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Effect of trifluoroethanol on the conformational stability of a hyperthermophilic esterase: a CD study

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Abstract

The conformational stability of the hyperthermophilic esterase AFEST from *Archeoglobus fulgidus* against the denaturing action of 2,2,2-trifluoroethanol (TFE) has been investigated by means of circular dichroism (CD) measurements. At room temperature far-UV and near-UV CD spectra point out the occurrence of a co-operative transition from the native structure to a denatured state characterized by a high content of α-helix. The TFE concentration at half-completion of the transition proves to be 3.5 M (25% v v^{-1}), by recording the molar ellipticity at both 222 and 276 nm. Thermal transition curves of AFEST in the absence and in the presence of TFE indicate a significant stability decrease on increasing the TFE concentration. The denaturation temperature is 99 °C for native AFEST, but becomes 85 °C at 1.4 M TFE (10% v v^{-1}), and 56 °C at 2.8 M TFE (20% v v^{-1}). It is also shown that, even though AFEST is very resistant to temperature, its resistance towards the denaturing action of TFE is similar to that of mesophilic proteins, including an esterase from *Escherichia coli*, AES. The proposal of a general mechanism for the TFE action on globular proteins leads to a reliable rationale of experimental data.

Keywords: Hyperthermophilic esterase; Conformational stability; TFE; Hydrophobic interactions

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Abbreviations: AFEST: esterase from *Archeoglobus fulgidus*; EST2: esterase from *Alicyclobacillus acidocaldarius*; AES: esterase from *Escherichia coli*; brefeldin A: esterase from *Bacillus subtilis*; H-group: hormone sensitive lipase-like group of the esterase/lipase super-family; TFE: 2,2,2-trifluoroethanol; CD: *circular dichroism*; GuHCl: *guanidine hydrochloride*; HPLC: high performance liquid chromatography; SDS–PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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1. Introduction

AFEST is a monomeric enzyme consisting of 311 residues, extracted from the hyperthermophilic micro-organism *Archeoglobus fulgidus*, whose optimal growth temperature is 83 °C [1]. It belongs to the H-group (hormone-sensitive lipase-like) of the esterase/lipase super-family [2]. The three-dimensional structure of AFEST has been solved by X-ray crystallography at 2.2-Å resolution [3]. The protein possesses the α/β hydrolase fold, with a central β-sheet consisting of eight strands flanked by several α-helical segments, characteristic of several lipases and esterases. The classic catalytic triad is constituted by Ser160, Asp255 and His285 [3,4], and, in line with its hyperthermophilic origin, AFEST has an optimal temperature for enzymatic activity of 80 °C [1].

In previous studies [5,6], we used CD and fluorescence measurements to investigate the stability of AFEST against temperature, urea and guanidine hydrochloride, GuHCl. It resulted that, at pH 7.5, the denaturation temperature is 99 °C, while the values of the denaturant concentration at half-completion of the transition are: 7.1 M urea and 2.0 M GuHCl. We concluded that AFEST is extra-stabilized against temperature by interactions fully active also in the presence of urea, but not in the presence of GuHCl. These findings suggested that the extra-resistance of native structure towards the denaturing action of temperature does not necessarily imply an extra-resistance towards chemical denaturants. To shed further light on this topic we have investigated the effect of TFE on the conformational stability of AFEST by means of CD measurements.

Even though TFE has been extensively used to stabilize the α-helical conformation in peptides and protein fragments [7,8], several studies have pointed out that it has a strong denaturing activity against the native structure of globular proteins [9]. At the best of our knowledge only one thermophilic enzyme, thermolysin, has been partially characterized in TFE-rich aqueous solution. Specifically, Fontana and co-workers [10] showed that the native structure of thermolysin is not affected by 50% v v^{-1} TFE at neutral pH, so that it can be successfully used in limited proteolysis studies. This evidence suggested that thermophilic enzymes could be TFE-resistant and prompted us to test this idea in the case of AFEST.

