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The Entropy Cost of Protein Association

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The temperature induced unfolding/dissociation of the dimeric subtilisin inhibitor from *Streptomyces* and its mutant D83C having an S–S crosslink between the subunits has been studied calorimetrically. Comparison of the entropies measured at different concentrations of dimer showed that the entropy cost of crosslinking is small. Its value at the standard concentration of 1 M is of the order of $-(5 \pm 4)$ cal/K·mol, i.e. it is more than one order of magnitude smaller than the values of translational entropies calculated on the base of statistical thermodynamics, using in particular the Sackur-Tetrode equation, and is close to the cratic entropy value suggested by classical mixing theory.

Keywords: protein association; translational entropy; cratic entropy; crosslinking; subtilisin inhibitor

Introduction

With the rapid rise of interest in phenomena of molecular recognition the thermodynamics of formation of macromolecular complexes is attracting wide attention. It is more or less clear that association of several independent molecular kinetic units into a single complex is driven by the same secondary forces which are responsible for protein folding, but in contrast to the folding of monomeric proteins the process of complex formation includes one extra negative entropy term associated with a significant decrease in the rotational and translational degrees of freedom. This entropy, and particularly its translational part, opposes formation of a complex, determines the concentration dependence of the association/dissociation process and correspondingly, the values of all the thermodynamic parameters specifying this process. To compare the energetics of formation of various complexes and understand their structural basis we have to eliminate this concentration dependence. This is usually done by extrapolating the experimental results to some standard concentration. In the physical chemistry of low molecular weight compounds, a standard concentration of 1 molar is conventionally assumed. The same standard is used in biophysical chemistry, although a 1 molar solution in the case of macromolecules makes little sense. The magnitude of the translational entropy at this standard concentration is still one of the most debated subjects in the case of macromolecular solutions.

According to the view, originally proposed by Gurney (1953) and later adopted by Kauzmann (1959), Tanford (1973), and many others, translational entropy is expressed by the cratic term which is just the entropy of mixing of the additional kinetic units appearing upon dissociation of a complex and is independent of the composition of the solution. For a 1 M standard aqueous solution (containing 55 M water) $\delta S^{\mathrm{cratic}} = R\ln(1/55) = 8.03$ cal/K·mol for dissociation of a dimer, and is supposed to be independent of the molecular weight of the solute. A value close in magnitude and opposite in sign was calculated by Steinberg & Scheraga (1963) for the association of two similar spherical particles, $-(10 \pm 8)$ cal/K·mol.

On the other hand, Doty & Myers (1953) calculated the entropy of dimerization of rigid particles of the mass and shape of insulin as −122 cal/K·mol. Similar values have been obtained by Finkelstein & Janin (1989) assuming that the translational entropy of macromolecules in aqueous solutions does not differ from that of small molecules in the gaseous phase and can be calculated by simple statistical mechanics using the Sackur-Tetrode equation. This gave for the translational

Abbreviations used: SSI, *Streptomyces* subtilisin inhibitor; WT, wild-type; D83C, mutant protein with Asp83 replaced by Cys; 1 cal = 4.184 J; $\delta S^0_{\mathrm{trans}}$, translational entropy under standard conditions; $\delta S^{\circ}_{\mathrm{rot}}$, rotational entropy under standard conditions; ΔH_t and $\Delta S_t = \Delta H_t/T_t$, the experiment enthalpy and entropy of transition; $\Delta\hat{S}^0$, standard entropy at 1 M concentration; $\delta\Delta\hat{S}^0$, difference between the standard entropies of unfolding of crosslinked and non-crosslinked dimers; CD, circular dichroism.

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entropy of a typical dimeric protein at 300 K and the 1 M standard concentration a value of 40 to 50 cal/K·mol depending on the molecular weight of the protein. According to the same authors the rotational entropy calculated from first principles is of the same order of magnitude. Therefore the $(\delta S^0_{\text{trans}} + \delta S^0_{\text{rot}})$ amounts to 100 cal/kmol with a positive sign for dissociation of dimer and a negative sign for its association (Finkelstein & Janin, 1989). Very similar values of the entropy effects of dimerization have been obtained by Tidor & Karplus (1994) using the statistical-thermodynamic approach suggested by Chandler & Pratt (1976). They calculated that dimerization of insulin results in decrease of the translational entropy by 43 cal/K·mol, decrease of the rotational entropy by 48 cal/K·mol, but found that it should be accompanied by increase of the vibrational entropy of 24 cal/K·mol. Therefore, according to these authors the overall change of the external entropy (i.e the entropy not associated with changes in conformation or hydration) upon dimerization of insulin should amount to −67 cal/K·mol. A value of entropy reduction of the order of 50 to 100 cal/K·mol has been widely used by many authors in the thermodynamic analysis of the formation of protein/protein and protein/DNA complexes (see e.g. Janin & Chothia, 1990; Janin, 1995; Searle & Williams, 1992; Searle *et al*., 1992; Spolar & Record, 1994).

That the above two estimates of the translational entropy differ by one order of magnitude is a matter of considerable concern. According to Murphy *et al*. (1994) a loss of 8 cal/K·mol in translational entropy for the formation of a dimer, as estimated from the ideal mixing model, is the most appropriate for energy parameterization calculations. However, according to Holtzer (1995) this cratic correction to the entropy of complex formation has no ground in thermodynamics and statistical mechanics first of all because even dilute solutions of macromolecules are not ideal (see also Gilson *et al*., 1997). On the other hand, Amzel (1997) concludes that using the gas phase translational entropy given by the Sackur-Tetrode equation results in large overestimation of the translational entropy in aqueous solution because most components of the movement of ligand in the binding site of protein and in solvent are similarly restricted and cannot be described by a harmonic oscillator. Meanwhile, the other authors came to some intermediate values for the translational/rotational entropy change upon association of molecules into complexes. Horton & Lewis (1992) calculated the association energy of quaternary complexes starting from atomic coordinates and concluded that the entropy cost of fixing one subunit relative to the other is −20 cal/K·mol. Brady & Scharp (1997) analyzed the crystal packing and solvation of cyclic dipeptides and found that the association entropy penalty should be −14.1 cal/K·mol. Erickson & Pantaloni (1981) previously came to a similar conclusion by analyzing possible models of self-assembly of proteins. According to Amzel (1997) the translational entropy hardly exceeds 10 cal/K·mol. A critical review of the statistical-thermodynamic basis for computations of binding was given by Gilson *et al*. (1997).

There have been several attempts to verify experimentally the predicted translational/rotational entropies. The appropriate procedure for measuring the translational entropy is to compare the entropy change of an intermolecular reaction with that of an intramolecular reaction in which the two reactants are linked by a covalent bond. Studies of reactions, such as the formation of acetic anhydride *versus* the formation of succinic anhydride led to a translational/rotational entropy value of about 45 cal/K·mol (Page & Jenks, 1971; Jenks, 1975). However, it was unclear whether the results obtained on small molecular weight compounds in a reaction involving the rearrangement of covalent bonds can be extrapolated to the case of formation of macromolecular complexes which usually proceed without changing chemical structure. It is therefore of paramount importance to determine the translational entropy for macromolecular complexes.

