Thermal and conformational stability of Ssh10b protein from archaeon *Sulfolobus shibatae*

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The secondary structure of the DNA binding protein Ssh10b is largely unaffected by change in temperature between 25 °C and 85 °C, indicating that the protein is highly thermostable. Here, we report the temperature-dependent equilibrium denaturation of Ssh10b in the presence of guanidine hydrochloride (GdnHCl). It was found that the transition midpoint values of the temperature (T_m), and changes of enthalpy (ΔH_m) and entropy (ΔS_m) of Ssh10b unfolding were linearly decreasing with increasing GdnHCl concentration. The true values of the thermodynamic parameters, $T_m = 402$ K, $\Delta H_m = 590 \pm 40$ kJ · mol⁻¹ and $\Delta S_m = 1.4 \pm 0.15$ kJ · T⁻¹ · mol⁻¹, were obtained by linear extrapolation to 0 M GdnHCl. The value of the heat capacity change of Ssh10b unfolding, $\Delta C_p = 3.8 \pm 0.2$ kJ · T⁻¹ · mol⁻¹ (approx. 19 J T⁻¹ ·

mol residue⁻¹), was obtained from the measured thermodynamic parameters. This is significantly smaller than that of the average value for mesophilic proteins (50 J · K⁻¹ · mol residue⁻¹) or the value calculated from the Ssh10b structural data (64 J T⁻¹ · mol residue⁻¹). A consequence of the small ΔC_p is that the ΔG of Ssh10b is larger than that of mesophilic proteins, while the values of ΔH and $T^* \Delta S$ are smaller. The small ΔC_p of Ssh10b appears to result mainly from the presence of compactness in the denatured state.

Key words: compact denatured state, enthalpy change, entropy change, equilibrium denaturation, hyperthermophilic protein, thermodynamic parameters.

INTRODUCTION

Hyperthermophiles constitute a group of micro-organisms with an optimum growth temperature of between 80 °C and 100 °C. They are generally found close to the root of the phylogenetic tree, which suggests that they preceded their mesophilic counterparts in the course of evolution [1–3]. Proteins isolated to date from the hyperthermophiles are composed of the 20 common amino acids. Furthermore, homologous hyperthermophilic and mesophilic proteins typically show 40–85 % sequence similarity, and their three-dimensional structures are superimposable [3]. Hyperthermophilic proteins show considerable thermal stability and thereby provide important models for the investigation of fundamental problems in structure biology, such as the stability and folding of proteins [4–7]. However, although a number of hyperthermophilic and thermophilic proteins have been purified and compared with their mesophilic counterparts, progress in understanding the physicochemical basis for the stability of extremely thermophilic proteins has been hampered by the very limited structural and thermodynamic data available [8]. Attempts to obtain thermodynamic information about thermostable proteins have been plagued by irreversibility of unfolding and/or inaccessibility of the unfolding reversion to physical measurement. Hence, studies of the thermodynamic stability of thermophilic and hyperthermophilic proteins have been reported for only a few select examples [9–25].

It has been shown that a higher transition temperature (T) in a hyperthermophilic protein could be obtained by three theoretical mechanisms: (1) by shifting the stability curve to increase the overall unfolding free energy at any temperature; (2) by decreasing the heat capacity change of unfolding (ΔC_p) between the fol-

ded and unfolded states to flatten the stability curve; or (3) by shifting the stability curve toward higher temperature [9,10]. Comparison of the change in free energy upon unfolding (ΔG) versus temperature (T) curves for mesophilic, thermophilic and hyperthermophilic proteins demonstrates that thermophilic and hyperthermophilic proteins use various combinations of these three mechanisms to reach their superior thermodynamic stabilities [3]. For example, the greater protein stability and resistance to higher temperature of Sac7d [11] and Sso7d [12] were found to stem from an increase in stability at all temperatures, with a broader maximum, as compared with their mesophilic homologues (mechanisms 1 and 2, above). The thermophilic homologue of phosphoglycerate kinase [9,10] is stabilized by a small ΔC_p (mechanism 2). The maximum stability of cytochrome *c*-552 [9,10] and cold-shock protein (CspTm) [13] is increased and shifted to higher temperature compared with their mesophilic homologues (mechanisms 1 and 3). In the case of glutamate dehydrogenase from the hyperthermophile *Thermotoga maritima* [15], interdomain interactions play a significant role in its enhanced stability (mechanism 1). Deutschman and Dahlquist [22] found that changes in both ΔH and ΔC_p are important contributors to the thermal stability of TmY as compared with BsY (mechanisms 1 and 2).