Experimental measurements indicate that this hyperthermophilic esterase is not so resistant to the denaturing action of TFE: at approximately 4.2 M TFE (30% v v^{-1}), the native tertiary structure is entirely lost at room temperature, leading to a non-compact helical state. Also the thermal stability of AFEST decreases significantly with TFE. The data are interpreted by considering that the TFE action is mainly due to its ability in weakening hydrophobic interactions.

2. Experimental

2.1. Protein purification and sample solutions

AFEST and AES were overexpressed in *E. coli* and purified as previously described [1,11]. The purity of homogeneous preparations was checked by SDS–PAGE and reversed-phase HPLC. Protein samples were dialyzed against appropriate buffers and concentrated by using an Amicon ultrafiltration apparatus for the following analyses. The enzymes were dissolved in a buffer solution, consisting of 20 mM sodium phosphate at pH 7.5, and the concentration determined spectrophotometrically using an extinction coefficient of 39 000 M^{-1} cm^{-1} at 278 nm, and 53 200 M^{-1} cm^{-1} at 280 nm for AFEST and AES, respectively, calculated from the amino acid sequence [12]. 2,2,2-trifluoroethanol was a Romil product of the highest purity grade. Protein solutions for CD measurements were exhaustively dialyzed by using Spectra Por MW 15 000–17 000 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 protein solutions were prepared in the buffer solution to be 10 times the requisite final protein concentration. Buffer, TFE and 10–20 μl of protein stock solution to give a final volume of 0.5 ml were added to 1.5 ml siliconized Eppendorf tubes. This yielded final TFE concentrations from 0 to 7 M, and the desired final protein concentration. Each sample was mixed by vortexing and was incubated overnight at 4 °C. Longer incubation times produced identical CD signals.

2.2. Circular dichroism

CD spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier type temperature control system (Model PTC-348WI). The instrument was calibrated with an aqueous solution of D-10-(+)-camphorsulfonic acid at 290 nm [13]. Molar ellipticity per mean residue, $[\theta]$ in deg cm^2 dmol^{-1}, was calculated from the equation: $[\theta]=[\theta]_{\mathrm{obs}}\cdot \mathrm{mrw}/10\cdot l\cdot C$, where $[\theta]_{\mathrm{obs}}$ is the ellipticity measured in degrees, mrw is the mean residue molecular weight, 113 Da, C is the protein concentration in g L^{-1} and l is the optical path length of the cell in cm. A 0.2- and 1-cm path length cell and a protein concentration of approximately 0.1 and 1 mg ml^{-1} were used in the far-UV and near-UV region, respectively. CD spectra were recorded 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 corrected by subtracting a buffer spectrum. The TFE-induced denaturation curves at constant temperature were obtained by recording the CD signal at 222 nm and 276 nm in the far-UV and near-UV, respectively, for each independent sample. Thermal unfolding curves were recorded in the temperature mode at 222 nm in the far-UV, and at 276 nm in the near-UV region, from 25 up to 105 °C with a scan rate of 1.0 K min^{-1}.

3. Results

The effect of TFE on the conformational stability of AFEST has been investigated by means of CD measurements at pH 7.5, 20 mM phosphate buffer and $T=20$ °C. In these experimental conditions the detected conformational changes are reversible, as demonstrated by suitable dilutions of fully denatured protein samples.

