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Folding of Class A β-Lactamases Is Rate-Limited by Peptide Bond Isomerization and Occurs via Parallel Pathways†

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ABSTRACT: Class A β-lactamases (M_r ~29000) provide good models for studying the folding mechanism of large monomeric proteins. In particular, the highly conserved *cis* peptide bond between residues 166 and 167 at the active site of these enzymes controls important steps in their refolding reaction. In this work, we analyzed how conformational folding, reactivation, and *cis*/*trans* peptide bond isomerizations are interrelated in the folding kinetics of β-lactamases that differ in the nature of the *cis* peptide bond, which involves a Pro167 in the BS3 and TEM-1 enzyme, a Leu167 in the NMCA enzyme, and which is missing in the PER-1 enzyme. The analysis of folding by spectroscopic probes and by the regain of enzymatic activity in combination with double-mixing procedures indicates that conformational folding can proceed when the 166−167 bond is still in the incorrect *trans* form. The very slow *trans* → *cis* isomerization of the Glu166−Xaa167 peptide bond, however, controls the final step of folding and is required for the regain of the enzymatic activity. This very slow phase is absent in the refolding of PER-1, in which the Glu166−Ala167 peptide bond is *trans*. The double-mixing experiments revealed that a second slow kinetic phase is caused by the *cis*/*trans* isomerization of prolines that are *trans* in the folded proteins. The folding of β-lactamases is best described by a model that involves parallel pathways. It highlights the role of peptide bond *cis*/*trans* isomerization as a kinetic determinant of folding.

Class A β-lactamases (260−280 residues; M_r ~29000) provide good models for studying the folding mechanisms of monomeric large globular proteins. The folding of the PC1 and TEM-1 enzymes, in particular, has been examined in detail (*1*−*9*). As observed with most large proteins (> 100 residues), the folding kinetics do not obey a two-state mechanism, and partially folded intermediates are observed. β-Lactamases (Figure 1A) consist of two structural domains (*10*−*16*), which are not arranged in a consecutive manner, like pearls on a string, but one domain is inserted into the other. The all-α domain of, e.g., BS3 consists of about 148 residues (arranged in five α-helices and four 3_{10} helices connected by loops) and is inserted between residues 66 and 215 of the α/β domain, which is formed by the 36 N-terminal and the 76 C-terminal residues of the protein chain. In the folded protein, the α/β domain uses its five-stranded antiparallel β-sheet to pack closely onto the all-α domain. The catalytic pocket is located at the interface between these two domains and contains the active serine (Ser70).

A distinct feature of class A β-lactamases is the presence of a long Ω-loop (*17*, *18*) of ~16 residues (residues 164−179, Figure 1), which is packed against the main body of the native enzyme, near the active site. The loop shows few contacts with the rest of the protein structure, mostly mediated by water molecules, and it is stabilized by a salt bridge between Arg164 and Asp179 at the ends of the loop. Remarkably, the Ω-loop contains a *cis* peptide bond preceding residue 167, which usually is a proline, although non-prolyl residues are also found in some enzymes. The precise conformation of the loop and the *cis* configuration of the bond to residue 167 are important for the correct positioning of residues that are essential for catalytic activity (*19*), particularly of the strictly conserved Glu166 (*12*, *20*, *21*). The Ω-loop and the *cis* peptide bond between residues 166 and 167 were observed in all structures solved to date, with a single exception, the PER-1 enzyme from *Pseudomonas aeruginosa*. In this β-lactamase, the Ω-loop shows a different fold (Figure 1B (*16*)) which, together with the insertion of four amino acids into strand β3 (positions 240a−d), enlarges the substrate binding pocket. This structural adjustment probably explains the high catalytic activity of the PER-1 enzyme toward cephalosporins with bulky substituents (*16*). Most remarkably, the 166−167 peptide bond (Glu166−Ala167) is *trans*, unlike in all other β-lactamases. Despite the substantial reorganization of the Ω-loop in the PER-1 enzyme, the side chain of the catalytic residue Glu166 is in the same position as in the other β-lactamases. This is shown by the superimposition of the Ω-loops of the PER-1, BS3, and TEM-1 enzymes (Figure 1B).

The detailed kinetic analysis of the folding mechanisms of the TEM-1 and PC1 enzymes and their respective P167T and I167P variants (*6*−*9*) suggested that the very slow (rate-limiting) step

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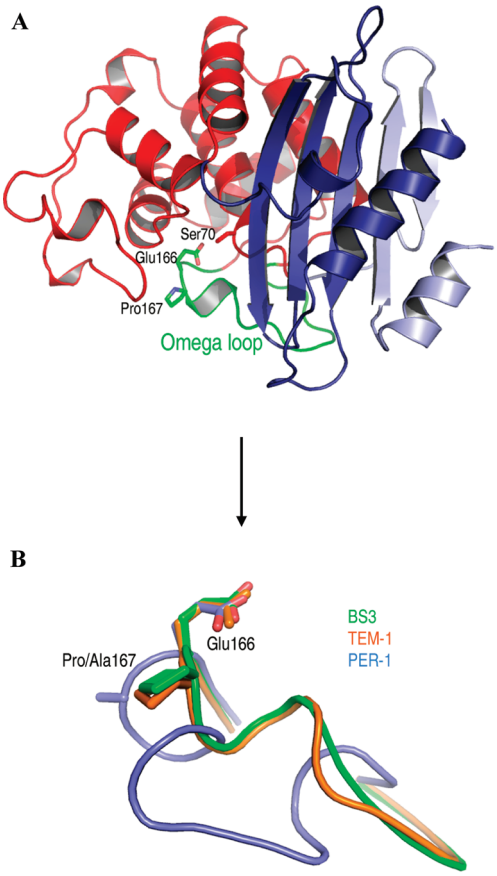

FIGURE 1: Schematic representation of the structure of the BS3 β-lactamase (*22*). (A) Ribbon representation with the helical (all-α) domain in red and the N-terminal and C-terminal parts of the α/β domain in light and dark blue, respectively. The Ω-loop is shown in green. The active site serine (Ser70), Glu166, and Pro167 (see text) are indicated. (B) Enlarged view of the BS3 Ω-loop (i.e., colored green in panels A and B) and comparison with the corresponding structural element in TEM-1 (in yellow) and PER-1 (in blue).

observed in the folding of class A β-lactamases (with time constants ($\tau = 1/k$) in the range of 3−15 min at 20−25 °C) is due to the *trans* → *cis* isomerization of the Glu166−Xaa167 peptide bond. For the TEM-1 enzyme (12 prolines), another slow kinetic phase was observed (τ = 20−30 s at 25 °C (*6*)), which probably reflects *cis*−*trans* isomerizations of additional proline residues (*6*, *7*).

In this work, we have characterized the slow folding kinetics of the PER-1 enzyme, which is devoid of a *cis* peptide bond in the Ω-loop and compared it with the folding mechanism of the NMCA β-lactamase and its Leu167Pro variant, which contain a *cis* Leu167 (*15*) and a *cis* Pro167, respectively, and of the BS3 enzyme, which contains a *cis* Pro167 (*22*). In addition, we have analyzed the refolding kinetics of TEM-1 and BS3 (both with a Pro167) at different temperatures. The results indicate that the folding of these enzymes involves two parallel pathways in which the enzymatically active conformation is recovered at very different rates.

MATERIALS AND METHODS

Enzymes and Chemicals. Ultrapure guanidinium chloride (GdmCl)[1] and cephalothin were obtained from Sigma Chemical Co. Benzylpenicillin was from Rhône-Polenc (Paris, France). All other chemicals were of reagent grade.

Wild-type TEM-1 β-lactamase was produced (*Escherichia coli* strain RB791) and purified as described (*23*).

