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Thermal Unfolding of the DNA-binding Protein Sso7d from the Hyperthermophile *Sulfolobus solfataricus*

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Thermal unfolding of the small hyperthermophilic DNA-binding protein Sso7d was studied by circular dichroism spectroscopy and differential scanning calorimetry. The unfolding transition can be described by a reversible two state process. Maximum stability was observed in the region between pH 4.5 and 7.0 where Sso7d unfolds with a melting temperature between 370.8 to 371.9 K and an unfolding enthalpy between 62.9 and 65.4 kcal/mol. The heat capacity differences between the native and the heat denatured states obtained by differential scanning calorimetry (620 cal/(mol K)) and circular dichroism spectroscopy (580 cal/(mol K)) resulted in comparable values. The thermodynamic reason for the high melting temperature of Sso7d is the shallow stability curve with a broad free energy maximum, corresponding to the relatively small heat capacity change which was obtained. The calculated stability curve shows that Sso7d has, despite of its high melting temperature, an only moderate intrinsic stability, which reaches its maximum ($\approx$7 kcal/mol) at 282 K. Sso7d is particularly poorly stabilized ($\approx$1 kcal/mol) at the maximum physiological growth temperature of *Sulfolobus solfataricus*. Sso7d has furthermore untypically low specific enthalpy (0.99 kcal/(mol residue)) and entropy (2.99 cal/(mol K)) values at convergence temperatures. No significant differences in thermal stability of the partially methylated Sso7d from *Sulfolobus solfataricus* and the cloned non-methylated form of the protein expressed in *Escherichia coli* were observed.

Keywords: thermostability; thermal unfolding; Sso7d, *Sulfolobus solfataricus*; differential scanning calorimetry; CD-spectroscopy

Introduction

A steadily increasing number of hyperthermophilic prokaryotes have recently been isolated from geothermally heated environments, which grow at or above the boiling point of water and under extreme pH conditions (Stetter *et al*., 1990). Proteins expressed in these organisms exhibit properties of particular importance for industrial processes and are suitable model systems to study protein stability and thermotolerance. A large number of thermostable proteins have been purified and compared to their mesophilic counterparts (Adams, 1993). However, insights into the stability of extremely thermophilic proteins have been hampered due to very limited structural and thermodynamic data available. In spite of numerous attempts to achieve a fundamental understanding of extreme protein thermostability, it is not sufficiently clear which energetic and kinetic mechanisms are responsible for the thermal adaptation of proteins from hyperthermophiles. It is a common opinion that the stabilization of thermophilic proteins results from an appropriate combination of the weak non-covalent forces and protein solvent interactions which are also typical for mesophilic proteins.

Several characteristics of Sso7d make it an attractive model for protein stability studies at high temperatures: Sso7d is a single domain protein which behaves as a monomer even at high protein concentrations, it contains no metal binding sites and disulphide bridges. It can be purified in sufficiently large amounts and its three-dimensional structure is known (Baumann *et al*., 1994). Sso7d is highly expressed in the hyperthermophilic archaeon *Sulfolobus solfataricus*. It belongs to a

Abbreviations used: DSC, differential scanning calorimetry; CD, circular dichroism.

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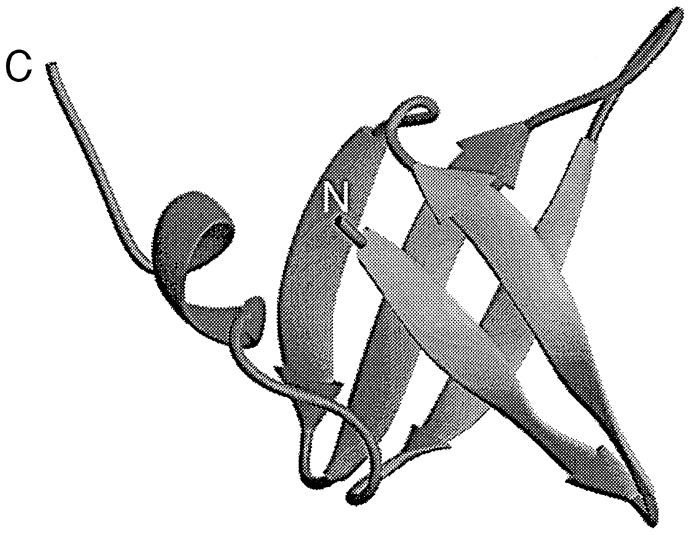
family of small basic proteins which probably play a role in the structural organization of DNA into a chromatin-like structure and which increase the melting temperature of DNA by non-specific binding. These so-called histone-like proteins share physical properties with eukaryotic histones but have no sequence homology (Dijk & Reinhardt, 1986; Drlica & Rouviere-Yaniv, 1987; Travers *et al*., 1994).

The family of small histone-like proteins in *Sulfolobus* can be classified according to their molecular weights into groups around 7000, 8000 and 10,000 daltons (Dijk & Reinhardt, 1986; Kimura *et al*., 1984; Grote *et al*., 1986; Choli *et al*., 1988a). The 7 kDa class of histone-like proteins from *Sulfolobus acidocaldarius* has been described first. It consists of five very similar proteins which have been named 7a to 7e according to their relative basicity (Choli *et al*., 1988a). However, it was recently shown that there are only two different genes responsible for the expression of these proteins called Sac7d and Sac7e (McAfee *et al*., 1995). In *Sulfolobus solfataricus* only Sso7d has been described as a member of the 7 kDa histone-like protein family so far (Choli *et al*., 1988b). The 64 residues long sequence of Sso7d is very similar to the Sac7 protein family from *S. acidocaldarius*, extremely basic and very rich in lysine residues (Figure 1).

Another interesting feature of Sso7d and Sac7 is the partial ϵ-mono-methylation of some of the lysine residues (Choli *et al*., 1988a,b). Mono-methylation of proteins has been detected in several other proteins present in *Sulfolobus*. Several authors have discussed a possible involvement in protein stabilization (Maras *et al*., 1992; McAfee *et al*., 1995; Baumann *et al*., 1994; Choli *et al*., 1988a). McAfee *et al*. (1995) have reported that the T_m value of methylated Sac7d at pH 4 is 6°C higher than the T_m value of the cloned non-methylated protein.

The tertiary structure of Sso7d, which has recently been determined by NMR spectroscopy (Baumann *et al*., 1994), comprises a triple-stranded β-sheet onto which a double-stranded sheet is packed and a small α-helix at the C terminus (Figure 1), with a similar topology to eukaryotic Src-homology 3 (SH3) domains. Similar tertiary structure motifs have also been found in the cyanobacterial photosystem I complex (Falzone *et al*., 1994) and in the biotin biosynthetic operon repressor (BirA) from *Escherichia coli* (Wilson *et al*., 1992). It is striking that this folding motif has emerged very early in evolution and has been conserved in all three primary domains of life: Eucarya, Bacteria and Archaea (Woese *et al*., 1990). The tertiary structure of Sac7 has the same fold as Sso7d except for a C-terminal extension of the α-helix (Edmondson *et al*., 1995).

