Denaturing action of urea and guanidine hydrochloride towards two thermophilic esterases

Pompea DEL VECCHIO^{*1}, Giuseppe GRAZIANO[†], Vincenzo GRANATA^{*}, Guido BARONE^{*}, Luigi MANDRICH[‡], Mosè ROSSI[‡] and Giuseppe MANCO[‡]

^{*}Department of Chemistry, University of Naples Federico II, Via Cintia, 45-80126 Naples, Italy, [†]Faculty of Science, University of Sannio, Via Port'Arsa, 11-82100 Benevento, Italy, and [‡]Institute of Protein Biochemistry, CNR, Via P. Castellino-80131 Naples, Italy

The stability of two thermophilic esterases, AFEST from *Archaeoglobus fulgidus* and EST2 from *Alicyclobacillus acidocaldarius*, against the denaturing action of urea and guanidine hydrochloride has been investigated by means of steady-state fluorescence and circular dichroism measurements. Experimental results indicate that the two enzymes, even though very resistant to temperature and urea, show a resistance to guanidine hydrochloride weaker than expected on the basis of data collected so far for a large set of globular proteins. Structural information

available for AFEST and EST2 and ideas that emerged from studies on the molecular origin of the greater thermal stability of thermophiles allow the suggestion of a reliable rationale. The present results may be an indication that the optimization of charge–charge interactions on the protein surface is a key factor for the stability of the two esterases.

Key words: chemical denaturant, electrostatic interactions, fluorescence measurement, thermophilic esterase.

INTRODUCTION

In the last few years, a great deal of work has been devoted to identify the interactions that determine the stability of the native structure of proteins extracted from thermophiles and hyperthermophiles at temperatures close to, or above, the boiling point of water [1]. The task is important both to resolve the protein folding problem and to increase the thermal stability of enzymes for biotechnological applications. Jaenicke [2] suggested that a principle of ‘corresponding states’ should be operative for homologous proteins extracted from organisms that have adapted to survive in different environmental conditions. In particular, the combination of thermodynamic stability and flexibility of a thermophilic protein at the ‘high’ optimum growth temperature (T_{opt}) of the thermophile has to be similar to the combination of thermodynamic stability and flexibility of the mesophilic counterpart at the ‘low’ T_{opt} of the mesophile. Since the folded structure is characterized by a marginal stability with respect to the unfolded conformations [3], the validity of the principle of corresponding states implies that several small favourable contributions may significantly enhance the stability against temperature.

It is a hard challenge, however, to identify such small contributions in the delicate and intricate balance of enthalpic and entropic factors to the stabilization Gibbs energy. The comparison of the amino acid sequences and the three-dimensional structures of several thermophilic proteins with those of their mesophilic counterparts has led to some suggestions on which interactions should account for the greater stability against temperature of the former [4–7]. However, more extensive experimental investigations are needed to reach definitive conclusions. The present work is a contribution in such direction.

AFEST [8] and EST2 [9] are two esterases extracted from the hyperthermophile *Archaeoglobus fulgidus* ($T_{opt} = 83^{\circ}\text{C}$), and the thermophile *Alicyclobacillus acidocaldarius* ($T_{opt} = 65^{\circ}\text{C}$) respectively. The two proteins are monomeric (311 residues for AFEST and 310 for EST2) with 42 % of sequence identity. They belong to the hormone-sensitive lipase group of the esterase/lipase super-family (H-group). The three-dimensional structures of AFEST and EST2 have been solved by X-ray crystallography at 2.2 Å and 2.6 Å (1 Å = 0.1 nm) resolution respectively [10,11]. Both proteins possess the α/β -hydrolase fold, with a central β -sheet consisting of eight strands, characteristic of several lipases and esterases. The two three-dimensional structures are very similar exhibiting a root-mean-square-deviation of 2.2 Å over 297 corresponding C α atoms [10]. The classic catalytic triad is constituted by Ser¹⁶⁰, Asp²⁵⁵ and His²⁸⁵ in AFEST [10,12], and Ser¹⁵⁵, Asp²⁵² and His²⁸² in EST2 [11,13].

