performed $H/^2H$ exchange experiments monitored by ESI-MS. Our results suggest that the Cys-Ala β-lase binds to GroEL in two distinct sets of conformations. β-lase shows no protection against $H/^2H$ exchange when the complex is formed from GdmCl-denatured enzyme, indicating an unfolded state bound to GroEL. In contrast, when the complex was formed from thermally destabilized enzyme, significant protection against $H/^2H$ exchange was observed. Thus, the chaperonin is able to recognize very different states, ranging from unfolded to native-like conformations, even of the same protein, and these structural characteristics may depend on the general hydrophobic surface properties of the folding intermediates.

Results

Reversible complex formation of GdmCl denatured Cys-Ala β-lase

When Cys-Ala β-lase is equilibrium-denatured in GdmCl and then diluted into refolding buffer in the presence of GroEL, the enzyme binds tightly to the chaperonin, and folding is prevented (Gervasoni & Plückthun, 1997). Figure 1a shows the refolding kinetics of Cys-Ala β-lase, monitored by the regain of enzymatic activity, when GdmCl-denatured enzyme was diluted into refolding buffer. In the absence of the chaperonin (open circles) two molar equivalents of bovine serum albumin (BSA) were added to one of Cys-Ala β-lase, since BSA stabilizes the Cys-Ala β-lase at high dilution (Laminet & Plückthun, 1989; Walker & Gilbert, 1995). The filled circles indicate folding in the presence of two equivalents of GroEL and four equivalents of GroES and 1 mM ATP. Both curves were fitted to a sum of two exponentials. The first phase of folding was too fast to be correctly obtained from the data of Figure 1a, but could be obtained by performing the folding reaction directly in the enzymatic activity assay buffer

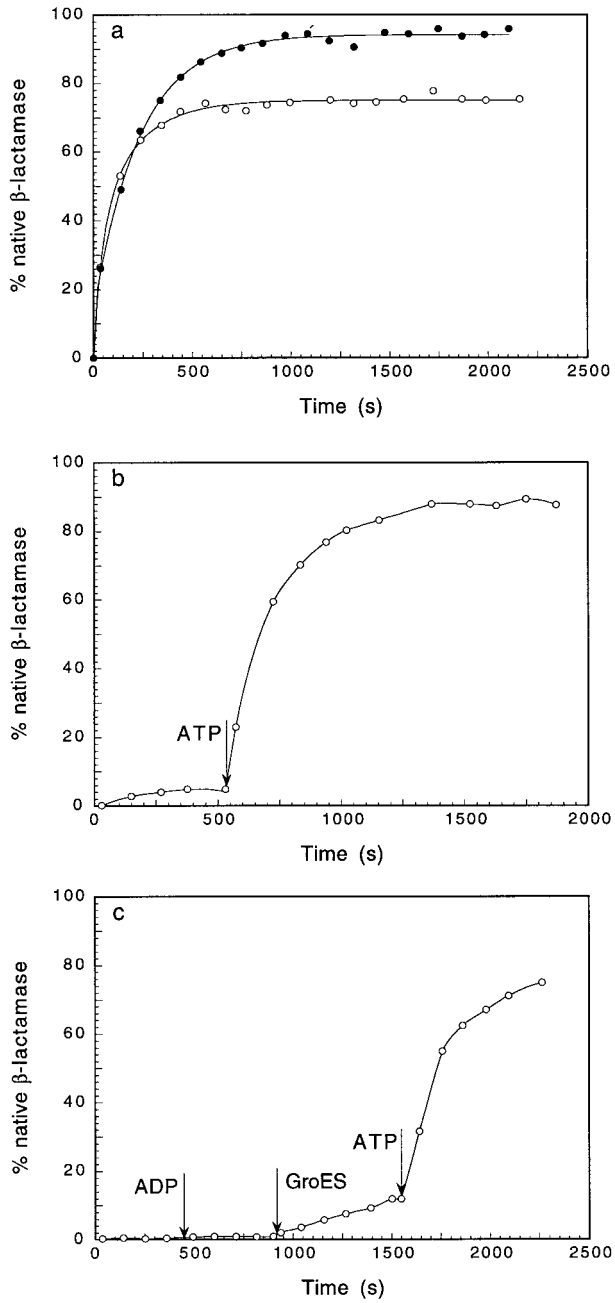

Figure 1. a, Folding of Cys-Ala β-lase in the presence or absence of GroEL/GroES/ATP. Cys-Ala β-lase was diluted 40-fold from a 4 M GdmCl solution into refolding buffer (final concentration 0.05 μM) containing 0.25 μM GroEL, 0.5 μM GroES and 1 mM ATP (●), or 0.5 μM BSA (○) (all final concentrations). The folding reaction was followed by withdrawing aliquots and immediately assaying for β-lase activity. b,c, Effect of the addition of GroES, ATP and ADP to GroEL-complexed Cys-Ala β-lase. The complex was formed by diluting GdmCl-denatured Cys-Ala β-lase (0.05 μM final concentration) into refolding buffer in the presence of a tenfold molar excess of GroEL over β-lase. At the time indicated by the arrows, 1 mM ADP, 1 μM GroES and 1 mM ATP (all final concentrations) were added to the refolding solution. At the time indicated by the experimental points, aliquots were withdrawn and immediately assayed for β-lase activity.

(Gervasoni & Plückthun, 1997), resulting in the same fast folding constant $k = 1.2 \times 10^{-2}\ s^{-1}$ for the folding reaction performed in the presence of GroEL, GroES and ATP as in their absence (data not shown). The rate constant of the second phase in the presence of GroEL, GroES and ATP was slightly slower than in their absence ($k = 3.6 \times 10^{-3}\ s^{-1}$ against $k = 6.0 \times 10^{-3}\ s^{-1}$).

After a stable complex between GroEL and Cys-Ala β-lase has formed, addition of 1 mM ATP causes its dissociation, and the enzyme folds to the native state and becomes enzymatically active (Figure 1b). Neither addition of ADP (Figure 1c) nor of AMP-PNP (data not shown) cause complex dissociation, suggesting the importance of ATP hydrolysis for releasing the enzyme from GroEL. When two molar equivalents of GroES were added to one of GroEL in the presence of ADP, only a slow complex dissociation could be measured by β-lase enzymatic activity (Figure 1c). In conclusion, Cys-Ala β-lase binds specifically to GroEL when the enzyme is diluted from a GdmCl solution, and dissociation of the complex can be specifically induced by addition of ATP, quantitatively regenerating native enzyme.