Our main goal in this work was to describe the thermal stability of Sso7d in the acidic region quantitatively. We have investigated two forms of Sso7d, the ϵ-mono methylated protein purified from *Sulfolobus solfataricus* (m-Sso7d) and the cloned non-methylated (c-Sso7d) using circular dichroism (CD) spectroscopy and differential scanning calorimetry (DSC). Both forms of the protein are highly soluble and unfold reversibly in

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                    10        20        30        40        50        60
Sso7d[a]  - A T V K F K Y K G E E K Q V D I S K I K K V W R V G K M I S F T Y D E G G G K T G R G A V S E K D A P K E L L Q M L - E - - K Q K K
Sac7d[b]  M V K V K F K Y K G E E K E V D I S K I K K V W R V G K M V S F T Y D D N - G K T G R G A V S E K D A P K E L L D M L A R A E R E K K
Sac7e[c]  M A K V R F K Y K G E E K E V D I S K I K K V W R V G K M V S F T Y D D N - G K T G R G A V S E K D A P K E L M D M L A R A E - K K K
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Figure 1. Secondary structure elements of Sso7d generated with the program RIBBON (Carlson, 1991) and alignment of Sso7d with Sac7d and Sac7e from *S. acidocaldarius*. The partially methylated lysine residues are marked with bold letters.

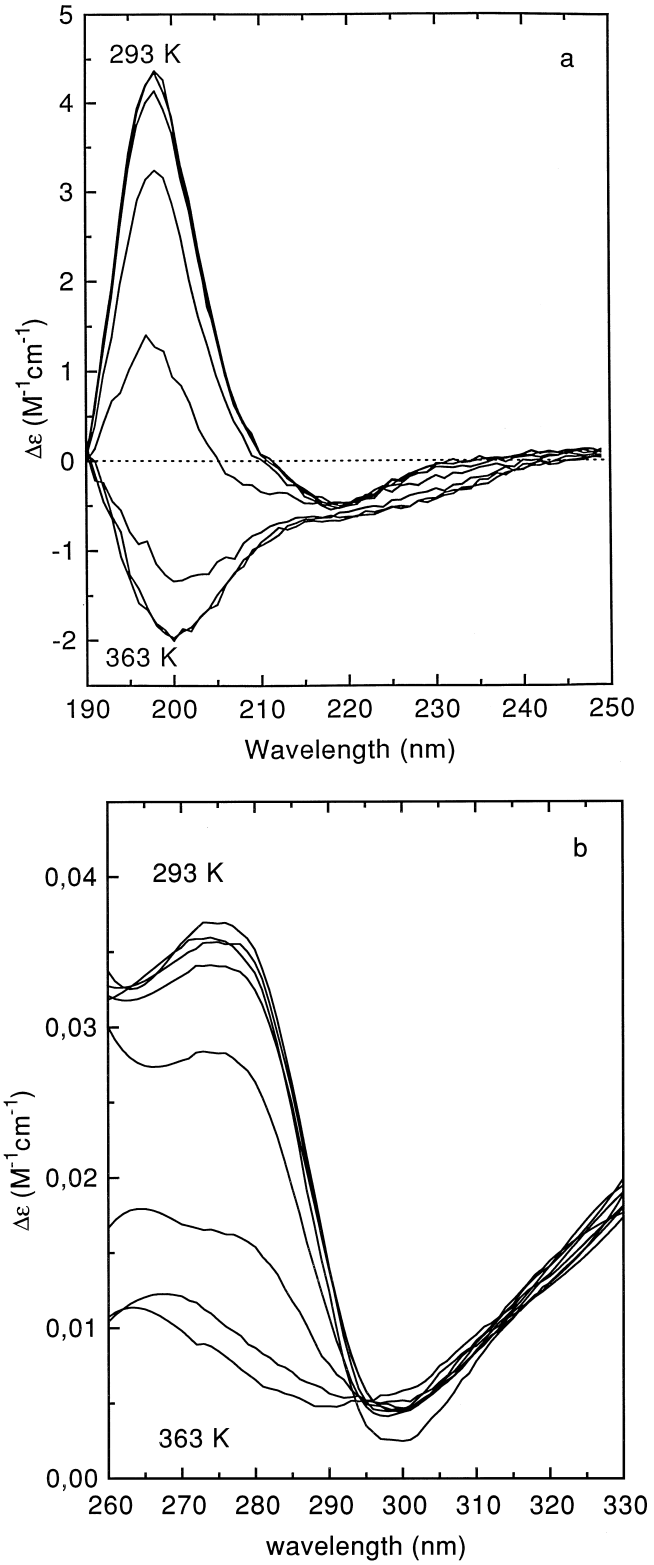

Figure 2. Temperature dependence of CD-spectra of Sso7d at pH 2.5. Spectra were recorded using a 0.1 cm cuvette in the temperature range between 298 and 368 K in 10 K steps and a two seconds averaging time in 0.5 nm intervals. The molar circular dichroism $\Delta\epsilon$ (M^{-1} cm^{-1}) per peptide bond is displayed. The spectra were recorded in 5 mM sodium phosphate buffer (pH 2.5). a, Region of the CD spectra reporting changes in the secondary structure (190 to 250 nm). The protein concentration was 0.2 mg/ml. b, Region of the CD spectra reporting changes in the environment of aromatic residues (260 to 330 nm), thus representing changes in the tertiary structure. The protein concentration was 2.5 mg/ml.

the acidic region. Our analysis indicated that Sso7d has a melting temperature near the boiling point of water with a T_m of 371.5 K at neutral pH, but is only moderately stabilized at the relevant physiological temperature in terms of the free energy of unfolding. No changes of T_m due to methylation of lysine residues were detected.

Results

We have characterized the thermal unfolding of Sso7d using DSC and CD-spectroscopy. Our analysis was based on the assumptions, that (1) the unfolding transition is a reversible two state process and (2) ΔC_p is independent of temperature within the experimental accuracy (Griko & Privalov, 1992; Pace & Laurents, 1989). We performed a series of experiments to demonstrate that these assumptions are indeed valid.

Experimental conditions

Two state transition

The presence of only two states in the thermal unfolding transition is supported by isodichroic points in CD spectra collected at different temperatures in the thermal transition region. The isodichroic points obtained here are shown in Figure 2a and b where a set of spectra collected in the temperature range between 298 K and 368 K are displayed. Two isodichroic points are clearly present at 292 nm and 190 nm.

The independence of the unfolding transition of the wavelength indicates that the secondary structure elements monitored at 200 nm (Figure 3a) break down together with the tertiary structure (275 nm; Figure 3b) without the presence of any detectable intermediates. The fitted functions (equation (1)) of these data resulted in indistinguishable values for $T_m = 342.5(\pm 0.34)$ K and $\Delta H_m = 39.6(\pm 0.4)$ kcal/mol. This is graphically demonstrated in the normalized plot of the two sets of data (Figure 3c). The data shown in Figure 3a are representative for all data collected in the spectral region at 200 nm reporting secondary structure.

The two state nature of the thermal unfolding transition could also be demonstrated by differential scanning calorimetry. The experimental data of the thermal scans shown in Figure 5c fit perfectly a two state model.

