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Temperature and Urea Have Opposing Impacts on Polyproline II Conformational Bias

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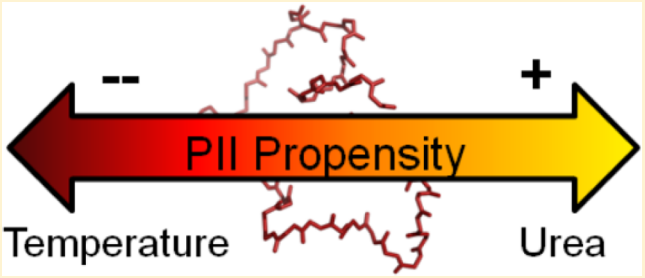
ABSTRACT: The native states of globular proteins have been accessed in atomic detail by X-ray crystallography and nuclear magnetic resonance spectroscopy, yet characterization of denatured proteins beyond global metrics has proven to be elusive. Denatured proteins have been observed to exhibit global geometric properties of a random coil polymer. However, this does not preclude the existence of nonrandom, local conformational bias that may be significant for protein folding and function. Indeed, circular dichroism (CD) spectroscopy and other methods have suggested that the denatured state contains considerable local bias to the polyproline II (PII) conformation. Here, we develop predictive models to determine the extent that temperature and the chemical denaturant urea modulate PII propensity. In agreement with our predictive model, PII propensity is observed experimentally to decrease with an increase in temperature. Conversely, urea appears to promote the PII conformation as determined by CD and isothermal titration calorimetry. Importantly, the calorimetric data are in quantitative agreement with a model that predicts the stability of the PII helix relative to other denatured state conformations based upon solvent accessible surface area and experimentally measured Gibbs transfer free energies. The ability of urea to promote the PII conformation can be attributed to the favorable interaction of urea with the peptide backbone. Thus, perturbing denatured states by temperature or cosolutes has subtle, yet opposing, impacts on local PII conformational biases. These results have implications for protein folding as well as for the function of signaling proteins that bind proline-rich targets in globular or intrinsically disordered proteins.



At present, the Protein Data Bank[1] contains more than 85000 protein structures, each of which represents an atomic-level characterization of the native state. Understanding how proteins arrive at the native state through the folding process requires knowledge of the denatured state.[2] Beyond the process of protein folding, characterization of the denatured state also has functional implications, both for globular proteins that utilize locally unfolded states to modulate their activity[3,4] and for intrinsically disordered proteins.[5−7] Experimental studies[8,9] have shown that chemically denatured proteins obey random coil statistics as described by Flory.[10] However, the observation that the denatured state behaves globally as a random coil does not preclude local bias to certain conformations.[11] Indeed, even a contrived denatured state model containing 92% of positions in their native conformation has been shown to obey random coil statistics.[12]

Tiffany and Krimm hypothesized that the denatured state contains local structure.[13] They arrived at this hypothesis after observing the resemblance of the circular dichroism (CD) spectra of denatured proteins to the CD spectra of proline homopolymers,[13] a peptide that was known to have a strong propensity for forming the polyproline II (PII) conformation.[14,15] Importantly, the PII conformation is also adopted by amino acids other than proline. Similar spectra were also observed for small homopolymers of glutamate and lysine, strongly suggesting that PII bias is present in sequences lacking proline.[16] The observation of PII bias in segments lacking proline has since been confirmed by surveys of the Protein Data Bank[17,18] and coil libraries.[19] In the past decade, both experimental and computational approaches have provided evidence that the PII conformation is highly populated in the denatured state.[11,20−27] However, it has been known since the work of Tiffany and Krimm that the propensity for PII in the denatured state is sensitive to solution conditions.[28]

Several groups have studied the effects of various solvent conditions on PII propensity using model peptide systems.[29−32] Of particular interest is work by Creamer and colleagues that uses CD spectroscopy to measure the impact of temperature variation and chemical denaturants, guanidine hydrochloride (GdnHCl) and urea, on PII propensity.[29,32] Collectively, their work provides experimental evidence that PII propensity has a weakly negative temperature dependence,[29] while the PII propensities of several model peptides exhibit significant increases in the presence of GdnHCl and urea.[29,32] Further, they suggest that the increase in PII content observed in the

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presence of urea is characteristic not only of model peptides but also of a sample of widely studied globular proteins.[32] Other studies of the temperature dependence of PII propensity in intrinsically disordered proteins[33] suggest that PII propensity is temperature-dependent. Together, these studies support the hypothesis that temperature and solvent perturbations have the capacity to modulate the local structure of the denatured state.

In this study, we examine the ability of temperature variation and urea to modulate the local backbone structure, specifically with respect to the PII conformation. We employ the system previously described[34−36] that utilizes the binding of a Src-homology domain (SH3) to a proline-rich ligand (SosY) derived from its biological binding partner, the son of sevenless protein. Importantly, the SH3 domain binds the SosY peptide specifically in the PII conformation.[37] Using isothermal titration calorimetry (ITC), differences in binding observed for the SosY peptides at different temperatures and solute concentrations can be interpreted in terms of changes in PII propensity. In agreement with predictive models, PII propensity is observed to decrease with an increase in temperature, while PII propensity is observed to increase with an increase in urea concentration. The observation that these two perturbants, commonly used to denature proteins, have opposite effects on the local PII structure of the denatured state has implications for protein folding and for the evolution and functional sensitivity of locally unfolded or intrinsically disordered (ID) proteins.