Recognition of thermally unfolded Cys-Ala β-lase by GroEL

Thermal denaturation of Cys-Ala β-lase was followed by measuring both enzymatic activity and mean residue molar ellipticity at 222 nm. Figure 2a shows the CD spectra of GdmCl-denatured (line 1), thermally destabilized (line 2) and native Cys-Ala β-lase (line 3). Compared to the chemically denatured enzyme, the enzyme exposed to 61°C still contains a significant amount of ordered structure, as was already described for other proteins (Aune *et al.*, 1967). In thermal unfolding, there was a steady increase of enzymatic activity up to 42°C, followed by an unfolding transition, with a midpoint of 47°C (Figure 2b and c, open circles). By following the CD signal, the transition midpoint of unfolding was also found to be 46°C (Figure 2b, small open diamonds). When GroEL was present in two molar equivalents with respect to Cys-Ala β-lase, there was an increase in the activity only up to 31°C, and a shift to lower temperatures by seven degrees for the unfolding transition midpoint (Figure 2c). Thus, GroEL recognizes thermally destabilized Cys-Ala β-lase, as it does wild-type β-lase (Zahn & Plückthun, 1994), below its denaturation temperature of 47°C. However, in contrast to wild-type enzyme, the Cys-Ala β-lase also allows stable complex formation from GdmCl denatured states, and thus a comparison of these bound states is possible.

The kinetic analysis of the thermal denaturation of Cys-Ala β-lase was performed by measuring the disappearance of its enzymatic activity (Figure 3). The denaturation rates of Cys-Ala β-lase were mono-exponential for all temperatures tested and not dependent on the presence of two molar

equivalents of GroEL (0.1 μM final concentration). At 42°C the rate of denaturation in the presence and in the absence of GroEL was found to be $1.1 \times 10^{-3}\ s^{-1}$ and $1.0 \times 10^{-3}\ s^{-1}$, respectively (Figure 3a). At 48°C in the absence of GroEL the kinetics of β-lase denaturation were best fitted by a single exponential decay plus a linear decrease (probably caused by slow aggregation). The unfolding rates at 48°C were also found to be indistinguishable within experimental error

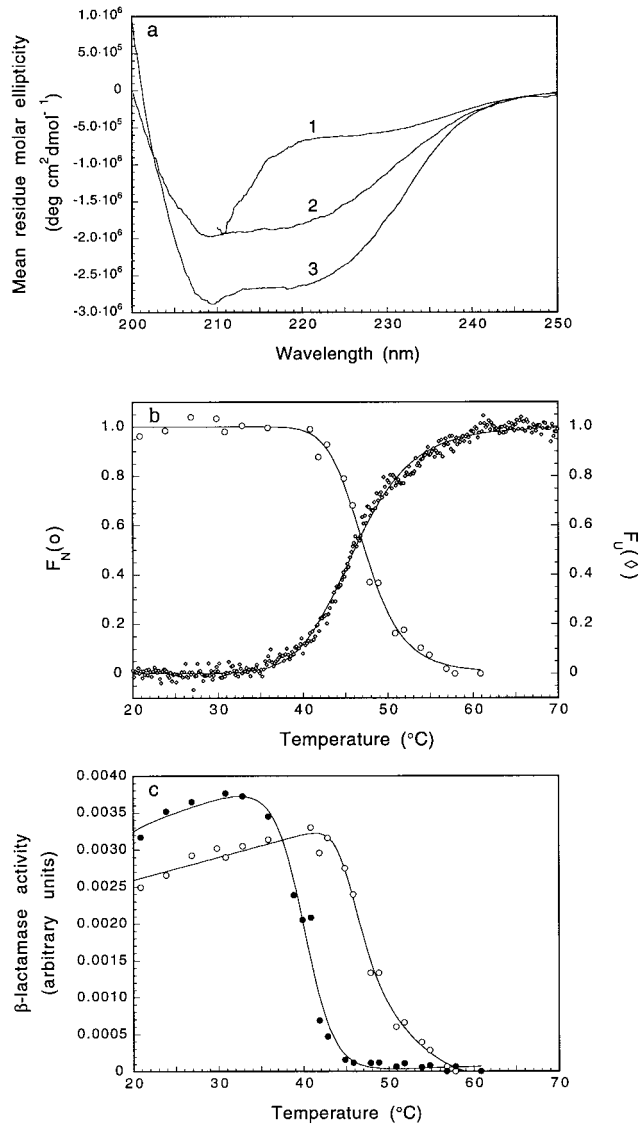

Figure 2. a, Far UV circular dichroism spectra of Cys-Ala β-lase (30 μM). Curve 1, in 4 M GdmCl pH 8.0; curve 2, 50 mM potassium phosphate buffer (pH 7.2) at 61°C; curve 3, in 50 mM potassium phosphate buffer (pH 7.2) at 6°C. b, Thermal unfolding of Cys-Ala β-lase. F_N, fraction of Cys-Ala β-lase (0.07 μM) in the native state, obtained by measuring enzymatic activity (○), measured after 15 minutes of incubation at the temperature indicated. The temperature of the enzymatic activity assay was the same as in the folding buffer. F_U, fraction of Cys-Ala β-lase (10 μM) in the unfolded state, obtained by measuring the mean residue molar ellipticity (◇, small symbols) as described in Experimental Procedures. Data from both thermal unfolding curves were fitted as described by Zahn & Plückthun (1994). c, Recognition of thermally destabilized Cys-Ala β-lase by GroEL. Enzymatic activity was measured as in b, in the absence (○) or in the presence (●) of two molar equivalents of GroEL.