AFEST and EST2 proved to be a good system to perform a careful comparative investigation of their stability against temperature, urea and guanidine hydrochloride (GuHCl) [14]. In our previous work [14], by performing CD measurements in the far-UV region at pH 7.5, we determined that the denaturation temperature of AFEST is 99 °C and that of EST2 is 91 °C. In addition, the two esterases proved to be very resistant to urea, but only weakly resistant to GuHCl. These results suggested that the interactions rendering AFEST and EST2 thermostable are not so effective in circumventing the GuHCl action. A further investigation of the stability of the two esterases against urea and GuHCl, using a different experimental technique, should be useful to confirm such evidence.

CD measurements in the far-UV region, detecting changes in the secondary structure, and steady-state fluorescence measurements, detecting changes in the tertiary structure, are com-

Abbreviations used: AFEST, esterase from *Archaeoglobus fulgidus*; ASA, accessible surface area; DBM, denaturant binding model; EST2, esterase from *Alicyclobacillus acidocaldarius*; GuHCl, guanidine hydrochloride; H-group, hormone-sensitive lipase group of the esterase/lipase super-family; LEM, linear extrapolation model; T_{opt} , optimum growth temperature.

¹ To whom correspondence should be addressed (e-mail delvecchio@chemistry.unina.it).

plementary tools to investigate the conformational stability of globular proteins. Thus, in the present work, urea- and GuHCl-induced unfolding curves of AFEST and EST2 have been determined by means of steady-state fluorescence measurements at a constant temperature of 20 °C. By combining CD and fluorescence experimental data with available structural information, we conclude that the weak resistance of the two thermophilic esterases against GuHCl is because electrostatic interactions among surface charged groups play a fundamental role for the tolerance to high temperature of both AFEST and EST2.

EXPERIMENTAL

Protein preparation

AFEST and EST2 were overexpressed in *Escherichia coli* and purified as previously reported [8,9]. SDS/PAGE and reversed-phase HPLC were used to check the purity of homogeneous preparations. An Amicon ultrafiltration apparatus was used to concentrate protein samples after exhaustive dialysis. Protein concentration was measured spectrophotometrically using $\epsilon = 39000$ and $43300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 278 nm for AFEST and EST2, respectively, calculated from absorption of tyrosine and tryptophan residues [15].

Sample solutions

A buffer solution, consisting of 20 mM sodium phosphate at pH 7.5, was used in all denaturation experiments. Chemical denaturants, urea and GuHCl were from Sigma (Sigma Chemicals, St. Louis, MO, U.S.A.). Urea was used after recrystallization from ethanol/water (1:1) mixtures; urea solutions were prepared immediately before use and the concentration was determined by refractive index measurements [16]. A commercial 8 M solution was used for GuHCl. Protein solutions were exhaustively dialysed, using Spectra Por MW 15000–17000 membranes, against buffer solution at 4 °C. The water used for buffer and sample solutions was double-distilled. The pH was measured at 25 °C with a Radiometer pH meter model PHM93. Stock solutions of urea and GuHCl, in different amounts, were mixed with protein solution to give constant, fixed final protein concentration. The final concentrations ranged from 0 to 9 M for urea, and from 0 to 6 M for GuHCl. Since high urea or GuHCl concentrations change the pH, the final pH for each sample was corrected by addition of concentrated solutions of HCl or NaOH. Each sample was mixed by vortexing and was incubated overnight at 4 °C. Longer incubation times produced identical spectroscopic signals.

Fluorescence measurements

Steady-state fluorescence measurements were conducted with a Jasco FP-750 spectrofluorimeter equipped with thermostat-controlled cell holders and the temperature was kept constant using a circulating water bath. The protein concentration ranged from 0.01 to 0.02 mg · ml⁻¹. The excitation wavelength was set at 280 nm in order to include the contribution of tyrosine residues to the overall fluorescence emission. The experiments were performed at 20 °C by using a 1 cm sealed cell and a 5 nm emission slit width, and corrected for background signal. Both the change in fluorescence intensity and the shift in fluorescence maximum wavelength were recorded to monitor the unfolding transition.

