CMLS, Cell. Mol. Life Sci. 54 (1998) 372–377
1420-682X/98/040372-06 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

β-Lactamases as models for protein-folding studies

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Abstract. This review traces some of the key features of the folding of β-lactamases and their relevance to the way proteins fold in general. Studies on the enzymes have highlighted the nature and role of equilibrium and transient condensed states. The kinetics of folding are multiphasic, and when monitored by acrylamide quenching of the tryptophan fluorescence, an early phase provides evidence for the transient accumulation of a nonnative intermediate involving burial of tryptophan in a nonpolar environment. Intermediate phases can be understood in terms of progressive folding of different parts of the molecule. The later, slow phases are associated with proline isomerization in the TEM-1 enzyme and, in its P167T mutant form, with isomerization from trans to *cis* of the E166 T167 peptide bond. Coupled with kinetic and X-ray crystallographic studies of the β-lactamase from *Staphylococcus aureus* and its D179Q mutant, it appears that the final stage of folding is that of collapse and packing of the Ω-loop on to the main body of the protein.

Key words. Protein folding; β-lactamase; *cis-trans* isomerization; folding intermediates; molten globule.

Although Jean-Marie Ghuysen would probably not regard himself as one of the folding community, the fact that protein folding offers one of the most technically and intellectually challenging problems in biochemistry has, by definition, brought it within his special sphere of interest. Coupled with his direct involvement with β-lactamases, it seems appropriate to offer this review as a tribute marking his achievements. In 1968, most of the substantial investigations of mechanisms of protein folding, notably those of Anfinsen on ribonuclease and staphylococcal nuclease, had been carried out on relatively small proteins. Staphylococcal β-lactamase PC1 was chosen for study first on the grounds of its larger size, which was at that time thought to offer better opportunities for identifying folding intermediates, and second because its folding would be dependent solely on noncovalent interactions and unconstrained by the presence of disulphide bonds. Further attractions were that its sequence had recently been determined by Ambler [1], diffraction data had been obtained from crystals and Novick [2] had produced two interesting mutants with much-reduced activities using chemical mutagenesis. In the event, the crystal structure did not appear until 1987 [3], no additional mutants could be made, despite efforts by several groups, until the early 1990s, the pattern of intermediates and their transitions has turned out to be little more complex than for some smaller proteins studied subsequently and the TEM-1 enzyme, with its single disulphide bond, shows similar folding behaviour to PC1. Nevertheless, PC1 has proved a useful model for folding studies, not least because of its high solubility and stability, and of the high degree of reversibility of denaturation.

The β-lactamase structure [3, 4] poses an interesting folding problem. The central section of the sequence folds into a globular unit that contains most of the α-helical secondary structure. The N- and C-terminal stretches are interleaved to form a five-stranded β-sheet which is contributed to by both chain segments and is sandwiched between the surface of the helical unit and

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another group of small helices. The problem may be visualized by grasping the N- and C-termini and pulling to unravel/unfold this β-structure 'domain'. Neither terminus can be pulled apart without destroying the fold of the other. This structure raises questions as to whether folding proceeds through a consecutive pathway with the β-unit folding on to the α-unit or whether there is a general collapse to a globule within which both units fold more or less independently.

β-lactamase also acts as a good model with which to tackle the question of how loops fold, since it contains a large Ω-loop of 16 residues (164 to 179) which packs relatively inefficiently against the main body of the enzyme, contacts being made largely through nine water molecules. It is located near the active site, and its precise conformation is important for orienting Glu-166, which is involved in catalysis. It is unusual for a loop in that it contains a *cis*-peptide bond between residues 166 and 167, the latter being proline in the TEM-1 β-lactamase but isoleucine in the staphylococcal PC1 enzyme.

In this review, we shall describe some of the interesting features and conclusions that have emerged from in vitro studies on the folding of β-lactamases, initially PC1 from *Staphylococcus aureus* and, more recently, TEM-1 and the class A enzyme from *Bacillus cereus*.