Far-UV CD spectra of AFEST in the absence and in the presence of increasing concentrations of TFE from 0 up to 7 M TFE (i.e. from 0 up to 50% v v^{-1}) are reported in Fig. 1. The spectrum of the native protein is characteristic of a well-structured α/β protein: approximately 40% of α-helix and 20% of β-sheet, in agreement with the X-ray structure [3]. The different curves clearly show that the addition of TFE modifies the spectrum: the intensity of the α-helical bands at 208 and 222 nm rises significantly and abruptly on increasing the TFE concentration above 2.8 M (20% v v^{-1}). Such an effect levels off at TFE concentrations of approximately 5.6 M (40% v v^{-1}). The near-UV CD spectra of AFEST in the absence and in the presence of TFE, reported in Fig. 2, show that the tertiary structure drastically changes in TFE-rich aqueous solutions. At 3.5 M TFE (25% v v^{-1}), a strong diminution of the near-UV CD signal is observed, indicating a global loss of tertiary structure, with disruption of the asymmetric environment of aromatic chromophores. These results point out that the behavior of AFEST with respect to the action of TFE differs strongly from that of thermolysin, even though both enzymes are thermophilic.

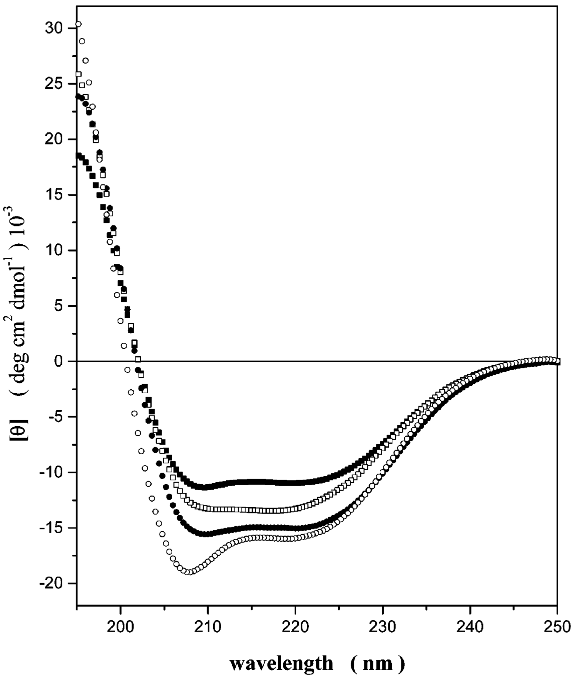

Fig. 1. Far-UV CD spectra of AFEST in the absence [filled squares] and in the presence of 25% [open squares], 30% [filled circles], and 50% [open circles] (v v^{-1}) TFE, recorded at pH 7.5 and 20 °C.

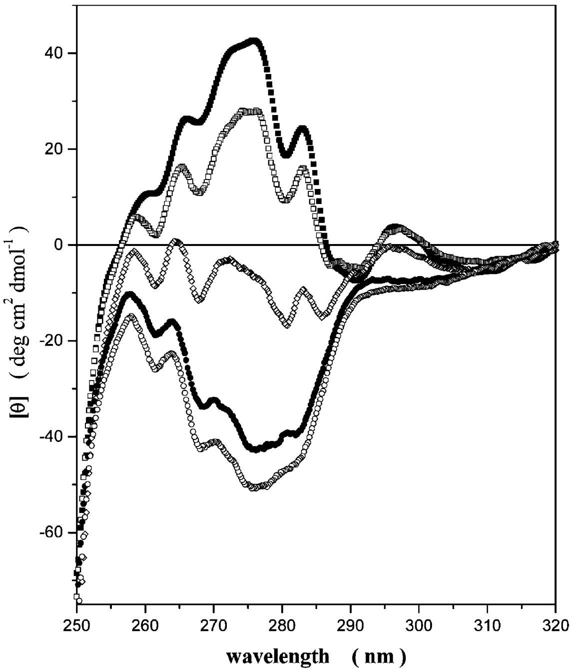

Fig. 2. Near-UV CD spectra of AFEST in the absence [filled squares] and in the presence of 20% [open squares], 25% [open diamonds], 30% [filled circles], and 50% [open circles] (v v^{-1}) TFE, recorded at pH 7.5 and 20 °C.