CD spectroscopy

CD spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier type temperature control system (PTC-348WI model). The instrument was calibrated with an aqueous solution of d-(+)-10-camphorsulphonic acid at 290 nm [17]. Molar ellipticity per mean residue, $[\theta]$ in deg · cm² · dmol⁻¹, was calculated from the equation $[\theta] = [\theta]_{\text{obs}} \cdot \text{mrw}/10 \cdot l \cdot C$, where $[\theta]_{\text{obs}}$ is the ellipticity measured in degrees, mrw is the mean residue molecular mass, 113 Da, C is protein concentration in g · l⁻¹ and l is the optical path length of the cell in cm. Cuvettes of 0.2 and 1 cm path length and protein concentrations of approx. 0.1 and 1 mg · ml⁻¹ were used in the far-UV and near-UV regions, respectively. CD spectra were recorded at 20 °C with a time constant of 4 s, a 2 nm band width and a scan rate of 5 nm · min⁻¹, were signal-averaged over at least five scans, and baseline was corrected by subtracting the reference spectrum.

RESULTS

The conformational stability of AFEST and EST2 against the denaturing action of urea and GuHCl has been investigated by means of steady-state fluorescence measurements at pH 7.5, 20 mM phosphate buffer and a temperature of 20 °C (i.e., the two esterases show the maximal enzymatic activity in the selected experimental conditions [8,9]). Both enzymes possess several fluorophore residues. In particular there are three tryptophan residues in AFEST and four tryptophan residues in EST2, and 16 tyrosine residues in both esterases. Fluorescence measurements were performed by using a 280 nm excitation wavelength in order to detect the contribution of both tyrosine and tryptophan

Figure 1 Fluorescence emission spectra of native AFEST at pH 7.5 (■), and of unfolded AFEST in 8 M urea (●) and 6 M GuHCl (▲)

Figure 2 Urea-induced equilibrium unfolding curves of EST2 at pH 7.5 and 20 °C obtained by recording the change of the fluorescence intensity at 337 nm ($\square$), and the shift in the wavelength corresponding to the maximum of the fluorescence spectrum ($\bullet$)

residues. This choice should be advantageous in having fluorescence signals that reflect global conformational changes of the tertiary structure, rather than local modifications, because AFEST and EST2 are two sufficiently large proteins. Measurements were performed after an overnight incubation of the samples at 4 °C (longer incubation times gave rise to identical results).

The fluorescence emission spectrum of AFEST shows a maximum at 335 nm that, when the tertiary structure is completely unfolded, shifts to 347 nm in 9 M urea and to 348 nm in 6 M GuHCl (see Figure 1); that of EST2 shows a maximum at 337 nm that shifts to 350 nm when the protein is completely unfolded. Upon increasing the concentration of both denaturants the signal intensity increases in the case of AFEST, but decreases in the case of EST2. It is well known that fluorescence spectra of proteins with a maximum around 335 nm are characteristic of tryptophan residues well buried in the hydrophobic core, whereas fluorescence spectra with a maximum around 350 nm are characteristic of tryptophan residues exposed to the aqueous solvent [18]. Therefore, both AFEST and EST2 have the tryptophan side chains buried in the native structure, but well exposed to the aqueous solvent in the denaturant-induced unfolded state.

In the selected experimental conditions, the denaturation induced by urea or GuHCl is a reversible process for both AFEST and EST2. Fully denatured samples, after exhaustive dialysis to eliminate urea or GuHCl, show fluorescence spectra equal to those of native proteins, and give denaturant-induced transition curves superimposable with those of freshly prepared samples.

EST2 shows sigmoidal transition curves recording both the change in fluorescence intensity at 337 nm I_{337} , and the shift in the wavelength corresponding to the maximum of fluorescence

Figure 3 GuHCl-induced equilibrium unfolding curves of EST2 at pH 7.5 and 20 °C obtained by recording the change of the fluorescence intensity at 337 nm ($\square$), and the shift in the wavelength corresponding to the maximum of the fluorescence spectrum ($\bullet$)

Table 1 Values of the urea and GuHCl concentration at half-completion of the unfolding transition for both AFEST and EST2, determined by means of CD spectroscopic [14] and fluorescence measurements at pH 7.5 and 20 °C

Three independent measurements were performed for each experimental condition. The reported numbers are the mean values from such measurements; the uncertainties are approx. 5% of the reported numbers.