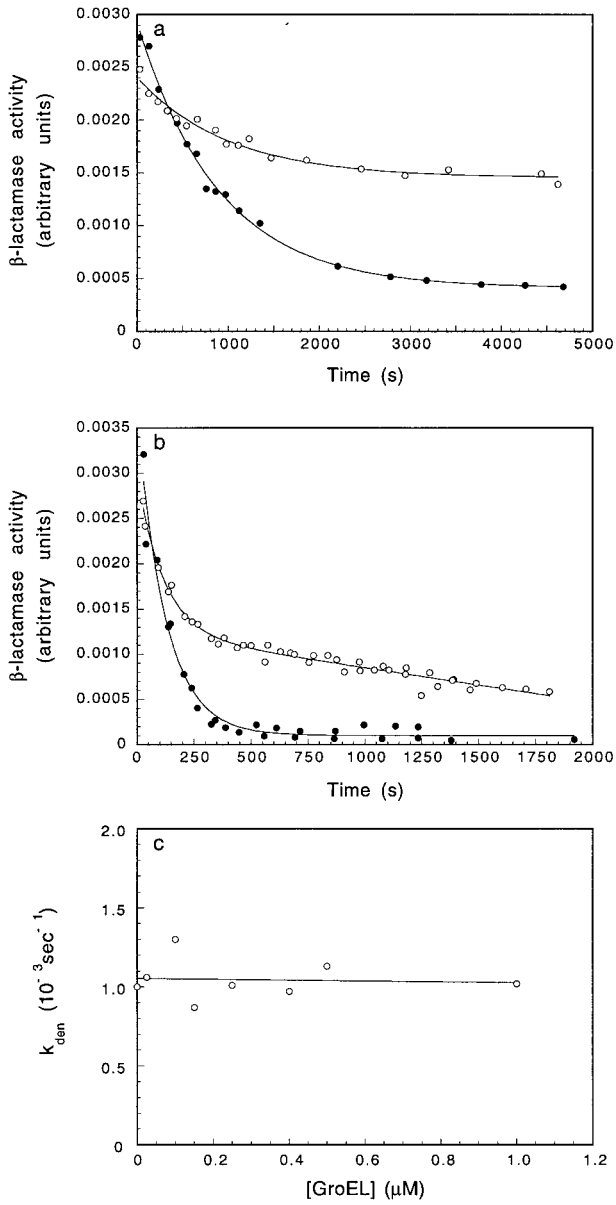

Figure 3. a,b, Unfolding kinetics of 0.07 μM Cys-Ala β-lase in the absence (○) or in the presence (●) of two molar equivalents of GroEL (0.14 μM) in the refolding buffer at (a) 42°C; (b) 48°C. Time courses of unfolding were followed by measuring the enzymatic activity. The curves were fitted by a single exponential, except the open circles in b, which were fitted by a single exponential plus a linear phase. The temperature of the enzyme assay buffer was 25°C for all experiments. c, Kinetics of apparent rate constants of unfolding (k_{den}) of Cys-Ala β-lase as a function of the GroEL concentration at 42 °C in refolding buffer. Cys-Ala β-lase final concentration was 0.05 μM for all measurements. The denaturation curves were analyzed according to first-order kinetics as in a.

(8.6×10^{-3} s^{-1} in the absence and 8.1×10^{-3} s^{-1} in the presence of GroEL; Figure 3b). At 25°C, the enzymatic activity did not decrease even in the presence of a higher ratio of GroEL over Cys-Ala β-lase and after longer incubation times (data not shown). Unfolding rates measured at 42°C were found to be independent of the GroEL concentration (Figure 3c). Taken together, all unfolding kinetics indicate that Cys-Ala β-lase unfolding is the rate-limiting step, followed by rapid binding to GroEL, consistent with previous results on the wild-type enzyme (Zahn & Plückthun, 1994).

Complex formation as a function of GroEL concentration and temperature

The extent of the Cys-Ala β-lase-GroEL complex formation at different temperatures, formed from chemically denatured β-lase (diluted from GdmCl into a GroEL solution) and from temperature destabilized Cys-Ala β-lase (by incubating native enzyme at high temperature with GroEL) were calculated by measuring the amount of free native β-lase as a function of GroEL concentration (Figure 4). When the complex was formed from thermally destabilized Cys-Ala β-lase, the apparent dissociation constant K'_D could be estimated from the fits of the curves to equation (2) (see Experimental Procedures) (Figure 4a). The K'_D is the product of the dissociation constant of the denatured form and the equilibrium constant of folding (equation (1)). When the enzyme was denatured in GdmCl, and refolding was started by dilution into refolding buffer in the presence of GroEL, the enzyme was kinetically partitioned between binding to the chaperonin and refolding to the native state. Consequently, the partitioning ratio K_P was obtained by fitting the data with equation (6) (see Experimental Procedures). We found for both complexes an exponential decrease in K'_D or K_P with increasing temperature in the measured range. When native Cys-Ala β-lase was incubated with GroEL below 36°C, K'_D was too large to be measured with this method. The previously measured K'_D of thermally destabilized wild-type β-lase shows a very similar temperature dependence (Zahn & Plückthun, 1994).

Double-jump refolding experiments

Folding of Cys-Ala β-lase shows a two-phase behavior in manual mixing (Vanhove *et al.*, 1997; Gervasoni & Plückthun, 1997). Interestingly, both phases are rate-limited by *cis-trans* isomerization around Xaa-Pro bonds (Vanhove *et al.*, 1997), even though the enzyme only contains one single *cis*-proline residue (Jelsch *et al.*, 1993). In order to investigate the role played by this single *cis*-proline in the interaction with GroEL, a double-jump experiment was performed. The Cys-Ala β-lase was first denatured for 15 seconds at 4°C in 4 M GdmCl and then immediately renatured by 100-fold dilution into refolding buffer at 25°C. The refolding process was followed by regain of β-lase enzymatic activity. When a tenfold molar excess of GroEL over β-lase was present in the refolding buffer, no activity at all was regained and refolding was thus quantitatively stopped (data not shown),

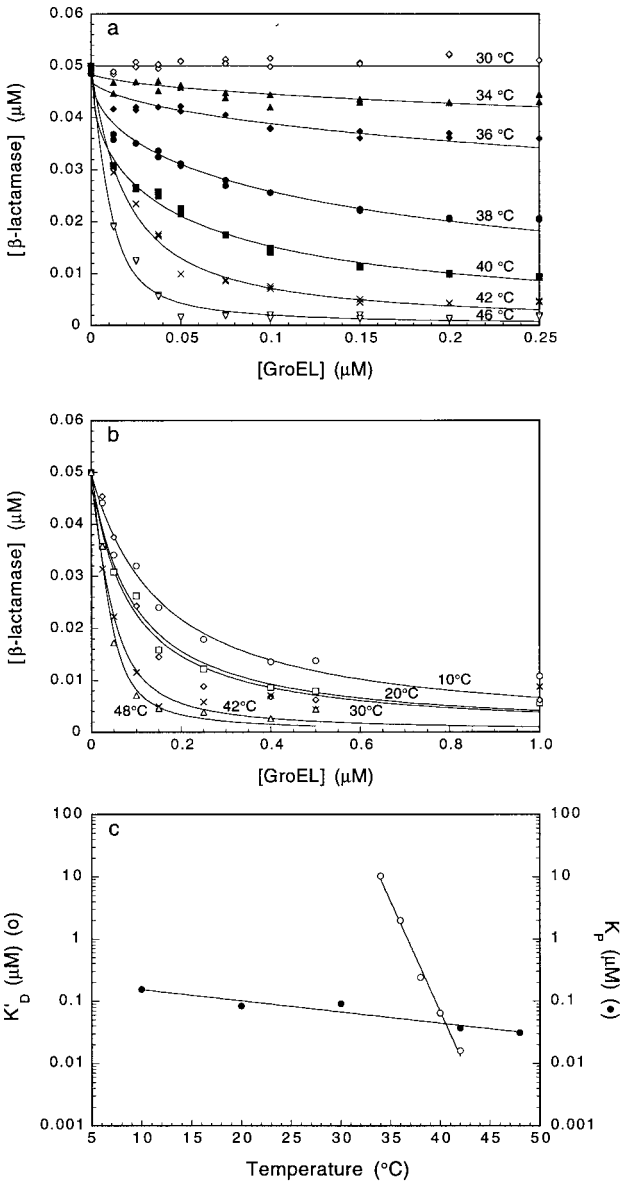

Figure 4. a,b, Titration of Cys-Ala β-lase (final concentration 0.05 μM) with GroEL. The temperatures used are indicated in the Figures. The concentration of free native Cys-Ala β-lase was determined by enzymatic activity after incubation at the indicated temperature with different total GroEL concentrations. a, Complex formed with thermally destabilized Cys-Ala β-lase. b, Complex formed with chemically denatured Cys-Ala β-lase. Fits in a and b assume that one molecule of Cys-Ala β-lase is bound to GroEL. c, Apparent dissociation constant K'_D and partitioning factor K_P as a function of temperature, as determined from the fitted curves in a (○) with equation (2), and from b (●) with equation (6), as described in Experimental Procedures.

demonstrating that Cys-Ala β-lase can bind to GroEL regardless of whether its four proline residues are in the correct native conformation. The interaction of Cys-Ala β-lase with GroEL must therefore involve large surfaces and not only small structural features around the *cis*-proline.

