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Urea unfolding of peptide helices as a model for interpreting protein unfolding

(model peptide helix/helix–coil transition/denaturant effects)

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ABSTRACT **To provide a model system for understanding how the unfolding of protein α-helices by urea contributes to protein denaturation, urea unfolding was measured for a homologous series of helical peptides with the repeating sequence Ala-Glu-Ala-Ala-Lys-Ala and chain lengths varying from 14 to 50 residues. The dependence of the helix propagation parameter of the Zimm–Bragg model for helix-coil transition theory (s) on urea molarity ([urea]) was determined at 0°C with data for the entire set of peptides, and a linear dependence of ln s on [urea] was found. The results were fitted by the binding-site model and by the solvent-exchange model for the interaction of urea with the peptides. Each of these thermodynamic models is able to describe the data quite well and we are not able to discern any difference between the ability of each model to fit the data. Thus a linear relation, $\ln s = \ln s_0 - (m/RT)\cdot[\text{urea}]$, fits the data for α-helix unfolding, just as others have found for protein unfolding. When the m value determined here for α-helix unfolding is multiplied by the number of helical residues in partly helical protein molecules, the resulting values agree within a factor of 2 with observed m values for these proteins. This result indicates that the interaction between urea and peptide groups accounts for a major part of the denaturing action of urea on proteins, as predicted earlier by some model studies with small molecules.**

We use helix–coil theory to examine how the α-helix to random coil transition depends on urea molarity for a homologous series of peptides. We address four specific questions. (*i*) Does urea denature proteins by interacting with the peptide group (1–3) or by solubilizing nonpolar side chains, especially aromatic groups (4–6)? (*ii*) Can the urea dependence of the α-helix unfolding reaction be explained quantitatively by the popular models [binding-site model (7, 8) and solvent-exchange model (9, 10)] for the solvent denaturation of proteins? (*iii*) Can the urea-induced unfolding transition of the α-helix be used as a simple model system to discriminate between these thermodynamic models? (*iv*) Can the dependence on urea molarity of native protein and molten globule unfolding be considered to result from the action of urea on protein α-helices and other elements of secondary structure?

The observation that short, alanine-based peptides form monomeric helices in aqueous solution (11) has permitted the direct determination of the energetics of helix formation [see review by Scholtz and Baldwin (12)]. Thermally induced helix–coil transitions in these peptides have been shown by spectroscopy and calorimetry (13, 14) to conform to helix–coil theory, which predicts that peptide helix unfolding is cooperative and multistate (15, 16), and have provided estimates of Gibbs energies and enthalpies of helix unfolding (13, 14).

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The peptides examined here consist primarily of alanine;‖ thus side-chain interactions are minimal, and the primary structural and thermodynamic changes during helix unfolding should result from the peptide backbone. Because these helical peptides lack hydrophobic cores and long-range tertiary interactions, the thermodynamics of solvent denaturation of secondary structure can be measured directly. Although secondary structure formation must be an integral part of the protein folding reaction, these other factors (hydrophobic core formation and long-range tertiary interactions) obscure the contributions of individual structural elements such as helix formation to the thermodynamics of protein folding. Furthermore, the use of simple sequences helps to limit the number of ways urea can interact with the helical and unfolded conformations. For this reason, the unfolding transitions of simple peptides better satisfy the basic assumption of the thermodynamic models for the interaction of urea with peptides that all denaturant binding sites are uniform than do the unfolding transitions of globular proteins, which contain all 20 amino acids arranged in a complex tertiary structure.

MATERIALS AND METHODS

Peptides Studied. The peptides, ranging in length from 14 to 50 residues, have the general sequence Ac-Tyr-(Ala-Glu-Ala-Ala-Lys-Ala)$_k$-Phe-NH$_2$. The details of the design, synthesis, and purification of the peptides are described in an earlier report (13).

Measurement of α-Helix Formation. Helix content was measured by circular dichroism (CD) spectroscopy with an Aviv Associates (Lakewood, NJ) model 60DS or 62DS spectropolarimeter equipped with a temperature control unit. Cuvettes with 10-mm pathlengths were employed for all measurements. Ellipticity at 222 nm is reported as mean residue ellipticity, $[\theta]$ (deg·cm^2·dmol^{-1}), and was calibrated with (+)-10-camphorsulfonic acid. Samples were prepared by diluting an aqueous peptide stock solution into a buffer consisting of 0.1 M NaCl and 1 mM each sodium phosphate, sodium borate, and sodium citrate (CD buffer). A stock solution of ≈8 M urea in CD buffer was prepared fresh daily. The concentration of urea in this stock solution was determined with refractive index measurements (17). In all cases, the pH was adjusted to 7.0 at room temperature by the addition of HCl or NaOH.

