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Urea–diketopiperazine interactions: A model for urea induced denaturation of proteins

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Abstract

The solubility of diketopiperazine (DKP) in aqueous urea (U) solutions with molalities ranging from 0 to 16 mol kg^{-1} (corresponding to urea activities ranging from 0 to 10 mol kg^{-1}) has been measured as a function of the urea activity at 298.15 K. In accordance with a previous study the solubility of diketopiperazine increases with increasing urea activity but drops sharply at a urea activity of 5.7 ± 0.2 mol kg^{-1}. This drop in solubility can be attributed to the formation of a DKP · U_2 cocrystal. The solubility data were fitted to a simple model based on the stoichiometry of the DKP · U_2 to yield an intrinsic equilibrium constant κ describing the interactions occurring between a urea molecule and a peptide group of diketopiperazine in aqueous solution, its value being $\kappa = 0.0447 \pm 0.0007$ kg mol^{-1}. When the activity of water is taken into account, κ has a lower value of 0.0398 ± 0.0007 kg mol^{-1}.

Keywords: Diketopiperazine; Aqueous urea solution; Solubility; Equilibrium constant; Protein denaturation

1. Introduction

Though the urea induced denaturation of proteins is yet to be fully understood, it is generally believed that one of two mechanisms is responsible for the ability of urea to destabilize the native state of globular proteins. In the first model urea interacts with the backbone peptide groups through the formation of hydrogen bonds, whereas in the second model aqueous urea dissolves the apolar sidechains buried in the interior of a native globular protein by decreasing the hydrophobic effect. A wide variety of model systems have been studied in establishing the importance of each of these mechanisms [1–7].

Solid diketopiperazine dissolution studies have been used to gain insight into interactions occurring between peptide groups inside a protein [8]. Likewise, here we present data on the solubility of diketopiperazine in aqueous urea solutions to determine the role of urea interacting with peptide groups. These studies allow us to understand the interactions that occur between urea and the peptide groups of a protein in its denatured state, and thus further delineate the two mechanisms by which urea denatures proteins.

In a previous study on the solubility of diketopiperazine in aqueous urea, it was shown that diketopiperazine, at urea concentrations above

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ca. 6 mol dm^{-3}, forms a cocrystal with urea, its stoichiometry being one diketopiperazine and two ureas [9]. Only recently has the crystal structure of DKP · U_2 been resolved (see preceding paper) [10]. We have extended the solubility data of diketopiperazine in aqueous urea to concentrations well above 6 mol dm^{-3} urea. In addition we have fitted the solubility data to a simple equilibrium model, based on the stoichiometry of the DKP · U_2 cocrystal.

The results of this model compound study and the crystallographic data are discussed in terms of hydrogen bonding interactions between urea and diketopiperazine in aqueous solution as well as in the crystalline state, and in terms of their importance for understanding urea induced denaturation of proteins.

2. Experimental

Urea was recrystallized from a water–ethanol mixture. Diketopiperazine (Bachem) was recrystallized from water. The solubility of diketopiperazine was determined as follows. Saturated diketopiperazine solutions in 2 cm^3 screw-capped test tubes were submerged in a constant temperature bath, held at 25.00 ± 0.01 °C, for at least 48 hours. The solution phase was separated from the crystal phase by centrifuging at 25.0 ± 0.1 °C (Beckman, 50 000 rpm, 1 h). An aliquot of ca. one gram of the solution was withdrawn with a syringe and diluted with water in a 100 cm^3 measuring flask. The concentration of the diketopiperazine of the diluted solution was measured using HPLC (Rabbit HP-Rainin HPX, column: Alltech

Table 1

Solubility of diketopiperazine as a function of the activity of urea at 298.15 K

Activity [U] (mol kg^{-1})	Solubility [DKP] (g (kg solvent)$^{-1}$)	Activity [U] (mol kg^{-1})	Solubility [DKP] (g (kg solvent)$^{-1}$)
(*Low urea concentration*)		(*High urea concentration*) [a]	
0	16.28	6.071	24.08
0	16.76	6.140	24.46
0	16.67	6.538	20.13
1.224	18.16	6.539	21.40
1.224	18.18	6.542	21.15
1.714	19.18	6.672	19.36
1.714	19.22	6.735	21.18
2.394	20.16	6.812	20.47
2.394	20.27	7.482	17.29
3.340	21.81	7.567	16.31
3.340	21.71	7.589	16.18
4.685	24.20	7.909	13.77
4.685	24.34	8.108	13.18
4.691	24.03	8.624	12.81
4.691	24.05	8.694	12.91
		9.880	9.71
		9.937	10.12
	(*Intermediate urea concentration*) [b]		
5.520	25.22	5.709	25.48
5.559	26.38	5.896	25.64
5.626	25.02	5.896	25.12

[a] The activity of urea at high urea concentrations was corrected for the amount of urea incorporated in the DKP · U_2 crystal.
[b] These data were not used in the fit.

