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Differential Scanning Calorimetric Study of the Thermal Stability of Xylanase from *Streptomyces halstedii* JM8†

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ABSTRACT: The thermal stability of two xylanases with molecular masses of 45 (Xys1L) and 35 (Xys1S) kDa has been characterized thermodynamically by high-sensitivity scanning microcalorimetry in the pH range 3.0−9.0. Thermal denaturation of Xys1L reveals three thermodynamically independent domains, and that of Xys1S, which is a proteolytic fragment of Xys1L (without a C-terminal part), reveals two thermodynamically independent domains, each of which follows a two-state thermal unfolding process under our experimental conditions. Nevertheless, the thermodynamic parameters of unfolding for each domain do not fit some of the correlations obtained for most compact globular proteins. It is known that if $\Delta H_{res}(T)$ and $\Delta S_{res}(T)$ are plotted against temperature for a number of water-soluble compact globular proteins, they all have a common value at approximately 110 °C (383 K). Calculation of the variations in the enthalpy and entropy of unfolding per residue for each domain of xylanase with temperature gave us $\Delta H_{res}(383)$ and $\Delta S_{res}(383)$ values of approximately 3 kcal/(mol of residue) and 9 cal/(K·mol of residue), respectively. This is practically 2-fold larger than those apparent for most medium-sized globular protein values. These descrepancies might be related to features of the folded and/or unfolded states of the protein.

The study of protein stability is important both in the academic and applied fields. Most often, estimates of conformational stability are based on an analysis of thermal unfolding [see, for instance, Pace et al. (1989)]. The thermal analysis technique allows the determination of the extent of denaturation or unfolding of the entire protein or specific domains of it. Any physical technique that accurately reflects the extent of unfolding can be used, although differential scanning calorimetry (DSC)[1] is most commonly employed. Information regarding the presence of domains, the strength of the interactions between domains, and the effects of substrate binding on domain stability and interactions can be determined with this method (Privalov, 1982; Sturtevant, 1987; Brandts et al., 1989; Lepock et al., 1990). If domain transitions are well separated on the temperature axis, then thermodynamic parameters may be obtained directly for each domain. Even when transitions overlap severely in temperature, the available software allows deconvolution of the transition contour to obtain estimates of individual-domain parameters. Bearing this in mind, we carried out an investigation of xylanase by the DSC method.

It is known that xylan is hydrolyzed by the cooperative actions of several enzymes, among which endo-β-1,4-xylanases (1,4-β-D-xylanohydrolase, EC 3.2.1.8) are the most important. Despite their industrial applications and their role in the bioconversion of renewable plant cell materials, little is known on structure and mechanisms of catalysis of xylanase which have been proposed to act by general acid catalysis involving amino acid carboxy groups (Bray & Clarke, 1990; Okada, 1989).

Streptomyces halstedii JM8, isolated from straw, produces and secretes into the culture supernatant at least two proteins of 45 (Xys1L) and 33−35 (Xys1S) kDa that have hydrolytic activity against xylan. Both proteins are encoded by the same gene, and the smallest form is originated from the larger one by proteolytic cleavage on the C-terminus.

As shown in this work, thermal denaturation of Xys1L reveals three thermodynamically independent domains, and that of Xys1S reveals two thermodynamically independent domains, each of which follows a two-state transition. Nevertheless, the thermodynamic parameters of unfolding for each domain do not fit some of the correlations obtained for most compact globular proteins.

MATERIALS AND METHODS

Materials. All the culture conditions were as described by Ruiz-Arribas et al. (1994). Briefly, the supernatant from 600 mL of an 84-h culture of *Streptomyces parvulus* transformed with the plasmid pJM9 (Ruiz-Arribas et al., 1994) grown in 1% yeast extract and 0.3 M sucrose supplemented with 0.5% oat spelt xylan (Sigma) was separated from mycelia by centrifugation at 6000*g* for 15 min and concentrated in a Minitan system (Millipore) using 10000 NMWL polysulfone membranes (Millipore, ref. PTNK OMP 04). The concentrate was dialyzed against

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[1] Abbreviations: DSC, differential scanning calorimetry; Xys1L, xylanase with a molecular mass of 45 kDa; Xys1S, xylanase with a molecular mass of 35 kDa; MES, 4-morpholineethanesulfonic acid.