Reversibility

CD spectra recorded of Sso7d before and after thermal denaturation in the acidic region were identical, indicating the reversibility of the unfolding transition (Figure 4a). However, if the sample was kept for a longer time in the denatured state like in the slow up- and down-scan, shown in

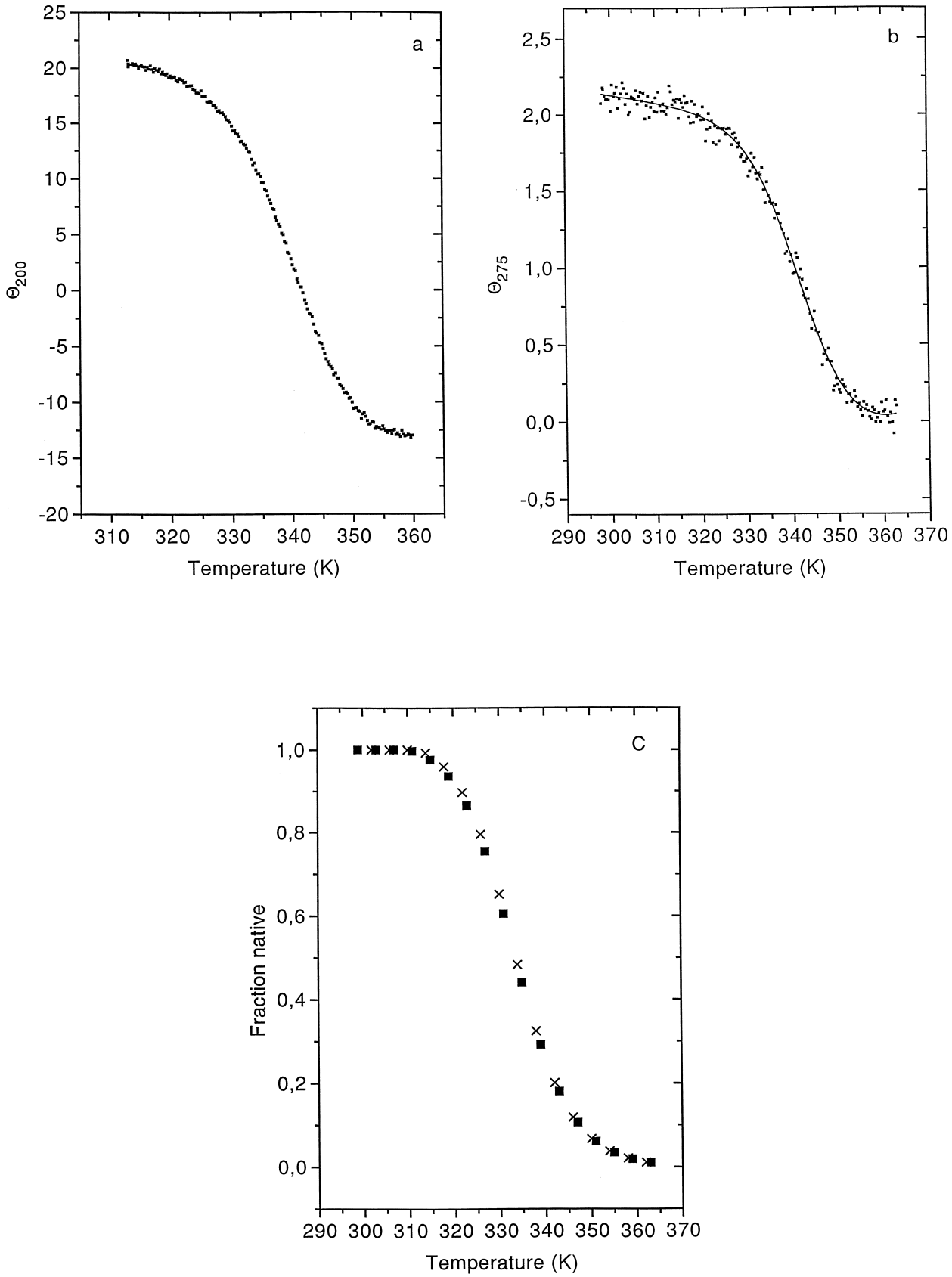

Figure 3. Thermal denaturation of Sso7d measured with CD-spectroscopy at different wavelengths at pH 2.5 using 5 mM sodium phosphate buffer. Only every tenth datapoint is displayed. a, Raw data measured at 200 nm. A protein concentration of 0.2 mg/ml was used. b, Raw data measured at 275 nm and fitted function (equation (1)). A protein concentration of 2.5 mg/ml was used. c, Normalized fitted function calculated from data measured in a (x) and b (■).

Figure 4b (40 K/hour), a small percentage of the protein denatured irreversibly probably due to aggregation. The irreversible processes which took place at high temperatures after the thermal transition did not affect the enthalpy and T_m evaluated from CD-measurements since fitting the data in Figure 4b to equation (1) resulted in a ΔH_m of 39.8 kcal/mol with a T_m of 342.3 K, whereas the data of the back scan of the same sample resulted in an enthalpy of 40.6 kcal/mol with a T_m of 341.9 K.

Similar results were obtained by differential scanning calorimetry (Table 1). The ratio $\Delta H^{cal}/\Delta H^{vH}$ at a scan rate of 60 K/hour was between 0.92 and 0.95 which is consistent with the assumption of a reversible two state unfolding of Sso7d. Typical values for the reversibility of the unfolding transitions for both proteins (c-Sso7d and m-Sso7d) were between 90 and 98%, which was judged by comparisons of the enthalpies of two subsequent temperature scans. The unfolding transition was not completely reversible under slower temperature scan conditions (40 K/hour) and some protein denatured irreversibly during each scan whereas the midpoint temperature of the unfolding transition remained constant in all scans.

The reversibility of the unfolding transition at neutral pH was still high (90%) but decreased dramatically above pH 7.5. The unfolding transition was 100% irreversible at pH 8.0 due to aggregation (not shown).

Since the reversibility of the transition decreased when Sso7d was incubated for a long time at high temperatures, the scan rate needed to be optimized for the CD and DSC experiments. The effects of different scan rates are compared in Table 1. A scan rate of 50 to 60 K/hour was found to be optimal for both DSC and CD spectroscopy.

Concentration dependence

The enthalpy as well as the midpoint of denaturation (T_m) was found to be independent of the protein concentration over a wide range (Figure 6), indicating that the transition is mono molecular and aggregation did not affect the measurements reported.

Buffer dependence

Changes in protonation associated with the thermal unfolding of a protein might give rise to additional heat effects due to the ionization heat of the buffer used in calorimetric measurements. This effect was shown to be relatively small for phosphate and carboxylic acid buffers, where the heat of ionisation is close to zero under the condition used in the calorimetric experiments (Christensen *et al*., 1976). This is not true for buffers carrying amino groups like Tris/Cl^-. Furthermore, the stability of a protein could depend on the buffer used (Johnson *et al*., 1992). We have investigated the thermal unfolding of c-Sso7d in a variety of buffers to rule out buffer specific effects. Data from these experiments are summarized in Table 2. In the acidic region, temperature scans at pH 2.5 using sodium acetate and phosphate buffer were compared and shown to be indistinguishable within the experimental error. At pH 6.5, sodium phosphate, Tris/Cl^- and Mes/K^+ were used and were also found to have little effect on T_m or ΔH^{cal}. Thus, under the chosen conditions we did not observe pronounced buffer effects on the enthalpy, stability or reversibility of the unfolding transition of Sso7d.

Thermal unfolding

Non-methylated Sso7d (c-Sso7d)

Thermal denaturation curves were recorded at 200 nm and at a protein concentration of 0.2 mg/ml at selected pH values between 2.5 and 3.5 in 5 mM sodium phosphate buffer using CD-spectroscopy (Table 3). Despite the limited pH range we observed a considerable difference in T_m from 337.4 K at pH 2.5 to 365.4 K at pH 3.5. Above pH 3.5 the denaturation temperature of Sso7d was too high to be precisely measured in aqueous solution. Scans at pH values higher than 3.5 resulted in short and noisy baselines of the denatured state and were therefore not included in this study.

