International Journal of Biological Macromolecules 34 (2004) 195–201

INTERNATIONAL JOURNAL OF Biological Macromolecules STRUCTURE, FUNCTION AND INTERACTIONS

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# Guanidine-induced unfolding of the Sso7d protein from the hyperthermophilic archaeon *Sulfolobus solfataricus*

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Accepted 5 April 2004

**Abstract**

The unfolding induced by guanidine hydrochloride of the small protein Sso7d from the hyperthermophilic archaeon *Sulfolobus solfataricus* has been investigated by means of circular dichroism and fluorescence measurements. At neutral pH and room temperature the midpoint of the transition occurred at 4 M guanidine hydrochloride. Thermodynamic information was obtained by means of both the linear extrapolation model and the denaturant binding model, in the assumption of a two-state N $\Leftrightarrow$ D transition. A comparison with thermodynamic data determined from the thermal unfolding of Sso7d indicated that the denaturant binding model has to be preferred. Finally, it is shown that Sso7d is the most stable against both temperature and guanidine hydrochloride among a set of globular proteins possessing a very similar 3D structure.


*Keywords:* Thermophilic protein; SH3 folding topology; Unfolding transition; Thermodynamic stability

## 1. Introduction

Guanidine hydrochloride (GuHCl) is a strong denaturant of globular proteins and is widely used to obtain quantitative information on their thermodynamic stability by performing equilibrium unfolding measurements [1,2]. To accomplish this task two different models are in use: the linear extrapolation model, LEM, and the denaturant binding model, DBM [3]. Several lines of evidence have suggested that the LEM model works well in the case of urea but not for GuHCl [3,4]. To further clarify this point, in the present study the stability of the hyperthermophilic protein Sso7d against the denaturing action of GuHCl has been investigated by means of circular dichroism, CD, and fluorescence measurements.

Sso7d is an abundant, small (62 residues) and basic (p$I$ = 10.2) protein isolated from the hyperthermophilic archaeon *Sulfolobus solfataricus* living at about 80 °C and pH 2–3 in volcanic hot springs [5,6]. Sso7d shows a variety of activities: (a) it is a non-sequence-specific DNA-binding protein, increasing the melting temperature of the DNA double-helix [7–9]; (b) it promotes the annealing of complementary DNA strands [10]; (c) it induces negative supercoiling [11], and a kink with unwinding of the DNA double helix [12,13]; (d) it shows a ribonuclease activity [14]; (e) it rescues aggregated proteins in an ATP hydrolysis-dependent manner [15].

The 3D structure of the protein in solution has been solved by means of NMR [7] (PDB codes 1SSO and 1JIC), and that of the protein complexed with oligonucleotides also by means of X-ray diffraction [13] (PDB codes 1BNZ and 1BF4). The structure is formed by the orthogonal packing of two β-sheets: the first one constituted by two antiparallel strands (residues 2–7 and 10–15, respectively), and the second consisting of three antiparallel strands (residues 21–25, 28–33 and 41–46, respectively). A C-terminal amphipatic

*Abbreviations: Abbreviations:* Sso7d and Sac7d, 7 kDa DNA-binding proteins from *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*, respectively; SH3, Src-homology 3 domain; Csp, cold shock protein; CD, circular dichroism; DSC, differential scanning calorimetry; MALDI-TOF/MS, matrix assisted laser desorption ionisation-time of flight mass spectrometry; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; GuHCl, guanidine hydrochloride

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doi:10.1016/j.ijbiomac.2004.04.002

α-helix (residues 52–61) caps the five-stranded incomplete β-barrel, abutting the β3–β4–β5 strands. Sso7d possesses a well-developed hydrophobic core, mainly constituted by Val3, Phe5, Ileu19, Val22, Phe31, Tyr33, Val45 and Leu54, with the aromatic side-chains in herringbone geometry. The surface of the protein is characterized by the presence of a lot of charged residues: 2 Arg, 13 Lys, 6 Glu and 3 Asp. There are no asparagines, histidines and cysteines. The thermal stability of Sso7d has been deeply investigated, showing that the protein has a denaturation temperature close to the boiling point of water [16–20]. However, the stability of Sso7d against the denaturing action of GuHCl has not been characterized.