H/^{2}H exchange: folding of wild-type and Cys-Ala β-lase in the absence of GroEL

In order to determine the number of hydrogen bonds resistant to exchange against deuterons from the solvent during the refolding of wild-type and Cys-Ala β-lase we carried out H/^{2}H exchange experiments monitored by ESI-MS. The folding of the denatured and deuterated proteins was initiated by dilution into refolding buffer in ^2H_2O (pH 8.0). After different periods of time aliquots were withdrawn and further diluted sixfold into H_2O until the folding reaction was complete (the final pH was 5.0). This allows H/^{2}H exchange of still unprotected deuterons to take place and the folding reaction to go to completion. The final number of protected deuterons was measured by ESI-MS and corrected for residual ^2H_2O in the final solvent. Figure 5 shows the time course of the number of deuterons that become protected during the folding process in the absence of chaperonin. Both wild-type and Cys-Ala β-lase show a bi-phasic deuterium incorporation. As observed by tryptophan fluorescence spectroscopy and enzymatic activity (Vanhove *et al.*, 1995, 1997; Gervasoni & Plückthun, 1997), both wild-type and Cys-Ala β-lase show a similar folding behavior, and the two phases at 0.1 M GdmCl final concentration were not significantly different between the two forms of the enzyme. About 60 deuterons become protected during the first two minutes of folding, followed by a slow phase in which only a slight increase of the number of protected deuterons was detected. Thus, stable secondary structures are formed early during the refolding of both wild-type and Cys-Ala β-lase.

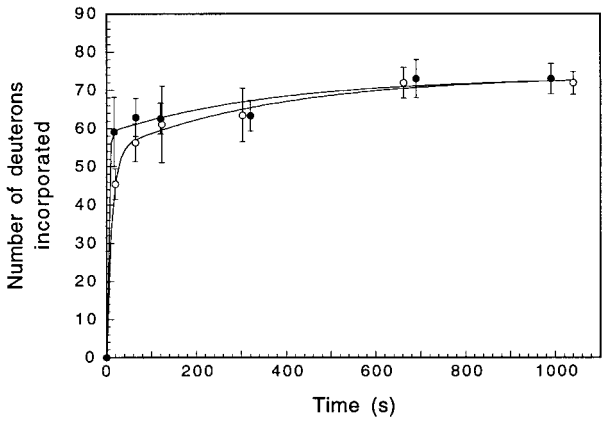

Figure 5. Rates of deuterium incorporation for Cys-Ala β-lase (○) and wild-type β-lase (●). Error bars were derived from the +18 to +30 charge states from two mass spectra obtained for each time point. Data were acquired and analyzed as described in Experimental Procedures.

H/^{2}H exchange of the GroEL-bound Cys-Ala β-lase

Complex formed from GdmCl denatured Cys-Ala β-lase

In order to investigate the nature of the intermediate of Cys-Ala β-lase bound to GroEL during refolding H/^{2}H exchange experiments were performed. Cys-Ala β-lase was completely denatured in 4 M GdmCl in ^2H_2O, and complex formation or refolding at 25°C was started by dilution into 100 mM potassium phosphate (pH 7.2) in ^2H_2O in the presence or absence of a tenfold molar excess of GroEL over β-lase. After 30 seconds, the complex was diluted 20-fold into folding buffer in H_2O to label the protein at those sites that are not protected in the complex with GroEL. After 45 seconds of labeling, Cys-Ala β-lase was then dissociated from GroEL by addition of ATP and allowed to fold to completion. Regain of β-lase activity (50% in the absence and 95% in the presence of GroEL) confirmed complex dissociation and complete β-lase refolding in the presence of GroEL. The number of deuterons which become protected during the first 30 seconds of refolding in ^2H_2O, in the presence and in the absence of GroEL, were quantified by ESI-MS. In the absence of GroEL, a molecular weight of Cys-Ala β-lase of 28,896 Da was measured, corresponding to 48(±4.2) captured deuterons. This is in agreement with the results of Figure 5 (30 seconds time point, open circle) described above, from which we conclude that stable secondary structure is formed early. In the presence of GroEL, the molecular weight of Cys-Ala β-lase was increased by only 4(±1.8) mass units, and thus, essentially no protons at all are protected under these conditions and the enzyme must be bound immediately. When Cys-Ala β-lase is refolded in H_2O from the ^2H_2O denatured state in the absence of GroEL, a broad distribution of masses results (the peak width at 50% intensity is 67 Da, data not shown), since a very different number of protons is trapped by different molecules upon folding. When the protein is released from GroEL, however, a very sharp mass of the protonated species results (the peak width at 50% intensity is only 30 Da, data not shown), indicating that a different process is observed, namely that of an exchange prior to folding. This strengthens the conclusion of exchange occuring in the complex and not afterwards. These results imply that GroEL prevents the refolding of the Cys-Ala β-lase by forming a stable complex with the enzyme during its early stages of folding, when intermediates with stable native hydrogen bonds have not yet formed.

Complex formed from thermally destabilized Cys-Ala β-lase

In order to investigate the conformation of the Cys-Ala β-lase at high temperature $H/^2H$ exchange experiments were performed. The exchange reaction was started by tenfold dilution of completely deuterated native β-lase, previously equilibrated at the indicated temperature, into buffered H_2O (pH 6.2) in the absence or presence of two molar equivalents of GroEL, equilibrated at the same temperature. Aliquots were taken at different time points and rapidly injected into the mass spectrometer without further purification steps. Figure 6 shows the reconstructed mass spectra of the $H/^2H$ exchange reaction, performed at 42°C, of Cys-Ala β-lase alone (Figure 6a) and Cys-Ala β-lase in the presence of GroEL (Figure 6b). It can be seen that, both in the absence and presence of GroEL, the initial peak first moves to a lower molecular weight and an additional peak subsequently appears at a low molecular weight and in a constant position. After two hours a single peak, with a centroid at 28,879 Da for Cys-Ala β-lase alone and 28,882 Da for Cys-Ala β-lase in complex with GroEL, was observed. Taking into account the residual ^2H_2O present during the experiment, 15 deuterons were calculated to be protected against $H/^2H$ exchange for Cys-Ala β-lase alone and 18 deuterons for Cys-Ala β-lase in complex with GroEL after two hours. Thus, the $H/^2H$ exchange experiments, performed with thermally destabilized Cys-Ala β-lase at 42°C (below its thermal midpoint of denaturation) suggest a conformation bound to GroEL with 18 hydrogen bonds intact. At 48°C the $H/^2H$ exchange kinetics were completed within 10 to 15 minutes and reached an equilibrium of 15 deuterons of Cys-Ala β-lase protected in the complex (data not shown).

