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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

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Abstract

The solubilities of cyclo(L-alanylglycine), cyclo(L-alanyl-L-alanine), cyclo(glycyl-L-leucine), cyclo(L-valyl-L-valine) and cyclo(glycyl-L-phenylalanine) were determined in water and in aqueous urea solutions up to concentrations of 9 molar urea at 298.15 K. The solubilities of all cyclic dipeptides increase with increasing urea concentration. A simple equilibrium model, taking into account the activity of urea and that of water, fits the solubility data yielding apparent equilibrium constants describing the interactions occurring between urea and the peptide groups plus the alkyl groups that are next to these peptide groups. The apparent equilibrium constants were converted to Gibbs energy parameters for each amino acid residu which were then used to make a quantitative estimate of the contribution of urea to the denaturation of proteins.

Key words: Cyclic dipeptide; Aqueous urea solution; Solubility; Apparent equilibrium constant; Protein denaturation

1. Introduction

This paper is a sequel to our studies concerning the solubility of cyclo(glycylglycine), or diketopiperazine, in aqueous urea solutions [1]. The previous study showed that the solubility of diketopiperazine increased with increasing urea concentration, but dropped sharply at urea concentrations above ≈ 6 mol dm^{-3}. This was attributed to the formation of a cocrystal of one diketopiperazine and two urea molecules at high urea concentrations [2,3]. Using this information we have developed a simple equilibrium model which, when applied to the solubility data, yields an intrinsic apparent equilibrium constant κ describing the interactions between urea and the peptide group of diketopiperazine. In order to correctly analyze the data it was essential to use the activity of urea combined with that of water as an independent variable [4]. The equilibrium constant κ was used to calculate the Gibbs energy of transfer of a peptide group of diketopiperazine from water to 8 molar urea. This was then used to estimate the effect of urea, from its interactions with peptide groups, on the denaturation of

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proteins. The main conclusion was that the interaction of urea with a peptide group is intrinsically weak. However, at high urea concentrations and due to the large number of buried peptide groups within a folded protein, the interactions of urea with the peptide groups of a protein are sufficiently large to contribute significantly to the overall Gibbs energy of denaturation of a protein.

One of the problems in dealing with these types of model systems is to separate the interactions of urea with peptide groups from those with apolar groups. In the case of diketopiperazine, it was not possible to separate the interactions of urea with a peptide group from those with an apolar group next to the peptide group. Therefore, we have extended our experiments to cyclic dipeptides that have larger alkyl groups than diketopiperazine allowing a better understanding of the interactions of urea with both types of functional groups.

In this paper, we present the solubilities of five cyclic dipeptides, cyclo(L-alanylglycine), cyclo(L-alanyl-L-alanine), cyclo(glycyl-L-leucine), cyclo(L-valyl-L-valine) and cyclo(glycyl-L-phenylalanine) determined in water and in aqueous urea solutions up to concentrations of 9 molar urea at 298.15 K. The results will be discussed in terms of the Gibbs energies of transfer of the amino acid residues from water to 8 molar urea. Finally, we will address the denaturation of proteins in aqueous urea in view of our results.

2. Experimental

The cyclic dipeptides were purchased from Bachem Bioscience (Philadelphia) and used as received. Urea was recrystallized from a water–ethanol mixture. The solubilities of the cyclic dipeptides were determined as follows. A saturated dipeptide solution (in water or in aqueous urea) containing an excess of crystals was put into the bottom chamber of a dialysis cell (see Fig. 1). The upper chamber of the cell was filled with water (or aqueous urea), or with a solution of a dipeptide. The two chambers were screwed together, separated by a membrane (Spectra/Por® Membranes, MWCO:1000). The dialysis cell was submerged and rotated continuously in a constant temperature bath, held at 25.00 ± 0.01°C, for at least seven days which was sufficient time for the solutions to reach equilibrium concentrations. The saturated solution in the upper part of the cell, free of crystals, was analyzed by HPLC (Hewlett Packard) (see also Ref. [1]). This method of determining the solubilities of diketopiperazines was compared with the previous one [1]: the results for diketopiperazine reported here are in good agreement with those of Ref. [1] (see Table 2 and Fig. 2a).