The concentration of the peptide stock solution was determined by ultraviolet absorbance of the single tyrosine chromophore with the extinction coefficient given by Brandts and Kaplan (18). The effect of urea on the helicity of the peptides

Abbreviation: LEM, linear extrapolation method.

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‖One glutamate and one lysine are included for every four alanines to enhance water solubility.

was determined by adding an aliquot of the concentrated urea solution in CD buffer to the sample of peptide in CD buffer and adjusting for volume changes after each addition. The reversibility of the urea-induced transition was determined by diluting a sample of peptide in concentrated urea into CD buffer. In all cases the reversibility of the transition was ≥98%.

Use of the Zimm–Bragg Model to Quantify Helix Formation. The data were fitted by the Zimm–Bragg model for the helix–coil transition (15). To determine the equilibrium constant for helix formation (termed the s value), the observed mean residue ellipticity, $[\theta]_{obs}$, must be converted to fractional helicity, f_H, for each of the peptides:

$$f_H = \frac{[\theta]_{obs} - [\theta]_C}{[\theta]_H - [\theta]_C}, \quad [1]$$

where $[\theta]_H$ and $[\theta]_C$, the $[\theta]$ values for helix and coil, respectively, are defined as

$$[\theta]_H = H_0 \cdot \left(1 - \frac{2.5}{n}\right) + H_U \cdot [\text{urea}] \quad [2]$$

$$[\theta]_C = C_0 + C_U \cdot [\text{urea}]. \quad [3]$$

The coefficients of the last two terms in Eqs. **2** and **3**, H_U and C_U, provide the urea dependence of the ellipticities of the helix and coil, respectively, and the H_0 and C_0 terms are the ellipticities of the helix and coil at 0°C in the absence of urea. A chain length dependence $(1 - 2.5/n)$ for H_0 is also included (14, 19).

CD data were fitted by the above equations using a version of the nonlinear least-squares procedure described by Johnson and Frasier (20) which has been implemented for a Macintosh personal computer by Brenstein (21). The best fit values for all of the variable parameters and their 67% confidence intervals (22) were determined in fitting the data by the indicated model (see below).

The observed fraction helix at any solution condition, f_H, calculated as shown in Eq. **1**, is used to determine the Zimm–Bragg helix–coil transition-theory parameters s and σ:

$$f_H = \frac{\sigma \cdot s}{(s-1)^3}\left(\frac{n \cdot s^{n+2} - (n+2)s^{n+1} + (n+2)s - n}{n\{1 + [\sigma \cdot s/(s-1)^2]\,[s^{n+1} + n - (n+1)s]\}}\right), \quad [4]$$

where n is the number of amide groups in the peptide, s is the propagation parameter, and σ is the helix nucleation parameter. This equation is from the Zimm–Bragg model for helix formation (15), in which peptides are treated as homopolymers and only one helical stretch of residues is allowed in each chain. This model is identical to the full treatments of helix formation for peptides of these chain lengths (see ref. 23). Besides the propagation parameter, s, the other parameter that is required for the analysis in Eq. **4** is σ, the helix nucleation constant. The value of σ has been determined previously (14, 24), and we find that its absolute value does not affect either s_0, the urea dependence of s_0, or the quality of the overall fitting. Therefore, we hold σ constant in all fittings at the previously determined value of 0.0030 (14, 24).

Models for Urea-Induced Unfolding. There are two main thermodynamic models that are used to analyze urea- and guanidine-induced protein denaturation curves (8, 10, 17): a binding model, in which the interaction of the denaturant with the protein can be treated as specific binding (7, 8, 25), and a solvent-exchange model, in which the interactions of both the solvent (water) and the cosolvent (urea) with the protein are treated explicitly in an expression that involves the interchange between both components at a particular interaction "site" on the protein (9). For urea denaturation of helical peptides, these two models have the general following forms:

(binding)

$$\ln s = \ln s_0 - \Delta n \cdot \ln(1 + k \cdot a) \quad [5]$$

(solvent exchange)

$$\ln s = \ln s_0 - \Delta n \cdot \ln(A_1 + K \cdot A_3) \quad [6]$$

$$= \ln s_0 - \Delta n[\ln f_1 + \ln(1 + (K' - 1) \cdot x_3)], \quad [7]$$

where s_0 is the s value for the homopolymer in the absence of urea, Δn is the difference in the number of binding or interaction sites between the coil and helix forms of a residue, k is the binding constant for urea, a is the molar activity of the urea solution, A_1 and A_3 are mole fraction activities of water and urea, f_1 is the mole fraction activity coefficient for water, x_3 is the mole fraction of the urea solution, and K and K' are the exchange equilibrium constants for the indicated concentration scale. Urea activity and mole fractions are determined from the relationships between urea molarity, mole fraction activity, and molar activity given by Pace (17). Mole fraction activity coefficients for water in the urea/water system were calculated from the data of Stokes (26). The derivations of these models and the physical basis for their forms have been given by Schellman (8–10).

An alternative method for quantifying urea-denaturation curves for proteins is called the linear extrapolation method (LEM) (27–29). This empirical method expresses the Gibbs energy of folding as a linear function of denaturant molarity. For the helix–coil transition, the LEM has the general form:

$$\ln s = \ln s_0 - \frac{m \cdot [\text{urea}]}{RT}, \quad [8]$$

where m is the change in the Gibbs energy of helix propagation per residue as a function of urea molarity, T is absolute temperature, R = 1.987 cal·mol^{-1}·K^{-1}, and the other terms have the same meanings as above. It can be shown that each of the physical models (Eqs. **5** and **6**) for the effect of the urea/water system on protein stability can be represented by the LEM equation under certain conditions (see below).

RESULTS

CD was used to monitor the helix contents of the series of peptides as a function of urea molarity at 0°C (Fig. 1). The addition of urea to solutions of these peptides caused a gradual loss of helical structure, as evidenced by the changes in $-[\theta]_{222}$ with increasing urea concentration. This urea dependence of

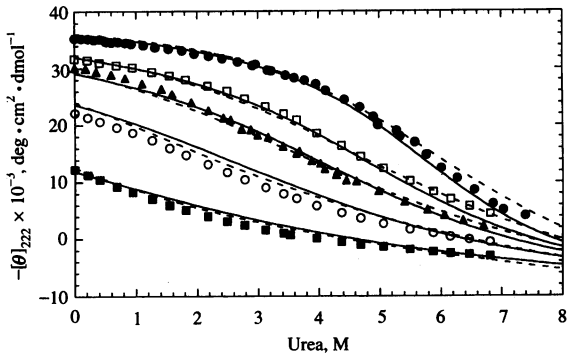

FIG. 1. Variation in mean residue ellipticity ($[\theta]_{222}$) with urea molarity for the five peptides of the general sequence Ac-Tyr-(Ala-Glu-Ala-Ala-Lys-Ala)$_k$-Phe-NH$_2$ with chain lengths of 14 (■), 20 (○), 26 (▲), 32 (□) and 50 (●) residues. The solutions contained 0.1 M NaCl and 1 mM each sodium phosphate, sodium borate, and sodium citrate at pH 7.0 and the measurements were performed at 0°C. The curves through the data were generated from the LEM (solid line) and the solvent-exchange model (dashed lines) using the best fit parameters given in Table 1.

helical structure on urea molarity was used to test various models of urea-induced unfolding and to quantify the effects of urea on the helix unfolding reaction. The fitted curves through the data are discussed below.

In the popular models for urea denaturation of proteins, the Gibbs energy of unfolding, obtained as a logarithm of the unfolding equilibrium constant, is plotted as a function of urea molarity. According to the Zimm–Bragg model for unfolding of α-helical peptides, the equivalent Gibbs energy parameter is proportional to the logarithm of s, the equilibrium constant for adding a residue to an α-helix. This quantity can be obtained from the fraction helix for a collection of peptides at a given urea concentration. The helix contents of the five peptides studied here were used simultaneously to determine s at various urea concentrations, with Eq. **4**. Since the helix–coil transition is cooperative, peptides with different chain lengths and identical composition differ in their helix contents (Fig. 1). By using five such peptides, we were able to include data for peptides showing a wide range of helix content, as well as data for peptides that approach either the 100% helix baseline (low urea concentration, 50-residue peptide) or the 0% baseline (high urea concentration, 14-residue peptide), at every urea concentration (Fig. 1) in our determination of s. This greatly increases the accuracy with which s can be measured and allows us to study the effect of urea on the unfolding transition over a very broad range of urea molarity, including the limit of no urea.