The recombinant *Bacillus licheniformis* BS3 β-lactamase, cloned in a pET22b plasmid (containing the kanamycin resistance gene), was transformed into the BL21 (DE3) strain of *E. coli* (Novagen, Madison, WI). Following inoculation with 500 mL of an approximately 17 h preculture, the enzyme was expressed in a 20 L fermentor (BioFlo; New Brunswick) containing 15 L of Luria−Bertani (LB) medium and 50 $\mu g \cdot mL^{-1}$ kanamycin. The culture was grown at 37 °C, pH ~7, and cell development was followed by monitoring both oxygen consumption and turbidimetry. At $A_{600} \approx 0.6$, expression was induced by addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG), and cell growth was allowed to proceed for another 3 h at 37 °C. The cells, harvested by centrifugation (5000*g* for 20 min at room temperature), were resuspended in 10 mM Tris, pH 7.2 (buffer A), and lysed with the help of a disintegrator (Series Z; Constant System, Warwick, U.K.). After centrifugation (9000 rpm for 1 h at 4 °C), the supernatant containing the periplasmic proteins was dialyzed overnight against 15 L of buffer A, filtered on a 0.45 μm membrane, and then loaded onto a 150 mL Q-Sepharose FF XK 26/40 column (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A. BS3 was eluted with a linear NaCl gradient (0−0.175 M) in buffer A, and the enzymatic activity of the fractions was assayed using benzylpenicillin as substrate. The active fractions were pooled, and a second purification step was performed using a 470 mL size exclusion Sephacryl S100 HR XK 26/100 column (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A containing 50 mM NaCl. Active fractions were pooled, dialyzed three times against milli-Q water, and finally lyophilized. No trace of contaminating protein was found by mass spectrometry analysis (M_r = 29472), indicating a homogeneity greater than 98% (consistent with SDS−PAGE analysis). Mass spectrometry revealed, however, that the enzyme exhibited microheterogeneity, most probably due to ragged N-termini, as observed with the *B. licheniformis* 749/C β-lactamase and three other class A enzymes (*24*). Thus, with the *B. licheniformis* enzyme, chromatofocusing and N-terminal sequencing indicated that the mature β-lactamase consisted of at least five different molecular species (*24*). The final BS3 preparation (630 mg) was stored at 4 °C.

Wild-type NMCA β-lactamase was produced by *E. coli* strain BL21 (DE3) containing the NMCA gene on a pET22b(+) derivative plasmid and purified in two steps as described (*15*). The L167P variant was constructed using a two-step method including PCR and *Dpn*I digestion (QuickChange site-directed mutagenesis kit from Stratagene). For high-level expression, the corresponding gene was cloned into the expression vector pET22bK^r (*25*) using the *Nde*I and *Bam*HI restriction enzyme sites. The pET22bK^r vector is the pET22b vector containing the kanamycin resistance gene. Recombinants containing the gene coding for the L167P NMCA variant were thus detected on the basis of their ability to confer kanamycin resistance to *E. coli*. That no unwanted mutation was present in the L167P expression plasmid was confirmed by sequencing. Finally, *E. coli* strain BL21 (DE3) was transformed by the pET22bK^r/L167P vector for overproduction and purification of the variant enzyme as described (*15*).

The recombinant PER-1 β-lactamase gene, cloned in the pRAZ1 expression vector described by Nordmann et al. (*26*)

[1]Abbreviations: CD, circular dichroism; GdmCl, guanidinium chloride; PCR, polymerase chain reaction; UV, ultraviolet; ANS, 1-anilino-8-naphthalenesulfonate.

and transformed into *E. coli* JM109 (Promega, Madison, WI), was used for expression of the wild-type enzyme. Following inoculation with 250 mL of an overnight preculture, the enzyme was expressed in a 20 L fermentor (BioFlo; New Brunswick) containing 12 L of brain−heart infusion (BH) medium and 25 $\mu g \cdot mL^{-1}$ kanamycin. The culture was grown at 37 °C, pH ~7, and cell development was followed by monitoring both oxygen consumption and turbidimetry. The cells, harvested by centrifugation (5000*g* for 20 min at room temperature), were resuspended in ~600 mL of 30 mM Tris-HCl, pH 8, containing 30% saccharose. Lysozyme lysis was used to extract the protein of interest from the periplasm. EDTA (80 μL of 0.5 M) and 400 μL of 2 $mg \cdot mL^{-1}$ lysozyme were added to the 630 mL of cell suspension, and lysis was allowed for 15 min on ice; the reaction was stopped by addition of 40 mL of 1 M $CaCl_2$. After centrifugation (12000 rpm for 1 h at 4 °C), the supernatant containing the periplasmic proteins was dialyzed overnight at 6 °C against 30 L of 20 mM Tris-HCl, pH 7.8, containing 0.5 mM dithiothreitol and 0.06% sodium azide (buffer A). The protein solution was then filtered sequentially on 1.2, 0.7, and 0.45 μm membranes (Millipore, DIAFLO) and loaded onto a 170 mL Q-Sepharose high-performance column (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A. The PER-1 β-lactamase was eluted with a linear NaCl gradient (0−0.5 M) in buffer A, and the enzymatic activity of the fractions was assayed using cephalothin as substrate. The active fractions were pooled, and a second purification step was performed using a 470 mL size exclusion Sephacryl S100 HR XK 26/100 column (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A. The active fractions were pooled, and a final polishing purification step was performed using a 19 mL Source 15 Q HR 26/10 column (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A. PER-1 was eluted with a linear NaCl gradient (0−75 mM) in buffer A. No trace of contaminating protein was found, either by SDS−PAGE, N-terminal amino acid sequencing (Q-S-P-L-L), or mass spectrometry analysis (M_r = 30850), indicating a homogeneity greater than 98%.

Concentrations of protein solutions were determined by measuring the absorbance at 280 nm, using molar extinction coefficient values of 24750, 28085, 30500, and 34950 $M^{-1} \cdot cm^{-1}$, for BS3, TEM-1, NMCA, and PER1, respectively.

Buffers. All experiments were performed in 50 mM sodium phosphate buffer, pH 7, in the presence (TEM-1, NMCA) or absence (BS3, PER-1) of 50 mM NaCl. Denaturant (GdmCl and urea) solutions were prepared in the same buffer, and pH was checked and, if necessary, adjusted to pH 7 with NaOH or HCl.

Determination of Enzyme Kinetic Parameters. Kinetic parameters were determined at 30 °C using a UVIKON 860 spectrophotometer (Kontron Instrument, Zürich, Switzerland) as described (*27*).

Denaturant-Induced Unfolding Transitions. Equilibrium unfolding was studied at 25 °C. Samples at various GdmCl and urea concentrations were left to equilibrate for at least 12 h. Unfolding was followed by the changes in intrinsic fluorescence (λ_{ex} = 280 nm (BS3, TEM-1 and PER-1) or 284 nm (NMCA); λ_{em} = 338 nm (BS3 and NMCA) or 340 nm (TEM-1 and PER-1)) and in far-UV CD at 220−222 nm as described (*28*) using a Perkin-Elmer LS50B spectrofluorometer and a Jasco J-810 spectropolarimeter. Denaturant concentrations were determined from refractive index measurements (*29*) using a R5000 hand refractometer from Atago (Japan). Intrinsic fluorescence was measured at protein concentrations ranging from 0.3 to 4 μM, and concentrations of 5−10 μM were used for far-UV CD measurements.

Kinetics of Refolding in the Manual Mixing Mode. All experiments were carried out at 25 °C. Kinetics of refolding were measured by fluorescence and CD as described for the TEM-1 enzyme (*6*) with protein concentrations of 0.3−16 μM. Refolding kinetics were also monitored by the recovery of enzymatic activity as described (*6*). Benzylpenicillin (with BS3 and TEM-1) and cephalothin (with NMCA) were used as substrates, and concentrations of the refolded samples were in the range of 1.7−3.4 and 1.6−2.1 nM, respectively.

Double-Mixing Experiments. Manual double-mixing experiments were performed as described (*6*). The dead time was about 10 s.

Stopped-flow double-mixing experiments were performed using a DX.17MV sequential stopped-flow spectrometer from Applied Photophysics (Leatherhead, U.K.). The NMCA β-lactamase (90 μM) was first unfolded by a 6-fold dilution with 1.44 M GdmCl in 50 mM glycine/HCl, pH 2.4 (under these conditions NMCA unfolds with τ ~3 ms), and then refolded after 50 ms by a second 6-fold dilution with the refolding buffer to a final protein concentration of 2.5 μM and a final GdmCl concentration of 0.2 M. The time course of refolding was followed by the change in the fluorescence emission above 300 nm, after excitation at 284 nm (3.25 nm bandwidth). The path length of the observation chamber was 0.2 cm. The photomultiplier was set to 750 V in all experiments.

Control experiments were performed to demonstrate that 1:5 mixing of the protein with 1.44 M GdmCl in 50 mM glycine/HCl, pH 2.4, resulted in a concentration of denaturant sufficient to completely unfold the protein (measured by monitoring fluorescence and ellipticity at 225 nm).

