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RESEARCH ARTICLES

Urea Effects on Protein Stability: Hydrogen Bonding and the Hydrophobic Effect

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ABSTRACT **The effects of urea on protein stability have been studied using a model system in which we have determined the energetics of dissolution of a homologous series of cyclic dipeptides into aqueous urea solutions of varying concentration at 25°C using calorimetry. The data support a model in which urea denatures proteins by decreasing the hydrophobic effect and by directly binding to the amide units via hydrogen bonds. The data indicate also that the enthalpy of amide hydrogen bond formation in water is considerably higher than previously estimated. Previous estimates included the contribution of hydrophobic transfer of the α-carbon resulting in an overestimate of the binding between urea and the amide unit of the backbone and an underestimate of the binding enthalpy. Proteins 31:107–115, 1998.** © 1998 Wiley-Liss, Inc.

Key words: calorimetry; desolvation; linear extrapolation model; binding; denaturation

INTRODUCTION

The effect of urea as a protein denaturant has made it a common tool for analyzing protein stability,[1] but the molecular mechanism of its effect is not well understood. Efforts to better understand how urea denatures proteins have focused on transfer studies of protein constituent groups between water and solutions of high urea concentration.[2,3] Such studies have shown that urea increases the solubility of both apolar and polar groups, i.e., the ΔG° of transfer is negative for both types of groups. However, the enthalpic and entropic components of the transfer process were not determined in these studies, thus making it difficult to develop a molecular model for these effects.

Other studies have determined the enthalpies of interaction of urea with constituent groups either from temperature-dependent properties[4–6] or from direct calorimetric studies.[6–9] These studies suggest that the urea–amide unit interaction is exothermic and the urea–apolar interaction is endothermic. However, the temperature-dependent studies suffer from a lack of precision and none of these studies has looked at urea concentration dependence or examined a homologous series of compounds from which group contributions to the thermodynamics could be extracted.

Other studies have looked at the interaction of urea with proteins.[10–16] However, like the model compound studies, the protein studies have generally focused on free energy changes, and even in the calorimetric studies, the effects are difficult to interpret.[17,18]

In this study we investigate the thermodynamics of dissolution of a homologous series of cyclic dipeptides as a function of aqueous urea concentration at 25°C. The cyclic structure of these compounds precludes complications of end group effects, such as ionic interactions of zwitter ions, and the homologous series permits separation of group contributions. The resulting data allow us to determine the enthalpic and entropic contribution of apolar and amide groups to urea interactions and aid in the interpretation of urea denaturation experiments. These data also illuminate basic features of the hydrophobic effect and hydrogen bonding as they relate to protein stability.

MATERIALS AND METHODS

The cyclic dipeptides c(AG) and c(AA) were purchased from Bachem Bioscience (Philadelphia, PA) and c(GG) and c(LA) from Sigma (St. Louis, MO). (Cyclic dipeptides are designated c(XY), where X and Y are standard one-letter abbreviations for the amino acid residues and c indicates the cyclic structure. All

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residues, except glycine, are the L conformer.) Urea was purchased from Mallinckrodt Chemicals (Paris, KY). All solutions were prepared using distilled deionized water.

Solubility Determination

Sijpkes et al.[19] determined the solubilities of c(GG), c(AA), and c(AG) at 298 K in units of grams per kilogram solvent as a function of urea activity. Molar solubilities as a function of molar urea concentration were calculated from their data using the density and activity values of Stokes[20] and fit to a third-order polynomial. Solubilities at the required urea concentrations were then determined by interpolation.

The solubilities of c(LA) at 298 K were determined by differential refractive index (DRI) measurements.[21] Concentrations of aqueous urea solutions were determined by refractometry[1] on a Abbe Mark II Digital Refractometer (Reichert Instruments, Buffalo, NY).

Dissolution Energetics

The molar dissolution free energy changes were determined as

$$\Delta G^\circ = -R\,T\,\ln K_{sol} \qquad (1)$$

where R is the gas constant, T the absolute temperature, and K_{sol} the molar solubility. The dissolution heats at 298 K were determined by phase equilibrium perturbation calorimetry (PEPC) as previously described[21,22] using a Model 4200 Isothermal Titration Calorimeter (Calorimetry Sciences Corporation, Pleasant Grove, UT). Between 2 and 10 µl of solvent were injected into the cell of a titration microcalorimeter containing saturated solution with excess solid. The molar dissolution enthalpy change (ΔH°) was determined as

$$\Delta H^\circ = \frac{q}{vK_{sol}} \qquad (2)$$

where q is the heat observed upon injection and v is the volume of the injected solvent.

RESULTS

A representative PEPC experiment is shown in Figure 1 for the dissolution of c(LA) into 1 M urea solution. Each peak represents an injection of 10 µl of solvent. The area under the peak is the heat, q, for that injection. Table 1 lists the K_{sol}, ΔG°, ΔH°, and ΔS° at 298 K for each compound as a function of urea concentration.

Figure 2 shows ΔH° as a function of molar urea concentration for each compound. In every case ΔH° is endothermic, but becomes less so with increasing urea concentration. The decrease in ΔH° with urea concentration is linear within the range of concentrations studied. The slope of this line represents the excess enthalpy, ΔH_{ex}, in 1 M urea, which is equivalent to the enthalpy of transfer from water to 1 M urea. As seen in Figure 2, the magnitude of this slope decreases with increasing size of the hydrophobic side chain. This is more clearly seen in Figure 3, in which ΔH_{ex} is plotted against the number of apolar hydrogens, N_{aH}, (i.e., hydrogens bonded to carbon).