The natural logarithm of s, calculated from the helix content of the set of five peptides at various urea concentrations, is plotted in Fig. 2 as a function of urea. In this figure, each point results from an independent fitting of CD data using Eqs. **1** and **4**. The value of s, so calculated, decreases monotonically with urea concentration, as expected from the urea-induced helix unfolding seen in Fig. 1. These data have been analyzed with both thermodynamic models for the effect of urea on helix formation (Eqs. **5** and **7**), and those results are also shown on the plot. For the binding model (Eq. **5**), the best fit values of s_0, k, and Δn (1.36, 0.08, and 0.77 sites per residue, respectively) were determined with nonlinear least-squares procedures. Likewise, the solvent-exchange model (Eq. **7**) gave these best fit values of s_0, K', and Δn: 1.37, 10.5, and 0.28 sites per residue, respectively. The interaction and exchange constants for the two models are related by the concentration of water, ≈55 M, such that $k = K'/55$. Each of these models, which provides an identical fit to the data, is indicated by the dashed line. For comparison, the LEM (Eq. **8**) gives s_0 and m of 1.35 and 27.4 cal·mol^{-1}·M^{-1} per residue, respectively; these values were determined by using a simple linear fit to the data (solid line).

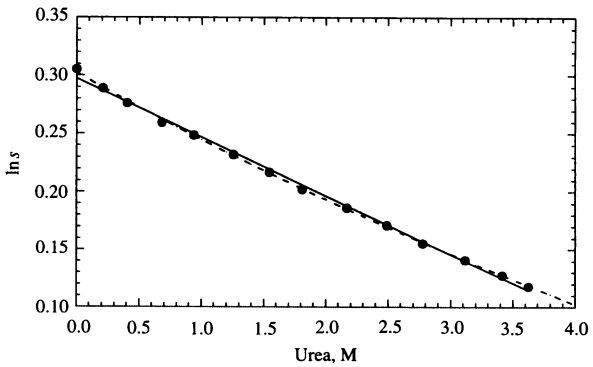

FIG. 2. Variation of the logarithm of the helix propagation parameter s with urea molarity as determined from Eqs. **1** and **4**, using the data provided in Fig. 1 for all the peptides. The filled circles show the measured values of s. The curves through the data points are derived from the LEM (solid line) and the two thermodynamic models for the effect of urea on helix formation (dashed lines). See the text for complete details.

All three curves fit the data very well, indicating that the thermodynamic models for urea-induced unfolding of proteins can be applied to simple helix unfolding reactions, and furthermore, the LEM appears to describe our system very well. The inability of the data to discriminate between these two thermodynamic models is discussed below.

To obtain accurate estimates of the model-based parameters for helix unfolding and associated uncertainties, we performed a global analysis of all the data shown in Fig. 1. Parameters determined from nonlinear least-squares fitting of the binding-site model (Eq. **6** with Eqs. **1–4**) are given, along with confidence intervals, in Table 1. Curves derived from these two fittings are shown in Fig. 1 for the LEM (solid lines) and the solvent-exchange model (dotted lines). The binding model gives a fit to the data that is nearly identical to that given by the solvent-exchange model (not shown).

These parameters are able to describe the urea-induced helix unfolding (Fig. 1) quite well. Fitting the data with the LEM produced an m value of the α-helix of 23 cal·mol^{-1}·M^{-1} per residue. This parameter represents the change in Gibbs energy of helix propagation of a *single residue* with urea molarity. Therefore, a typical helix in a protein (10–12 residues in length) would contribute to the observed m value about 250 cal·mol^{-1}·M^{-1}. This point is discussed below in comparing the helix m value with m values from proteins.

DISCUSSION

Models for the Effect of Urea on Helix Formation. Use of helix–coil theory to describe the effect of urea on helix stability in short, defined-sequence, alanine-based peptides produces a nearly linear decrease in ln s with urea molarity (Fig. 2). This linear dependence is similar to that seen for protein unfolding reactions and demonstrates that the LEM (Eq. **8**) can be used to describe the urea dependence of our helix unfolding reaction (solid line). This result is in stark contrast to that found for the effect of urea on helix formation in long homopolymers of L-glutamic acid (30), where a nonlinear dependence of the Gibbs energy of helix formation on urea molarity was observed. Our data in Fig. 2 can also be fitted reasonably well with either thermodynamic model: the binding-site model (Eq. **5**) or the solvent-exchange model (Eqs. **6** and **7**). It may seem surprising that fitting the data does not distinguish between these models, since s is determined directly over a wide range of urea molarity, including low concentrations where these models are predicted to give different fits of protein stability data (17). One explanation might be the small range in ln s, 0.1–0.3 (Fig. 2), for our peptides compared with the 20-times larger range of ln K observed in protein studies and the studies on long polymers of L-glutamic acid.