EXPERIMENTAL PROCEDURES

Models for the Modulation of PII by Temperature and Urea. The PII propensities of alanine (30%) and glycine (10%) have been calorimetrically determined.[34−36] The COREX algorithm[38] was used to estimate the SosY peptide reference enthalpy and heat capacity of unfolding. This was accomplished by excising the SosY ligand from the X-ray crystal structure in complex with the *Caenorhabditis elegans* Sem-5 (sex muscle five) Src-homology 3 (SH3) domain.[37] From these data, the temperature dependence of the difference in conformational free energy between alanine and glycine was modeled. Using the same peptide X-ray coordinates, an algorithm (http://best.bio.jhu.edu/mvalue/) was used to estimate the expected free energy for unfolding the SosY peptide in 1 M urea based upon experimentally determined Gibbs transfer free energies (TFEs).[39,40] This algorithm calculates the solvent accessible surface area of the SosY peptide (taken as the native state) and determines the free energy of unfolding based on the expected difference in solvent accessible surface areas of denatured state models.[41−43] Model scripting and data analysis were performed in the R Project.

Isothermal Titration Calorimetry. The *C. elegans* Sem-5 (sex muscle five) Src-homology 3 (SH3) domain was expressed and purified as described previously.[37] The son of sevenless (SosY) peptide ligand and mutant peptides (Ac-VPPXVPPR-RRY, where X is P, A, or G) were obtained from Genscript USA Inc. and Neo Biosciences Inc., with their identities and purities (>98%) verified by mass spectrometry. SosY peptide solutions were prepared using the buffer from the final protein dialysis. SH3 protein and SosY peptide concentrations were determined using the Edelhoch method[44] using a Shimadzu UV-1800 spectrophotometer and the extinction coefficients of 13940 and 1280 M^{-1} cm^{-1} for SH3 and SosY peptides, respectively. Isothermal titration calorimetry (ITC) experiments were performed on a MicroCal VP-ITC[45] instrument in phosphate buffer [20 mM sodium phosphate and 200 mM sodium chloride (Fisher)] at pH 7.5 and 25 °C as described by Ferreon and Hilser.[34] c values of the titrations ranged from 1 to 20. Solutions were thoroughly degassed, and an initial injection of 2 μL was included to correct for pretitration mixing. For all conditions tested, the ligand was titrated into identical buffer lacking the SH3 protein and the subsequent SH3−SosY titration heats were corrected for the heats of ligand dilution, which were small (~100 cal/mol or less) compared to the heat of binding. Peptide concentrations were normally ~10 times that of the SH3 protein. We used approximately 34 injections of 8 μL of ligand for our titrations, with a 280 s equilibration time between injections. The temperature and urea concentrations were varied as needed for the respective assays. Data were fit in Origin 7 (OriginLab Corp.) using nonlinear least-squares regression. Fit values for N (the apparent binding stoichiometry) were near 1.0 with a typical error of ±0.04. Fit values for K varied for each experiment, but the error in the fitting was typically only 1−2% of the fit value. The small error in fitting for K is crucial, as our free energy comparisons come directly from these values. Our error in $\Delta\Delta G$ is ±30 cal/mol, corresponding to ~5% of our typically observed $\Delta\Delta G$ of 600−700 cal/mol.

Circular Dichroism Spectroscopy. All CD scans were performed on a Jasco J-720 spectropolarimeter. SosY peptide samples were prepared by diluting ITC ligand solutions (with identical buffer) to concentrations suitable for CD, ~0.1 mg/mL. This dilution was necessary to avoid aggregation during temperature scan experiments. Buffer conditions remained identical to those for ITC [20 mM sodium phosphate and 200 mM sodium chloride (pH 7.5)]. Spectra were measured at 298 K from 200 to 250 nm, at a scan rate of 10 s/nm. Data were collected in nanometer increments and represent an average of three scans. Temperature scans were performed at a rate of 1 °C/min. Urea concentrations were varied as needed for the respective assays. At the high concentrations of urea used in our study, the quality of the data as the wavelength scans approached 200 nm was reduced because of the absorbance of the cosolute. Below 205 nm, noise from urea absorbance contributes significantly to the signal.

