FEBS Letters 401 (1997) 138–142 FEBS 18055

Folding intermediates of β-lactamase recognized by GroEL

Pietro Gervasoni, Andreas Plückthun*

Biochemisches Institut der Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland

Received 9 September 1996; revised version received 2 December 1996

Abstract β-Lactamase, from which the disulfide bond was removed by two Cys → Ala mutations, forms stable complexes with GroEL only during the first 30 s of folding, while wild-type β-lactamase forms no stable complex under these conditions. The 3-phasic kinetics of folding are very similar between wild-type and mutant. After 4 s, Trp-210 is already juxtaposed to the disulfide bond, but proline *cis-trans* isomerization has not yet taken place and almost no enzymatic activity is observed. This shows that GroEL is unable to bind late folding intermediates and also discriminates between the degree of unfolding possible in wild-type disulfide-containing β-lactamase and the Cys-Ala mutant.

Key words: Molecular chaperone; Protein folding; β-Lactamase

1. Introduction

Molecular chaperones are thought to guide the folding of proteins inside the cell. In *E. coli*, the main cytoplasmic chaperones are the DnaK/DnaJ/GrpE and the GroEL/GroES systems [1,2]. While the global function of molecular chaperones – preventing aggregation under unfavorable folding conditions – has been well documented, there is controversy in how they carry out their action [3–5]. In the case of GroEL, folding intermediates are thought to bind to the chaperone and are probably released only when they have reached more native-like forms [1–6]. Aggregation-prone intermediates may also be released, but would rebind. It is thus crucial to understand in which states a substrate protein can bind to GroEL.

We have used RTEM β-lactamase (β-lase) as a model system to study the interaction with GroEL [7]. While in the *E. coli* cell only the precursor may interact with GroEL before it is transported to the periplasm [8,9], this model system offers unique insight into chaperone guidance of protein folding because of differences in interaction between GroEL and various forms of β-lase. The precursor of β-lase is known to bind very tightly [10], while the mature form binds much more weakly [7], and differences between reduced and oxidized β-lase are also apparent (this study). The thermodynamics and kinetics of β-lase folding have been extensively characterized [7,10–13]. β-Lase is able to fold to the native state both in the cytoplasm and in the periplasm, and it does not need its disulfide bond to reach the native state [14], which can thus serve as an optional constraint in the structure. In the crystal structure [15] a *cis*-proline at position 167 has been detected, and the folding kinetics are consistent with a rate-limiting proline *cis-trans* isomerization and a kinetic intermediate [11,16].

In this paper, we have examined the interaction of Cys-Ala β-lase (mature wild-type β-lase with both Cys residues forming the disulfide bond mutated to Ala residues) with GroEL, and we find that the chaperonin forms a stable complex with the enzyme at early stages of folding, but is unable to interact with a late folding intermediate, which is populated before the slow phases of folding. We have used a comparison of wild-type and Cys-Ala β-lase to characterize the degree of structure formation in the intermediates.

2. Materials and methods

2.1. Proteins

Wild-type β-lase and the Cys-Ala double mutant β-lase were both produced and purified by methods that have been described elsewhere [17]. The chaperonin GroEL was purified as described previously [7].

2.2. Folding kinetics measured by fluorescence

The folding of β-lase was followed by monitoring its intrinsic fluorescence. Manual mixing refolding experiments were carried out at 25°C. The excitation wavelength was 280 nm (bandwidth 5.0 nm), the emission wavelength was 340 nm (bandwidth 10 nm). Refolding experiments were initiated by dilution of denatured β-lase (in 4 M GdmCl), into refolding buffer. The final concentration of β-lase was 0.2 μM, that of the denaturant was 0.1 M. Stopped-flow refolding experiments were carried out at 10°C with a SF61 stopped-flow spectrofluorimeter (HI-TECH Scientific, UK). The excitation wavelength was 280 nm and the emission light was filtered with a cut-off filter of 320 nm. Refolding experiments were initiated by a 20-fold dilution of β-lase denatured in 4 M GdmCl into refolding buffer. The final concentration of β-lase was 1.8 μM, that of the denaturant was 0.2 M.