Data Analysis. Equilibrium unfolding curves were analyzed on the basis of a two-state-model (N ⇌ U) for the unfolding transitions, according to eq 1, by assuming that the differences in free energy between the N and U species exhibit a linear dependence on denaturant concentration (*30*, *31*):

$$y_{obs} = (y_N + y_U \exp a)/(1 + \exp a) \quad (1)$$

with

$$a = -(\Delta G°(H_2O)_{N-U} + m_{N-U}[\text{GdmCl}])/(RT)$$

y_{obs} is the measured parameter at a given denaturant concentration, and y_N and y_U are the values of this parameter for the native and unfolded states at the same denaturant concentration. The observed linear dependence of some of these parameters on denaturant concentration was taken into account as described (*28*). $\Delta G°(H_2O)_{N-U}$ is the difference in free energy between N and U under physiological conditions; m_{N-U} is the slope, $\delta(\Delta G°)/\delta[\text{GdmCl}]$, of the corresponding linear plot of the free energy against denaturant concentration. R is the gas constant, and T is the absolute temperature. The midpoint of the transition, i.e., the denaturant concentrations at which [U]/[N] = 1, is given by $C_m = -\Delta G°(H_2O)/m$. The equilibrium folding data are presented as the fractional change in signal as a function of GdmCl concentration, calculated as follows (eq 2):

$$\text{fractional change} = (y_{obs} - y_N)/(y_U - y_N) \quad (2)$$

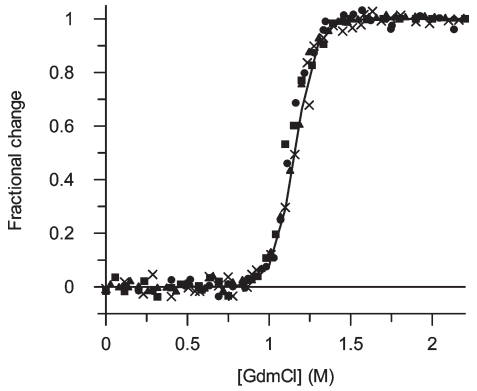

FIGURE 2: GdmCl-induced equilibrium unfolding transition of BS3 β-lactamase at pH 7, 25 °C, monitored by the change in fluorescence intensity at 338 nm (×), the change in ellipticity at 222 nm (▲) and 273 nm (●), and activity measurements (■). Data were analyzed on the basis of a two-state model (eq 1), and the solid line was drawn using the parameters obtained in fluorescence experiments (Table 1). Data are presented as the fractional change in signal (calculated according to eq 2) as a function of GdmCl concentration.

The kinetics of folding were analyzed according to a single exponential term (eq 3) or to the sum of two ($i = 2$), three ($i = 3$), and four ($i = 4$) exponentials (eq 4).

$$F_t = F_\infty + A \exp(-kt) \quad (3)$$

$$F_t = F_\infty + \sum A_i \exp(-k_i t) \quad (4)$$

The temperature dependence of the folding kinetics was analyzed according to the Eyring transition theory using the following equation (see refs *32* and *33*):

$$\ln(k/T) = -\Delta H^{\ddagger}/(RT) + \ln(k_B \kappa/h) + \Delta S^{\ddagger}/R \quad (5)$$

where k is the reaction rate at any given temperature, k_B is the Boltzmann constant, h is the Planck constant, T is the absolute temperature, R is the gas constant, κ is the transmission coefficient (taken as unity; see ref *34*), $\Delta S^{\ddagger}$ is the entropy of activation, and $\Delta H^{\ddagger}$ is the enthalpy of activation.

The program Grafit 5.0.10 (Erithacus Software Ltd.) was used for nonlinear least-squares analysis of the data. Errors are calculated as standard deviations throughout.

RESULTS

Equilibrium Unfolding Experiments. The stability of the four β-lactamases was derived from GdmCl- or urea-induced unfolding transitions. Unfolding of the TEM-1 enzyme (*6*) was reported to be reversible, and the noncoincidence of the transitions obtained by intrinsic fluorescence emission and far-UV CD measurements indicated that an intermediate species (H) is populated in the course of equilibrium unfolding. State H is thermodynamically stable in the presence of 1.5 M GdmCl and has been described as a molten globule species (*6*). Based on a three-state model, the Gibbs free energy of unfolding of TEM-1 (ΔG°_{N-U}) was calculated to be 44 ± 3 kJ·mol^{-1}. In contrast, the GdmCl-induced equilibrium unfolding transition of BS3 (Figure 2) reveals that this enzyme unfolds cooperatively in an apparent two-state transition, where only the native and unfolded states are significantly populated. Similar results were obtained for the GdmCl-induced unfolding of NMCA and the urea-induced unfolding of PER-1. For all three enzymes, superimposable transition curves were obtained by intrinsic fluorescence and far-UV CD, indicating that secondary and tertiary structures are destabilized coincidently. For the BS3 enzyme (Figure 2), transition curves obtained by near-UV CD and activity measurements coincide as well, confirming that this enzyme follows a simple two-state reversible unfolding transition. Table 1 compares the stabilities of the three β-lactamases of this study with those measured previously for the TEM-1 and PC1 enzymes.

Table 1: Thermodynamic Parameters of Unfolding of Class A β-Lactamases at pH 7, 25 °C, As Obtained from Analysis of Equilibrium Transitions Monitored by both Intrinsic Fluorescence and Far-UV CD Measurements[a]

enzyme	ΔG°_{N-U} (kJ·mol^{-1})	$-m_{N-U}$ (kJ·mol^{-1}·M^{-1})	C_m (M)
TEM-1[b]	44 ± 3[d]	34 ± 2.5[d]	–[d]
BS3[b]	44 ± 4[e]	39 ± 4[e]	1.14 ± 0.02[e]
NMCA[b]	30 ± 2	38 ± 3	0.8 ± 0.1
NMCA L167P[b]	33 ± 4	33 ± 4	1.0 ± 0.13
PER-1[c]	39 ± 1.5	8.5 ± 0.7	4.6 ± 0.1
PC1[b]	32 ± 4[f]	36 ± 4[f]	–[f]

[a]Measured in 50 mM sodium phosphate containing 50 mM NaCl (NMCA, TEM-1, and PC1). [b]In the presence of GdmCl. [c]In the presence of urea. [d]TEM-1 unfolds according to a three-state process (*6*) with $\Delta G^{\circ}_{N-H} = 20 \pm 2$ kJ·mol^{-1} and $\Delta G^{\circ}_{H-U} = 24 \pm 1$ kJ·mol^{-1}, $m_{N-H} = 23 \pm 2$ kJ·mol^{-1}·M^{-1} and $m_{H-U} = 11 \pm 0.4$ kJ·mol^{-1}·M^{-1}, and $C_{m,N-H} = 0.9 \pm 0.02$ M and $C_{m,H-U} = 2.24 \pm 0.02$ M. [e]Transition also monitored by near-UV CD and catalytic activity measurements. [f]PC1 unfolds according to a three-state process (*9*) with $\Delta G^{\circ}_{N-H} = 14 \pm 2$ kJ·mol^{-1} and $\Delta G^{\circ}_{H-U} = 18 \pm 2$ kJ·mol^{-1}, $m_{N-H} = 22 \pm 3$ kJ·mol^{-1}·M^{-1} and $m_{H-U} = 13.5 \pm 1.4$ kJ·mol^{-1}·M^{-1}, and $C_{m,N-H} = 0.64 \pm 0.02$ M and $C_{m,H-U} = 1.31 \pm 0.02$ M.

Refolding Kinetics of BS3 and TEM-1 β-Lactamases. The refolding kinetics of BS3 at a final GdmCl concentration of 0.26 M, monitored by intrinsic fluorescence and by far-UV CD spectroscopy at 25 °C, gave identical results (Figure 3). After a burst phase, which was complete within the dead time of mixing (ca. 10 s), a slow phase with a time constant of about 15 s and a very slow phase with a time constant of 600 s were observed. Refolding of BS3, followed by the recovery of enzyme activity (Figure 4), was also biphasic, with τ values in agreement with those obtained by optical measurements. The slow phase was difficult to measure at 25 °C, in particular by CD, because its time constant is close to the dead time of manual mixing. Its existence and its time constant value of ~15 s, could, however, be confirmed by stopped-flow measurements ($\tau = 15 \pm 1$ s (*35*)). The very slow phase can be measured reproducibly by the three methods, resulting in $\tau = 600 \pm 100$ s at 25 °C.

These kinetic experiments reveal that a transient intermediate accumulates in the dead time of manual mixing (10 s). It shows a substantial amount of secondary structure, as indicated by the regain of ~70% of the helix CD at 222 nm. It presumably also contains partial tertiary structure, as shown by the ~50% of recovery of the native fluorescence. It is, however, enzymatically inactive, as suggested by the data in Figure 4. This was confirmed by activity measurements performed immediately after dilution (40−60-fold) of the unfolded enzyme (in 2 M GdmCl) with the refolding buffer in the presence of substrate. A similar kinetic intermediate species (termed I) was observed for TEM-1 β-lactamase (*6*). It accumulates because its final folding to the native state is decelerated by the *cis*−*trans* isomerization of non-native Xaa−Pro peptide bonds (TEM-1 contains 12 proline residues (*6*, *7*)).