RESULTS

Temperature Disfavors the PII Conformation. Prior to measuring the temperature dependence of PII propensity, we developed a simple model to predict the outcomes of ITC experiments. To estimate the temperature dependence, reference values were needed for the enthalpy of unfolding a PII helix as well as the heat capacity of the PII helix. Via extraction of the structure of the SosY peptide bound to an SH3 domain[37] for use as a "native" PII helix, reference values for the unfolding enthalpy and heat capacity of the PII helix (Table 1) were calculated using the COREX algorithm.[38] The PII propensities of alanine (Ala) and glycine (Gly) were previously[34,46] determined to be ~30 and 10%, respectively, corresponding to a bias in Ramachandran space to the highlighted region of Figure 1a (φ = −75 ± 10°, and ψ = 145 ± 10°). The ratios of the populations for Ala and Gly in either unfolded or PII conformations could be used to calculate reference entropies (Table 1). From these parameters, the change in conformational enthalpy, entropy, free energy, and equilibrium constant was computed over a range of temperatures. This allowed for the prediction of the temperature dependence of the difference in the free energy observed for Ala- and Gly-substituted SosY peptides (Figure 1b). The

Table 1. Prediction of PII Temperature Dependence

SosY		$\Delta H_u{}^a = -1176$		$\Delta C_p{}^a = -10.4$	
	U/PII[b]	$\Delta S_{conf}{}^c$	$\Delta G_{conf}{}^d$	$K_{conf}{}^e$	$\Delta\Delta G_{app}{}^f$
Ala	70/30	−2.26	−503	2.3	−650
Gly	90/10	0.42	−1302	9.0	

[a]Reference unfolding enthalpy and heat capacity of SosY estimated by COREX.[38] [b]Ratios of probability unfolded to probability PII determined calorimetrically[34] at 25 °C. [c]$\Delta S_{conf} = \Delta S_{ref} + \Delta C_p \ln(T/T_{ref})$, where $\Delta S_{ref} = [-RT \ln(U/PII) - \Delta H_U]/-T_{ref}$, where $T = T_{ref} = 25$ °C. [d]$\Delta G_{conf} = \Delta H_{conf} - T\Delta S_{conf}$, where $\Delta H_{conf} = \Delta H_{ref} + \Delta C_p(T - T_{ref})$, where $T = T_{ref} = 25$ °C as described above. [e]$K_{conf} = e(-\Delta G_{conf}/RT)$, where $T = 25$ °C as described above. [f]$\Delta\Delta G_{app} = -RT \ln[(1 + K_{conf,Gly})/(1 + K_{conf,Ala})]$, where $T = 25$ °C.

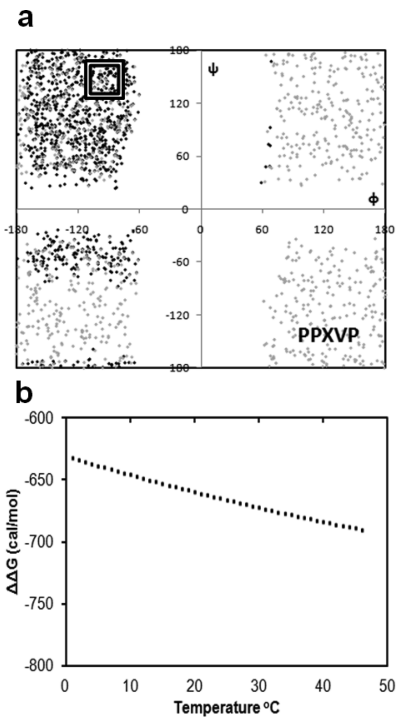

Figure 1. (a) Ramachandran plot of ϕ and ψ angles at position X in 1000 conformers of PPXVP for alanine (black) and glycine (gray). The window marks the region of Ramachandran space corresponding to the PII conformation ($\varphi = -75 \pm 10°$, and $\psi = 145 \pm 10°$). (b) Predicted temperature dependence of the difference in free energy of binding between alanine and glycine SosY peptides.

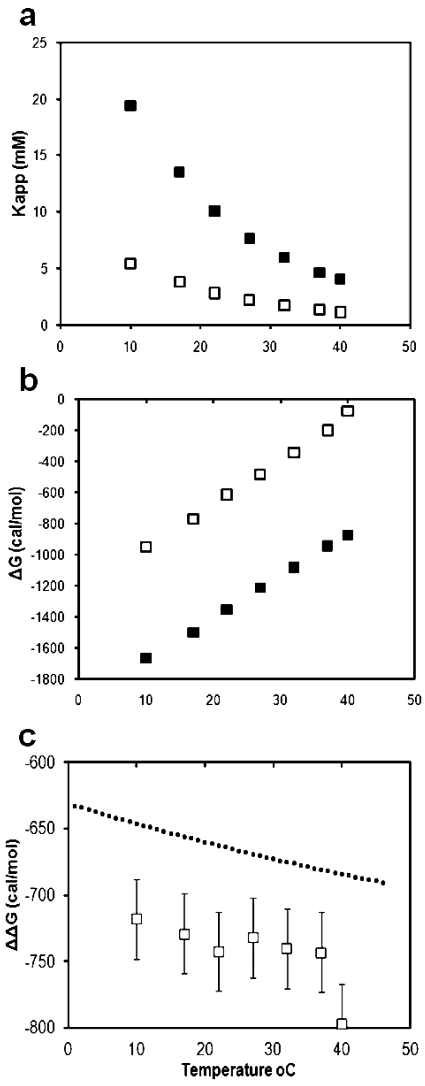

Figure 2. (a) Calorimetrically measured binding constants for the SH3 binding of proline (■) and alanine (□) SosY peptides in phosphate buffer (pH 7.5). (b) Binding free energies for proline (■) and alanine (□) calculated from the fit binding constants using the relationship $\Delta G = -RT \ln(K_{app})$. (c) Experimentally determined temperature dependence of the difference in free energy of binding (□) with model prediction for comparison (···). The error bars are the standard error in $\Delta\Delta G \pm 30$ cal/mol.

negative slope observed in Figure 1b suggests that increasing the temperature disfavors the PII conformation. In addition to interpreting the sign of the predicted slope, the predicted temperature dependence also provided an important insight to guide experiments. Specifically, it informs on the magnitude of the expected change and indicates the range of temperatures to investigate using ITC (10−40 °C), over which an observable difference would be detected that is outside of error in the determination of $\Delta\Delta G$.