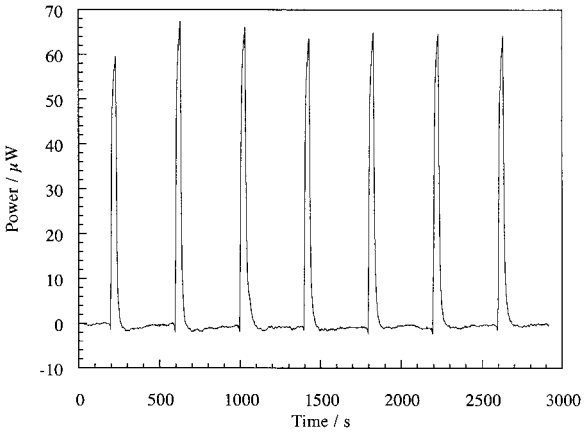

Fig. 1. Representative phase equilibrium perturbation calorimetry experiment for the dissolution of c(LA) into 1 M urea solution using 10 µL injections.

Figure 3 indicates that ΔH_{ex} is a linear function of the number of apolar hydrogens and thus directly proportional to the hydrophobic surface area. The slope of the line gives the ΔH of transfer of one apolar hydrogen from water to 1 M urea, while the intercept gives the ΔH of transfer of the two amide units of the dipeptide. Note that in this separation of the group contributions, the amide unit is defined as the carbonyl carbon and oxygen along with the amino nitrogen and hydrogen (CONH). The amide does *not* include the α-carbon of the peptide backbone which, instead, contributes to the hydrophobic groups.

The positive slope in Figure 3 indicates that hydrophobic transfer is enthalpically unfavorable, whereas the negative intercept indicates that the amide unit transfer is enthalpically favorable.

Similar effects are seen in the entropy of dissolution, ΔS°. For each compound, ΔS° decreases linearly with increasing urea in the concentration range studied, and the magnitude of the slope decreases with increasing size of the hydrophobic side chain (Fig. 4). As seen in Figure 5, ΔS_{ex}, the ΔS of transfer from water to 1 M urea, is negative but increases as N_{aH} increases. The positive slope indicates that the transfer of hydrophobic surface from water to 1 M urea is entropically favorable, whereas the negative intercept indicates that the transfer of the two amide units is entropically unfavorable.

The excess free energy, ΔG_{ex}, represents the change in free energy upon transfer from water to 1 M urea. The contributions of hydrophobic and amide groups can be calculated from the ΔH_{ex} and ΔS_{ex} contributions using the standard relationship

$$\Delta G = \Delta H - T\,\Delta S \qquad (3)$$

TABLE I. Thermodynamics of Dissolution of Cyclic Dipeptides Into Aqueous Urea Solutions at 25°C

Compound	Urea concentration/M	K_{sol}*/M^{-1}	ΔG°/kJ mol^{-1}	ΔH°/kJ mol^{-1}	ΔS°/J K^{-1} mol^{-1}
c(GG)†	0.00	0.145	4.79	26.2	71.8
	2.00	0.173	4.35	23.7	64.9
	4.00	0.207	3.91	21.3	58.4
c(AG)	0.00	0.561	1.43	17.7	54.6
	0.05	0.563	1.42	17.3	53.4
	0.25	0.574	1.38	17.3	53.5
	0.50	0.587	1.32	16.8	51.8
	1.00	0.613	1.21	16.3	50.8
	2.00	0.671	0.990	15.3	48.0
	3.00	0.733	0.769	14.2	45.2
c(AA)	0.00	0.181	4.24	13.7	31.7
	0.50	0.190	4.12	13.1	30.0
	1.00	0.199	4.00	12.8	29.6
	2.00	0.218	3.78	11.9	27.3
	3.00	0.237	3.57	11.3	25.9
c(LA)	0.00	0.0162	10.2	11.9	5.79
	0.50	0.0176	10.0	11.4	4.56
	1.00	0.0194	9.77	11.1	4.30
	2.00	0.0224	9.41	10.8	4.78
	3.00	0.0265	9.00	9.97	3.26

*Calculated from the data of Sijpkes et al.[19] except for c(LA) as described in the text.
†Data from Gill et al.[4]

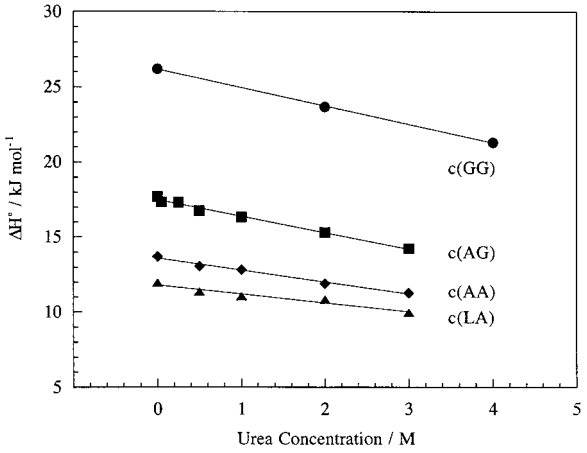

Fig. 2. The dissolution ΔH° as a function of molar urea concentration for cyclic dipeptides.

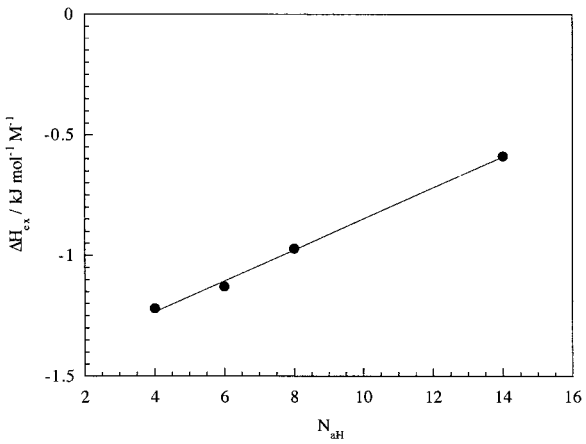

Fig. 3. The ΔH° of transfer from water to 1-M urea, ΔH_{ex}, for cyclic dipeptides as a function of the number of apolar hydrogens, N_{aH} (i.e., hydrogens bonded to aliphatic carbons).