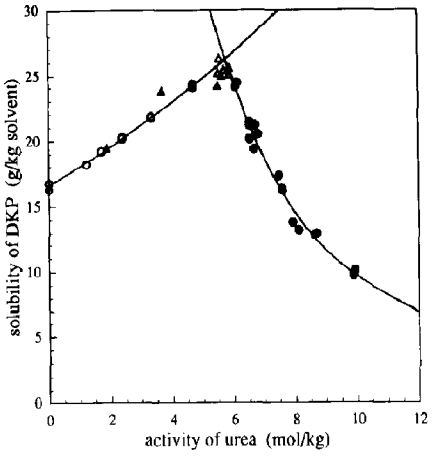

Fig. 1. The solubility of diketopiperazine as a function of the activity of urea: (○) at low urea activity, (●) at high urea activity, (△) at intermediate urea activity, and (▲) data from Ref. [9]. The lines represent the data calculated using the equilibrium constants determined without including the activity of water.

HPLC, Econosphere C18 5U, 250 mm × 4.6 mm) connected to a UV detector (Tracor 970A, monitored at 215 nm). This technique was used in order to separate urea from diketopiperazine and quantify peak areas. (Prior to the determination of the concentrations of a series of diketopiperazine solutions a calibration line was made using solutions of known diketopiperazine concentrations.)

3. Results

The experimental data, collected in Table 1, and plotted in Fig. 1, show the solubility of diketopiperazine as a function of the activity of urea. The activity of urea on a molality scale basis was calculated using the data of Bower and Robinson [11]. The lines drawn in Fig. 1 represent the two models presented below. The phase transition between the two forms of crystalline diketopiperazine appears at a urea activity of 5.7 ± 0.2 mol kg^{-1}.

Ideally, in order to calculate the true equilibrium constants of the equilibria in Schemes 1a and 1b the activities of both urea and diketopiperazine should be known as a function of both urea and diketopiperazine concentrations [12]. However, since the saturation concentration of diketopiperazine in water is relatively low at 0.145 mol kg^{-1}, we have assumed its activity to be equal to its concentration. (The activity coefficient of an analogous, linear dipeptide *N*-acetylglycine methylamide, at molalities between 0 and 1 mol kg^{-1}, is 1.00 ± 0.02 [13].) Also we have assumed that diketopiperazine does not have a significant effect on the activity of urea. Due to a lack of appropriate data on diketopiperazine, this was validated by calculating the activity of urea at a molality of glycylglycine equal to the maximum saturation concentration of diketopiperazine at a given urea molality [14]. For instance, at a urea molality of 7.5 mol kg^{-1}, the activity of urea changes by 1% when the molality of glycylglycine is 0.25 mol kg^{-1}; at a urea molality of 16 mol kg^{-1}, the molality of glycylglycine being 0.1 mol kg^{-1}, the change in urea activity is 10%. (We assumed that diketopiperazine being a neutral molecule should affect the activity of urea to a lesser extent than glycylglycine which exists as a zwitterion in solution.)

4. Association / dissociation model

The stoichiometry of the solution equilibria between diketopiperazine and urea reflects the stoichiometry of the $DKP \cdot U_2$ cocrystal. Diketopiperazine, in a high molarity aqueous urea solution, when in the transition from solution to solid $DKP \cdot U_2$ exists presumably in a form where it is to some extent bound to urea molecules.

In Scheme 1a the diketopiperazine begins in its pure crystalline state (DKP_{cr}). The first step is the dissolution of diketopiperazine into aqueous solution (DKP_{aq}), characterized by a solubility constant K_s. The aqueous diketopiperazine then successively interacts with one urea ($(DKP \cdot U)_{aq}$) and a second urea ($(DKP \cdot U_2)_{aq}$), characterized by association constants K_1 and K_2, respectively. As the concentration of diketopiperazine is relatively low we assumed only diketopiperazine monomers to be present in the solution. (The amount of dimer is $< 1\%$ at 0.15 mol dm^{-3} [15].) If we assume independent binding sites, an in-