Isotherms were measured for WT and Ala-substituted SosY peptides binding to an SH3 domain as previously described.[34−36] From an isotherm, the apparent stoichiometry, molar enthalpy of binding, and binding constant are directly obtained from a nonlinear least-squares regression. The temperature dependence of the fit binding constants for WT and Ala-substituted SosY peptides is observed to decrease with an increase in temperature (Figure 2a). A decrease in the level of binding is expected, as the increasing temperature likely shifts the ensemble of the SH3 domain to more unfolded, binding-incompetent states. However, the binding constants in Figure 2a represent a convolution of the impact of temperature on the SH3 domain and on the SosY peptide ligand. Resolving the impact of temperature on the PII propensity of the SosY peptide requires examination of the difference in binding free energies, where the effect of temperature on the SH3 domain is assumed to be the same in both experiments. We note that the decrease in the level of binding is particularly dramatic for the WT SosY peptide (Figure 2a). Correspondingly, there is an increase in the apparent binding free energies for both peptides (Figure 2b). While the trends in the binding free energies for both peptides appear to be nearly linear, the slopes of the temperature dependencies are different. As shown in Figure 2c, the temperature dependence of the difference in free energy of binding has a modest negative slope. Also, the difference in free

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energies measured at the extremes of the temperature range is outside of the error characteristic of $\Delta\Delta G$ (±30 cal/mol). Importantly, there is a fair agreement with the experimentally determined differences in binding free energy and those predicted by the model (Figure 2c). Though the magnitude of the experimentally determined difference is greater (by <100 cal/mol) than expected, the temperature dependence is essentially exactly as predicted.

CD spectroscopy was used to provide an independent confirmation of our interpretation of the ITC data. The molar ellipticity at 228 nm has been interpreted previously to report on the presence of PII in model peptides and in proteins.[13,20,29,32,47,48] Under identical solution conditions, temperature scans of WT and Ala-substituted SosY peptides were observed to have a decreasing molar ellipticity at 228 nm (Figure 3). Further, the decrease in the magnitude of the PII

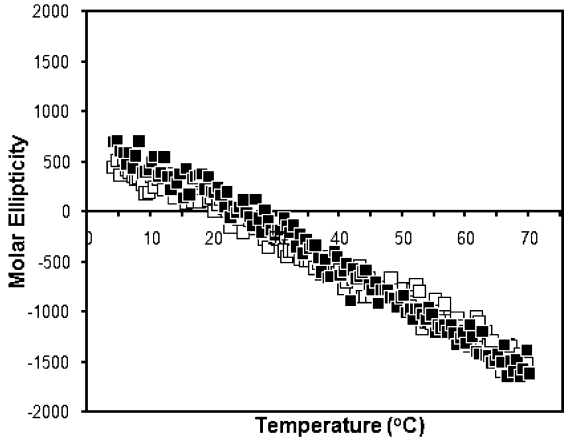

Figure 3. CD temperature scans of the molar ellipticity (degrees square centimeters per decimole) at 228 nm of proline (■) and alanine (□) SosY peptides in phosphate buffer (pH 7.5).

signal in the WT SosY peptide was observed to decrease at a faster rate than that of the Ala-substituted SosY peptide, consistent with the binding trends in the ITC data. Qualitatively, we interpret the decrease in the magnitude of the signal as a shift in the PII propensity of the conformational ensemble, and the absence of any transition suggests that even at high temperatures the peptide still possesses some PII bias.

Collectively, both the ITC (Figure 2) and CD (Figure 3) experiments indicate that PII propensity has a temperature dependence. Specifically, in our experimental system, PII propensity decreases with an increase in temperature. These results are in agreement with our predictive model (Figure 1) and with a prior study by Creamer and colleagues.[29]

Urea Promotes the PII Conformation. A model was also developed to predict the effects of urea on the PII propensity of the SosY peptide. Using the same crystal structure of the SosY peptide employed for prediction of temperature modulation of PII (Figure 1), the solvent accessible surface area was computed for the constituent members of the peptide: the peptide backbone and the side chains (Figure 4a). The stability of the PII helix, taken as the native state, relative to three denatured state models[41−43] was estimated on the basis of a summation of the constituent transfer free energies (TFEs) determined from model compound studies[39,40] for the PII helix and the denatured state models, which have different solvent accessible surface areas. The results of the model are summarized in Table