2.3. β-Lase enzymatic activity assay

The β-lase activity was determined spectrophotometrically at 486 nm at 25°C using the chromogenic substrate nitrocefin (BBL Microbiology Systems, Cockeysville, MD) [14]. β-Lase solution (typically 5 μl) was mixed with 1 ml of refolding buffer (10 mM MOPS, 50 mM KCl, 5 mM Mg-acetate, pH 7.2) containing 0.2 mM nitrocefin and 0.5% DMSO (from the nitrocefin stock). The final concentration of GdmCl in refolding buffers was 0.1 M and kept constant for all the experiments. BSA was found to stabilize the Cys-Ala β-lase [14] and therefore added to all refolding buffers at 1 μM concentration. Initial rates of hydrolysis were measured at 486 nm during 30 s [14].

2.4. Folding kinetics measured by enzymatic activity (discontinuous)

For carrying out folding experiments wild-type and the Cys-Ala double mutant β-lase were both first denatured overnight in 4 M GdmCl pH 7.2. After dilution 1:500 into refolding buffer the regain of activity was followed by withdrawing an aliquot and monitoring the change in absorbance as described in Section 2.3. The final concentration of the wild-type and the double mutant β-lase in the cuvette was always 8.0 nM.

2.5. Folding kinetics measured by enzymatic activity (continuous, with optional prefolding)

Denatured β-lase was first diluted 1:40 in folding buffer (Section 2.3) and allowed to refold for different lengths of time (prefolding). This premixed solution was further diluted 1:14 into refolding buffer

*Corresponding author. Fax: (41) (1) 257 5712.
E-mail: plueckthun@biocfebs.unizh.ch

Abbreviations: GdmCl, guanidinium hydrochloride; β-lase, β-lactamase

PII S0014-5793(96)01449-4

containing 0.2 mM nitrocefin and 0.5% DMSO in the presence of a 10-fold molar excess of GroEL over β-lase. In all folding buffers the concentration of the denaturant was 0.1 M and BSA was added to 1 μM. Identical experiments were performed in the absence of GroEL. The folding of the enzyme is biphasic [11] and can be given [18] by

$$\frac{dE}{dt} = E_{tot} - f_A(E_{tot}-E_0)e^{-k_1 t} - (1-f_A)(E_{tot}-E_0)e^{-k_2 t} \quad (1)$$

where E_{tot} is the total amount of enzyme added in denatured form, E_0 is any enzyme folded during the prefolding step, and k_1 and k_2 are the rate constants of folding. f_A is the fractional amplitude of the first phase. Product P is due to the accumulated enzyme

$$P(t) = k_{cat}\int_0^t \left(\frac{dE}{dt}\right)dt =$$

$$E_{tot}k_{cat}t - k_{cat}(E_{tot}-E_0)\left[\frac{f_A}{k_1}(1-e^{-k_1 t}) + \frac{(1-f_A)}{k_2}(1-e^{-k_2 t})\right] \quad (2)$$

where k_{cat} is the turnover number of the enzyme ($k_{cat} = 742\ s^{-1}$ for the Cys-Ala double mutant β-lase and $k_{cat} = 806\ s^{-1}$ for the wild-type β-lase [14]), and substrate is assumed to be saturating. Upon fitting the experimental data to Eq. 2, E_{tot}, E_0 and both k_1 and k_2 are obtained. In the absence of GroEL, E_{tot} equals the total enzyme added in denatured form, while in its presence smaller values were obtained, since part of β-lase forms a stable complex with GroEL and escapes folding. The amount of enzyme bound to GroEL as a function of prefolding time was estimated by comparing E_{tot} in the presence and absence of GroEL. In the absence of prefolding, E_0 was found to be less than 1% of E_{tot} and Eq. 2 could then be simplified by setting $E_0 = 0$, without significantly changing the result.

3. Results

Wild-type β-lase, which contains a disulfide bond between Cys-77 and Cys-123, and the Cys-Ala double mutant β-lase both fold to the native state with multiphasic kinetics (Fig. 1), which can be observed by Trp-fluorescence (excitation at 295 nm). The peak of the wild-type β-lase shifts from 352 nm to 342 nm, while that of the Cys-Ala β-lase shifts from 352 nm to 340 nm upon folding, consistent with transferring the Trp residues from a hydrophilic to a more hydrophobic environment (Fig. 2). The fluorescence of both proteins, denatured in 4 M GdmCl, is lower than that of the corresponding native proteins, consistent with previous reports [7,11]. The peak maximum of the native Cys-Ala β-lase is 20% higher than that of the native wild-type protein (Fig. 2), which suggests that the intrinsic fluorescence of Trp-210 is quenched by the presence of the disulfide bond (Fig. 3). The similar structure of native wild-type and Cys-Ala β-lase is demonstrated by the similar peak maxima, and by the very similar kinetic parameters of the enzymatic activity for both proteins [14].

