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Folding and aggregation of TEM β-lactamase: Analogies with the formation of inclusion bodies in *Escherichia coli*

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Abstract

The enzyme TEM β-lactamase has been used as a model for understanding the pathway leading to formation of inclusion bodies in *Escherichia coli*. The equilibrium denaturation of TEM β-lactamase revealed that an intermediate that has lost enzymatic activity, native protein fluorescence, and UV absorption, but retains 60% of the native circular dichroism signal, becomes populated at intermediate (1.0–1.4 M) concentrations of guanidium chloride (GdmCl). This species exhibits a large increase in bis-1-anilino-8-naphthalene sulfonic acid fluorescence, indicating the presence of exposed hydrophobic surfaces. When TEM β-lactamase was unfolded in different initial concentrations of GdmCl and refolded to the same final conditions by dialysis a distinct minimum in the yield of active protein was observed for initial concentrations of GdmCl in the 1.0–1.5 M range. It was shown that the lower reactivation yield was solely due to the formation of noncovalently linked aggregates. We propose that the aggregation of TEM β-lactamase involves the association of a compact state having partially exposed hydrophobic surfaces. This hypothesis is consistent with our recent findings that TEM β-lactamase inclusion bodies contains extensive secondary structure (Przybycien TM, Dunn JP, Valax P, Georgiou G, 1994, *Protein Eng* 7:131–136). Finally, we have also shown that protein aggregation was enhanced at higher temperatures and in the presence of 5 mM dithiothreitol and was inhibited by the addition of sucrose. These conditions exert a similar effect on the formation of inclusion bodies in vivo.

Keywords: β-lactamase; inclusion bodies; molten globule; protein folding

In recent years it has become apparent that the tendency of proteins to aggregate is of considerable significance for the physiology of the cell as well as for numerous applications in biotechnology (DeYoung et al., 1993). Protein aggregates are formed in vivo as a result of mutations that affect the folding pathway, expression of heterologous polypeptides, or exposure of the cell to certain environmental stresses. Expression of heterologous genes in *Escherichia coli* and other gram-negative bacteria is often accompanied by the formation of micron-size aggregates or inclusion bodies (Mitraki & King, 1989; De Bernardez-Clark & Georgiou, 1991). Whether a protein will fold to its native state or aggregate to form inclusion bodies depends on a variety of physicochemical and physiological parameters, including interactions with chaperones, the growth temperature, level of protein synthesis, and the concentration of small solutes in the in vivo folding environment (Schein & Noteborn, 1988; Bowden & Georgiou, 1990; Blum et al., 1992).

Little is known regarding the mechanism of protein aggregation in the cell. It is generally accepted that aggregation and folding are competing processes with the former exhibiting higher order kinetics and thus becoming favored at elevated protein concentrations. A central question is at what point does the folding pathway branch off. Is aggregation the result of specific complementary interactions between late, nativelike, folding intermediates, or is it simply a consequence of the insolubility of either the unfolded or native state? Earlier in vitro studies produced somewhat conflicting answers (London et al., 1974; Goldberg et al., 1991). However, there is mounting evidence that in vivo protein aggregation, as manifested by the formation of inclusion bodies, is a highly specific process resulting from the association of intermediates having appreciable secondary struc-

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Abbreviations: Bis-ANS, bis-1-anilino-8-naphthalene sulfonic acid; GdmCl, guanidium chloride; DTT, dithiothreitol; OD_{600}, optical density measured at 600 nm; FTIR, Fourier transform infrared spectroscopy.

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ture. Seminal studies by King and coworkers revealed that conditional mutations in the tailspike protein of phage P22 influence the extent of aggregation in vivo and folding pathway in vitro but not the stability and solubility of the native state (Mitraki et al., 1991, 1993). Amino acid substitutions that specifically affect the folding pathway in the cell and are manifested by a change in the propensity to form inclusion bodies have also been isolated in other proteins (Mitraki & King, 1992; Rinas et al., 1992; Chrunyk et al., 1993; Wetzel, 1994).

The TEM β-lactamase is exported across the cytoplasmic membrane and is found in soluble form in the periplasmic space when expressed from its native promoter in pBR322-type vectors. Inclusion bodies are formed upon overexpression from a strong promoter and at elevated temperatures when TEM β-lactamase is secreted via an OmpA leader peptide (Georgiou et al., 1986; Bowden & Georgiou, 1990). Signal peptide deletions that abolish export lead to massive aggregation in the cytoplasm in the form of highly regular, cylindrical inclusion bodies (Bowden et al., 1991). Conditions that affect the formation of TEM β-lactamase inclusion bodies include the growth temperature, the addition of nonmetabolizable sugars in the culture medium, and host mutations that impair the formation of disulfide bonds (Bowden & Georgiou, 1990; Chalmers et al., 1990; M. Ostermeier, unpubl. obs.).