A second reason is suggested by the magnitude of the interaction constants k and K' and the range of urea concentrations investigated. Because k is in the range 0.08–0.13, and the urea concentration ranges from 0 to about 8 M (Fig. 1), the product of $k{\cdot}a$ is usually <1. The Taylor series expansion of $\Delta n{\cdot}\ln(1 + k{\cdot}a)$ (Eq. **5**) for $k{\cdot}a < 1$ provides

$$\Delta n{\cdot}\ln(1 + k{\cdot}a) \approx \Delta n\left[k{\cdot}a - \frac{(k{\cdot}a)^2}{2} + \frac{(k{\cdot}a)^3}{3} - \ldots\right]. \quad [9]$$

The higher-order terms in Eq. **9** can be neglected for small values of $k{\cdot}a$; thus, a comparison of the LEM (Eq. **8**) and the binding model (Eq. **5**) provides

$$\frac{m{\cdot}[\text{urea}]}{RT} \approx \Delta n{\cdot}k{\cdot}a. \quad [10]$$

This result has two consequences: (*i*) there should be a linear correlation between ln s and urea molarity regardless of which formalism is appropriate and (*ii*) we cannot determine k or Δn

Table 1. Parameters determined for the effect of urea on helix formation in the peptides

	LEM*			Exchange model†			Binding model‡		
Parameter	Best	Low	High	Best	Low	High	Best	Low	High
s_0	1.34	1.34	1.35	1.39	1.38	1.41	1.39	1.39	1.40
m	23.0	22.9	23.3	—	—	—	—	—	—
K'	—	—	—	12.9	12.3	13.4	—	—	—
k	—	—	—	—	—	—	0.14	0.14	0.14
Δn	—	—	—	0.32	0.28	0.36	0.52	0.52	0.53
H_0	−44,000	−43,600	−44,630	−43,190	−42,350	−43,880	−42,500	−42,130	−42,860
H_U	320	110	460	500	−15	470	−620	−490	−750
C_0	4,400	4,000	5,500	4,050	3,500	4,540	5,090	4,750	5,400
C_U	340	89	440	360	330	410	280	210	340

Units for the parameters are as follows: m, cal·(mol res)$^{-1}$·M(urea)$^{-1}$ (mol res, mole of amino acid residues); Δn, site per residue; H_0 and C_0, deg·cm^2·dmol^{-1}; H_U and C_U, deg·cm^2·dmol^{-1}·M(urea)$^{-1}$.

*LEM as given by Eq. 8.

†Solvent-exchange model as described by Eq. 7. The mole fraction of the urea solution was calculated from the molar concentration by using the relationship given by Pace (17), and the activity coefficient of water on the mole fraction scale (f_1) was calculated from the data of Stokes (26).

‡Binding-site model as described by Eq. 5. The urea molar activity was calculated from the molar concentration by using the relationship given by Pace (17).

separately, but only the product $\Delta n \cdot k$. Similar conclusions are found when one considers the LEM and the solvent-exchange model.

Relationship to Urea Denaturation of Proteins. Proteins for which both x-ray crystal structures and m values for urea have been determined are listed in Table 2. For each protein, the expected urea dependence from helical structure is calculated on the basis of the number of α-helical residues and the m value determined here for helix unfolding (23 cal·mol^{-1}·M^{-1} per residue). For proteins containing a large amount of helical structure (>40%), the calculated m value is as large as that observed for protein unfolding. For proteins containing a smaller amount of helical structure, the calculated m value is smaller than the observed value, showing that, as expected, other factors contribute to the overall m value for protein unfolding. Since the disruption of β-sheet will expose peptide groups to urea, it seems likely that the disruption of β structure upon unfolding also contributes substantially to the overall m value.