The thermodynamics of transfer from water to 1 M urea are summarized in Table 2. The ΔG of transfer is favorable both for apolar hydrogens and for amide groups, although the driving forces are quite different, as indicated by the ΔH and ΔS values. The values per methyl group (equal to three times the value per apolar hydrogen) are given in parenthesis. It can be seen that the ΔG per methyl and per amide unit are comparable values.

DISCUSSION

The transfer free energies of the amide units and hydrophobic groups from water to urea are both negative, indicating that both hydrophobic and hydrophilic groups contribute to urea denaturation of proteins, as has been noted in previous studies.[3,5,7,23] The partitioning of these transfer free energies into enthalpic and entropic components suggest the molecular basis for the favorable transfer.

Hydrophobic Contributions

The apolar transfer is characterized by a positive ΔH° and a positive ΔS°, indicating that the apolar interaction with aqueous urea is enthalpically unfavorable but entropically favorable. This is in agreement with previous studies of the transfer of compounds from water into 6 M urea by Kresheck and

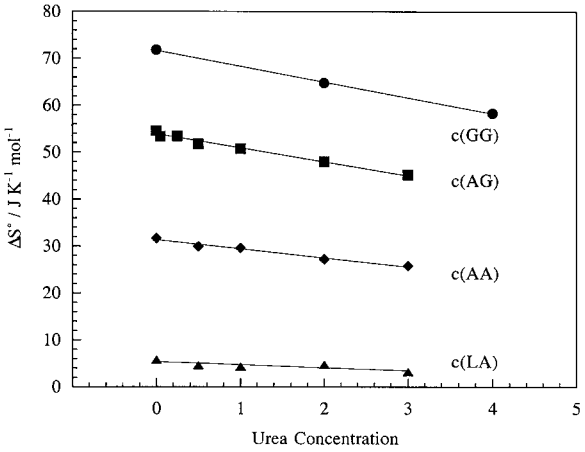

Fig. 4. The dissolution ΔS° as a function of molar urea concentration for cyclic dipeptides.

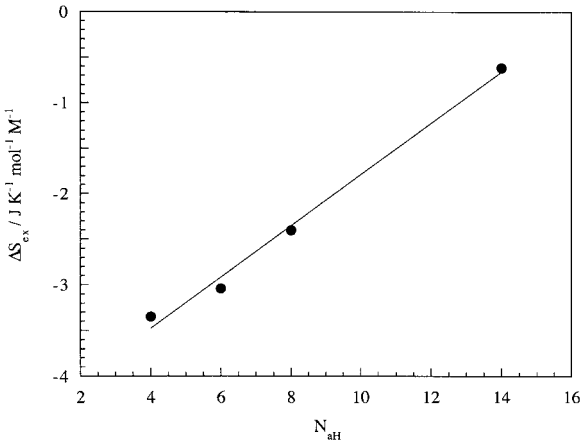

Fig. 5. The ΔS° of transfer from water to 1-M urea, ΔS_{ex}, for cyclic dipeptides as a function of the number of apolar hydrogens, N_{aH} (i.e., hydrogens bonded to aliphatic carbons).

TABLE II. Group Contributions to the Excess Energetics in 1-M Urea at 25°C*

	Apolar hydrogen	Amide unit
ΔH_{ex}/J mol^{-1}	75 ± 16 (225)	−727 ± 75
ΔS_{ex}/J K^{-1} mol^{-1}	0.30 ± 0.04 (0.90)	−2.3 ± 0.2
ΔG_{ex}/J mol^{-1}	−16 ± 4 (−48)	−54 ± 19

*Numbers in parentheses are the contributions per methyl group.

Benjamin[7] and into 7 M urea by Wetlaufer et al.[5] However, the data of Wetlaufer et al. are derived from temperature-dependent solubility studies and are less reliable than those of Kresheck and Benjamin. Kresheck and Benjamin find a ΔH° of transfer for an isobutyl group (N_{aH} = 9) from water to 6 M urea of 3.49 kJ mol^{-1} and a ΔS° of 14.6 J K^{-1} mol^{-1}. Our values from Table 2 predict a ΔH° of 4.0 kJ mol^{-1} and a ΔS° of 16 J K^{-1} mol^{-1}. The excellent agreement indicates two points: one, the ΔH of transfer of hydrophobic groups is a linear function of molar urea concentration, since our values are calculated from transfer from water to 1 M urea and compare well to values for transfer to 6 M urea; and two, the apolar contribution for transfer to urea is additive.

The observation that the favorable transfer of hydrophobic groups to urea solution arises from a favorable entropy change suggests that urea diminishes the hydrophobic effect. The question naturally arises as to how urea does this. Assuming that the hydrophobic effect reflects the 'structure-making' tendency of hydrophobic groups in water,[24] one might suppose that urea acts as a structure-breaker. However, spectroscopic data suggest that urea has little effect on water structure, as O–D stretching frequencies in HDO are unaffected by urea.[25] This conclusion is supported by molecular dynamics simulations which show that urea has little effect on the O–O radial distribution function,[26,27] and by the observation that urea and water mix as nearly ideal solutions.[20] Consequently, urea's diminution of the hydrophobic effect cannot be attributed to urea's action as a structure breaker.

Alternatively, it has been suggested that urea's action is simply the result of its ability to interact with water without perturbing the water structure.[27–29] As urea is a larger molecule than water, it is able to displace several water molecules from the solvation shell around apolar groups; these displaced waters regain the entropy normally associated with hydrophobic hydration and thus the transfer of hydrophobic groups from water to urea solution is accompanied by an increase in entropy.

