Contribution of Translational and Rotational Entropy to the Unfolding of a Dimeric Coiled-Coil

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Significant controversy surrounds the estimation of the translational and rotational entropy of a macromolecule in aqueous solutions. To address this issue, the entropy of intersubunit cross-linking of a complex was determined in this study by comparing the calorimetrically measured unfolding entropies of a synthetic two-stranded α-helical coiled-coil both with and without a disulfide bond between the two polypeptide chains. The experimental cross-linking enthalpy and entropy are equivalent. In contrast, ideal gas statistics predict that the translational and rotational enthalpy and entropy differ by an order of magnitude. After the vibrational component is subtracted from the experimental cross-linking entropy, the resultant translation and rotational entropy is 5 ± 8 eu (1 eu = 1 cal·K^{-1}mol^{-1} = 4.184 J·K^{-1}mol^{-1}) at a standard state concentration of 1 M. This value is more than an order of magnitude smaller than estimates in the literature based on statistical mechanics of ideal gas models, which are 90−100 eu. This makes it doubtful that the translational and rotational entropy of macromolecules in aqueous solutions can be treated using ideal gas statistics.

Introduction

For a polyatomic molecule of n atoms, there are $3n$ degrees of freedom. In theoretical studies of these polyatomic molecules, it is a common practice to divide the $3n$ degrees of motion into 3 center of mass (COM) translational degrees of freedom, 3 rotational degrees of freedom of the whole molecule, and $3n-6$ internal vibrational/rotational degrees of freedom.[1] Depending on the questions at hand, such a division of motions may or may not be advantageous. For the following questions, this division has its merit. One is to compare reactions of different orders, such as the unfolding of a dimeric coiled-coil with that of a trimeric coiled-coil. Another is to correlate solution thermodynamic data on proteins and other biomacromolecules with their structures. In both cases, to make a meaningful comparison/correlation, the contributions from translational and rotational motions of the whole molecule have to be subtracted so that the resultant thermodynamic data correspond to reactions between molecules with fixed COM and orientations.

Because the translational and rotational motions of macromolecules at ambient temperatures can be treated classically, the enthalpy of the these motions, $\Delta H°_{t,r}$, can be easily estimated by applying the equi-partition theorem[1,2] as $3RT + P\Delta\bar{V}$. P is the pressure and $\Delta\bar{V}$ is the difference between the partial molar volumes of the products and the reactants. For dimeric dissociation reactions in ideal gas, $P\Delta\bar{V} = RT$. Thus $\Delta H°_{t,r}$(ideal gas) is $4RT$. For dimeric dissociation reactions in aqueous solutions, the $P\Delta\bar{V}$ term is negligible and $\Delta H°_{t,r}$(aqueous solution) is about $3RT$. The situation with the entropy of the translational and rotational motions, $\Delta S°_{t,r}$, is much more complex because the evaluation of it requires some knowledge of the potential function of the system. Clearly, the total potential function of a macromolecular aqueous solution is quite different from that of a macromolecular ideal gas. It is unclear whether and how this difference in potential function affects the evaluation of $\Delta S°_{t,r}$, particularly its volume term. Much controversy has been generated with regard to the estimation of $\Delta S°_{t,r}$(aqueous solution). The contentious point is whether $\Delta S°_{t,r}$(ideal gas) is a good approximation of $\Delta S°_{t,r}$(aqueous solution).

One view is that the ideal gas results are applicable to macromolecules in solution.[3−7] For typical protein molecules with molecular weight ranging from 5 to 25 kD, the translational and rotational motions each give 45−50 entropy units (1 eu = 1 cal·K^{-1}mol^{-1} = 4.184 J·K^{-1}mol^{-1}) at a standard state concentration of 1 M. This gives $\Delta S°_{t,r}$(aqueous solution) a value in the range of 90−100 eu, equivalent to $45-50RT$. The most notable feature of ideal gas statistics is that they predict that the entropic effect of the translational and rotational motions is an order of magnitude larger than the enthalpic effect ($4RT$).

A second view on this matter is to equate the translational entropy to the cratic entropy, given by the functional form $-R$ ln x, where x is the mole fraction of the solute.[8] The cratic entropy was originally proposed by Gurney[9] to denote the part of the mixing entropy that depends only on the numbers of distinct particles that have been mixed, i.e., it is actually the mixing entropy of solutes in ideal solutions. For a 1 M aqueous solution (containing 55 M water), the cratic term for the solute

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is given by $\delta S^{\text{cratic}} = -R \ln (1/55) = 8$ eu. The same value was then assumed to be correct for the cratic entropy term of proteins[10−12] at the fictitious 1 M standard state. However, the physical foundation of equating translational entropy with the cratic entropy is problematic from both thermodynamic[13] and statistical mechanical[14] points of view.

A third view on this matter,[13] proposed as a criticism of the second view, is to regard the motion of every atom as translational with equal kinetic energy. Then the momentum part of the translational motion of each individual atom exactly cancels out because each atom is necessarily present in both the reactant and the product. This essentially is an application of the equipartition theorem and requires that all the motions in a molecule can be treated classically. This view was echoed in a recent review by Gilson and co-workers[14] which discussed the theoretical framework for calculating binding affinities. This approach does not separate the $3n$ degrees of freedom into translational, rotational, and vibrational portions.

Several computational approaches to this problem have also been suggested. Invariably, these computational methods attack the problem from the protein−ligand binding point of view and always obtain the combined effect of the translational and rotational entropy loss upon binding. Hermans and Wang[15] recently developed a method of molecular dynamic simulation to estimate free energy for the binding of benzene molecule to a T4 lysozyme mutant. The simulation gives a value of $T\Delta S^\circ_{t,r}$(aqueous solution) as 7 kcal·mol^{-1} at 1 M standard state, which is equivalent to about 23 eu at 300 K.

Another type of computational approach is to compare the empirically calculated binding free energy with the measured values and then set the difference equal to the loss of translational and rotational entropy. According to Murphy et al.[11] and Gomez and Freire,[12] $\Delta S^\circ_{t,r}$(aqueous solution) is close to the cratic entropy value, i.e., about 8 eu. In another study,[16] $\Delta G^\circ_{t,r}$(aqueous solution) was varied systematically from 0 to 41.8 kJ·mol^{-1}. It was concluded that the 95% confidence interval for $\Delta G^\circ_{t,r}$(aqueous solution) is ±8 kJ·mol^{-1}, equivalent to ±6.5 eu at 298 K, with the optimum value being 4.3 eu, about half the value of the cratic entropy.

These conflicting theoretical thoughts and computational results suggest the need for an extensive experimental evaluation of $\Delta S^\circ_{t,r}$(aqueous solution) for macromolecules. The most direct approach is to measure the transfer entropy of macromolecules from gaseous phase to water. In fact, this has been used to get the correction on results from ideal gas statistics for small organic molecules, using Trouton's rule for nonassociating liquids.[10] However, this approach has serious drawbacks because not only is it very difficult to measure transfer quantities of macromolecules from gas to water, but also the transfer quantities will contain a large hydration term that is orders of magnitude larger than the translational and rotational effect. This is hardly surprising because water and the macromolecule strongly associate, therefore violating the basic premise of Trouton's rule.[17] Making matters worse was the very likelyhood that upon transferring from gaseous phase to water, the macromolecule will have conformational changes. The dissolution of protein crystals into aqueous solutions is an even more complicated transfer process because an additional factor, the crystal lattice entropy, has to be reckoned with. These factors make it impractical to obtain an accurate estimate of $\Delta S^\circ_{t,r}$(aqueous solution) for macromolecules from transfer measurements.