Thermodynamically stable partially denatured states

Experimental studies have traditionally centred on the search for intermediates as a route to defining pathways of folding [5]. PC1 was one of the first proteins found to exhibit a thermodynamically stable state under mild denaturing conditions [6, 7]. Termed 'state H' on account of its substantial content of secondary structure, optical and nuclear magnetic resonance (NMR) spectra showed that few, if any, persistent tertiary interactions remain. Its radius, judged by viscosity, gel electrophoresis and gel exclusion chromatography, is appreciably larger than that of the native protein, thus making it untypical of the compact molten globule [8]. It does, however, bind 1-anilino-8-naphthalenesulfonic acid (ANS) and must therefore contain substantial elements of condensed structure. A ''cheese-spread'' model of the enzyme as a three-domain molecule which separates into molten globulelike units in mild denaturant was proposed [9] but not proven.

The stability of state H relative to the fully unfolded state is close to half the free energy of stabilization of the native states, $\Delta G'_{N\text{-}U,0}$*, which is 20 kJ mol^{-1} for PC1 [10] and 45 kJ mol^{-1} for TEM-1 [11].

*The suffix '0' shows that the value of the parameter has been extrapolated to zero concentration.

Sulphate ions show a remarkable effect on the stability of PC1. In the presence of unfolding concentrations of urea, the addition of sulphate progressively reverses the transitions U$\leftrightarrow$H and H$\leftrightarrow$N [10]. The fact that the same values of $\Delta G'_0$ are obtained from these folding transitions as from the urea unfolding transitions proves the thermodynamic reversibility in this mixed solvent system. The effect of sulphate is greater on the H$\leftrightarrow$N than on the U$\leftrightarrow$H transition, the values of $\Delta G'_{N\text{-}H,0}$ and $\Delta G'_{H\text{-}U,0}$ being made more negative by 41 kJ mol^{-1} and 20 kJ mol^{-1}, respectively, per mole l^{-1} of sulphate. The increase in stability of H with respect to N is reflected entirely in the unfolding rate constant being decreased by a factor of 10^{11} in the presence of 1.4 M sulphate. The difference in effect of sulphate on the two transitions indicates that a higher proportion of nonpolar interactions is broken on going from N to H than from H to U, which is consistent with the model for state H described above.

Reversible denaturation at low pH has been studied by Fink and co-workers [12]. At ionic strengths sufficient to cause charge shielding of the positively charged groups, the protein unfolds partially to a molten globule state, A. At low ionic strength, however, the mutual repulsion of the charges at low pH further destabilizes the structure to result in a fully unfolded state. At high pH the protein again partially and reversibly unfolds to give a compact structure, B, similar to state H.

All three partially denatured states, H, A and B, refold to the active native state with a slow phase having the same rate constant as the slow phase of refolding of the fully unfolded protein, ($5 \cdot 10^{-4}$ s^{-1}), suggesting that all four states refold through a common intermediate and that the partially unfolded states are closely similar and different from the transient, kinetic intermediate.

Transient kinetic intermediates

While the final slow step of folding of PC1 is kinetically two state, as shown by the use of a variety of probes as monitors [13], the overall folding was recognized early to involve the accumulation of intermediates [7]. With the addition of stopped-flow techniques, these are now observable as a series of five kinetic phases. The last intermediate to accumulate interconverts to the native state only slowly, being observable for up to an hour at low temperatures. Termed 'I', it exhibits the characteristic spectroscopic properties of the equilibrium state H, but by a variety of criteria [13, 14], it differs in having the considerably more compact conformation of the classical molten globule. There are two possible contributing factors to the greater Stokes radius of I compared with N. One is the molten nature of the main body of the enzyme as shown by spectroscopic properties; the other is the Ω-loop, which, due to the bond before residue 167 being in the nonnative *trans* configu-

ration, is probably disordered (see later). It is frequently assumed, and in one case supported by spectroscopic evidence [15], that the equilibrium and kinetic 'molten globules' are identical. Although β-lactamase may be unique, the Stokes radii of other proteins need to be measured before making generalizations. It is not surprising to find differences, since one exists in denaturing solvents and the other in native-stabilizing solvents.

The question as to whether states of intermediate conformation such as H and I lie on a folding pathway or whether they are off-pathway species has been discussed [9, 10, 16] but not resolved satisfactorily.