Several years ago, Muller published a model for the effects of urea on the hydrophobic effect consistent with the above ideas.[29] In this model Muller assumes that the entropy of transfer is directly proportional to the amount of water displaced from the hydration shell. From his calculations of the ΔH° and ΔS° of transfer of several hydrocarbons from water to 7 M urea, one can determine values of ΔH_{ex} and ΔS_{ex} per apolar hydrogen. These values are 40 ± 2 J mol^{-1} for ΔH_{ex} and 0.24 ± 0.01 J K^{-1} mol^{-1} for ΔS_{ex}, compared to the values of 70 J mol^{-1} for ΔH_{ex} and 0.3 J K^{-1} mol^{-1} for ΔS_{ex} determined here.

There is very good agreement between our value of ΔS_{ex} and that calculated from Muller's model, which suggests the appropriateness of his approach. In contrast, the agreement for ΔH_{ex} is much poorer. In calculating ΔH of transfer, Muller included two empirically derived terms intended to reflect the difference in van der Waals interactions of the solute with urea and water.[29] The terms were chosen to produce the best agreement with the data of Wetlaufer et al.[5] The relatively poor agreement between the measured value of ΔH_{ex} and Muller's calculated value probably reflects the variability of the ΔH values determined by Wetlaufer et al. As noted above, these values were determined from the tem-

perature dependence of the solubility rather than by direct calorimetric methods. The excellent agreement between our values and the calorimetrically determined values of Kresheck and Benjamin[7] supports this interpretation.

The positive ΔH of transfer of hydrophobic groups into urea solution is notable, since the hydrophobic effect is generally considered to be purely entropic at 25°C.[30] The transfer of apolar groups from crystalline cyclic dipeptides[21,31] and from liquid amides[32,33] into water is characterized by a negative ΔH° and a negative ΔS°. The negative ΔH° at 25°C associated with hydrophobic transfer in these systems contrasts to the near-zero value generally assumed,[30] but is consistent with the positive ΔH of transfer into urea solution. The conclusion that the hydrophobic effect is purely entropic at 25°C is based on the transfer thermodynamics of liquid hydrocarbons.[34,35] However, the liquid hydrocarbons studied consist of a combination of aromatic, cyclic, and linear alkanes, which do not represent a homologous series. In particular, the inappropriate designation of aromatic groups as purely hydrophobic has recently been noted.[36] While other explanations for the positive ΔH of transfer of hydrophobic groups into urea are possible, the data are consistent with the idea that transfer of hydrophobic groups into water is exothermic at 25°C.

Hydrogen Bonding and Amide Interactions

The amide unit transfer is characterized by a negative ΔH and a negative ΔS, indicating that the amide interaction with aqueous urea is enthalpically favorable and entropically unfavorable. The negative ΔH and ΔS suggest that the amide–urea interaction is a specific interaction, most likely mediated through hydrogen bonding. It must be emphasized here again that we are defining the amide unit as consisting of those four atoms involved in the amide bond (the carbonyl carbon and oxygen and the amino nitrogen and hydrogen), but not including the α-carbon and its hydrogens.

The idea that the amide–urea interaction is, in fact, a hydrogen bonding interaction is supported by several lines of evidence. The structure of a cocrystal of urea and c(GG) has been determined and shows extensive hydrogen bonding interactions[37] with a stoichiometry of one urea per amide unit (i.e., two ureas per c(GG)). The crystal structure of dihydrofolate reductase in the presence of urea shows that most of the urea interactions with the protein are through hydrogen bonds.[38] Molecular dynamics simulations of barnase unfolding in urea show that nearly all of the urea molecules in the first solvation shell show at least one hydrogen bond with the protein.[39] Combined with the negative ΔH and ΔS of transfer of the amide unit from water to urea, the conclusion that the urea hydrogen bonds to the amide in aqueous solution seems very reasonable.

TABLE III. Thermodynamics of Amide Binding Interactions at 25°C

Interaction	κ	ΔH°/kJ mol^{-1}	ΔG°/kJ mol^{-1}	ΔS°/J K^{-1} mol^{-1}
Urea–amide	0.023 ± 0.11	−34 ± 3	9.3 ± 1.2	−145 ± 11
Amide–amide*	0.018	−29	10	−130

*Data from Murphy and Gill.[42]

If the amide–urea interaction can be considered as a one-to-one binding reaction mediated through hydrogen bonds, then a binding constant can be calculated from ΔG_{ex} using the following equation[40]:

$$\Delta G_{ex} = -R\,T \ln (1 + \kappa x) \qquad (4)$$

where R is the gas constant, T the absolute temperature, κ the binding constant, and x the urea activity. At 1 M urea, ΔG_{ex} is -54 ± 19 J mol^{-1} and the urea activity is 0.965, yielding a value of κ of 0.023 ± 0.011 M^{-1}. This value is smaller than previous estimates for urea interaction with the amide, which average around 0.04.[6,41] The reason for this difference is that previous authors have included the α-carbon of the glycine residue as part of the interaction. If we include the contribution of the two apolar hydrogens of the α-carbon, we have a ΔG_{ex} of -94 J mol^{-1}, resulting in an estimated κ of 0.037, which is in excellent agreement with previous studies. However, the α-carbon contributes favorably to transfer into urea solutions due to the diminution of the hydrophobic effect, as discussed above, and not through a binding reaction. Consequently, as our goal here is to understand the binding between urea and the amide unit, it is appropriate to consider only the carbonyl and amino groups. The binding constant of 0.023 is thus a more accurate estimate for urea–amide interactions.

Using the binding constant determined from Equation (4), the ΔH° of binding can also be determined since the ΔH_{ex} is given by the following:[6,17]

$$\Delta H_{ex} = (\Delta H^\circ - L_3)\frac{\kappa x}{1 + \kappa x} \qquad (5)$$

where L_3 is the excess enthalpy of the urea solution at the urea activity x. Using the equations of Schellman and Gassner,[17] L_3 is found to be -0.7 kJ mol^{-1} at 1 M urea, so that ΔH° of binding is calculated to be -34 ± 3 kJ mol^{-1}. The thermodynamics of urea binding to the amide unit are summarized in Table 3.