It is important to note that the folding topology of Sso7d is very similar to that of Src-homology 3 (SH3), domains and cold shock proteins [21]. Actually, the SH3 folding topology is adopted by a large number of very different sequences characterized by a specific pattern of hydrophobic and polar residues upon structure-based alignment. A comparison of the stability of such proteins against both temperature and GuHCl may provide useful information. The present experimental data indicate that: (a) Sso7d is very resistant to the denaturing action of GuHCl; (b) the DBM model has to be preferred to the LEM one; (c) there is a correlation between the denaturation temperature and the GuHCl concentration at the midpoint of the unfolding transition for a set of proteins all possessing the SH3 folding topology.

## 2. Materials and methods

### 2.1. Protein and sample preparation

Sso7d from *S. solfataricus* was expressed in *Escherichia coli* and purified as described [22]. The recombinant protein proved to be indistinguishable from its natural counterpart on the basis of its DNA-binding and ribonuclease activities and stability against temperature, even though no lysine monomethylation was found [20]. The purity of the protein was confirmed by means of SDS–PAGE and MALDI–TOF mass spectrometry. Before further measurements, sample solutions were dialyzed against the required buffer at 4 °C for 24 h. Dialysis tubes with a cutoff limit of 3500 Da were used. The Sso7d concentration of dialyzed samples was determined spectrophotometrically using $\varepsilon_{280} = 8300\,M^{-1}\,cm^{-1}$, calculated by the method of Gill and von Hippel [23].

All measurements were performed at pH 7.0, 20 mM sodium phosphate buffer. The pH was measured at 25 °C with a Radiometer pHmeter, model PHM93. The phosphate buffer has a low protonation enthalpy [24], so that the solution pH depends little upon temperature. Doubly deionized water was used throughout. A commercial 8 M solution from Sigma was used for GuHCl. Stock protein solutions were prepared in the buffer solution to be 10 times the requisite final protein concentration. Buffer, GuHCl stock solution 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 GuHCl concentrations from 0 to 7 M, and the desired final protein concentration. Since high GuHCl concentrations change the pH, the final pH for each sample was corrected by addition of NaOH. Each sample was mixed by vortexing and was incubated overnight at 4 °C. Longer incubation times produced identical spectroscopic signals.

### 2.2. CD measurements

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 [25]. Molar ellipticity per mean residue, $[\theta]$ in deg $cm^2\,dmol^{-1}$, was calculated from the equation: $[\theta] = [\theta]_{obs}\cdot mrw/10lC$, where $[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular weight, $C$ is the protein concentration in $g\,L^{-1}$ and $l$ is the optical path length of the cell in cm. Cuvettes of 0.2 cm path length and protein concentrations of 0.2 mg $mL^{-1}$ were used in the far-UV region. 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. Thermal unfolding curves were recorded in the temperature mode, over the range 5–110 °C, with a scan rate of 1.0 K $min^{-1}$. Samples were rapidly cooled after the first heating run and scanned for a second time to estimate the reversibility of the unfolding transition.

### 2.3. Fluorescence measurements

Steady-state fluorescence measurements were perfomed with a JASCO FP-750 spectrofluorimeter equipped with thermostated cell holders and temperature was kept constant by a circulating water bath. The protein concentration was 0.1–0.15 mg $mL^{-1}$. The excitation wavelength was set at 290 nm; the experiments were performed at 20 °C, using a 1 cm sealed cell and a 5 nm emission slit width, and corrected for background signal. The change in fluorescence intensity at 350 nm was recorded to monitor the unfolding transition.

