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Interactions of α-chymotrypsinogen A with alkylureas

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Abstract

Solvation of α-chymotrypsinogen A (α-ctg A) in aqueous urea, methylurea, *N,N'*-dimethylurea and ethylurea was studied by density measurements. From the densities at constant molality and at constant chemical potential the preferential solvation parameters were determined. In urea and methylurea preferential solvation was observed, whereas in *N,N'*-dimethylurea and ethylurea at higher concentration water is preferentially bound. From preferential solvation data Gibbs free energy of transfer of α-ctg A from water to urea and alkylurea solutions were calculated. Since the enthalpies of transfer were determined previously, the entropies of transfer could also be obtained so that a complete thermodynamic description is available. An attempt is made to interpret the values of the thermodynamic quantities in terms of various interactions involved in solvation as well as to calculate the exchange constant by using the model of weak interactions. In solvation of alkylureas the hydrophobic nature of the alkyl groups is clearly reflected.

Keywords: Urea; Alkylureas; Solvation; Gibbs free energy

1. Introduction

In previous publications we reported on studies of binding of some alkylureas (methylurea, *N,N'*-dimethylurea and ethylurea) to β-lactoglobulin (β-lg) [1] and human serum albumin (HSA) [2]. In this article we present the results of studies on the binding of alkylureas to α-chymotrypsinogen A (α-ctg A). Preferential interactions of urea to α-ctg A and β-lg, as it was found before, are stronger than those of alkylureas, although the thermal stability of β-lg [3] and α-ctg A (N. Poklar, unpublished data) decreases with increasing size of the alkyl group. The reason for the different behaviour of urea and alkylureas can be found in the different types of interactions of urea and alkylureas with amino acids on the surface of protein. It is to be noted that by 'binding' we understand all kinds of interactions between solvent, denaturant and protein. The quantity which is thermodynamically unobjectionable and readily determined at dialysis equilibrium by density measurements is preferential binding or solvation that is the measure of an excess or deficiency of a solvent component in the domain of the protein molecule. This does not apply to the total binding of water and denaturant for which determination other methods have to be used. Recently, a theoretical analysis of the thermodynamic meaning of preferential solvation in terms of exchange between molecules of water and ligand on the protein surface has been published

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[4–6] and was used here to calculate the exchange constant, K_{ex}. This model for solvation in mixed solvents has been extended to cover the thermal properties, enthalpy, entropy and heat capacity [7].

2. Theory

2.1. Thermodynamic treatment of preferential interactions

According to conventional notation component 1 is water, component 2 is protein and component 3 is the additive. The preferential solvation parameter ξ_3 (the preferential solvation parameter in grams of component 3 bound preferentially to 1 gram of component 2) is obtained from densities at constant chemical potential and constant molality by using the following relation [8–10]

$$\xi_3 = \left(\frac{\partial g_3}{\partial g_2}\right)^0_{T,\mu_1,\mu_3} = \frac{\left(\frac{\partial \rho}{\partial g_2}\right)^0_{T,\mu_1,\mu_3} - \left(\frac{\partial \rho}{\partial g_2}\right)^0_{T,P,m_3}}{\left(\frac{\partial \rho}{\partial g_3}\right)_{T,P,m_2}} \tag{1}$$

where g_3 is the concentration of component i in g/g of principal solvent, water; μ_i is the chemical potential of component i; ρ is the density of the solution in g/ml. The superscript ^{0} indicates infinite dilution of the protein. The two terms in the numerator were obtained from density data at constant chemical potential (μ) and constant molality (m), respectively. The term in the denominator is obtained from densities measured in the absence of protein. The preferential solvation parameter on a molal basis, $(\partial m_3/\partial m_2)^0_{T,\mu 1,\mu 3} = \Gamma_{23}$, i.e. the number of moles of component 3 bound preferentially to one mole of component 2 is obtained from the relation:

$$\Gamma_{23} = \left(\frac{\partial m_3}{\partial m_2}\right)^0_{T,\mu_1,\mu_3} = \left(\frac{M_2}{M_3}\right)\left(\frac{\partial g_3}{\partial g_2}\right)^0_{T,\mu_1,\mu_3} \tag{2}$$

where M_i denotes the molar weight.