It is important to note the ramifications of excluding the α-carbon in the definition of the amide unit as it applies to hydrogen bonding in aqueous solution. According to Equation (5), the same ΔH_{ex} is interpreted as giving a smaller ΔH° as κ is increased. Additionally, the hydrophobic groups contribute posi-

tively to ΔH_{ex} so that the magnitude of ΔH_{ex} is also decreased by inclusion of the α-carbon in the definition of the interacting group. These two factors combine to underestimate significantly the ΔH° of amide hydrogen bond formation in solution, a problem previously noted by Némethy and Scheraga[43] in commenting on the interpretation of thermodynamics of dimerization of N-methyl acetamide by Klotz and Franzen.[44] Our results indicate that the amide hydrogen bond has a very favorable ΔH° of formation in aqueous solution.

Ideally, the binding of urea to the amide unit should be treated using an exchange model that recognizes the role of water in weak interactions.[17,45] Currently, expressions for the exchange model have only been derived for the case in which the binding of one urea molecule only displaces one water molecule. It seems unlikely that a one-to-one exchange is realistic given the relative sizes of the two molecules. We have therefore not attempted to correct for the effects of water activity. In a previous study of the effects of urea on the solubility of c(GG), application of the exchange model resulted in a reduction of κ by ten percent. [41]

Table 3 also shows the thermodynamics of dimerization of c(GG) as modeled from heats of dilution.[42] The values are nearly identical to those for the binding of urea to the amide unit, again suggesting that urea's interaction with the amide is mediated through hydrogen bonds, and that the urea–amide binding ΔH° reflects the enthalpy of amide hydrogen bond formation in aqueous solution.

As the peptide bond in the cyclic dipeptides is in the *cis* conformation, a one-to-one complex between urea and the amide can have either one or two hydrogen bonds. Molecular dynamics studies suggest that the lowest energy state of the urea dimer in aqueous solution is the cyclic dimer with two hydrogen bonds.[26] It seems likely that the cyclic structure with two hydrogen bonds would also be favored in the urea–amide interaction here. Consequently, the ΔH° of formation of an amide hydrogen bond would be estimated to be at least -17 kJ mol^{-1}, but not more than -34 kJ mol^{-1}.

It has been suggested that the ΔH° of amide hydrogen bond formation may be favorable in water, but that the enthalpic cost of desolvating the hydrogen-bonded groups upon burial in the protein interior will be at least as large, if not larger, than the ΔH° of amide hydrogen bond formation.[46–49] We have studied previously the thermodynamics of aqueous dissolution of the cyclic dipeptides as a model to estimate the contributions of amide hydrogen bonds and the hydrophobic effect to the enthalpy of protein folding. The formation of an amide hydrogen bond and burial of the hydrogen-bonded pair in the crystal has a ΔH° of -13.5 ± 2.7 kJ mol^{-1}.[31] Comparison of this value and the ΔH° of amide hydrogen bond formation in aqueous solution suggests that the 'desolvation penalty' is between 3.5 and 20.5 kJ mol^{-1}, depending on the number of hydrogen bonds in the urea–amide interaction. It must be noted that the desolvation enthalpy can be offset by other factors upon burial of the hydrogen bonding groups such as van der Waals and electrostatic interactions.

The above results can be compared to recent calculations on the energetics of dissolution and sublimation of cyclic dipeptides by Brady and Sharp.[50] They calculate that the association ΔS° upon transfer from solution to the crystal is -122 J K^{-1} mol^{-1} for c(GG), which includes losses in translational and rotational degrees of freedom. This is compared to the -145 J K^{-1} mol^{-1} for association between urea and the amide unit in solution from Table 3. The reasonable agreement between the two numbers supports the assertion that there are two hydrogen bonds formed between the urea and the peptide, since the loss of rotational degrees of freedom upon forming the second hydrogen bond would make the binding process more analogous to the process of transfer from solution to the crystal.

It is more difficult to compare the enthalpic terms. Brady and Sharp[50] calculate a contribution to ΔH° from inter- and intramolecular terms of -103 kJ mol^{-1} for the transfer from solution to the crystal. This corresponds to -51.5 kJ mol^{-1} per peptide. However, this value includes van der Waals interactions from packing as well as hydrogen bonding and long-range electrostatic terms, but excludes solvation. Their solvation contribution of 77.4 kJ mol^{-1} is calculated by assuming that all the solvation enthalpy is due to solvation of polar groups. This assumption may be incorrect based on arguments given above. While the signs and magnitudes of the terms agree reasonably well with the binding energetics determined in this study, a careful comparison must await calculations of interaction in the solution phase.

In summary, the analysis of the interactions between urea and the amide unit suggests that hydrogen bonding between amide groups in aqueous solution is enthalpically very favorable but is offset by a large, unfavorable ΔS°. The ability of urea to enhance the solvation of protein amide units is due to urea itself being a soluble amide.

Implications for the Interpretation of Chemical Denaturation Studies

The general applicability of the cyclic dipeptide model to understanding the effects of urea on protein stability is confirmed by comparison to protein studies. In particular, it is observed here that the dissolution ΔH°, analogous to the unfolding ΔH°, decreases with increasing urea concentration. This is in agreement with previous studies on the thermal denaturation of proteins in urea solutions, which show that the ΔH° of unfolding decreases with increasing urea concentration.[11,12,16]

The current data bear also on the interpretation of data from urea denaturation studies of proteins. Chemical denaturation studies have generally been

analyzed using either the binding model or the linear extrapolation method (LEM).[1,51] We will discuss the implications of the current results for both these approaches, beginning with the binding model.

The denaturant binding model

The binding model assumes that the unfolded state has more sites at which denaturant can bind and that all of these sites are identical and independent.[51] Using this model, the unfolding equilibrium constant, K, is a function of the denaturant activity as[51]

$$K = K_0(1 + \kappa x)^{\Delta n} \quad (6)$$

where K_0 is the equilibrium constant in the absence of denaturant, κ the binding constant for denaturant to sites on the protein, x the denaturant activity, and Δn the additional number of binding sites in the unfolded protein relative to the native state.