To better emphasize the TFE-induced conformational changes, transition curves have been constructed by recording the molar ellipticity at 222 nm (Fig. 3, panel a), and at 276 nm (Fig. 3, panel b). Both transition curves have a sigmoidal shape indicating the occurrence of a cooperative process. In addition, they are characterized by the same value of the TFE concentration at half-completion of the transition: $[\mathrm{TFE}]_{1/2} = 3.5$ M (i.e. 25% v v^{-1}). Since the two CD signals detect different levels of protein structure (i.e. at 222 nm the secondary structure and at 276 nm the tertiary one), the process can be assimilated to a two-state (all-or-none) transition. This finding indicates that the overall breakdown of the tertiary structure and the formation of a helical state in TFE-rich aqueous solutions are two cooperative and simultaneous processes. Therefore, as advocated by Thomas and Dill [14], the non-local interactions are weakened and the local ones are favored by the addition of TFE to protein aqueous solution, leading to the disruption of the native fold and to the stabilization of a non-compact helical state.

An exchange model in which the TFE and water molecules exchange binding sites on the polypeptide chain has been developed by Fersht and co-workers [15], but there is no structural evidence of specific binding sites. For this reason, we preferred not to fit the transition curves of Fig. 3 with respect to such a model, and to provide only the value of $[\mathrm{TFE}]_{1/2}$. The latter has to be considered an operational measure of the protein stability.

Thermal transition curves in the absence and in the presence of TFE have been obtained by means of both far-UV (see Figs. 4 and 5) and near-UV (see Fig. 6) CD measurements, using a scan rate of 1.0 K min^{-1}, at pH 7.5, 20 mM phosphate buffer. The denaturation induced by temperature is always an irreversible process [5], and this prevents a complete analysis by means of equilibrium thermodynamics. AFEST in the native state has a denaturation temperature $T_d = 99$ °C, close to the boiling point of water. On adding small amounts of TFE there is a significant destabilization of the native structure. In fact both far-UV and near-UV thermal transition curves provide the following values: $T_d = 92$ °C at 0.7 M TFE (5% v v^{-1}), and $T_d = 85$ °C at 1.4 M TFE (10% v v^{-1}).

In the far-UV region no sigmoidal transition curves have been detected at TFE concentrations higher than 1.4 M, as shown in Fig. 5. On increasing the TFE concentration the molar ellipticity at 222 nm becomes more negative at 20 °C, and then shows a continuous rise with temperature, with no inflection points. This means that the helical state characteristic of TFE-rich aqueous solutions does not undergo a co-operative transition induced by temperature. However, the measurements in the near-UV region show a co-operative transition also at 2.8 M (20% v v^{-1}) and 3.5 M (25% v v^{-1}) TFE, characterized by $T_d = 56$ °C and 40 °C, respectively (see Fig. 6). In all probability the discrepancy with the results obtained in the far-UV region is due to the different level of protein structure monitored by the two probes. In any case also in the near-UV region no sigmoidal transition curve has been detected at TFE concentrations higher than 3.5 M. This result