Early stages in folding

It was realized early that secondary structure is formed much more rapidly than tertiary structure in β-lactamase [7]. Stopped-flow studies have since shown that secondary structure and collapse occur very fast for both β-lactamases, over 60% of the native far-ultraviolet (UV) ellipticity at 230 nm being generated in less than 10 ms [17, 18] and the adsorption of ANS becoming maximal by 50 ms [13] in PC1 and less than 3 ms for TEM-1 [18]. These results are characteristic of the many proteins that have now been studied and, coupled with the results of H/D exchange experiments, support the thesis that a molten globule (in its broader sense) collapsed structure is formed very early and contains a substantial amount of nativelike topology, and that packing and possibly repacking within this collapsed form account for the subsequent phases of folding.

The early stages of folding of TEM-1 show features that bear on the debate as to whether folding is hierarchical or not. The tryptophan residues in this β-lactamase are largely exposed to solvent. A stopped-flow study showed that the quenching of intrinsic fluorescence by acrylamide decreased within the burst phase and then increased to the value characteristic of the native protein [18]. This shows that tryptophan residues were, on average, less accessible in early intermediates than in the native enzyme. This strongly suggests that one or more tryptophan residues are buried in a nonpolar environment from which they have to escape during subsequent folding, thus arguing for a nonhierarchical folding pathway. Based on the folding of the mutant enzyme lacking a disulphide bond, Gervasoni and Plückthun [19] assigned the quenching of fluorescence prior to the minimum to the location of W210 in its native position close to the disulphide bond. The monotonic increase in acrylamide quenching following the burst phase must therefore be assigned to the increasing exposure to solvent of tryptophan(s) other than W210. An attractive model would involve the C-terminal W290 being buried in a nonnative conformation in the burst phase. Based on the structural features described above, early intermediates can be proposed as taking the form of a collapsed and partially folded (molten globule-like) α-helical domain, with its N- and C-terminal extensions probably collapsed but not yet intertwined in the native β-sheet structure of the α/β domain. In this dynamic structure the C-terminal tryptophan could well find a lower energy state by becoming buried in the hydrophobically collapsed α-domain. In order to fold and generate the native α- and β-secondary structures, this tryptophan would then have to be extracted from the α-domain core and become exposed to solvent. This would be consistent with the observed slower formation of 35 to 40% of the native ellipticity (complete in about 60 s) prior to the final, Xaa-Pro isomerization-dependent changes in tertiary structure. Accumulation of this intermediate is explained in terms of the relative stability of the interaction of the tryptophan.

Along with the early and transient formation of nonnative α-helical structure in β-lactoglobulin [20] and the similar, early hydrophobic interactions of tryptophan formed in lysozyme [21–24], these results provide further evidence that relatively stable, nonnative intermediates can be formed during folding. Whether they are off-pathway intermediates or whether their formation enables folding of other parts of the protein, followed by breakdown of the nonnative interaction by another route towards the native structure, remains to be discovered.

The region around the disulphide bond is nativelike at an early stage of folding

The enzymes of the TEM family contain a single disulphide which connects Cys-77 (α2-helix) and Cys-123 (α4-helix) in the all-α domain [25, 26]. Interestingly, this disulphide bond is not essential for enzymatic activity or correct folding [19, 27–30], but its formation contributes 14 kJ mol^{-1} to the overall stability of the protein [27, 29]. Refolding from the reduced unfolded state has been shown to involve a competition between disulphide bond formation and folding of the molecule into a compact, catalytically active conformation that buries the two cysteines in the core of the enzyme [30, 31]. Based on decreasing accessibility to *N*-ethylmaleimide, half of the burial of the thiol groups occurs at a very early stage of folding [31]. In agreement with these reports, other lines of evidence suggest that the region around the disulphide bond is folded in a nativelike pattern early during folding: (i) Trp-210 comes close to its native topology, next to the disulphide bond, within 4 s [19], and (ii) the kinetics of the phases leading to the native conformation are hardly affected by removal of the disulphide bond [29, 32].

Table 1. The multiphasic folding of TEM-1 β-lactamase*.

