Kinetic and thermodynamic consequences of the removal of the Cys-77–Cys-123 disulphide bond for the folding of TEM-1 β-lactamase

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Class A β-lactamases of the TEM family contain a single disulphide bond which connects cysteine residues 77 and 123. To clarify the possible role of the disulphide bond in the stability and folding kinetics of the TEM-1 β-lactamase, this bond was removed by introducing a Cys-77 → Ser mutation, and the enzymically active mutant protein was studied by reversible guanidine hydrochloride-induced denaturation. The unfolding and refolding rates were monitored using tryptophan fluorescence. At low guanidine hydrochloride concentrations, the refolding of the wild-type and mutant enzymes followed biphasic time courses. The characteristics of the two phases were not significantly affected by the mutation. Double-jump experiments, in which the protein was unfolded in a high concentration of guanidine hydrochloride for a short time period and then refolded by diluting out the denaturant, indicated that, for both the wild-type and mutant enzymes, the two refolding phases could be ascribed to proline isomerization reactions. Equilibrium unfolding experiments monitored by fluorescence spectroscopy and far-UV CD indicated a three-state mechanism (N ↔ H ↔ U). Both the folded mutant protein (N) and, to a lesser extent, the thermodynamically stable intermediate, H, were destabilized relative to the fully unfolded state, U. Removal of the disulphide bond resulted in a decrease of 14.2 kJ/mol (3.4 kcal/mol) in the global free energy of stabilization. Similarly, the mutation also induced a drastic increase in the rate of thermal inactivation.

INTRODUCTION

Since the factors that stabilize the folded state of proteins are almost completely offset by those that favour the unfolded state, native proteins are only marginally stable. Typically, the free-energy difference between the native and denatured states is about 21–84 kJ/mol (5–20 kcal/mol) [1]. Disulphide bonds play a unique role in stabilizing proteins, providing covalent cross-links between segments of the polypeptide chain. Disulphide bonds can supply a substantial contribution to the stability of a protein [2,3]. Although the mechanism by which disulphide bonds confer stability is not yet known in detail, one major aspect is the decrease in the conformational chain entropy of the unfolded polypeptide [4–6]. Nevertheless, enthalpic and native-state effects cannot be neglected [7].

Class A β-lactamases catalyse the hydrolysis of the β-lactam ring of penicillins and related antibiotics, and are thus responsible for many antibiotic-resistance phenomena. The TEM-1 class A β-lactamase is a monomeric protein produced by various Gram-negative bacteria and contains a single disulphide bond between Cys-77 and Cys-123 [8,9]. The folding of this protein shows the following characteristics [10]. (i) Equilibrium guanidine hydrochloride (GdmCl)-induced unfolding revealed the existence of a thermodynamically stable intermediate, referred to as state H, which lies between the native and fully unfolded states of the protein. State H retains a high content of secondary structure but very little specific tertiary organization. (ii) A compact and highly ordered transient intermediate (I) is formed early during the folding reaction. (iii) The final step of the folding process is rate-limited by *cis*/*trans* isomerization of two or more Xaa–Pro peptide bonds, one of which involves Pro-167 [11].

Interestingly, the disulphide bond of RTEM β-lactamase is not essential for enzymic activity. Both the reduced protein and a double Cys → Ala mutant protein fold to give active species [12], the thiols of the former being resistant to reaction with alkylating agents and to oxidation [13]. Walker and Gilbert [13] have proposed that oxidation and catalysis by protein disulphide isomerase require the presence of an equilibrium between the folded reduced protein and a less folded species in which the thiols are able to react with oxidizing and alkylating agents [13].

We have therefore made a detailed study of the equilibria and kinetics of folding of a Cys-77 → Ser mutant of the TEM-1 β-lactamase. The rate-limiting steps of folding and unfolding have been shown to be little affected by the mutation. By contrast, both the native and thermodynamically stable intermediate states were destabilized relative to the fully unfolded protein. The additional stabilization conferred by the disulphide bond in the wild-type enzyme is shown to be associated with a drastic decrease in the sensitivity of the protein to heat-induced inactivation.

MATERIALS AND METHODS

Materials

Dithiothreitol was purchased from Janssen Chimica (Beerse, Belgium). Ultrapure GdmCl was from ICN Biomedicals Inc.