The BS3 enzyme contains 11 proline residues, and it is likely that isomerizations of prolyl peptide bonds determine the folding

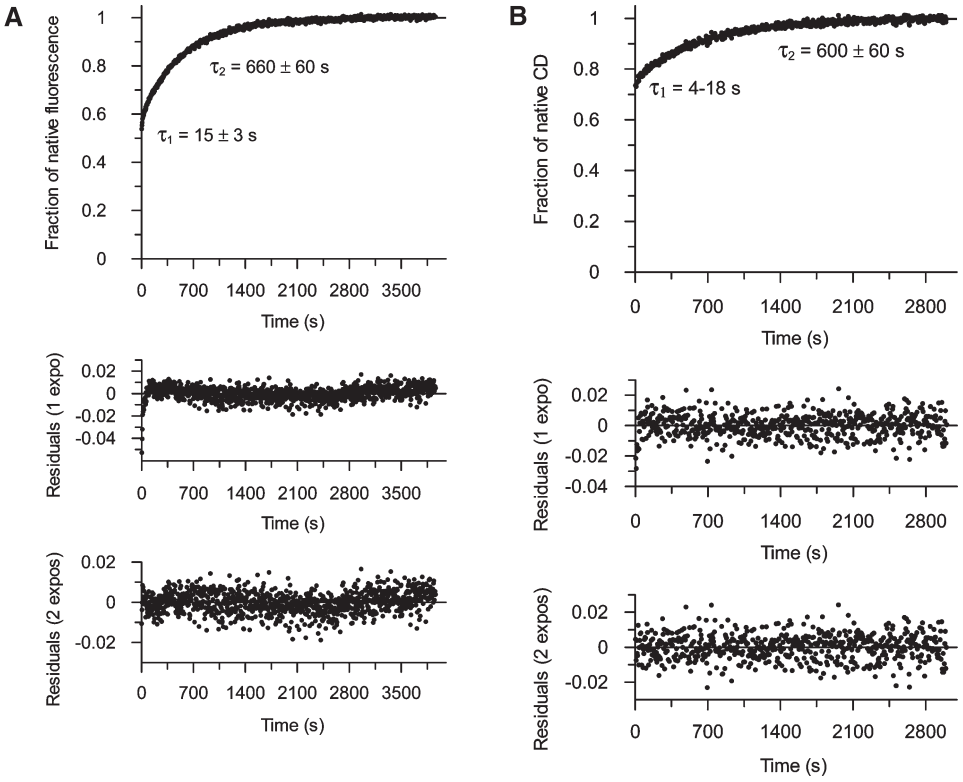

FIGURE 3: Refolding kinetics of BS3 β-lactamase at pH 7, 25 °C, in the presence of 0.26 M GdmCl monitored by (A) intrinsic fluorescence at 338 nm (after excitation at 280 nm) and (B) CD at 222 nm. Double exponential functions (eq 4) have been fitted to the data, which have been normalized so that the value of the native state is 1 and that of the denatured state (under the refolding conditions) is 0. Note that for CD experiments reliable time constant values could be obtained for the second phase only (see text). In both cases, the patterns of residuals from the fits of single or double exponential functions to the experimental data are shown for comparison.

kinetics of this protein as well. In a double-mixing experiment (*6*, *36*), we therefore unfolded the protein in 6.9 M GdmCl at 25 °C for 5−10 s only. This time is sufficient for conformational unfolding to go to completion, but the prolyl bonds remain in their native-like isomeric state, because the unfolding time is too short to allow significant isomerization (τ values of 60 s for the *cis* to *trans* isomerization and of 300 s for the reverse reaction, both at 25 °C, are given by Kiefhaber et al. (*37*)). In the subsequent refolding reaction (in 0.26 M GdmCl) both the optical and catalytic properties of the native BS3 enzyme were regained within the dead time of manual mixing (~10 s). This observation strongly suggests that the slow refolding reactions of the BS3 enzyme are limited in rate by prolyl isomerizations.

From the temperature dependences of the two slow refolding phases (Figure 5), activation enthalpies ($\Delta H^{\ddagger}$) of 92 ± 14 and 96 ± 4 kJ·mol^{-1} were calculated for the slow and the very slow folding reactions, respectively. These values are consistent with the high activation enthalpies (80−90 kJ·mol^{-1} (*38*)) associated with *cis*−*trans* isomerization of Xaa−Pro peptide bonds.

For the TEM-1 enzyme, the two slow refolding phases (see ref *6*) were measured as a function of temperature as well. Refolding was followed by intrinsic fluorescence (not shown) and by enzymatic activity (Figure 6). At low temperatures (≤20 °C), biphasic kinetics were observed by both probes (see Figure 6A), whereas at 30 °C (Figure 6B) and above, the kinetics could be represented by a single exponential function, indicating that only the very slow phase is observed under these conditions. The corresponding Eyring plots in Figure 7 give activation enthalpies of 65 ± 8 kJ·mol^{-1} for the slow and 67 ± 4 kJ·mol^{-1} for the very slow refolding phases. These values are lower than those measured for the slow phases in BS3 folding but still in the range of values observed for rate-limiting *cis*−*trans* isomerizations in other protein folding reactions.

The refolding data obtained for BS3 β-lactamase can be interpreted based on the kinetic model proposed by Vanhove et al. (*7*) for the TEM-1 enzyme (Figure 8). For both, the accumulation of state I is followed by activity regain in two distinct reactions, the slow and the very slow reactions (Figures 4 and 6). The BS3 enzyme refolds via these two parallel pathways at all temperatures tested. For TEM-1, the two pathways can be discriminated clearly only below 30 °C (Figure 6A). At higher temperature, the native enzyme is apparently formed in a single very slow phase (Figure 6B). At 10 °C, about 20% of the fully denatured TEM-1 molecules use the slow ($\tau \approx 110$ s) refolding pathway. Presumably, these are the $^{c}I^{c}$ molecules that possess a native-like *cis* Pro167 (Figure 8). Accordingly, in the 80% very slowly refolding $^{t}I^{t}$ molecules ($\tau \approx 1300$ s), Pro167 is *trans*. For the BS3 enzyme, the proportion of slow and very slow refolding molecules is different; ~55% of molecules refold along the slow track (i.e., via $^{c}I^{c}$) and ~45% of molecules refold along the very slow track (i.e., via $^{t}I^{t}$) at 10 °C (with τ ~70 and ~5000 s, respectively; Figure 4A).

Refolding Kinetics of NMCA β-Lactamase. The NMCA enzyme contains a *cis* Glu166−Leu167 instead of a *cis* Glu166−Pro167 bond (as in the TEM-1 and BS1 enzymes). Its folding was also followed at 25 °C, in the presence of 0.2 M GdmCl, by measuring the regain of the native fluorescence (at 338 nm) and CD (at both 220 and 270 nm). The kinetics measured by fluorescence (Figure 9A) and near-UV CD (not shown) were biphasic, with time constant values of 36 ± 4 s for the slow phase and 900 ± 90 s for the very slow phase, whereas the far-UV CD (not shown) monitored the slow phase (42 ± 3 s) but not the very

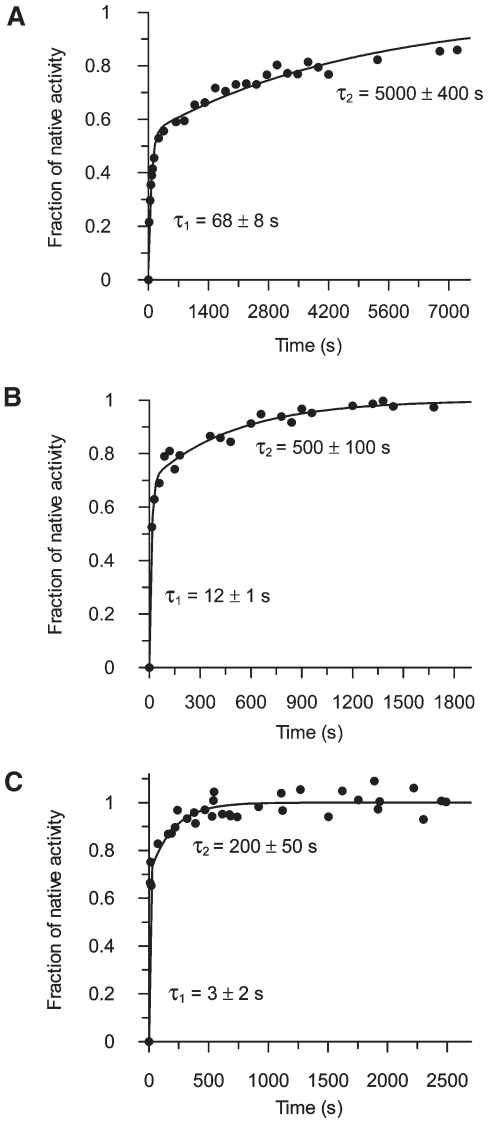

FIGURE 4: Refolding kinetics of BS3 β-lactamase at pH 7 at a final GdmCl concentration of 0.26 M, followed by recovery of enzymatic activity at (A) 10 °C, (B) 25 °C, and (C) 40 °C. The solid lines have been obtained by fitting double exponential functions to the data (eq 4). Note that the lack of activity at $t = 0$ was determined experimentally (see text). Data have been normalized as indicated in the legend of Figure 3.