Technique	Information	Phase number					
		1†	2	3	4	5	6§
Kinetics	τ	<1 ms	150 ms	600 ms	5 s	30 s	300 s
Intrinsic fluorescence	kinetics	+	−	−	+	+	+
Acrylamide quenching	solvent accessibility of Trp and Tyr	−	+	+	+	+	−
ANS binding	exposure of hydrophobic surfaces	+	−	−	−	−	−
Far-UV CD	formation of secondary structure	+	+	+	+	0	0
Recovery of activity	formation of native state	no	no	no	no	yes	yes
Sensitivity to double jump‡	involvement of proline isomerization	no	no	no	no	yes	yes

+, − and 0 indicate increase, decrease and no change respectively in the signal.
*Data from refs. 11, 18 and 33. †The 'burst' phase within the dead-time of the instrument. ‡Protein unfolded for a short time such that refolding took place from a species in which all prolines were in the native configuration. §Ascribed to *trans* → *cis* isomerization of the E166–P167 peptide bond.

Subsequent folding events

Following the burst phase, and similar to PC1, the folding of TEM-1 exhibits five measurable phases with time constants of 150 ms, 600 ms, 5 s, 30 s and 300 s [18]. Acrylamide quenching experiments indicated that the tryptophan residues that are buried in the burst phase (see above) are subsequently slowly re-exposed as folding proceeds. Gervasoni and Plückthun [19] showed that Trp-210 comes close to its native topology within 4 s of folding, suggesting that the increase in tryptophan accessibility probed by acrylamide quenching involves one or more of the three remaining tryptophans. It is probable, therefore, that a major contribution to the increase in the intrinsic fluorescence observed during the three slower phases is associated with formation of the aromatic interaction between Trp-229 and Trp-290.
The significance of the different phases observed during the folding of TEM-1 β-lactamase is shown in table 1.

Proline isomerization and folding of the Ω-loop

Formation of the native state of TEM-1, monitored by recovery of enzymatic activity and by fluorescence, occurs with biphasic kinetics [19, 33], indicating that the polypeptide chain folds via two parallel pathways. In order to investigate the possible role of the unusual configuration of residue 167 in determining the kinetics of folding, the P167T mutant was constructed [11]. It was found to fold with a single phase only, leading to the conclusion that the slower phase of the wild-type enzyme is associated with the isomerization of the E166 P167 peptide bond in the Ω-loop. The faster phase ($\tau = 30$ s) reflects isomerization of still unassigned proline residue(s) in the body of the enzyme [11, 33]. Analysis of the kinetic behaviour and sequences of other class A β-lactamases, including the enzymes produced by *Staphylococcus aureus*, *Streptomyces albus*, *B. licheniformis* and the NMCA β-lactamase from *Enterobacter cloacae* (A. Lejeune, M. Vanhove, R. H. Pain and J.-M. Frère, unpublished observations), suggests Pro-62 and Pro-145 as possible candidates. The models in figure 1 account for the experimentally observed kinetics of folding of the wild-type and mutant TEM-1 proteins.
PC1, with the *cis*-peptide bond in the Ω-loop being between E166 and I167, folds with a single, slow phase, as monitored by enzyme activity, circular dichroism (CD) and gel exclusion chromatography [10, 13]. Using the traditional double-jump technique of Brandts et al. [34], this phase was shown not to be associated with proline isomerization [10]. On the basis of the structure of the PC1 and P54 enzymes, however, Herzberg et al. [35] proposed that the *trans* → *cis* isomerization of the E166 I167 bond should be the rate-limiting step in the conversion of the kinetic intermediate I to the native state. In the light of the TEM-1 results, this appears a likely explanation and one which could be tested by studying an I167P mutant of PC1.

The Staphylococcal P54 mutant enzyme

One of the two mutants made by Novick, P54, has the Asp-179 in PC1 replaced by asparagine. This results in an enzyme with much-reduced activity, with reduced intensity of the near-UV CD spectrum and a slightly greater Stokes radius compared with PC1 [35, 36]. In the crystal structure [35], the Ω-loop is disordered, due presumably to the loss of the main stabilizing electrostatic interactions of D179 with the side chain of R164 and the main-chain nitrogens of V163 and R164. The main body of the enzyme, however, is closely similar to that of PC1, with rms deviations for all atoms being 0.76 Å and, for main-chain atoms, 0.45 Å. The former are due primarily to surface side chains, especially lysine, while the only main-chain atoms that deviate more than 1 Å from the positions in PC1 are located in