Another approach is to measure the dissociation entropy of two macromolecules in an aqueous solution. This is essentially the dissolution of microcrystals with the microcrystal being the dimer. The drawback is that in a dissociation reaction, many factors other than the translational and rotational motions contribute to the dissociation entropy and it is difficult to separate these factors. One such factor is hydration of the interface of the dimer.

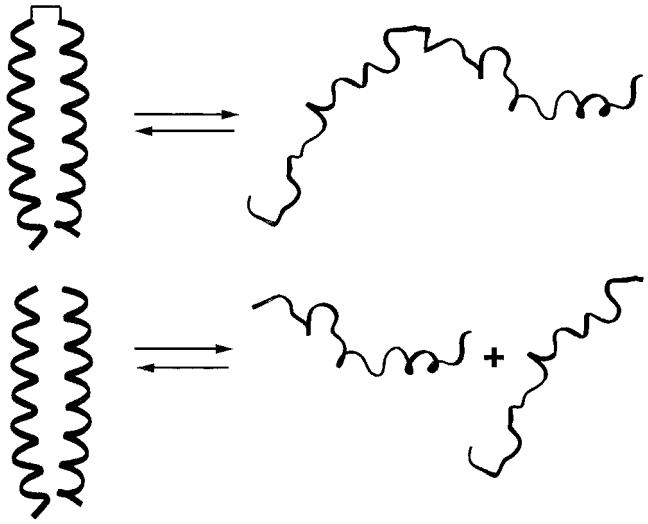

Figure 1. Schematic depiction of the equilibria for the unfolding of a cross-linked two-stranded parallel α-helical coiled-coil (top) as well as for the unfolding of its non-cross-linked counterpart (bottom). Note that the bottom forward reaction involves a dissociation process.

A third approach is a remedy to this second approach. It eliminates factors other than the translational and rotational motions from the dissociation reaction by comparing intermolecular reaction with its intramolecular counterpart where the two reactants are linked by a covalent bond (Figure 1). The challenge is to find a pair of such reactions in which the cross-link causes minimum disturbance to the folded and unfolded states.

In this study, we designed a peptide sequence made of repetitive heptad units which is capable of forming a homodimeric coiled-coil. A cross-link was then introduced through a disulfide bond at the hydrophobic interface. Structural features of the two peptides were characterized by analytical ultracentrifugation and circular dichroism (CD) spectroscopy. The differences between the enthalpy and entropy of unfolding of the cross-linked coiled-coil and that of the non-cross-linked coiled-coil were determined by calorimetric measurements of the partial heat capacity functions of the peptides in the temperature range 5−115 °C, over which their reversible unfolding/dissociation takes place under the given solvent conditions.

Experimental Procedures

The peptides were synthesized using standard solid-phase methodology and purified by reversed-phase HPLC.[18−20] The disulfide bond was formed by air oxidation in 100 mM NH_4HCO_3 at pH 8.[21] The authenticity of the peptides was verified by mass spectrometry. The sequences of the two peptides are shown in Figure 2. Compared to the cross-linked sequence, cysteine is substituted by serine in the non-cross-linked sequence. This conservative substitution is necessary because the free thio group will oxidize at high temperature even at pH as low as 2.0 (unpublished observation).

Sedimentation equilibrium studies were performed at 20 °C on a Beckman model E analytical ultracentrifuge equipped with Rayleigh interference optics as described previously.[22] The

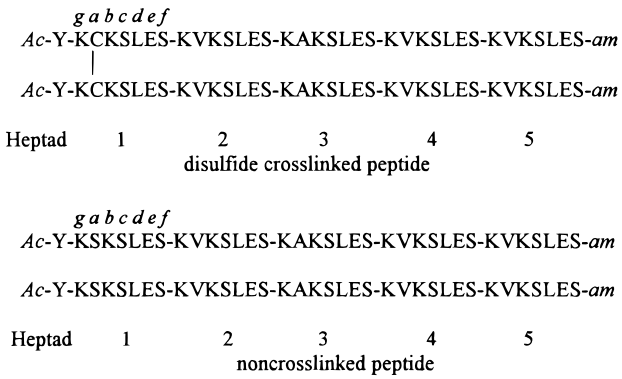

Figure 2. Sequences of the cross-linked and non-cross-linked peptides. The *N-*, *C*-termini were acetylated and amidated, respectively. *a, b, c, d, e, f, g* denote different positions in a heptad. Each chain has five heptads as labeled. The only difference between the two peptides is that in the non-cross-linked molecule, serine replaces the cysteine.

samples were dialyzed for 48 h against 100 mM Na_2SO_4 at pH 2.0 prior to loading into the ultracentrifuge cell. The radial equilibrium concentrations were analyzed using the program NONLIN[23] as described previously.[22]

The ellipticity of the peptides was measured using a Jasco-710 spectropolarimeter, calibrated with D-10-camphorsulfonate. The concentrations of the peptides were determined from their UV absorption at 275 nm in 5−6 M guanidinium hydrochloride at pH 6.5. The extinction coefficients at 275 nm are 2900 $M^{-1}\cdot cm^{-1}$ for the non-cross-linked and 3045 $M^{-1}\cdot cm^{-1}$ for the cross-linked peptide, calculated from their amino acid compositions according to Gill and von Hippel.[24] Light scattering was corrected for according to Winder and Gent.[25] Throughout this work, all peptide concentrations are expressed in molarity of the dimer.

Calorimetric measurements were carried out on the Nano-DSC microcalorimeter manufactured by Calorimetric Science Corporation (CSC). All measurements were conducted at a scanning rate of 1 $K\cdot min^{-1}$. The partial heat capacity of the peptides in solution was determined as described by Privalov and Potekhin,[26] using the value 0.764 $mL\cdot g^{-1}$ for the partial specific volumes of both peptides, calculated from their amino acid compositions according to Makhatadze et al.[27]

For the calorimetric study, solvent conditions were chosen as 100 mM Na_2SO_4 brought to pH 2.0 by phosphoric acid. Under this condition, the cross-linked and non-cross-linked coiled-coils unfold in the temperature range 5−115 °C. pH 2.0 was chosen to avoid chemical modification of the disulfide bond in the cross-linked peptide which occurs at high temperature in neutral and alkaline buffers.[28] To increase the temperature range of calorimetric experiments, they were all conducted under 3 atm excess pressure.