The translational entropy on formation of a macromolecular complex can be estimated experimentally by comparing the entropy of unfolding/dissociation of the complex with the entropy of its unfolding without dissociation, i.e. unfolding of the same complex having covalently linked subunits. Most specific macromolecular associates, such as protein/protein and protein/DNA complexes, dissociate upon unfolding of the subunits if these subunits are not covalently linked. If unfolding is induced by temperature increase (heat denaturation), the entropy of this process can be measured directly by the heat capacity calorimeter since:

$$\Delta S = \int_{T_1}^{T_2} \frac{\langle C_p(T)\rangle dT}{T} = \int_{T_1}^{T_2} \langle C_p(T)\rangle d\ln T \qquad (1)$$

where $\langle c_P(t)\rangle$ is the excess heat absorption in the temperature zone (T_1,T_2) of the reaction. The idea of such an experiment is therefore simple, but there are two practical problems in its realization. First, the calorimetric instrument and the whole experiment needs to be precise enough to reliably register the small differences between the large entropies of unfolding of the two species, when covalently bonded and when not. Second, the species studied should differ by only a single covalent crosslink which holds the subunits together without any deformation, and the temperature induced unfolding of these species should be highly reversible so as to be able to treat it thermodynamically. The first requirement has been met by the appearance of highly sensitive scanning microcalorimeters, particularly those having a highly stable baseline (e.g. the Nano-DSC of the Calorimetric Science Corporation), which is

especially important for precision in determining the heat of a reaction that proceeds over a broad temperature range. More difficult is the problem of finding an appropriate object for such experiments: a dimeric protein which unfolds reversibly and can be crosslinked without noticeable stress. One such protein is *Streptomyces* subtilisin inhibitor (SSI). In the native state SSI exists as a homodimer and dissociates to monomers upon heat denaturation (Takahashi & Sturtevant, 1981; Tamura *et al.*, 1991a,b). With replacement of Asp83 by Cys this mutated dimer (D83C) was crosslinked with a disulfide bond (Tamura *et al.*, 1994). It was believed that this replacement and introduction of an intersubunit disulfide bond does not noticeably affect the structure of the dimer since both proteins have a very similar inhibitor constant. The wild-type SSI and its D83C mutant therefore appear to be perfect objects for the calorimetric study of the thermodynamic effect of crosslinking the subunits of a complex and particularly for evaluation of the translational entropy. In the first calorimetric experiments carried out by one of us (Tamura *et al.*, 1994) it was shown that formation of the inter-subunit disulfide bond leads to significant stabilization of the SSI complex, but because of insufficient precision of the calorimetric measurements we failed to estimate quantitatively the entropy effect of crosslinking. This study is now repeated on the same objects using a more precise instrument, more sophisticated treatment of the experimental data and what is particularly important, using a much wider range of protein concentrations. We also undertake a detailed CD and NMR investigation of WT SSI and the D83C mutant in the folded and unfolded states to be sure that they do not differ in conformation. This has permitted us to evaluate the loss of external entropy at protein association.

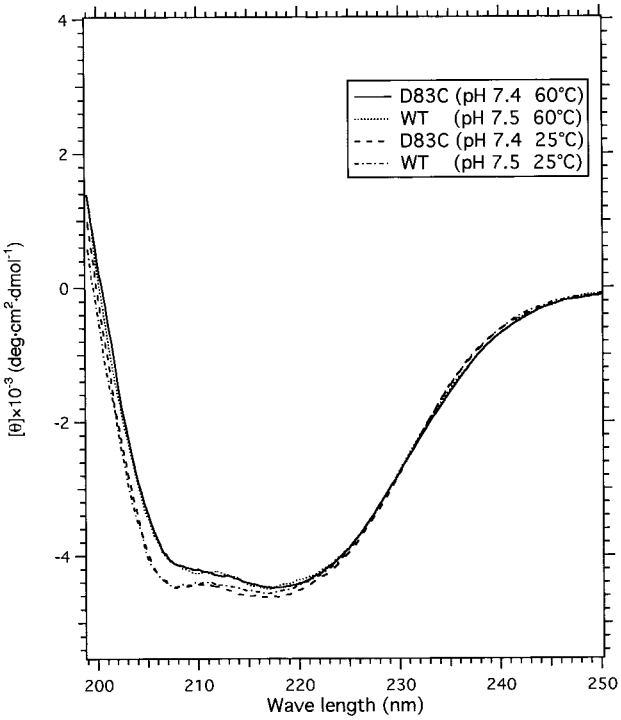

Figure 1. CD spectra in the far UV of WT SSI and the D83C mutant under conditions where both are in the folded (native) state: D83C at 25°C, pH 7.4 (broken curve); WT SSI at 25°C, pH 7.5 (dot-and-dash curve); D83C at 60°C, pH 7.4 (continuous curve); WT at 60°C, pH 7.5 (dotted curve).

Results

Specification of the folded and unfolded states

CD spectra for the native form of WT SSI and the D83C mutant in the far UV region are closely overlapping each other at both 25 and 60°C (Figure 1), indicating that the mutant retains the same secondary structure as the wild-type SSI.

According to the ^{1}H-NMR spectra for the native state (Figure 2 (a) and (b)) as well as two-dimensional spectroscopy (COSY and NOESY; data not shown), most of the chemical shifts for the side-chains, both the aliphatic and aromatic residues, were identical. A small shift (of about 0.2 ppm) was found only for the few β-protons near Asp83 which is replaced by Cys, but most of the assigned side-chain signals, such as Met70,73, and Tyr75 in the reactive site segment, His43 and Tyr93 in the hydrophobic core, Met103 in the α-helix, Leu12 and Trp86 in the β-sheet, showed the same chemical shifts suggesting that the tertiary structure of the D83C mutant is nearly identical to that of WT SSI.

NMR measurements for the denatured states also showed similarity (Figure 2 (c) and (d)), suggesting that the conformation of D83C is nearly identical to that of WT SSI. This is symbolically shown for the alpha proton of X residue in the X-Pro sequence where Pro is in the *trans* form, observed at around 5 ppm, indicating that even the *cis-trans* equilibrium for proline residues is the same.

Specification of the unfolding reaction

In contrast to the previous study by Tamura *et al.* (1994) which was done over a broad pH range so as to obtain information on the protonation effects upon unfolding of SSI and its mutants, we choose for this study just two solvent conditions: pH 3.0, 25 mM glycine buffer and pH 6.0, 25 mM phosphate buffer, but varied the protein concentration over a broad range from 450 μM to 15 μM. This 30-fold variation of concentration was needed because the concentration dependence of the effect of crosslinking on the stability of the dimer is of principal importance for evaluation of the translational entropy.