### 2.4. Analysis of transition curves

Thermal unfolding transitions were analyzed with the two-state N ⇔ D model whose equilibrium constant is given by [26]:

$$K_d(T) = \exp\left\{\frac{-\Delta_d H(T_d)}{R}\times\left[\left(\frac{1}{T}\right)-\left(\frac{1}{T_d}\right)\right]\right\} \quad (1)$$

where $T_d$ is the denaturation temperature at which $K_d = 1$ and $\Delta_d H(T_d)$ is the denaturation enthalpy change. The de-

naturation heat capacity change, $\Delta_d C_p$, is considered zero because it cannot reliably be determined from CD measurements [26]. Correspondingly the observed molar ellipticity is:

$$[\theta] = \frac{[\theta]_N + [\theta]_D K_d}{1 + K_d} \quad (2)$$

where $[\theta]_N$ and $[\theta]_D$ are the molar ellipticities of the native and denatured states, respectively, which are assumed to depend linearly on temperature. A non-linear least-squares regression was carried out to estimate the unknown parameters associated with the unfolding transition, using the Levenberg–Marquardt algorithm, as implemented in the Optimization Toolbox of MATLAB. Since Sso7d has a denaturation temperature close to 100 °C around neutral pH [16], and the CD instrument works up to 110 °C, incomplete thermal unfolding transitions have been recorded. The reliability of the thermodynamic parameters obtained from such incomplete transitions was analyzed in detail in a previous article [19].

GuHCl-induced unfolding transition curves were analysed in the assumption of a two-state N ⇔ D transition, by means of two models [3]. The linear extrapolation model, LEM, assumes that the standard denaturation Gibbs energy change is a linear function of the denaturant concentration:

$$\Delta_d G = \Delta_d G_{H2O} - m[\text{GuHCl}] \quad (3)$$

where $\Delta_d G_{H_2O}$ is the value of $\Delta_d G$ in the absence of denaturant and $m$ is a measure of the dependence of $\Delta_d G$ on denaturant concentration. Furthermore, $\Delta_d G_{H_2O}$=$m[\text{GuHCl}]_{1/2}$, where $[\text{GuHCl}]_{1/2}$ is a measure of the midpoint of the denaturation region. The denaturant binding model, DBM, assumes that protein unfolding is due to the fact that there are a greater number of identical and non-interacting binding sites for the denaturant on the unfolded state than there are on the native state. As a consequence:

$$\Delta_d G = \Delta_d G_{H_2O} - \Delta n RT \ln(1 + k \times a) \quad (4)$$

where $\Delta n$ is the difference in the number of denaturant binding sites, $k$ the equilibrium constant for binding at each site, and $a$ is the activity of the denaturant. The equation to calculate the molarity-based activity for GuHCl from the molar concentration was provided by Pace [3]. Clearly, $K_d = \exp(-\Delta_d G/RT)$ and Eq. (2) is used for both LEM and DBM, in the assumption that the molar ellipticity or the fluorescence intensity of the native and unfolded states vary linearly with denaturant concentration. A non-linear least-squares regression was carried out to estimate the unknown parameters associated with the unfolding transition (see above).

The thermodynamic parameters obtained from the temperature-induced unfolding allow the calculation of the denaturation Gibbs energy change as a function of temperature [27]:

$$\Delta_d G(T) = \Delta_d H(T_d)\left[1 - \left(\frac{T}{T_d}\right)\right] + \Delta_d C_p\left[T - T_d - T\ln\left(\frac{T}{T_d}\right)\right] \quad (5)$$

which is strictly correct in the assumption that $\Delta_d C_p$ is temperature-independent. In order to have consistency, the value of $\Delta_d G$ (20 °C) should agree with the $\Delta_d G_{H_2O}$ estimates obtained by means of the LEM and DBM models.