Scheme 1a

The solubility of diketopiperazine in aqueous urea solutions as a function of the urea activity, at low urea concentrations (from 0 to 6 mol dm^{-3})

$$DKP_{cr} \overset{K_s}{\rightleftharpoons} DKP_{aq}$$

$$DKP_{aq} + U \overset{K_1}{\rightleftharpoons} (DKP \cdot U)_{aq}$$

$$(DKP \cdot U)_{aq} + U \overset{K_2}{\rightleftharpoons} (DKP \cdot U_2)_{aq} \quad (1a)$$

The total amount of diketopiperazine in solution is

$$DKP_{T,aq} = DKP_{aq} + (DKP \cdot U)_{aq} + (DKP \cdot U_2)_{aq} \quad (2a)$$

From the equilibrium conditions of eq. (1a) it follows that ($K_1 = 2\kappa$ and $K_2 = \frac{1}{2}\kappa$, see text)

$$DKP_{aq} = K_s \quad (3a)$$

$$(DKP \cdot U)_{aq} = 2\kappa [U] DKP_{aq} = 2 K_s \kappa [U] \quad (4a)$$

$$(DKP \cdot U_2)_{aq} = \tfrac{1}{2}\kappa [U] (DKP \cdot U)_{aq} = K_s \kappa^2 [U]^2 \quad (5a)$$

Finally, combination of eqs. (2a)–(5a) yields

$$DKP_{T,aq} = K_s \left[1 + 2\kappa [U] + \kappa^2 [U]^2\right] \quad (6b)$$

Scheme 1b

The solubility of $DKP \cdot U_2$ in aqueous urea solutions as a function of the urea activity, at high urea concentration (from 6 to 10 mol dm^{-3})

$$(DKP \cdot U_2)_{cr} \overset{K_s'}{\rightleftharpoons} (DKP \cdot U_2)_{aq}$$

$$(DKP \cdot U_2)_{aq} \overset{K_1'}{\rightleftharpoons} (DKP \cdot U)_{aq} + U$$

$$(DKP \cdot U)_{aq} \overset{K_2'}{\rightleftharpoons} DKP_{aq} + U \quad (1b)$$

The total amount of diketopiperazine in solution is

$$DKP_{T,aq} = (DKP \cdot U_2)_{aq} + (DKP \cdot U)_{aq} + DKP_{aq} \quad (2b)$$

Making use of the equilibrium conditions in eq. (1b) gives ($K_1' = 2\kappa'$ and $K_2' = \frac{1}{2}\kappa'$, see text)

$$(DKP \cdot U_2)_{aq} = K_s' \quad (3b)$$

$$(DKP \cdot U)_{aq} = 2\kappa' [U]^{-1} (DKP \cdot U_2)_{aq} = 2 K_s' \kappa' [U]^{-1} \quad (4b)$$

$$DKP_{aq} = \tfrac{1}{2}\kappa' [U]^{-1} (DKP \cdot U)_{aq} = K_s' \kappa'^2 [U]^{-2} \quad (5b)$$

Combining eqs. (2b)–(5b) finally yields

$$DKP_{T,aq} = K_s' \left[1 + 2\kappa' [U]^{-1} + (\kappa')^2 [U]^{-1}\right] \quad (6b)$$

trinsic binding constant κ can be introduced that is related to K_1 by $K_1 = 2\kappa$, and to K_2 by $K_2 = \frac{1}{2}\kappa$ [16].

In Scheme 1b the diketopiperazine begins in its cocrystalline form $(DKP \cdot U_2)_{cr}$. The dissolution process involves the equilibrium between the crystalline $(DKP \cdot U_2)_{cr}$ and the aqueous $(DKP \cdot U_2)_{aq}$ characterized by a solubility constant K_s'. When in solution, $(DKP \cdot U_2)_{aq}$ dissociates into $(DKP \cdot U)_{aq}$ and DKP_{aq}, characterized by dissociation constants K_1' and K_2', respectively. Again, introducing an intrinsic binding constant κ' gives $K_1' = 2\kappa'$ and $K_2' = \frac{1}{2}\kappa'$ [16].

The equilibrium constants K_s, κ and K_s', κ' were calculated by a nonlinear least squares anal-

Table 2

Literature values of solubility constants (K_s) of DKP in water and of equilibrium constants (κ) pertaining to urea-peptide interactions in aqueous solution (all at 298.15 K)

Constant		Type of interaction	Reference
K_s (g (kg solvent)$^{-1}$)	16.62 ± 0.06		This work
	16.6		[9]
	16.6		[18]
κ (kg mol^{-1})	0.0447 ± 0.0007 [a]	Urea–peptide	This work
	0.0398 ± 0.0007 [b]	Urea–peptide	This work
	0.035 ± 0.06 [c]	Urea–peptide	This work
	0.038	Urea–peptide	[19]
	0.061 ± 0.010	Urea–polar group	[21]
	0.041	Urea–urea	[1]
	0.052	Urea–urea	[20]

[a] Without activity of water.