Figures 3 and 4 give the partial molar heat capacities of WT SSI and its D83C mutant at pH

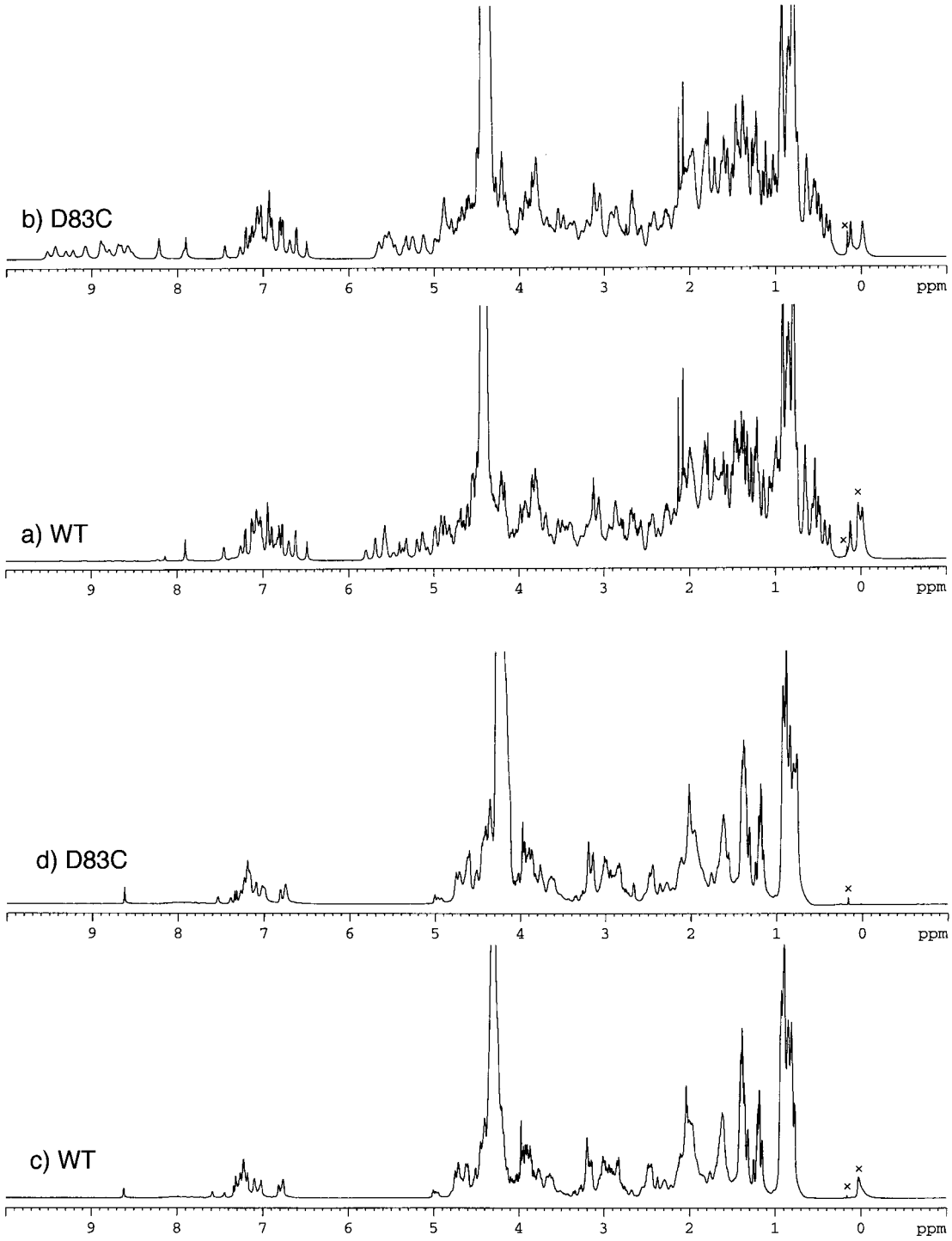

Figure 2. One-dimensional 1H-NMR spectra for WT SSI and the D83C mutant under conditions where both are folded (a and b) and unfolded (c and d): (a) WT SSI in 25 mM phosphate buffer, pH 6.6, 60°C; (b) D83C mutant at pH 6.4, 60 °C; (c) WT SSI in 25 mM glycine buffer, pH 2.6, 70 °C; (d) D83C mutant at pH 2.2, 75 °C.

6.0 and pH 3.0 determined at various concentrations of protein. They look very similar to those obtained previously by Tamura *et al*. (1994) and completely confirm the earlier conclusions that: (a) the temperature induced unfolding of the D83C mutant and the unfolding/dissociation of SSI are both highly reversible cooperative processes; (b) the temperature of unfolding of the S–S cross-linked D83C mutant is much higher than that of the WT SSI and does not depend on concentration (Tables 1 and 2). Thus unfolding of the D83C mutant is certainly a monomolecular reaction. For a two-state bimolecular reaction one should expect the following relationship between the transition

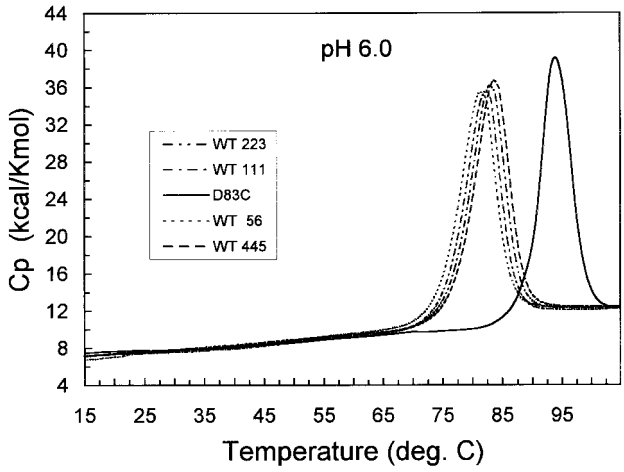

Figure 3. Temperature dependencies of the partial molar heat capacity of WT SSI and the D83C mutant in pH 6.0 solutions and different concentrations of protein. Numbers in the box indicate initial concentrations of dimer in μ M.

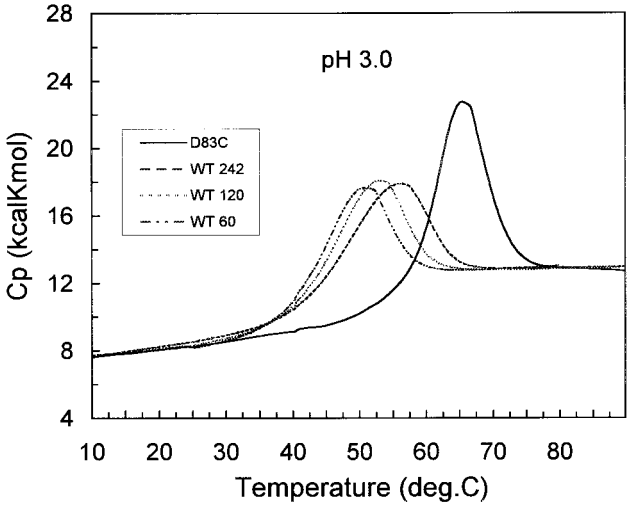

Figure 4. Temperature dependencies of the partial molar heat capacity of WT SSI and the D83C mutant in pH 3.0 solutions and different concentrations of protein. Numbers in the box indicate initial concentrations of dimer in μ M.

temperature and concentration of dimer, N (Breslauer, 1995):

$$\frac{\partial(10^3/T_t)}{\partial \log_{10}[N]} = -\frac{2.303R}{\Delta H^{vH}} \qquad (2)$$

were ΔH^{vH} is the effective or van't Hoff enthalpy of this process and T_t is the temperature of half transition. The plot of the $1/T_t$ *versus* the negative logarithm of concentration, pN, indeed shows an almost linear dependence for WT SSI at pH 6.0 with a slope of about 180 kcal/mol (Figure 5). The plot for WT SSI at pH 3.0 has a much larger slope than that at pH 6.0 and, since it covers a much broader temperature range, reveals noticeable deviation from linearity. This might mean that the enthalpy depends on temperature and its value at pH 3.0 is smaller than that at pH 6.0, being about 100 kcal/mol at the mid temperature. Comparison of these enthalpies with the calorimetrically estimated values (Tables 1 and 2) shows that these effective van't Hoff enthalpies are in a good correspondence with the real ones. This represents a strong argument that the unfolding of WT SSI is indeed a bimolecular two-state reaction.