slow phase. Major changes occurred also during the dead time of manual mixing (~60% of fluorescence intensity; ~50% and ~70% of near- and far-UV CD signals, respectively), indicating that a partially folded species with optical properties reminiscent of state I (see above) accumulates during the folding of the NMCA enzyme as well. Reactivation (Figure 9B) showed no burst phase (i.e., state I is inactive). The entire catalytic activity was regained in the very slow phase ($\tau = 830 \pm 70$ s), indicating that all molecules must pass through the slowest refolding phase to reach the final native state. The noncoincidence of kinetic traces observed by fluorescence, CD, and activity measurements suggests the presence of a second kinetic species that is significantly populated on the folding pathway. These observations can also be accounted for by the kinetic model in Figure 8, but assuming that $^{t}I^{c}$ is significantly populated whereas $^{c}I^{c}$ is not. $^{t}I^{t}$ is formed in the slow phase (~40 s) and is already highly similar to the fully folded state (100% of far-UV CD signal and ~90% of near-UV CD and fluorescence intensities), but it is devoid of catalytic activity.

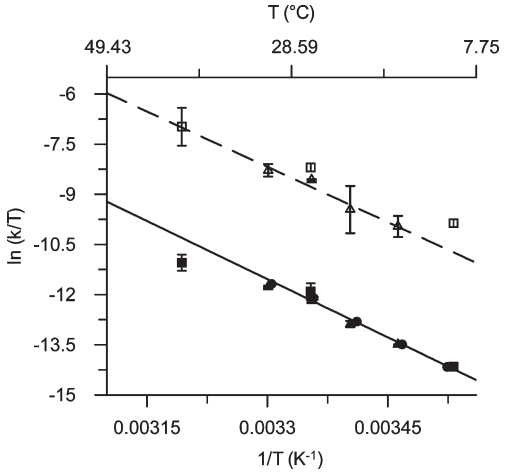

FIGURE 5: Temperature dependence of the rate constants for the two slow phases observed during BS3 refolding, measured by intrinsic fluorescence (Δ for the slow phase and ▲ for the very slow phase), far-UV CD (●), and recovery of enzyme activity (□ for the slow phase and ■ for the very slow phase), shown as an Eyring plot. k has the unit s^{-1}, and T has units of K. The continuous line (very slow phase) is the best fit of eq 5 to the CD data with $\Delta H^{\ddagger} = 96 \pm 4$ kJ·mol^{-1}, and the dashed line (slow phase) is the best fit of eq 5 to the fluorescence data with $\Delta H^{\ddagger} = 92 \pm 14$ kJ·mol^{-1}.

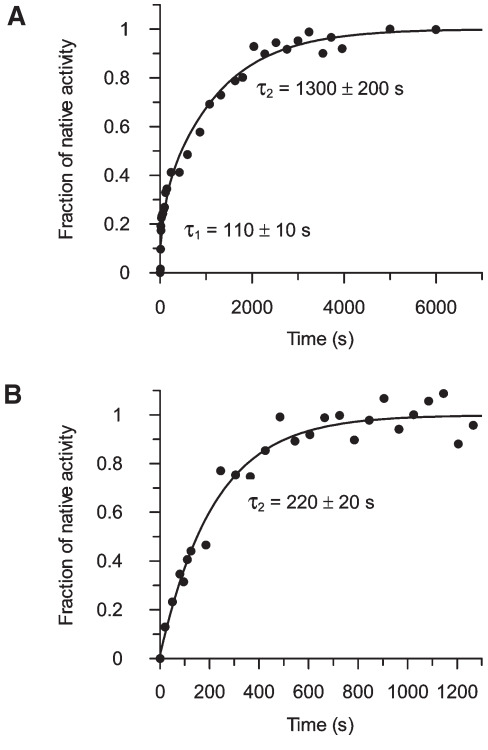

FIGURE 6: Refolding kinetics of TEM-1 β-lactamase at pH 7 at 0.18 M GdmCl as monitored by recovery of enzymatic activity at (A) 10 °C and (B) 30 °C. The solid lines were obtained by fitting double and single exponential functions to the experimental data obtained at 10 and 30 °C, respectively. Note that the lack of activity at $t = 0$ was determined experimentally. Data have been normalized as indicated in the legend of Figure 3.

In refolding experiments above 0.6 M GdmCl, the slow phase vanished, indicating that the $^{t}I^{c}$ intermediate is destabilized under these conditions and that only the very slow phase is observed.

NMCA β-lactamase contains eight proline residues. To examine how prolyl isomerizations contribute to the slow refolding

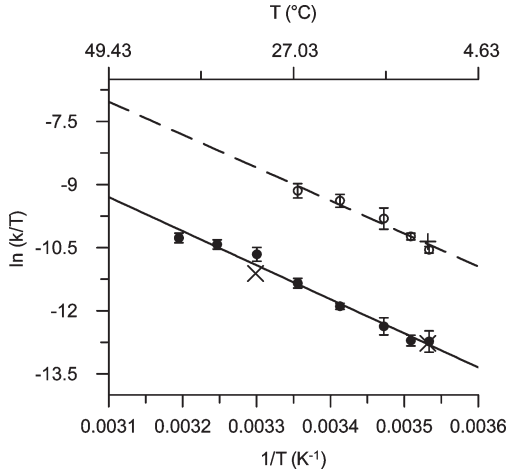

FIGURE 7: Temperature dependence of the rate constant value for the two slow phases observed during TEM-1 refolding, measured by intrinsic fluorescence (○ for the slow phase and ● for the very slow phase) and recovery of enzyme activity (+ for the slow phase and × for the very slow phase), shown as an Eyring plot. k has units of s^{-1}, and T has units of K. The continuous line (very slow phase) is the fit of eq 5 to the fluorescence data with $\Delta H^{\ddagger} = 67 \pm 4$ kJ·mol^{-1}, and the dashed line (slow phase) is the fit of eq 5 to the fluorescence data with $\Delta H^{\ddagger} = 65 \pm 8$ kJ·mol^{-1}.

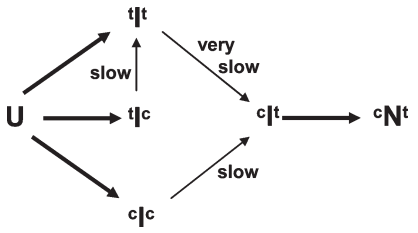

FIGURE 8: Kinetic model for the folding of class A β-lactamases at 10−40 °C, pH 7. The superscripts preceding and following the capital letter, which identifies the state of folding of the protein, refer to the configurations of the 166−167 peptide bond and the other Xaa−Pro bond(s) in the protein, respectively, whose isomerization accounts for the observed phases of folding. The steps represented by bold arrows are fast compared with the mixing dead time. With all enzymes, conversion of $^{c}I^{t}$ to $^{c}N^{t}$ is very fast (*6*, *7*). The $^{t}I^{t}$ species is significantly populated with all enzymes, whereas $^{c}I^{c}$ accumulates for those enzymes (BS3, TEM-1, I167P PC1, L167P NMCA) displaying a Pro at position 167 only. Although it remains possible that $^{t}I^{c}$ might be formed in a nonnegligible proportion with all enzymes (see ref *7*), the transition $^{t}I^{c} \rightarrow {}^{t}I^{t}$ is observed with NMCA only. PC1 and P167T TEM-1 appear to fold according to linear pathways. Note that states U and I correspond to large ensembles of conformers, and the experimental parameters are averaged over these ensembles.

phases, we performed manual double-mixing experiments in which the time of unfolding was successively increased before refolding was initiated. These experiments gave a clear result (Figure 10). The slow refolding reaction ($\tau \approx 40$ s) was absent when the protein was unfolded for a short time only. Its amplitude increased when the duration of unfolding was increased, and the kinetics of this slow increase showed $\tau \approx 300$ s (at 10 °C; Figure 10). This constitutes good evidence that the 40 s refolding phase is caused by prolyl isomerizations in the unfolded protein and is limited in rate by their reisomerizations (*36*, *37*). In contrast, the very slow refolding reaction ($\tau \approx 900$ s) was present with its full amplitude in all refolding traces, even after unfolding times as short as 10 s, when all unfolded molecules still contain the native-like prolyl isomers. This very slow refolding reaction is thus not caused by the reisomerization of incorrect prolyl bonds.