The free energy of stabilization, $\Delta G = \Delta H - T^* \Delta S$, of a protein is the difference between the free energy of the folded and the unfolded states of that protein, where $\Delta H = \Delta H^0 + \Delta C_p(T - T^0)$; $\Delta S = \Delta S^0 + \Delta C_p \ln(T/T^0)$; ΔH^0 and ΔS^0 are the standard enthalpy and entropy of unfolding at $T^0 = 298$ K respectively; and ΔC_p is the heat capacity change between the native and unfolded states of the system. The ΔC_p is relatively independent of temperature within the range 20–80 °C [26].

Abbreviations used: ΔASA_{ap} , the change in solvent-accessible surface area on unfolding of apolar groups; ΔASA_{pol} , the change in solvent-accessible surface area on unfolding of polar groups; cal, calculated; ΔC_p , change in heat capacity upon unfolding; DLS, dynamic light scattering; ex, experimentally determined; ΔG , change in free energy upon unfolding; GdnHCl, guanidine hydrochloride; ΔH , change in enthalpy upon unfolding; ΔH^0 , standard enthalpy of unfolding at $T^0 = 298$ K; IPTG, isopropyl β -D-thiogalactoside; ΔS , change in entropy upon unfolding (the addition of a subscript 'm' to any parameter, e.g. ΔS_m , indicates the transition midpoint value); ΔS^0 , standard entropy of unfolding at $T^0 = 298$ K.

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Thus, ΔH^0 , ΔS^0 and ΔC_p are the intrinsic parameters for describing the thermodynamic properties of protein folding. Furthermore, these three macroscopic thermodynamic parameters of protein folding have direct microscopic interpretations. ΔH^0 is the measurement of changes (at standard temperature 298 K) in packing forces of side chains, while ΔS^0 reflects the numbers of accessible conformational states between the native and the unfolded states, including the contribution of solvent. ΔC_p is the measurement of change in accessible surface area between the native and the unfolded states of the protein. A microscopic understanding of hyperthermophilic protein stability in terms of protein structure should compare the parameters ΔH^0 , ΔS^0 and ΔC_p of the hyperthermophilic protein with those of the mesophilic protein.

The DNA binding protein Ssh10b from the archaeon *Sulfolobus shibatae*, a member of the sac10b family, is conserved in most thermophilic and hyperthermophilic archaeal genomes that have been sequenced to date. The protein constitutes approx. 4–5 % of the cellular protein, binds double-stranded DNA without apparent sequence specificity and is capable of constraining negative DNA supercoils in a temperature-dependent fashion. This binding ability is weak at 25 °C, but is enhanced substantially at 45 °C or higher temperature. Ssh10b is a dimeric protein composed of two identical subunits. The monomer of Ssh10b consists of 97 amino acid residues with no disulphide bonds, and the protein is highly thermostable [27]. The crystal structure of Ssh10b reveals a mixed α/β structure comprising four β -strands and two α -helices [28]. In the present study, we report the temperature-dependent equilibrium denaturation of Ssh10b in the presence of guanidine hydrochloride (GdnHCl), monitored by CD spectra. We found that in the presence of GdnHCl, protein unfolding is fully reversible and the temperature of the unfolding transition becomes accessible to physical measurement. The thermodynamic parameters, ΔH_m , T_m and ΔS_m , for Ssh10b unfolding were found to decrease with increasing GdnHCl concentration. The true values for these thermodynamic parameters were obtained by linear extrapolation to 0 M GdnHCl. The value of ΔC_p of Ssh10b unfolding is $3.8 \pm 0.2 \text{ kJ} \cdot \text{T}^{-1} \cdot \text{mol}^{-1}$, which is significantly smaller than that of the average value for mesophilic proteins. Our results suggest that the small ΔC_p of Ssh10b results from the presence of compactness in the denatured state.

MATERIALS AND METHODS

Materials

GdnHCl was from Gibco BRL, IPTG (isopropyl β -D-thiogalactoside) from Merck, and foline phenol was from Sigma. The *ssh10b* gene was provided by Professor Jinfeng Wang (Institute of Biophysics, Academia Sinica, Beijing, China) and Professor Li Huang (State Key Laboratory of Microbial resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China) [27].