In this work we have investigated the equilibrium denaturation, the folding kinetics, and the aggregation of TEM β-lactamase in vitro. We present evidence that aggregation is initiated by the association of a molten globule-like folding intermediate. This result is consistent with the spectroscopic analysis of the secondary structure of TEM β-lactamase in *E. coli* inclusion bodies, which revealed the presence of appreciable nativelike structure in the aggregated polypeptide chains (Przybycien et al., 1994). Thus, the aggregation of TEM β-lactamase, both in vitro and in vivo, appears to arise from specific interactions of nativelike intermediates. Furthermore, we demonstrate that conditions which have been shown to inhibit the formation of inclusion bodies in *E. coli* exert a similar effect on aggregation in vitro.

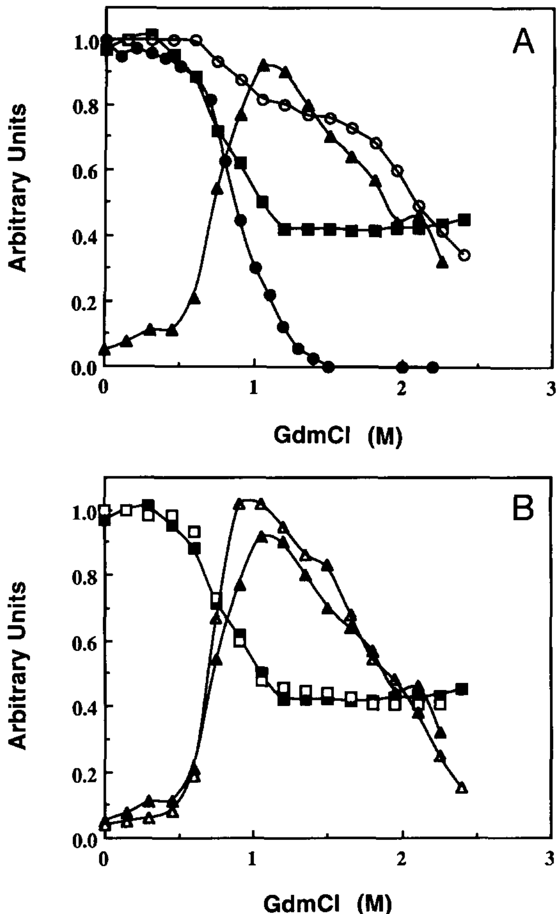

Fig. 1. A: Equilibrium denaturation curves for TEM β-lactamase as measured by fluorescence intensity (■); CD at 222 nm (○); enzymatic activity (●); and Bis-ANS fluorescence (▲). **B:** Comparison of equilibrium curves measured by fluorescence intensity (squares) and Bis-ANS binding (triangles). Open symbols: data obtained with 5 mM DTT; closed symbols: data obtained without DTT.

Results

Folding equilibrium

The GdmCl-induced unfolding transition of purified TEM β-lactamase at room temperature (23 °C) was monitored by different techniques and the results are shown in Figure 1A. Unfolding was found to be completely reversible up to concentrations of 4 mg/mL (see below). The change in fluorescence intensity as a function of denaturant concentration could be represented reasonably well by a sigmoidal transition with a midpoint at 0.87 M GdmCl. No change in the protein fluorescence was observed at denaturant concentrations higher than 1.2 M. A coincident, 2-state-like transition ($C_m = 0.86$ M) was also detected by UV difference spectroscopy at 286.5 nm, the wavelength for $\Delta\epsilon_{max}$ (Valax & Georgiou, 1991). The change in β-lactamase specific activity using penicillin G as the substrate also exhibited a similar dependence on the concentration of denaturant with detectable activity measurable in up to 1.5 M GdmCl.

Complex changes in the protein were detected by far-UV circular dichroism (Fig. 1A). The initial transition which accounted for approximately 40% of the total signal, was sigmoidal and centered around 0.8 M GdmCl. This was followed by a plateau between 1.0 and 1.5 M GdmCl and a second, broader transition. The presence of the plateau in the CD signal indicates the existence of a populated intermediate state having appreciable secondary structure. The characteristics of this intermediate were further examined by the binding of the fluorescent probe Bis-ANS. This reporter molecule exhibits little fluorescence in aqueous solutions but becomes highly fluorescent upon binding to organized hydrophobic regions in proteins (Horowitz & Butler 1993). The wavelength for maximum Bis-ANS fluorescence was independent of the GdmCl concentration indicating that the addition of the probe did not cause conformational changes in the protein. However, the Bis-ANS fluorescence intensity increased sharply at concentrations beyond 0.5 M with a maximum observed at 1.1 M GdmCl. Higher concentrations of denaturant led to a gradual decrease in the Bis-ANS fluorescence, which paralleled the loss of the far-UV CD signal and presumably reflects the global unfolding of the protein.

To investigate the significance of the single disulfide bond of β-lactamase in unfolding, the protein was reduced by incubating in 5 mM DTT for 12 h. At that point, over 95% of the cys-

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teines were present in the free thiol form as determined by sulfhydryl titration using Ellman's reagent. The denaturation curves obtained by monitoring the change in fluorescence intensity (Fig. 1B), the far-UV CD signal, and the UV adsorption of the protein in DTT (data not shown) were indistinguishable from those of the oxidized protein. The overall profiles of Bis-ANS fluorescence were also quite similar, but the reduced protein exhibited a small, yet reproducible increase in the Bis-ANS fluorescence in the 0.7–1.4 M GdmCl region. The maximum Bis-ANS fluorescence was observed in 1.0 M GdmCl and was 15% higher following reduction with DTT.