There is another procedure for calculating ξ_3, which is quite equivalent to Eq. 1, by applying $(\partial\rho/\partial c_2)^0_{T,\mu 1,\mu 3}$, and the partial specific volumes of component 2 and 3, $\bar{v}_2^0$ and $\bar{v}_3^0$ in g/ml, respectively [11]. Where c_2 is the concentration of protein in g/ml and ρ the density of the solution in g/ml.

$$\left(\frac{\partial \rho}{\partial c_2}\right)^0_{T,\mu_1,\mu_3} = \left(1 - \bar{\bar{v}}_2^0 \rho_{sol}\right) + \xi_3\left(1 - \bar{\bar{v}}_3^0 \rho_{sol}\right)$$

$$= (1 + \xi_3) - \rho_{sol}\left(\bar{\bar{v}}_2^0 + \xi_3 \bar{\bar{v}}_3^0\right) \tag{3}$$

where ρ_{sol} is the density of the solvent (solution of component 3 and 1 without protein) in g/ml.

The isopotential specific volume, $v_2^{\backslash 0}$ (the partial specific volume of component 2 defined at constant temperature, T, and chemical potential of components 1 and 3, μ_1 and μ_3, respectively) is defined at $c_2 \to 0$ [11,12] as follows:

$$v_2^{\backslash 0} = \frac{1}{\rho_{sol}}\left[1 - \left(\frac{\partial \rho}{\partial c_2}\right)^0_{T,\mu_1,\mu_3}\right]. \tag{4}$$

From Eqs. 2, 3 and 4 the following relation is then obtained:

$$\xi_3 = \frac{\rho_{sol}\left(\bar{\bar{v}}_2^0 - v_2^{\backslash 0}\right)}{1 - \rho_{sol}\bar{\bar{v}}_3^0}. \tag{5}$$

The preferential interaction parameter can also be expressed [13–16] as:

$$\left(\frac{\partial m_3}{\partial m_2}\right)^0_{T,P,\mu_3} = -\frac{\left(\frac{\partial \mu_3}{\partial m_2}\right)^0_{T,P,m_3}}{\left(\frac{\partial \mu_3}{\partial m_3}\right)^0_{T,P,m_2}}. \tag{6}$$

With the minor approximation [17]:

$$\left(\frac{\partial m_3}{\partial m_2}\right)^0_{T,P,\mu_3} = \left(\frac{\partial m_3}{\partial m_2}\right)^0_{T,\mu_1,\mu_3}. \tag{7}$$

This parameter is equal to the binding measured experimentally at dialysis equilibrium and by using Euler's theorem

$$\left(\frac{\partial \mu_3}{\partial m_2}\right)^0_{T,P,m_3} = \left(\frac{\partial \mu_2}{\partial m_3}\right)^0_{T,P,m_2} \tag{8}$$

it follows from Eq. 6:

$$\left(\frac{\partial m_3}{\partial m_2}\right)^0_{T,P,\mu_3} = -\frac{\left(\frac{\partial \mu_2}{\partial m_3}\right)^0_{T,P,m_2}}{\left(\frac{\partial \mu_3}{\partial m_3}\right)^0_{T,P,m_2}} = -\left(\frac{\partial \mu_2}{\partial \mu_3}\right)^0_{T,P,m_2} \quad (9)$$

The perturbation of chemical potentials leads to a redistribution of solvent components in the domain of the protein [18–20].

From Eq. 9 and applying that:

$$\left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2} = RT\left(\frac{\partial \ln a_3}{\partial m_3}\right)_{T,P,m_2}$$

and $\Delta G^0_{tr} = n\Delta\mu^0_2$

it follows that

$$\Delta G^0_{tr} = \mu^0_{2(den)} - \mu^0_{2(H_2O)} = -RT\int_{m_3=0}^{m_3}\left(\frac{\partial m_3}{\partial m_2}\right)^0_{T,\mu_1,\mu_3} d\ln a_3 \quad (10)$$

where ΔG^0_{tr} is the Gibbs free energy of transfer of protein from water to aqueous denaturant solutions, a_3 is the activity of the denaturant, the subscripts (H_2O) and (den) refer to the protein in water and in denaturant solution, respectively, and the superscript^0 indicates infinite dilution of protein.