Protein purification

An overnight culture of *Escherichia coli* (BL21) cells containing the target plasmid was added to 1 litre of Luria–Bertani media plus ampicillin (60 $\mu\text{g}/\text{ml}$) and incubated at 37 °C with shaking at 220 r.p.m. At $D_{600} = 0.8$, expression was induced with 1 mM IPTG. After an additional 3 h at 37 °C, the cells were harvested by centrifugation (6000 g at 4 °C for 20 min). The cell pellet was then resuspended in 5 vol. of buffer (20 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 6.8, 1 mM EDTA) and the cells were sonicated on ice at 40 W

for 10 min. The lysate was centrifuged at 6000 g for 15 min at 4 °C. The supernatant was maintained at 80 °C for 20 min in a temperature controller (Huber CC130) and then centrifuged at 12 000 g for 15 min at 4 °C. Ammonium sulphate was added gradually to the supernatant to a final concentration of 70 % (w/v). After centrifugation at 6000 g for 30 min at 4 °C, the precipitate was dissolved in 5 ml of buffer A (20 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.5, 1 mM EDTA) and dialysed against the same buffer. After centrifugation at 13 500 g , the sample was applied to an UNO S6 column (Biorad), which had been equilibrated in buffer A. Proteins were eluted with buffer B (20 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.5, 1 M NaCl, 1 mM EDTA) [27]. The concentrated sample was then dialysed against buffer A. The purity of protein was verified by SDS/PAGE.

Unfolding studies

Unfolding of Ssh10b by GdnHCl was monitored by CD using a Jasco-J720 spectropolarimeter. The CD signal in the region 190–250 nm (far-UV CD) was monitored using a rectangular quartz cuvette with a pathlength of 1 mm. The temperature was increased from 298 K to 358 K, monitored using a thermometer attached to the thermosatted cell holder. The samples containing various concentrations of GdnHCl were equilibrated for 3–6 h before experiments were performed. Samples were heated at 1 °C per minute from 25 °C to 85 °C in a cell sealed with parafilm to prevent evaporation, while keeping the pressure relatively constant. At the end of each experiment the samples were cooled to room temperature to check the reversibility.

Analysis of the denaturation data

The thermodynamic properties of Ssh10b were calculated assuming a two-state denaturation process $N_2 \xrightleftharpoons{k} 2U$. Concentrations of the folded protein $[N_2]$ (in dimer units) and the unfolded protein $[U]$ (in monomer units) at different temperatures or denaturant concentrations were calculated [29]:

$$[N_2] = P_t/2 \times \frac{y - y_U + m_U T[D]}{y_N + m_N T[D] - y_U + m_U T[D]} \quad (1)$$

$$[U] = P_t \frac{y_N + m_N T[D] - y}{y_N + m_N T - y_U + m_U T[D]} \quad (2)$$

where P_t is the total protein concentration in monomer units; y is the experimentally measured signal value at a given temperature T or given denaturant concentration $[D]$; y_N and y_U are the intercepts; and m_N , and m_U are the slopes of the native and unfolded baselines, respectively. The apparent equilibrium constant (K) and the corresponding free energy change (ΔG) at temperature T or denaturant concentration $[D]$ were calculated according to:

$$K = \frac{2 \times P_t}{y_N + m_N T[D] - y_U + m_U T[D]} \times \frac{[y_N + m_N T[D] - y]^2}{y - y_U + m_U T[D]} \quad (3)$$

$$\Delta G = -RT \ln K \quad (4)$$

where R is the gas constant and T is the absolute temperature. According to the linear free energy model [30–33], the changes in free energy, enthalpy, and entropy changes that occur on unfolding are expected to vary linearly with the denaturant concentration, $[D]$:

$$\Delta G = \Delta G(\text{H}_2\text{O}) - m_G[D] \quad (5)$$

$$\Delta H = \Delta H(\text{H}_2\text{O}) - m_{\text{H}}[\text{D}] \quad (6)$$

$$\Delta S = \Delta S(\text{H}_2\text{O}) - m_{\text{S}}[\text{D}] \quad (7)$$

where $\Delta G(\text{H}_2\text{O})$, $\Delta H(\text{H}_2\text{O})$ and $\Delta S(\text{H}_2\text{O})$ represent the free energy, enthalpy and entropy changes of unfolding in the absence of denaturant; and m_{G} , m_{H} and m_{S} are the slopes of the transition for the free energy, enthalpy and entropy changes respectively. The midpoint of the transition (C_{m}), where 50% of the protein was unfolded, is a function of protein concentration P_{t} :

$$C_{\text{m}} = C_{\text{m}}^0 + \frac{RT \ln(P_{\text{t}})}{m_{\text{G}}} \quad (8)$$

where $C_{\text{m}}^0 = \frac{\Delta G(\text{H}_2\text{O})}{m_{\text{G}}}$ is the transition midpoint at protein concentration $P_{\text{t}} = 1$ M. For thermal unfolding, assuming that the heat capacity change (ΔC_{p}) between the native and unfolded states of the system is relatively independent of temperature within the range of the transition, then [34,35]:

$$\begin{aligned} \Delta G(T) &= -RT \ln(K) \\ &= \Delta H_{\text{m}} - T \Delta S_{\text{m}} + \Delta C_{\text{p}} \left((T - T_{\text{m}}) \right. \\ &\quad \left. + T \ln \left(1 - \frac{T - T_{\text{m}}}{T} \right) \right) \end{aligned} \quad (9)$$

where ΔH_{m} , and ΔS_{m} are the enthalpy and entropy changes, respectively, at the transition midpoint, where $T = T_{\text{m}}$. Within the transition range, where $T \ln(1 - \frac{T - T_{\text{m}}}{T}) \approx T_{\text{m}} - T$ from Equation 9 can be simplified to the van't Hoff plot:

$$\ln(K) = \frac{\Delta S_{\text{m}}}{R} - \frac{\Delta H_{\text{m}}}{RT} \quad (10)$$