## 3. Results

The GuHCl-induced unfolding of Sso7d has been investigated by recording: (a) the molar ellipticity at 222 nm, $[\theta]_{222}$; (b) the change in fluorescence intensity at 350 nm, $I_{350}$. The measurements were performed at 20 °C, 20 mM phosphate buffer, pH 7.0, after an overnight incubation of the samples at 4 °C (longer incubation times gave rise to identical signals), and were independent of protein concentration in the range 0.5–0.05 mg mL$^{-1}$. Since Sso7d consists almost entirely of β-sheets, its unfolding transitions have been usually monitored at 200 nm. However, it is not possible to obtain reliable values of $[\theta]_{200}$ on increasing the concentration of GuHCl. In addition, Sso7d contains only one trypthophan residue, Trp23, whose side-chain is well exposed to the solvent and plays a role in the DNA-binding activity of the protein [7]. As a consequence of the water exposure of Trp23, the fluorescence emission spectrum of native Sso7d presents a maximum at 350 nm and there is no appreciable shift of such a maximum upon unfolding.

The GuHCl-induced unfolding of Sso7d is reversible: renaturation of completely unfolded samples upon suitable dilution, showed a full recovery of all the spectroscopic features of the native protein. The transition curves are shown in Fig. 1. They have a sigmoidal shape and the GuHCl concentration at the transition midpoint $[\text{GuHCl}]_{1/2} = 4.0$ M, regardless of the spectroscopic probe used to monitor unfolding. This finding should be considered an indication that the GuHCl-induced unfolding of Sso7d is a two-state N ⇔ D process. It was not possible to study the urea-induced unfolding of Sso7d because the corresponding transition curves were not yet completed at 9 M urea.

The GuHCl-induced transition curves have been analysed by means of the LEM and DBM models, Eqs. (3) and (4). Both models are able to fit with good accuracy the experimental curves and the results are collected in Table 1. The numbers indicate that there is no agreement between LEM and DBM for the $\Delta_d G_{H_2O}$ estimates, but there is agreement between the estimates obtained by each model from the analysis of the two spectroscopic probes. Specifically: (a) from $[\theta]_{222}$, $\Delta_d G_{H_2O}$ [in kJ mol$^{-1}$] = 26.8 by LEM and 35.7 by DBM; (b) from $I_{350}$, $\Delta_d G_{H_2O}$ [in kJ mol$^{-1}$] = 28.0 by LEM and 36.5 by DBM. The obtained values for the parameters

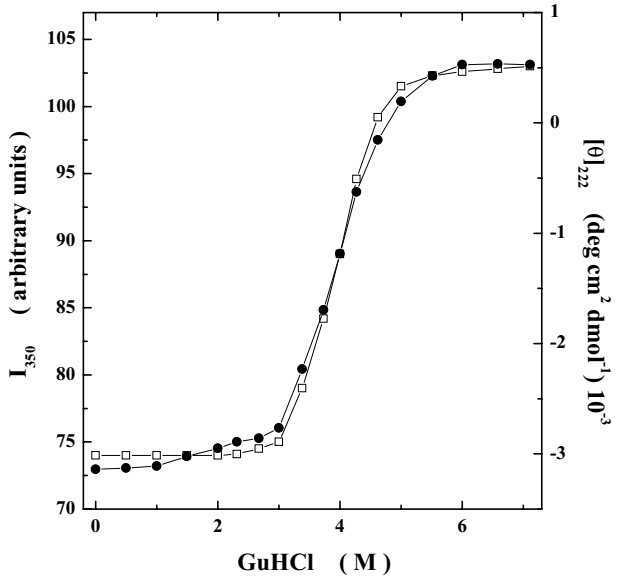


Fig. 1. GuHCl-induced unfolding curves of Sso7d at pH 7.0 and 20 °C obtained by recording the change in molar ellipticity at 222 nm (filled circles) and the change in fluorescence intensity at 350 nm (open squares).