We then wished to elucidate the mechanism of $H/^2H$ exchange of GroEL bound Cys-Ala β-lase in the framework of the following minimal scheme:

$$\begin{array}{ccccc} \mathrm{Bla_c\text{-}H} & \underset{k_c}{\overset{k_o}{\rightleftarrows}} & \mathrm{Bla_o\text{-}H} & \xrightarrow{k_{ex}} & \mathrm{Bla_o\text{-}^2H} \underset{k_o}{\overset{k_c}{\rightleftarrows}} \mathrm{Bla_c\text{-}^2H} \\ & & k_{on}\downarrow\uparrow k_{off} & & k_{on}\downarrow\uparrow k_{off} \\ & & \mathrm{GroEL\text{-}Bla_o\text{-}H} & \xrightarrow{k_{ex}} & \mathrm{GroEL\text{-}Bla_o\text{-}^2H} \end{array}$$

$H/^2H$ exchange takes place when structural fluctuations of the protein (characterized by an opening rate k_o and a closing rate k_c) cause exposure of the protons to solvent, and those protons will exchange with an intrinsic exchange rate k_{ex}. The observed rate constant for exchange k_{obs} is given by $k_{obs} = k_o k_{ex}/(k_{ex} + k_c)$ (Hvidt & Nielsen, 1966) and two limits can be defined: when $k_c \gg k_{ex}$ (the EX_2 limit) the equation is simplified to $k_{obs} = k_o k_{ex}/k_c$ and when $k_{ex} \gg k_c$ (the EX_1 limit) the equation becomes $k_{obs} = k_o$. Under the EX_2 mechanism the exchange rate k_{obs} is a function of the intrinsic solvent exchange rate (k_{ex}) and of the unfolding/folding equilibrium constant, which in a two-state model is equal to the ratio of the equilibrium concentrations $[Bla_o]_{eq}/[Bla_c]_{eq}$. Under the EX_1 mechanism the rate constant for exchange k_{obs} is also a direct measure of the rate of protein unfolding.

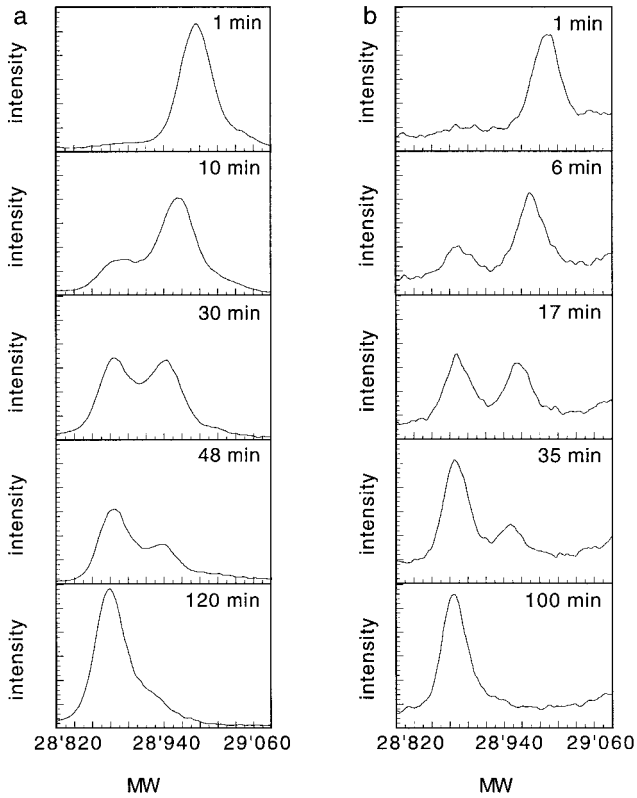

Figure 6. Time course of $H/^2H$ exchange. Reconstructed ESI-MS spectra after $H/^2H$ exchange against H_2O at pH 6.2 and 42°C of deuterated Cys-Ala β-lase in the absence (a) and in the presence (b) of two molar equivalents of GroEL. The time period of exchange is specified in the Figure.

By analyzing the transformed mass spectra these two limits can be distinguished (Miranker *et al.*, 1993), and information can be obtained on whether $H/^2H$ exchange takes place by local or global unfolding (Chung *et al.*, 1997). If $H/^2H$ exchange takes place by a pure EX_2 mechanism, a single peak, whose mass will shift with time, will be detected. If $H/^2H$ exchange takes place by a pure EX_1 mechanism, two peaks will be observed, with masses reflecting the molecules that did not undergo exchange at all, and the molecules that underwent complete exchange, with their intensities reflecting the amount of $H/^2H$ exchange. Under the conditions tested only a shift of the peak to lower molecular weight, indicating a pure EX_2 mechanism, was observed when the $H/^2H$ exchange was performed at 25°C (data not shown). In contrast, a mixture of the two limiting cases described was observed at 42°C (Figure 6) and 48°C (data not shown), both in the presence and absence of GroEL, suggesting that the intrinsic

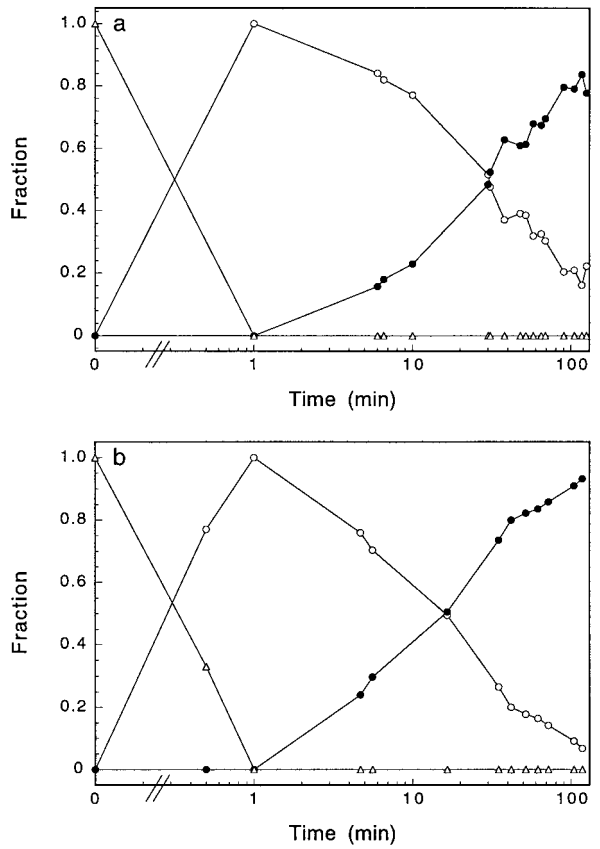

Figure 7. a,b, Time course of the population of the three principal states of Figure 6 ((Δ), native Cys-Ala β-lase deuterated at all exchangeable sites N; (●), final state protected against further H/^{2}H exchange U; (○), all intermediate states between N and U, observed in the H/^{2}H exchange behavior of the deuterated Cys-Ala β-lase at 42 °C in the absence (a) and in the presence of GroEL (b)). Because of the complex H/^{2}H exchange kinetics, which are not reducible to a limited sum of exponential terms, the data points have been connected to each other only for illustrative purposes. For clarity, in a and b a logarithmic time scale is used.