Abbreviations used: GdmCl, guanidine hydrochloride; N, H and U, native folded state, thermodynamically stable intermediate and fully unfolded state respectively of the enzyme.

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(Aurora, OH, U.S.A.). The origin of the β-lactam antibiotics was as described previously [14]. All other reagents were of analytical grade.

Enzymes

The *Escherichia coli* strain producing the C77S mutant of the TEM-1 β-lactamase was described previously [15]. The protein was produced at 25 °C in LB medium; about 10 mg of pure enzyme was obtained from 15 litres of culture with a yield of 30 %. The mutant enzyme was purified as follows. Cells were isolated by centrifugation and then suspended in 30 mM Tris/HCl buffer, pH 7, containing 1 mM EDTA and 27 % (w/v) sucrose. The periplasmic contents of cells were released by lysozyme treatment as previously described [16]. Cell debris was removed by centrifugation, and the supernatant was dialysed against 10 mM Tris/HCl buffer, pH 7.5. The crude extract was applied to a Q-Sepharose Fast Flow column (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. The enzyme was eluted by a linear NaCl gradient (0–1 M). Active fractions were pooled and dialysed extensively against 100 mM sodium phosphate, pH 8. The protein was then applied to a Ni^{2+}-nitrilotriacetate–agarose column (Affiland, Ans, Belgium) equilibrated in 100 mM sodium phosphate, pH 8, containing 1 M NaCl. The TEM-1 β-lactamase contains six histidine residues, and four of them form two patches on the surface of the protein which can interact with the metal ion of the column. The protein was eluted by 40 mM imidazole. The enzyme solution was concentrated to > 1 mg/ml and dialysed against 50 mM sodium phosphate, pH 7.

Production and purification of the wild-type TEM-1 β-lactamase was as previously described [16].

Determination of enzymic activity

The kinetic parameters were determined at 30 °C in 50 mM sodium phosphate, pH 7, as described for the wild-type enzyme [17].

Equilibrium studies of GdmCl-induced unfolding

Equilibrium unfolding induced by GdmCl was monitored by following the changes in tryptophan fluorescence or by far-UV CD using a Perkin–Elmer LS50 luminescence spectrophotometer and a Jobin–Yvon CD6 dichrograph respectively [10]. Experiments were performed at 25 °C in 50 mM sodium phosphate, pH 7, containing 50 mM NaCl and supplemented with 10 mM dithiothreitol to prevent intermolecular disulphide formation. The transition curves obtained by fluorescence spectroscopy and CD were analysed using eqns. (1) and (2) respectively, assuming a three-state model ($N \leftrightarrow H \leftrightarrow U$, where N is the native state, H is the thermodynamically stable intermediate and U is the fully unfolded state):

$$F_{obs} = \{(F_N + p[GdmCl]) + (F_H + F_U \exp b)\exp a\} / [1 + (1 + \exp b)\exp a] \quad (1)$$

$$\theta_{obs} = (\theta_N + \{\theta_H + (\theta_U + q[GdmCl])\exp b\}\exp a) / [1 + (1 + \exp b)\exp a] \quad (2)$$

where:

$$a = \{-\Delta G_{H-N}(0) + m_{HN}[GdmCl]\}/\boldsymbol{R}T$$

and:

$$b = \{-\Delta G_{U-H}(0) + m_{UH}[GdmCl]\}/\boldsymbol{R}T$$

$\Delta G_{H-N}(0)$ and $\Delta G_{U-H}(0)$ are the differences in free energy between H and N and between U and H respectively in the absence of denaturant, and m_{HN} and m_{UH} are the slopes of the transitions. As for the wild-type enzyme, the terms $(F_N + p[GdmCl])$ and $(\theta_U + q[GdmCl])$ were used to account for the observed linear dependence of the fluorescence of the native protein and of the far-UV CD signal of the denatured state respectively on the denaturant concentration [10]. The values of p and q are thus entirely empirical.