Results

The sedimentation equilibrium data obtained for the cross-linked (Figures 3a and 3b) and non-cross-linked (Figures 3c and 3d) coiled-coils are adequately described by a single species model as indicated by the small square root of variance (respectively 8.0×10^{-3} and 6.3×10^{-3} for the cross-linked and non-cross-linked) and the scattered distribution of the residuals. The apparent MW for the cross-linked and non-cross-linked peptides are respectively 10 500 and 9500, assuming a partial specific volume of 0.73 $mL\cdot g^{-1}$. These values are slightly higher than the expected MW for the two-stranded cross-linked monomer (8166) and the non-cross-linked (8140) dimers (Figure 1). On the other hand, the data sets could not be fitted to a self-association model. This indicates that within the range of concentration observed (which covers the range of concentrations used for the calorimetric studies) the cross-linked and the non-cross-linked peptides can be assumed to exist predominantly as covalently and noncovalently linked two-stranded coiled-coils, respectively. The slightly higher MW could then be explained by some nonideal behavior because of the highly charged nature of the coiled-coils (+20/molecule) at pH 2.0.

In the presence of 100 mM Na_2SO_4 pH 2.0, CD spectra of both peptides show typical characteristics of coiled-coils[18−21] (Figure 4). The ellipticity of neither peptide showed a concentration dependency over the experimental concentration range (inset to Figure 4). The average ellipticity at 222 nm (Θ_{222}) of the cross-linked peptide is $-35\,540 \pm 520$ $deg\cdot cm^2\cdot dmol^{-1}$ and that of the non-cross-linked peptide is $-29\,400 \pm 770$ $deg\cdot cm^2\cdot dmol^{-1}$ at 4 °C. There is no qualitative difference between these two spectra, both of which are indicative of the formation of α-helical coiled-coil. Because helicity is proportional to ellipticity for helical peptides,[29] the helicity of the non-cross-linked peptide is about 83% of that of the cross-linked peptide.

The lack of concentration dependency of the ellipticity of the non-cross-linked peptide means that its population is a homogeneous one in which each molecule is 83% as helical as the cross-linked peptide. This is consistent with the sedimentation equilibrium result of the non-cross-linked peptide, where only one population with the dimer molecular weight is observed. In contrast, the alternative scenario, in which an equilibrium exists between fully helical and fully random molecules, will show a concentration-dependent ellipticity in CD spectroscopy and two populations in sedimentation equilibrium, with one population having the molecular weight of the monomer and the other having the molecular weight of the dimer.

Figure 5 shows some of the partial molar heat capacity profiles of the cross-linked and non-cross-linked peptides at the highest experimental concentrations. Although the absolute value of the partial molar heat capacity can be measured accurately only at high concentrations, the temperature at which the partial molar heat capacity reaches maximum, T_{max}, can be reliably obtained at considerably lower concentrations. Tables 1 and 2 list T_{max} at all experimental concentrations for the cross-linked and non-cross-linked peptides, respectively. For the cross-linked peptides T_{max} is about 94.4 °C and independent of concentration, as would be expected for a monomeric unfolding process. T_{max} of the non-cross-linked peptide, on the other hand, is significantly lower than that of the cross-linked peptide and depends on peptide concentration. Such a concentration dependency of T_{max} is expected for a dimeric or any multimeric unfolding/dissociation processes and $1/T_{max}$ approximates a linear function of the logarithm of the peptide concentration (Figure 6). The relatively lower T_{max} of the non-cross-linked peptide indicates that, at experimental concentrations, the cross-link stabilizes the coiled-coil conformation by increasing the effective concentration.

Unfolding transitions of both the cross-linked and the non-cross-linked peptides fit the two-state transition model (Figure 5). From the fitting procedure, the standard unfolding enthalpy and entropy at T_{max}, $\Delta H°(T_{max})$, and $\Delta S°(T_{max})$, as well as heat capacity change at arbitrary temperature T, $\Delta C_p(T)$, can be determined (for detail, see Appendix A). Throughout this work, the standard state concentration is chosen as the conventional 1 M. These standard thermodynamic quantities are given in

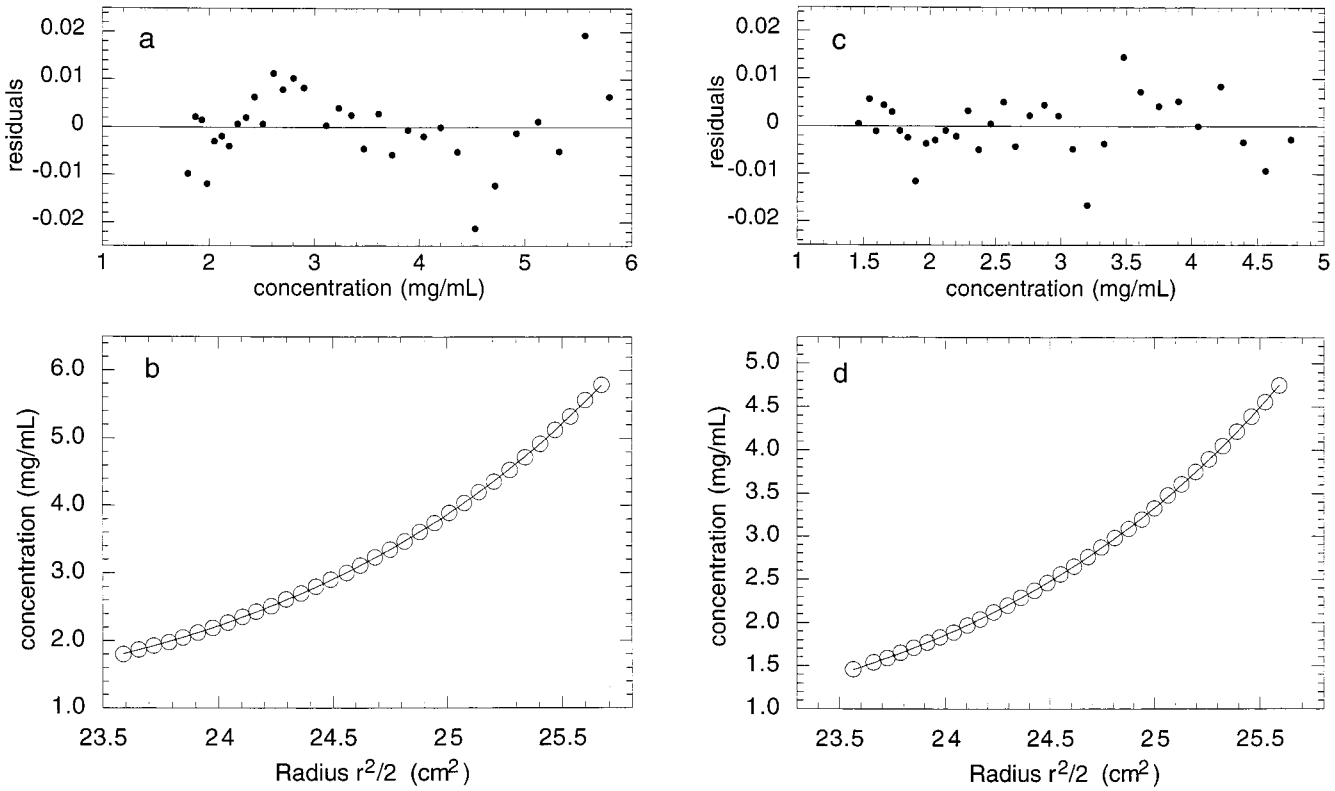

Figure 3. Sedimentation equilibrium studies of the cross-linked and the non-cross-linked coiled-coil. Both runs were performed in 100 mM Na_2SO_4 brought to pH 2.0 by phosphoric acid. The centrifuge speed used for the cross-linked coiled-coil (panels a and b) was 22 000 rpm and for the non-cross-linked coiled-coil (panels c and d) the speed was 24 000 rpm. The raw data and the curves fitted to a single species model are shown in panels b and d. The corresponding residuals are shown in panels a and c. The single species model is shown to fit adequately the raw data indicating that within the observed concentration range, both the cross-linked and the non-cross-linked peptides can be assumed to exist respectively as a covalently and noncovalently linked two-stranded coiled-coils.