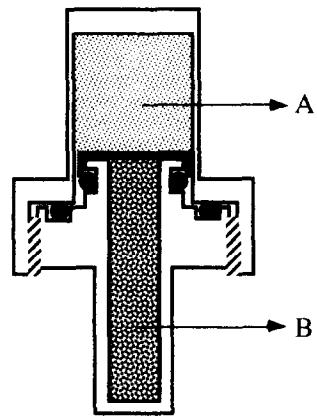

Fig. 1. Dialysis cell used for the determination of solubilities of cyclic dipeptides in water. Chamber A, having a volume of ≈ 1.5 cm^3, contained a solution of cyclic dipeptide whose concentration was analyzed by HPLC and chamber B, having a volume of ≈ 1.0 cm^3, contained a saturated solution with crystals.

3. The exchange equilibrium model

In order to evaluate the solubility data of the cyclic dipeptides in aqueous urea solutions we have used an exchange model similar to the one presented earlier [1], where we assumed each urea interacting with a binding site, i.e. a peptide group, to be exchanged by one water molecule, and vice versa, via the formation of hydrogen bonds.

In Scheme 1, a dipeptide begins in its crystalline state denoted by DIP_{cr}. The first step is the dissolution into aqueous solution to give [DIP $\cdot (H_2O)_2]_{aq}$, characterized by a solubility constant

K_s. Here we assumed that initially the dipeptide is solvated by water molecules only. The aqueous dipeptide then successively interacts with one urea to form $[DIP \cdot (U,H_2O)]_{aq}$, and a second urea to form $[DIP \cdot U_2]_{aq}$, characterized by apparent equilibrium constants K_1 and K_2, respectively.

Table 1
The solubilities of cyclic dipeptides (DIP) as a function of the activity of urea (U) at 298.15 K [a]

Act. U (mol kg^{-1})	Sol. DIP (g (kg solvent)$^{-1}$)	Act. U (mol kg^{-1})	Sol. DIP (g (kg solvent)$^{-1}$)	Act. U (mol kg^{-1})	Sol. DIP (g (kg solvent)$^{-1}$)
cyclo(glycylglycine)					
0	16.3	2.09	19.4	3.20	21.1
0	16.8	2.09	19.8	4.23	23.4
0	16.9	3.20	22.1	4.23	23.7
cyclo(L-alanylglycine)					
0	71.2	0.91	77.7	2.85	90.7
0	71.5	1.48	82.4		
0	72.6	2.63	87.5		
4.21	*95.8*	*5.65*	*95.7*	*8.09*	*93.3*
4.31	*92.7*	*7.26*	*94.3*	*9.21*	*94.7*
cyclo(L-alanyl-L-alanine)					
0	24.9	1.27	29.2	3.08	33.5
0	25.2	1.27	29.7	3.89	34.3
0	25.5	2.09	30.6	3.89	34.3
0	26.3	2.09	30.7	3.89	35.1
1.27	28.7	3.08	32.6		
5.09	*36.0*	*6.51*	*38.1*	*8.44*	*37.4*
5.09	*36.3*	*8.44*	*37.1*	*8.44*	*39.0*
cyclo(L-valyl-L-valine)					
0	0.250	1.80	0.292	4.83	0.447
0	0.252	1.80	0.328	5.19	0.476
0	0.258	3.25	0.362	5.19	0.481
1.09	0.288	3.25	0.401		
1.09	0.297	4.83	0.445		
6.55	*0.521*	*8.48*	*0.578*	*9.24*	*0.611*
8.48	*0.576*	*9.24*	*0.581*		
cyclo(glycyl-L-leucine)					
0	8.14	2.25	10.5	4.41	13.2
0	8.19	2.25	10.7	4.41	13.4
0	8.22	2.25	10.9	5.52	14.5
0.96	8.49	3.62	12.1		
0.96	8.71	3.62	12.6		
cyclo(glycyl-L-phenylalanine)					
0	1.32	2.03	1.97	3.63	2.54
0	1.38	2.03	1.99	3.63	2.66
1.34	1.60	2.03	2.01	4.41	3.01
1.34	1.66	2.84	2.13		
1.34	1.68	2.84	2.21		

[a] The data printed in italics were not included in the data analysis (see text).
[b] The solubilities of cyclo(L-alanylglycine) were measured using the centrifuge method [1].