The conclusion that the unfolding/dissociation of the WT SSI dimer is a two-state bimolecular transition and that the unfolding of the D83C mutant is a two-state monomolecular reaction fol-

Table 1. Calorimetric data for the wild-type SSI and S–S crosslinked mutant (D83C) at pH 6.0

Protein	$N \times 10^{-6}$	T_t	ΔH_t	ΔS_t	$\Delta H(80)$	$\Delta S(80)$	$\Delta \hat{S}^o$ (80)
	445	83.6	185.9	521	178.8	501	487
	223	82.8	184.8	519	179.2	503	488
	111	82.1	183.5	517	179.3	504	487
WT SSI	55.7	81.3	181.0	511	178.4	504	486
	54.5	81.3	181.4	512	178.8	505	487
	27.8	80.4	179.2	507	178.4	506	486
Average					**178.8**		**487**
	97.7	94.2	197.8	538	179.1	482	
D83C	113.4	94.0	198.6	541	180.1	485	
	11.0	94.2	–	–	–	–	
Average					**179.6**	**483**	**483**

$N = N^o/N^{st}$, where N^o is the initial concentration of dimer and N^{st} is the standard concentration (1 M). T_t, the transition temperature in °C; ΔH in kcal/mol; ΔS in cal/K·mol. ΔH_t and $\Delta S_t = \Delta H_t / T_t$, the experimental enthalpy and entropy of transition; $\Delta H(80)$ and $\Delta S(80)$, the enthalpy and entropy of transition reduced to 80°C; $\Delta \hat{S}$ (80), standard entropy of unfolding/dissociation at 80°C.

Table 2. Calorimetric data for the wild-type SSI and S–S crosslinked mutant (D83C) at pH 3.0

Protein	$N \times 10^{-6}$	T_t	$\Delta H'_t$	$\Delta S'_t$	ΔH_t	ΔS_t	$\Delta H(60)$	$\Delta S(60)$	$\Delta \hat{S}$ (60)
	455	59.1	104.1	315.1	118.4	335	120.7	342	328
	242	54.9	92.6	284.7	106.9	305	120.7	347	332
	120	52.1	85.0	261.0	99.3	281	121.4	348	331
WT SSI	59.7	49.8	77.8	241.0	92.1	261	120.7	348	330
	29.6	48.1	72.8	226.6	87.1	247	120.4	349	329
	14.6	46.3	–	–	–	–	–	–	
Average							**120.9**		**330**
D83C	92.4	65.1	116.9	345.6	131.0	366	**117.8**	**324**	**324**
	11.6	65.1	–	–	–	–		–	

$N = N^o/N^{st}$, where N^o is the initial concentration of dimer, and N^{st} is the standard concentration (1 M); T_t, transition temperature in °C; ΔH in kcal/mol; ΔS in cal/K·mol; $\Delta H'_t$ and $\Delta S'_t = H'_t/T_t$, calorimetrically measured enthalpy and entropy of unfolding at transition temperature; ΔH_t and ΔS_t, enthalpy and entropy of transition corrected on two histidines protonation effect; $\Delta H(60)$ and $\Delta S(60)$, enthalpy and entropy of transition corrected on two histidines protonation and reduced to 60°C; $\Delta \hat{S}^o$ (60), standard entropy of unfolding/dissociation at 60°C.

lows also from comparison of the calorimetric enthalpy with the van't Hoff enthalpy determined from the sharpness of the temperature induced process, as previously reported (Tamura *et al.*, 1994). More accurately it can be shown by the deconvolution of the observed excess heat capacity function, as described in Materials and Methods. The excess heat effect of WT SSI unfolding is indeed perfectly described by a bimolecular two-state transition (Figure 6(a) and (b)).

The enthalpies of unfolding

The enthalpies of unfolding of WT SSI and the D83C mutant given in Tables 1 and 2 are close but not identical to those previously reported by Tamura *et al.* (1994). This difference results mainly from differences in the intrinsic heat capacity func-

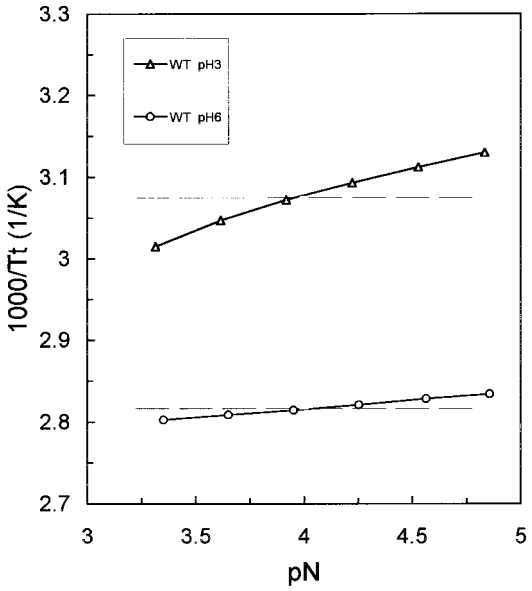

Figure 5. Plot of $1/T_t$ *versus* the negative logarithm of concentration, p*N*, for WT SSI at pH 6.0 and pH 3.0.