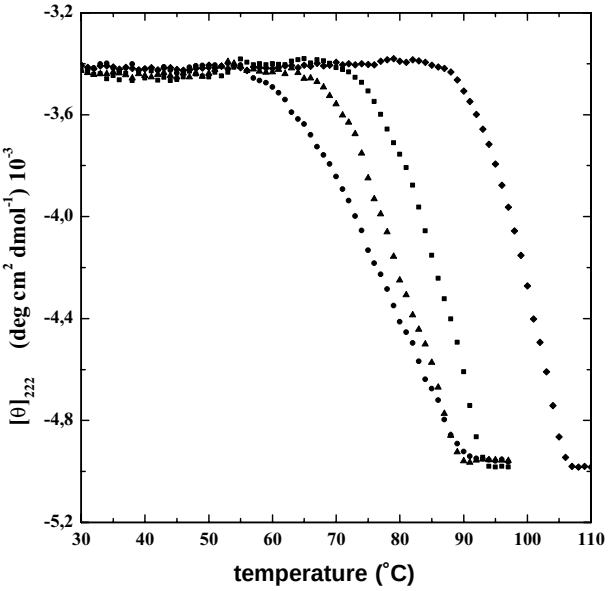


Fig. 2. Temperature-induced unfolding curves of Sso7d at pH 7.0 in the absence (diamonds) and in the presence of 1 M (squares), 1.5 M (triangles), and 2.7 M GuHCl (circles).

$m$ and $\Delta n$ are in line with those expected for a protein of this size. The estimates of the binding constant determined by the fitting procedure with the DBM model, $k = 0.54$ and $0.56\,M^{-1}$, agree with the mean value of $0.60\,M^{-1}$ obtained by various authors from studies on both model compounds and globular proteins [3,28].

The temperature-induced unfolding of Sso7d was investigated by recording the molar ellipticity at 222 nm in both the absence and presence of GuHCl at pH 7.0. The experimental transition curves are reported in Fig. 2. The process is reversible in the selected conditions according to the reheating criterion, and has been analysed by means of the two-state N ⇔ D model. The latter fits well the experimental transition curves. The numerical results, collected in Table 2, indicate that both the denaturation temperature and the denaturation enthalpy change decrease significantly on increasing the concentration of GuHCl: (a) $T_d$ passes from 99 °C for Sso7d alone to 85 °C at 1 M GuHCl and 76 °C at 2.7 M GuHCl; (b) $\Delta_d H(T_d)$ passes from $270\,kJ\,mol^{-1}$ for Sso7d alone to $200\,kJ\,mol^{-1}$ at 1 M GuHCl and $130\,kJ\,mol^{-1}$ at 2.7 M GuHCl. It is worth noting that the thermodynamic parameters obtained for Sso7d in the absence of GuHCl are close to those determined by means of differential scanning calorimetry, DSC, measurements by Ladenstein and co-workers [16], and those determined by CD measurements at 200 nm by us [29]. This is a strong indication that the temperature-induced unfolding of Sso7d is a two state N ⇔ D transition. Furthermore, it is worth mentioning that Sso7d

Table 2
Thermodynamic parameters characterizing the thermal unfolding of Sso7d at pH 7.0, 20 mM phosphate buffer, in the absence and presence of different GuHCl concentrations, monitored by changes of $[\theta]_{222}$

| [GuHCl] (M) | $T_d$ (°C) | $\Delta_d H(T_d)$ (kJ mol$^{-1}$) |
|---|---|---|
| 0 | 99 | 270 |
| 1.0 | 85 | 200 |
| 1.5 | 80 | 155 |
| 2.7 | 76 | 130 |

The data were analysed as described in the text. Each figure is the average of the values calculated by the nonlinear regression procedure over three independent measurements. The uncertainties in the estimates for $T_d$ and $\Delta_d H(T_d)$ amount to 2 and 10%, respectively, of the reported values. The quality of the fit was good, as indicated by the small values of the standard deviation of the calculated points from the experimental ones.