Because the concentration of the dipeptides is low we assumed only dipeptide monomers to be present in solution [1].

When a dipeptide is symmetric, as is the case for GG, AA and VV, and assuming independent binding sites, an intrinsic apparent equilibrium constant κ can be introduced, so that $K_1 = 2\kappa$, and $K_2 = \frac{1}{2}\kappa$ (see Eq. (3a) in Scheme 1) [5]. Throughout this paper we will use κ in case of symmetric cyclic dipeptides.

When a dipeptide is asymmetric, as is the case for AG, GL and GF, the value for K_1 (representing the glycine residue) was fixed at a value equivalent to $\kappa = 0.0398$ kg mol^{-1} obtained earlier for diketopiperazine [1]. This is necessary since fitting the data using Eq. (4a) in Scheme 1 does not resolve K_1 and K_2 without fixing one of these parameters.

Scheme 1. The solubility of a cyclic dipeptide (DIP) in aqueous area solutions (see text):

$$\mathrm{DIP_{cr}} + n\mathrm{H_2O} \overset{K_s}{\rightleftharpoons} \left[\mathrm{DIP(H_2O)_2}\right]_{aq},$$

$$\left[\mathrm{DIP(H_2O)_2}\right]_{aq} + \mathrm{U} \overset{K_1}{\rightleftharpoons} \left[\mathrm{DIP}\cdot(\mathrm{U}, \mathrm{H_2O})\right]_{aq} + \mathrm{H_2O},$$

$$\left[\mathrm{DIP}\cdot(\mathrm{U}, \mathrm{H_2O})\right]_{aq} + \mathrm{U} \overset{K_2}{\rightleftharpoons} \left[\mathrm{DIP}\cdot\mathrm{U_2}\right]_{aq} + \mathrm{H_2O}. \quad (1a)$$

The total amount of cyclic dipeptide in solution is

$$\mathrm{DIP_{T,aq}} = \left[\mathrm{DIP(H_2O)_2}\right]_{aq} + \left[\mathrm{DIP}\cdot(\mathrm{U}, \mathrm{H_2O})\right]_{aq} + \left[\mathrm{DIP}\cdot\mathrm{U_2}\right]_{aq}. \quad (2a)$$

Combining Eqs. (1a) and (2a) and assuming two equal and independent binding sites yields:

$$\mathrm{DIP_{T,aq}} = K_s[1 + 2\kappa X + \kappa^2 X^2]. \quad (3a)$$

In the case of two different binding sites the equivalent of Eq. (3a) becomes

$$\mathrm{DIP_{T,aq}} = K_s\left[1 + (K_1 + K_2)X + K_1K_2X^2\right], \quad (4a)$$

where $X = [\mathrm{U}]/[\mathrm{W}]$ is the independent variable, [U] is the activity of urea and [W] is the activity of water.

4. Results

The experimental data, collected in Table 1 and plotted in Figs. 2a–2f, show the solubilities of the cyclic dipeptides as a function of the activity of urea. The activity of urea on a molality scale basis was calculated from the data of Bower and Robinson [6].

The apparent equilibrium constants κ, K_1 and K_2 were calculated by a nonlinear least-squares analysis of the experimental data of Table 1 using Eq. (3a) or (4a) of Scheme 1. (The actual independent variable was the activity of urea divided by that of water, [U]/[W], see Scheme 1.) The results are summarized in Table 2 together with some literature values. The solubility constants are mostly in good agreement with previously determined values (Table 2). However, for GL and VV there is substantial deviation. This has been attributed to experimental problems in the original solubility measurements of these two compounds [8,9]. No literature values for the apparent equilibrium constants κ, K_1 and K_2 are known as far as we are aware. The lines drawn in Figs. 2a–2f represent the equilibrium model presented in Section 3.