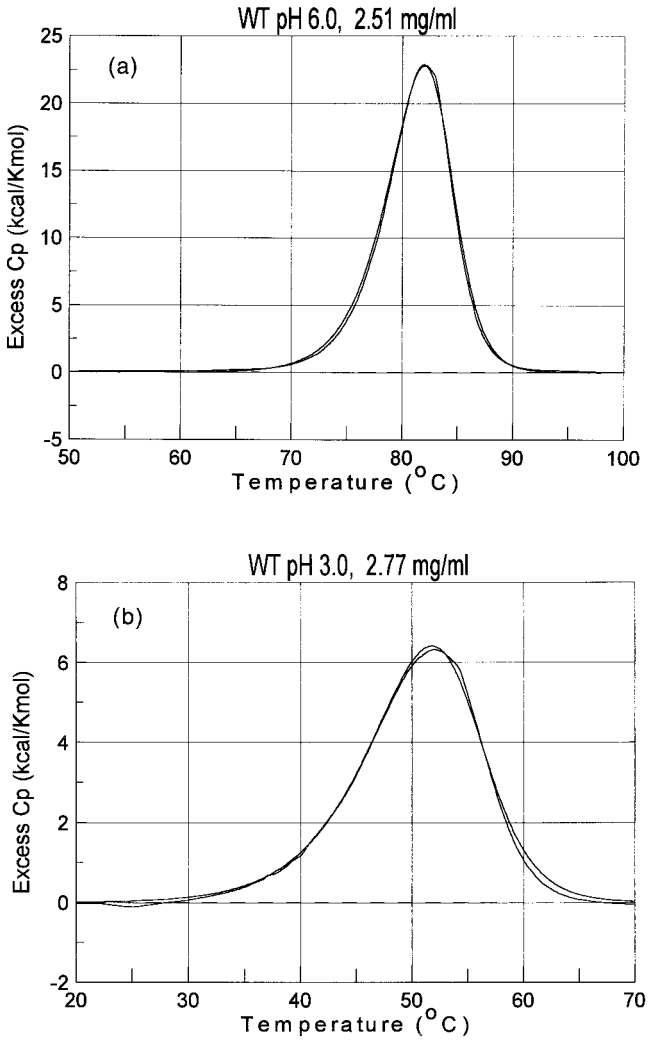

Figure 6. Simulation of the calorimetrically determined excess heat absorption of WT SSI assuming that the temperature induced process represents a bimolecular two-state transition: (a) at pH 6.0, $T_t = 81.3$°C, $\Delta H = 182.5$ kJ/mol; (b) at pH 3.0, $T_t = 50.7$, $\Delta H = 88.6$ kJ/mol. In both cases the simulated curve almost completely coincides with the experimental one.

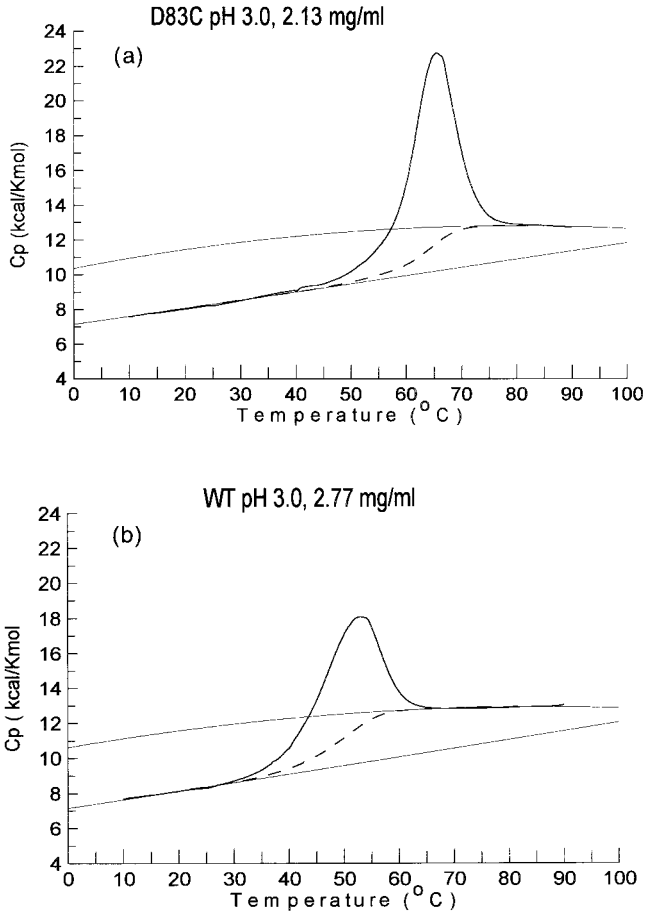

Figure 7. Temperature dependence of the partial heat capacity of WT SSI and the D83C mutant at pH 3.0 (continuous line) and the heat capacity functions for the native and unfolded states (light lines) which are used for estimation of the intrinsic heat capacity in the transition zone (broken lines).

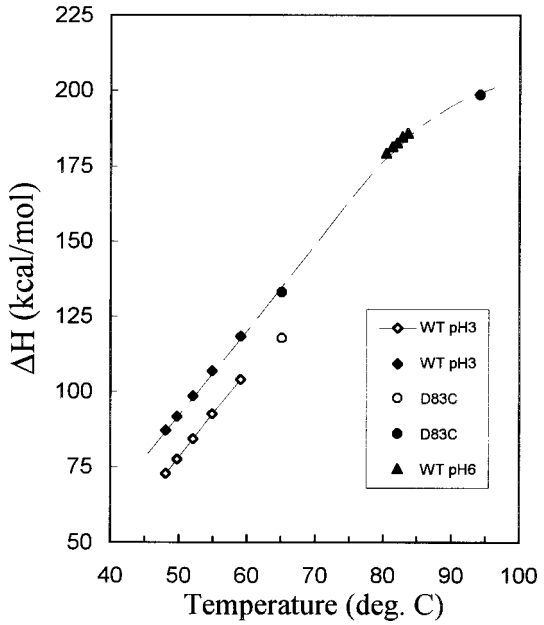

Figure 8. The enthalpies of unfolding of WT SSI and the D83C mutant measured at different concentration and pH (corrected for the histidine protonation effect in the case of low pH) and plotted against transition temperature. Empty symbols, including protonation heat, filled symbols, protonation heat subtracted.

tion used in the transition zone, which in our case was calculated from the known temperature dependencies of the heat capacities of the native and unfolded forms (Figure 7) and not on the extrapolation of the initial and final slopes of the heat capacity function to the transition zone (see Materials and Methods).

In the case of the solution at pH 3.0 the measured enthalpies were corrected for the effect of protonation of histidine. As in the previous paper of Tamura *et al.* (1994), this correction was assumed to be independent of temperature and equal to −7.14 kcal/mol (Izatt & Christensen, 1970). Using this correction we find that the enthalpy of unfolding of SSI and the D83C mutant is represented by the same universal function of temperature, notwithstanding at what pH the calorimetric experiment was carried out (Figure 8). With temperature increase the enthalpy function asymptotically approaches some constant value, as is usual for all globular proteins, which presumably is about 210 kcal/mol at 125 °C, i.e. about 9 cal/g. It is known that the asymptotic value of the specific unfolding enthalpy of a compact globular protein at this temperature is between 8 and 12 cal/g (Makhatadze & Privalov, 1995).

The asymptotic dependence of the unfolding enthalpy of globular proteins on the temperature results from the temperature dependence of the heat capacity increment of unfolding, which decreases with temperature increase and approaches zero above 125°C (Makhatadze & Privalov, 1995). This dependence of the heat capacity increment on temperature follows from the different temperature dependence of the heat capacity of the native (compact) and unfolded states of proteins: while the partial heat capacity of the native protein in aqueous solution is a linearly increasing function of temperature, the heat capacity of the unfolded state is not a linear function of temperature (Privalov & Makhatadze, 1990, 1992; Makhatadze & Privalov, 1995; Freire, 1995). These functions for the considered proteins are given in Figure 7 and in analytical form they are given by equations (11) and (12) in Materials and Methods. For the native proteins the heat capacity functions were determined by calorimetric measurements under conditions providing the highest stability to proteins and the highest precision for calorimetric determination of the