H/^{2}H exchange mechanism of Cys-Ala β-lase is not perturbed by the chaperonin. Figure 6 clearly shows first a shift of the centroid of Cys-Ala β-lase to lower masses (characteristic of an EX_2 mechanism), followed by the appearance of a second peak (characteristic of an EX_1 mechanism), corresponding to the protected final state.

Figure 7 shows the time course of the intensities of the three principal states (initial, intermediate mass, final), obtained from the reconstructed ESI-MS spectra of Figure 6. While the time courses in the presence and absence of GroEL are similar, they are not identical (Figures 6 and 7). Exchange is slightly faster in the presence of GroEL (after 30 minutes H/^{2}H exchange only 50% of the Cys-Ala β-lase molecules have reached the protected final state in the absence of GroEL (Figure 7a), as compared to 75% in its presence (Figure 7b)), perhaps because GroEL stabilizes the bound open state. If the on-rate k_{on} is fast, relative to the rate at which open forms are populated which can form a stable complex, the equilibrium between the open and the closed form of the enzyme is shifted to the exchange competent state. Consequently, under the EX_2 exchange mechanism the observed exchange rate k_{obs} increases, even if the intrinsic exchange rate (k_{ex}) is not effected by GroEL. Under an EX_1 mechanism, k_{obs} is only accelerated if k_o itself is faster. In the simplified model described above we assume that the form of Cys-Ala β-lase that undergoes H/^{2}H exchange is also the form recognized by GroEL. However, the principal ideas of this model also hold in a more complicated situation. If the enzyme binds to GroEL at a patch not relevant for hydrogen exchange, any more open form of the enzyme would subsequently be stabilized by additional interactions with hydrophobic residues of GroEL, thus accelerating an EX_2 exchange. Most likely, such binding would also lower the transition state of unfolding, thereby increasing the rate in an EX_1 exchange.

Discussion

We present here an analysis of the interaction of the Cys-Ala double mutant β-lase with GroEL under two distinct sets of conditions. When GdmCl-denatured Cys-Ala β-lase was incubated with an excess of GroEL during refolding, a stable, specific and reversible complex was formed (Figure 1). When this complex was subjected to H/^{2}H exchange, no protection could be detected by ESI-MS, suggesting a bound state lacking stable native-like secondary structure. The stable complex formed from GdmCl-denatured Cys-Ala β-lase thus appears to be loosely packed, and must be able to make hydrophobic contacts with GroEL by exposing hydrophobic amino acid residues. In contrast, when H/^{2}H exchange was performed with complexes formed from thermally destabilized Cys-Ala β-lase at 42°C, about 18 protons remained protected over two hours when the enzyme was bound to GroEL (Figures 6 and 7). When H/^{2}H exchange experiments had been performed with wild-type β-lase under similar conditions (except that a temperature of 48°C was used), it was shown that the enzyme retained 49 protons and thus a considerable amount of native-like structure when bound to GroEL (Gervasoni *et al.*, 1996). Thus, Cys-Ala β-lase is able to bind to GroEL in two different sets of conformations.

It is important to note that GdmCl-denatured wild-type β-lase does not form stable complexes with GroEL (data not shown), indicating that the disulfide bond prevents a conformation crucial for binding under these conditions. Previous experiments, using reduced urea-unfolded wild-type β-lase and stoichiometric amounts of GroEL showed no influence on folding (Laminet *et al.*, 1990; Zahn & Plückthun, 1992, 1994), while a small

excess of GroEL showed some retardation of folding (Zahn & Plückthun, 1994). Both wild-type and Cys-Ala β-lase fold through a partially folded intermediate of the molten globule type, and exist in three stable states in urea or GdmCl equilibrium folding experiments (Georgiou *et al.*, 1994; Zahn & Plückthun, 1994; Vanhove *et al.*, 1995, 1997; Frech *et al.*, 1996). Removal of the disulfide bond results in a decrease of 14.2 kJ mol^{-1} in the global free energy of stabilization (Vanhove *et al.*, 1997). The transition from the equilibrium folding intermediate H to the native protein N was less affected by the disulfide bond ($\Delta\Delta G_{N\text{-}H} = 3.7$ kJ mol^{-1}) than the transition from the fully unfolded protein U to H ($\Delta\Delta G_{H\text{-}U} = 10.4$ kJ mol^{-1}; Vanhove *et al.*, 1997). The transition U to H is probably more complex than a two-state process, and additional intermediates might exist between U and H (Uversky & Ptitsyn, 1994). The kinetics of interconversion between N and H are also independent of the presence of the disulfide bridge (Frech *et al.*, 1996; Vanhove *et al.*, 1997). By observing the PDI- and DsbA-mediated oxidation of β-lase during refolding, it was suggested that the region around the disulfide bond in the kinetic intermediate I should already be in a native-like conformation, before the slow rate-limiting phase of folding takes place (Walker & Gilbert, 1995; Frech *et al.*, 1996). These results are consistent with the observation obtained with wild-type β-lase from Trp-fluorescence kinetics, where it was shown that Trp210 and the disulfide bond, after few seconds refolding, must be already juxtaposed as in the native conformation (Gervasoni & Plückthun, 1997). The Trp-fluorescence kinetics experiments and the H/^{2}H exchange experiments both suggest that GroEL is able to capture unfolded Cys-Ala β-lase which exposes sufficient hydrophobic surface to lead to stable binding, but only during the first seconds of folding. In contrast, GroEL is not able to capture wild-type β-lase under the same conditions, because of the constraining effect of the disulfide bond. Furthermore, our double-jump folding experiments have shown that the enzyme forms a stable complex with GroEL whether or not the three *trans*- and the single *cis*-proline residue are in the native conformation.

A thermodynamic partitioning model, in which GroEL mediates unfolding of a substrate protein by binding to its unfolded form and thereby shifting the overall equilibrium toward the unfolded state, was proposed (Zahn & Plückthun, 1994; Zahn *et al.*, 1994b; Walter *et al.*, 1996). From this model it follows that GroEL promotes unfolding only if the free energy of binding is similar or larger than the conformational stability of the intermediate. This reaction can be observed only if the intrinsic rate of unfolding is sufficiently high. Analysis of the thermal denaturation experiment (Pace, 1990) of Cys-Ala β-lase monitored by measuring the mean residue molar ellipticity gave a free energy of unfolding at 42°C of approximately −3.4 kJ mol^{-1}. The overall binding equilibrium between Cys-Ala β-lase and GroEL (when the complex was formed starting from thermally destabilized Cys-Ala β-lase at 42°C), was derived from:

$$\mathrm{Bla_N} \rightleftarrows \mathrm{Bla_U}$$

$$\frac{\mathrm{Bla_U + GroEL} \rightleftarrows \mathrm{Bla_U \cdot GroEL}}{\mathrm{Bla_N + GroEL} \rightleftarrows \mathrm{Bla_U \cdot GroEL}}$$