Table 1
Parameter values obtained by the LEM and DBM analyses of GuHCl-induced unfolding of Sso7d, monitored by changes of $[\theta]_{222}$ and $I_{350}$ at pH 7.0, 20 mM phosphate buffer, and 20 °C

| | LEM | | | DBM | | |
|---|---|---|---|---|---|---|
| | $[GuHCl]_{1/2}$ (M) | $m$ (kJ M$^{-1}$mol$^{-1}$) | $\Delta_d G_{H_2O}$ (kJ mol$^{-1}$) | $\Delta n$ | $k$ (M$^{-1}$) | $\Delta_d G_{H_2O}$ (kJ mol$^{-1}$) |
| $[\theta]_{222}$ | 4.0 | 6.7 | 26.8 | 22.8 | 0.56 | 35.7 |
| $I_{350}$ | 4.0 | 7.0 | 28.0 | 22.9 | 0.54 | 36.5 |

The data were analysed as described in the text. Each figure is the average of the values calculated by the nonlinear regression procedure over three independent measurements. The uncertainties in the estimates for the various parameters amount to 10% of reported values. For all the examined transition curves the standard deviation of the calculated points from the experimental ones was small, indicating the good quality of the fit.

shows a marked dependence of $T_d$ on the solution pH over the pH range 2.5–4.5, whereas $T_d$ is practically constant over the pH range 5–8 [16]. Such pH dependence was rationalized by means of a thermodynamic model linking the unfolding transition to the proton binding on two acidic side-chains possessing anomalously low p$K_a$ values [30]. Recently, NMR measurements and electrostatic calculations have confirmed the correctness of the thermodynamic analysis [31].

Even though the CD data do not allow a reliable determination of $\Delta_d C_p$, we can use the value obtained for Sso7d by DSC investigations [16,19], 2.7 kJ K$^{-1}$ mol$^{-1}$. Since the magnitude of $\Delta_d C_p$ is very sensitive to solvent conditions [28,32,33], we use the above value solely to perform calculations for Sso7d in the absence of GuHCl. The existence of a large positive heat capacity change associated with the unfolding of globular proteins implies that $\Delta_d H$ has a strong temperature dependence. The knowledge of $\Delta_d C_p$, in the assumption that it is temperature-independent, allows the calculation of $\Delta_d H$ at any temperature [27]. In particular, at $T = 76\,^\circ$C, the denaturation temperature of Sso7d in the presence of 2.7 M GuHCl, the denatutation enthalpy change of Sso7d in the absence of GuHCl should amount to $270 + 2.7(76–99) = 208$ kJ mol$^{-1}$. This number has to be contrasted against that obtained from the thermal transition curve in the presence of 2.7 M GuHCl, 130 kJ mol$^{-1}$. The difference between the two values, 78 kJ mol$^{-1}$, should reflect the energy gained for the establishment of favourable interactions between guanidinium ions and Sso7d. In other words, this simple analysis, even though crude, suggests that there is a direct exothermic interaction between GuHCl and the protein.

It is also possible to calculate the Gibbs energy change associated with the unfolding of Sso7d by means of Eq. (5), using the values of $T_d$ and $\Delta_d H(T_d)$ determined from the thermal transition curves and the value of $\Delta_d C_p$, 2.7 kJ K$^{-1}$ mol$^{-1}$, from DSC investigations [16,19]. In general, such a curve has a parabolic-like profile because $\Delta_d C_p$ is a large positive quantity, and it is called the stability curve of the protein [34]. By performing a quantitative comparison at 20 °C, it results that: (a) the DBM estimate of $\Delta_d G_{H_2O}$, $36 \pm 4$ kJ mol$^{-1}$, agrees quantitatively with that determined from the temperature-induced unfolding of Sso7d, 33 kJ mol$^{-1}$; (b) the LEM estimate of $\Delta_d G_{H_2O}$, $28 \pm 3$ kJ mol$^{-1}$, underestimates the thermodynamic stability of Sso7d. This finding is in line with the results of Makhatadze, who showed that, in general, it is not correct to use the LEM model to obtain reliable estimates of $\Delta_d G_{H_2O}$ from GuHCl-induced unfolding curves [4]. Also Filimonov and colleagues [35] concluded that the LEM model produces too low estimates of $\Delta_d G_{H_2O}$.