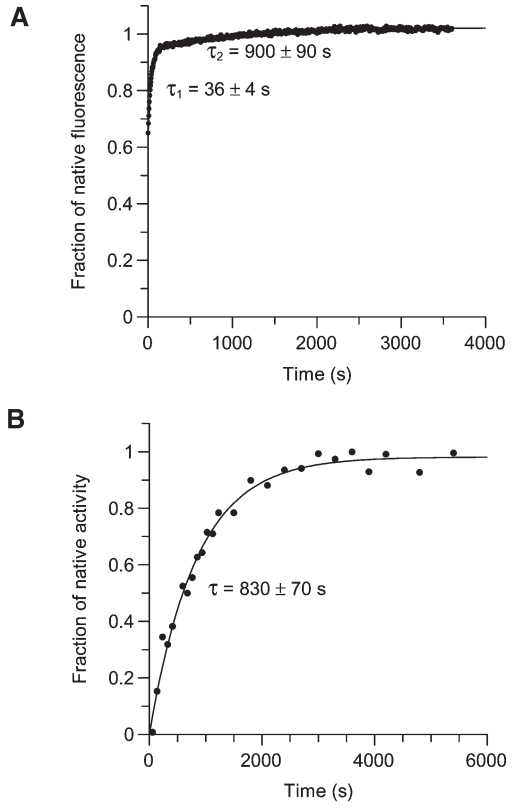

FIGURE 9: Refolding kinetics of the NMCA β-lactamase at pH 7, 25 °C, in the presence of 0.20 M GdmCl, followed by (A) intrinsic fluorescence measurements ($\lambda_{ex} = 284$ nm and $\lambda_{em} = 338$ nm) and (B) recovery of enzymatic activity. A double exponential function has been fitted to the fluorescence data, normalized as described in the legend of Figure 1, whereas the solid line for enzymatic activity measurements was obtained by fitting a single exponential function to the data.

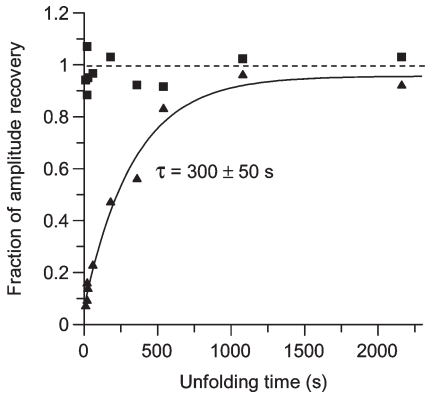

FIGURE 10: Time course of formation of the slow (▲) and very slow (■) refolding phases of NMCA β-lactamase after rapid unfolding at 10 °C. After various periods of denaturation in 4.0 M GdmCl, 50 mM sodium phosphate, and 50 mM NaCl, pH 7.0, samples were refolded by a 20-fold dilution with the same buffer, without GdmCl, at 25 °C. Refolding was followed by fluorescence. The amplitudes of the slow and the very slow refolding reactions are shown as a function of the incubation time under unfolding conditions. The amplitudes are given relative to the values observed for a sample that was unfolded for 1 h. The solid line represents a monoexponential function, fitted to the data obtained for the slow phase. The broken line drawn at 1 indicates complete amplitude recovery in the dead time of the double-mixing experiment.

In the folded form, the NMCA enzyme also contains a *cis* peptide bond (Glu166−Leu167). The *cis* to *trans* isomerization of

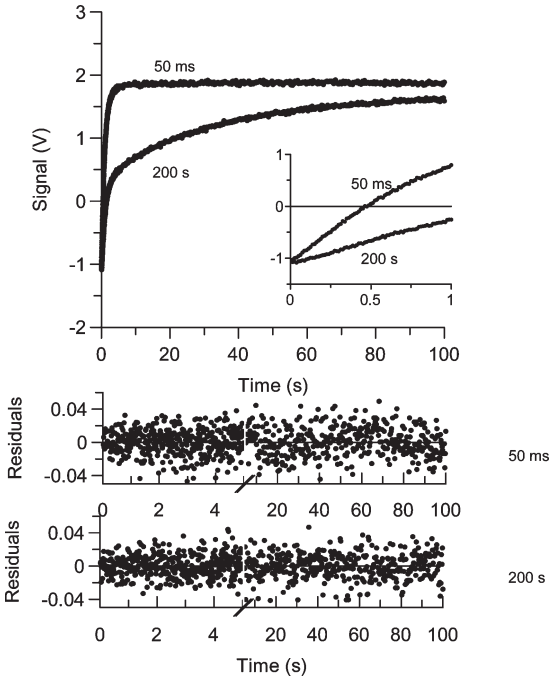

FIGURE 11: Refolding kinetics of NMCA β-lactamase at 25 °C after short-term or long-term unfolding. In a stopped-flow double-mixing experiment, the protein was unfolded in 1.44 M GdmCl in 50 mM glycine/HCl, pH 2.4, for 50 ms (upper curve) or 200 s (lower curve) and then refolded by a 6-fold dilution to 0.2 M GdmCl in 50 mM sodium phosphate, pH 7, containing 50 mM NaCl. The kinetics of refolding monitored by the change in fluorescence above 300 nm are shown. The inset shows the first second of the reaction. The data were analyzed as the sum of three (refolding kinetics after 50 ms unfolding) or four (200 s unfolding) exponential functions using eq 4 with $\tau_1 = 0.2 \pm 0.04$ s, $\tau_2 = 1.03 \pm 0.06$ s, $\tau_3 = 4 \pm 1$ s, and $\tau_4 = 36 \pm 4$ s. Note that the very slow phase ($\tau \sim 900$ s) observed after manual mixing is not observed in these stopped-flow experiments. The residuals from the fits of the sum of three (50 ms) or four (200 s) exponential functions to the experimental data are shown.

such non-prolyl bonds after unfolding is known to be much faster, with $\tau < 1$ s (*39*). To examine whether the very slow refolding reaction might be correlated with non-prolyl *cis*/*trans* isomerization, we repeated the double-mixing experiments by using stopped-flow equipment, which allowed refolding to be followed after very short unfolding intervals. In fact, after 50 ms of unfolding, both the slow and the very slow refolding reactions were absent, and the protein was fully folded in about 5 s (Figure 11). This provides good evidence that the very slow refolding reaction reflects the *trans* to *cis* isomerization of the Glu166−Leu167 bond. It originates from unfolded molecules with the incorrect *trans* form of this bond, which are formed within a few seconds after unfolding. Virtually all refolding molecules must go through this reaction, because more than 99% of all unfolded molecules contain an incorrect *trans* Glu166−Leu167 bond. Both the slow (manual mixing) and fast (stopped-flow) double jump experiments provide evidence that NMCA refolds according to the kinetic model in Figure 8, with both $^{t}I^{c}$ and $^{t}I^{t}$ as major intermediate species. The $^{c}I^{c}$ species is not populated because a non-prolyl *cis* peptide bond at positions 166−167 is too destabilizing and thus occurs in presumably less than 1% of all unfolded molecules.

Prolyl isomerases accelerate the isomerization of prolyl peptide bonds but not of non-prolyl peptide bonds (*40*). In fact, the human peptidyl−prolyl *cis*−*trans* isomerase cyclophilin accelerated the slow refolding phase of NMCA β-lactamase (Figure 12)

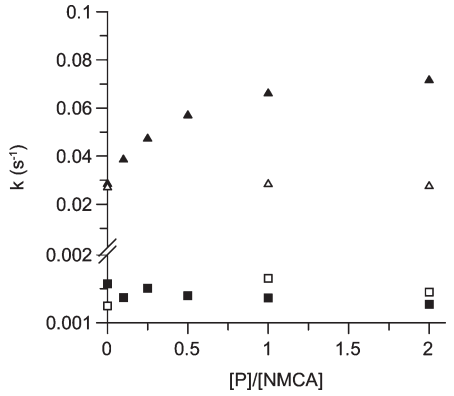

FIGURE 12: Effect of the prolyl isomerase cyclophilin on NMCA refolding. The rate constant values of the slow (triangles) and very slow (squares) refolding phases of NMCA β-lactamase (1 μM) were measured at 25 °C in 50 mM sodium phosphate, pH 7, containing 50 mM NaCl and 0.2 M GdmCl, in the presence of various cyclophilin (filled symbols) or lysozyme (open symbols) concentrations (indicated as [P]/[NMCA] for the cyclophilin or lysozyme to β-lactamase ratio).

but had no effect on the very slow phase. The specificity of cyclophilin is shown by the absence of effect on β-lactamase folding when it is replaced by lysozyme. This supports the conclusion that the slow (40 s) refolding phase is caused by prolyl isomerizations, whereas the very slow (900 s) phase originates from a non-prolyl isomerization.