Thermal unfolding of Sso7d in the pH region $2.5 < pH < 7$ was studied by DSC with a calorimeter equipped to function up to a temperature of 400 K (130°C). Representative DSC measurements (raw data as well as a fit to a two state model) are shown in Figure 5. The heat capacity change at constant pressure, ΔC_p was directly obtained from the individual thermal scans (Table 3) and from the slope of plots of ΔH_m *versus* T_m (Figure 7) (Privalov & Khechinashvili, 1974; Pace & Laurents, 1989).

ΔC_p upon denaturation for c-Sso7d determined from CD data was 580 cal/(mol K). This value is in good agreement with a ΔC_p of 620 cal/(mol K) determined by DSC (Figure 7).

Methylated Sso7d (m-Sso7d)

Methylation of lysine residues has been reported for several proteins in *Sulfolobus* (Maras *et al*., 1992; McAfee *et al*., 1995; Baumann *et al*., 1994; Choli *et al*., 1988a) and some authors have discussed an increase in thermotolerance caused by partial ϵ-monomethylation of some of the lysine residues. To investigate the influence of lysine methylation on the melting temperature of Sso7d, m-Sso7d was purified from *Sulfolobus solfataricus* and was compared with the non-methylated c-Sso7d using CD-spectroscopy and DSC. Both proteins were dialysed against identical buffers in the same beaker.

Data were measured at several pH values and were included for a direct comparison in Table 3 and Figures 7 and 8. The comparison of the denaturation enthalpies and temperatures at the transition midpoint revealed no significant difference between the methylated and non-methylated forms of the protein. Heat capacity changes

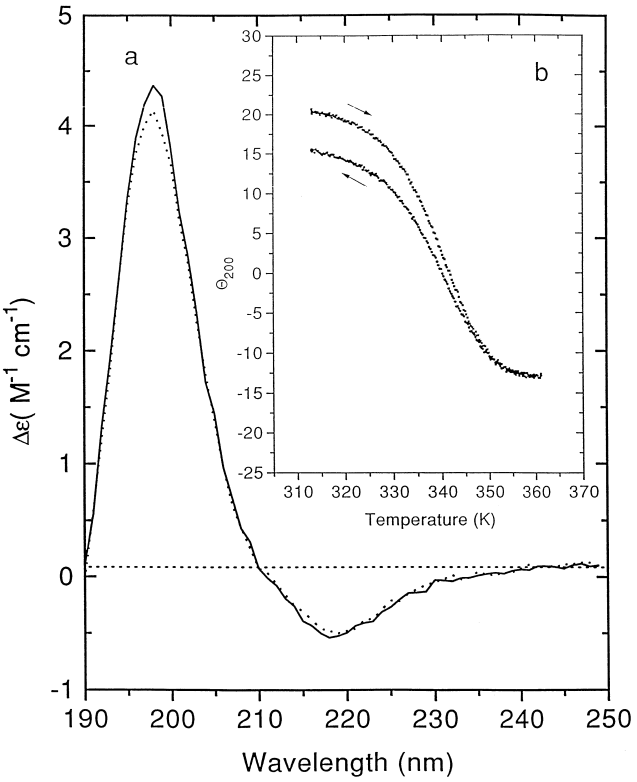

Figure 4. Reversibility of the c-Sso7d unfolding transition in the acidic region (pH 2.5) using 5 mM sodium phosphate buffer. a, CD-spectra of Sso7d before (continuous line) and after (dotted line) the sample was rapidly heated (600 K/hour) to a final temperature of 368 K. The spectra were recorded at 298 K. b, CD spectra recorded at 200 nm using a scan rate of 40 K/hour. A temperature up and downscan is shown and indicated by arrows.

calculated for the methylated form of Sso7d were also comparable with those determined for the non-methylated protein.

DSC scans of methylated Sso7d did not differ in T_m and ΔH^{cal} from the cloned non-methylated protein (Figures 7 and 8). Thus, based on our study of Sso7d, it appears that lysine methylation is not a general mechanism used by *Sulfolobus* to increase the denaturation temperature of endogenous proteins.

Discussion

The energetics of thermal unfolding of the DNA binding protein Sso7d was studied by CD spectroscopy and differential scanning microcalorimetry. Initial calorimetric studies have been reported for several proteins from the hyperthermophilic Archaeons *Pyrococcus furiosus* (Klump *et al*., 1992) and *Sulfolobus acidocaldarius* (McAfee *et al*., 1995). To our knowledge, this work represents the first quantitative thermodynamic characterization of a protein from a hyperthermophilic Archaeon.

The thermodynamic analysis of the thermal unfolding process shows unambiguously that, under the chosen experimental conditions, Sso7d behaves as a single-domain protein. Its structure unfolds in an essentially two-state process without any detectable intermediates. We have, however, made the observation that the calorimetric C_p endotherms become asymmetric, when Sso7d is heated at pH > 7.0 and at high ionic strength. It is possible that under these conditions the distortion of the measured heat capacity peak is caused by a nonspecific aggregation. At pH > 8.0, the unfolding process is irreversible due to aggregation, which restricted our investigation to the acidic region. Maximum stability was observed in the pH region between pH 4.5 and 7.0 where the unfolding of Sso7d is only moderately dependent on pH and ionic strength. In this pH region, DSC revealed averaged values of $T_m = 371.5$ K and $\Delta H^{cal} = 63.3$ kcal/mol.

The heat capacity difference between the native and the denatured state was determined directly from individual calorimetric scans, as well as from plots of ΔH_m *versus* T_m. As shown in Table 3, there is a significant spreading of ΔC_p. On the contrary, the plot $\Delta H_m/T_m$ (Figure 7) is essentially linear (R-value 0.98), suggesting that ΔC_p is a constant in the temperature range studied. Such a discrepancy between ΔC_p obtained calorimetrically and assessed from ΔH_m *versus* T_m plots has been analysed earlier (Liu & Sturtevant, 1996). These authors have pointed out that ΔC_p depends strongly on the circumstances under which the variation of ΔH^{cal} with respect to T_m is observed. ΔC_p determined by this way is sensitive to inaccuracies in baseline determinations, whereas the enthalpy, as the integral over the whole curve, is less prone to this inaccuracy. The average ΔC_p calculated from individual scans (633 cal/(mol K)) is comparable to a ΔC_p obtained by a linear fit of a ΔH_m *versus* T_m plot, suggesting, that at least for this study, the baseline inaccuracy is the likely reason for the observed discrepancy. Interestingly, the dependence of ΔH_m on T_m remains linear in the entire temperature range which was experimentally accessible (Figure 7). It is a common opinion based on investigations of mesophilic proteins (Griko & Privalov, 1992; Pace & Laurents, 1989) that the linearity holds to about 350 K. Above this temperature ΔH_m is expected to converge to a certain value, thus forcing ΔC_p to tend to zero. Experimental evidence, that enthalpy functions deviate from linearity at higher temperature was provided by a study by Wintrode *et al*. (1994). However, our study shows that the upper limit of the temperature range, where ΔC_p is constant is extended in the case of Sso7d by about 20 K up to 370 K.