Under the assumption that at equilibrium the amount of free, unfolded Cys-Ala β-lase (Bla_U) or bound native Cys-Ala β-lase ($Bla_N \cdot EL$) is negligible and since the total GroEL concentration (EL_t) is larger than that of Cys-Ala β-lase (Bla_t) to ensure complete complex formation, the overall K_D of the complex ($Bla_U \cdot EL$) could be calculated to be 1.06×10^{-8} M at 42°C, and the observed free energy of complex formation from Bla_N and GroEL was approximately −3.3 kJ mol^{-1} at the concentrations used. Consequently, the intrinsic free energy of complex formation is sufficient to shift the equilibrium toward the complex. Indeed, by following the thermal denaturation kinetics at 42°C of Cys-Ala β-lase in the presence of GroEL we found that GroEL binds to thermally destabilized Cys-Ala β-lase and shifts the equilibrium toward the complex, but that it does not affect the rate constant of unfolding (Figure 3). Nevertheless, GroEL does not further unfold Cys-Ala β-lase to a completely denatured state. As determined by H/^{2}H exchange experiments Cys-Ala β-lase in complex with the chaperonin still shows a significant number of deuterons protected against exchange after two hours (Figures 6 and 7). Although we cannot exclude *a priori* that a minority of the Cys-Ala β-lase molecules binds to GroEL in a fully unfolded state, which are in equilibrium with a more native-like conformation, we cannot detect such molecules under the conditions used.

When urea-unfolded or thermally inactivated DHFR was presented to GroEL, different protease-resistent conformations of DHFR were detected, suggesting that the experimental conditions used may contribute to differences in the formation of the complex (Gorovits & Horowitz, 1997). Our experiments performed with β-lase confirm this observation and show that GroEL is able to bind thermally destabilized Cys-Ala β-lase in a conformation that still contains stable secondary structure, and by doing so avoids protein aggregation; this behavior can be well explained by a thermodynamic partitioning model (Zahn & Plückthun, 1994; Zahn *et al.*, 1994b; Walter *et al.*, 1996). But GroEL is also able to transiently bind an early intermediate lacking stable secondary structure during the folding of GdmCl-denatured Cys-Ala β-lase, increasing the final yield of native protein in the presence of ATP and GroES. A kinetic partitioning model (Hardy & Randall, 1991; Katsumata *et al.*, 1996; Ranson *at al.*, 1997) was used to describe the ability of the chaperonin to block refolding of Cys-Ala β-lase. In conclusion, the cha-

peronin GroEL binds to Cys-Ala β-lase in at least two different conformations, depending on the experimental conditions used to form the substrate-protein GroEL complex, and on the intrinsic stability of the intermediate recognized by GroEL. Experiments to determine if the observed different conformations of the GroEL-bound Cys-Ala β-lase are interchangeable, are in progress.

Experimental Procedures

Protein preparation

Wild-type and Cys-Ala β-lase were produced and purified by methods that have been described elsewhere (Laminet & Plückthun, 1989; Gervasoni *et al.*, 1996). The chaperonins GroEL and GroES were purified from the *Escherichia coli* strain W3110 harboring the plasmid pOF39 (Fayet *et al.*, 1989) as described previously (Zahn *et al.*, 1994a,b) except for an additional FPLC Mono-Q Sepharose (Pharmacia) step in 50 mM Tris, 0.1 mM EDTA, 1 mM DTT (pH 7.2), using gradient elution with 0 to 500 mM NaCl. Protein concentrations were measured using the bicinchoninic acid assay (Pierce) and are always given for the oligomeric state. All measurements in this work, unless stated otherwise, were carried out in folding buffer (50 mM Mops, 50 mM KCl, 5 mM $MgCl_2$, pH 7.2) at 25°C.

Enzymatic activity assay

The β-lase enzyme assay was performed at 25°C as described previously (Laminet & Plückthun, 1989; Gervasoni & Plückthun, 1997) except for the experiment presented in Figure 2c, where the temperature of the assay was the same as in the folding buffer.

Circular dichroism spectroscopy

The decrease in secondary structure of the Cys-Ala β-lase (10 μM in 50 mM potassium phosphate, pH 7.2) during thermal denaturation was recorded by circular dichroism (CD) spectroscopy on a JASCO-500 spectropolarimeter using a thermostated cell with 1 mm pathlength. The temperature was increased at a linear rate of 0.5°C/min. The spectra were evaluated as described elsewhere (Zahn *et al.*, 1996b).

Dissociation constant of Cys-Ala β-lase with GroEL

In order to determine the apparent equilibrium constant for dissociation (K'_D) titration experiments were performed with thermally destabilized Cys-Ala β-lase and GroEL. Cys-Ala β-lase was incubated with GroEL at temperatures between 34 and 42°C. The K'_D of the complex, formed from thermally destabilized Cys-Ala β-lase, which is the product of both the dissociation constant of the complex and the folding equilibrium of Cys-Ala β-lase (equation (1)), was determined from a one-parameter fit using equation (2), which was derived from the law of mass action:

$$K'_D = \frac{[\mathrm{Bla_N}][\mathrm{EL_{free}}]}{[\mathrm{Bla_U} \cdot \mathrm{EL}]} \quad (1)$$

$$[\mathrm{Bla_N}] = [Bla_t] - \frac{[Bla_t] + K'_D + n[\mathrm{EL_t}]}{2} - \frac{\sqrt{-4[\mathrm{Bla_t}]n[\mathrm{EL_t}] + (-[\mathrm{Bla_t}] - K'_D - n[\mathrm{EL_t}])^2}}{2} \quad (2)$$

$[\mathrm{Bla_t}]$ and $[\mathrm{EL_t}]$ are the total concentrations of Cys-Ala β-lase and GroEL, respectively; $[\mathrm{Bla_U} \cdot \mathrm{EL}]$ is the concentration of the complex between non-native Cys-Ala β-lase and GroEL; and n, which was set to 1, is a defined number of GroEL-binding sites. The concentration of free native Cys-Ala β-lase $[\mathrm{Bla_N}]$ was obtained by measuring the enzymatic activity one hour after incubation, since experiments had shown that there is no further change after this time.

Kinetic partitioning ratio of Cys-Ala β-lase

The ability of GroEL to block the refolding of chemically denatured Cys-Ala β-lase is due to a kinetic partitioning of the enzyme between folding and association to GroEL. Therefore, to obtain the kinetic partitioning ratio K_P of the complex, formed starting from GdmCl-denatured Cys-Ala β-lase, as a function of GroEL concentration and temperature, the experimental data were analyzed with equation (6), on the basis of the competition between folding (equation (3)) and binding (equation (4)):

$$\mathrm{Bla_U} \xrightarrow{k_f} \mathrm{Bla_N} \quad (3)$$

$$\mathrm{Bla_U} + \mathrm{GroEL} \xrightarrow{k_b} \mathrm{Bla_U} \cdot \mathrm{GroEL} \quad (4)$$

Assuming that one molecule of Cys-Ala β-lase is bound to GroEL and that folding can only occur in free solution, it follows equation (5):

$$\frac{[\mathrm{Bla_N}]}{[\mathrm{Bla_U} \cdot \mathrm{EL}]} = \frac{[\mathrm{Bla_N}]}{[\mathrm{Bla_t}] - [\mathrm{Bla_N}]} = \frac{k_f}{k_b[\mathrm{EL_{free}}]} \quad (5)$$

By solving equation (5) for $[\mathrm{Bla_N}]$, by setting $[\mathrm{EL_{free}}] = [\mathrm{EL_t}]$, since $[\mathrm{EL_t}]$ is present in large excess, and by defining $K_P = k_b/k_f$, equation (6) is obtained:

$$[\mathrm{Bla_N}] = \frac{[\mathrm{Bla_t}]}{(K_p[\mathrm{EL_t}] + 1)} \quad (6)$$

Chemically denatured Cys-Ala β-lase (in 4 M GdmCl for two hours at 20°C) was diluted into refolding buffer containing increasing amounts of GroEL (β-lase final concentration was 0.05 μM, GdmCl final concentration was 0.1 M) at different temperatures between 10 and 48°C. $[\mathrm{Bla_N}]$ was obtained by measuring β-lase enzymatic activity 15 minutes after refolding was started.