As was pointed out in the previous paper, to calculate the apparent equilibrium constant of the interactions between urea and a given amino acid residue, the thermodynamic activities of urea, water and dipeptide should be known as a function of the concentrations of urea and of the dipeptide [1,11]. The activity of water was calculated from the activity data of urea using the Gibbs–Duhem relation [6]. Because the saturation concentrations of the dipeptides are low they were considered to be ideal solutes with regard to water and urea at low urea concentrations [1].

However for some dipeptides, and at high urea concentrations, it is clear from Fig. 2 that the experimental solubilities are smaller than the predicted solubilities calculated using the apparent equilibrium constants of Table 2 and the solubility data at low urea concentrations. This is most apparent for cyclo(L-alanylglycine) which has the highest solubility of the dipeptides studied here and whose solubility deviates already at relatively low urea concentrations. The reason for the devi-

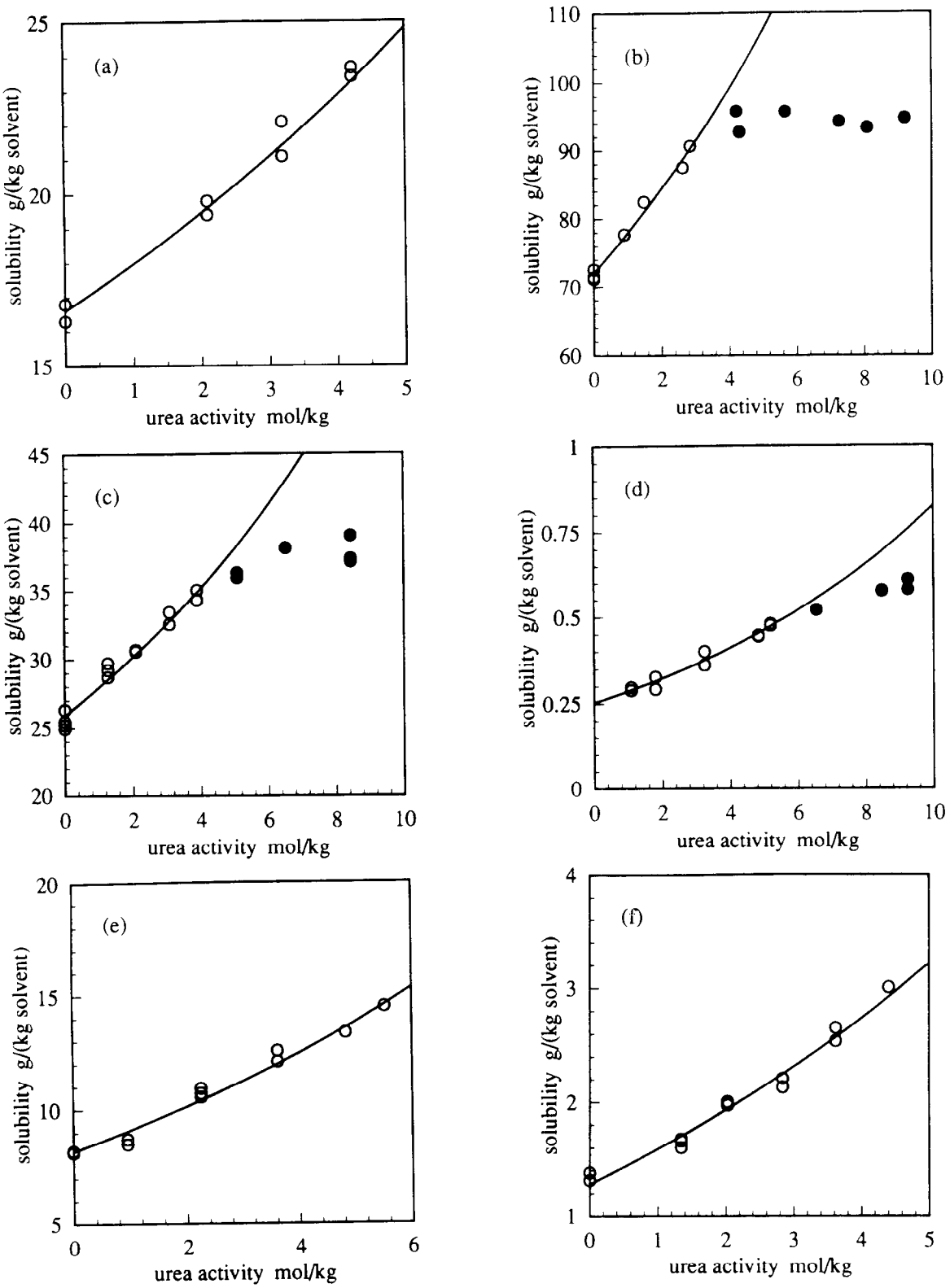

Fig. 2. The solubilities of cyclic dipeptides as a function of the activity of urea: (a) cyclo(glycylglycine), (b) cyclo(L-alanylglycine), (c) cyclo(L-alanyl-L-alanine), (d) cyclo(L-valyl-L-valine), (e) cyclo(glycyl-L-leucine) and (f) cyclo(glycyl-L-phenylalanine). The open circles (○) denote the data that were included in fitting the data, the closed symbols (●) denote data that were excluded from the analysis (see text). The lines were calculated using the apparent equilibrium constants determined from the analysis of the data in terms of Eqs. (3a) or (4a).

Table 2
Solubility constants K_s and apparent equilibrium constants κ, K_1 and K_2 of cyclic dipeptides

Peptide	K_s (g (kg solvent)$^{-1}$)	κ (kg mol^{-1})	K_1 [a] (kg mol^{-1})	K_2 (kg mol^{-1})
GG	16.61 ± 0.07 [b] 16.6 ± 0.2 (16.6) [c]	0.0398 ± 0.0007 [b] 0.0411 ± 0.0019		
AG	72.0 ± 0.5 (71.6) [d]		0.0398	0.0395 ± 0.0044
AA	25.9 ± 0.3 (26.6) [d]	0.0380 ± 0.0021		
VV	0.253 ± 0.005 (0.420) [d]	0.0631 ± 0.0033		
GL	8.19 ± 0.15 (10.4) [d]		0.0398	0.069 ± 0.006