[b] With activity of water.

[c] From solubility data at high urea concentration.

ysis of the experimental data of Table 1 using eqs. (6a) and (6b) (we have not used the data at intermediate urea concentrations because the phase transition is not well defined and it is therefore not clear what points should be included in either set of data):

$K_s = 16.50 \pm 0.07$ g (kg solvent)$^{-1}$ and

$\kappa = 0.0447 \pm 0.0006$ kg mol^{-1};

$K_s' = 0.07 \pm 0.09$ g (kg solvent)$^{-1}$ and

$\kappa' = 104 \pm 68$ mol kg^{-1}.

The reciprocal value of κ' ($1/\kappa' = 0.0096 \pm 0.0062$ kg mol^{-1}) differs significantly from κ and also has a large error. This would be an indication that the model is inappropriate. However, the deviation between κ and $1/\kappa'$ is presumably due to the greater influence of water activity at high urea concentrations where $1/\kappa'$ is determined. We will show that when the activity of water is included in the analysis, i.e. instead of using an association–dissociation model an exchange model [17] is used (see Section 5), the value of $1/\kappa'$ is much closer to the value of κ.

The solubility constant for diketopiperazine (K_s) is in good agreement with previously determined values (see Table 2) [9,18]. In similar studies where equilibrium constants describing the interactions occurring between urea and a peptide group have been determined the activity of water has not been included [1,19,20]. Our conditions for determining κ most closely matches these experiments. Comparison with the literature values in Table 2 shows general good agreement. Moreover, studies of urea induced denaturation of proteins involving application of a simple multiple site interaction model, taking into account interactions between urea and all polar groups of the protein, yield an equilibrium constant that is in agreement with the κ derived from the solubility studies presented here [21].

5. Exchange model

The model presented in Section 4 has an essential shortcoming. One imagines that in solution all DKP species are hydrated to some extent and we may tentatively assume that each of the binding sites of DKP, these being its peptide groups, are occupied by at least one water molecule. Instead of an association–dissociation process, what is more likely to occur is an exchange equilibrium where a urea molecule displaces a water molecule at a given binding site, or, vice versa, a urea molecule is displaced by a water molecule. This has been extensively discussed by Schellman [17]. If one assumes that one urea molecule displaces one water molecule at a binding site, the binding site being a peptide group of DKP, the equilibrium eq. (1a) in Scheme 1a becomes

$$\mathrm{DKP_{cr}} + n\mathrm{W} \overset{K_s}{\rightleftharpoons} (\mathrm{DKP \cdot W_2})_{aq}$$
$$(\mathrm{DKP \cdot W_2})_{aq} + \mathrm{U} \overset{K_1}{\rightleftharpoons} (\mathrm{DKP \cdot (U, W)})_{aq} + \mathrm{W}$$
$$(\mathrm{DKP \cdot (U, W)})_{aq} + \mathrm{U} \overset{K_2}{\rightleftharpoons} (\mathrm{DKP \cdot U_2})_{aq} + \mathrm{W} \qquad (1a')$$

(n is an unknown and comes in the form of the subscripts$_{aq}$) and, for example, the equilibrium constant K_1 becomes

$$K_1 = \frac{(\mathrm{DKP \cdot U})_{aq}[\mathrm{W}]}{\mathrm{DKP_{aq}}[\mathrm{U}]}, \qquad (1)$$

where [W] is the activity of water. The other equilibria can be treated similarly. The activity of water as a function of the molality of urea can be calculated using the Gibbs–Duhem relation and the data of Bower and Robinson [11]. As discussed above, we assume all DKP species to be ideal solutes, i.e. their activity coefficients have a value of one. In eqs. (6a) and (6b) the independent variable now becomes [U]/[W] in place of [U]. As a result, the following equilibrium constants emerge:

$K_s = 16.62 \pm 0.06$ g (kg solvent)$^{-1}$ and

$\kappa = 0.0398 \pm 0.0007$ kg mol^{-1};

$K_s' = 0.9 \pm 0.3$ g (kg solvent)$^{-1}$ and

$\kappa' = 28.8 \pm 5.3$ mol kg^{-1}.