Unfolding and refolding kinetics

The unfolding and refolding kinetics were monitored by following the intrinsic fluorescence of the protein, as described for the wild-type enzyme [10]. Unfolding kinetics were fitted to a single-exponential equation (eqn. 3, where F_0 and F_∞ are the observed fluorescence of the protein at zero and infinite time respectively). Refolding traces were analysed using eqn. (3) or according to eqn. (4), assuming a double-exponential signal. Refolding was also followed by estimating the recovery of enzymic activity, using 200 μM cephalothin as substrate. Experiments were performed at 25 °C in 50 mM sodium phosphate, pH 7, containing 50 mM NaCl and 10 mM dithiothreitol.

$$F_t = [(F_0 - F_\infty)\exp(-kt)] + F_\infty \quad (3)$$

$$F_t = [X_1 \exp(-k_1 t) + X_2 \exp(-k_2 t)] + F_\infty \quad (4)$$

Double-jump experiments

Double-jump experiments were performed as previously described [10]. The protein was first fully unfolded for 5–10 s in 4.5 M GdmCl and then immediately refolded by diluting out the denaturant. The refolding reaction was followed either by fluorescence spectroscopy or by measurement of recovery of enzymic activity. All solutions were supplemented with 10 mM dithiothreitol.

Assay of irreversible heat inactivation

The enzyme (0.1 mg/ml) was incubated at various temperatures in 50 mM sodium phosphate, pH 7. Samples, withdrawn after various periods of time, were subsequently left at room temperature for at least 1 h before the residual activity was determined with 1 mM benzylpenicillin as substrate. This 1 h incubation was sufficient to allow complete renaturation of any reversibly unfolded protein.

RESULTS

Enzymic activity measurements

The kinetic parameters of the C77S mutant were measured with various substrates. The k_{cat}/K_m values of the mutant enzyme for benzylpenicillin, cephalothin, cefazolin and cephalosporin C were not significantly different from those of the wild-type enzyme. By contrast, a 5-fold decrease and a 3-fold increase in the k_{cat}/K_m value were observed for nitrocefin and carbenicillin respectively.

Equilibrium unfolding experiments

GdmCl-induced unfolding curves obtained by fluorescence spectroscopy and CD are shown in Figure 1. These experiments support a three-state model ($N \leftrightarrow H \leftrightarrow U$) in which a partially unfolded state (H) is significantly populated at intermediate GdmCl concentrations [10].

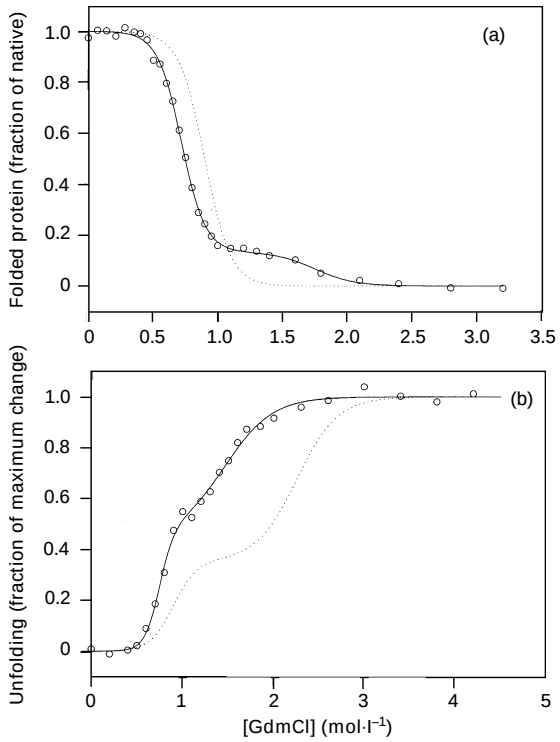

Figure 1 Equilibrium GdmCl-induced denaturation of the C77S mutant enzyme monitored by (a) fluorescence spectroscopy ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 340$ nm) and (b) CD at 220 nm

The broken lines are results for the wild-type enzyme [10]. The solid lines represent the theoretical curves corresponding to a three-state mechanism, and were obtained using the thermodynamic parameters given in Table 1. All studies were carried out at 25 °C in 50 mM sodium phosphate, pH 7, containing 50 mM NaCl and 10 mM dithiothreitol. Protein concentrations were 0.2 μM and 5 μM for the fluorescence and CD experiments respectively.