Refolding Kinetics of the cis-Leu167Pro NMCA β-Lactamase. To examine the role of the 166−167 *cis* peptide bond in the folding of NMCA further, we substituted Leu167 by a proline to generate a *cis* Glu166−Pro167 bond, as in most other β-lactamases. Both the CD spectra (in the near- and far-UV regions; not shown) and the thermodynamic parameters (Table 1) of the wild-type and variant proteins are identical within the error limit, indicating that the mutation did not result in significant changes in the three-dimensional structure and stability of the enzyme. The catalytic parameters of the variant and wild-type enzymes were measured with five substrates and compared (Table 2). Variations in specificity constant (k_{cat}/K_m) values were smaller than 1 order of magnitude, suggesting that substitution of Leu by Pro in the Ω-loop of NMCA induced only minor structural changes in the enzyme active site. Most importantly, the high activity of the variant enzyme provides strong evidence that the 166−167 peptide bond retains a *cis* orientation.

Comparison of the slow refolding kinetics of the wild-type enzyme and the Leu167Pro variant at 0.2 M GdmCl (Figure 13A) shows that, in both cases, about 60% of the native fluorescence is recovered within the dead time of manual mixing, indicating that both proteins form an intermediate during folding. The slow part of the refolding kinetics was different. Whereas the wild-type enzyme exhibited two slow phases (as shown above), the Leu167Pro variant exhibited an apparently monoexponential refolding reaction with a time constant of ~37 s. The reactivation of the Leu167Pro variant at 0.2 M GdmCl was fast and complete within ~2 min. Thus, it could not be measured accurately, but apparently it follows the same time course as the fluorescence-detected kinetics in Figure 13A. It was at least 10-fold faster than the reactivation of the wild-type protein, which showed a time constant of 830 s under these conditions (Figure 13B).

At higher (0.5 M) GdmCl concentration, reactivation of the Leu167Pro variant could be monitored (Figure 13B), however, and confirms that refolding of NMCA was strongly accelerated

Table 2: Kinetic Parameter Values for the Wild-Type and L167P NMCA β-Lactamases[a]

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	k_{cat}/K_m (% of wild-type)
benzylpenicillin	250 (40)	310 (300)	8×10^5 (1.3×10^5)	600
cephalothin	650 (170)	650 (120)	10^6 (1.4×10^6)	71
cefotaxime	nd (nd)	> 700 (> 200)	2.3×10^3 (6.2×10^3)	37
imipenem	300 (220)	60 (600)	5×10^6 (3.6×10^5)	1350
cefoxitin	nd (4)	> 900 (340)	1.1×10^3 (1.2×10^4)	9

[a]Values in parentheses are for the wild-type enzyme. All standard deviations were within 10−20% of the average values (n = 3). nd = not determined.

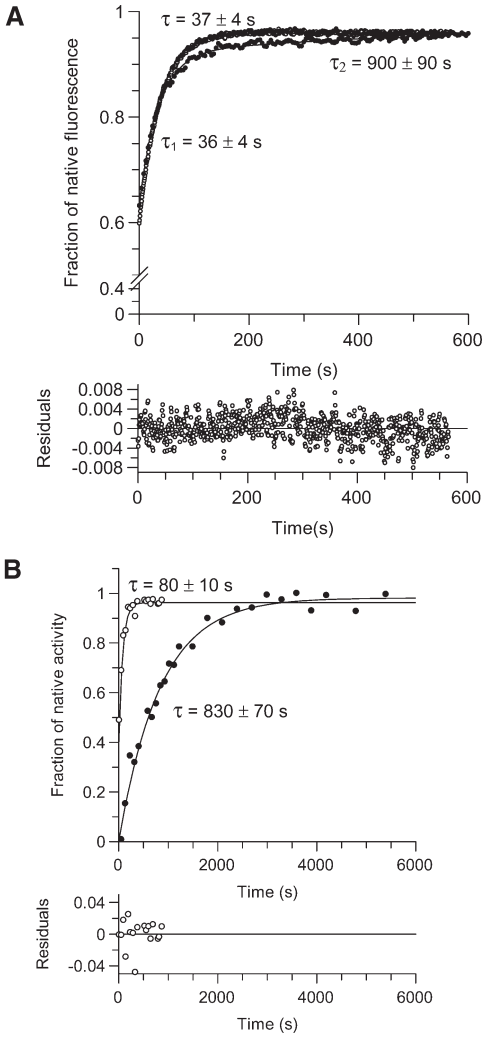

FIGURE 13: Refolding kinetics of wild-type NMCA β-lactamase (filled symbols) and the L167P variant (open symbols) at pH 7, 25 °C, followed by (A) intrinsic fluorescence measurements in 0.2 M GdmCl and (B) recovery of enzymatic activity in 0.2 and 0.5 M GdmCl for the wild-type and variant enzyme, respectively. In (A), double (lower trace) or single (upper trace) exponential functions were fitted to the kinetics of the wild-type and mutated enzymes, respectively, and normalized as described in the legend of Figure 3. In (B), single exponential functions were fitted to the data. The patterns of residuals from the fits to the data obtained with the variant NMCA are shown.

by the Leu167Pro substitution. Under these conditions, ~40% of the refolding molecules acquire enzymatic activity within the dead time of the experiment, whereas the ~60% remaining molecules refold with a time constant of ca. 80 s (identical to the value determined by fluorescence measurements performed under the same conditions). This behavior is consistent with the occurrence of parallel folding routes, as described in Figure 8 for

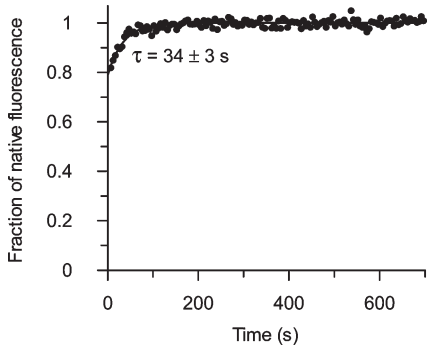

FIGURE 14: Refolding kinetics of PER-1 β-lactamase at pH 7, 25 °C, in the presence of 0.8 M urea monitored by recovery of native fluorescence (λ_{ex} = 280 nm and λ_{em} = 340 nm) at 25 °C. A single exponential function has been fitted to the data, normalized as described in the legend of Figure 1.

the BS3 and TEM-1 enzymes. Manual double-mixing experiments (data not shown) confirm that the Leu167Pro variant of NMCA becomes similar to the last two enzymes.

Refolding Kinetics of PER-1 β-Lactamase. As described before (see Figure 1B), the peptide bond between residues 166 (Glu) and 167 (Ala) in the Ω-loop of PER-1 is in the *trans* conformation.

Figure 14 shows the refolding kinetics of the PER-1 β-lactamase (13 prolines), as measured by fluorescence at 340 nm. In the presence of 0.8 M urea, ca. 85% of the native signal was recovered during the dead time of manual mixing. Following this burst phase event, a single kinetic phase, with τ = 34 ± 3 s (at 25 °C), was observed. When the protein was unfolded in 7 M urea for only 10 s and then refolded (in a double-mixing experiment), the enzyme was fully renatured (in 0.8 M urea) within the dead time of manual mixing. This shows again that the slow phase (τ = 10−40 s at 25 °C) observed with most class A enzymes is likely to be associated with proline isomerization. The very slow refolding phase observed with all class A β-lactamases studied so far (τ = 200−1000 s at 20−25 °C) was not observed for the PER-1 enzyme.

Finally, investigation of the temperature dependence of the slow refolding phase observed in PER-1 refolding (in the 10−30 °C range; data not shown) allowed computation of an activation enthalpy ($\Delta H^{\ddagger}$) value of 58 ± 4 kJ·mol^{-1}, which is also compatible with folding being rate-limited by *cis*−*trans* isomerization of proline-containing peptide bonds.