The accuracy of ΔC_p determinations using CD spectroscopy or DSC is considered to be around 10%. Thus, the heat capacity changes upon unfolding of 580 cal/(mol K) determined by CD-spectroscopy and 620 cal/(mol K) measured by DSC are statistically indistinguishable. The calorimetric measurements were made over a larger temperature interval (almost 30 K). Therefore the value of 620 cal/(mol K) was used for all further calculations discussed below.

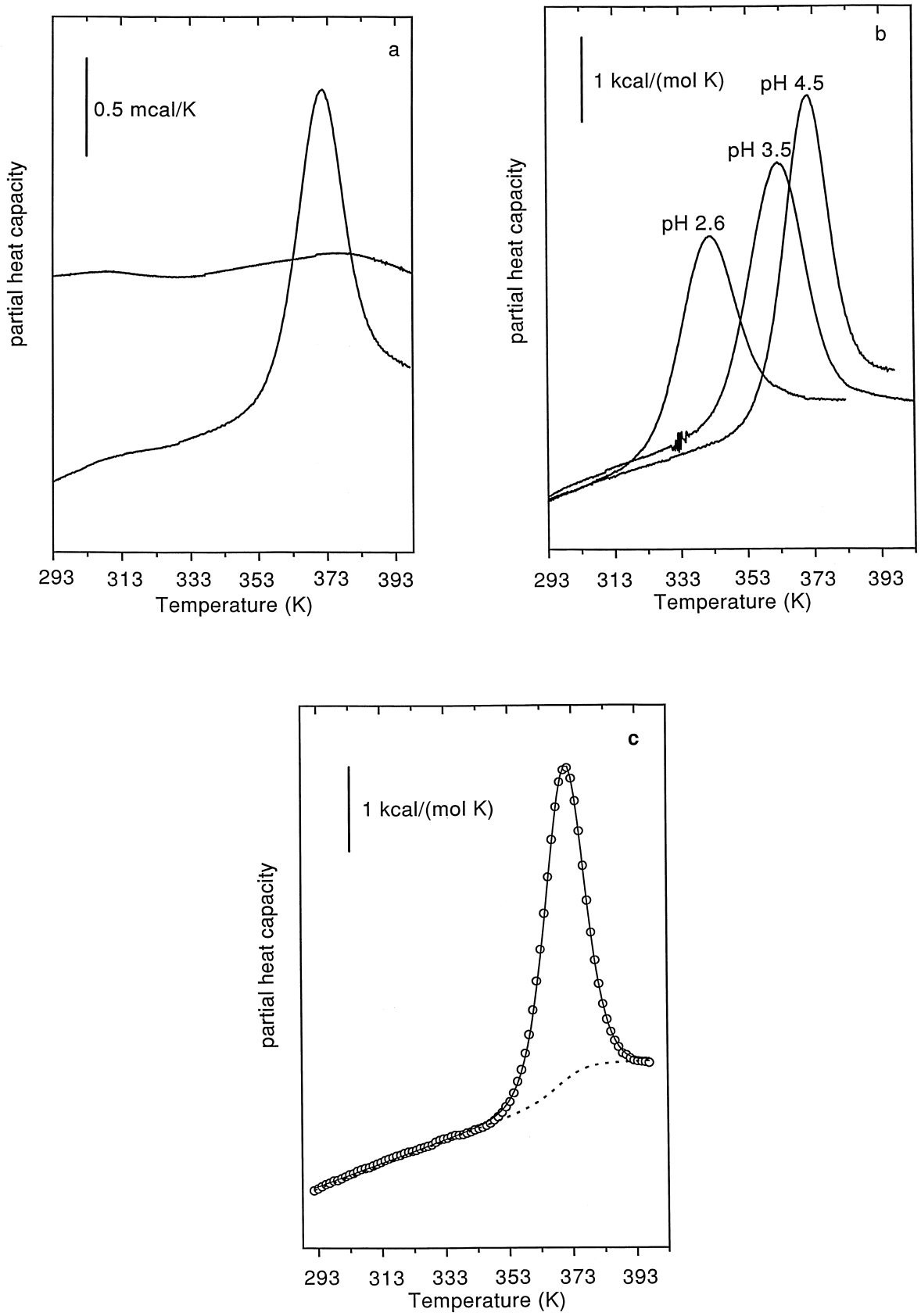

Figure 5. Representative DSC experiments of c-Sso7d. a, Raw data; temperature scan and the corresponding buffer baseline (5 mM sodium phosphate buffer, pH 5.0). b, Three representative melting curves measured at different pH values after subtraction of the corresponding buffer baseline and concentration normalization. c, Fitting to a two state model using the software supplied by MicroCal. The fitted curve is displayed as a continuous line. Only every third datapoint (O) is displayed. The progress baseline is shown as a dotted line.

Table 1. Reversibility and optimization of the scan rate

DSC Scan rate (K/hour)	Scan no.	T_m (K)	ΔH^{cal} (kcal/mol)	$\Delta H^{cal}/\Delta H^{vH}$	Rev.[c] (%)
90	1	339.1	39.6	0.81	—
	2	339.1	39.1		98.7
75	1	338.8	41.6	0.87	—
	2	339.1	40.1		96.4
60	1	338.7	42.0	0.93	—
	2	338.7	40.1		95.4
	3	338.8	33.1		79.3
	4	339.4	25.7		61.2
50	1	337.3	42.2	0.91	—
	2	337.6	35.1		84.0
40	1	337.2	43.0	0.98	—
	2	338.3	33.6		78.0

CD spectroscopy[a] Scan rate	T_m	ΔH_m	Rev.[c]
600	342.9	40.8	100
300	339.3	40.8	100
120	337.9	39.5	100
60	336.5	38.9	97
60	336.5	38.8	96
60	336.5	38.7	79[b]
45	336.7	39.6	92
30	336.4	40.4	68

Scans were performed in 5 mM sodium phosphate buffer (pH 2.5) from 298 to 373 K using a protein concentration of 2 mg/ml.

[a] Scans at 200 nm with a protein concentration of 0.2 mg/ml in 5 mM sodium phosphate buffer (pH 2.5) from 298 to 363 K. The cooling rate was 20 K/min.

[b] The sample remained at 363 K for 20 minutes.

[c] Reversibility was calculated as described in Material and Methods.

Specific ΔC_p values calculated per mol of amino acid residue for small proteins have been reported to lie in the range between 10.4 cal/(mol K) per residue (ribonuclease A) and 17.8 cal/(mol K) per residue (myoglobin) (Privalov & Gill, 1988). The value determined for Sso7d, 9.7 cal/(mol K), is at the lower level of ΔC_p per mol and residue found in globular proteins (Swint & Robertson, 1993; Alexander *et al*., 1992). The heat capacity change is thought to arise from the hydration of non-polar residues which are exposed upon unfolding and has been shown to be directly proportional to the exposed hydrophobic surface area (Pfeil & Privalov, 1976; Privalov & Makhatadze, 1990). Small proteins, which are unable to form a large hydrophobic core due to their size, show small ΔC_p values. Indeed, the small heat capacity change of Sso7d may result from the large proportion of hydrophobic residues, which are already exposed in the native state (Baumann *et al*., 1994). Another explanation would be an only partial exposure of hydrophobic residues in the unfolded state. The temperature dependence of the free energy of unfolding, $\Delta G(T)$, is completely specified by the three parameters T_m, ΔH_m and ΔC_p or equivalently, ΔH_m, ΔS_m and ΔC_p; with the slope given by ΔS_m and the curvature by $\Delta C_p/T$ (Becktel & Schellman, 1987). Since Sso7d is a protein with a particularly high melting temperature, we were interested in how the thermotolerance of a protein is reflected by these parameters. The melting temperature, T_m, can be increased by variations in enthalpy, entropy and ΔC_p that result in a certain combination of lifting and shifting the stability curve and/or flattening its curvature, i.e. increasing its breadth (Nojima *et al*., 1977). In more recent studies it was reported that the unfolding free energy curve of thermostable proteins seems to be flattened rather than shifted, without a reasonable change of the ΔG maximum (Jaenicke, 1991). On the other hand, the often observed high melting temperature of ''small'' proteins can be a consequence of the small size of the folding unit, as Alexander *et al*. (1992) have pointed out. However, the small size of a protein and consequently a small heat capacity change is often not sufficient to make the protein thermostable, i.e. not all small proteins are thermostable. For example, small proteins like barnase and RNase T_1 have melting temperatures of about 330 K (Yu *et al*., 1994).