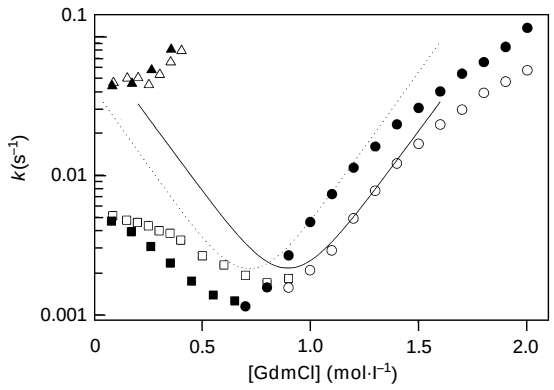

Figure 2 Apparent rate constants for the unfolding (○, ●) and refolding (□, ■, △, ▲) kinetics of the wild-type (○, □, △) and C77S mutant (●, ■, ▲) TEM-1 β-lactamase

Values for the wild-type protein are from [10]. The solid line (wild-type) and the broken line (mutant) represent the rate constants calculated on the basis of the equilibrium results and of the unfolding rates obtained between 1.1 and 1.3 M GdmCl (wild-type) or 0.9 and 1.1 M GdmCl (mutant), assuming a two-state model.

The thermodynamic parameters describing the equilibrium folding process of the C77S mutant (Table 1) were obtained from fitting of the experimental equilibrium unfolding curves (results not shown) using eqns. (1) and (2). Replacement of Cys-77 by Ser, with loss of the disulphide bond between residues 77 and 123, resulted in a 14.2 kJ/mol (3.4 kcal/mol) $[\Delta\Delta G_{H-N}(0) + \Delta\Delta G_{U-H}(0)]$ decrease in the global free energy of stabilization of the protein. Interestingly, both the mid-points of the transitions and the differences in free energy were less affected for the first transition (N ↔ H) than for the second one (H ↔ U). By contrast, no significant changes were observed for the m_{HN} and m_{UH} values.

Unfolding and refolding kinetics

Refolding of the C77S mutant was monitored by fluorescence spectroscopy and by regain of enzymic activity. Similarly to those for the wild-type enzyme [10], the refolding kinetics of the mutant were characterized by two major slow phases below 0.5 M GdmCl, and were fitted using eqn. (4) (see the Materials and methods section). At higher denaturant concentrations, the refolding kinetics became monoexponential, and the experimental traces were fitted to eqn. (3). Figure 2 shows the dependence of the apparent rate constants for unfolding and refolding on the GdmCl concentration for the mutant enzyme compared with those of the wild-type protein. Figure 2 also shows the 'V-shaped' curve representing the rate constants calculated according to a two-state model, as described by Matouschek et al. [18]. A divergence between the theoretical and experimental curves is evident, and clearly invalidates the two-state approximation at all GdmCl concentrations.

It was also shown previously, from double-jump experiments, that the two major slow refolding phases of the wild-type enzyme were rate-limited by *cis*/*trans* isomerization around Xaa–Pro bonds [10,11]. Under the same conditions, the C77S mutant exhibited a similar behaviour, the two slow refolding phases being sensitive to double-jump experiments (results not shown). This,

Table 1 Parameters characterizing equilibrium unfolding of wild-type TEM-1 β-lactamase and the C77S mutant

Cm represents the concentration of GdmCl at the transition mid-point, and was calculated from $Cm = \Delta G/m$. Values (means ± S.D.) were obtained from equilibrium unfolding monitored by fluorescence spectroscopy and fitting to eqn. (1) (*), and from equilibrium unfolding monitored by CD and fitting to eqn. (2) (**) (see the Materials and methods section). Note: 1 kcal = 4.18 kJ. Values for the wild-type protein are from [10].

	$\Delta G_{H-N}(0)$ (kJ/mol)*	m_{HN} (kJ/mol)*	Cm_{H-N} (M)*	$\Delta G_{U-H}(0)$ (kJ/mol)**	m_{UH} (kJ/mol)**	Cm_{U-H} (M)**
Wild-type	21.7 ± 1.7	24.2 ± 1.7	0.90 ± 0.02	23.8 ± 0.8	10.9 ± 0.4	2.24 ± 0.02
C77S	18.0 ± 1.7	25.1 ± 1.7	0.71 ± 0.02	13.4 ± 0.4	9.6 ± 0.4	1.41 ± 0.06