absolute values of the partial heat capacity at the given temperatures. These are concentrated protein solutions (from 2 to 10 mg/ml) and neutral pH. In acidic solutions one can determine this function only for the D83C mutant. It was found that the heat capacity functions for the native state of WT SSI and the D83C mutant are indistinguishable. For the WT SSI at pH 3.0, the temperature dependence of the native state heat capacity cannot be determined properly because unfolding starts at too low temperatures, but the heat capacity of the protein at 20°C is exactly the same as that in pH 6.0 solutions and equals 7.9(±0.3) cal/kmol, i.e. the specific heat capacity is 0.34(±0.02) cal/K·g, which is in the range expected for globular proteins. However, the temperature dependence of the heat capacity of the native WT SSI and D83C mutant dimers, (which is 0.0021 cal/K^2g) is almost twice as much as for monomeric globular proteins (Privalov & Makhatadze, 1990). For the unfolded proteins the heat capacity function was calculated from the sequence as proposed by Privalov & Makhatadze (1990) and checked by comparing with experimental values at the temperatures at which the proteins are in the unfolded state. The correctness of the difference between the heat capacities of the unfolded and native states was checked also by comparing the calculated values with the heat capacity increment observed at the transition and evaluated by simulation of the experimental heat capacity function under all the studied conditions. These calculations gave us confidence that the measured heat capacity increment of unfolding is correct. Its decrease with increasing temperature explains the discrepancy previously found: that the enthalpy of unfolding of the D83C mutant is lower than that of the WT SSI if compared at the same temperature (Tamura *et al.*, 1994). The enthalpy of unfolding of the D83C mutant appeared to be lower than that of WT SSI not because of some structural effect of crosslinking (deformation of the native conformation or incompleteness of unfolding) but because the unfolding of the D83C mutant takes place at much higher temperatures than that of the WT SSI where the heat capacity increment is smaller. In that case one cannot use a simple linear extrapolation to compare the enthalpy values measured at different temperatures. This is especially true for the experiments at pH 6.0 where unfolding occurs at temperatures where the decline of the heat capacity increment with temperature rise is steeper.

If we take into account the temperature dependence of the heat capacity increment and recalculate the unfolding enthalpy values to the same temperature, chosen as a standard, T_o:

$$\Delta H(T_o) = \Delta H(T_t) + \int_{T_t}^{T_0} \Delta C_p(T)\mathrm{d}T \qquad (3)$$

we find that they are very similar (Tables 1 and 2). We used different standard temperatures for the results obtained at pH 6.0 and pH 3.0 only so as to decrease the error arising from extrapolation from distant temperatures. The deviation between the values for the WT SSI and D83C at each of these standard temperatures (i.e. for two different solvent conditions) does not exceed the experimental error. The conclusion is that crosslinking does not lead to noticeable changes in the unfolding enthalpy of WT SSI.

Determination of the unfolding entropy

If the enthalpy function of some temperature induced process is known we can determine the entropy of this process. For the monomolecular two-state transition this can be done using the following modification of the general equation (1):

$$\Delta S(T_t) = \frac{\Delta H(T_t)}{T_t} \qquad (4)$$

where T_t is the half transition temperature in the absolute Kelvin's scale (see Materials and Methods). Since a monomolecular reaction does not depend on concentration this entropy can be considered as a standard, $\Delta\hat{S}^o$ (T_t).

The entropy of dissociation of a homodimer depends on the concentration and at the standard concentration, N^{st} , the standard entropy is given by the equation:

$$\Delta\hat{S}^o(T_t) = \frac{\Delta H(T_t)}{T_t} + R\ln(2N) \qquad (5)$$

Here the first term represents the entropy of the temperature induced transition at the concentration used in the experiment irrespective of the nature of the transition, $\Delta S(T_t)$. The second term accounts for the stoichiometry of the considered reactions; $N = N^o/N^{st}$ is a dimensionless relative initial concentration of the dimer (see Materials and Methods).

The entropies for the unfolding of the WT SSI and the D83C mutant at pH 6.0 and pH 3.0 are given in Tables 1 and 2. Since unfolding in acidic solutions proceeds with protonation of one histidine in each monomer, we have to correct the measured entropies, as we did with the enthalpy, to make them compatible with the results obtained in solutions at pH 6.0 in which histidine residues are not protonated. Assuming that the pK of histidine protonation at 298.15 K (25°C) is 6.0 and knowing the enthalpy of protonation, we can estimate its entropy at pH 3.0 as:

$$\delta S^{pr}(T) = \frac{\delta H^{pr} - 2.3RT(\mathrm{p}K - \mathrm{pH})}{T} \qquad (6)$$

This gives for δS^{pr} at 25°C (298.15 K) the value −10 cal/K·mol. Assuming that this correction does not depend significantly on temperature we find that the corrected entropy of unfolding of the

D83C mutant in acidic solution at 65.1°C equals 368 cal/K·mol. On the other hand if the entropy of unfolding of the D83C mutant in pH 6.0 solutions is reduced to the same temperature of 65.1°C (338.3 K) using the equation:

$$\Delta S(T) = \Delta S(T_t) + \int_{T_t}^{T} \Delta C_p(T) \mathrm{d} \ln T \qquad (7)$$

we obtain the value of 387 cal/K·mol. This difference might result from a too distant extrapolation (over 30 K) without knowing the possible heat capacity effect of protonation. Also, one cannot expect that protein unfolding in the pH 6.0 and pH 3.0 solutions would be absolutely identical entropically. Consequently, to exclude a possible error of too far extrapolation the results obtained at pH 6.0 and pH 3.0 are considered separately and reduced to different standard temperatures.

In Table 1 we give the values of the unfolding entropies of the WT SSI and the D83C mutant in pH 6.0 solutions reduced to the standard temperature of 80°C (353.2 K) and in Table 2 give the unfolding entropies of these proteins in pH 3.0 solutions, corrected for the protonation effect and reduced to the standard temperature of 60°C (333.2 K). It is clear that since protonation effects in WT SSI and D83C are identical they should not affect the difference in their unfolding entropies. The last columns in these two Tables include the standard entropies of unfolding for 1 M standard concentration.

Discussion

As it follows from Tables 1 and 2, the entropies of unfolding of the D83C mutant reduced to standard temperatures do not depend on concentration, as it should be for a monomolecular reaction. For the WT SSI, unfolding of which precedes with dissociation, the unfolding entropies are significantly larger and clearly increasing with decrease of concentration, as one would expect for a bimolecular reaction. The standard entropy of unfolding of WT SSI at 1 M standard concentration shows no dependence on concentration, but surprisingly its value appears to be very close to the entropy of unfolding of the D83C mutant which can be regarded as a standard since it does not depend on concentration. The difference between these two standard entropies, that of the D83C mutant and WT SSI, is of the order of 4 or 6 cal/K·mol at both standard temperatures, 60 and 80°C, i.e. under both solvent conditions, pH 6.0 and pH 3.0.