β-Lactamase aggregation upon refolding

Unfolding of the reduced protein in 3 M GdmCl followed by dilution with buffer to a final protein concentration of 3 mg/mL resulted in the appearance of light scattering material as soon as the concentration of GdmCl was reduced below 1.5 M (M. Ostermeier, unpubl. data). The soluble TEM β-lactamase obtained after dilution had a specific activity identical to the authentic protein and eluted as a single symmetric peak by gel filtration HPLC (Valax, 1993). Light scattering material consisted of large protein aggregates, which could be easily collected by centrifugation. Nonreducing SDS-PAGE revealed no evidence of intermolecular disulfide bonds among the polypeptide chains in the aggregate. Prolonged incubation in phosphate buffer did not result in any appreciable release of protein from the aggregates (P. Valax, unpubl. obs.). Light scattering material did not form even after prolonged incubation in 3 M GdmCl.

Aggregation depends strongly on the protein concentration, which changes in the course of refolding experiments initiated by dilution from denaturant solutions. It was of interest to examine the extent of aggregation and the reversibility of the refolding process under conditions where the protein concentration is kept constant throughout the experiment. For this purpose, known amounts of TEM β-lactamase were first equilibrated in 3 M GdmCl and then the concentration of denaturant was reduced to the same final value (0.02 M GdmCl) by dialysis. Within experimental error, the fraction of the protein that was soluble after refolding was equal to the reactivation yield. The reactivation yield is defined as the enzymatic activity following refolding over the activity in a sample of identical protein concentration that was treated in the same way as the samples that were subjected to refolding except that the denaturant was omitted.

Figure 2 shows the fraction of the initial β-lactamase activity recovered at 23 °C and 37 °C as a function of the protein concentration. As expected, the extent of reactivation of TEM β-lactamase depended strongly on both the temperature and the protein concentration. At 37 °C and protein concentrations higher than 0.5 mg/mL, the decrease in the yield of active protein was accompanied by the formation of aggregated material. In contrast, at 23 °C aggregation could not be detected up to 4 mg/mL. At both temperatures, under conditions where refolding was no longer completely reversible, the recovery yield upon refolding was linearly dependent on the protein concentration. Irreversibilities were solely due to aggregation, as there was no evidence of multimeric species in solution and the specific activity of the refolded protein was identical to the native TEM β-lactamase.

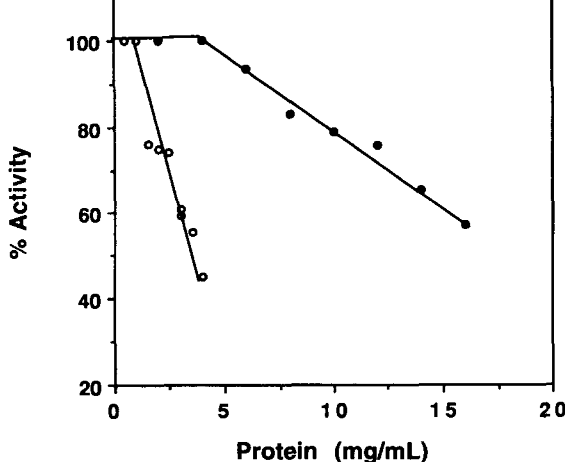

Fig. 2. Effect of protein concentration on the recovery of enzymatic activity of reduced TEM β-lactamase unfolded in 3 mM GdmCl for 3 h followed by removal of the denaturant by dialysis to a final denaturant concentration of 0.02 M. ●, 23 °C; ○, 37 °C.

The activity recovered upon refolding at 37 °C was strongly dependent on the initial GdmCl concentration (Fig. 3). Incubation of native protein in GdmCl concentrations between 1.0 and 1.4 M prior to dialysis resulted in only about 5% recovery of the enzymatic activity compared to 60% when the initial GdmCl concentration was 3 M or higher. However, no such minimum in the recovered activity was observed at 23 °C even though the protein concentration for these experiments was 3 times higher.

All subsequent experiments were conducted at 37 °C to allow direct comparison with the formation of TEM β-lactamase inclusion bodies in the periplasmic space of *E. coli*. When unfolding and dialysis were conducted under nonreducing conditions, the activity yield was substantially higher for all initial GdmCl concentrations (Fig. 4). Nevertheless, both in the absence and in the presence of DTT, the refolding yield exhibited a minimum at an initial GdmCl concentration of 1.4 M.