## 4. Discussion

The molecular mechanism of the denaturing action of GuHCl is not definitely established [1,2]. Two main possibilities exist: (a) an indirect mechanism related to the modifications caused by the addition of GuHCl to the properties of water; (b) a direct mechanism related to the occurrence of direct favourable interactions between the polypeptide chain and the guanidinium ions. The finding of an exothermic interaction between Sso7d and GuHCl and the reliability of the thermodynamic parameters obtained by means of the DBM model seem to support the validity of the direct mechanism. This is in line with the results of a recent neutron diffraction study of the hydration structure of the guanidinium ion [36], suggesting its direct interaction with the protein surface. However, as pointed out by Schellman [1,2], it is not strictly correct to speak of "binding" because of the weakness of the interaction and the high concentration of the denaturant. One should speak of a solvent exchange mechanism in which the denaturant molecules substitute water molecules on the protein surface. In addition, the difference in the excluded volume effect between pure water and aqueous solutions of denaturants or stabilizers seems to be a fundamental factor that should be taken into account properly [2,37–39].

It should be meaningful to compare the stability of Sso7d against temperature and GuHCl with that of proteins possessing the same folding topology: Sac7d from *Sulfolobus acidocaldarius*, SH3 domains and cold shock proteins, Csp [35,40–46]. All such proteins are constituted by about 60 residues and do not contain disulfide bridges. On this basis, even though $\Delta_d G_{H_2O}$ is the correct measure of the thermodynamic stability, we can use the values of $[GuHCl]_{1/2}$, that are directly determined from the experimental data and should not be affected by errors due to the use of the LEM model, as an "operational" measure of protein stability. The data, collected in Table 3, indicate that there is a correlation

Table 3
Comparison of the stability against GuHCl, measured by $[GuHCl]_{1/2}$, and temperature, measured by $T_d$, for a set of globular proteins all possessing the SH3 folding topology

| | $N_{res}$ | $[GuHCl]_{1/2}$ (M) | $T_d$ (°C) | $D + E$ | $K + R$ | $N_{chg}/N_{res}$ (%) |
|---|---|---|---|---|---|---|
| Sso7d[a] | 62 | 4.0 | 99 | 3 + 6 | 13 + 2 | 39 |
| Sac7d[b] | 66 | 2.8 | 91 | 5 + 7 | 14 + 4 | 45 |
| Spc-SH3[c] | 62 | 2.0 | 66 | 6 + 4 | 8 + 2 | 32 |
| Sem5-SH3[d] | 60 | 2.4 | 79 | 4 + 4 | 3 + 3 | 23 |
| Btk-SH3[e] | 64 | 2.4 | 80 | 5 + 7 | 4 + 3 | 30 |
| Bs-CspB[f] | 67 | 1.5 | 54 | 1 + 8 | 5 + 2 | 24 |
| Bc-Csp[f] | 66 | 2.7 | 77 | 2 + 10 | 5 + 1 | 27 |
| Tm-Csp[g] | 66 | 3.3 | 94 | 3 + 8 | 10 + 1 | 33 |

The data refer to pH 7.0 or to the pH conditions of maximal stability. See text for further details. For each protein the total number of residues and the charged ones are also reported.

[a] Present work
[b] Ref. [40].
[c] Ref. [41].
[d] Ref. [42].
[e] Refs [21,43].
[f] Refs [44,45].
[g] Refs [44,46].