There are two primary problems with using this model in analyzing protein unfolding. First is the assumption that denaturants such as urea act through specific binding to the protein. Second is the assumption that all sites have identical binding constants. The present work addresses both issues.

The interactions of urea with hydrophobic groups and with the amide unit clearly result from different mechanisms. The interaction with hydrophobic groups appears to be largely a result of steric factors which produce a diminution of the hydrophobic effect. In contrast, the interaction of urea with the amide unit appears to be a genuine binding interaction. Consequently, the inclusion of hydrophobic groups in the empirical definition of the 'binding site' results in a larger apparent binding constant that inaccurately reflects the binding interaction. This is true even though the binding model may provide a good fit to the experimental data.

The fact that urea interacts differently with different functional groups necessarily results in heterogeneity in the 'binding sites' on a protein. The difficulties with interpreting data in which heterogeneous sites are analyzed as though they were homogeneous have been discussed. In particular, Schellman and Gassner[17] and DeKoster and Robertson[18] have noted that the binding constant and $\Delta H°$ of binding between urea and protein sites derived by Makhatadze and Privalov[12] inaccurately reflect the actual binding interactions.

Makhatadze and Privalov obtain a binding constant of 0.06 ± 0.01 for the interaction of urea with protein sites, and a $\Delta H°$ of -9 ± 2 kJ mol^{-1}. Their binding constant is considerably larger and their $\Delta H°$ is considerably smaller in magnitude than those obtained in the current study. The reasons for this difference are the same as those noted above for the binding of urea to the peptide backbone. Because Makhatadze and Privalov's binding sites are empirically defined, they include hydrophobic contributions. The favorable $\Delta G°$ of transfer of hydrophobic groups into urea solution results in a larger estimate of the binding constant. The $\Delta H°$ of binding is underestimated when using an overestimated binding constant as seen in Equation (5). The underestimate of the magnitude of $\Delta H°$ is exacerbated again by the fact that the hydrophobic groups contribute positively to ΔH of transfer into urea solutions, further diminishing the magnitude of the estimated binding $\Delta H°$.

In summary, the use of the binding model may, in fact, give a good fit to experimental unfolding data, but the resulting parameters cannot be interpreted as physically meaningful. All of the model parameters (i.e., the binding constant, κ; the change in the number of binding sites, n; and the binding enthalpy, $\Delta H°$) will reflect some average properties of the heterogeneous system, but cannot provide information relevant to interpreting the physical interaction of urea with protein functional groups.

The linear extrapolation method

The linear extrapolation method is the most common means of analyzing chemical denaturation experiments. This model assumes that the free energy of unfolding, $\Delta G°_{unf}$, is linearly dependent on denaturant concentration as

$$\Delta G°_{unf} = \Delta G°_{water} - m\,[den] \quad (7)$$

where $\Delta G°_{water}$ is the free energy of unfolding in the absence of denaturant, [den] is the molar denaturant concentration, and m is the magnitude of the slope of a plot of $\Delta G°_{unf}$ vs. [den]. The value of $\Delta G°_{water}$ is determined from the extrapolated intercept. The use of the LEM has been generally validated by showing that the value of $\Delta G°_{water}$ determined by extrapolation agrees with that determined from differential scanning calorimetric experiments.[52]

The magnitude of the slope, often referred to as the m-value, has been interpreted as indicating the amount of surface area exposed upon unfolding.[53] This relationship has been extremely important in interpreting the effects of point mutations on protein stability[54,55] and in interpreting equilibrium folding pathways from the effects of denaturant on hydrogen exchange kinetics.[56,57]

The m-value for c(GG), calculated from the ΔG_{ex} values in Table 2, is 172 ± 20 J mol^{-1} M^{-1}. This gives a value of 86 ± 10 J mol^{-1} M^{-1} per residue, which is in very good agreement with the m-value determined for peptide helices by Scholtz, et al.[58] of 96 ± 1 J mol^{-1} M^{-1} per residue. In contrast, the average m-value in proteins is 52 ± 12 J mol^{-1} M^{-1} per residue[53], which is considerably smaller than the value for the model compound or helix studies. This difference is not due to the *cis* conformation of the peptide in the cyclic

dipeptides, since the values from these compounds and from the helix, which has *trans* peptides, agree. Rather, the difference likely reflects the nature of the unfolded state of the proteins, which probably prevent full saturation of amide binding sites by the urea due to steric effects.

While the values presented here do not predict the m-values of proteins, the current results support the general use of the LEM in analyzing chemical denaturation curves. The empirical observation that $\Delta G°_{unf}$ is a linear function of molar urea concentration can result in two ways. First, it is possible that both the $\Delta H°$ and $\Delta S°$ are linear functions or molar urea concentration, suggesting an underlying physical basis supporting the LEM. Alternatively, $\Delta H°$ and $\Delta S°$ could be curvilinear functions of molar urea concentration, which, through compensating effects, can result in a linear response of $\Delta G°$. Such an observation would suggest that the linear $\Delta G°$ results from complex physical interactions and would make the use of the LEM less physically interpretable. Our results show that both $\Delta H°$ and $\Delta S°$ of dissolution are linear functions of molar urea concentration, thus supporting the use of the LEM.

CONCLUSION

The effects of urea on the thermodynamics of dissolution of cyclic dipeptides result in three important conclusions.

One, urea denatures proteins because it diminishes the hydrophobic effect by displacing water in the solvation shell and because it specifically binds to amide units.