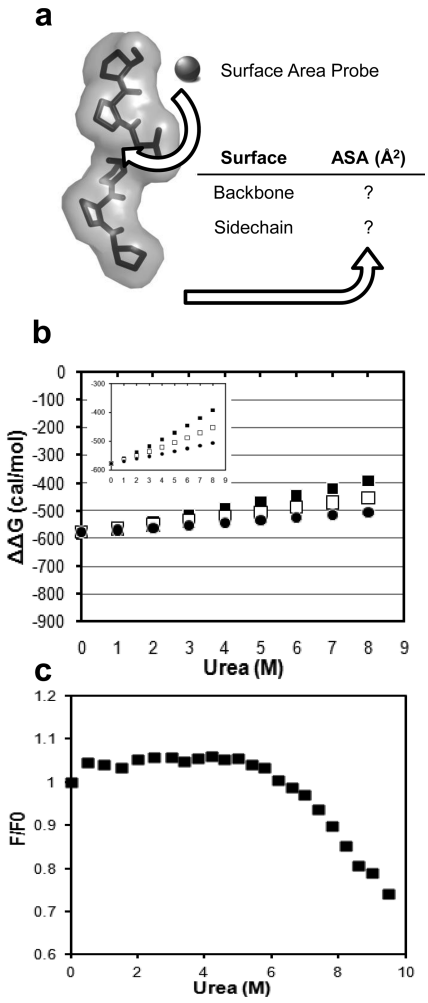

Figure 4. (a) Schematic of the solvent accessible surface area calculation using the crystal structure of SosY as a template.[37] The surface area is subdivided into backbone and individual amino acids. (b) Predicted dependence of the difference in free energy of binding between alanine and glycine SosY peptides on urea concentration for three denatured state models. In terms of surface area, the denatured state models are extended (■),[41,42] intermediate (□),[43] and compact (●).[41,42] (c) Unfolding of SH3 upon titration of urea using the change in the fluorescence of interior tryptophans (residues 38 and 39) as a reporter for denaturation.

2. It is notable that the ability of urea to promote the PII conformation is consistent regardless of the denatured state

Table 2. Predicted *m* Values for SosY in 1 M Urea

	Gibbs TFEs (cal mol^{-1} M^{-1})			
	backbone	side chain	total	N → D
native (PII)	−244	126	−118	
denatured				
compact	−138	111	−27	91
intermediate	−170	118	−53	65
extended	−203	125	−78	40

model used. The predicted dependence of PII propensity on the concentration of urea is shown in Figure 4b. As with our temperature dependence model, this prediction also provided several insights to guide our experimental approach. First, the model predicts that urea will promote the PII conformation

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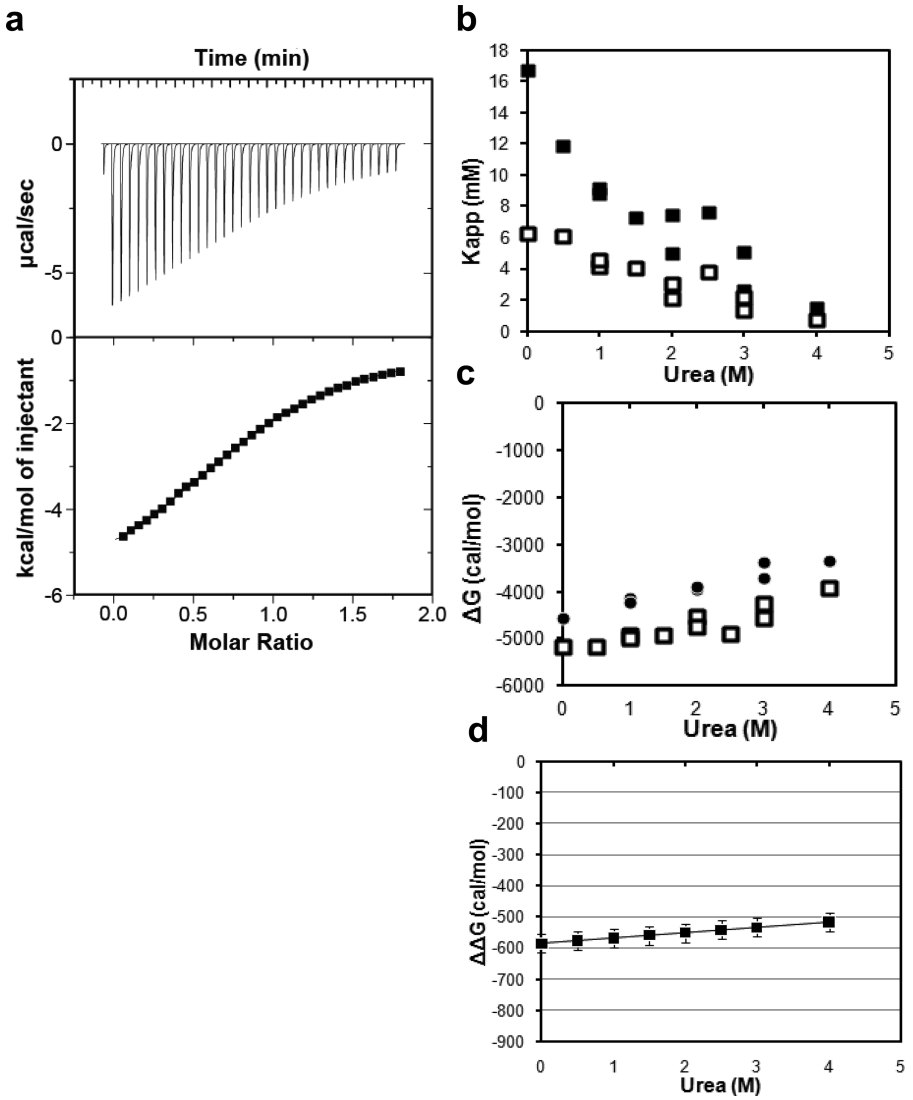