2.2. The solvent exchange model

In the case that the interactions are very weak and the solutions are concentrated and non-ideal as in most cases which include protein denaturation or unfolding, binding must be understood as a competition between the ligand and the principal solvent [6]. Schellman has shown that the preferential interaction per exchangeable site, i, is related to the exchange equilibrium constant, K'_{ex} expressed on mole fraction scale, where x_i is the mole fraction of component i [5]. An overview of Schellman's theory is given in Appendix A.

$$\left(\frac{\partial m_3}{\partial m_2}\right)^{i,0}_{T,P,\mu_3} = \frac{x_3(K'_{ex}-1)}{x_1 + K'_{ex}x_3} \quad (11)$$

This equation is meant to describe all cases of solvent interchange at one exchangeable site. If exchange is not possible or is exceedingly unlikely, then $K'_{ex} = 0$. For non-exchangeable sites the expression: $-x_3/x_1 = -m_3/m_1$ (subscripts 1,2 and 3 indicate principal solvent (water), protein and cosolvent (ligand or denaturant), respectively) is then obtained (see Appendix A). The effect of the solvent on the thermodynamic state of the protein, however, is given by the global preferential interactions over the entire protein molecule. These must include the contributions from non-exchangeable sites as well as from exchangeable ones [20,21]. After multiplying Eq. 11 by the number of sites and dividing them into contributions of exchangeable (n') and non-exchangeable sites (B_1^{Nex}) the following expression is obtained:

$$\left(\frac{\partial m_3}{\partial m_2}\right)^0_{T,P,\mu_3} = n\frac{(K'_{ex}-1)m_3}{m_1 + K'_{ex}m_3} - \frac{m_3}{m_1}B_1^{Nex} \quad (12)$$

where B_1^{Nex} is the number of non exchangeable water molecules found at loci on the protein molecule. The complete relation between binding at the n exchangeable sites and global preferential interactions must therefore, also contain a term for the non exchangeable water [20].

The problem still remains how to determine the exact number of exchangeable and non-exchangeable sites on the surface of the molecule, because the surface of the molecule presented to the solvent depends on conformation and so the nature and number of sites depend on conformation [7]. One possible way to obtain B_1 (absolute hydration; actual amount of water bound to the protein) is from the relation between the preferential binding measured by dialysis equilibrium and the effective total numbers of ligand and water molecules actually found at loci on the protein molecule B_3 (absolute solvation) and B_1, respectively [9,22,23]:

$$\left(\frac{\partial m_3}{\partial m_2}\right)^0_{T,P,\mu} = B_3 - \frac{m_3}{m_1}B_1 \quad (13)$$

It should be noted that, while $(\partial m_3/\partial m_2)^0_{T,\mu_1,\mu_3}$ is a true thermodynamic quantity, B_1 and B_3 are not [20].

Eisenberg has shown recently [24] that hydration of proteins (B_1) does not change extensively upon

denaturation after testing the so called 'invariant particle' model which requires a linear relation between the density increments and the solution density. This leads to a linear relationship between interaction parameter ξ_1 (the preferential hydration parameter in grams of component 1 bound preferentially to 1 gram of component 2) and inverse cosolvent molality, and values for the solvent and cosolvent exclusion parameters. Interaction parameter, ξ_1, is defined as $\xi_1 = -\xi_3/g_3$, where g_3 is the number of grams of component 3 per gram of component 1, and it is in the case of bovine serum albumin (BSA) in the presence of high concentration of NaCl and guanidiniumhydrocloride and in the case of halophilic malate dehydrogenase (hMDH) [25], a linear function of reciprocal cosolvent weight molality g_3^{-1} at high values of g_3. Studies ranging from very low to high cosolvent or denaturant concentrations in a protein unfolding process may require the application of a more complex analysis by using powerful methods such as X-ray, nuclear magnetic resonance and neutron diffraction. Recently, it has been shown by Chalikian [26] that compressibility measurements provide insight into hydration changes in proteins during denaturation processes or ligand binding and might allow in the future an exact determination of the degree of hydration of protein in the presence of different concentrations of added denaturant.

3. Materials and methods

3.1. Materials

α-Chymotrypsinogen A (α-ctg A) (Type II from bovine pancreas) used was a six-times crystallised and lyophilised powder supplied by Sigma Chemical Co. (St. Louis, MO). Before use, it was dissolved in three times distilled water thoroughly dialysed against it and filtered through Millipore filters (type HA, pore size 0.45 μm) and lyophilised. Ultra pure urea was a product of Kemika (Zagreb, Croatia). Methylurea, *N,N'*-dimethylurea and ethylurea were supplied by Fluka (Buch, Switzerland). Before use, alkylureas were recrystallized from hot ethanol and dried in vacuum over P_2O_5 at 50°C for 48 h.