Sso7d has its maximum stability at a temperature of around 282 K with a free energy ΔG^{max} of only about 7 kcal/mol. At the temperature of optimal growth of *S. solfataricus* ($\approx$350 K), it is only moderately stabilized with a ΔG $\approx$2.8 kcal/mol and at the maximum growth temperature ($\approx$363 K) ΔG drops to values around 1 kcal/mol (Figure 9). Thus, Sso7d certainly does not belong to the group of proteins that have a particularly stable native conformation at physiological growth temperatures of *Sulfolobus*. However, the unfolding thermodynamics of Sso7d described here in a simple *in vitro*

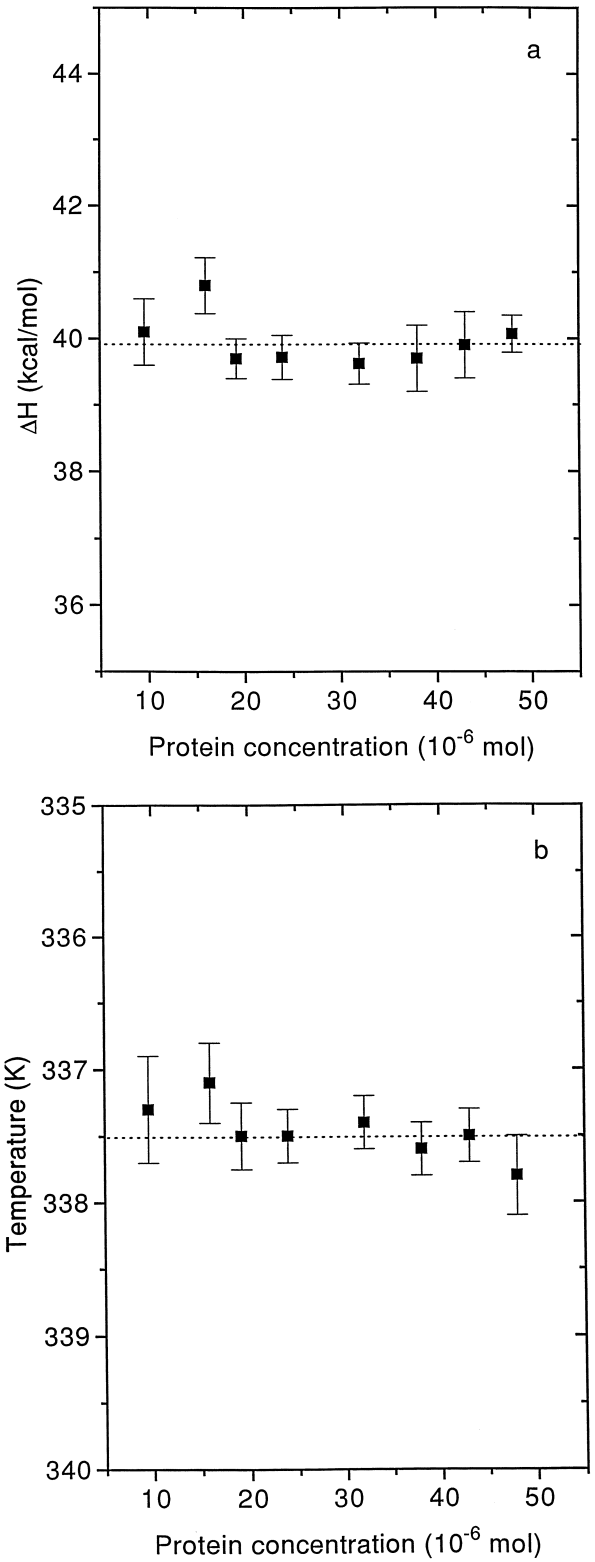

Figure 6. Dependence of ΔH (a) and T_m (b) on the protein concentration obtained by CD spectroscopy. All temperature scans were performed using sodium phosphate buffer at pH 2.5.

system could be different from the *in vivo* situation, where Sso7d interacts with DNA and possibly other intracellular solutes, which can stabilize its conformation.

Denaturation enthalpies calculated for Sso7d at room temperature (298 K) of 0.28 kcal/mol per residue and entropies of 0.5 cal/(mol K) are in the usual range calculated for small proteins (Privalov & Gill, 1988). Murphy *et al*. (1990) have pointed out that the specific enthalpies and entropies converge for most proteins around 380 K at values of 1.5 kcal/(mol residue) and 4.2 cal/(mol K residue), respectively. However, with an increasing number of calorimetric studies on small globular proteins the convergence of specific enthalpy and entropy functions is not as evident as with an initially smaller set of proteins (Makhatadze & Privalov, 1994, 1995). Particularly Sso7d shows at convergence temperatures (T_h^* = 377 K, T_s^* = 385 K; Ragone & Colonna, 1994) significantly lower specific enthalpy and entropy values of ΔH^* = 1.04 kcal/(mol residue) and ΔS^* = 2.99 cal/(mol K residue), respectively.

Contrary to the often discussed opinion that an increased melting temperature correlates with a high stability, we have observed that T_m is increased in spite of a relatively low ΔG_{max} value. Thus, the reason for the high melting temperature of Sso7d is a shallow stability curve with a broad ΔG maximum, corresponding to the small ΔC_p value. The consequence is that a protein which is less energetically stabilized may show a higher T_m. Proteins with significantly higher enthalpy and entropy values at convergence temperatures like barnase (Griko *et al*., 1994) and RNase T_1 (Yu *et al*., 1994; Makhatadze & Privalov, 1994) show relatively large unfolding free energies yet relatively low melting temperatures. Our results demonstrate clearly that an increased thermal stability (T_m) is not necessarily accompanied by an increased thermodynamic stability (ΔG).

We compared the lysine ϵ-monomethylated Sso7d with the non-methylated cloned protein and found no significant differences in the denaturation temperature or the heat capacity change. Sso7d and its analogue from *Sulfolobus acidocaldarius*, Sac7d, are extremely homologous in both amino acid sequence (Figure 1) and tertiary structure. The native Sac7d protein, which is partially methylated at lysines 4 and 6, shows a similar denaturation temperature as Sso7d at pH 4 and in the neutral region (McAfee *et al*., 1995). However, for the cloned and non-methylated Sac7d a 6.5 K lower T_m has been reported and it has been proposed that the partial methylation of the two lysine residues is responsible for the shift in T_m towards higher temperature. Our analysis of the methylated and non-methylated forms of Sso7d did not provide significant evidence which would support the conclusion that an increase in T_m could be the consequence of lysine methylation. Several other authors discuss an involvement of protein methylation in the repair of damaged proteins (Clarke, 1993; Geiger & Clarke, 1987; McFadden & Clarke, 1982). Therefore remains the role of lysine mono methylation, at least in Sso7d, still unknown.