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distilled water in the same system. Finally, the sample (55 mL) was filtered through 0.22-μm syringe filters (Gelman Sciences) and equilibrated with 10 mM MES/NaOH, pH 6.0, used as buffer for purification. Purification was performed on a fast performance liquid chromatograph (Pharmacia) equipped with a Q-HR 5/5 (Pharmacia) anionic-exchange column. The column was equilibrated with 10 mM MES/NaOH, pH 6.0, and a NaCl gradient from 0 to 0.15 M was used for protein elution. Residual protein was eluted in the presence of 1 M NaCl. Xylanolytic activity of the different fractions was detected on 1.5% agar plates containing 0.1% Remazol-Brilliant-Blue xylan (Sigma). Xylanase activity was measured by the 3,5-dinitrosalicylic acid method (Bernfeld, 1951) using xylose as standard. Xylanase concentrations were determined by the Peterson (1977) method.

DSC Measurements. The buffers used for the DSC experiments were 60 mM sodium phosphate for pH 3.6 and 6−7.6 and glycine-NaOH for pH 8.5−8.9. DSC experiments were performed on a MicroCal MC-2D differential scanning microcalorimeter (MicroCal Inc., Northampton, MA) with cell volumes of 1.22 mL, at a scanning rate of 1 K/min, interfaced with a personal computer (IBM compatible). Before measurement, samples were degassed with stirring in an evacuated chamber for 15 min at room temperature and then immediately loaded into the calorimeter cell; the final dialysis buffer (also degassed) was loaded into the reference cell. A pressure of 2 atm of dry nitrogen was always kept over the liquids in the cells throughout the scans to prevent any degassing during heating. A background scan collected with buffer in both cells was subtracted from each scan. The reversibility of the thermal transitions was verified by checking the reproducibility of the calorimetric trace in a second heating of the sample immediately after cooling from the first scan. The temperature dependence of the molar heat capacity of xylanase was further analyzed and plotted using a Windows-based software package (Origin), also supplied by MicroCal.

Calculation of Thermodynamic Stability. Protein stability referred by Becktel and Schellman (1987) as function of the free energy of unfolding, ΔG, versus temperature was calculated from the experimental transition enthalpies at T_m, $\Delta H(T_m)$, the transition temperatures, (T_m), and the heat capacity changes, ΔC_p, which is the difference in heat capacity between the native and unfolded state and, in a first approximation, is independent of the solution conditions and temperature between 10 and 70 °C (Privalov, 1979, 1990) using the modified Gibbs−Helmholtz equations (Becktel & Schellman, 1987):

$$\Delta H(T) = \Delta H(T_m) + \Delta C_p(T - T_m) \quad (1)$$

$$\Delta S(T) = \Delta S(T_m) + \Delta C_p \ln(T/T_m) \quad (2)$$

$$\Delta S(T_m) = \Delta H(T_m)/T_m \quad (3)$$

$$\Delta G(T) = \Delta H(T) - T\Delta S(T) \quad (4)$$

$$\Delta G(T) = \Delta H(T_m)(1 - T/T_m) - \Delta C_p(T_m - T) - \Delta C_p T \ln(T/T_m) \quad (5)$$

where T refers to absolute temperature.

RESULTS AND DISCUSSION

DSC Results. The original calorimetric recording of the heat capacity changes of heated Xys1L solution and a buffer−buffer baseline at pH 8.9 are given in Figure 1.

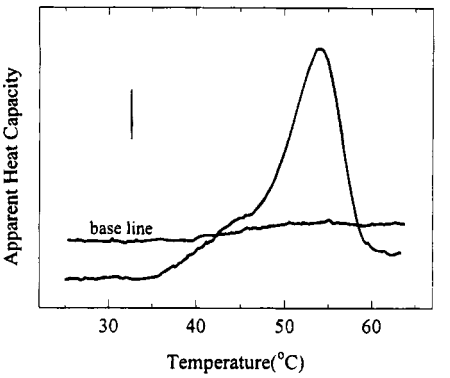

FIGURE 1: Original calorimetric recording of heat absorption of Xys1L solution at 1 K/min and pH 8.9 and buffer−buffer base line. Protein concentration: 1.0 mg/mL. The vertical bar is 2 mcal/K.