The temperature of the transition midpoint (T_{m}) is a function of the protein and denaturant concentration, as follows:

$$T_{\text{m}} = \frac{\Delta H_{\text{m}}}{\Delta S_{\text{m}} - R \ln(P_{\text{t}})} = \frac{\Delta H_{\text{m}}(\text{H}_2\text{O}) - m_{\text{H}}[\text{D}]}{\Delta S_{\text{m}}(\text{H}_2\text{O}) - m_{\text{S}}[\text{D}] - R \ln(P_{\text{t}})} \quad (11)$$

Hydrodynamic radius measurements

Dynamic light scattering (DLS) measurements were performed at a protein concentration of 10 mg/ml at pH 6.8 at various concentrations of GdnHCl. Before the DLS measurements, the solutions were centrifuged at 14 000 *g* for 10 min. All experiments were performed at a 90° scattering angle on a DynaPro-LSR (Protein Solution, Charlottesville, VA, U.S.A.) at a wavelength of 781.8 nm. During the measurements, the temperature was maintained at 20 °C using a built-in temperature controller. The diffusion coefficient (D) was calculated from the autocorrelation function, using the accessory software DYNAMICS provided with the instrument. Since the structure of the protein does not change at GdnHCl concentrations lower than 4 M, and the viscosity of GdnHCl solutions differs significantly from that of pure water, the diffusion coefficient D was corrected not to pure water, but to 0.5 M GdnHCl. The experimentally measured hydrodynamic radius, R_{H}^{ex} , of the protein was determined from the Stokes–Einstein relationship [36]:

$$R_{\text{H}}^{\text{ex}} = \frac{kT}{6\pi\eta D} \quad (12)$$

Figure 1 CD spectra of 50 μM Ssh10b protein in phosphate buffer at pH 6.8

Line 1, native protein measured at 25 °C; line 2, native protein measured at 85 °C; line 3, denatured protein in 6 M GdnHCl measured at 25 °C; line 4, denatured protein in 6 M GdnHCl measured at 85 °C; line 5, Ssh10b protein denatured in 7 M GdnHCl and then refolded by 10-fold dilution to give final concentrations of 0.7 M for denaturant and 50 μM for protein; line 6, Ssh10b protein denatured in 3.5 M GdnHCl at 85 °C and then refolded by re-equilibrating to 25 °C and diluting 7-fold to give final concentrations of 0.5 M for denaturant and 50 μM for protein.

Here k is the Boltzmann's constant, T is the absolute temperature and η is the solvent viscosity. The hydrodynamic radius R_{H} of the protein was calculated using the native protein (in 0.5 M GdnHCl) as a reference molecule:

$$R_{\text{H}} = R_{\text{H}}^{\text{ex}} \frac{R_{\text{H}}^{\text{cal}}(N)}{R_{\text{H}}^{\text{ex}}(N)} \quad (13)$$

where $R_{\text{H}}^{\text{ex}}(N)$ is the experimentally determined hydrodynamic radius of the native protein and $R_{\text{H}}^{\text{cal}}(N)$ is the calculated hydrodynamic radius of the native protein dimer.

The theoretical hydrodynamic radius of the native and fully denatured protein were calculated according to the method of Wilkins et al. [36]:

$$R_{\text{H}}^{\text{cal}} = (4.75 \pm 1.11)N^{0.29 \pm 0.02} \quad (14)$$

for native protein (dimer) and:

$$R_{\text{H}}^{\text{cal}} = (2.21 \pm 1.07)N^{0.57 \pm 0.02} \quad (15)$$

for fully denatured protein (monomer), where N is the number of residues in the protein.

The compaction factor C is defined as [36]:

$$C = [R_{\text{H}}(\text{D}) - R_{\text{H}}]/[R_{\text{H}}(\text{D}) - R_{\text{H}}(N)] \quad (16)$$

where $R_{\text{H}}(\text{D})$ and $R_{\text{H}}(N)$ represent the predicted hydrodynamic radii of the fully denatured and native protein, respectively.