DISCUSSION

The β-lactamases studied in this work showed cooperative two-state unfolding transitions unlike the PC1 and TEM-1 enzymes (*6*, *41*). Under similar experimental conditions (i.e., pH 7 and 20−25 °C), these two enzymes populated a thermodynamically stable partially unfolded state (termed H) at intermediate GdmCl

Table 3: Overview of Slow Kinetic Phases Observed with Class A β-Lactamases

enzyme	residue at position 167	conformation of 166−167 bond	τ_1 (slow phase)[b,c] (s)[a]	τ_2 (very slow phase)[d] (s)[a]
TEM-1[e]	Pro	*cis*	20[b]	200
TEM-1 P167T[f]	Thr	*cis*	nd	350
BS3	Pro	*cis*	15[b]	600
NMCA	Leu	*cis*	40[c]	900
NMCA L167P	Pro	*cis*	<10[b]	80
PC1[g]	Ile	*cis*	≤ 10[c]	500
PC1 I167P[g]	Pro	*cis*	<10[b]	170
PER-1	Ala	*trans*	35[c]	nd

[a]All measurements at 25 °C, except PC1 (20 °C), and in GdmCl (0.1−0.5 M), except PER-1 (urea 0.8 M). [b] $^{c}I^{c} \rightarrow {}^{c}I^{t}$. [c] $^{t}I^{c} \rightarrow {}^{t}I^{t}$. [d] $^{t}I^{t} \rightarrow {}^{c}I^{t}$. [e]Reference *6*. [f]Reference *7*. [g]Reference *9*. nd, not detected.

concentrations, which showed properties of a molten globule. Our data confirm that the class A β-lactamases differ strongly in the Gibbs free energy of stabilization between 20 and 30 kJ·mol^{-1} for PC1 (*5*, *9*, *41*) to ~45 kJ·mol^{-1} for TEM-1 (*6*) and BS3 (this work). Interestingly, the *m* values of the cooperative transitions of the NMCA and BS3 enzymes are similar to the sum of the *m* values for the two stages of unfolding as observed for the TEM-1 and PC1 enzymes (Table 1). This indicates that the initial, native and the final, fully unfolded forms of these β lactamases are very similar to each other in their exposure of solvent-accessible surface area upon unfolding (*42*).

With the sole exception of PER-1, all class A β-lactamases display in the Ω-loop a *cis* peptide bond between the catalytic residue Glu166 and residue 167. Only in the PER-1 enzyme is the Glu166−Ala167 bond *trans*, and this difference in backbone conformation is accompanied by a substantial reorganization of the Ω-loop (see Figure 1B). Still, the γ-carboxylate group of Glu166 is at the same position as in all other β-lactamases.

The folding characteristics of the β-lactamases of this study confirm that the slow isomerization of the Glu166−Xaa167 peptide bond is a critical rate-limiting step for folding and activation of these enzymes (see Table 3). An incorrect (*trans*) isomer of this bond does not prevent folding to start. In fact, partially folded species (I) accumulate rapidly in the folding of all class A β-lactamases (PC1, TEM-1, NMCA, BS3, and PER-1 (*6*, *43*) and this work). The rapidly formed kinetic intermediates are of the molten globule type, containing at least 70% of the far-UV ellipticity of the native protein, weak tertiary structure, and are devoid of catalytic activity. Using stopped-flow techniques (refs *4*, *8*, *9*, and *44* and Figure 11 in this work), the structural changes occurring in the dead time of manual mixing experiments could be decomposed into a series of distinct kinetic phases, each of which represents a well-defined, cooperative transition. These fast events are associated with substantial secondary structure formation and thus can be described as conformational folding. In contrast, the following slow phase(s) observed with all enzymes can be associated with prolyl or non-prolyl peptide bond isomerization.

State I is probably a heterogeneous mixture of partially folded molecules that differ in the isomeric state of prolyl peptide bonds (*7*) and the extent of folding. The major kinetic intermediates are presumably the $^{t}I^{t}$, $^{c}I^{c}$, $^{t}I^{c}$ species (see Figure 8). They differ in stability from each other and for the different β-lactamases. In particular, the occurrence of a non-prolyl residue at position 167 significantly destabilizes species $^{c}I^{c}$ with a correct, but thermodynamically very unfavorable, *cis* peptide bond at positions 166−167 and incorrect *cis* isomers at other Pro sites. Increasing the temperature (or the final GdmCl concentration, see ref *6*) also destabilizes the $^{c}I^{c}$ intermediate during TEM-1 refolding. At 30 °C (or >0.5 M GdmCl), the whole reaction is channeled through $^{t}I^{t}$. In comparison with TEM-1, the $^{c}I^{c}$ intermediate species on the BS3 folding pathway appears to be more strongly populated and stable. Thus, the slow isomerization of the Glu166−Pro167 peptide bond, which is likely to occur concomitantly with folding and collapse of the Ω-loop residues onto the main body of the enzyme (*8*, *9*), appears to be highly dependent on local structural effects. TEM-1 and BS3 differ at seven positions in the Ω-loop (Figure1).

The occurrence of the $^{t}I^{c}$ state in model 1 was inferred by Vanhove et al. (*7*) but could not be proven. The refolding kinetics of NMCA β-lactamase now provide support for the existence of this intermediate species. In the kinetic model presented in Figure 8, $^{t}I^{c}$ could be significantly populated, as is the case for NMCA, but this does not appear to influence the refolding, except of NMCA. With the latter, however, the slow refolding phase disappears at GdmCl concentrations greater than 0.6 M, indicating that $^{t}I^{c}$ is largely destabilized and that $^{t}I^{t}$ remains the only significantly populated species. These data, together with the destabilization of the $^{c}I^{c}$ intermediate during TEM-1 refolding under some conditions, indicate that $^{t}I^{t}$ is the most stable species on the folding pathways of most class A β-lactamases. Finally, data obtained with NMCA also indicate that $^{t}I^{t}$ displays optical properties virtually identical to those of the native state, in excellent agreement with the view that this incompletely folded species corresponds overall to the native structure of the enzyme, lacking a well-defined native conformation only in the Ω-loop. This species probably resembles the native state in stability, whereas intermediate species with non-native *cis* isomers of certain *trans* prolines probably show a substantially reduced stability relative to the native state.

The slow and the very slow reactions differ by about 1 order of magnitude, and for both the BS3 and the TEM-1 enzyme, these reactions lead to enzymatically active molecules (Figures 4 and 6). This demonstrates that these enzymes refold via two major parallel pathways as described by the model in Figure 8.

The slow phase, which is formed slowly in the unfolded state (>10 s), is caused by the *cis* → *trans* isomerization of prolyl peptide bonds that are *trans* in the native state (denoted $I^{c} \rightarrow I^{t}$ in Figure 8). The observed refolding time constants (τ_1) in the 10−40 s range (Table 3) and slow isomerization (*trans* → *cis*) in the unfolded state are consistent with the values of 60 s for the *cis* to *trans* isomerization and of 300 s for the reverse reaction, respectively, both at 25 °C, given by Kiefhaber et al. (*37*).

The very slow phase is caused by the *trans* → *cis* isomerization of the 166−167 peptide bond, which is *cis* in the native state. When residue 167 is a proline, this reaction is about 10-fold slower than the slow phase, which reflects *cis* → *trans* isomerizations. This is consistent with *cis:trans* ratios near 1:10 for prolyl peptide bonds. Similar rates were observed for the very slow reaction, irrespective of whether the *cis* peptide bond is formed to a proline (such as Pro167 in TEM-1 or BS3) or to a non-prolyl residue (such as Ile167 in PC1 or Leu 167 in NMCA; see Table 3). This result strengthens the view (*7*, *39*) that the strong shift of the *cis*/*trans* equilibrium toward *trans* for non-prolyl peptide bonds (*cis*/*trans* ratio of 1:100 to 1:1000) results mainly from a dramatic (100−1000-fold) acceleration of the *cis* → *trans* reaction and not from a deceleration of the *trans* → *cis* reaction. This is confirmed

by the observation (see Figures 10 and 11) that when the *cis* peptide bond is formed with Leu at position 167 (NMCA), the species with incorrect *trans* isomers are formed very rapidly in the unfolded state with a time constant < 1 s (*39*).

PER-1 β-lactamase does not contain a *cis* peptide bond in its Ω-loop. The absence of the very slow phase in the refolding and reactivation of this β-lactamase completes the evidence that the isomerization of the Glu166−Xaa167 peptide bond dictates the rate of the very slow step in the folding of most class A β-lactamases. When other non-native prolines are absent, folding can proceed to a native-like, but inactive form of these enzymes. It remains to be elucidated whether the *trans* → *cis* isomerization right at the active site is also used as a switch to regulate the enzymatic activity of β-lactamases.

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