Reversibility of Transitions for Xys1L. The extent of reversibility, measured by relative area recovery, seen on the second scan of the Xys1L at different pH depended on the temperature at which the first scan was terminated before cooling the samples in preparation for the second scan. In all cases, the low-temperature transition was fully reversible. If heating was terminated at the temperatures before the main peak maximum, then the second scan showed about 90% reversibility and became irreversible when the heating was maintained at higher temperatures. Recently, some authors [see, for example, Sánchez-Ruiz et al. (1988), Galisteo et al. (1991), and Sánchez-Ruiz (1992)] have advised against trying to estimate thermodynamic parameters from DSC data on proteins which show little or no reversibility in the second scan. We found, however, that the results obtained in this work were practically independent of the scan rate, suggesting that the denaturation process is not kinetically determined. We conclude from this that the data could still be analyzed semiquantitatively using thermodynamic models (Hu & Sturtevant, 1987; Lin et al., 1994).

Dependence of T_m on pH. The variation with temperature of the apparent molar heat capacity of Xys1L solutions at three pH values (solid lines) as well as the results of deconvolution using the software provided by Microcal under the assumption of a two-state model of unfolding (interrupted lines) are given in Figure 2, and Table 1 summarizes the thermodynamic data for individual transitions. These results indicate that Xys1L denatures in three steps. There are two possible mechanisms to account for the three-step transition: (1) the protein undergoes three conformational unfoldings spread throughout; (2) three discrete domains unfold, each through a single step. On the basis of the results offered below, we believe that the three transitions would be due to the unfolding of three discrete domains of Xys1L. In fact, calorimetric recordings of the heat capacity changes for the fragment of xylanase without the C-terminus, Xys1S, show only two transitions. Figure 3 and Table 1 summarize the thermodynamic data for individual transitions of Xys1S obtained by analysis of the experimental contours under the assumption of a two-state model of unfolding, as in the case of Xys1L. Comparison of data for Xys1L and Xys1S presented in Table 1 suggests that the first and second transitions of Xys1S are more similar to the first and third transitions of Xys1L. This finds support in the comparative analysis of the behavior of the thermodynamic functions of

Table 1: Thermodynamic Data for the Individual Transitions of Xylanase

	Xys1L						Xys1S			
	transition 1		transition 2		transition 3		transition 1		transition 2	
pH	T_m (°C)	ΔH (T_m) (kcal/mol)	T_m (°C)	ΔH (T_m) (kcal/mol)	T_m (°C)	ΔH (T_m) (kcal/mol)	T_m (°C)	ΔH (T_m) (kcal/mol)	T_m (°C)	ΔH (T_m) (kcal/mol)
3.6	38.5 ± 0.2	66 ± 4	43.5 ± 0.4	84 ± 4	47.6 ± 0.1	108 ± 5				
4.1							43.2 ± 0.3	57 ± 3	50.3 ± 0.2	100 ± 4
6.3	51.7 ± 0.2	100 ± 2	59.3 ± 0.2	150 ± 5	64.3 ± 0.1	223 ± 6				
7.5	52.4 ± 0.3	93 ± 7	55.4 ± 0.4	137 ± 8	58.4 ± 0.1	187 ± 8	60.5 ± 0.2	99 ± 7	64.0 ± 0.1	200 ± 4
8.9	44.5 ± 0.1	77 ± 4	51.5 ± 0.1	118 ± 5	54.6 ± 0.1	157 ± 6	46.6 ± 0.2	72 ± 3	53.8 ± 0.1	136 ± 2