Sample preparation for H/^{2}H exchange

Wild-type and Cys-Ala β-lase were deuterated at all exchangeable sites by exposure to 4 M GdmCl in ^2H_2O for two hours at pH 7.0 (values given in the text for pH and p^{2}H were taken directly from the pH-meter). Protein samples were then freeze-dried, redissolved in ^2H_2O and incubated for two hours at 25°C. This procedure was repeated three times to ensure complete deuteration at all labile sites. Protein samples were then desalted on a PD-10 column (Pharmacia) equilibrated with 100 mM potassium phosphate (pH 7.2) in ^2H_2O and allowed to

refold at 4°C for two hours. The yield of correctly refolded β-lase was checked by measuring the enzymatic activity.

H/^{2}H exchange: folding of β-lase in the absence of GroEL

20 μM wild-type or Cys-Ala β-lase was denatured in 4 M GdmCl ^2H_2O buffer (pH 8.0) at 25°C for two hours. The folding reaction was initiated by a 100-fold dilution of the protein samples into 50 mM Tris (pH 8.0) in ^2H_2O at 10°C. Within a 20-minute period aliquots were withdrawn at different time points and further diluted sixfold into citrate buffer (pH 4.9) in H_2O. The final pH after mixing was 5.0. The folding process was then allowed to proceed to completion for two hours at 10°C. The protein solutions were buffer-exchanged against ammonium acetate (pH 6.0) in H_2O by a gel filtration step on a PD-10 column (Pharmacia) and concentrated with a Centricon-10 concentrator (Amicon) to a final concentration of 20 μM. Molecular masses were determined immediately by ESI-MS as described below. The final number of protected deuterons was corrected for residual ^2H_2O (17%), still present in the refolding buffer after the second dilution into H_2O, using the equation $y + (n-y) \times 0.17 = x$, where y is the number of protons which become protected during folding due to the formation of stable hydrogen bonds; n is the number of deuterons becoming protected during the whole folding reaction (73 for both wild-type and Cys-Ala β-lase); x is the measured number of deuterons incorporated, obtained from the difference between the measured and the theoretical molecular weight of the enzyme in H_2O (28,845 Da for Cys-Ala β-lase, 28,906 for wild type β-lase). When the refolding experiment was performed at 10°C, the reconstructed mass spectra of the Cys-Ala β-lase, taken at different time points after refolding was started, showed a minor second peak corresponding to the protein's theoretical molecular weight in H_2O. At 25°C this was more pronounced than at 10°C and might reflect the fraction of molecules unable to fold correctly to the native state, since only 50% enzymatic activity was regained when GroEL was not present in the refolding buffer at 25°C. This behavior was not observed with wild-type β-lase.

H/^{2}H exchange: kinetic complex formation

Deuterated Cys-Ala β-lase that had been denatured in 4 M GdmCl in ^2H_2O during two hours at 25°C was diluted 40-fold into 100 mM potassium phosphate (pH 7.2) in ^2H_2O containing a tenfold molar excess of GroEL over β-lase. GroEL had been previously buffer exchanged on a PD-10 column (Pharmacia) equilibrated with 100 mM potassium phosphate (pH 7.2) in ^2H_2O. After 30 seconds of incubation in ^2H_2O (complex formation is observed under these conditions, as verified by the absence of β-lase enzymatic activity) the β-lase-GroEL complex was further diluted 20-fold into H_2O (50 mM Mops, 50 mM KCl, 5 mM $MgCl_2$, pH 7.2) to allow labeling at all non-protected sites of β-lase. Because of the high amount of GroEL over β-lase it was impossible to obtain good mass spectra of Cys-Ala β-lase at this stage. The H/^{2}H exchange was therefore quenched by refolding of Cys-Ala β-lase, and the native enzyme was separated from GroEL by a gel filtration step. To induce folding ATP was added to 0.8 mM final concentration 45 seconds after the 20-fold dilution into H_2O; the subsequent complex dissociation and quantitative β-lase refolding was checked by measuring enzymatic activity during one hour. The protein samples were then concentrated to 50 μl final volume with a Centricon-10 concentrator (Amicon) and loaded on a G3000SWXL (7.8 mm × 30 cm) gel filtration column, equilibrated with the ESI-MS compatible ammonium acetate buffer (pH 6.2) in H_2O. This step simultaneously allows separation of β-lase from GroEL and desalting. The whole experiment was performed at 25°C. As a control the same experiment was also performed without adding GroEL to the first dilution step. Molecular masses were determined immediately by ESI-MS as described below and corrected for 5% residual ^2H_2O in refolding buffer.

H/^{2}H exchange: thermal complex formation

To initiate the H/^{2}H exchange reaction, 20 μM Cys-Ala β-lase, deuterated at all exchangeable sites, was diluted tenfold into H_2O (pH 6.2) in the presence or absence of two molar equivalents of GroEL at 42°C and at 48°C. Aliquots were taken at different time points within a two-hour period. The degree of deuterium incorporation into β-lase was deduced from the protein's mass shift during the course of the experiment as described below.

ESI-mass spectrometry

All ESI mass spectra were recorded using an API III$^+$ triple quadrupole mass spectrometer equipped with an atmospheric pressure ionization (API) source and a pneumatically assisted electrospray interface (PE-SCIEX, Thornhill, ON, Canada). The mass resolution of the spectrometer was tuned to give a constant peak width of approximately 1 Da (full width at half-maximum) across the mass range of interest. Mass spectra were acquired using a step size of 0.15 Da and a dwell time of 0.7 ms across the m/z range of 800 to 1800 Da. Spectra represent averages of five second scans, averaged over one minute. Protein samples were rapidly mixed with an equal volume of 50% methanol, 50% H_2O and 0.5 % formic acid (by vol.) precooled to 4°C. Typically, 5 μl of the quenched samples were injected into the ion source within ten seconds at a flow rate of 7 μl/min and at an ion spray voltage of 5000 V. The conditions of the ESI mass spectrometer were carefully controlled to study H^2/H exchange in labile states. This includes continually flushing the enclosed ionization chamber with dry nitrogen and pre-cooling the delivery solvent (50% methanol, 0.25% formic acid) to 4°C.

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