In a detailed analysis of the manual mixing folding kinetics (Fig. 1A,B), both wild-type and Cys-Ala β-lase gave the same rate constant for the slow phase ($k = 5.5\times10^{-3}\ s^{-1}$). The fast phase of folding was 1.5 times more rapid for the wild-type ($k = 4.6\times10^{-2}\ s^{-1}$ vs. $3.0\times10^{-2}\ s^{-1}$), however, suggesting

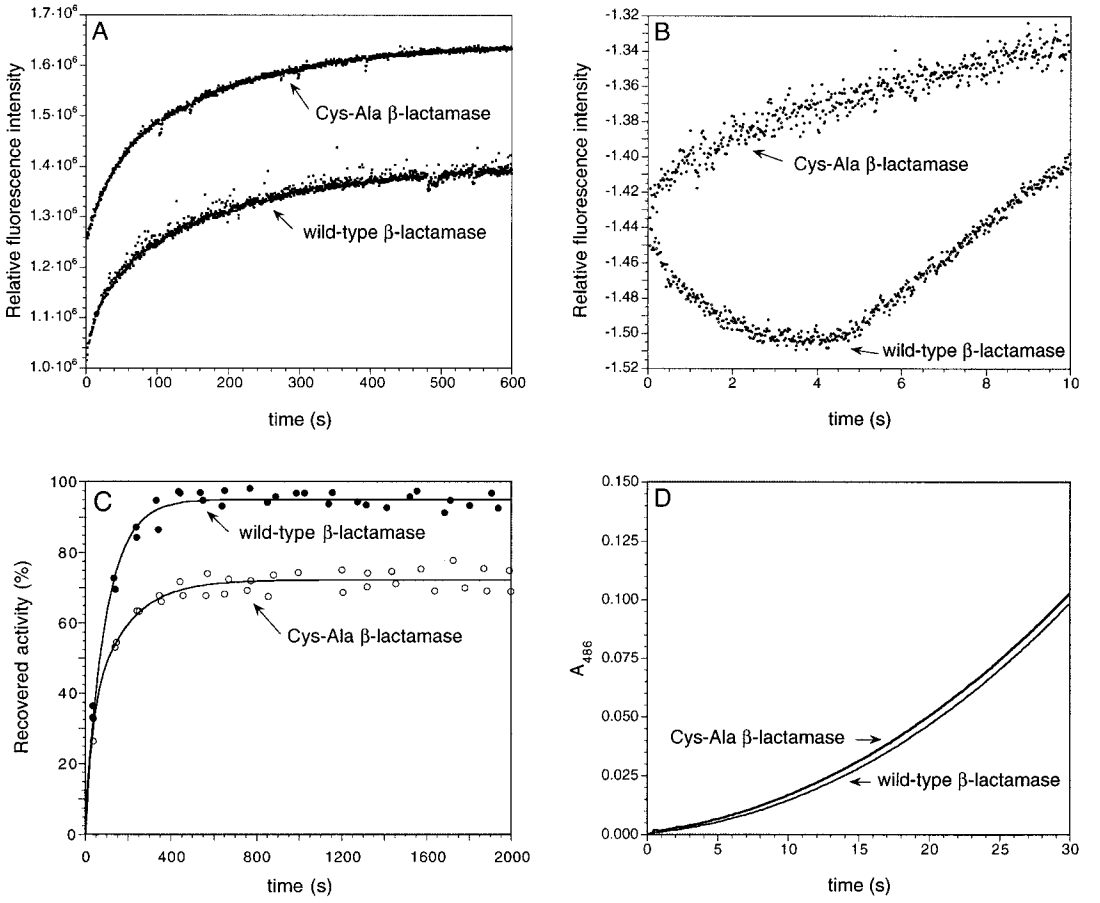

Fig. 1. Refolding of wild-type and Cys-Ala β-lase followed by fluorescence spectroscopy (A and B) and by recovery of enzymatic activity (C and D). A: Manual mixing refolding kinetics at 25°C; the mixing dead time was about 10 s. B: Stopped-flow refolding kinetics at 10°C (see Section 2.2). C: Refolding kinetics of the regain of enzymatic activity. After initiation of refolding by dilution, aliquots were withdrawn and the yield of native β-lase was measured by enzymatic activity. D: Regain of enzymatic activity after dilution of the denatured enzyme into refolding buffer containing the substrate nitrocefin, monitored by the absorbance change of nitrocefin at 486 nm (see Section 2.5).