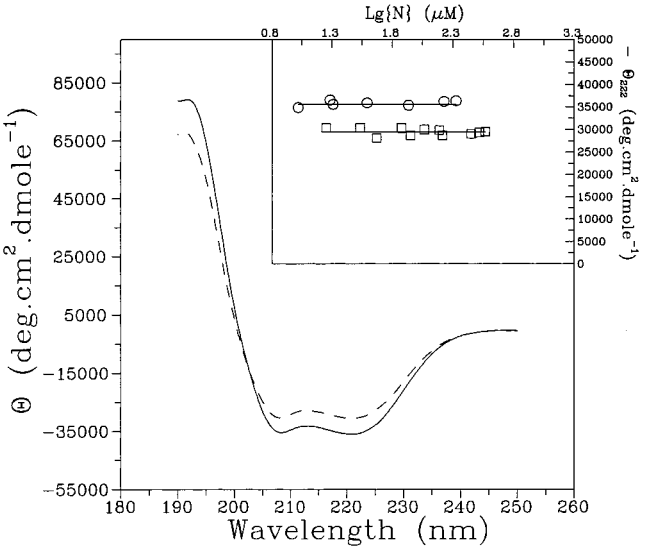

Figure 4. Circular dichroism spectra of the cross-linked (solid line) and non-cross-linked (dashed line) peptides at 2 °C, pH 2.0 in phosphoric acid containing 100 mM Na_2SO_4. Inset: Neither peptide's ellipticity (Θ_{222}) is concentration dependent. Circles represent the cross-linked and squares the non-cross-linked peptide.

Tables 1 and 2 for the cross-linked and non-cross-linked peptides, respectively.

For the non-cross-linked peptide, a van't Hoff unfolding enthalpy and entropy, $\Delta H°_{vH}$ and $\Delta S°_{vH}$, can be extracted from the linear relationship between $1/T_{max}$ and the logarithm of the peptide concentration (see Appendix B for detail). This van't Hoff enthalpy and entropy corresponds to the middle of the transition temperature range covered, which is 60 °C. The results are given in Table 2.

$\Delta H°(T)$ and $\Delta S°(T)$ at arbitrary temperature T can be calculated from $\Delta H°(T_{max})$, $\Delta S°(T_{max})$, and $\Delta C_p(T)$ (see Appendix A for detail). Figure 7 plots $\Delta H°(T)$ and $\Delta S°(T)$ of the cross-linked peptide (the solid lines), as well as $\Delta H°(T_{max})$ and $\Delta S°(T_{max})$ of the non-cross-linked peptide. The difference between the unfolding enthalpy (entropy) of the non-cross-linked peptide and the unfolding enthalpy (entropy) of the cross-linked peptide gives the apparent cross-linking enthalpy (entropy). At 70 °C, the apparent cross-linking enthalpy, $\delta\Delta H°$, is -14 ± 14 kJ·mol^{-1} and the apparent cross-linking entropy, $T\delta\Delta S°$, is -14 ± 12 kJ·mol^{-1} (equivalent to -10 ± 8 eu). The common temperature of 70 °C is chosen for comparison to minimize error in extrapolation caused by uncertainty in $\Delta C_p(T)$. Also plotted in Figure 7 (the dashed lines) are $\Delta H°(T)$ and $T\Delta S°(T)$ of the cross-linked peptide normalized to the same helicity of the non-cross-linked peptide. The difference between the unfolding enthalpy (entropy) of the non-cross-linked peptide and the normalized unfolding enthalpy (entropy) of the cross-linked peptide gives the corrected cross-linking enthalpy (entropy). The normalization is justified on the grounds that the peptide is made of repetitive units. At 70 °C, the corrected cross-linking enthalpy, $\delta\Delta H°$, is 21 ± 14 kJ·mol^{-1} and the corrected cross-linking entropy, $T\delta\Delta S°$, is 19 ± 12 kJ·mol^{-1} (equivalent to 13 ± 8 eu). Structurally, the 17% helicity difference between the cross-linked and non-cross-linked peptides is presumably due to end fraying at the *N*-terminal of the non-cross-linked peptide, which is eliminated by the disulfide bond in the cross-linked peptide.[19,20] Energetically, this means that the vibrational modes of the folded state are perturbed by the cross-link. For the