The possible error in our entropy difference estimates depends primarily on the error in the estimates of the difference in the enthalpies of unfolding of SSI and the D83C mutant. If the crosslinking does not affect the enthalpy of dimer unfolding, neglecting the heat capacity increment dependence on temperature the entropy effect of crosslinking is expressed as:

$$\begin{aligned}\delta\Delta S(T_1) &= \Delta S(T_{t,1}) - \Delta S^{ss}(T_{t,1}) \\ &\approx \frac{\Delta H(T_{t,1})}{T_{t,1}} - \frac{\Delta C_p(T_{t,2} - T_{t,1})}{T_{t,1}} \\ &\quad - \frac{\Delta H(T_{t,2})}{T_{t,2}} + \Delta C_p \ln\left(\frac{T_{t,2}}{T_{t,1}}\right) \\ &= \frac{\Delta T}{T_{t,1}}\left(\frac{\Delta H(T_{t,2})}{T_{t2}} - \frac{\Delta C_p \Delta T}{2T_{t,1}}\right) \qquad (8)\end{aligned}$$

where $\Delta T = T_{t,2} - T_{t,1}$ is the difference in the transition temperatures of the crosslinked and non-crosslinked dimer. It appears that the accuracy in the determination of $\delta\Delta S$ depends on the accuracy of the determination of ΔT, which can be done with high precision bearing in mind its rather large value (5 to 10 K). There is, however, some uncertainty in the identity of the unfolding enthalpy functions of WT SSI and the D83C mutant. We can be sure that the deviation between these enthalpies does not exceed 3 kcal/mol. Therefore the uncertainty in the obtained value of the crosslinking entropy is about 4 cal/K·mol.

The question now is how we should interpret the estimated entropy effect of crosslinking in the dimer. If we assume that the crosslinking of the native dimer does not lead to significant change of its entropy and the entropies of crosslinked and non-crosslinked native dimers are identical, than it follows that the entropy difference which we get at the standard concentration corresponds to the standard entropy of crosslinking of two unfolded polypeptide chains. It is clear that the unfolded crosslinked dimer does not dissociate and its translational freedom is the same as that of the monomer. But its rotational freedom is also not twice that of the monomer. Therefore, to the entropy effect of crosslinking should contribute not only restrictions on the translational freedom but also, at least to some extent, the restrictions on the rotational freedom. Nevertheless, the value of the crosslinking entropy which we measured is one order of magnitude smaller than the value predicted by statistical mechanic consideration for the loss of translational entropy (−50 cal/K·mol) and more so for the (translational + rotational) entropy (−100 J/K·mol) on the formation of a protein dimer at the standard 1 M concentration (Finkelstein & Janin, 1989; Tidor & Karplus, 1994). If the translational entropy were 50 cal/K·mol, the heat of unfolding of WT SSI would be 15 % higher than that measured, or even more if the rotational entropy makes a contribution. This difference in the heat effect exceeds our experimental error by at least one order of magnitude.

The value of the crosslinking entropy which we obtained is close in magnitude to the value predicted by the classical mixing theory for the loss in translation entropy on formation of a dimer, −8 cal/K·mol. One might explain the discrepancy

between our results and the estimates based on statistical-mechanic calculations by assuming that crosslinking changes the vibrational modes of the dimer and decreases its entropy (Tidor & Karplus, 1993). However, it is difficult to imagine that a single crosslink might decrease the vibrational entropy of a very stable and rigid dimer to such an extent as to balance almost completely the translational/rotational entropy effect which is expected to be of the order of 100 cal/K·mol. If crosslinking significantly affects the vibrational modes of a dimer, then that should appear in the temperature dependence of the native state heat capacity function: however, in the case when these functions are precisely determined over a broad temperature range for the crosslinked and non-crosslinked dimer (i.e. in solutions at pH 6.0) we do not see the difference in their slopes. Furthermore, no difference is seen between the $\delta\Delta\hat{S}^{o}$ values determined for two different temperatures, 60 and 80°C, a difference which should certainly appear if crosslinking induces significant changes in vibrational modes of a dimeric molecule.

The discrepancy between the value obtained for the crosslinking entropy and the translational entropy calculated by the Sackur-Tetrode equation cannot be explained either by the assumption that the unfolded crosslinked and non-crosslinked polypeptides, although similar in conformation, differ in their excluded volumes and the probabilities of intramolecular contacts. The Sackur-Tetrode equation does not take these circumstances into account. However, the larger excluded volume and more extensive intramolecular contacts of the crosslinked polypeptides could only decrease their entropy relative to the non-crosslinked polypeptides, i.e. result in a larger value of the net crosslinking entropy. By the same reason the small value of the obtained crosslinking entropy cannot be explained by the assumption that a disulfide bond reduces configurational freedom of the crosslinked residues in the polypeptide chains. If this effect of the reduction of configurational freedom takes place indeed it could only increase the observed crosslinking entropy, i.e. without this effect the $\delta\Delta S$ value would be even smaller than what we get. The additional bond which is introduced at crosslinking perhaps might increase the entropy of a dimer in the unfolded state by $R\ln 3 = 2$ cal/K·mol. If so, the net entropy reduction due to restrictions in the translational and rotational freedom upon formation of dimer should be higher than this value, i.e. it should be (7 ± 4) cal/K·mol. This, however, does not change much our conclusion that the entropy price of freedom lost upon complex formation is small.

The entropy of complex formation is smaller than the predicted perhaps because of various compensating effects and limitations which are not taken into account at the simplified statistical mechanic considerations. These might be the restrictions on the motion in the solvent, the unharmonic nature of these motions (Amzel, 1997), the change in the vibrational modes upon association (Tidor & Karplus, 1993), etc. Indeed, the calculated entropy loss upon protein association decreases in magnitude with the elaboration of statistical-mechanic analysis of this process and in the most recent publications (see Amzel, 1997; Brady & Sharp, 1997) it does not differ dramatically from the value which we determined experimentally.

Materials and Methods

Proteins

Streptomyces subtilisin inhibitor (SSI) and its mutant D83C were prepared as described by Tamura *et al.* (1994). A molecular mass of 23,000 was used for the wild-type (WT) SSI and D83C dimers (Hiromi *et al.*, 1985). For the calorimetric experiments two solvent conditions were used: 25 mM glycine buffer (pH 3.0) and 25 mM phosphate buffer (pH 6.0). The concentrated stock solutions of protein were exhaustively dialyzed against solvent and the concentration determined spectrophotometrically, using an absorption of 0.796 at 280 nm for a 1 mg/ml solution (Hiromi *et al.*, 1985). Solutions of lower concentration were obtained by accurate dilution of the stock solution and their concentrations were checked spectrophotometrically.

CD measurements

Circular dichroism (CD) spectra were measured on Jasco J-720 spectropolarimeter equipped with a variable-temperature accessory using a cell with a light path of 1 mm. Protein concentration in 25 mM phosphate buffer was 0.37 mg/ml for WT SSI and that for D83C was 0.33 mg/ml.

NMR measurements

One-dimensional, two-dimensional correlated spectroscopy (COSY), and nuclear Overhauser effect spectroscopy (NOESY) 1H-NMR spectra were measured on a Bruker DMX-750 spectrometer. Temperature values given are those of the sample solution in the NMR probe, calibrated with a thermocouple prior to the NMR measurements. SSI samples were dissolved in either 25 mM deuterated glycine buffer or 25 mM deuterated phosphate buffer at a concentration of 10 mg/ml. The pH values are direct readings of pH meter calibrated against $^{1}H_{2}0$ buffers.