that the presence of the disulfide bond accelerates this phase. In addition to this phase, another faster phase can be detected by stopped-flow measurements. In the wild-type enzyme, first a decrease of fluorescence is seen, with a kinetic constant of about $k = 0.22$ s^{-1}, and a subsequent slower phase with increasing fluorescence ($k = 2.6 \times 10^{-2}$ s^{-1}). In contrast, under identical conditions, the Cys-Ala β-lase gives rise to a fast stopped-flow phase with increasing fluorescence ($k = 1.1$ s^{-1}) followed by a slower phase ($k = 4.9 \times 10^{-2}$ s^{-1}) also with increasing fluorescence. The slower phases seen in stopped-flow kinetics may be identical to the corresponding fast manual phases, but since a cut-off filter had to be used in the stopped-flow apparatus, they are not directly comparable. However, the ratio of the kinetic constants of the wild-type β-lase and Cys-Ala β-lase is 1.53 for the respective slow stopped-flow phases and 1.61 for the respective fast manual phases. Thus, it appears that the disulfide bond may quench the fluorescence of Trp-210 within the first 4 s. While a similar fast phase is also seen in the mutant enzyme, no quenching is observed. Therefore, Trp-210 must come close to the disulfide bond within the first 4 s. Fig. 3 shows the structure of the wild-type β-lase [15] in which Trp residue 210 is clearly seen overlaying the disulfide bond.

Fig. 1C,D shows refolding of β-lase followed by recovery of enzymatic activity. The curves in Fig. 1C were fitted to the sum of two exponential terms, both with relative amplitudes and rate constants similar to those obtained by fluorescence measurements ($k = 8.0 \times 10^{-3}$ s^{-1} and $k = 6.9 \times 10^{-3}$ s^{-1} for the slow phase of wild-type and Cys-Ala β-lase, respectively). To obtain the fast folding rate constant in the regain of activity (too fast to be correctly obtained from the data of Fig. 1C), the method of Gray et al. [18] was used, modified to fit double-exponential curves (Eq. 2) (Fig. 1D). Refolding of wild-type and Cys-Ala β-lase denatured in 4 M GdmCl was started directly by dilution into refolding buffer in the presence of the substrate nitrocefin. Accumulated product was measured during the first 30 s of folding. From the fits to Eq. 2, using the amplitudes from Figs. 1A and 1C and taking into account that the denaturant already inhibits the reaction at a low concentration (0.1 M) and can be considered a competitive inhibitor ($K_i = 0.38$ M) [11], the fast folding rate constants for the regain of enzymatic activity were obtained. For the wild-type, a rate constant of $k = 5.0 \times 10^{-2}$ s^{-1} and for the Cys-Ala mutant a rate constant of $k = 4.7 \times 10^{-2}$ s^{-1} was determined. The initial enzymatic activities calculated for $t = 0$ are insignificant (1.5% and 0.2%, respectively). Taken together, these results suggest that the folding mechanism of wild-type and Cys-Ala enzymes is very similar. Thus, while we have to use the Cys-Ala mutant for the binding studies to GroEL, the comparison to the wild-type enzyme allows some conclusion about the folding events within the first few seconds of folding, leading to a better interpretation of the GroEL binding data.

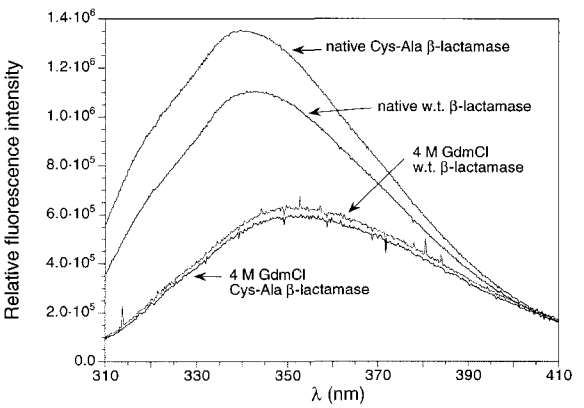

Fig. 2. Trp fluorescence spectra of the wild-type and the Cys-Ala β-lase using an excitation wavelength of 295 nm at 25°C. All protein solutions were at 0.2 μM.