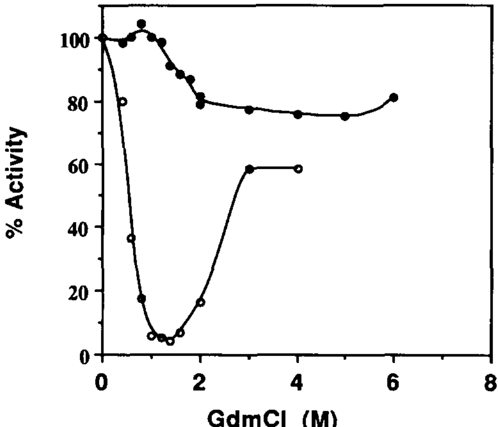

Fig. 3. Recovery of enzymatic activity of reduced TEM β-lactamase unfolded in different initial concentrations of GdmCl and refolded by dialysis to a final denaturant concentration of 0.02 M. ●, 23 °C, 10 mg/mL protein; ○, 37 °C, 3 mg/mL protein.

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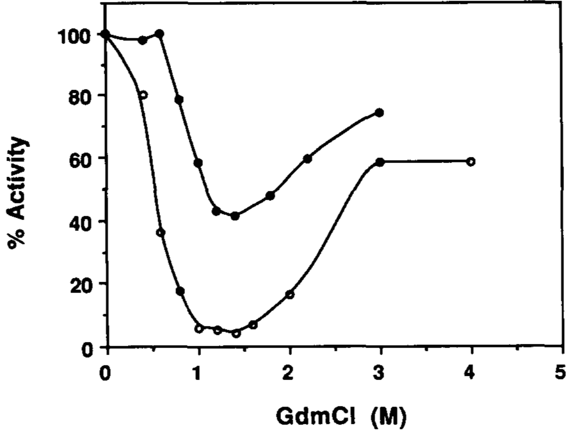

Fig. 4. Recovery of enzymatic activity of β-lactamase at 37 °C in the presence or absence of 5 mM DTT. A solution of 3 mg/mL of protein was incubated in different initial concentrations of GdmCl and refolded by dialysis to a final denaturant concentration of 0.02 M. ●, with 5 mM DTT; ○, without DTT.

The addition of moderate concentrations of sucrose in the growth medium has been shown to inhibit the aggregation of TEM β-lactamase in the periplasmic space of *E. coli* grown at 37 °C (Bowden & Georgiou, 1990). At a TEM β-lactamase concentration of 3 mg/mL, the presence of 0.6 M sucrose resulted in a moderate increase in the recovery of correctly folded, active protein upon refolding from solutions containing >0.5 M GdmCl (Fig. 5). The effect of sucrose was most pronounced when refolding was initiated from the completely unfolded state. For protein unfolded in 3 M GdmCl, the protein concentration at which aggregation became apparent increased with increasing concentrations of sucrose in the range of 0–0.6 M (Valax, 1993). Surprisingly however, the addition of sucrose to protein samples equilibrated in less than 0.5 M GdmCl resulted in a decrease in the refolding yield. This result is puzzling because at such low denaturant concentrations the native conformation is well populated (Fig. 1) and sucrose is known to enhance the stability of the native state.

Folding kinetics

The reactivation of β-lactamase from denaturant solutions, as monitored by fluorescence, exhibits a fast phase, which accounts for around 50% of the final specific activity and is complete within the dead time of the experiment, followed by a slower phase (Laminet & Plückthun, 1989). The kinetics of the slow phase in refolding from 3 M GdmCl were monitored by the change in fluorescence intensity at 345 nm. For all final GdmCl concentrations tested, the data for the recovery of more than 90–95% of the fluorescence intensity of the native protein could be fitted by a first-order rate expression. Both the amplitude and the rate constant for the slow phase in folding were dependent on the final GdmCl concentration (Table 1). The amplitude decreased to zero for final GdmCl concentrations >0.6 M. The slow phase in the folding of the reduced protein also exhibited first-order kinetics, but the amplitude was larger and the rate constant smaller. At low final GdmCl concentrations, folding was faster for the oxidized TEM β-lactamase, but the rate constants in the presence and absence of DTT became indistinguishable when the protein was diluted into 0.75 M GdmCl.

The formation of folding intermediates that bind to Bis-ANS were determined as follows. The protein was equilibrated in 3 M GdmCl and then refolding was initiated by dilution to different final concentrations of denaturant. Bis-ANS was added to the refolding mixture at different times following dilution and the fluorescence intensity at 500 nm was determined. For $t = 0$ min, Bis-ANS was present in the dilution buffer. A Bis-ANS binding species was found to form within the dead time for these experiments. Figure 6 shows that the maximum amount of Bis-ANS binding increases for the first few minutes after the initiation of folding and then decreases slowly. The intensity and time of addition of the probe for maximum of Bis-ANS fluorescence was a function of the final GdmCl. Dilution of β-lactamase into 1.8 M GdmCl did not result in any increase in Bis-ANS fluorescence over the basal level at $t = 0$.