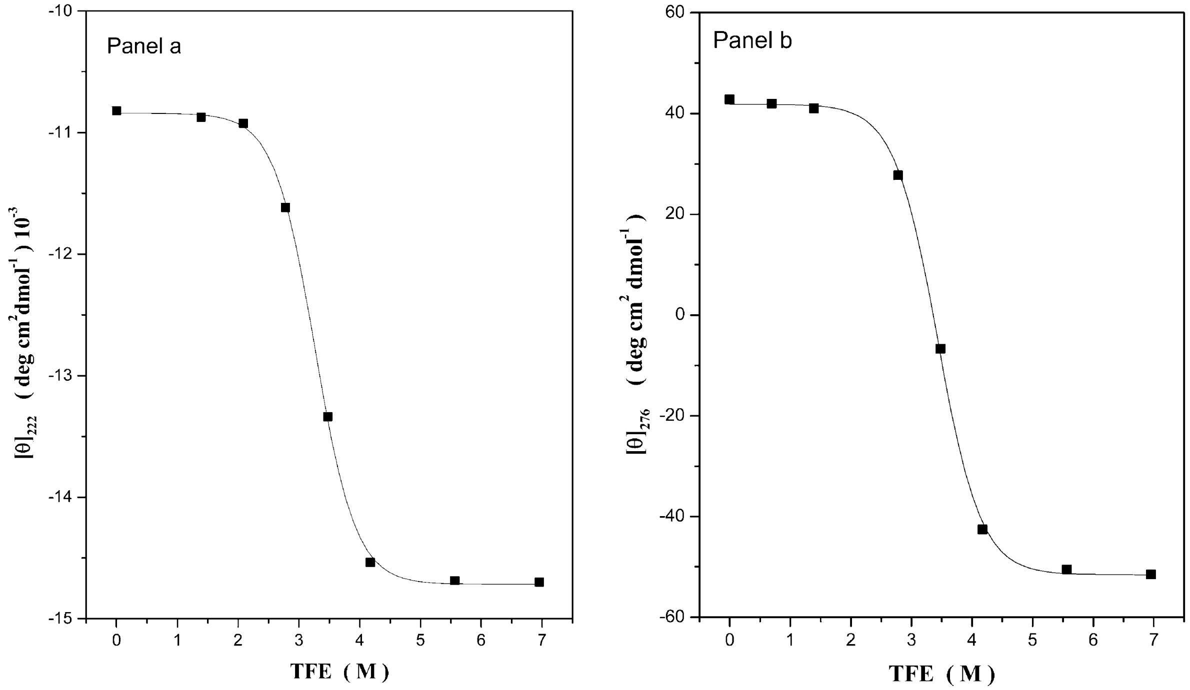

Fig. 3. TFE-induced transition curves of AFEST at pH 7.5 and 20 °C obtained by recording the change of the CD signal at 222 nm (panel a) and 276 nm (panel b). The continuous curves through the experimental points are not the result of a mathematical fitting with respect to a selected model. They serve only as a guide for eyes to appreciate the sigmoidal nature of the transition and to estimate its mid-point.

is expected by considering that such TFE concentration corresponds to the mid-point of the transition curves determined for AFEST at room temperature.

It is worth noting that, the CD spectra in both the far-UV and near-UV regions well emphasize that the conformational features of the denatured state of AFEST depend markedly upon the solvent composition (i.e. compare the end points of the thermal transition curves shown in Fig. 6). This emphasizes the need of a better characterization of non-compact conformations, since the denatured state, being the other half of the folding equation, plays a role in protein stability [16].

4. Discussion

AFEST, being a hyperthermophilic enzyme, is very resistant to temperature [5]. However, TFE is very effective in denaturing this esterase since at room temperature $[TFE]_{1/2}=3.5$ M. In order to gain perspective, the latter number has to be contrasted against those determined for GuHCl- and urea-induced denaturation of AFEST [5,6]: at room temperature $[GuHCl]_{1/2}=2.0$ M and $[urea]_{1/2}=7.1$ M. These values indicate that TFE, widely used to enhance the helical content of peptides, is actually a strong denaturant of globular proteins, twice more effective than urea.

To perform a useful comparison, we have determined the effect of TFE on the conformational stability of the esterase AES from *E. coli*, that can be considered the mesophilic counterpart of AFEST [11]. The TFE-induced transition curve obtained for AES, by recording the molar ellipticity at 222 nm in the same experimental conditions adopted for AFEST, is shown in Fig. 7. It results $[TFE]_{1/2}=3.5$ M for AES, the same value obtained for AFEST, even though the denaturation temperature of AES is 68 °C, 31 °C lower than that of