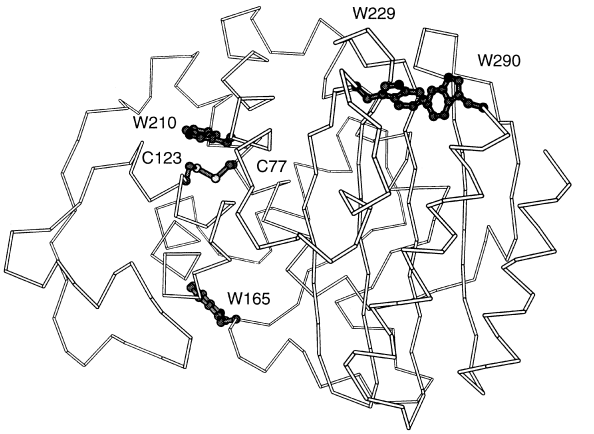

Fig. 3. Polypeptide backbone of RTEM β-lase showing the position of the disulfide bridge between Cys-77 and Cys-123 and the four Trp residues (Trp-165, Trp-210, Trp-229, Trp-290).

To investigate up to which time point GroEL can interact with Cys-Ala β-lase and block its refolding, this protein, denatured in 4 M GdmCl, was first premixed with refolding buffer in the absence of GroEL and then allowed to refold for different periods of time. After this prefolding step the protein solution was diluted again in refolding buffer, now in the presence of GroEL and the β-lactamase substrate nitrocefin. The regain of β-lase activity was then measured directly in this mixture. The increase in enzymatic rate over time is a direct measure of the increase of active enzyme due to folding. The curves were fitted to Eq. 2 which describes lag kinetics under the assumption that the substrate (nitrocefin) interacts only with the native enzyme. That GroEL is not able to denature either native wild-type or Cys-Ala β-lase was shown in control experiments (Gervasoni and Plückthun, unpublished results). From the fits to Eq. 2 the percentage of β-lase bound to GroEL was determined as a function of prefolding time (Fig. 4). Without prefolding ($t = 0$), the amount of Cys-Ala β-lase bound to GroEL was 90%, which decreased to 20% after the mutant enzyme was allowed to prefold for 30 s prior to adding GroEL (Fig. 4). After 30 s refolding in the absence of GroEL, however, only 25% of Cys-Ala β-lase is active, suggesting that 55% of the enzyme is still in an inactive conformation which is not recognized by the chaperonin. This result suggests that GroEL only binds effectively to the Cys-Ala β-lase during the early phases of folding. In contrast, no binding of wild-type β-lase to GroEL was observed upon starting the refolding either from urea [7,10] or from GdmCl (data not shown) at 25°C.

4. Discussion

GroEL can discriminate between non-native and native

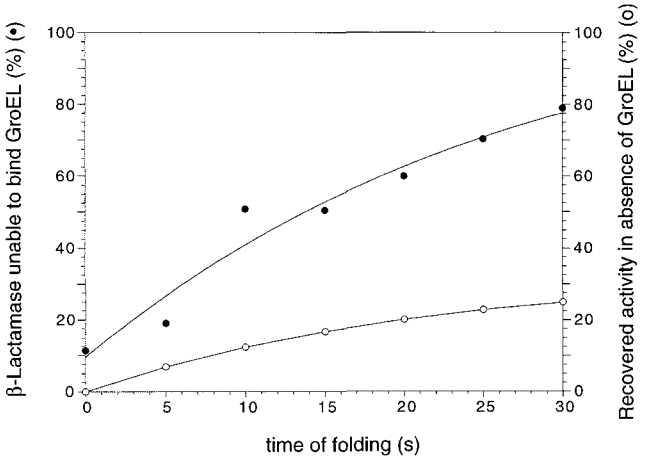

Fig. 4. Refolding of Cys-Ala β-lase measured by following the regain of enzymatic activity during the first 30 s of folding (open circles). Percent of Cys-Ala β-lase unable to bind to GroEL as a function of prefolding time (closed circles), calculated from Eq. 2 (see Section 2.5).

states of substrate proteins. Non-native forms of pre-β-lase [10], a carbamidomethylated variant of RNase T1 [19], DHFR [20], rhodanese [21] and α-lactalbumin [22] among others, have been shown to interact and bind tightly to GroEL, although for DHFR these results have not been confirmed by more recent works [23,24]. For the latter three proteins it has been proposed that they are bound in a molten globule state [20,25], while for an antibody Fab fragment a very late folding intermediate was suggested to bind to GroEL [26]. However, cyclophilin becomes globally destabilized [27], and barnase [28] can transiently bind to GroEL in a completely unstructured conformation. Thus, very different proposals for the bound states have been made, and this is almost certainly related to intrinsic differences in the substrate proteins.