Two, the binding of urea to the amide unit is mediated through hydrogen bonds and exemplifies amide–amide hydrogen bonding in water. This interaction is characterized by a large, favorable $\Delta H°$ of binding (-34 kJ mol^{-1}) at 25°C, which probably corresponds to two hydrogen bonds with a $\Delta H°$ of formation of -17 kJ mol^{-1}. Comparison of this value with the contribution of the amide hydrogen bond to the dissolution of cyclic dipeptides suggests that the enthalpic penalty of desolvating a hydrogen bond pair upon burial in the protein interior is not overly large.

Three, the linearity of $\Delta H°$ and $\Delta S°$ of dissolution with molar urea concentration supports the use of the linear extrapolation model in analyzing chemical denaturation curves.

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REFERENCES

1. Pace, C.N. Determination and analysis of urea and guanidine hydrocholride denaturation curves. Methods Enzymol. 131:266–280, 1986.
2. Robinson, D.R., Jencks, W.P. The effect of compounds of the urea–guanidinium class on the activity coefficient of acetyltetraglycine ethyl ester and related compounds. J. Am. Chem. Soc. 87:2462–2470, 1965.
3. Nandi, P.K., Robinson, D.R. Effects of urea and guanidine hydrochloride on peptide and nonpolar groups. Biochemistry 23:6661–6668, 1984.
4. Gill, S.J., Hutson, J., Clopton, J.R., Downing, M. Solubility of diketopiperazine in aqueous solutions of urea. J. Phys. Chem. 65:1432–1435, 1961.
5. Wetlaufer, D.B., Malik, S.K., Stoller, L., Coffin, R.L. Nonpolar group participation in the denaturation of proteins by urea and guanidinium salts: Model compound studies. J. Am. Chem. Soc. 86:508–514, 1964.
6. Schönert, H., Stroth, L. Thermodynamic interaction between urea and the peptide group in aqueous solutions at 25°C. Biopolymers 20:817–831, 1981.
7. Kresheck, G.C., Benjamin, L. Calorimetric studies of the hydrophobic nature of several protein constituents and ovalbumin in water and in aqueous urea. J. Phys. Chem. 68:2476–2486, 1964.
8. Savage, J.J., Wood, R.H. Enthalpy of dilution of aqueous mixtures of amides, sugars, urea, ethylene glycol, and pentaerythritol at 25°C: Enthalpy of interaction of the hydrocarbon, amide, and hydroxyl functional groups in dilute aqueous solutions. J. Solution Chem. 5:733–750, 1976.
9. Sijpkes, A.H., Somsen, G., Lilley, T.H. Enthalpies of interacton of some N-acetyl amino acid amides in aqueous urea solutions at 298.15 K. J. Chem. Soc. Farady Trans. 86:2943–2949, 1990.
10. Brandts, J.F., Linda, H. The thermodynamics of protein denaturation. III. The denaturation of ribonuclease in water and in aqueous urea and aqueous ethanol mixtures. J. Amer. Chem. Soc. 89:4826–4838, 1967.
11. Johnson, C.M., Fersht, A.R. Protein stability as a function of denaturant concentration: The thermal stability of barnase in the presence of urea. Biochemistry 34:6795–6804, 1995.
12. Makhatadze, G.I., Privalov, P.L. Protein interactions with urea and guanidinium chloride: A calorimetric study. J. Mol. Biol. 226:491–505, 1992.
13. Neri, D., Billeter, M., Wider, G., Wüthrich, K. NMR determination of residual structure in a urea-denatured protein, the 434 repressor. Science 257:1559–1563, 1992.
14. Pace, C.N., Laurents, D.V., Thomson, J.A. pH dependence of the urea and guanidine hydrochloride denaturation of ribonuclease A and ribonuclease T1. Biochemistry 29:2564–2572, 1990.
15. Pace, C.N., Laurents, D.V., Erickson, R.E. Urea denaturation of barnase: pH dependence and characterization of the unfolded state. Biochemistry 31:2728–2734, 1992.
16. Paz-Andrade, M.I., Jones, M.N., Skinner, H.A. The enthalpy of interaction of urea with some globular proteins. Eur. J. Biochem. 66:127–131, 1976.
17. Schellman, J.A., Gassner, N.C. The enthalpy of transfer of unfolded protiens into solutions of urea and quanidinium chloride. Biophys. Chem. 59:259–275, 1996.
18. DeKoster, G.T., Robertson, A.D. Calorimetrically derived parameters for protein interactions with urea and guanidine-HCl are not consistent with denaturant m-values. Biophys. Chem. 64:59–68, 1997.
19. Sijpkes, A.H., van de Kleut, G., Gill, S.C. The solubilities of five cyclic dipeptides in water and in aqueous urea at 298.15 K: A quantitative model for the denaturation of proteins in aqueous urea solutions. Biophys. Chem. 52:75–82, 1994.
20. Stokes, R.H. Thermodynamics of aqueous urea solutions. Aust. J. Chem. 20:2087–2100, 1967.
21. Murphy, K.P., Gill, S.J. Group additivity thermodynamics for dissolution of solid cyclic dipeptides into water. Thermochim. Acta 172:11–20, 1990.
22. Murphy, K.P., Gill, S.J. Calorimetric measurement of the enthalpy of dissolution of diketopiperazine in water as a function of temperature. Thermochim. Acta 139:279–290, 1989.