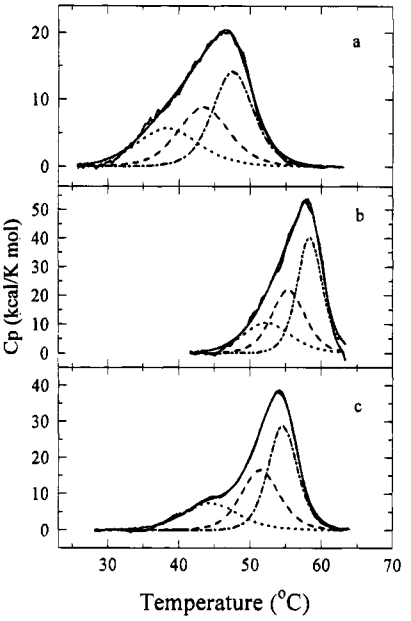

FIGURE 2: DSC thermograms of Xys1L at pH 3.6 (a), 7.5 (b), and 8.9 (c). The solid lines are experimental data, while the broken lines resulted from fitting the data to the two-state model. Protein concentrations were 0.015 mM in panels a and b and 0.022 mM in panel c. The scan rate was 1 K/min.

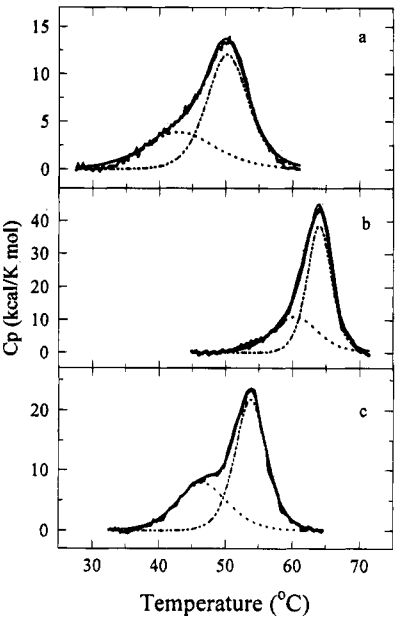

FIGURE 3: DSC thermograms of Xys1S at pH 4.1 (a), 7.5 (b), and 8.9 (c). The solid lines are experimental data, while the broken lines resulted from fitting the data to the two-state model. Protein concentrations were 0.01 mM in panel a, 0.012 mM in panel b, and 0.015 mM in panel c. The scan rate was 1 K/min.

individual transitions with temperature for Xys1S (see Figures 5 and 6, thin lines) and for Xys1L (thick lines in these figures). Thus, the second transition of Xys1L would be connected with unfolding of the C-terminal domain. Measurements of the changes in enzymatic activity of Xys1L and Xys1S with temperature (Figure 4) show that a concomitant loss of enzymatic activity takes place strictly in the temperature region of the last high-temperature transition. The foregoing suggests that Xys1L has three thermodynamically independent domains.

Temperature Dependence of the Free Energy of Unfolding. It is known that for globular proteins the heat capacity of the unfolded state is greater than that of the folded state (Privalov, 1979). This difference in heat capacity (ΔC_p) has been shown to be correlated with the increase in the exposure of the hydrophobic surface area when unfolding takes place (Privalov & Khechinasvili, 1974). If ΔC_p is known, the free energy of unfolding can be calculated at temperatures beyond the experimentally measurable range using eq 5. Figure 5 shows the variation with temperature of the individual transition enthalpies obtained from deconvolution of the initial contours. Transition temperatures were varied by changes in pH. Within the accuracy of the measurements of $\Delta H(T_m)$, we obtained three linear plots for individual transitions over the experimental temperature range. Ac-

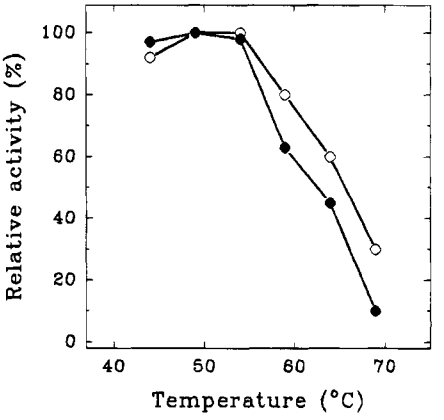

FIGURE 4: Temperature dependence of the activities of Xys1L (●) and Xys1S (○) at pH 7.5. Activity was assayed by the 3,5-dinitrosalicylic acid method (Bernfeld, 1951) after taking aliquots at different temperatures when the xylanase solution was heated at a rate of 1 K/min.