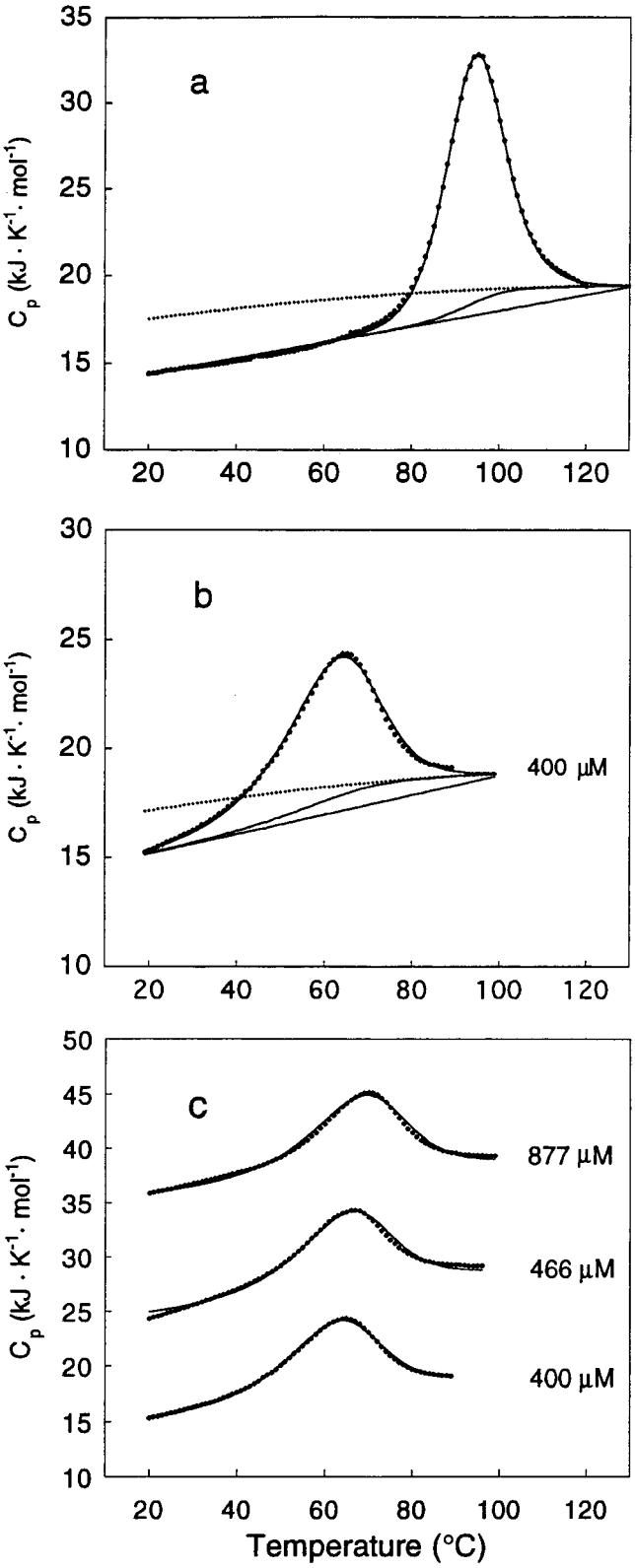

Figure 5. Partial molar heat capacity of the cross-linked (a) and non-cross-linked (b, c) peptides. Dark dots are the experimental data and solid line through the dark dots is the fitting of two-state transition model. In (a) and (b), dashed line is the calculated heat capacity of the unfolded state and solid line is fitting of the heat capacity of the native state. (c) The partial molar heat capacity of the non-cross-linked peptide at different concentrations (see Appendix A for detail).

purpose of obtaining $\Delta S°_{t,r}$(aqueous solution), this perturbation of the folded state can either be eliminated at this step by the normalization procedure or can be eliminated together with perturbation of the unfolded state at a later stage. Either way the same result should be obtained within experimental error.

TABLE 1: Thermodynamic Data of the Unfolding of the Crosslinked Peptide[a]

conc. (μM)	T_{max} (°C)	$\Delta H°$ (T_{max})	$\Delta S°$ (T_{max})	$\Delta H°$ (70 °C)	$\Delta S°$ (70 °C)	$\Delta \hat{H}°$ (70 °C)	$\Delta \hat{S}°$ (70 °C)
350	94.4	250	690	209	566	174	470
188	94.3	249	680	205	554	170	460
66	94.4	–	–	–	–	–	–
average				207 ± 11	560 ± 30	172 ± 11	465 ± 30

[a] $\Delta H°$ in kJ·mol^{-1}; $\Delta S°$ in J·K^{-1}·mol^{-1}, standard state concentration is 1 M. $\Delta \hat{H}° = 0.83\Delta H°$, the enthalpy value normalized to the helicity of non-cross-linked peptide. $\Delta \hat{S}° = 0.83\Delta S°$, the entropy value normalized to the helicity of non-cross-linked peptide. Errors in $\Delta \hat{H}°$ and $\Delta \hat{S}°$ are due to accumulation of error in $\Delta C_P(T)$ during extrapolation.

TABLE 2: Thermodynamic Data for the Unfolding of the Non-Cross-Linked Peptide[a]

conc. (μM)	T_{max} (°C)	$\Delta H°$ (T_{max})	$\Delta S°$ (T_{max})	$\Delta H°$ (70 °C)	$\Delta S°$ (70 °C)
877	69.1	195	521	195	522
466	66.0	188	505	194	522
400	64.1	186	500	196	525
217	61.6	–	–	–	–
137	59.7	–	–	–	–
103	57.7	–	–	–	–
55	54.0	–	–	–	–
25	50.4	–	–	–	–
–	60.0[b]	173[b]	461[b]	189[b]	508[b]
average				193 ± 3	519 ± 6

[a] $\Delta H°$ in kJ·mol^{-1}; $\Delta S°$ in J·K^{-1}·mol^{-1}, standard state concentration is 1 M. [b] Values obtained from the concentration dependence of T_{max} (see Appendix B for detail). Errors in $\Delta H°$ and $\Delta S°$ are standard deviations.

Of all the calorimetric data, T_{max}, $\Delta H°(T_{max})$, $\Delta S°(T_{max})$, and $\Delta C_p(T)$, T_{max} is the most reliable and $\Delta C_p(T)$ is the least reliable. The error in T_{max} is less than 0.3% (1 K). The uncertainty in $\Delta H°(T_{max})$ and $\Delta S°(T_{max})$ is mainly caused by error in concentration determination. An objective criterion in judging the uncertainties in T_{max}, $\Delta H°(T_{max})$, and $\Delta S°(T_{max})$ is to compare the values obtained for the cross-linked peptide at different concentrations, because these quantities are concentration independent. From Table 1, we can see that actual errors in all three quantities are less than 1%. The errors in $\Delta C_p(T)$ are about 5%, which correspond to the relative errors obtained for the fitted parameters in $C_{P,N}(T)$, and $C_{P,U}(T)$ (see Appendix A for detail). The uncertainty in $\delta\Delta H°$ ($\pm$ 14 kJ·mol^{-1}) and $T\delta\Delta S°$ ($\pm$12 kJ·mol^{-1}) is a combination of the accumulation of error in $\Delta C_p(T)$ as a result of extrapolation and standard deviation of different measurements which are caused by errors in concentration determination and reproducibility of calorimetric measurements.

Discussion

The purpose of this work is to find out how good is $\Delta S°_{t,r}$(ideal gas) as an approximation of $\Delta S°_{t,r}$(aqueous solution). The most notable feature of our results is that the cross-linking entropy, $T\delta\Delta S°$, is about equal to the cross-linking enthalpy, $\delta\Delta H°$ (apparent or corrected). This is in sharp contrast to the prediction from ideal gas statistics that $T\Delta S°_{t,r}$ is an order of magnitude larger than $\Delta H°_{t,r}$. More quantitative results depend on how large a vibrational component is contained in $\delta\Delta H°$ and $\delta\Delta S°$. Formally, $\delta\Delta H°_{vi}$, the vibrational component of $\delta\Delta H°$, and $\delta\Delta S°_{vi}$, the vibrational component of $\delta\Delta S°$, can be

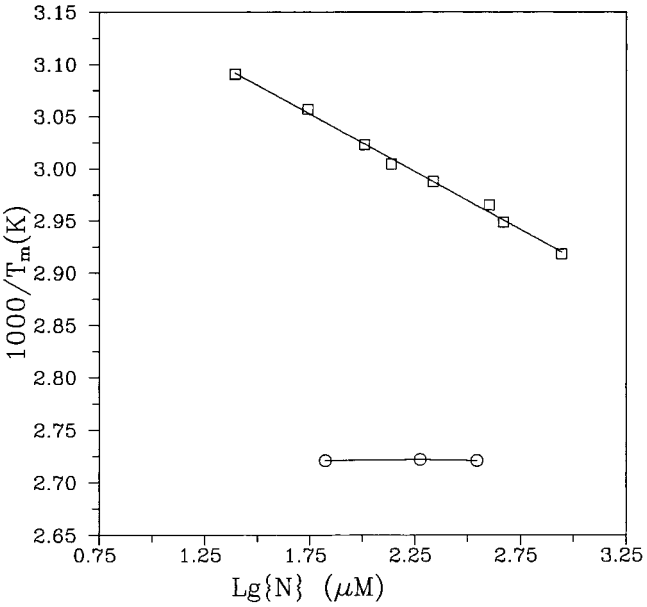

Figure 6. Although T_{max} of the cross-linked peptide (circles) shows no concentration dependency, that of the non-cross-linked (squares) does depend on concentration, expressed here as $10^3/T_{max}$ vs lg[N]. $10^3/T_{max}$ and lg[N] satisfy the linear relationship: $10^3/T_{max} = -0.1104$ lg[N] + 3.2454. From the slope of this linear relationship, the van't Hoff enthalpy of the transition, $\Delta H°_{vH}$, can be obtained as 173.2 kJ·mol^{-1}, corresponding to the middle of the transition temperature range covered, which is 60 °C. [N] at this temperature is 161 μM. Fraction of unfolded molecule is 0.65. These data are then used to calculate the transition entropy $\Delta S°_{vH}$ at this temperature. The result is 461 J·K^{-1}·mol^{-1}. See Appendix B for detail.