GF	1.28 ± 0.04 (1.58) [e]		0.0398	0.189 ± 0.015

[a] K_1 was fixed to the value of κ of diketopiperazine (see text). [b] Determined with the centrifuge method [1]. [c] Refs. [2,9]. [d] Ref. [8]. [e] Calculated from $\Delta_s^{\infty}G_m^0 = 22$ kJ mol^{-1} [9,10].

ation is probably caused by the non-ideal behavior of the dipeptides regarding the urea and water activities at high urea concentrations. Consequently, the model cannot be used in this region and these points were left out of the analysis. (In Table 1 these data are printed in italics.) Unlike in the case of diketopiperazine [3], no proof was found for the formation of cocrystals with urea.

The results of our solubility study show that urea's interaction with a peptide group is strongly influenced by the alkyl group next to it. In the series of residues A–V–L–F the apparent equilibrium constant κ or K_2 increases from 0.0388 kg mol^{-1} (A) (which is the average value of the alanine residues of AG and AA) to 0.189 kg mol^{-1} (F). The exception to this pattern is the change when going from G (0.0398 kg mol^{-1}) to A (0.0388 kg mol^{-1}). This immediately shows that a simple additivity scheme does not apply for this limited set of model compounds.

Table 3
Values of k and K_2 for the various peptide groups and their corresponding Gibbs energies of transfer from water to 8 molar urea, $\Delta_{tr}G_m^0$, calculated from Eq. (1)

Peptide group	κ or K_2 (kg mol^{-1})	$\Delta_{tr}G_m^0$ (J mol^{-1})
G	0.0398	−327
A	0.0388 [a]	−311
V	0.0631	−682
L	0.069	−764
F	0.189	−2028

[a] The average value for the residue of alanine from cyclo(L-alanylglycine) and cyclo(L-alanyl-L-alanine).

5. Discussion

The method to measure the solubilities of amino acids or amino acid derivatives in aqueous urea solutions to determine the interactions occurring between these solutes and urea has been used before [12–14]. However, in these studies the influence of the activity of urea and that of water has not been taken into account. Also, the data have been analyzed in terms of Gibbs energies of transfer of these model compounds without the use of a specific molecular model.