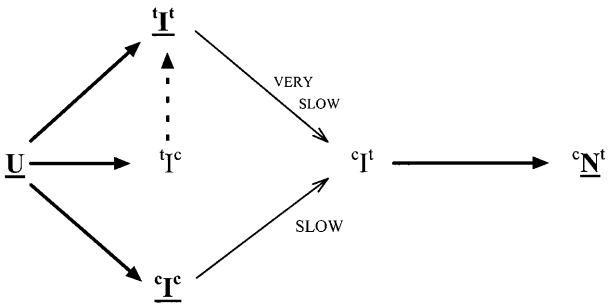
close proximity to the W-loop and are associated with the active site depression.

The kinetics of folding of P54 are dramatically different from those of PC1, in that the slow, rate-determining step is completely lacking. While the fast phases of folding are indistinguishable from those of PC1 (H. Christensen and R. H. Pain, unpublished observations), the slow phase characteristic of PC1 is absent, as monitored by near-UV CD. Urea-gradient gel electrophoresis supports these results, showing that the rapidly formed collapsed state H is in fast equilibrium with the still more condensed, fully folded P54 protein. Based on this kinetic behaviour, it was suggested that the folded state closely resembles the transient intermediate I observed in PC1 [36]. That it is not the same as I is evident from the near-UV CD spectrum [36] and subsequently from the crystal structure [35], the main body of the enzyme being folded sufficiently stably to impart optical asymmetry to one or more tyrosines, while the disordered W-loop accounts for the observed increase in Stokes radius. These results support the suggestion [35], strengthened by the kinetic studies on TEM-1 [11], that the slow folding phase of PC1 is due to isomerization of the D166–I167 peptide bond. The last step in folding is hence the folding and collapse of the Ω-loop residues on to the main body of the enzyme.

The fact that P54, unlike the intermediate I, behaves as a stable globular protein, despite having lost the stabilizing interactions involving D179, would appear to be due to the compensating formation of an alternative interaction between the amide N of D179 and the main-chain N of A69 [35]. The other Novick mutation of T40 to I in P2 results in a folded species that is very much on the borders of being a stable globular protein [36]. From the structure of PC1 [4], the mutation would be expected to prevent the W-loop from 'bedding down' on to the main body of the enzyme. In this case there are not likely to be any compensating stabilizing interactions.

Perhaps the most interesting feature of the folding of P54 is that the main body of the enzyme, which contains nine proline residues, folds rapidly from the equilibrium unfolded polypeptide. This demands further investigation.

WILD-TYPE ENZYME



P167 MUTANT

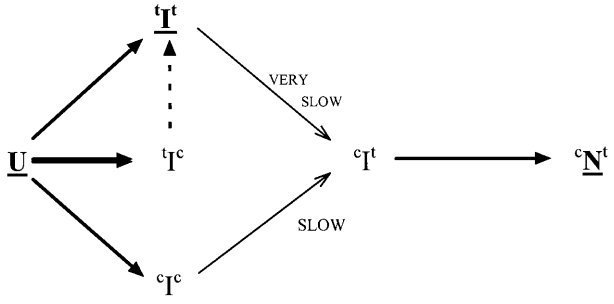


Figure 1. Kinetic models for the folding of the wild-type and mutant TEM-1 enzymes. The underlined symbols in bold type represent the forms which can be present in nonegligible amounts during the folding process. The superscripts preceding and following the capital letter which identifies the state of folding of the protein refer to the configurations, respectively, of the 166–167 peptide bond and the other Xaa–Pro bond(s) whose isomerization accounts for the observed phase of folding. The steps represented by bold arrows are fast compared with the mixing dead time. It remains possible that $^{t}I^{c}$ might be formed in a nonnegligible proportion but, in that case, the transition $^{t}I^{c} \rightarrow {}^{t}I^{t}$ (bold, dashed arrow) should be faster than both $^{t}I^{t} \rightarrow {}^{c}I^{t}$ and $^{c}I^{c} \rightarrow {}^{c}I^{t}$ [11].

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