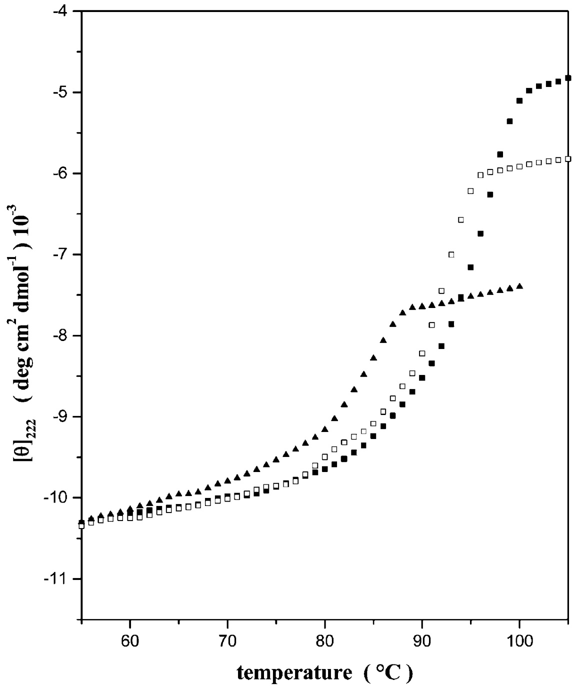

Fig. 4. Thermal transition curves obtained by following the change in the CD signal at 222 nm for AFEST in the absence [filled squares] and in the presence of 5% [open squares], and 10% [filled triangles] (v v^{-1}) TFE, at pH 7.5.

AFEST (a complete report on the AES conformational stability will be presented elsewhere). The $[TFE]_{1/2}$ values obtained for AFEST and AES are also similar to those determined for several mesophilic proteins. In fact, $[TFE]_{1/2} = 2.8$ M for α-lactalbumin, 4.2 M for hen egg-white lysozyme, 5.5 M for both tendamistat and ribonuclease A [17–19]. This indicates that, while AFEST has an extra-stability against temperature with respect to mesophilic globular proteins, it does not have an extra-stability against the action of TFE. Therefore, understanding the molecular mechanism of the TFE action would be useful to shed light on the interactions rendering thermostable AFEST.

Despite considerable effort, there is as yet no general consensus on a single mechanism accounting for all the effects TFE has on globular protein conformations [9]. On the basis of several studies it emerges that TFE is a drastic modifier of the interplay between local interactions and non-local ones [9,14,17–19]. The addition of TFE to a protein aqueous solution, by weakening non-local interactions and favoring local ones, leads to the disruption of the native tertiary structure and to the stabilization of helical conformations. Among the non-local interactions determining the stability of the native folded state, the hydrophobic effect is generally considered the principal factor [20]. It seems that TFE affects mainly the strength of hydrophobic interactions with respect to neat water. In fact, non-polar compounds are significantly more soluble in liquid TFE than in water [21]. Thus, the weakening of hydrophobic interactions destabilizes the compact native state, favoring more extended, non-compact conformations and seems to be a pre-condition for helix induction. The latter phenomenon would be an indirect effect of tertiary structure breakdown.

Along these lines a detailed rationalization of the combined action of TFE would be the follow-