Discussion

The changes in the fluorescence intensity, enzymatic activity, and UV adsorption during the unfolding of TEM β-lactamase in GdmCl followed a sigmoidal transition with a mid-point

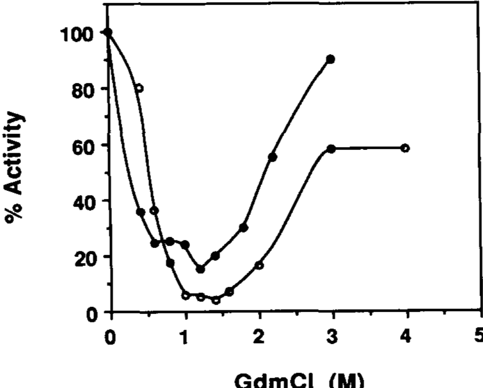

Fig. 5. Recovery of the enzymatic activity of TEM β-lactamase in 5 mM DTT and in the presence (●) or absence (○) of 0.6 M sucrose. The experimental conditions were as in Figure 4.

Table 1. *Amplitudes of the fast phase and rate constants for the slow phase in the folding of β-lactamase from 3 M GuHCl to the indicated final concentrations in the presence or absence of 5 mM DTT*

GuHCl (M)	DTT	Amplitude (fraction of the total transition)	k (min^{-1})
0.30	+	0.43 ± 0.015	0.120 ± 0.003
0.45	+	0.36 ± 0.02	0.107 ± 0.001
0.60	+	0.31 ± 0.012	0.089 ± 0.002
0.75	+	0.28 ± 0.025	0.083 ± 0.003
0.30	−	0.31 ± 0.01	0.182 ± 0.002
0.45	−	0.14 ± 0.01	0.131 ± 0.001
0.60	−	0.03 ± 0.005	0.098 ± 0.006
0.75	−	0.02 ± 0.01	0.078 ± 0.002

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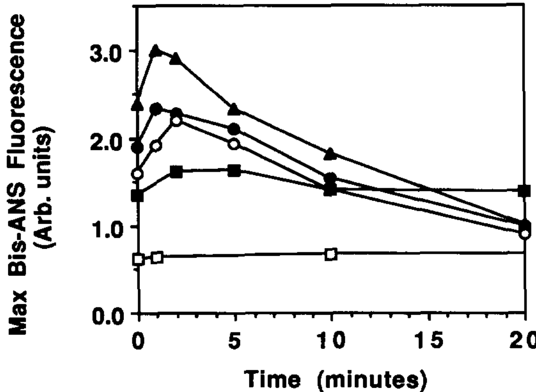

Fig. 6. Maximum fluorescence due to Bis-ANS binding. A final concentration of 1 μM Bis-ANS was added at different times after dilution of a solution of 0.3 mg/mL of reduced β-lactamase unfolded in 3 M GdmCl to different final concentrations of denaturant. ▲, 0.3 M; ●, 0.45 M GdmCl; ○, 0.45 M GdmCl, no DTT; ■, 0.75 M GdmCl; □, 1.85 M GdmCl.

around 0.85–0.9 M. The loss of enzymatic activity and tryptophan fluorescence indicate that the end of this transition is marked by a significant change in tertiary structure. However, the far-UV CD signal revealed the existence of an intermediate state, which is populated at GdmCl concentrations between 1.1 and 1.5 M and has about 60% of the secondary structure of the native protein. The appearance of this intermediate is accompanied by a marked increase in Bis-ANS fluorescence, a probe that binds preferentially to organized hydrophobic surfaces. The presence of significant secondary structure and exposed hydrophobic surfaces concomitant with the loss of native tertiary structure is consistent with the appearance of a compact intermediate or "molten-globule" state (Ptitsyn et al., 1990; Fink, 1994). Compact folding intermediates have also been detected in 3 other class A β-lactamases, from *Staphylococcus aureus*, *Bacillus licheniformis*, and *Bacillus cereus* under conditions of low denaturant concentration, in acid or high salt (Robson & Pain, 1976; Goto & Fink, 1989; Ptitsyn et al., 1990; Calciano et al., 1993). These enzymes share a high degree of topological similarity with the TEM β-lactamase, but with several subdomains existing in different relative orientations (Jelsch et al., 1992; Strynadka et al., 1992). The compact intermediate form of the *B. licheniformis* enzyme at low pH has been characterized in detail (Calciano et al., 1993). It has a Stokes radius that is only about 10% larger than that of the native protein, binds ANS, and has a high degree of secondary structure. The characteristics of the compact state of *B. licheniformis* β-lactamase suggest that subdomains with native secondary structure are present, but at least some tertiary interactions have been lost, leading to some expansion of the molecule (Fink, 1994). Given the homology and structural similarity between the native *B. licheniformis* enzyme and TEM β-lactamase, it is reasonable to postulate that the intermediate we detected in low GdmCl concentrations adopts a similar conformation.

TEM β-lactamase contains a single disulfide bond between Cys^{77} and Cys^{123}. Replacement of one or both cysteines by site-specific mutagenesis affects the stability but not the enzymatic activity of the protein (Schultz et al., 1987; Laminet & Plückthun, 1989). When the disulfide bond was reduced by DTT, the denaturation transition was not affected except that an increase in Bis-ANS binding at low GdmCl concentrations was observed. Cys^{77} and Cys^{123} form a disulfide bond connecting helices h2 and h4 (Jelsch et al., 1992). Reduction of the disulfide bond presumably leads to loss of stabilizing interactions in the helix-dominated subdomain of β-lactamase (residues 61–211) and increased solvent exposure of the hydrophobic residues in helix h2.