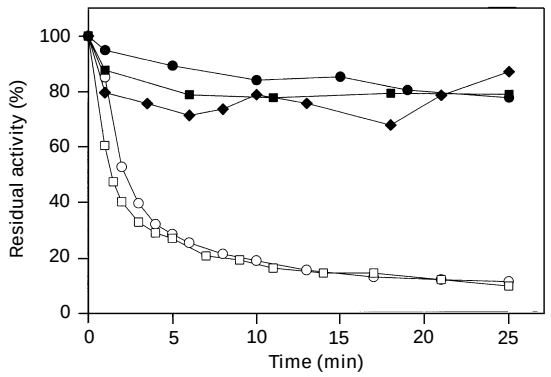

Figure 3 Heat-induced inactivation of the wild-type TEM-1 β-lactamase and the C77S mutant

Wild-type: ●, 55 °C; ■, 75 °C; ◆, 90 °C; mutant: ○, 55 °C; □, 60 °C. The enzyme concentration was 0.1 mg/ml. Experiments were performed in 50 mM sodium phosphate, pH 7. S.D.s were within 10% of the experimental values.

together with the unfolding/refolding kinetics, suggests a key role for the same proline residues in the refolding of the two enzymes.

Finally, the refolding kinetics of both the wild-type and mutant enzymes as assessed by fluorescence spectroscopy indicated a significant recovery in the intrinsic fluorescence during the dead-time of manual mixing. This phase accounted for about 55% of the total enzyme fluorescence, and suggested the rapid formation of a highly ordered intermediate, referred to as state I [10]. Preliminary stopped-flow experiments indicated that this phenomenon was not extremely rapid, but occurred with rate constants of approx 0.1–0.2 s^{-1}, which were not significantly different for the wild-type and mutant proteins.

Irreversible heat inactivation

The results of the heat-inactivation experiments for the wild-type TEM-1 β-lactamase and the C77S mutant are presented in Figure 3. The wild-type enzyme was found to be extremely resistant to inactivation. It retained 80–90% of its activity after a 25 min incubation at 55, 75 and even 90 °C. However, even at 55 °C the enzyme was unfolded (the melting temperature for the wild-type enzyme is 50.1 °C [19]), emphasizing the high degree of reversibility of thermal unfolding. By contrast, the mutant protein was inactivated to a large extent within a few minutes at 55 °C. The inactivation of the mutant enzyme was not due to the presence of proteases, as shown by the fact that the wild-type enzyme retained 80–90% of its activity after a 25 min incubation at 60 °C in the presence of an equimolar amount of the mutant enzyme (results not shown).

DISCUSSION

Effect of the disulphide bond on the stability of TEM-1 β-lactamase

The equilibrium unfolding transitions of the C77S mutant indicate that the disulphide bond makes a significant contribution to the intrinsic stability of the protein. The effect of disulphide bonds on the stability of proteins has been attributed to an increase of entropy of the unfolded state [20,21]. The magnitude of the stabilization (S) would depend on the number of residues (n) in the loop that is cross-linked by the disulphide, and can be estimated from eqn. (6) [22]:

$$S = -2.1 - (3/2)\boldsymbol{R}\ln n \quad (6)$$

This approach gives a decrease of 14.6 kJ/mol (3.5 kcal/mol) in the free energy of stabilization of the C77S mutant enzyme at 25 °C, in good agreement with the experimental results. However, the main components of the stabilizing effect of disulphide bonds have also been suggested to be enthalpic [23], and the complexity of the thermodynamics associated with solvation and restriction of internal rotation and vibration, and their mutual compensation, render a definitive interpretation difficult.

As for the wild-type [10] and reduced [24] enzymes, the GdmCl-induced equilibrium unfolding of the C77S mutant occurred in two discrete steps, indicating the existence of a thermodynamically stable intermediate state, referred to as state H. State H exhibits the properties of a molten globule: substantial secondary structure content but very few specific tertiary interactions. For the C77S mutant, both the N↔H and H↔U transitions were observed by fluorescence spectroscopy, while only the N↔H transition was observed for the wild-type enzyme using this probe. This, however, is due to the fluorescence spectra for the wild-type fully unfolded (U) and partially unfolded (H) states being closely similar [10], unlike the case with the mutant enzyme (results not shown). Thus this difference does not reflect a fundamentally modified behaviour of the mutant enzyme.