RESULTS

Denaturant-induced unfolding

Figure 1 shows typical CD spectra of the native protein, consistent with the native CD spectrum of Ssh10b reported previously [27], measured at 25 °C and pH 6.8 (Figure 1, line 1) and 85 °C (Figure 1, line 2). The spectra are nearly identical, indicating that the

Figure 2 GdnHCl induced unfolding curves

The concentration (P_1) of Ssh10b was 50 μ M at pH 6.8 at 15 °C ($\square$), 20 °C ($\circ$), 25 °C ($\diamond$), 45 °C ($\triangle$), 65 °C (∇), 75 °C ($\bullet$), 80 °C ($\blacksquare$) and 85 °C ($\blacktriangle$). The inset shows the protein concentration dependence of unfolding at 7 μ M ($\blacksquare$), 50 μ M ($\bullet$) and 70 μ M ($\blacktriangle$).

protein is thermally stable up to 85 °C. Upon incubation in 8 M urea for 24 h, Ssh10b underwent no reduction in backbone CD signal (results not shown), indicating that Ssh10b is resistant to urea-induced denaturation. At 25 °C and pH 6.8 in 6 M GdnHCl, Ssh10b loses approx. 80% of its CD signal (Figure 1, line 3). The CD spectrum of the denatured protein measured at 25 °C was found to undergo no further change when the concentration of GdnHCl was increased to 8 M, or when the pH was varied between 2 and 10 (results not shown). The fact that the CD spectra do not change with further changes in the denaturing conditions, suggests that the residual structure may not be native-like structure, but rather an ensemble of compact states. The CD signal of the 6 M GdnHCl denatured protein increases with increasing temperature (Figure 1 line 4), implying that there is more residual structure at higher temperature. A similar phenomenon was observed in denaturation of Sac7d [11], but the reason remains unclear.

GdnHCl-induced unfolding of Ssh10b was studied at pH 6.8, at several constant temperatures between 15 °C and 85 °C, by measuring far-UV CD at 222 nm (Figure 2). The transitions were characterized by a single sharp change in the ellipticity that looks like a typical two-state transition. The observed denaturation midpoint decreases with increasing temperature (Table 1), indicating a decrease in the free energy of unfolding with increasing temperature. As shown in the inset to Figure 2, protein concentration dependence of the transition curves was observed at GdnHCl concentrations between 3 and 4.5 M, but a much smaller protein concentration dependence was seen at GdnHCl concentrations higher than 4.5 M. The free energy of unfolding, $\Delta G(\text{H}_2\text{O})$, and the m value for the GdnHCl-induced unfolding curve were obtained from the fit to Equation 9. As shown in Figure 3, $\Delta G(\text{H}_2\text{O})$ and the m value increased slightly with increasing temperature between 15 °C and 45 °C, then reached a maximum, before starting to decrease with further increase in temperature.

Heat-induced unfolding in the presence of different concentrations of GdnHCl

As Ssh10b is thermally stable up to 85 °C (Figure 1, lines 1 and 2), the thermal unfolding of the protein was carried out in

Table 1 Thermodynamic parameters derived from GdnHCl denaturation for the conformational stability of Ssh10b

Protein concentration, $P_1 = 50 \mu\text{M}$.

T (K)	$\Delta G(\text{H}_2\text{O})$ ($\text{kJ} \cdot \text{mol}^{-1}$)	m_G ($\text{kJ} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$)	C_m^* (M)
288	128	20	4.9
293	131	21	4.85
298	126	23	4.3
318	137	24	4.19
338	102	21	3.5
348	83	19	2.93
353	55	12	2.12
358	49	11	1.68

* Calculated according to equation 8.

Figure 3 The unfolding free energy of Ssh10b (ΔG) versus temperature (T)

Inset shows the unfolding m value of Ssh10b versus temperature. The concentration (P_1) of Ssh10b was 50 μ M.

the presence of different concentrations of GdnHCl. At pH 6.8, increasing the temperature from 25 °C to 85 °C in the presence of GdnHCl results in unfolding of the protein by an apparent two-state mechanism (Figure 4). In certain cases, although the unfolding curve of a protein shows a single observable step, the transition may involve intermediates. Computer simulation of the Ssh10b experimental data could be fitted to both 2-state and 3-state equations within experimental error. However, the 3-state values of $\Delta G(\text{H}_2\text{O})$ and ΔH_m are only approx. 10% larger than those obtained by 2-state fitting. Thus, the choice of model does not significantly affect the thermodynamic parameters obtained. The values obtained by fitting to a two-state model were used in the subsequent analysis.