The suggestion that urea-induced protein unfolding results from exposure of peptide groups to solvent is difficult to test directly with proteins because protein unfolding is highly cooperative and intermediates are not populated to high levels. A few globular proteins do exhibit two resolved stages in equilibrium unfolding, in which an intermediate conformation is well populated. An intermediate of apomyoglobin has been observed at pH 4.5 that contains a high level of α-helical structure but lacks a close-packed tertiary structure (see ref. 33 and references therein). The use of the LEM for urea-induced unfolding of this intermediate has given an experimental m value of 1 kcal·mol^{-1}·M^{-1} (48), as compared with the predicted value of 1.4 kcal·mol^{-1}·M^{-1} based on the helix m value determined here. Although this agreement is encouraging, the test is not stringent, because nonpolar groups appear to be

Table 2. Comparison between predicted and observed m values for protein unfolding

Protein	Protein Data Bank code	No. of residues	No. of helical residues (% helix)	Helix m value*	Exp. m value	Helix/Exp. ratio, %	Ref.
Calbindin D9K	3ICB	75	52 (69)	1200	940	128	31
Trp aporepressor	3WRP	107 (2×)	72 (67)	3310	2900	114	32
Apomyoglobin (whale)		153	76 (50)	1750	2000	88	33
Thioredoxin	1THO	109	48 (44)	1100	1300	85	34
					1320	83	29
β-Lactamase	3BLM	257	94 (37)	2160	3200†	68	35
HPr (*Bacillus subtilis*)	2HPR	89	31 (35)	710	1050	68	‡
RNase A (bovine)	9RSA	124	33 (27)	760	1300	58	36
					1100	69	37
					1140	67	38
					1400	54	39
					1410	54	40
Dihydrofolate reductase	5DFR	159	43 (27)	990	1900	52	41
Hen egg-white lysozyme	6LYZ	129	31 (24)	710	1120	63	37
					1070	66	42
					1290	55	38
Staphylococcal nuclease	2SNS	149	34 (23)	780	2360	33	43
RNase Ba	1RNB	110	22 (20)	510	1905	27	44
					1940	26	45
RNase T1	6RNT	104	17 (16)	390	1210	32	46
α-Chymotrypsin	4CHA	241	29 (12)	670	2070	32	38
Chymotrypsinogen A	1CHG	245	17 (7)	390	2030	19	47

*Determined by multiplying the number of helical residues by our m value for helix unfolding, 23 cal·mol^{-1}·M^{-1} per residue.

†Deviates from two-state behavior.

‡J.M.S., unpublished results.

partly buried in this intermediate (33) and could also contribute to the observed m value. Also, unfolding of the apomyoglobin intermediate was assumed to be a two-state reaction.

Comparison with Model Compound Studies. Even though our results indicate that we cannot separate the number of interaction sites (Δn) from the binding or interaction constant (k or K') for the two models, we can nonetheless compare the products $\Delta n \cdot k$ or $\Delta n \cdot K'$ with the results from model compound studies. Fitting the binding-site model to our data produces a urea binding constant of $k = 0.14$ with $\Delta n = 0.52$ site per residue, which gives $\Delta n \cdot k = 0.07$ per residue (where urea concentration is expressed on the molar activity scale). Likewise, the solvent-exchange model provides $\Delta n \cdot K' = 4.1$ per residue (where the urea concentration is expressed as a mole fraction). This interaction constant is nearly identical to that determined from a variety of different studies on model compounds ($K = 1.8$–3.3), including studies of the interaction of urea with peptide groups (49), the binding of urea to diketopiperazines (50), the dimerization of urea in aqueous solution (1), and the osmotic coefficients of urea/water solutions (51). This interaction constant also agrees with the observed values for some protein unfolding reactions (17) and is identical to that measured calorimetrically by Makhatadze and Privalov (52) for the binding of urea to three unfolded proteins. Although we cannot directly determine Δn itself, it is interesting that the value of Δn we find, 0.3–0.5 urea binding site per residue, is similar to that determined by Robinson and Jencks (2) for small peptides and is in good agreement with estimates from proteins (6, 52).

The observation that the efficacy of urea as an unfolding agent of the α-helix is equal to that of proteins makes somewhat puzzling the suggestion that protein unfolding is caused by the interaction of urea with hydrophobic groups (6). It is possible that observed m values for proteins are small values that reflect the difference between a large number of stabilizing and destabilizing interactions between urea and the constituent groups of the unfolded protein, analogous to the large number of stabilizing and destabilizing interactions that largely offset each other to determine the stability of folded proteins in water. More work is needed to understand this observation.

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