Esterase	Probe	[Urea] _{1/2} (M)	[GuHCl] _{1/2} (M)
AFEST	$[\theta]_{222}$	7.1	2.0
	$\lambda_{\max}$	7.1	2.1
EST2	$[\theta]_{222}$	5.9	1.9
	I_{337}	5.9	1.9
	$\lambda_{\max}$	5.8	2.0

spectrum, $\lambda_{\max}$. The urea-induced transition curves are reported in Figure 2; the GuHCl-induced transition curves are shown in Figure 3. The values of the denaturant concentration at half-completion of the transition, indicated as $[\text{den}]_{1/2}$, proved to be: (i) $[\text{urea}]_{1/2} = 5.9$ M from I_{337} and 5.8 M from $\lambda_{\max}$ and (ii) $[\text{GuHCl}]_{1/2} = 1.9$ M from I_{337} and 2.0 M from $\lambda_{\max}$. Thus both fluorescence signals afford very similar values of $[\text{den}]_{1/2}$. Moreover, the latter agree with those previously determined by recording the molar ellipticity at 222 nm in CD measurements [14]: $[\text{urea}]_{1/2} = 5.9$ M and $[\text{GuHCl}]_{1/2} = 1.9$ M (see Table 1). Therefore the transition curves constructed with three different experimental probes, by monitoring the conformational changes in the secondary and tertiary structures respectively, afford the same $[\text{den}]_{1/2}$ value for urea and GuHCl respectively. On this basis, it is reasonable to conclude that the denaturant-induced unfolding of EST2 is a reversible two-state $N \leftrightarrow D$ process

Figure 4 Change of the fluorescence intensity at 335 nm for AFEST at increasing the concentration of urea: there is no evidence of a sigmoidal transition

Figure 5 Urea- (■) and GuHCl-induced (●) equilibrium unfolding curves of AFEST at pH 7.5 and 20 °C obtained by recording the shift in the wavelength corresponding to the maximum of the fluorescence spectrum

(where 'N' and 'D' represent 'native' and 'denatured' respectively).

In the case of AFEST, no sigmoidal transition curves have been obtained by recording the change in the fluorescence intensity at 335 nm, I_{335} . As an example, the experimental results of I_{335} versus urea concentration are reported in Figure 4. Nevertheless, AFEST shows sigmoidal transition curves by recording $\lambda_{\max}$ versus the concentration of both denaturing agents, as shown in Figure 5. The values of $[\text{den}]_{1/2}$ proved to be $[\text{urea}]_{1/2} = 7.1$ M and $[\text{GuHCl}]_{1/2} = 2.1$ M; these values are in agreement with those determined in the previous study [14] by recording the molar ellipticity at 222 nm in CD measurements: $[\text{urea}]_{1/2} = 7.1$ M and $[\text{GuHCl}]_{1/2} = 2.0$ M (see Table 1). This indicates that also the denaturant-induced unfolding of AFEST is a reversible two-state $N \rightleftharpoons D$ process.

The finding that the fluorescence intensity decreases for EST2 but increases for AFEST on increasing the concentration of both denaturants may appear strange in view of the strict similarity of the three-dimensional structures of the two esterases. The far-UV CD spectra of the two esterases in 20 mM phosphate buffer, pH 7.5, and at a temperature of 20 °C (Figure 6a) indicate that the content of secondary structure is very similar (i.e. the average values obtained using different methods of estimating protein secondary structure from the spectra of both proteins are 40 % of α -helix and 20 % of β -sheet). However, the near-UV CD spectra of the two proteins under the same experimental conditions (Figure 6b) are significantly different. This means that the aromatic side chains are located in different positions in the three-dimensional structures; the exact location can be easily verified using the coordinate files deposited in the Protein Data Bank (accession code 1jji for AFEST and 1evq for EST2). Specifically, tryptophan residues occupy positions 139, 208 and

217 in the sequence of AFEST, and positions 85, 134, 213 and 229 in the sequence of EST2. The sequence structure alignment of the two proteins demonstrates that there is correspondence solely between Trp¹³⁹ and Trp²¹⁷ of AFEST and Trp¹³⁴ and Trp²¹³ of EST2 [12]. Moreover, it reveals that in AFEST only two tryptophan residues have close-by tyrosine residues (i.e. Tyr¹³⁴ for Trp¹³⁹ and Tyr²²² for Trp²¹⁷), whereas in EST2 all four tryptophan residues have proximal tyrosine residues (i.e. Tyr⁷⁹ and Tyr⁸⁰ for Trp⁸⁵, Tyr¹²⁹ for Trp¹³⁴, Tyr²¹⁸ for Trp²¹³, and Tyr²²⁵ for Trp²²⁹). Therefore it should not be surprising that the behaviour of the fluorescence intensity signal could be different for the two proteins in response to the perturbation induced by urea and GuHCl.