A protein species that exhibits strong binding to Bis-ANS is formed very early upon dilution from 3 M GdmCl (Fig. 6). When the probe is added directly in the diluting buffer, a rapid increase in fluorescence occurs and is complete within 10 s (within the dead time for these experiments). An additional, more gradual increase in fluorescence is complete within 45 s, depending on the final GdmCl concentration, followed by a gradual decrease as the protein reaches the native state. Ptitsyn et al. (1990) have observed similar changes in the binding of Bis-ANS during the folding of the *S. aureus* β-lactamase. They proposed that the Bis-ANS binding species, which is formed early on in the reaction, corresponds to a folding intermediate with the characteristics of the molten globule. It is tempting to speculate that the binding of Bis-ANS to TEM β-lactamase reflects the formation of a molten globule-like species, in analogy with the *S. aureus* enzyme.

The maximum Bis-ANS binding was dependent on the final GdmCl concentration and on the time of addition of Bis-ANS. Maximum Bis-ANS fluorescence was observed within the first few minutes after folding commences. This reflects a rapid increase in organized hydrophobic surfaces during the early steps in the folding transition, which coincides with the fast phase detected by the change in protein intrinsic fluorescence. The early steps are followed by a slower transition manifested by progressively lower Bis-ANS binding and a first-order change in the protein intrinsic fluorescence.

Both the amplitude and the rate constant for the transition monitored by the intrinsic fluorescence depend on the final GdmCl concentration. The rate constant for the slow phase observed with the reduced protein is greater than for the oxidized TEM β-lactamase. A possible explanation is that the presence of the disulfide bond facilitates alignment of the 2 helices in the active site subdomain of the protein and expedites the formation of native secondary structure. Interestingly, the amplitude of the fast phase was greater for the reduced protein than for the oxidized protein.

At relatively higher protein concentrations (3 mg/mL), dilution of the unfolded protein to GdmCl concentrations lower than 1.5 M resulted in the formation of light scattering material in the solution. The parameters that influence protein aggregation upon refolding were investigated by employing diafiltration to lower the denaturant concentration. This allowed the refolding to proceed to the same final conditions while keeping the protein concentration constant throughout the experiment. Neither aggregation nor any decrease in the reactivation of reduced β-lactamase upon refolding from 3 M GdmCl were evident even at relatively high protein concentrations: 0.5 mg/mL at 37 °C and 4.0 mg/mL at 23 °C. At higher protein concentration the reactivation yield varied linearly with the amount of protein. The slope of the reactivation versus protein concentration curve increased substantially at 37 °C.

The reactivation yield was strongly dependent on the initial GdmCl concentration at which TEM β-lactamase had been in-

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cubated prior to refolding. At 37 °C, starting with 3 mg/mL of protein that had been equilibrated in 1.0–1.4 M GdmCl, the recovery of active β-lactamase was only 5% compared to over 60% recovery when refolding was initiated from the unfolded state (i.e., in 3 M GdmCl). A similar dependence of the reversibility of refolding on the initial concentration of denaturant has been observed with other proteins, such as horse muscle phosphoglycerate kinase (Mitraki et al., 1987), rhodanese (Horowitz & Criscimagna, 1986), and human growth hormone (De Felippis et al., 1993). The reactivation yield of horse muscle phosphoglycerate kinase at 23 °C exhibited a sharp trough when refolding was initiated from 0.7 ± 0.1 M GdmCl. This phenomenon was shown to result from the aggregation of an intermediate that becomes populated in moderate denaturant concentrations and exhibits a reduced degree of α-helix formation relative to the native state. In the case of rhodanese, the aggregation of the sulfur-free enzyme in GdmCl induced minor changes in overall structure, but led to a substantial increase in the exposure of apolar surfaces of the protein as evidenced by the binding of ANS. In a similar fashion the aggregation of TEM β-lactamase is enhanced drastically in the presence of around 0.9–1.4 M GdmCl, conditions that favor the formation of the compact intermediates having exposed hydrophobic surfaces. The near absence of aggregation at the lower temperatures further supports the hypothesis that the self-association of β-lactamase is driven by hydrophobic forces (Mitraki et al., 1987).

The reduced protein exhibited both higher Bis-ANS binding and showed a lower protein yield upon refolding. As discussed above, this greater tendency to aggregation may be related to increased exposure of the hydrophobic face of helix h2 in the reduced protein. Also, the addition of sucrose resulted in some increase in the reactivation yield, the magnitude of which depends on the initial denaturant concentration. Sugars can affect the folding reaction in a complex manner (Hurle et al., 1987). Specifically, the inhibition of protein aggregation may be related to one of the following processes: (1) viscosity effects that lower the rate constant for the high-order reactions responsible for protein self-association; (2) stabilization of the native state; (3) acceleration of the rate-limiting step in folding effectively decreasing the concentration of the aggregation-prone intermediate. Although we have not attempted to determine the relative contribution of these 3 mechanisms, preliminary results in our laboratory have shown that sucrose increases the rate of refolding of β-lactamase from 3 M GdmCl as well as the stability of the native state (Valax & Georgiou, 1991; Valax, 1993).