23. Roseman, M., Jencks, W.P. Interactions of urea and other polar compounds in water. J. Am. Chem. Soc. 97:631–640, 1975.
24. Frank, H.S., Evans, M.W. Free volume and entropy in condensed systems. III. Entropy in binary liquid mixtures, partial molal entropy in dilute solutions, structure and thermodynamics in aqueous electrolytes. J. Chem. Phys. 13:507–532, 1945.
25. Swenson, C.A. Effects of protein denaturants of the urea–guanidinium class on bulk water structure. Arch. Biochem. Biophys. 117:494–498, 1966.
26. Kuharski, R.A., Rossky, P.J. Molecular dynamics study of solvation in urea–water solution. J. Am. Chem. Soc. 106: 5786–5793, 1984.
27. Tsai, J., Gerstein, M., Levitt, M. Keeping the shape but changing the charges: A simulation of urea and its isosteric analogs. J. Chem. Phys. 104:9417–9430, 1996.
28. Kuharski, R.A., Rossky, P.J. Solvation of hydrophobic species in aqueous urea solution: A molecular dynamics study. J. Am. Chem. Soc. 106:5794–5800, 1984.
29. Muller, N. A model for the partial reversal of hydrophobic hydration by addition of a urea-like cosolvent. J. Phys. Chem. 94:3856–3859, 1990.
30. Baldwin, R.L. Temperature dependence of the hydrophobic interaction in protein folding. Proc. Natl. Acad. Sci. U.S.A. 83:8069–8072, 1986.
31. Habermann, S.M., Murphy, K.P. Energetics of hydrogen bonding in proteins: A model compound study. Protein Sci. 5:1229–1239, 1996.
32. Spolar, R.S., Livingstone, J.R., Record, M.T., Jr. Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water. Biochemistry 31:3947–3955, 1992.
33. Barone, G., Del Vecchio, P., Giancola, C., Graziano, G. The liquid amide transfer model and the unfolding thermodynamics of small globular proteins. Int. J. Biol. Macromol. 17:2–51257, 1995.
34. Gill, S.J., Nichols, N.F., Wadsö, I. Calorimetric determination of enthalpies of solution of slightly soluble liquids. II. Enthalpy of solution of some hydrocarbons in water and their use in establishing the temperature dependence of their solubilities. J. Chem. Thermo. 8:445–452, 1976.
35. Gill, S.J., Wadsö, I. An equation of state describing hydrophobic interactions. Proc. Natl. Acad. Sci. U.S.A. 73:2955–2958, 1976.
36. Makhatadze, G.I., Privalov, P.L. Energetics of interactions of aromatic hydrocarbons with water. Biophys. Chem. 50:285–291, 1994.
37. Thayer, M.M., Haltiwanger, R.C., Allured, V.S., Gill, S.C., Gill, S.J. Peptide–urea interactions as observed in diketopiperazine–urea cocrystal. Biophys. Chem. 46:165–169, 1993.
38. Dunbar, J., Yennawar, H.P., Banerjee, S., Luo, J., Farber, G.K. The effect of denaturants on protein structure. Protein. Sci. 6:1727–1733, 1997.
39. Tirado-Rivas, J., Orozco, M., Jorgensen, W.L. Molecular dynamics simulations of the unfolding of barnase in water and 8-M aqueous urea. Biochemistry 36:7313–7329, 1997.
40. Schellman, J.A. Macromolecular binding. Biopolymers 14: 999–1018, 1975.
41. Sijpkes, A.H., van de Kleut, G.J., Gill, S.C. Urea–diketopiperazine interactions: A model for urea-induced denaturation of proteins. Biophys. Chem. 46:171–177, 1993.
42. Murphy, K.P., Gill, S.J. Thermodynamics of dissolution of cyclic dipeptide solids containing hydrophobic side groups. J. Chem. Thermodynamics 21:903–913, 1989.
43. Némethy, G., Scheraga, H.A. The structure of water and hydrophobic bonding in proteins. III. The thermodynamic properties of hydrophobic bonds in proteins. J. Phys. Chem. 66:1773–1789, 1962.
44. Klotz, I.M., Franzen, J.S. Hydrogen bonds between model peptide groups in solution. J. Am. Chem. Soc. 84:3461–3466, 1962.
45. Schellman, J.A. A simple model for solvation in mixed solvents: Application to the stabilization and destabilization of macromolecular structures. Biophys. Chem. 37:121–140, 1990.
46. Yang, A.-S., Sharp, K.A., Honig, B. Analysis of the heat capacity dependence of protein folding. J. Mol. Biol. 227: 889–900, 1992.
47. Makhatadze, G.I., Privalov, P.L. Energetics of protein structure. Adv. Protein Chem. 47:307–425, 1995.
48. Makhatadze, G.I., Privalov, P.L. Contribution of hydration to protein folding thermodynamics. I. The enthalpy of hydration. J. Mol. Biol. 232:639–659, 1993.
49. Honig, B., Yang, A.-S. Free Energy balance in protein folding. Adv. Prot. Chem. 46:27–58, 1995.
50. Brady, G.P., Sharp, K.A. Energetics of cyclic dipeptide crystal packing and solvation. Biophys. J. 72:913–927, 1997.
51. Tanford, C. Protein denaturation. Adv. Protein Chem. 24:1–95, 1970.
52. Santoro, M.M., Bolen, D.W. A test of the linear extrapolation of unfolding free energy changes over an extended denaturant concenration range. Biochemistry 31:4901–4907, 1992.
53. Myers, J.K., Pace, C.N., Scholtz, J M. Denaturant m-values and heat capacity changes: Relation to changes in accessible surface areas of protein unfolding. Protein. Sci. 4:2138–2148, 1995.
54. Dill, K.A., Shortle, D. Denatured states of proteins. Annu. Rev. Biochem. 60:795–825, 1991.
55. Shortle, D. The denatured state (the other half of the folding equation) and its role in protein stability. FASEB J. 10:27–34, 1996.
56. Bai, Y., Sosnick, T.R., Mayne, L., Englander, S.W. Protein folding intermediates: Native-state hydrogen exchange. Science 269:192–197, 1995.
57. Chamberlain, A.K., Handel, T.M., Marqusee, S. Detection of rare partially folded molecules in equilibrium with the native conformation of RNaseH. Nature Struct. Biol. 3:782–787, 1996.
58. Scholtz, J.M., Barrick, D., York, E.J., Stewart, J.M., Baldwin, R.L. Urea unfolding of peptide helices as a model for interpreting protein unfolding. Proc. Natl. Acad. Sci. U.S.A. 92:185–189, 1995.