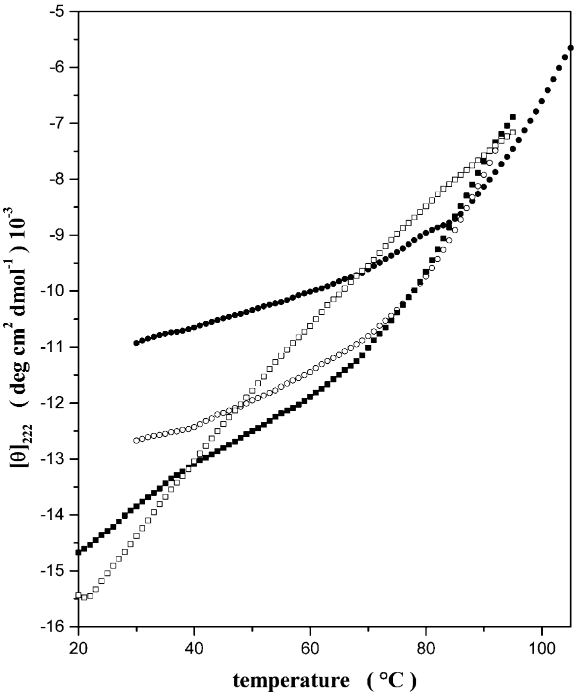

Fig. 5. Thermal transition curves obtained by following the change in the CD signal at 222 nm for AFEST in the presence of 15% [filled circles], 25% [open circles], 30% [filled squares], and 50% [open squares] (v v^{-1}) TFE, at pH 7.5.

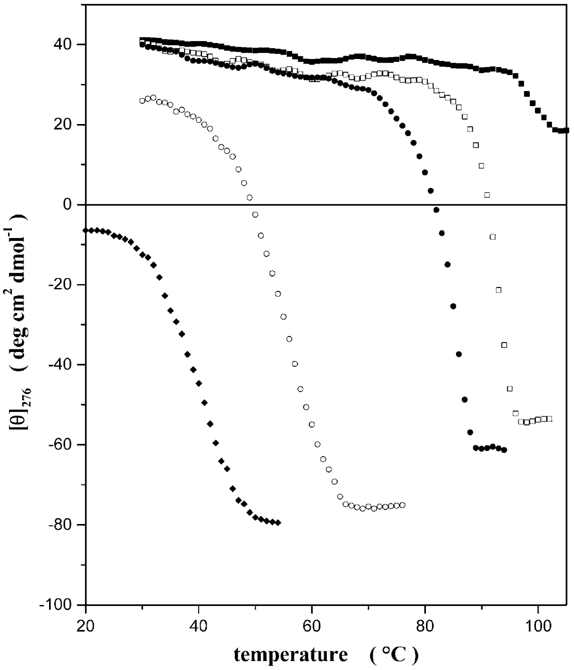

Fig. 6. Thermal transition curves obtained by following the change in the CD signal at 276 nm of AFEST in the absence [filled squares] and in the presence of 5% [open squares], 10% [filled circles], 20% [open circles], and 25% [filled diamonds] (v v^{-1}) TFE, at pH 7.5.

ing. The Gibbs energy cost to create a cavity suitable to host the polypeptide chain is a fundamental component of the hydrophobic effect [22–24]. In fact the work to create a cavity of given radius is larger in water than in common organic solvents, and the gap rises on increasing the cavity radius [23,25]. A macroscopic measure of this difference can be inferred from the surface tension values [26]. The surface tension, in dyne cm^{-1}, at 25 °C, is 71.99 for water, 28.22 for benzene, 24.65 for *c*-hexane, 21.97 for ethanol, and 17.89 for *n*-hexane [27]. Such fundamental difference between water and common organic solvents is widely recognized, even though there is still debate on what is the actual feature of water determining it: the small size of the water molecules, or the strength of the H-bonding network of the liquid [23,24].

According to small angle X-ray scattering measurements [28], globular proteins in the native state have a radius of gyration substantially smaller than in the unfolded state (i.e. $R_g = 15.0$ Å and 27.3 Å for the native and unfolded state of RNase A, respectively; $R_g = 18.0$ Å and 44.4 Å for the native and unfolded state of α-lactoglobulin, respectively). Therefore, the Gibbs energy cost of cavity creation strongly favors the native compact state in water. However, the work of cavity creation in TFE–water mixtures is smaller than in pure water [29], and, in fact, the surface tension of water is decreased on introduction of TFE [30]. This can be directly verified by means of scaled particle theory [31], in the assumption that a water molecule corresponds to a sphere of 2.8 Å diameter, and a TFE molecule to a sphere of 4.9 Å diameter [32]. This fact causes a drastic destabilization of the native tertiary structure of globular proteins when dissolved in TFE-rich aqueous solutions. Such a mechanism distinguishes TFE from both