3.2. Methods

Solutions for density measurements were prepared on a precise balance (Sartorius). Dry protein and denaturant were weighted in. Protein concentrations before and after dialysis were determined on a Cary 1 spectrophotometer (Varian) at 280 nm at 25°C. Absorption coefficient values for α-ctg A in aqueous urea and alkylurea solutions were determined with the same spectrophotometer. The values are assembled in Table 1. The densities at 25°C were measured

Table 1
Absorbance coefficients, ε (g of protein/g of solution)$^{-1}$ of α-chymotrypsinogen A in urea and alkylurea solutions at 280 nm at 25°C in a 1-cm cuvet

Denaturant	Conc. (M)	ϵ_{280} [a]	Denaturant	Conc. (M)	ϵ_{280}
Urea			*N,N'*-dimethylurea		
	0	1997		2	2021
	2	2032		4	2075
	3	2095		5	2129
	4	2153		6	2134
	5	2074		7	2150
	6	2034		8	2166
	7	2101			
	8	2117			
Methylurea			Ethylurea		
	2	2036		1	2007
	4	2046		2	2055
	6	2056		3	2082
	8	2138		4	2092
				5	2055

[a] Relative error is estimated to be 1%.

with a DMA 620 precision densitometer (Anton Paar, Graz). The temperature of the cell compartment was controlled to ± 0.02°C with a Heto circulation thermostat. The instrument was calibrated with air and water. For each denaturant solution more than five measurements at different protein concentration were made.

4. Results and discussion

4.1. Preferential solvation

The preferential solvation parameters obtained by using Eq. 1 are given in Table 2. Examination of Table 2 reveals that in urea and methylurea solutions the denaturant is preferentially bound, since all values of the preferential solvation parameter ξ_3 are positive. For urea the values are taken from literature [27]. Further examination of the table reveals that in the ethylurea solutions negative values of ξ_3 appear at concentration above 3 M whereas in *N,N'*-dimethylurea the limit appears at concentration above 6 M, which correlates quite well with the midpoint of the conformational transition of α-ctg A obtained by following the change in position of fluorescence emission maxima [28]. In these solutions water is preferentially bound.

Similar behavior was observed in the previous studies of interactions between alkylureas and β-lg [1] and HSA [2]. In both cases urea and methylurea are preferentially bound in the whole concentration range, whereas in *N,N'*-dimethylurea and ethylurea solutions at higher denaturant concentration water is preferentially bound to β-lg [1] but not to HSA [2]. The preferential hydration means that the solvent in the domain of the protein is enriched in water with respect to the bulk solvent. The reason why *N,N'*-dimethylurea and ethylurea bind to β-lg and α-ctg A in a different way than to HSA could be found in the physico-chemical nature of the surface of protein molecule that is in contact with solvent (protein composition and structure). HSA is the most polar of the proteins examined and has a very hydrophilic surface [2].

Timasheff and co-workers have published a long series of papers reporting preferential interaction for a variety of stabilising and destabilising cosolvents. See Ref. [21] and references therein. Measurements of a number of proteins in 8 M urea (ribonuclease-

Table 2
Preferential solvation parameters of α-chymotrypsinogen A in aqueous alkylurea solutions and urea pH 7.0, at 25°C

Denaturant	Conc. (M)	$(\partial\rho/\partial g_2)^0_{T,\mu 1,\mu 3}$	$(\partial\rho/\partial g_2)^0_{T,P,m3}$	$(\partial\rho/\partial g_3)_{T,P,m2}$	$(\partial g_3/\partial g_2)^0_{T,\mu 1,\mu 3}$ [b]
Methylurea	2	0.2137	0.2099	0.1263	0.03
	4	0.1875	0.1827	0.0955	0.05
	6	0.1209	0.1168	0.0676	0.06
	8	0.0897	0.0865	0.0453	0.07
N,N'-dimethylurea	2	0.2104	0.2077	0.0906	0.03
	4	0.1639	0.1626	0.0542	0.02
	6	0.1166	0.1170	0.0253	−0.02
	8	0.0770	0.0771	0.0074	−0.01
Ethylurea	1	0.2319	0.2308	0.1156	0.01
	2	0.2153	0.2150	0.0815	0.00
	3	0.1974	0.1981	0.0685	−0.01
	4	–	0.1612	0.0537	–
Urea [a]	1	–	–	–	0.00
	2	–	–	–	0.03
	3	–	–	–	0.07
	5	–	–	–	0.08
	7	–	–	–	0.04
	8	–	–	–	0.02

[a] Data are from literature [27].
[b] Relative error is estimated to be about 30%.