The van't Hoff plots (Figure 4, inset) demonstrate a good linear relationship between $\ln(K)$ and $1/T$ for each of the GdnHCl concentrations measured. The slope of the van't Hoff plot is $\Delta H_m/R$ and the intercept is $\Delta S_m/R$ (Equation 10). The values of ΔH_m and ΔS_m thus obtained are observed to decrease linearly with increasing GdnHCl concentration (Figure 5), as expected (Equations 6 and 7). Linear extrapolation to zero GdnHCl concentration gives the values $\Delta H_m = 590 \pm 40 \text{ kJ} \cdot \text{mol}^{-1}$ and $\Delta S_m = 1.4 \pm 0.15 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$.

The observed unfolding transition temperature, T_m , also appears to decrease linearly with increasing GdnHCl concentration

Figure 4 Temperature dependence of ellipticity at 222 nm at pH 6.8 of Ssh10b in the presence of different concentrations of GdnHCl

(a) 2.5 M GdnHCl, (b) 3.0 M GdnHCl, (c) 3.3 M GdnHCl, (d) 3.5 M GdnHCl, (e) 4.0 M GdnHCl, (f) 4.3 M GdnHCl, (g) 5.0 M GdnHCl, (h) 6.0 M GdnHCl, (i) 7.0 M GdnHCl. The inset shows the plots of $\ln(K)$ versus $1/T$.

Figure 5 The GdnHCl concentration dependence of the (A) enthalpy change, ΔH_m , (B) entropy change, ΔS_m , and (C) melting temperature, T_m , of Ssh10b

The broken line in (C) was calculated using Equation 11 with the values $\Delta H_m = 590 \text{ kJ} \cdot \text{mol}^{-1}$, $m_H = 104 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$, $\Delta S_m = 1.4 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ and $m_S = 0.26 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$.

(Figure 5C), giving an extrapolated value of $T_m = 402 \text{ K}$ (129°C) in the absence of denaturant. According to Equation 11, the dependence of the T_m value on denaturant concentration is expected to show curvature (broken line in Figure 5C). The T_m was also calculated according to Equation 9 using the following values: $\Delta H_m = 590 \text{ kJ} \cdot \text{mol}^{-1}$, $m_H = 104 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$, $\Delta S_m = 1.4 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$, $m_S = 0.26 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$ and $P_i = 50 \mu\text{M}$. The T_m value obtained by this calculation is 398 K , which is in good agreement with the value obtained by linear extrapolation

Figure 6 The reconstructed stability curves for Ssh10b and Arc dimmer

Solid lines are for Ssh10b (a–c) and broken lines are for Arc repressor (1–3). (a, 1) ΔG versus T , (b, 2) ΔH versus T , (c, 3) ΔS^*T versus T .

Figure 7 The GdnHCl concentration dependence of the hydrodynamic radius of Ssh10b

The protein concentration was 10 mg/ml at pH 6.8. The DLS measurement was performed at 20°C . The data shown are corrected for the solution viscosity.

(402 K). Thus, the results are not influenced by the model chosen. The value of the heat capacity change, $\Delta C_p = 3.8 \pm 0.2 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$, was obtained by fitting the $\Delta G(\text{H}_2\text{O})$ values to Equation 9 using $T_m = 398 \text{ K}$, $\Delta H_m = 590 \text{ kJ} \cdot \text{mol}^{-1}$ and $\Delta S_m = 1.4 \text{ kJ} \cdot \text{T}^{-1} \cdot \text{mol}^{-1}$.

There are no structural or thermodynamic data currently available for a mesophilic homologue of Ssh10b. Therefore, the Arc repressor was chosen as a suitable example of a typical mesophilic protein that has been well-characterized [37]. The Arc repressor is a dimeric DNA binding protein with 53 residues per monomer, and is thus the closest to Ssh10b in size, structure and function among the mesophilic proteins for which thermodynamic parameters have been determined. Figure 6 shows a comparison of the calculated stability curves for Ssh10b (solid lines) and Arc repressor (broken lines). (The plots of ΔG versus T , ΔH versus T and $T^* \Delta S$ versus T for Arc repressor were constructed from the published thermodynamic parameters [37] as follows: $T_m = 327 \text{ K}$, $\Delta H_m = 284 \text{ kJ} \cdot \text{mol}^{-1}$, $\Delta C_p = 6.4 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ and $\Delta S_m = 0.78 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$).

Hydrodynamic radius measurements in the presence GdnHCl

The hydrodynamic radius of Ssh10b as a function of GdnHCl concentration is shown in Figure 7. A single transition is observed in the range $4\text{--}5 \text{ M}$ GdnHCl, which is in good agreement with the CD results (Figure 2). The average hydrodynamic radius of the denatured protein is approx. 2.57 nm , which is significantly smaller than the predicted value of 3.05 nm (Equation 15). The

compaction factor (Equation 16) is approx. 57 %, indicating that the denatured state is non-random and compact, possibly an ensemble of compact structures.