Figure 5. (a) Representative isotherm of SH3 titrated with the SosY ligand. In this case, the buffer contained 2 M urea. The fit K value for this data was well-determined by the nonlinear least-squares regression (K = 8770 ± 102, an error of <2%). (b) Calorimetrically measured binding constants for the SH3 binding of proline (■) and alanine (□) SosY peptides in different concentrations of urea (pH 7.5). (c) Binding free energies for proline (●) and alanine (□) calculated from the fit binding constants using the relationship $\Delta G = -RT \ln(K_{app})$, as a function of urea concentration. (d) Difference in free energy of binding obtained from fits of the binding free energies in panel c. The trend line is shown to clarify the upward slope of the data. The error bars are the standard error in $\Delta\Delta G$ ± 30 cal/mol.

(decreasing magnitude of $\Delta\Delta G$ in Figure 4b). However, because the SosY peptide is small, the magnitude is expected to be modest. From the predicted urea dependence, the difference in $\Delta\Delta G$ at 0 and 4 M urea is greater than the experimental error (±30 cal/mol), which provides a target concentration range for ITC and CD experiments. Fortunately, the SH3 domain is expected to be >90% folded in 4 M urea (Figure 4c). However, even if the SH3 domain is perturbed by the urea, the impact is expected to be the same for WT and Ala-substituted SosY peptide experiments, as taking the difference in the free energy of binding effectively "cancels out" the impact of urea on the SH3 conformation.

Figure 5a shows a representative isotherm of titration of SosY into an SH3 solution, and the slope of the isotherm is directly related to the binding constant. The binding constants fit from ITC experiments in different concentrations of urea show a marked decrease (Figure 5b) similar to that observed with an increase in temperature (Figure 2a). This might be expected as urea could bias the SH3 ensemble toward unfolded, binding-incompetent states. An increasing (less favorable) free energy of binding is observed for both peptides (Figure 5c), reflecting the decrease in the level of binding. The urea dependence of the binding free energy for each peptide can be fit linearly, revealing slightly different slopes. The difference between the linear fits from Figure 5c is shown in Figure 5d. The positive trend in the data (Figure 5d) indicates that, as predicted by the surface area transfer model (Figure 4b), urea does promote the PII conformation.

Our interpretation that urea promotes the PII conformation was confirmed by CD spectroscopy. Figure 6a shows a wavelength scan of the proline SosY peptide at different urea concentrations. The shape of the spectra corresponds to spectra proposed to characterize a denatured peptide ensemble with PII bias,[13,20,29,32,47,48] namely, a negative peak near 205 nm and a positive peak between 220 and 230 nm. Focusing on the wavelength range of 220−230 nm makes the differences in the

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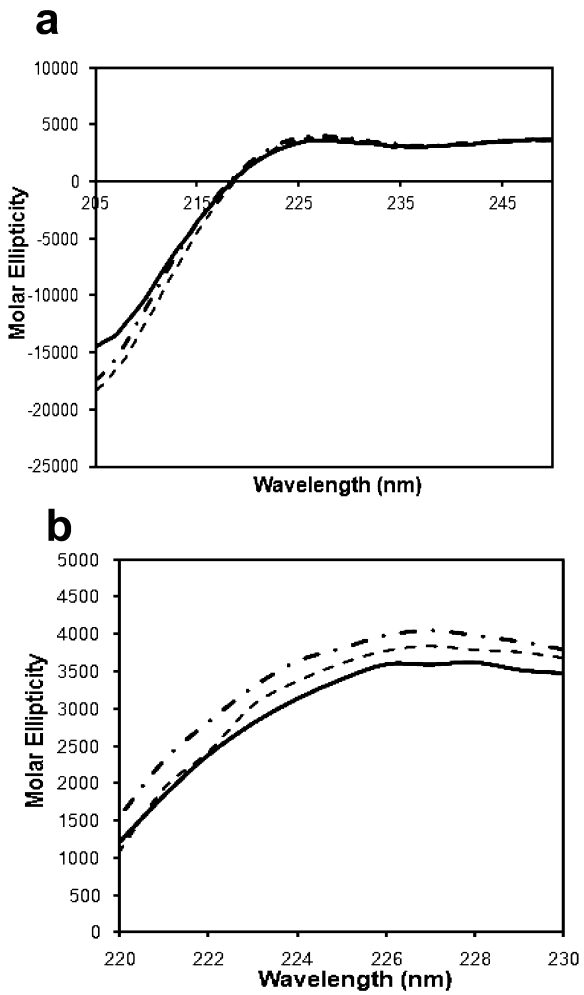