To obtain more information, we have now studied the stable binding of Cys-Ala β-lase during its folding to GroEL and related it to the folding pathway. The equilibrium folding of β-lase, both from *Staphylococcus aureus* [12] and from *E. coli* [11], follows a three-state behavior. The refolding kinetics reveal the existence of three phases and thus at least two transient intermediates (Fig. 1). Since refolding from the thermodynamically stable intermediate and the native state is also biphasic, the late kinetic intermediate is not identical to the thermodynamic one, but lies between the latter and the native state. The last slow refolding step is due to proline *trans-cis* isomerization [11]. Stopped-flow kinetics using the fluorescent probe ANS have shown that a transient molten globule state is formed within 0.1 and 0.2 s [13]. Furthermore, the reactive sulfhydryl groups were protected from alkylation during folding of the reduced β-lase during the manual mixing time, suggesting that the two Cys residues become buried in the core of the protein during the early stages of folding [29] and that the protein assumes a compact structure very fast.

At about 4 s into refolding of the wild-type enzyme, the Trp-210 fluorescence is fully quenched by the disulfide, and Trp-210 and the disulfide must be already juxtaposed (Fig. 1), and the topology of at least this part of the protein must be correctly formed. Because of the similar shape of the kinetics it is likely that the topology is formed to a similar degree in the Cys-Ala β-lase. At this point, the mutant enzyme is still able to interact with GroEL. At about 30 s, the ability of the mutant β-lase to bind to GroEL has decreased to 20%. This corresponds about to the time of formation of the intermediate visible in manual fluorescence kinetics (Fig. 1A). At this time, the enzyme has little or no activity, possibly because the *cis-trans* isomerization of Pro-167 is still to follow. The neighboring Glu-166 acts as a general base and probably needs a *cis* residue in position 167 to be in the right conformation for catalytic activity. After folding of Cys-Ala β-lase is started, the capacity of GroEL to interact with the enzyme, preventing regain of enzymatic activity, decreases exponentially with a rate constant of $k = 3.6 \times 10^{-2}$ s^{-1} (Fig. 4), which is similar to the rate constant of the faster manual phase of folding, measured by Trp fluorescence and by regain of enzymatic activity (Fig. 1). Therefore, the ability to bind to GroEL is lost with the conversion into the late intermediate.

Cys-Ala β-lase thus presents an example of a protein which can form stable complexes, but only in an early phase of folding. Nevertheless, it is not a completely unfolded state which binds, but an intermediate with partially native-like topology, since at least Trp-210 is at its approximate position in the first 4 s of folding when 80% of the protein can still form a complex. The late folding intermediate prior to the *cis-trans* isomerization does not interact with GroEL.

Even though the kinetic phases are very similar for wild-type and Cys-Ala β-lase, the early intermediate of wild-type may not be able to unfold enough to expose sufficient hydrophobic surface to lead to stable binding, probably because of the constraining effect of the disulfide bond. While the interaction of pre-β-lase with GroEL is very tight, probably because of additional hydrophobic interactions with the signal sequence, mature β-lase interacts much more weakly [7,10]. As long as the disulfide bond is intact, a stable complex for the mature β-lase is only formed at higher temperature [7], and no interaction is seen at room temperature. Proton exchange experiments show [17] that a native-like conformation is preserved under these conditions. When the refolding of denatured reduced β-lase was started by dilution into refolding buffer in the presence of one molar equivalent of GroEL, still no complex formation was detected by measuring enzymatic activity [10]. In the presence of a 10-fold molar excess of GroEL the refolding of the Cys-Ala β-lase can be quantitatively prevented, but only if GroEL is already present in the refolding buffer during the first seconds of refolding. GroEL can therefore discriminate between wild-type and Cys-Ala β-lase before the slow *cis-trans* isomerization phase. Taken together these data will be helpful in the further structural characterization of the bound state of β-lase.