It is striking that the topology of Sso7d is identical to that of Scr-homology 3 domains (SH3)

Table 2. Effect of buffer composition on thermodynamic parameters of Sso7d

pH	T_m[a] (K)	ΔH^{cal} (kcal/mol)	$\Delta H^{cal}/\Delta H^{vH}$ (kcal/mol)	Buffer (5 mM)
2.5	338.1	42.6	0.92	Acetate (adjusted with HCl)
2.5	338.7	42.0	0.93	Na-Phosphate
6.5	370.9	61.3	0.89	Mes/K^+
6.5	371.5	62.4	0.91	Tris/Cl^-
6.5	370.8	60.3	0.91	Na-Phosphate

[a] Errors in T_m were 0.2 K.

(Baumann *et al*., 1994). A search in the PDB database for similar three-dimensional structures and topology using the DALI program (Holm & Sander, 1993) revealed the conservation of this supersecondary structure in all three primary domains of life: Eucarya, Archaea and Bacteria, which indicates that this folding motif has evolved at high temperatures very early in evolution and has probably conserved its thermotolerance. A comparative investigation of the thermal stability of proteins with SH3 domain-like folding pattern is in preparation.

Material and Methods

Purification and cloning of Sso7d

Heterogeneously ϵ-mono-methylated Sso7d from *Sulfolobus solfataricus* was purified as described (Baumann *et al*., 1994). Non-methylated Sso7d was purified from over-expressing *E. coli* BL21 DE3 cells carrying a pET15b vector (Novagen) in which the PCR amplified Sso7d coding region was cloned in front of an inducible T7 promoter. The coding region was sequenced and the deduced amino acid sequence of the gene was identical to the protein sequence found by Edman degradation except for the N-terminal methionine present in the cloned protein (Baumann *et al*., 1994; Fusi *et al*., 1993).

E. coli BL21 DE3 cells were grown in LB medium and were induced for two hours at an absorbance at 600 nm (A_{600}) of 0.8 with 1 mM isopropyl β-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation at 6000 rpm in a Beckman JA 10 rotor and resuspended in 50 mM Tris-HCl buffer (pH 7.4) with 500 mM NaCl and lysed in a French press. Most of the *E. coli* proteins in the lysate were precipitated by heat treatment (353 K (80°C) for 20 minutes and subsequently removed by centrifugation using a Beckman rotor SW28 (20,000 rpm for 20

Table 3. Thermal unfolding of Sso7d

DSC pH	T_m[a] (K)	ΔH^{cal} (kcal/mol)	ΔH^{vH} (kcal/mol)	ΔC_p[f] (cal/(mol K))	ΔG^{298}[b] (kcal/mol)	ΔG^{353}[c] (kcal/mol)
2.5	338.3	42.4	46.0	457	3.4	–2.0
2.6	341.2	46.0	45.9	435	3.9	–1.7
3.0	351.9	48.2	51.4	415	4.5	–0.2
3.0[e]	351.6	49.4	50.9	1190	4.7	–0.2
3.1	356.2	52.9	48.4	836	5.4	0.4
3.5	362.1	57.3	52.1	459	6.2	1.4
4.0	368.3	60.3	62.0	865	6.8	2.3
4.5	371.0	64.5	65.6	551	7.7	2.8
4.75	371.6	62.6	67.1	397	7.3	2.8
5.5	371.9	63.1	68.4	419	7.4	2.9
6.0	371.1	62.7	64.7	528	7.3	2.8
6.0[e]	371.6	64.6	67.9	405	7.7	2.9
6.5	370.8	63.4	64.8	1055	7.4	2.8
CD						
7.0	371.2	65.4	71.6	859	7.8	2.9
2.5	337.5		39.9		3.1	–2.0
2.5[e]	339.4		39.8		3.1	–1.8
2.7	342.9		41.5		3.4	–1.3
2.8[e]	343.3		44.6		3.9	–1.3
2.9	349.5		44.6		4.0	–0.4
3.0[e]	353.0		48.3		4.6	–0.1
3.1	353.9		49.3[d]		4.7	0.1
3.3[e]	359.3		52.9[d]		5.4	0.9
3.5	365.5		54.9[d]		5.8	1.7

[a] Errors in T_m were 0.2 K.
[b] ΔG was calculated according to equation (2) with a ΔC_p of 620 cal/(mol K).
[c] 353 K is the optimal growth temperature of *Sulfolobus solfataricus*.
[d] Due to the short baseline of the denatured protein the value y_d and m_d were unreliable and were fixed to the value calculated in previous scans.
[e] m-Sso7d.
[f] The averaged value of the directly determined heat capacity change was 633 cal/(mol K).

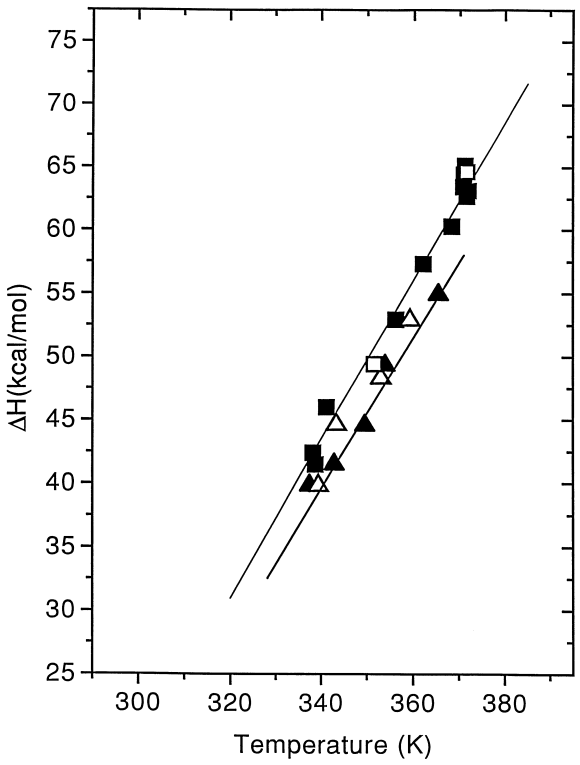

Figure 7. Temperature dependence of ΔH_m. Plots of ΔH_m *versus* T_m for m-Sso7d (▲) and c-Sso7d (Δ) measured by CD-spectroscopy and m-Sso7d (□) and c-Sso7d (■) measured by DSC, respectively. Sodium phosphate buffer was used in all scans. The lines show a linear fit of the data measured for c-Sso7d by DSC and CD spectroscopy, respectively. Data measured for m-Sso7d were not included in the fit. The heat capacity change (620 cal/(mol K) for DSC and 580 cal/(mol K) for CD-spectroscopic measurements were obtained by the slopes of the fitted lines.