defined by the following equations:

$$\delta\Delta H° = \Delta H°_{t,r}(\text{aqueous solution}) + \delta\Delta H°_{vi} \quad (1)$$

$$\delta\Delta S° = \Delta S°_{t,r}(\text{aqueous solution}) + \delta\Delta S°_{vi} \quad (2)$$

The vibrational component contains the inevitable perturbations of the unfolded state as a result of cross-linking. It also contains perturbations of the folded state if the apparent cross-linking enthalpy and entropy are used in eqs 1 and 2. These vibrational terms can be estimated and subtracted from the cross-linking enthalpy and entropy in the following way. As analyzed in the Introduction, $\Delta H°_{t,r}$(aqueous solution) can be obtained from the equi-partition theorem as $3RT$, or 8.6 kJ·mol^{-1} at 70 °C. This forms the basis of estimating the magnitude of the vibrational component in $\delta\Delta H°$ and $T\delta\Delta S°$. If we use the apparent cross-linking enthalpy and entropy, from eq 1, $\delta\Delta H°_{vi}$ is about -22.6 kJ·mol^{-1}. Considering the compensatory relationship that exists between enthalpy and entropy of vibrational motions,[30] $T\delta\Delta S°_{vi}$ should also be on the order of -22.6 kJ·mol^{-1}. This gives $\delta\Delta S°_{vi}$ a value of -66 J·K^{-1}·mol^{-1}. Substituting this value of $\delta\Delta S°_{vi}$ into eq 2, we obtain a $\Delta S°_{t,r}$(aqueous solution) value on the order of 6 ± 8 eu. If the corrected cross-linking enthalpy and entropy are used, the same procedure will give $\Delta S°_{t,r}$(aqueous solution) a value of 4.3 ± 8 eu. Within experimental error, these two approaches give the same result for $\Delta S°_{t,r}$(aqueous solution), with an average of 5 ± 8 eu. Therefore, it makes no difference whether perturbation of the vibrational modes in the folded state is corrected in advance by helicity normalization or corrected together with perturbation of the unfolded state, using the arguments of equi-partition of energy and enthalpy−entropy compensation.

The value for $\Delta S°_{t,r}$(aqueous solution), 5 ± 8 eu, obtained here is the same as that obtained by Tamura and Privalov,[31] which was 5 ± 4 eu. The protein used in that study is the subtilisin inhibitor (SSI) from *Streptomyces*. SSI, a globular protein, is much more robust than an α-helical coiled-coil such that the cross-link caused very little perturbation to the structure and the measured cross-linking enthalpy is within experimental error to the predicted $3RT$. Consequently, no correction was made to the cross-linking entropy and the resultant $\Delta S°_{t,r}$(aqueous solution) value has a smaller error range.

The $\Delta S°_{t,r}$(aqueous solution) values obtained in both studies are much smaller than those for $\Delta S°_{t,r}$(ideal gas), which is 90−100 eu.[3−7] These results cast a serious doubt on using $\Delta S°_{t,r}$(ideal

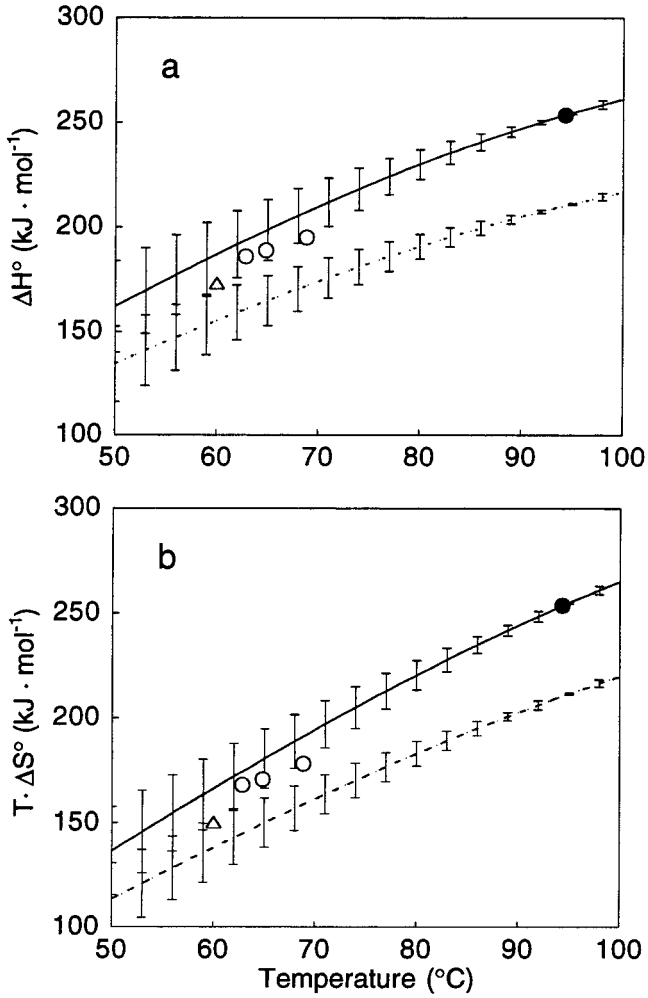

Figure 7. Comparison of unfolding enthalpy (a) and entropy (b). Solid circle is the average $\Delta H°(T_{max})$ or $T\Delta S°(T_{max})$ of the cross-linked peptide, whereas empty circles are $\Delta H°(T_{max})$ or $T\Delta S°(T_{max})$ of the non-cross-linked peptide at different T_{max} values as a result of different peptide concentrations. Empty triangle is $\Delta H°_{vH}$ and $\Delta S°_{vH}$ of the non-cross-linked peptide obtained from its transition temperature's concentration dependency (Figure 6). In (a), solid line is $\Delta H°(T)$ of the cross-linked peptide and dashed line is $0.83\Delta H°(T)$, i.e., the helicity normalized standard unfolding enthalpy of the cross-linked peptide. In (b), solid line is $T\Delta S°(T)$ of the cross-linked peptide and dashed line is $0.83T\Delta S°(T)$, i.e., the helicity normalized standard unfolding entropy of the cross-linked peptide. The difference between the empty symbols and the solid lines gives the apparent cross-linking enthalpy $\delta\Delta H°(T)$ and entropy $T\delta\Delta S°(T)$. The difference between the empty symbols and the dashed lines gives the corrected cross-linking enthalpy $\delta\Delta H°(T)$ and entropy $T\delta\Delta S°(T)$. As can be seen, $\delta\Delta H°(T)$ and $T\delta\Delta S°(T)$ are of similar magnitude. The size of the error bars reflects that the uncertainty in $\Delta C_P(T)$ is being accumulated through integration as the temperature moves away from T_{max}. The cross-linking enthalpy and entropy are obtained at 70 °C to minimize extrapolation. The error in $\Delta C_P(T)$ is about 5%. See Appendix A for detail.