Rnase, β-1g, α-ctg A, α-chymotrypsin, lysozyme and others) [29] and in 6 M GuHCl [8,30] show that urea and GuHCl are preferentially bound to all proteins except to Rnase, which gives in 6 M GuHCl $(\partial m_3/\partial m_2)^0_{T,\mu 1,\mu 3}$ value of essentially zero [30]. According to Tanford the observed zero thermodynamic binding of GuHCl at 6 M indicates that at this denaturant concentration the maximum unfolding of protein molecule takes place [30].

4.2. Thermodynamic analysis

The standard Gibbs free energy of transfer of α-ctg A from water to denaturant solution, ΔG^0_{tr}, was determined from the measured preferential solvation data by graphical integration of Eq. 10. The a_3 dependence on concentration needed in these calculation was obtained for urea and alkylurea from the literature [31,32]. The results are given in Table 3 and Fig. 1. Inspection of ΔG^0_{tr} values shows that those corresponding to urea are significantly more negative than those observed in alkylurea solutions. Furthermore, the ΔG^0_{tr} dependence on the denaturant concentration shows in *N,N'*-dimethylurea and ethylurea solutions a characteristic minimum, which corresponds to a maximum favourable interaction with the denaturant. Such a complex behaviour may be attributed to the fact that the measured overall

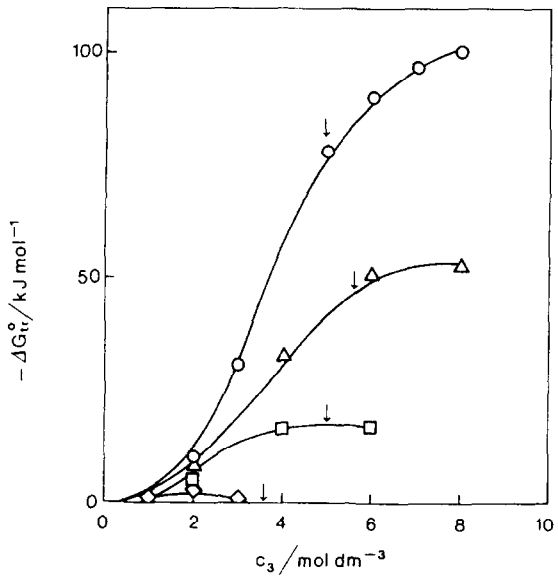

Fig. 1. The Gibbs free energies of transfer of α-chymotrypsinogen A from water to aqueous urea and alkylurea solution, ΔG^0_{tr}, at 25°C. (○) urea, (△) methylurea, (□) *N,N'*-dimethylurea and (◇) ethylurea. Arrows indicate the position of the half transition of α-ctg A in presence of different alkylurea solutions [28].

ΔG^0_{tr} is the sum of various contributions due to the local protein–solvent interaction. One should keep in mind that the surface of protein is highly diverse, consisting of aliphatic, aromatic, ionic, and polar

Table 3
Thermodynamic quantities (in kJ/mol) of transfer of α-chymotrypsinogen A from water to aqueous urea or alkylurea solutions at 25°C

Denaturant	Conc. (M)	ΔG^0_{tr} (kJ/mol)	ΔH^0_{tr} [a] (kJ/mol)	$T\Delta S^0_{tr}$ (kJ/mol)
Urea	1	0 ± 2	−70 ± 5	−70 ± 7
	2	−10 ± 3	−145 ± 10	−135 ± 15
	3	−31 ± 5	−200 ± 15	−169 ± 20
	5	−78 ± 15	−370 ± 20	−292 ± 30
	7	−97 ± 20	−600 ± 40	−503 ± 50
	8	−101 ± 20	−700 ± 50	−599 ± 60
Methylurea	2	−8 ± 2	−45 ± 3	−37 ± 4
	4	−33 ± 6	−125 ± 10	−92 ± 10
	6	−51 ± 10	−230 ± 15	−179 ± 20
	8	−53 ± 10	−350 ± 20	−297 ± 30
N,N-dimethylurea	2	−5 ± 2	80 ± 5	85 ± 10
	4	−17 ± 3	370 ± 30	387 ± 10
	6	−17 ± 3	2040 ± 50	2057 ± 100
Ethylurea	1	−1 ± 1	65 ± 3	66 ± 10
	2	−3 ± 2	280 ± 15	283 ± 30
	3	−1 ± 1	–	–

[a] Data are from literature [39].