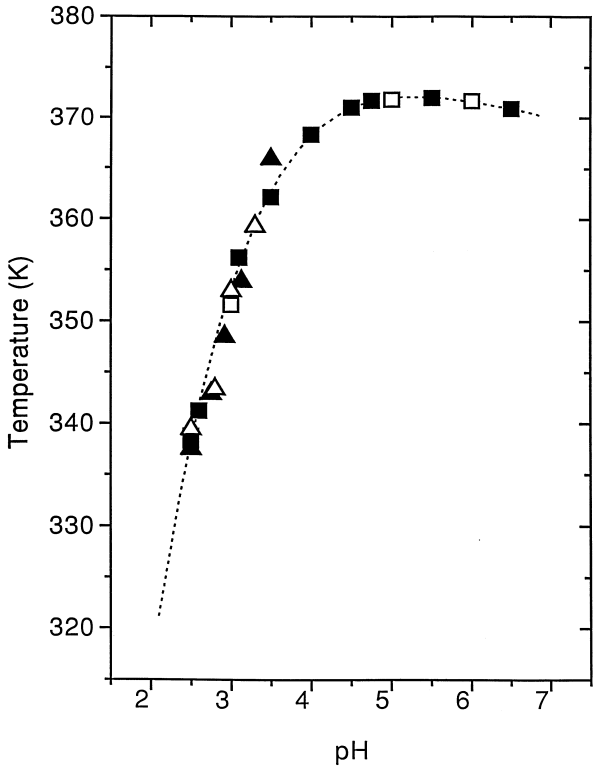

Figure 8. Dependence of the melting temperature (T_m) on pH using data calculated for m-Sso7d and c-Sso7d measured by DSC and CD-spectroscopy. DSC: c-Sso7d (■), m-Sso7d (□); CD-spectroscopy: c-Sso7d (▲) m-Sso7d (Δ).

minutes). The clear supernatant was dialysed overnight against 30 mM sodium phosphate buffer (pH 6.5), applied to a Mono-S ion exchange column (Pharmacia) equilibrated with the same buffer and eluted with a linear NaCl gradient (50 mM to 1000 mM). Sso7d eluted at a salt concentration of 300 mM. The purity of the protein was estimated by SDS-PAGE (Laemmli, 1970). Only a single band was visible on an overloaded gel stained with Coomassie brilliant blue (R). The purity was further confirmed by analytical reverse phase HPLC using a Vydac C4 column. Purified Sso7d eluted as a single symmetrical peak monitored at 280 and 214 nm. This purification procedure yielded about 20 mg purified protein per litre culture medium.

CD spectroscopy

The pure protein was concentrated to a concentration of 1 mg/ml and extensively dialysed against the buffer used in the individual CD experiments. The concentration of Sso7d was measured spectroscopically on a Cary 4E spectrophotometer using an extinction coefficient calculated from tyrosine ($\epsilon_{280\,nm}$ = 1400 M^{-1} cm^{-1}) and tryptophan ($\epsilon_{280\,nm}$ = 5500 M^{-1} cm^{-1}) absorption (Gill & Hippel, 1989). Chemicals used for buffers were of highest purity available.

A CD spectrophotometer (AVIV 62 DS) equipped with a thermoelectric cell holder was used to record thermal denaturation curves at a fixed wavelength and an averaging time of five seconds by heating the sample with a constant rate. The pH of the sample was measured before and after the scans and was found to be constant within 0.1 pH unit. 5 mM sodium phosphate buffer was used in all experiments. The various buffers (blanks) used in the experiments showed no ellipticity changes during heating and were therefore neglected in the data analysis. The sample was cooled rapidly (30 K/min) after the denaturation scan to determine reversibility of the unfolding-folding transition by measuring the ellipticities of the sample before and after heating at 298 K as described by Swint & Robertson (1993).

All data were evaluated using the ORIGIN (MicroCal Software) program package. Confidential intervals were calculated with a 95% confidence value. Data measured at high ionic strength were found to be more noisy at high temperatures and were weighted according to their statistical error. Both baselines and the transition region were fitted simultaneously using the equation:

$$y_{obs} = \frac{(y_n + m_n T_m) + (y_d + m_d T) + (\exp(\Delta H_m / R(1/T_m - 1/T)))}{(1 + \exp(\Delta H_m / R(1/T_m - 1/T)))}, \quad (1)$$

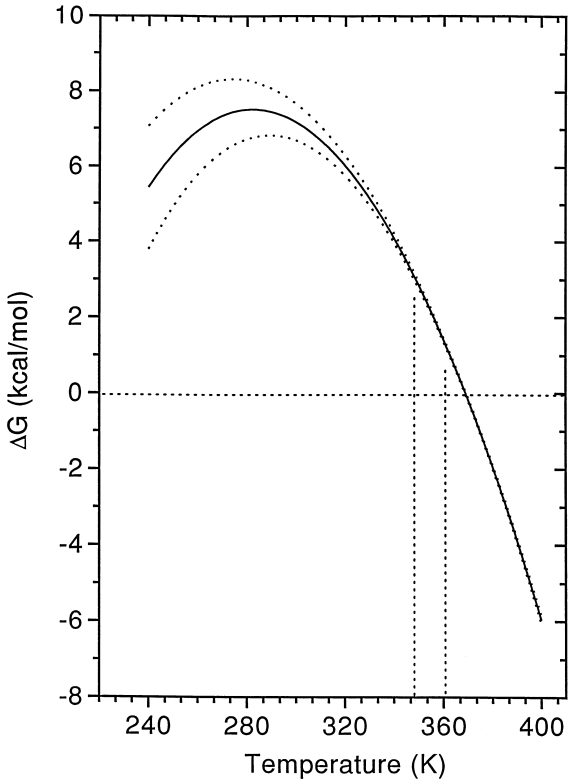

Figure 9. Stability curve (ΔG *versus* T) for Sso7d calculated under conditions where Sso7d shows maximum stability; a ΔC_p of 620 cal/(mol K) was used. Unfolding free energy of Sso7d calculated according to equation (2) (continuous curve). The error of the measurement ($\pm 10\%$) is indicated by the two dotted curves. The optimal and maximum growth temperatures are indicated by dotted lines.

where y_{obs} is the observed ellipticity, y_n and y_d are the y-intercepts of the baselines of the native and denatured protein and m_n, m_d are the slopes of these baselines. ΔH_m is the enthalpy at the transition temperature T_m (Santoro & Bolen, 1988; Swint & Robertson, 1993). Evaluations were based on the assumption that the unfolding transition is a two-state process and ΔC_p, representing the difference in heat capacity between the folded and the unfolded state, is constant in the temperature region measured.

The temperature dependence of ΔG was calculated at any temperature from the modified Gibbs-Helmholtz equation:

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln(T/T_m)] \quad (2)$$

Differential scanning microcalorimetry

Differential scanning calorimetry is the only direct method for obtaining ΔC_p, and was used in our study in parallel to the CD spectroscopic analysis. DSC was carried out on a MicroCal MCS calorimeter suitable for studies up to about 400 K (130°C) The calorimeter was controlled by the MCS OBSERVER program (MicroCal). The samples were extensively dialysed against the buffer used in the scanning experiment. The pH was measured at 298 K before and after the thermal scan. The samples were routinely degassed for five minutes before they were used for a calorimetric analysis. Two prescans were carried out before each scan to improve baseline stability (McAfee *et al*., 1995). Samples were heated during the prescans with the same rate used in the experiment but only to a temperature of 323 K where no thermal transition occurs. Each sample was scanned after the actual calorimetric scan for a second time to estimate the reversibility of the unfolding transition. Buffer baselines were measured under identical conditions and were subtracted from the corresponding data of the protein samples. All buffers used had a concentration of 5 mM.

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