gas) as an approximation of $\Delta S°_{t,r}$(aqueous solution) for macromolecules. These measured $\Delta S°_{t,r}$(aqueous solution) values are, on the other hand, close to the cratic entropy. Amzel[32] recently gave a theoretical explanation, based on the free volume theory of the liquid state,[33,34] as to why the cratic term is numerically close to the translational part of $\Delta S°_{t,r}$(aqueous solution). The numerical result obtained by Amzel for the translational entropy is 10.6 eu. Amzel's estimate poses a question rather than an explanation because the numerical result rests upon the premise that the solute molecule is the same size as the water molecule, which is invalid for macromolecular solutes such as proteins. In terms of the free volume theory of the liquid state, there is a qualitative difference between macromolecular solutes and small molecule solutes because the size of a macromolecular solute is much larger than that of the solvent cage, which is about 3.1 Å for aqueous solutions. It seems that a more refined theoretical analysis is needed to explain these experimental results.

Appendix A: Analysis of Calorimetric Data of Multimeric Molecules

For the two-state unfolding of a multimeric protein in the general form:

$$N \Leftrightarrow m_1D_1 + m_2D_2 + \cdots + m_kD_k \tag{A1}$$

the partial molar heat capacity, $C_P(T)$, at temperature T is given by[26]

$$C_P(T) = C_{P,N}(T) + \Delta H°(T)\cdot\frac{dF(T)}{dT} + F(T)\Delta C_P(T) \tag{A2}$$

where $F(T)$ is the fraction of unfolded molecules, $\Delta H°(T)$ is the standard unfolding enthalpy, $C_{P,N}(T)$ is the temperature-dependent partial molar heat capacity of the native state, and $\Delta C_P(T)$ is the heat capacity change upon unfolding, given by:

$$\Delta C_P(T) = C_{P,U}(T) - C_{P,N}(T) \tag{A3}$$

with $C_{P,U}(T)$ being the temperature-dependent partial molar heat capacity of the unfolded state.

To extract thermodynamic quantities out of $C_P(T)$, the following fitting procedure is used. First, $C_{P,U}(T)$ for both the cross-linked and the non-cross-linked peptides was calculated from their amino acid compositions.[35] They are both found to fit with 3% of the following quadratic functions of T:

$$C_{P,U} = A + B\cdot T + C\cdot T^2 \tag{A4}$$

where $A = -3.8$ kJ·K^{-1}·mol^{-1}, $B = 0.113$ kJ·K^{-2}·mol^{-1}, and $C = -0.0001374$ kJ·K^{-3}·mol^{-1}.

Then $C_{P,N}(T)$ is obtained from fitting the pretransition partial molar heat capacity of the cross-linked peptide to a linear function of T:

$$C_{P,N}(T) = D + E\cdot T \tag{A5}$$

where $D = 1.21$ kJ·K^{-1}·mol^{-1} and $E = 0.045$ kJ·K^{-2}·mol^{-1}.

To determine $\Delta H°(T)$ and $\Delta S°(T)$ from $C_P(T)$, it is first noted that $F(T)$ is not an independent variable, it is related to $\Delta H°(T)$, $\Delta S°(T)$, and $\Delta C_P(T)$ through the equilibrium constant of the reaction $K(T)$:

$$K(T) = \frac{F(T)^n[N]^{n-1}\prod_{i=1}^{k}(m_i)^{m_i}}{1 - F(T)} \tag{A6}$$

where m_i values are the stoichiometric coefficients, with $n = \Sigma m_i$. n is the order of reaction. $[N] = [N]^0/[N]^{st}$ is the dimensionless concentration with $[N]^0$ being the total concentration of the complex and $[N]^{st}$ being the concentration of the standard state.[26] In this study, $[N]^{st} = 1$ M. For monomeric reaction ($n = 1$, $m = 1$),

$$K(T) = \frac{F(T)}{1 - F(T)}$$

which gives

$$F(T) = \frac{K(T)}{1 + K(T)} \tag{A7}$$

For homodimeric reaction ($n = 2$, $m = 2$),

$$K(T) = \frac{4[N]\cdot F(T)^2}{1 - F(T)}$$

which gives

$$F(T) = \frac{-K(T) + (K(T)^2 + 16[N]K(T))^{1/2}}{8[N]} \tag{A8}$$

Furthermore, the temperature derivative of $F(T)$ can be obtained as:

$$\frac{dF(T)}{dT} = \frac{\Delta H°(T)}{RT^2}\cdot\frac{F(T)\cdot(1 - F(T))}{n - F(T)(n-1)} \tag{A9}$$

$K(T)$ is related to $\Delta H°(T)$ and $\Delta S°(T)$ through the following thermodynamic relationship:

$$-RT\ln K(T) = \Delta G°(T) = \Delta H°(T) - T\Delta S°(T) \tag{A10}$$

This allows $F(T)$ in the expression of $C_P(T)$ to be replaced by $\Delta H°(T)$ and $\Delta S°(T)$.

The transition temperature, T_t, can be replaced by T_{max}, defined as the temperature at which $C_P(T)$ reaches maximum. T_{max} is the most accurately determined quantity from calorimetric measurement. At this temperature, it can be shown that $F(T)$ satisfies the following equation:[26]

$$F(T_{max}) = \frac{n^{1/2}}{n^{1/2}+1} + \frac{n^{1/2}}{n-1}\cdot\left[1 - \frac{1}{(1+(n-1)X)^{1/2}}\right] \tag{A11}$$

with

$$X = \frac{RT_{max}[3T_{max}\Delta C_p(T_{max}) - 2\Delta H°(T_{max})]}{(\Delta H°(T_{max}))^2} \tag{A12}$$

For the unfolding of macromolecules, X is very small (0.05 for the cross-linked and 0.08−0.1 for the non-cross-linked). Therefore, eq A12 can be further simplified using series expansion up to the quadratic term in X:

$$F(T_{max}) = \frac{n^{1/2}}{n^{1/2}+1} + \frac{n^{1/2}}{2}X - \frac{3(n-1)\cdot n^{1/2}}{8}X^2 \tag{A13}$$

This gives the equilibrium constant at T_{max} as:

$$\ln K(T_{max}) = \ln \frac{F(T_{max})^n [\mathrm{N}]^{n-1} \prod_{i=1}^{k} (m_i)^{m_i}}{1 - F(T_{max})}$$

$$\approx \ln \frac{[\mathrm{N}]^{n-1} \cdot n^{n/2} \prod_{i=1}^{k} (m_i)^{m_i}}{(n^{1/2}+1)^{n-1}} + X \cdot \frac{n^{1/2} \cdot (n^{1/2}+1)^2}{2} \quad \text{(A14)}$$