How do these observations correlate with the formation of β-lactamase inclusion bodies in *E. coli*? We have shown earlier that the formation of inclusion bodies occurs when the protein is overexpressed, it is completely suppressed when the cells are grown at 23 °C, and it is enhanced in *dsbA* mutant strains where the formation of disulfide bonds is impaired (Bowden & Georgiou, 1990; Chalmers et al., 1990; Bowden et al., 1991; M. Ostermeier & G. Georgiou, unpubl. results). Clearly, these results bear strong similarities with the finding reported here for the purified protein. In vivo aggregation can also be inhibited completely by growing the cells in the presence of nonmetabolizable sugars. Bowden and Georgiou (1990) showed that the addition of sugars affects neither protein synthesis nor the kinetics of pre-β-lactamase processing and suggested they must influence the folding pathway of the mature polypeptide. We found that the addition of sucrose also inhibits aggregation in vitro; however, the effect was not as dramatic as would have been expected based on the in vivo data. Regardless, the analogies between the in vitro results and the formation of inclusion bodies is quite striking. It is tempting to speculate that the formation of β-lactamase inclusion bodies is dictated solely by the amino acid sequence of the protein. However, the generality of this hypothesis is tempered by observations that the expression of β-lactamase with a heterologous leader peptide results in a massive increase in inclusion body formation (Bowden & Georgiou, 1990). Thus, at least under some physiological conditions, other factors must play a role in the aggregation of TEM β-lactamase in vivo.

Inclusion bodies often contain minor amounts of other proteins. With some care, such protein aggregates can be separated from other cellular components and thus, the conformation of the aggregated polypeptide chains can be analyzed by spectroscopic techniques applicable to particulate samples. Recently, the secondary structure of β-lactamase and interleukin 1β in inclusion bodies was determined by Raman and attenuated total internal reflectance FTIR spectroscopies, respectively (Oberg et al., 1994; Przybycien et al., 1994). Aggregates of both proteins exhibited extensive secondary structure. Clearly, the aggregation of IL-1β and β-lactamase in *E. coli* must be the result of interactions among folding intermediates in which extensive secondary structure has already formed. For interleukin 1β, the secondary structure in inclusion bodies was very similar to the native protein. In contrast, the β-lactamase inclusion bodies exhibited an increase in β-sheet content at the expenses of helical structure. Such differences relative to the native protein are expected given that the aggregation of β-lactamase most likely involves partially folded intermediate(s). Evidently, this is not the case for the in vivo aggregation of interleukin 1β; for this protein, self-association must involve an almost completely native intermediate. Similarly, inclusion bodies formed by cellulase consist of essentially native protein and exhibit full enzymatic activity (Tokatlidis et al., 1991). It appears that there are at least 2 classes of inclusion bodies: the first class results from the association of nativelike intermediates as is the case for interleukin 1β and cellulase; the second class of inclusion bodies, which is represented by TEM β-lactamase, involves the association of a compact state having extensive secondary structure, but not necessarily nativelike tertiary interactions.

Although the completely denatured TEM β-lactamase in 3 M GdmCl was not susceptible to aggregation, the data presented in this work do not completely rule out the possibility that in vitro aggregation involves the self-association of the denatured state rather than a low solubility intermediate. This is because populations of denatured molecules can be present even under conditions that favor the native state (De Young et al., 1993). However, given that the inclusion bodies formed in *E. coli* have considerable secondary structure, and because protein aggregation in vivo and in vitro appear to have many similarities, the hypothesis that the aggregation of TEM β-lactamase involves an insoluble denatured state does not appear likely.

Materials and methods

β-Lactamase purification

β-Lactamase was purified from the periplasmic fraction of *E. coli* RB791(lacIq8) cells transformed with plasmid pJG108 (Bowden et al., 1991). The cells were grown at 30 °C in M9 salts