cording to the Kirchoff equation:

$$d(\Delta H)/d(T) = \Delta C_p \quad (6)$$

the slope of these plots represents ΔC_p, the molar heat capacity changes on unfolding. The numerical values 2.2, 4.1, and 6.7 kcal/mol·K for the first, second, and third

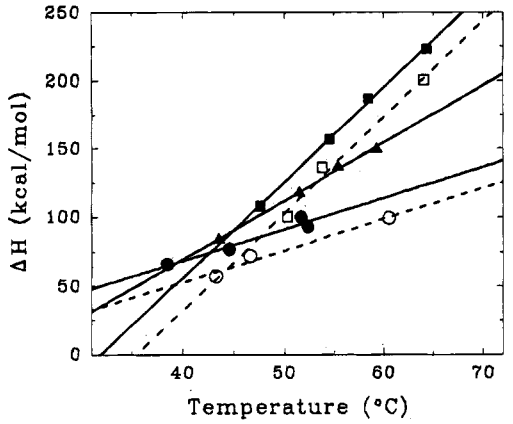

FIGURE 5: Temperature dependence of the molar calorimetric enthalpy for the first (●), second (▲), and third (■) transitions of Xys1L. Open symbols refer to the first (○) and second (□) transitions of Xys1S. Lines were fitted by linear regression. pH values were carefully adjusted before each measurement and controlled after measurement.

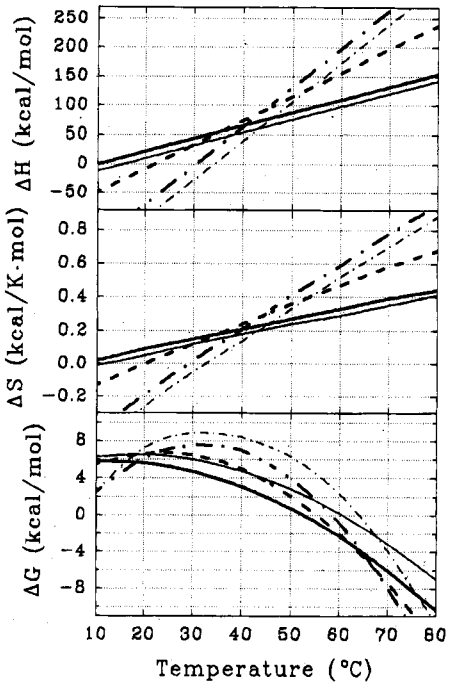

FIGURE 6: Thermodynamic functions for xylanase plotted *vs* temperature according to the eqs 1−5 (see text). (—) Calculated for the first transition of Xys1L (thick lines) and for Xys1S (thin lines); (- - -) for the second transition of Xys1L; and (-•-•-) for the third transition of Xys1L (thick lines) and second transition of Xys1S (thin lines).

individual transitions of Xys1L, respectively, were obtained. Using the reference values of $\Delta H(T_m)$ for individual transitions from Table 1 and the respective values of ΔC_p, $\Delta H(T)$, $\Delta S(T)$, and $\Delta G(T)$ were calculated (Figure 6). Becktel and Schellman (1987) have shown that the temperature of maximum stability, T_s*, where $\Delta S = 0$, can be calculated by

$$T_s^* = T_m \exp(-\Delta H(T_m)/T_m \Delta C_p) \qquad (7)$$

The calculated values of T_s* for the three curves in Figure 5 (thick lines) are 15, 23, and 30 °C, testifying to the relative hydrophobity of Xys1L (Pace & Laurents, 1989). The stability of Xys1L domains at 30 °C, the physiologically relevant temperature for *S. halstedii* JM8, is 5−9 kcal/mol,

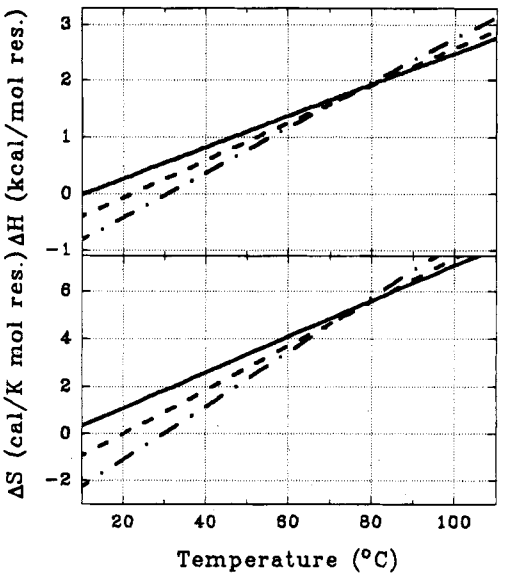

FIGURE 7: Variations in enthalpy (ΔH) and entropy (ΔS) per residue of Xys1L domains unfolding with temperature assuming a constant ΔC_p, (—) for the first, (- - -) for the second, and (-•-•-) third domain, accordingly.