The last expression is obtained through series expansion permitted by the small value of X. From this relationship, we obtain:

$$\Delta S^\circ(T_{max}) = \frac{\Delta H^\circ(T_{max}) - \Delta G^\circ(T_{max})}{T_{max}}$$

$$= \frac{\Delta H^\circ(T_{max})}{T_{max}} + R \ln K(T_{max})$$

$$= \frac{\Delta H^\circ(T_{max})}{T_{max}} + R \ln \frac{[\mathrm{N}]^{n-1} \cdot n^{n/2} \prod_{i=1}^{k} (m_i)^{m_i}}{(n^{1/2}+1)^{n-1}} + RX \cdot \frac{n^{1/2} \cdot (n^{1/2}+1)^2}{2} \quad \text{(A15)}$$

The above eqs A13, A14, and A15 are valid for any n, including $n = 1$. For monomeric reaction ($n = 1$, $m = 1$), the second term in eq A15 is zero. For homodimeric reaction ($n = 2$, $m_1 = 2$), the second term is Rln3.32[N]. The third term is practically zero for all the measurements. Equation A15 eliminates $\Delta S^\circ(T_{max})$ from the expression of $C_P(T)$. Therefore, only eight parameters remain in the actual fitting procedure. These are A, B, C, D, E, T_{max}, $\Delta H^\circ(T_{max})$, and [N]. Of these eight parameters, the B, C, E, T_{max}, and [N] are fixed. A and D values are allowed to float around the average value to account for uncertainty of the experimental partial molar heat capacities. The error on A is ± 0.3 kJ·K^{-1}·mol^{-1} and the error on D is ± 0.6 kJ·K^{-1}·mol^{-1}. $\Delta H^\circ(T_{max})$ and $\Delta S^\circ(T_{max})$ are then obtained by an in-house written least-squares fitting procedure.

From $\Delta H^\circ(T_{max})$, $\Delta S^\circ(T_{max})$, and $\Delta C_P(T)$, $\Delta H^\circ(T)$ and $\Delta S^\circ(T)$ at arbitrary temperature T can be obtained, using the following equations:

$$\Delta H^\circ(T) = \Delta H^\circ(T_{max}) + \int_{T_{max}}^{T} \Delta C_p(T)\, \mathrm{d}T$$

$$\Delta S^\circ(T) = \Delta S^\circ(T_{max}) + \int_{T_{max}}^{T} \frac{\Delta C_p(T)}{T}\, \mathrm{d}T \quad \text{(A16)}$$

Appendix B: the van't Hoff Analysis

From eq A6 it is clear that when $n \geq 2$, the fraction of unfolding molecule, F, is a function of both temperature T and total complex concentration [N]. T and [N] are independent variables. In contrast, T_{max} is a function of [N]. The dependency of T_{max} on [N] at constant pressure is given by the following equation, which is obtained by differentiating both sides of eq A6 with respect to $1/T_{max}$:

$$(n-1) \cdot \frac{\partial \ln[\mathrm{N}]}{\partial(1/T_{max})} = -\frac{\Delta H^\circ(T_{max})}{R} - \frac{\partial \ln \frac{F(T_{max})^n}{1 - F(T_{max})}}{\partial(1/T_{max})}$$

$$= -\frac{\Delta H^\circ(T_{max})}{R} - \frac{F(T_{max}) + n(1 - F(T_{max}))}{(1 - F(T_{max}))} \cdot \frac{\partial F(T_{max})}{\partial(1/T_{max})}$$

$$= -\frac{\Delta H^\circ(T_{max})}{R} - \frac{F(T_{max}) + n(1 - F(T_{max}))}{(1 - F(T_{max}))} \cdot \left[\frac{n^{1/2}}{2} - \frac{3(n-1) \cdot n^{1/2}}{8} \cdot X\right] \cdot \frac{\partial X}{\partial(1/T_{max})} \quad \text{(B1)}$$

The last expression follows from eq A13. Ignoring the small dependence of $\Delta C_P(T)$ on T, from the expression of X, we obtain

$$\frac{\partial X}{\partial(1/T_{max})} = \frac{2RT_{max}^2 \cdot (3T_{max}\Delta C_P(T_{max}) - \Delta H^\circ(T_{max})) \cdot (T_{max}\Delta C_P(T_{max}) - \Delta H^\circ(T_{max}))}{(\Delta H^\circ(T_{max}))^3} \quad \text{(B2)}$$

The second term in eq B1 is much smaller than the first term. For example, at the three highest concentrations where X can be calculated, the ratios of the second term to the first term are 0.012, 0.015, and 0.017. Therefore, the relationship between T_{max} and [N] can be simplified as:

$$\frac{\partial \ln[\mathrm{N}]}{\partial(1/T_{max})} = -\frac{\Delta H^\circ(T_{max})}{(n-1)R} \quad \text{(B3)}$$

or:

$$\frac{\partial(10^3/T_{max})}{\partial \lg[\mathrm{N}]} = -\frac{2303 \cdot (n-1)R}{\Delta H^\circ(T_{max})} \quad \text{(B4)}$$

This means that $(T_{max})^{-1}$ is, to a good approximation, a linear function of ln[N]. The linearity rests upon the values of $\Delta C_P(T_{max})$ and $\Delta H^\circ(T_{max})$. As long as $T_{max} \cdot \Delta C_P(T_{max})$ is not excessively larger than $\Delta H^\circ(T_{max})$ (2 or 3 orders of magnitude), the linearity will hold over a large experimental concentration range. The physical significance is that $F(T_{max})$ is close to a constant (0.64−0.65 in this study) in the experimental concentration range. In fact, such linearity holds between ln[N] and any temperature with a fixed F value. Marky and Breslauer[36] had previously derived such a linear relationship between ln[N] and $(T_t)^{-1}$ with the constraint that $\Delta C_P(T) = 0$. T_t is the temperature where $F = 0.5$. The analysis presented here shows that the constraint on ΔC_p is unecessary in most cases.

Such a transition temperature and total concentration dependence analysis allows one to obtain the van't Hoff transition enthalpy from the slope of the plot. This van't Hoff enthalpy is most logically assigned to the middle of the experimentally covered transition temperature range. From this enthalpy and the assigned temperature and corresponding [N], the reaction's equilibrium constant K and transition entropy can be calculated, using eqs A6 and A15, respectively.

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List of Notations

$\delta\Delta H°$	experimental cross-linking enthalpy
$\delta\Delta S°$	experimental cross-linking entropy
$\Delta H°_{t,r}$	enthalpy change due to translational and rotational motions
$\Delta S°_{t,r}$	entropy change due to translational and rotational motions
$\Delta H°_{t,r}$(ideal gas)	the $\Delta H°_{t,r}$ term in ideal gas phase
$\Delta S°_{t,r}$(ideal gas)	the $\Delta S°_{t,r}$ term in ideal gas phase
$\Delta H°_{t,r}$(aqueous solution)	the $\Delta H°_{t,r}$ term in aqueous solutions
$\Delta S°_{t,r}$(aqueous solution)	the $\Delta S°_{t,r}$ term in aqueous solutions
$\delta\Delta H°_{vi}$	vibrational component of the experimental cross-linking enthalpy
$\delta\Delta S°_{vi}$	vibrational component of the experimental cross-linking entropy

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