Acknowledgements: We acknowledge Marcus Jäger for helpful discussions, Dr. Antonio Baici for assistance with the stopped-flow spectrofluorimeter, and Dr. Annemarie Honegger for preparing Fig. 3.

References

[1] Hartl, F.-U. (1996) Nature 381, 571–580.
[2] Becker, J. and Craig, E.A. (1994) Eur. J. Biochem. 219, 11–23.
[3] Mayhew, M., da Silva, A.V.R., Martin, J., Erdjument-Bromage, H., Tempst, P. and Hartl, F.-U. (1996) Nature 379, 420–426.
[4] Todd, M.J., Lorimer, G.H. and Thirumalai, D. (1996) Proc. Natl. Acad. Sci. USA 93, 4030–4035.
[5] Corrales, F.J. and Fersht, A.R. (1996) Proc. Natl. Acad. Sci. USA 93, 4509–4512.

[6] Weissman, J.S., Rye, H.S., Fenton, W.A., Beechem, J.M. and Horwich, A.L. (1996) Cell 84, 481–490.
[7] Zahn, R. and Plückthun, A. (1994) J. Mol. Biol. 242, 165–174.
[8] Bochareva, E.S., Lissin, N.M. and Girshovich, A.S. (1988) Nature 336, 254–257.
[9] Kusukawa, N., Yura, T., Ueguchi, C., Akiyama, Y. and Ito, K. (1989) EMBO J. 8, 3517–3521.
[10] Laminet, A.A., Ziegelhoffer, T., Georgopoulos, C. and Plückthun, A. (1990) EMBO J. 9, 2315–2319.
[11] Vanhove, M., Raquet, X. and Frère, J.-M. (1995) Proteins Struct. Funct. Genet. 22, 110–118.
[12] Mitchinson, C. and Pain, R.H. (1985) J. Mol. Biol. 184, 331–342.
[13] Ptitsyn, O.B., Pain, R.H., Semisotnov, G.V., Zerovnik, E. and Razgulyaev, O.I. (1990) FEBS Lett. 262, 20–24.
[14] Laminet, A.A. and Plückthun, A. (1989) EMBO J. 8, 1469–1477.
[15] Jelsch, C., Mourey, L., Masson, J.-M. and Samama, J.-P. (1993) Proteins Struct. Funct. Genet. 16, 364–383.
[16] Craig, S., Hollecker, M., Creighton, T.E. and Pain, R.H. (1985) J. Mol. Biol. 185, 681–687.
[17] Gervasoni, P., Staudenmann, S., James, P., Gehrig, P. and Plückthun, A. (1996) Proc. Natl. Acad. Sci. USA (in press).
[18] Gray, T.E. and Fersht, A.R. (1993) J. Mol. Biol. 232, 1197–1207.
[19] Walter, S., Lorimer, G.H. and Schmid F.X. (1996) Proc. Natl. Acad. Sci. USA 93, 9425–9430.
[20] Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A.L. and Hartl, F.-U. (1991) Nature 52, 36–42.
[21] Mendoza, S.A., Rogers, E., Lorimer, G.H. and Horowitz, P.A. (1991) J. Biol. Chem. 266, 13044–13049.
[22] Hayer-Hartl, M.K., Ewbank, J.J., Creighton, T.E. and Hartl, F.-U. (1994) EMBO J. 13, 3192–3202.
[23] Viitanen, P.V., Donaldson, G.K., Lorimer, G.H., Lubben, T.H. and Gatenby, A.A. (1991) Biochemistry 30, 9716–9723.
[24] Clark, A.C., Hugo, E. and Frieden, C. (1996) Biochemistry 35, 5893–5901.
[25] Robinson, C.V., Groß, M., Eyles, S.J., Ewbank, J.J., Mayhew, M., Hartl, F.-U., Dobson, C.M. and Radford, S.E. (1994) Nature 372, 646–651.
[26] Lilie, H. and Buchner, J. (1995) Proc. Natl. Acad. Sci. USA 92, 8100–8104.
[27] Zahn, R., Spitzfaden, C., Ottiger, M., Wüthrich, K. and Plückthun, A. (1994) Nature 368, 261–265.
[28] Zahn, R., Perrett, S., Stenberg, G. and Fersht, A.R. (1996) Science 271, 642–645.
[29] Walker, K.W. and Gilbert, H.F. (1995) Biochemistry 34, 13642–13650.