Calorimetric measurements

Calorimetric measurements were performed on the prototype of the Nano-DSC (Calorimetric Science Corporation) which has been developed in the Biocalorimetry Center of the Johns Hopkins University (Privalov *et al.*, 1995). The capillary cells of this instrument of rather large volume (0.887 ml) provide an extremely stable baseline over a broad temperature range which is the main requirement for the determination of the partial heat capacity of a solute in dilute solution. The latter is determined at any given temperature by equation (9):

$$C_{p}(T)_{pr} = C_{p}(T)_{solv}[V(T)_{pr}/V(T)_{solv}] - \Delta C_{p}^{app}(T)/M(T)_{pr} \quad (9)$$

where $V(T)_{pr}$ and $V(T)_{solv}$ are the specific volumes of

protein and solvent at temperature T, $M(T)$ is the mass of protein in the calorimetric cell at temperature T, and $\Delta C_p^{app}(T)$ is the calorimetrically measured difference in the heat capacity of protein solution and solvent (Privalov & Potekhin, 1986; Privalov *et al.*, 1995).

The enthalpy of a temperature induced reaction is determined by integration of the excess heat capacity, $\langle C_p(T)\rangle$:

$$\Delta H = \int \langle C_p(T)\rangle \mathrm{d}T \tag{10}$$

The excess heat capacity function, $\langle C_p(T)\rangle$, is determined as the heat absorbed above the intrinsic heat capacity in the transition zone. The proper estimate of the intrinsic heat capacity in the transition zone is one of the most important aspects in the analysis of calorimetric data. It is evident that the simple connection of the heat capacity of the initial and final states by the straight line is incorrect. Only marginally better is the widely used extrapolation of the initial and final heat capacity functions to the midpoint of the transition because in most cases it is far from clear where the transition starts and ends. Furthermore, since the solubility of the unfolded state is lower the protein can aggregate at high temperatures. Even slight aggregation results in significant deformation of the heat capacity function and consequent errors in its extrapolation. This is especially true for bimolecular reactions for which the heat absorption functions are more asymmetric, having elongated low temperature shoulders (Freire, 1995; Breslauer, 1995). The straightforward extrapolation of the low temperature part of the heat capacity functions is therefore quite unjustified. In this study we first determined the partial heat capacity function of the native state over a broad temperature range and under conditions at which the proteins are the most stable (neutral pH, high concentration of protein). This showed that the partial molar heat capacity of the native SSI and D83C mutant are similar and are well described by the following linear function of temperature:

$$C_p{}^{N}(T) = AT + B \tag{11}$$

with $A = 0.0483$ kcal/K^2mol, $B = (6.86 + 0.X)$ kcal/K·mol and the variable X (between -0.3 and $+0.3$) which takes account of the uncertainty in the estimation of the absolute value of the heat capacity of the native state. The latter results from the specificity of the Nano-DSC that the slope of its base line is highly reproducible upon reloading the calorimetric cells but the level of base line can vary to some extent, being sensitive to the presence of microscopic bubbles. The heat capacity of the unfolded state was described by the parabolic function of temperature:

$$C_p{}^{U}(T) = AT^2 + BT + C, \tag{12}$$

with $A = -0.000314$ kcal/K^3mol, $B = 0.0592$ kcal/K^2mol and $C = (9.94 + 0.X)$ kcal/K·mol, which was calculated from the sequence of amino acid residues as described by Privalov & Makhatadze (1990) and was found to be in a very good correspondence with the experimentally determined values for SSI and the D83C mutant at high temperatures, where these proteins are in the unfolded state. Using these heat capacity functions of the native and unfolded states, the intrinsic heat capacity of protein in the transition zone was then calculated by iteration, assuming that it increases in proportion to the heat absorbed up to the given temperature (Privalov & Potekhin, 1986). It should be noted that the program for its calculation and the determination of the transition enthalpy and entropy values, comes with the Nano-DSC, together with a program for determination of the partial heat capacity function from the calorimetric results.

Entropy determination

The entropy of temperature induced monomolecular transitions was determined in two ways; (a) by integrating the excess heat capacity function in the logarithmic scale, in accordance with equation (1), and (b) by using the measured transition enthalpy assuming that at the half transition temperature, T_t, $\Delta G(T_t) = \Delta H(T_t) - T_t \Delta S(T_t) = 0$, and

$$\Delta S(T_t) = \frac{\Delta H(T_t)}{T_t} \tag{13}$$

The two methods give absolutely identical values.

For the multimeric temperature induced reaction:

$$X \Leftrightarrow m_1 U_1 + m_2 U_2 + \ldots + m_k U_k$$

we have for the equilibrium constant

$$K = \frac{(N)^{n-1} F^n}{1 - F} \prod_{i=1}^{k} (m_i)^{m_i} \tag{14}$$

here $n = \Sigma_{i=1}^{k} m_i$ is the order of reaction; $N = N^o/N^{st}$ is the dimensionless relative initial concentration of the complex, where N^o is the initial concentration and N^{st} is the standard concentration and F is the fraction of molecules that have undergone the transition at a given temperature T (Privalov & Potekhin, 1986). For the dissociation of a homodimer ($m = 2$, $n = 2$) at the half transition temperature, T_t, where $F = 1/2$:

$$K = (2N) \tag{15}$$

and

$$\Delta G(T_t) = -RT \ln(2N) \tag{16}$$

Therefore, for the standard entropy of unfolding/dissociation of a dimer at T_t we have:

$$\Delta \hat{S}^o(T_t) = \frac{\Delta H(T_t) - \Delta G(T_t)}{T_t} = \frac{\Delta H(T_t)}{T} + R \ln(2N) \tag{17}$$

It should be noted that if the temperature induced transition is sharp and the heat capacity increment is not big then instead of the half transition temperature T_t one can use the temperature at which the excess heat absorption equals half the total, $T_{1/2}$, or the temperature of the maximum of the heat absorption, T_{max}. The difference between them is not large (less then 1 K) in comparison to the value of the absolute transition temperature (Privalov & Potekhin, 1986). Therefore the error in the entropy estimation due to this approximation is less then 0.3% (i.e. is less then 1 cal/K·mol) and, being the same for both the WT SSI and D83C mutant, does not affect the difference between their unfolding entropies, $\delta\Delta\hat{S}^o$.

The entropy of a temperature induced multimeric reaction can also be determined at the reference temperature T_{ref} at which the Gibbs energy of the reaction is zero (Tamura *et al.*, 1994; Thompson *et al.*, 1993; Johnson *et al.*, 1995; Johnson & Freire, 1996). However, since T_{ref} is much higher than the actual transition temperature, the error in determination of the standard entropy at a temperature which is chosen as standard (usually the mid temperature for the cases which are compared) is much larger than that in the method used here.

Deconvolution of the heat capacity function

From a rigorous statistical thermodynamic point of view, the partial molar heat capacity of a macromolecule is given by the equation

$$C_p(T) = C_p{}^o(T) + \sum_{i=1}^{N} \Delta H_i \frac{\partial P_i}{\partial T} + \sum_{i=1}^{N} P_i \Delta C_{p,i} \qquad (18)$$

where $Cp^o(T)$ is the partial molar heat capacity of the native state, ΔH_i is the relative enthalpy of the state i, $\Delta H_i = H_i - H_o$ and P_i is the population of state i (Griko *et al*., 1995; Johnson & Freire, 1996). The program for the computer simulation of the experimental partial heat capacity function of macromolecules is included into a software package which comes with the Nano-DSC (Privalov *et al*., 1995). The simulation was used to double check the values of the enthalpy of unfolding and heat capacity increment determined as described above and to verify the model of unfolding, particularly the number of realized states.

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