Reversibility

Reversibility of GdnHCl-induced unfolding was confirmed by diluting protein denatured in GdnHCl concentrations of 2.5 M to 8 M and comparing the CD spectrum with that for the native protein. Figure 1 (line 5) shows a typical CD spectrum for the 10-fold dilution of 7 M GdnHCl denatured protein. Reversibility of heat-induced unfolding in the presence of GdnHCl was confirmed by comparing the CD signal at 222 nm after cooling the samples to 25 °C. Figure 1 (line 4) shows a typical CD spectrum after heat-induced unfolding at 85 °C in the presence of 3.5 M GdmHCl, followed by re-equilibration at 25 °C and 7-fold dilution. The refolded protein after thermal cycling was found to be identical to native protein by SDS/PAGE, implying that no deamidation occurred during the thermal cycling.

DISCUSSION

The function of the Ssh10b protein is to compact and regulate the availability of the genetic material in thermophilic archaea at their growth temperature, which is as high as 85 °C. The protein is very stable at ambient temperature ($\Delta G = 126 \text{ kJ} \cdot \text{mol}^{-1}$ at 25 °C) and shows more typical stability at high temperature ($\Delta G = 49 \text{ kJ} \cdot \text{mol}^{-1}$ at 85 °C). The protein has a weak ability to constrain DNA in negative supercoils at 25 °C, whereas this ability is greatly increased at 85 °C, supporting the speculative concept that there is a trade-off between stability and activity [26,38,39]. Our results demonstrate that the Ssh10b protein uses a combination of two mechanisms to increase its thermodynamic stability: (1) the free energy of the Ssh10b protein is higher than that of a typical mesophilic protein with similar functional properties (such as Arc repressor) at any temperature, and 2) the stability curve of the Ssh10b protein is more flat than that of a typical mesophilic protein.

Ssh10b is a dimer in solution; some dimeric proteins show a 3-state denaturation curve, with population of monomeric or dimeric intermediates, while others follow a 2-state model [29]. The experimental data suggest that the unfolding of Ssh10b follows a 2-state transition, implying that the monomeric subunits of Ssh10b are not significantly populated under the conditions of the denaturation experiments. The X-ray structure shows that the monomer of Ssh10b is stabilized by several salt bridges and an ion-pair network, all of which are on the 'back' of the Ssh10b dimer. In the process of GdnHCl denaturation, the dissociation of the dimer would expose these ion-pair residues to solution and destroy the interactions. This may explain why the monomeric subunits are not significantly populated during equilibrium denaturation in GdnHCl.

Protein amino acid composition has long been thought to be linked to thermostability. Statistical analysis comparing amino acid composition in mesophilic and thermophilic proteins indicate trends toward substitutions such as glycine $\rightarrow$ alanine and lysine $\rightarrow$ arginine [3]. The amino acid composition of Ssh10b shows such trends to only a minor extent: the protein consists of approx. 6.7 % alanine and 8.2 % arginine, compared with the average values for mesophilic proteins of 8.0 % and 4.3 % respectively [3]. The known Ssh10b homologues share 35 % to 60 % identity and 55 % to 82 % similarity at the amino acid sequence level [27]. Amino acid composition and sequence appears not to be the most important stabilizing factor in the case of Ssh10b.

It has also been suggested that protein stability is determined by the stability and tight packing of the hydrophobic core. The helices of thermophilic proteins are observed to contain a lower content of β -branched residues (valine, isoleucine and threonine), which leads to greater stability of the helices of thermophilic proteins compared with those of mesophilic proteins [40]. The structure of the Ssh10b monomer reveals a mixed α/β structure comprising four β -strands and two α -helices (Protein Database code 1h0x). The two α -helices in Ssh10b consist of approx. 15 % valine, 7.7 % threonine and 7.7 % isoleucine, similarly to the β -branched residue content of mesophilic proteins, suggesting that this is not a stabilization mechanism in the case of Ssh10b also. On the other hand, the Ssh10b structure is stabilized by four salt bridges and an ion-pair network involving four residues, and thus the number of ion pairs in Ssh10b is clearly higher than the average value, which is approx. 5 per 150 residues of protein [41]. This is a common feature of proteins from hyperthermostable organisms, and appears to be an important contributing factor to the remarkable stability of Ssh10b.