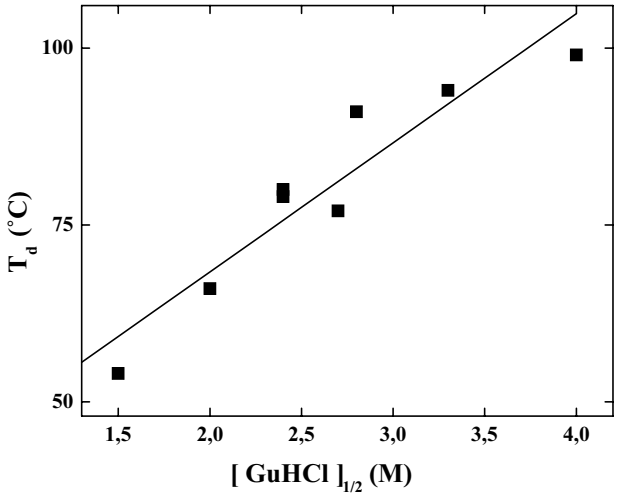


Fig. 3. Plot of $T_d$ vs. $[GuHCl]_{1/2}$ for the eight proteins listed in Table 3. The straight line is the result of the linear least-squares fit.

between the $T_d$ values and the $[GuHCl]_{1/2}$ values for this set of structurally similar proteins. CspB is the less stable against temperature, $T_d = 54\,^{\circ}C$, and is also the less stable against GuHCl, $[GuHCl]_{1/2} = 1.5\,M$. Sso7d is the most stable against temperature, $T_d = 99\,^{\circ}C$, and is also the most stable against GuHCl, $[GuHCl]_{1/2} = 4\,M$. The plot of $T_d$ versus $[GuHCl]_{1/2}$ is shown in Fig. 3 and a least squares fit gives a linear correlation coefficient of 0.94. The fact that, within the SH3 fold class, $T_d$ correlates with $[GuHCl]_{1/2}$ is an interesting observation that merits attention, and it would be interesting to see if such a correlation holds for other protein families.

Thermophilic and hyperthermophilic globular proteins possess an extra-stability against temperature with respect to their mesophilic counterparts. But this extra-stability against temperature could not correspond, in general, to an extra stability against the denaturing action of GuHCl. This is simply because temperature and GuHCl unfold globular proteins by means of distinct molecular mechanisms, even though not entirely clarified. A native structure with stabilizing interactions suitably optimised to resist high temperature could have a normal resistance against GuHCl. In order to gain perspective one has to consider that EST2 (310 residues), a thermophilic esterase from *Alicyclobacillus acidocaldarius*, and AFEST (311 residues), a hyperthermophilic esterase from *Archeoglobus fulgidus*, are characterized, at pH 7.5, by $T_d = 91$ and $99\,^{\circ}C$, respectively, but $[GuHCl]_{1/2} = 1.9\,M$ for both of them [26]. Instead, for SH3-like proteins the stability against GuHCl parallels that against temperature, as can be readily appreciated by looking at the three cold shock proteins in Table 3. The data point out that Sso7d is the most stable among these structurally similar proteins against both the temperature and GuHCl, even though there are other two hyperthermophilic proteins, Sac7d and Tm-Csp, in the set. To this regard, one has to note that Sso7d possesses an exceptional stability also against the denaturing action of pressure [17].

According to the thermodynamic and structural investigations on F31A-Sso7d [17–20], and the kinetic measurements on several mutant forms of Sso7d by Guerois and Serrano [47], it emerges that the residues constituting the hydrophobic core of the protein play a crucial role in order to render the native structure extra-stable against temperature, pressure and GuHCl. This does not contrast with the current idea that favourable electrostatic interactions among charged groups placed on the protein surface are the specific tool to cope with high temperature [48–50]; see also the percentage values of the ratio of the number of charged residues over the total for the SH3-like proteins listed in Table 3. In fact, the optimum placement of charges on the protein surface implies also the optimum burial of nonpolar groups to build up a compact hydrophobic core shielded from water contact [51]. The hydrophobic effect is the fundamental stabilizing interaction driving the folding of the polypeptide chain; when it is suitably coupled to favourable interactions on the surface, the native structure can become extra-stable.

## Acknowledgements

Work supported by grants from the Italian Ministry for the Instruction, University and Research (M.I.U.R., Rome).

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