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supplemented with 0.2% glucose, 0.2% casein, and 100 μg/mL of ampicillin. The cultures were induced with 10^{-4} M isopropyl-β-D-thiogalactoside at an OD_{600} of about 0.35. Under these conditions, β-lactamase accumulates in soluble form at a level exceeding 30% of the total cell protein and no accumulation of unprocessed pre-β-lactamase precursor is observed (Valax & Georgiou, 1993). After overnight growth, the cells were harvested by centrifugation at 10,000 × *g* for 10 min, resuspended in 10 mM Tris acetate buffer, pH 8.0, containing 0.75 M sucrose, and then converted to spheroplasts according to the procedure described by Osborn and Munson (1976). The spheroplasts were centrifuged at 8,000 × *g* for 15 min and the supernatant was saved and dialyzed overnight against 4 L of 100 mM sodium acetate, pH 7.5, with 400 mM NaCl. The protein was then purified on a Chelating Sepharose FF (Pharmacia, Inc.) column. Prior to loading, the column was washed with 300 mL of buffer A, pH 7.5, containing 50 mM EDTA, and equilibrated with 300 mL of solution A, pH 4.0. Zn^{+2} was loaded onto the packing by flowing 300 mL of buffer A, pH 4.0, containing 5 g/L of $ZnCl_2$. The spheroplast supernatant was loaded directly onto the column at a flow rate of 2 mL/min. After washing with 60 mL of buffer A, pH 7.5, to elute weakly bound protein, the column was eluted with a linear gradient from pH 7.5 to pH 4.0, developed over 80 min, and was finally washed with 60 mL of buffer A, pH 4.0. The active fractions were pooled and dialyzed against 50 mM potassium phosphate, pH 7.0. Approximately 250 mg of β-lactamase was recovered from a 2-L culture. β-Lactamase was found to be more than 95% pure as determined by densitometry of 15% acrylamide SDS-PAGE gels. For long-term storage, the enzyme was diluted in 50 mM KH_2PO_4 buffer, pH 7.0, at a concentration of 1 mg/mL, and was rapidly frozen in dry ice and kept at −70 °C. Under these conditions the enzyme remained fully active for several months.

Unfolding equilibrium measurements

Protein intrinsic fluorescence spectra were determined in an SLM SPF-500C spectrofluorometer using an excitation wavelength of 280 nm. The emission spectra were scanned from 310 to 480 nm. For equilibrium measurements, TEM β-lactamase at a concentration of 60 μg/mL was incubated with varying concentrations of GdmCl for 3 h. The temperature was controlled at 23 °C using a thermostated compartment and an associated water bath. The maximum difference in the fluorescence intensity of the native and denatured protein, in the presence or absence or 6 M GdmCl respectively, was detected at 345 nm (Valax & Georgiou, 1991).

Hydrophobic surface exposure to the solvent was monitored using the fluorescent probe Bis-ANS (Horowitz & Butler, 1993). TEM β-lactamase at a concentration of 60 μg/mL was equilibrated with GdmCl as above, Bis-ANS was added to a final concentration of 10 μM, and the fluorescence emission spectra were scanned from 400 and 600 nm using an excitation wavelength of 395 nm. CD spectra were recorded with a Jasco J500 spectropolarimeter with a model J-500 data processing unit. A protein concentration of 0.3 mg/mL was used for all experiments. The protein solution was equilibrated in the denaturant solution for at least 3 h prior to spectra collection.

The folding equilibria for the reduced TEM β-lactamase were monitored as described above except that all buffers were thoroughly degassed and the protein samples were equilibrated in the presence of 5 mM dithiothreitol. Sulfhydryl titration using Ellman's reagent confirmed that the single disulfide bond in β-lactamase is completely reduced under these conditions (Valax, 1993).

Folding kinetics

TEM β-lactamase was incubated for 3 h at room temperature in 50 mM potassium phosphate buffer containing 3 M GdmCl with or without 5 mM DTT. The samples were then diluted to various final GdmCl concentrations by the addition of potassium phosphate buffer, pH 7.0, with or without DTT and GdmCl. For all experiments, the final protein concentration after dilution was 60 μg/mL. Protein folding was monitored by measuring the change in fluorescence at 345 nm using an excitation wavelength of 280 nm.

To measure the kinetics of Bis-ANS binding, unfolded TEM β-lactamase in 3 M GdmCl was diluted with buffer to different final GdmCl concentrations as above, and Bis-ANS at a final concentration of 1 μM was added at different times after the initiation of refolding. The intensity of fluorescence emission was recorded at 500 nm with an excitation wavelength of 395 nm.

Protein renaturation

Known amounts of TEM β-lactamase were lyophilized and redissolved in 50 mM potassium phosphate, pH 6.0, containing various concentrations of GdmCl in the presence or absence of 5 mM DTT, as required. The samples were dialyzed against the same solution for 3 h at room temperature in a Pierce microdialyzer model 500. Subsequently, the protein was renatured by dialyzing against phosphate buffer without GdmCl. In all experiments, the final GdmCl concentration was 0.02 M. Following renaturation, the dialysates were centrifuged at 10,000 rpm for 20 min in an Eppendorf microcentrifuge tube at 4 °C and the activity remaining in the supernatant was determined. The pellets were washed in 50 mM potassium phosphate, pH 6.0, and resuspended in the same buffer by vortexing. For all experiments the activity in the wash was less than 5% of the activity found in the supernatant immediately after dialysis. No β-lactamase activity was detected after additional washing of the aggregated pellet.

General methods

β-Lactamase activities were determined spectrophotometrically using penicillin G as the substrate (Valax, 1993). All activity data reported are the average of 3 measurements. Protein concentrations were calculated using an extinction coefficient of ϵ_{281} = 29,400 M^{-1} cm^{-1} (Sigal et al., 1984).

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