The reciprocal value of κ' becomes $1/\kappa' = 0.035 \pm 0.006$ kg mol^{-1}. For K_s and κ, the results do

not change significantly. However, the value of $1/\kappa'$ is now equivalent to the value of κ within errors indicating that all equilibria in Schemes 1a and 1b are similar with respect to the interaction of urea with the peptide groups of DKP, i.e. all equilibria are governed by one intrinsic equilibrium constant, κ. Moreover, it indicates that the introduction of the activity of water into the analysis of the data is of paramount importance, especially when at high urea concentrations.

6. Discussion

One of the major difficulties in interpreting the interaction of urea with proteins in aqueous solution is the lack of structural information regarding direct interactions as well as an understanding of the influence of urea on the activity of water involved in the hydration of polar and apolar groups. As a result of this we generally do not have an appropriate model for interpreting urea interactions with various model compound systems or proteins. However, in the case of diketopiperazine–urea interactions part of the missing information is available from the cocrystal structure of diketopiperazine with urea, presented in the previous paper [10]. Of particular interest are the hydrogen bonding patterns that are present in DKP · U_2 cocrystal, indicating the importance of one aspect of urea-induced denaturation of proteins, namely the direct interactions of urea with protein peptide groups [10]. This study supports the notion that hydrogen bonds are formed between urea molecules and peptide groups of model compounds and proteins in an aqueous environment.

We are currently unable to separate hydration effects from hydrogen bonding and we have assumed as others have that the major contribution of urea to the solubility of DKP and DKP · U_2 is due to hydrogen bonding between urea and the peptide group. From our studies we have obtained a binding constant of 0.0447 ± 0.0007 kg mol^{-1} for the urea–peptide interaction (without inclusion of the water activity in the analysis of the data), in close agreement with comparable model compound studies [1,19,20] and studies of urea binding to peptide groups (or polar groups) of proteins [21].

The results of all of these studies show that the binding of a urea molecule interacting with a peptide group is intrinsically weak. Nevertheless, solutions of urea at high concentrations are quite sufficient to denature a large number of proteins.

In order to determine the Gibbs energetic contribution of the urea–peptide interaction to protein denaturation we consider the Gibbs energy of transfer of a peptide group from water to 8 M urea. Using our equilibrium constant of 0.0447 kg mol^{-1} one obtains a Gibbs energy of transfer of -720 J mol^{-1}, the Gibbs energy of transfer being defined as

$$\Delta_{tr}G = -RT \ln(1 + \kappa[U]) \quad (2)$$

and [U] = 7.54 mol kg^{-1} is the activity of urea at 8 M urea [11].

However, as pointed out in Section 5, it is more appropriate to consider an exchange equilibrium in place of a binding equilibrium. Equation (2) then becomes [17]

$$\Delta_{tr}G = -RT \ln([W] + \kappa[U]) \quad (3)$$

with [W] the activity of water being 0.841 at 8M urea [11]. Using the corrected value of $\kappa = 0.0398$ kg mol^{-1} (see Section 5) the Gibbs energy of transfer of a peptide group becomes -327 J mol^{-1}. As is obvious, this is a significantly lower value than the previous one of -720 J mol^{-1}.

Now if we use these results in the case of an actual protein, hen eggwhite lysozyme for example, a protein with 129 amino acids, application of the above analysis yields a contribution to the Gibbs energy of protein unfolding, from urea binding to all backbone peptide groups of the protein, of ca. -42 kJ mol^{-1} in 8 M urea.

To compare this with the Gibbs energy of hydration of the hydrophobic groups of lysozyme upon unfolding in 8 M urea we have used the solubility data of hydrocarbons in 7 M urea of Wetlaufer et al. [3]. For lysozyme the contribution of urea to the Gibbs energy of unfolding due to urea's influence on solvation of apolar groups is ca. -60 kJ mol^{-1}.

The sum of both contributions, ca. -102 kJ mol^{-1}, is not very far outside the range of stabil-

ity of most globular proteins of -20 to -80 kJ mol^{-1}. Moreover, the change in the Gibbs energy of denaturation of lysozyme in water relative to that in 8 *M* urea [22–24] appears to be -60 to -70 kJ mol^{-1}. Although our calculated value is too high, it is of the same order of magnitude as the experimental one. We conclude that protein denaturation is brought about through urea's interaction with polar groups as well as through hydration effects of apolar groups.

In summary, the analysis presented here provides a unique approach for determining the structural and thermodynamic relationships observed between urea and peptide groups in aqueous solution presumably through hydrogen bonding. Ultimately to obtain a complete picture of the interaction of urea with peptide groups will require an understanding of the influence of urea on the activity of water involved in hydration of peptide groups and the competition between water and urea for specific solvation sites on peptide groups [17]. A similar requirement is needed for interpreting data of urea's influence on the solubility properties of apolar groups.

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