Figure 6. (a) CD spectrum of the proline SosY peptide in different concentrations of urea at 25 °C. Data are shown for urea concentrations of 1 M (—), 2 M (– – –), and 3 M (·—·). (b) Close-up of the positive peak in panel a showing the increasing molar ellipticity (degrees square centimeters per decimole) of the positive peak between 220 and 230 nm for the proline SosY peptide as a function of urea concentration at 25 °C.

molar ellipticity more striking (Figure 6b). The height of the peak in Figure 6b follows a rank order with increasing urea concentration. Similar spectra were observed for Ala- and Gly-substituted SosY peptides (data not shown). When the change in PII propensity upon addition of urea is quantified using the method of Creamer and colleagues,[29] the data suggest that urea can significantly change the PII propensity of the peptide. The change in the maximal CD signal in the 220−230 nm region of the spectra ranges from 5 to 10% across urea concentrations (Table 3), well outside of our experimental measurement error of 4% from our multiple scans. If the molar ellipticity in this wavelength range is taken to report exclusively on the PII conformation (an assumption that may or may not be valid), the increase in PII propensity for the SosY peptide would be approximately 20% (Table 3).

We have elected not to analyze regions of the spectra outside of the 220−230 nm range for three primary reasons. First, Creamer and colleagues[20,29,32] have previously utilized this CD signal as a reporter for the PII conformation, and thus, we interpret our data similarly. Second, the quality of the CD spectra deteriorates in the regions below 210 nm because of the

Table 3. Increasing PII Propensity in Urea by CD

	proline	alanine	glycine
θ_{max}[a]			
1 M	3617	3576	3962
2 M	3791	3803	4223
3 M	3989	3782	4445
PII%[b]			
0 M	50	49	51
1 M	71	71	73
2 M	72	72	75
3 M	74	72	77
ΔPII%	21	22	22

[a]Molar ellipticity (degrees square centimeters per decimole) observed at 228 nm. [b]Determined using the method of Kelly et al.[29]

absorbance of urea that is present at high concentrations. Lastly, and most importantly, our spectra collectively lack an isodichroic point. Consequently, we interpret our peptide ensembles to be populated by more than two conformational species. The absence of an isodichroic point precludes interpreting the other regions of our CD spectra in terms of increasing or decreasing helicity or strand, random coil, or PII content. Regardless, the CD experiments provide an independent validation of our interpretation of the ITC data.

Together, both the ITC (Figure 5) and CD (Figure 6 and Table 3) experiments suggest that urea promotes the PII conformation. These results are in agreement with the results of Whittington et al.[32] and other studies that suggest the denaturant GdnHCl may also increase PII propensity in the denatured state.[28,29] In addition, a surface area transfer model is able to quantitatively predict the stability of the PII helix relative to other limiting denatured state models.

DISCUSSION

In our model for the prediction of the temperature dependence of PII propensity, a conformational equilibrium for the SosY peptide is defined between two states: PII (binding-competent) and other conformations that are binding-incompetent. This equilibrium was previously characterized in the SH3−SosY system,[34−36] which detects the population of the peptide in the PII conformation through binding linkage. The ability of the SH3 domain to directly bind peptide in the PII conformation represents a significant advantage for detection of PII and determination of PII propensity. However, as noted,[34] the calorimetrically determined PII propensities may be an underestimation of amino acid PII bias, as PII is detected only in the window of Ramachandran space that is binding-competent.

Because the SosY peptide is proline-rich (45% proline), it is expected to have a significant PII bias in solution. In two or three positions within the SosY peptide sequence, there are instances where one proline residue follows another. At these specific positions, we expect a significant bias to the PII conformation that is locally driven primarily by steric interactions.[49] Though the SosY peptide is a biological segment taken from the son of sevenless protein, its high intrinsic PII bias allows a detectable baseline CD signal similar to that observed in proline homopolymers. Despite this high intrinsic PII bias, the apparent binding constant measured by ITC is only in the millimolar range. Because PII helix formation has been proposed to be noncooperative,[50] the population of SosY peptide that is a complete PII helix may be extremely low (PII

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%^{n} ~ 0.3^{11}% ~ 0.0002%). The entropic penalty of restricting the SosY peptide to the PII conformation upon binding is known to be compensated by a substantial enthalpy, −8 kcal/mol.[35]

Several factors contribute to the agreement obtained between the experimentally determined differences in the free energy of binding measured by ITC and our model for PII temperature dependence (Figure 2c). First, the agreement indicates that the COREX estimates for the reference heat capacity and enthalpy of unfolding of the SosY peptide seem to be reasonable. Previously, the COREX algorithm has been used to thermodynamically characterize native[51] and denatured[52] ensembles of full-length proteins, but it has not been used for small peptides because of concerns about the accurate modeling of the ensemble where constituent members have very small differences in surface area. Second, the ability of the model to predict the magnitude of the temperature dependence also suggests that the conformational equilibria assumed for Ala- and Gly-substituted SosY peptides are reasonable. If the calculated ratios of unfolded to PII were incorrect for the peptides, the predicted temperature dependence would be very different from what is experimentally observed (Figure 2c and Table 1).