As was pointed out by Schellman [4], the Gibbs energy of transfer from water to 8 molar urea for

each amino acid residu can be calculated from the apparent equilibrium constants using

$$\Delta_{tr}G^{o}_{m} = -RT \ln([W] + K[U]), \quad (1)$$

where [W] is the activity of water at 8 molar urea ([W] = 0.841), [U] is the corresponding activity of urea ([U] = 7.54 mol kg^{-1}) and K is κ or K_2 from Table 2. The values of $\Delta_{tr}G^{o}_{m}$ for each residue are collected in Table 3.

It will be useful to see how the values of $\Delta_{tr}G^{o}_{m}$ can be used to predict the Gibbs energy of denaturation of proteins in aqueous urea. In a denaturation process, each amino acid residue that is buried or partially buried in the folded protein will become exposed to the solvent, which will contribute to the overall Gibbs energy of denaturation or unfolding. Therefore, the values of $\Delta_{tr}G^{o}_{m}$ of individual amino acid residues can in principle be used to predict the overall Gibbs energy of denaturation of a given protein when the amount and type of amino acid residues is known. Of course here we have only a limited set of data and therefore we can only give a rough estimate. Also the data were obtained from model compounds with cis-peptide groups, whereas in proteins they generally have the trans-conformation.

For several proteins, the Gibbs energies of denaturation are known as a function of the urea concentration [15,16] and this enables the calculation of the apparent Gibbs energies of denaturation in water ($\Delta_{D}G^{o}_{m,\phi}$ [water]) and that in, for instance, 8 molar urea ($\Delta_{D}G^{o}_{m,\phi}$ [8 M urea]). The difference of these two terms,

$$\Delta\Delta_{D}G^{o}_{m,\phi} = \Delta_{D}G^{o}_{m,\phi}[8\text{ M urea}] - \Delta_{D}G^{o}_{m,\phi}[\text{water}], \quad (2)$$

can be calculated for some proteins and varies from -29 to -39 kJ mol^{-1} for α-lactalbumin to -69 to -115 kJ mol^{-1} for α-chymotrypsin [15–17]. $\Delta\Delta_{D}G^{o}_{m,\phi}$ roughly depends on the number of amino acid residues in the protein. This parameter reflects the influence of urea (here at 8 molar urea) on the denaturation or unfolding process of a given protein in terms of the Gibbs energy.

The value of $\Delta\Delta_{D}G^{o}_{m,\phi}$ can in principle be estimated from the sum of the contributions of the Gibbs energies of transfer of all buried or partially buried residues from pure water to 8 molar urea yielding the Gibbs energy change of the hypothetical process of transferring a protein in its denatured state in pure water to that in 8 molar urea. As was mentioned above, there is not yet enough information for an exact estimate of $\Delta\Delta_{D}G^{o}_{m,\phi}$ for a given protein, because there are $\Delta_{tr}G^{o}_{m}$ values of only five (apolar) amino acid residues. Nevertheless, as an approximation, taking their average value from Table 3, $\Delta_{tr}G^{o}_{m}$(av) $= -820$ J (mol residue)$^{-1}$, and considering that ≈ 70 percent of the residues of a protein is buried [18], the calculated value of $\Delta\Delta_{D}G^{o}_{m,\phi}$ for a protein of 150 residues becomes -86 kJ mol^{-1}, which is well within the range of experimental values of -29 to -115 kJ mol^{-1} (see above) [15–17].

In conclusion, even in the absence of a definitive model for the interactions of urea molecules with peptide and alkyl groups, the analysis presented here allows for a reasonable estimate of $\Delta\Delta_{D}G^{o}_{m,\phi}$ for a given protein. Our tentative conclusion is that the sum of the Gibbs energies of interaction of urea with the polar and apolar groups of amino acid residues accounts for the destabilization of globular proteins in concentrated urea solutions. Further experiments, in combination with more advanced models, must be developed to yield better insight into aspects of stability and denaturation of proteins in aqueous systems.

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