The free energy of stabilization, ΔG (where $\Delta G = \Delta H - T^* \Delta S$), of a protein is the difference between the free energy of the folded and the unfolded states of that protein. As a protein folds it loses considerable entropy, which must be balanced by a gain of enthalpy for the free energy to favour folding. It is interesting to note that the ΔG per residue of Ssh10b is higher than that of the mesophilic Arc repressor protein at any temperature, but the values of ΔH and ΔS per residue of Ssh10b are lower than these for the mesophilic protein at temperatures higher than 298 K. This is because the ΔC_p ($19 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1} \cdot \text{residue}^{-1}$) of Ssh10b is significantly smaller than that for the Arc repressor or for mesophilic proteins in general (the average value is around $50 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1} \cdot \text{residue}^{-1}$ [42]). As a consequence of the small ΔC_p , the rate of increase in ΔH and ΔS of Ssh10b with increasing temperature is more gradual than for mesophilic proteins, especially in the case of the ΔS values. This means that the high thermal stability of the Ssh10b protein is due mainly to a low ΔC_p value, as has been observed for other hyperthermophilic proteins [8].

In general, the native state of a protein has many of its hydrophobic side chains shielded from water because they are packed within the hydrophobic core. Conversely, the hydrophobic side chains of the denatured protein are exposed to solvent and the water molecules stack around these in 'icebergs'. This results in a decrease in the entropy of the denatured state and compensates partly for the conformational entropy decrease associated with protein folding. The ΔC_p of unfolding principally reflects the melting of these icebergs. It has been shown that the ΔC_p of unfolding is described by [43,44]:

$$\Delta C_p = 0.45 \times \Delta ASA_{\text{ap}} - 0.26 \times \Delta ASA_{\text{pol}} \text{ (cal} \cdot \text{degree}^{-1} \cdot \text{mol}^{-1}) \quad (17)$$

where ΔASA_{ap} and ΔASA_{pol} are the change in solvent-accessible surface area on unfolding, for given apolar and polar groups respectively. The Dictionary of protein secondary structure: pattern (DSSP) file of Ssh10b (1h0x.dssp) [45] gives a ΔASA_{ap} value of $8952 \text{ \AA}^2$ ($1 \text{ \AA} = 0.1 \text{ nm}$) and a ΔASA_{pol} value of $3409 \text{ \AA}^2$, giving a ΔC_p of $64.7 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1} \cdot \text{residue}^{-1}$. The ΔASA values shown refer to the dimer-to-coil transition, which assumes a 2-state transition. However, as the area of the dimer interface ($1150 \text{ \AA}^2$ [28]) is only approx. 10 % of the total surface area, population of the folded monomer during denaturation would not affect the conclusions drawn.

The calculated ΔC_p is significantly larger than the measured value. In general, a low ΔC_p value reflects that the hydrophobic

side chains in the native and the denatured states of the protein are similarly accessible to the solvent water molecules. A measured ΔC_p value lower than predicted implies either that some hydrophobic side chains remain relatively solvent-exposed in the native state or remain relatively buried in the denatured state. When hydrophobic side chains in the native protein structure are accessible to solvent water molecules, this implies the presence of a cavity filled by water molecules within the core of the folded protein. This is unlikely to be the basis of the low value of ΔC_p for Ssh10b for the following reasons. (1) The cavity would need to be unrealistically large (approx. 2/3 total surface area according to Equation 1). (2) Increasing the temperature should allow the water molecules to escape from the cavity, which would perturb the protein structure. In contrast, the CD spectrum of Ssh10b indicates that no structural change takes place when the temperature is increased from 25 °C to 85 °C. (3) If the native state of Ssh10b contained ordered water molecules, then some of the entropic compensation of iceberg formation in the denatured state would be lost and a high ΔS of unfolding would be predicted. As the ΔS for Ssh10b is in fact lower than for the mesophilic Arc repressor, this therefore cannot explain the lower ΔC_p of the thermophilic protein.

As the presence of a water-filled cavity within the Ssh10b structure seems unlikely, the low ΔC_p is best explained by the presence of residual structure in the denatured state of Ssh10b. A compact denatured state would explain why the ΔC_p value is low both in comparison to the average value for all mesophilic proteins and in comparison to the value calculated from the Ssh10b structural data. Experimental evidence in support of a compact denatured state ensemble for Ssh10b are as follows. (1) The CD spectra of denatured Ssh10b indicates compactness of the denatured protein. (2) The compaction factor calculated from the hydrodynamic radius measurements is approx. 57%, indicating that the denatured state is non-random and compact, possibly an ensemble of compact structures. (3) A compact denatured state predicts that the folding entropy change, ΔS , should be smaller than that for a typical mesophilic protein, agreeing with the experimental observations. (4) As discussed above the number of ion pairs in Ssh10b is clearly higher than the average value. This is expected to lead to a high enthalpy of folding, ΔH^0 (298 K). However, the ΔH^0 (298 K) for Ssh10b is smaller than that of a typical mesophilic protein. This may reflect the presence of stabilizing interactions in the ensemble of compact structures that represent the denatured state.

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