The N↔H transition was less affected by the mutation than that between H and U. The latter transition was shifted to a lower concentration of denaturant by more than 0.8 M, and the difference in free energy between states H and U accounted for about 75% of the global free energy change. Although interpretation must remain tentative because of the reasons given above, this could be explained assuming that the entropy of the fully unfolded polypeptide is much more influenced than that of state H by the removal of the disulphide bond. By contrast, the slopes of the transitions were very similar, suggesting that no major change occurs in the degree of exposure of non-polar residues to solvent in the unfolded state on loss of the disulphide.

The free-energy values obtained here are lower than those measured by Zahn et al. [24], the difference being best accounted for by the stabilizing effect of the phosphate and sulphate ions included in their buffer [25]. Walker and Gilbert [13] estimated the free-energy difference between the native state and the state in which the thiols of the reduced enzyme are accessible to alkylation and oxidation to lie between 10.0 and 15.5 kJ/mol (2.4 and 3.7 kcal/mol). Using the results of Zahn et al. [24], they concluded that this state is more stable, and hence presumably more tightly packed, than state H. The new values reported here are still sufficiently large to support this conclusion.

Finally, with respect to the relative importance of disulphide bridges and other factors implicated in stabilizing proteins, it is interesting to remember that the *Bacillus licheniformis* β-lactamase, which is devoid of disulphide bridges and exhibits only 37% identity with the TEM enzyme, is significantly more stable than the latter [19].

Effect of the disulphide bond on the unfolding and refolding reactions

The kinetic studies of the reversible unfolding/refolding were performed to explore the role of the single disulphide bond in the mechanism of folding of the oxidized TEM-1 β-lactamase. The behaviour of the wild-type and mutant enzymes appeared to be similar. The refolding kinetics of both enzymes monitored by fluorescence spectroscopy indicated the appearance during the dead-time of manual mixing of a highly ordered intermediate previously referred to as state I [10].

Below 0.5 M GdmCl, the final refolding steps of the two

enzymes are dominated by two major slow phases. These originate from proline isomerization, as indicated by the double-jump experiments. Recent analyses suggest a key role for Pro-167 in the case of the wild-type enzyme [11].

In some proteins, disulphide bonds have marked effects on the folding kinetics [26,27]. In the present case, however, the apparent rate constants for the rate-limiting steps of the unfolding and refolding reactions (Figure 2) were not strongly influenced by the mutation, suggesting that the region around the disulphide bond is already folded in a native-like way before the final folding events take place.

The processes of collapse and secondary structure formation occur on the millisecond time-scale during protein folding [28]. Compared with these rates, the kinetics of the initial phase of fluorescence change, reflecting the decrease in solvent accessibility on forming state I, are rather slow (in the range 0.1–0.2 s^{-1}), so that it can be concluded that no significant burial of the tryptophan side chains occurs during the first few milliseconds. This is consistent with the tryptophans being located near the surface of the enzyme, with three of them (out of four) still being partly accessible to solvent in the native state [8,9]. The kinetics of folding of the reduced enzyme observed by Walker and Gilbert [13], based on decreasing accessibility to *N*-ethylmaleimide, showed a slow phase or phases with a rate constant in the region of 3×10^{-2} s^{-1}. Approximately half the burial of the thiols occurred, however, within the dead-time of the measurements. Comparing these results with those observed here for the fluorescence changes, it is reasonable to conclude that a significant proportion of the unfolded molecules can collapse rapidly to a state in which the thiols are buried but where no significant change in the tryptophan environment has occurred. The slow folding kinetics of the TEM β-lactamase have been shown to be limited by peptide bond isomerization [10]. It is interesting to speculate that the fast and slower folding conformers governing thiol burial are likewise defined by peptide bond isomerization, in this case at a much earlier stage of folding.

Effect of the disulphide bond on the heat-inactivation process

One of the more surprising consequences of the removal of the disulphide bond in the TEM-1 enzyme was the dramatically increased sensitivity to thermal inactivation. It was verified that proteases were not responsible for inactivation. These results are consistent with a physiological role for the disulphide bond which, due to its stabilizing effect, protects against reactions leading to inactivation, probably as a result of irreversible aggregation of partially unfolded states.

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