which is about 10 times the thermal energy (RT). This seems to be adequate because it is known that in biological processes energy is exchanged, as a rule, by portions of 4−8 kcal/mol, and hence the upper limit of the protein conformational stability should have the same value. A possible reason for this is that such high stability is not necessary, for example, to make protein degradation feasible (Pace, 1975) or to lead to a high degree of protein flexibility, which may be essential for function (Becktel & Schellman, 1987; Creighton, 1990; Fischer & Schmid, 1990).

Since until now there are no other data about the structure of xylanase, we have estimated the values of the molecular mass for Xys1L domains from the calorimetric data (Table 1) on the assumption of a constant mean heat sorption value per amino acid residue. The results of these estimations give molecular masses of 10, 14, and 21 kDa for the first, second, and third domains of Xys1L, respectively. Despite the arbitrary nature of our assumption (Privalov, 1979), the value of the molecular mass obtained from this estimation for the second domain of Xys1L (14 kDa) shows good agreement with the results obtained on comparing the calorimetric data for Xys1L and its fragment without the C-terminal part, Xys1S (see above).

The calculation of the variations in the enthalpy and entropy of unfolding per residue for each domain of Xys1L with temperature from the average value of 119 Da for the molecular mass of the amino acid residue is shown in Figure 7. It was found that on plotting $\Delta H_{res}(T)$ and $\Delta S_{res}(T)$ versus temperature for a number of water-soluble compact globular proteins they all have a common value at approximately 110 °C (383 K) (Privalov & Khechinashvili, 1974; Privalov, 1979; Privalov & Gill, 1988; Doig & Williams, 1992). In our case, we obtained values of aproximately 3 kcal/(mol of residue) and 9 cal/(K·mol of residue), respectively, for ΔH_{res}(383) and ΔS_{res}(383), considerably higher than apparent for most medium-sized globular protein values [$\Delta H_{res}(383) \simeq$ 1.5 kcal/(mol of residue) and $\Delta S_{res}(383) \simeq$ 4.5 cal/(K·mol of residue)] (Doig & Williams, 1992). There are not many examples of proteins with different values of ΔH_{res}(383) and

$\Delta S_{res}(383)$, although in this sense data have been offered for histones, which have noncompact folded structures (Tiktopulo et al., 1982), and certain small proteins such as neurotoxins from *Naja naja oxiana* venom (Khechinashvili & Tsetlin, 1984) for which $\Delta H_{res}(383)$ and $\Delta S_{res}(383)$ have relatively low values purported to be due to a smaller number of hydrogen bonds and/or nonpolar contacts in the folded proteins (Khechinashvili & Tsetlin, 1984; Privalov & Gill, 1988). Recently, the results of calorimetric research with barnase were published (Martínez et al., 1994); barnase was found to have a higher value than the average one for globular proteins of specific heat of unfolding at any temperature. To explain this, the authors propose that the temperature-induced unfolded state of barnase would have much less residual structure than that of other proteins, meaning that its unfolded state must be very specific and very close to random coil. If this were so, then a cooperative melting of all structural elements, including the secondary structure, would be accompanied by higher than average changes in enthalpy and entropy. The same situation can be applied to our case with xylanase domains. Probably, in the case of xylanase, the number of hydrogen bonds and/or nonpolar contacts in the folded state of the domains is considerably larger and the unfolded state is very close to a random coil in comparison with most medium-sized globular proteins. Of course, in the absence of other structural data this assumption needs further investigation because such a discrepancy may be connected with partial irreversibility of the unfolding of xylanase. This is now under study at our laboratory.

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