In the previous work [14], we analysed the urea- and GuHCl-induced transition curves of AFEST and EST2 by means of both the linear extrapolation model, LEM, and the denaturant binding model, DBM [16]. However, both methods failed to provide reliable and consistent values of the stabilization Gibbs energy in the absence of denaturant, the desired estimate of protein stability. This finding is due to the fact that such models are too crude to account fully for the complex action of urea and GuHCl, involving both a direct interaction with the protein surface and a modification of the solvent properties, as discussed below. As a consequence, in the present work, we preferred not to analyse the urea- and GuHCl-induced transition curves by means of LEM and DBM, and to provide only the values of $[\text{den}]_{1/2}$. The latter, being determined directly from the experimental data, have to be considered a reliable operational measure of protein stability.

Since $[\text{urea}]_{1/2} = 7.1$ M for AFEST and 5.8 M for EST2, the latter protein is less stable than the former with respect to the denaturing action of urea. On the other hand, since

Figure 6 Far-UV CD spectra (a), and near-UV CD spectra (b) of AFEST (●) and EST2 (■) recorded at pH 7.5 and 20 °C

$[\text{GuHCl}]_{1/2} = 2.1 \text{ M}$ for AFEST and 1.9 M for EST2, the former protein is only slightly more stable than the latter with respect to the denaturing action of GuHCl. Since AFEST is extracted from a hyperthermophilic micro-organism and EST2 is extracted from a thermophilic one, the expectation is that the first protein would be more stable than the second. In fact, at pH 7.5, the denaturation temperature of AFEST is 99 °C and that of EST2 is 91 °C [14]. Therefore, one can state that some stabilizing interactions are operative in the presence of urea so that AFEST proves to be more stable than EST2, but non-operative in the presence of GuHCl, so that the two esterases prove to have almost the same stability.

DISCUSSION

The fundamental result of the present study is the finding that both CD and fluorescence transition curves indicate that

$[\text{urea}]_{1/2} \cong 3 [\text{GuHCl}]_{1/2}$ for both AFEST and EST2. This means that GuHCl has a denaturing activity against these enzymes that is stronger than expected. In fact, the comprehensive analysis of literature performed by Pace and colleagues [19] pointed out that the relationship $[\text{urea}]_{1/2} \cong 2 [\text{GuHCl}]_{1/2}$ is generally valid for mesophilic globular proteins. In other words, GuHCl is usually twice as strong as urea in denaturing globular proteins, whereas in the case of the two thermophilic esterases, it is 3-fold stronger than urea. We suggest that this quantitative difference is not coincidental, but it may be a diagnostic tool in providing useful indications on the interactions playing a key role for the conformational stability of AFEST and EST2 against temperature.

In fact, Brunori and co-workers [20] found $[\text{urea}]_{1/2} = 6.4 \text{ M}$ and $[\text{GuHCl}]_{1/2} = 1.8 \text{ M}$, for cytochrome c_{551} and demonstrated that such a result is because the presence of GuHCl masks the stabilizing contribution of the Glu⁷⁰–Lys¹⁰ salt-bridge. Moreover, Hodges and co-workers [21] showed that, when the charge–charge interactions in designed two-stranded coiled-coils were changed stepwise from 20 attractions to 20 repulsions, the $[\text{urea}]_{1/2}$ values showed a proportional decrease, whereas the $[\text{GuHCl}]_{1/2}$ values were almost unaffected. Since the melting temperatures of the coiled-coils were consistent with the $[\text{urea}]_{1/2}$ values, it is clear that the contribution of electrostatic interactions is masked by the presence of GuHCl.

After the pioneering proposal by Perutz and Raidt [22,23], the pivotal role of electrostatic interactions in rendering the folded conformation of globular proteins more stable against temperature is emerging from several different studies [7,24–29]. In particular, experimental investigations, exploiting site-directed mutagenesis, have revealed that the optimization of charge–charge interactions on the protein surface is an effective strategy to enhance the thermal stability of globular proteins [30–32].