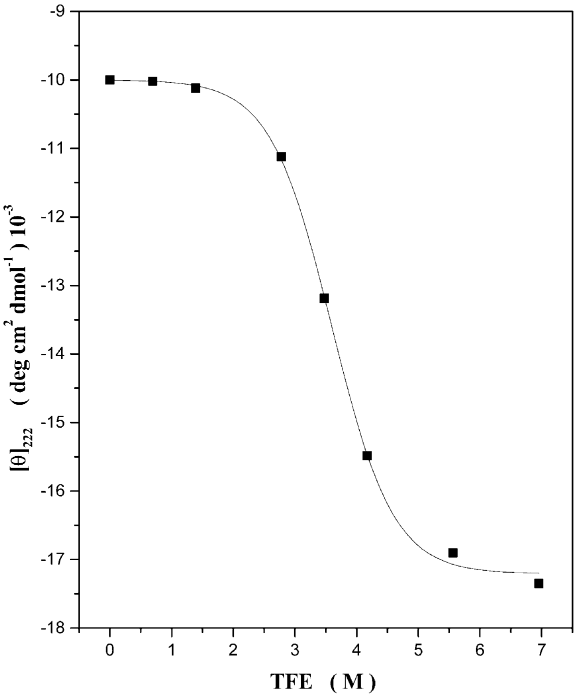

Fig. 7. TFE-induced transition curves of AES at pH 7.5 and 20 °C obtained by recording the change of the CD signal at 222 nm. For the meaning of the continuous curve see Fig. 3.

urea and GuHCl because their addition to water causes an increase of the surface tension [33].

It is worth noting that the TFE denaturing action is strictly linked to the mechanism by which TFE can stabilize helical conformations [34,35]. The large size of the TFE molecule with its bulky non-polar CF_3 group, and the fact that it has only a single H-bond donor/acceptor site imply that TFE is less capable of H-bonding to backbone peptide groups with respect to water (i.e. teflon coat model). Thus protein conformations in which the peptide groups form intramolecular H-bonds, such as the α-helix, are favored with respect to the random coil in TFE-rich aqueous solutions. Moreover, even though TFE–water mixtures have a lower dielectric constant than pure water (i.e. at room temperature TFE has a dielectric constant of 27, while that of water is 79 [27]), the electrostatic interactions, according to computer simulations [34], seem to be not affected because charged residues are preferentially solvated by water molecules.

Detailed analysis of AFEST structure [3] pointed out that it is rather compact with a shortening of the loops in comparison to a thermophilic esterase, EST2, from *Alicyclobacillus acidocaldarius*, and a mesophilic one, brefeldin A, from *Bacillus subtilis*. In addition, the number of ion pairs is larger on the AFEST surface with respect to both EST2 and brefeldin A [3]. Clearly an optimization of electrostatic interactions on the protein surface implies also an optimization of the burial and packing of non-polar groups in the protein interior [36]. Therefore, even though it is widely recognized that charge–charge interactions play a fundamental role for the resistance to high temperature [37–39], the contribution from hydrophobic interactions is still essential for the stability of the native tertiary structure. When the latter favorable contribution is decreased, as in TFE-rich aqueous solutions, there is the breakdown of native structure, and the polypeptide chain assumes non-compact conformations. Such an analysis explains the finding that TFE has a strong denaturing action on AFEST enzyme.

In conclusion, the present data demonstrate that AFEST, a hyperthermophilic enzyme, has a resistance against the denaturing action of TFE similar to that of its mesophilic counterpart AES and to that of other mesophilic proteins. A general mechanism for the TFE action on globular proteins is proposed, and it allows a reliable rationalization of the experimental results.

Acknowledgments

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