The model developed to predict the impact of cosolutes, specifically urea in this study, is conceptually straightforward. The solvent accessible surface areas calculated for the native state (SosY, a PII helix) are compared to those of three denatured state models: (1) a fully extended unfolded state with a maximal change in surface area,[41,42] (2) a compact denatured state modeled from the coil library,[41,42] and (3) an intermediate model approximating a self-avoiding random coil.[43] Because the changes in surface area between the PII helix and any of the three denatured state models are small, the dependence of the PII conformation on solvent is expected to be weak. However, one advantage of this approach is its generality. Importantly, this scheme could be used to predict the effect of any cosolute for which Gibbs transfer free energies have been measured for all amino acids and the peptide backbone.

The data reported in Table 2 provide insight into the mechanism by which urea promotes the PII conformation. The ability of urea to denature proteins has been attributed to the favorable interactions of urea with the peptide backbone.[39,53,54] It has been previously determined that the PII helix conformation has a substantial degree of backbone solvent accessible surface area,[19] which may explain why PII is stabilized by urea. In comparison, interaction of urea with the side chains of the SosY peptide tends to be unfavorable, but the unfavorable energy of side chain interactions is essentially outweighed in the PII helix by the considerable backbone interactions. This complex interplay between the favorable interactions of urea with the backbone and unfavorable interactions with the side chains may explain why a maximally extended denatured state model is less stable than the PII helix as the side chain solvent accessible surface areas are maximized.[19] In short, the experimental observation that urea increases PII propensity is completely consistent with the presence of high peptide backbone solvent accessible surface area in the PII conformation.

The extent to which structure persists in the denatured state has been a long-standing question in both protein folding and function. Partially denatured states in which tertiary contacts have been disrupted yet secondary structure persists, so-called molten globules,[55,56] represent extreme cases illustrative of the degree of structure that may exist outside of the lowest-energy native states. Indeed, as our ability to interrogate proteins improves, evidence that the denatured state contains nativelike structure and interactions is accumulating.[57−65] At present, the role of PII propensity in the folding process of globular proteins is not clear. Though the PII conformation is highly populated in the denatured state, proteins need not fold through the PII conformation.

Evidence of nativelike structure and significant conformational bias in the denatured state has led some researchers to question whether proteins are ever random coils.[66] This line of questioning brings the verbiage and criteria by which we define a "random coil" to the forefront. Since Tanford's demonstration[67] that a polypeptide in GdnHCl obeys global geometric properties of a random coil proposed by Flory,[10] the observed global random behavior has often been conflated with random local conformations within the polypeptide chain. PII propensity represents one form of local conformational bias in the denatured or unfolded state. The ability of temperature and cosolutes to modulate PII propensity need not disrupt the global random coil behavior of the polypeptide chain at high temperatures or high urea concentrations. Importantly, while urea appears to promote the PII conformation, we do not claim that peptides in high urea concentrations are PII helices, nor do we claim promoting PII bias is the mechanism by which urea denatures globular proteins.

Environmental stresses such as those used in this study are physiologically relevant. Significant urea concentrations are known to accumulate in cells within elasmobranch fishes (sharks and rays)[68] and in the mammalian renal medulla (kidney),[69] reaching levels that alter functional and structural properties of intracellular proteins. Fortunately, animals, plants, and microorganisms have adapted to urea denaturing stress, primarily via accumulation of small organic molecules that counteract urea.[70] Indeed, small molecule osmolytes are utilized throughout nature to cope with environmental stresses.[71] It may also be the case that homologous proteins in signal pathways reliant on PII binding in extremophiles have evolved to modulate the PII propensity of key protein segments through mutation, but this hypothesis has not been investigated.

In summary, the local conformations of the denatured state ensemble appear to be sensitive to the environment, whether it is temperature or solvent perturbations. Remarkably, the sensitivity appears to be in the PII propensity of the polypeptide, which has implications for protein function in cells. For example, the SH3−SosY peptide system used in this study is taken from the Ras signaling pathway that regulates cell proliferation and differentiation.[72] The pathway is mediated by the binding of a PII conformational switch in the son of sevenless protein.[73,74] Importantly, PII-mediated interactions are abundant in cell signaling, with Pawson and Nash reporting that as many as 253 SH3 domains and 115 SH2 domains (a relative that binds proline-rich phosphotyrosine peptide sites) may be encoded in the human genome.[75] In addition, there are estimated to be more than 100 other domains (WW, EVH1, and GYF) encoded in the human genome that also recognize proline-rich regions.[76] In fact, comparative genomic studies show that proline-rich segments are one of the most commonly encoded motifs in eukaryotes.[77] ID proteins that often contain low-complexity, proline-rich sequences[78] may also have their PII content modulated by environmental stresses. Modulation

of PII in ID proteins has implications for signaling as well because ID proteins are known to function as transcription factors[79] and as "hub" proteins in signaling pathways.[80,81] It follows that numerous biological signal networks that utilize recognition of the PII conformation or proline-rich sites are then directly sensitive to commonly occurring intracellular and extracellular stresses such as heat shock and osmotic shock.

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ABBREVIATIONS

CD, circular dichroism; GdnHCl, guanidine hydrochloride; ITC, isothermal titration calorimetry; PII, polyproline II; SH3, Src-homology 3; SosY, son of sevenless protein; TFE, transfer free energy.

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