The two thermophilic esterases are acid proteins highly charged at neutral pH: AFEST has 46 acid and 34 basic residues and EST2 has 41 acid and 28 basic residues. Analysis of their three-dimensional structures [10] reveals the presence of 24 ion pairs in EST2 and 26 ion pairs in AFEST, using a cut-off distance of 4 Å, and 39 ion pairs in EST2 and 42 ion pairs in AFEST, using a cut-off distance of 6 Å (the 4 Å cut-off distance is that usually adopted to define the existence of an ion-pair; the 6 Å cut-off distance is used to take into account weaker electrostatic interactions). These values, when normalized to the number of residues in each protein, prove to be larger than those determined for brefeldin A [10], an esterase isolated from *Bacillus subtilis*, that can be considered the mesophilic counterpart of AFEST and EST2. Furthermore, the ratio between the accessible surface area (ASA) of charged and polar residues and the ASA of non-polar residues is 2.05 for EST2 and 2.48 for AFEST, whereas it is 1.55 for brefeldin A [10]. Such numbers indicate that there is a larger exposure of charged groups in the two thermophilic esterases with respect to the mesophilic analogue. Therefore the optimization of electrostatic interactions on the protein surface appears to have a pivotal role for the thermal stability of AFEST and EST2.

We would like to show that this hypothesis can be helpful to rationalize the results of urea- and GuHCl-induced denaturation. The molecular mechanisms of the denaturing action by urea and GuHCl have not yet been clarified in detail [33,34]. Two fundamental but common features of the two denaturants are the following: (a) both urea and GuHCl interact preferentially with the backbone CONH groups and other polar groups in the side chains by forming multiple H-bonds [35–37], leading to the salting in of such groups in the aqueous solution; and (b) the addition of either urea or GuHCl to water causes an increase of

the surface tension [38], leading to salting out of non-polar groups from the aqueous phase. (Actually, the van der Waals energetic interactions of non-polar groups with urea and GuHCl are stronger than those with water, but are more than counter-balanced, at least for small aliphatic side chains [36], by the difference in the work of cavity creation that is larger in both urea-rich and GuHCl-rich aqueous solutions than in pure water. In this respect, the direct proportionality between the work of cavity creation in a liquid and its surface tension is well established [39]. In case of polar groups, the formation of multiple H-bonds with both urea and GuHCl dominates all other contributions). The first action is strongly destabilizing, while the second one stabilizes the folded conformation. However, neither of them allows the discrimination between the effectiveness of urea and that of GuHCl, and one has to consider other features.

Urea and the GuH^+ ion are very similar from the structural point of view, but the latter has a positive charge delocalized over the planar structure. The difference in the ionic character should be the relevant factor [20,21]. It is safe to assume that GuH^+ , being preferentially adsorbed on the protein surface due to its preferential interaction with H-bonding groups [36,37,40], can perturb and weaken the optimized electrostatic interactions between charged side chains. Clearly, such a mechanism is able to discriminate between urea and GuHCl, and to account for the stronger denaturing ability of the latter. In addition, it allows the rationalization of the weaker than expected resistance of the two thermophilic esterases against GuHCl, in the hypothesis that the optimization of charge-charge interactions is a main determinant of the high thermal stability for both AFEST and EST2.

It is worth noting that we tried to mimic the masking effect of GuHCl by recording urea-induced transition curves of both esterases in the presence of NaCl (results not shown). The values of $[\text{urea}]_{1/2}$ in the presence of 1 M NaCl proved to be slightly larger than those obtained in the absence of salt (i.e. there is a slight stabilization of both esterases). These results demonstrate that NaCl is not able to perturb the optimization of electrostatic interactions among charged side chains because sodium and chloride ions are not preferentially adsorbed on the protein surface.

In conclusion, experimental data indicate that AFEST and EST2, two thermophilic esterases, are very resistant to urea but weakly resistant to GuHCl. Even though it is widely accepted that virtually all proteins are more susceptible to GuHCl than to urea, we find a significant quantitative difference. In the case of AFEST and EST2, GuHCl is 3-fold stronger than urea, whereas, on the average, GuHCl is expected to be twice as strong as urea in denaturing globular proteins. This quantitative difference can be rationalized by referring to the pivotal role of electrostatic interactions in increasing the thermal stability of globular proteins, because their favourable contributions are suppressed by the ionic nature of GuHCl and its preferential adsorption on the protein surface. Since electrostatic interactions seem fundamental to both cope with and resist high temperatures, we suggest that the above result (i.e. $[\text{urea}]_{1/2}/[\text{GuHCl}]_{1/2} > 2$) should be a common feature of thermophilic proteins.

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