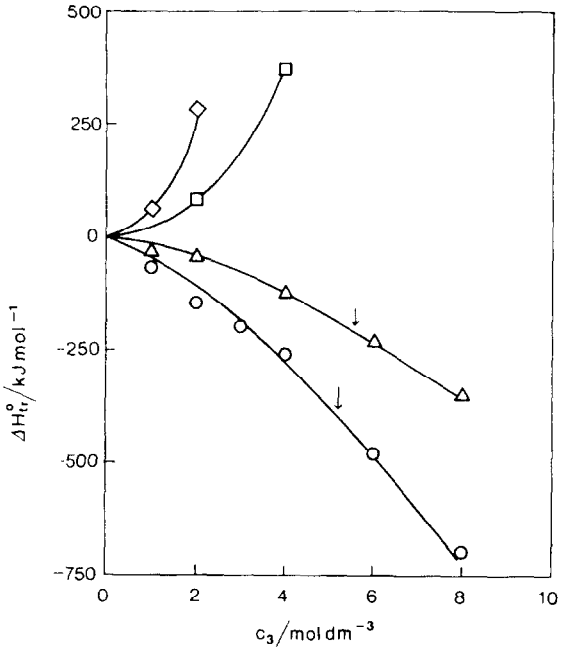

Fig. 2. The enthalpies of transfer of α-chymotrypsinogen A from water to aqueous urea and alkylurea solutions, ΔH^0_{tr}, at 25°C, data are from literature [39]. (○) urea, (△) methylurea, (□) *N,N'*-dimethylurea and (◇) ethylurea. Arrows indicate the position of the half transition of α-ctg A in presence of different alkylurea solutions [28].

groups. Solvent transfer studies indicate that all of these groups except aliphatic interact favourably with reagents like urea but not always with alkylureas [33–38]. Thus the total interaction of protein with a cosolvent should be conceived of as a large number of very small interactions involving essentially every group that makes direct contact with the solvent.

For a complete thermodynamics analysis of protein transfer from water to denaturant solutions, the enthalpy of transfer, ΔH^0_{tr}, and entropy of transfer, ΔS^0_{tr}, are needed. Since the enthalpies have been determined previously [39] we can use these data to calculate the entropies of transfer. All the values of thermodynamic quantities are presented in Table 3. When examining Table 3 we notice that values of the enthalpy of transfer of protein from water into urea solutions, ΔH^0_{tr}, are large and negative and become more negative with increasing concentration of urea (Fig. 2). From the energetic point of view the large exothermic ΔH^0_{tr} values indicate that in urea solutions the protein–solvent interactions are more favourable than in pure water. ΔH^0_{tr} may be also considered as a sum of several contributions accompanying the transfer of protein from water to denaturant solutions. These contributions will reflect in the first place the changes in solvation of aliphatic, aromatic, ionic and polar groups on the protein molecules [33–38]. As shown by model studies the interactions of urea molecules with peptide groups result in large exothermic values while their interactions with CH_2 groups produce enthalpies that are more positive [33,34]. Another contribution to ΔH^0_{tr}, important only when the denaturant concentration is high, is so-called protein stabilisation enthalpy, ΔH^0_D. Its value for α-ctg A determined at 25°C from DSC measurements is 295 kJ/mol (N. Poklar, unpublished data). As can be seen from Table 3 the introduction of ethyl and *N,N'*-dimethyl groups gives rise to hydrophobic interactions with nonpolar groups on the surface of protein molecules. The effect is endothermic and appears to be predominant in the case of *N,N'*-dimethyl and ethylurea.

Let us now consider the values of $T\Delta S^0_{tr}$ (Table 3, Fig. 3) which are negative for urea and methylurea and positive for *N,N'*-dimethyl and ethylurea in the whole concentration range. Large negative $T\Delta S^0_{tr}$ values observed in urea solutions may be ascribed mainly to urea — CONH interactions whose contribution is according to model studies [37] large and negative. The $T\Delta S^0_{tr}$ values observed in other denaturant solutions seem to be consistent with the results of the same model studies [37] which also show that the contributions due to CONH–CH_2 and CH_2–CH_2 interactions are both positive, the first one being smaller than the second one. Another contribution to $T\Delta S^0_{tr}$, important only when the denaturant concentration is high, is protein stabilisation entropy, $T\Delta S^0_D$, and for α-ctg A its value determined at 25°C from DSC measurements is 245 kJ/mol (N. Poklar, unpublished data). To summarise, it seems that in urea and methylurea the main contribution to the $T\Delta S^0_{tr}$ is due to interactions of urea with peptide groups while in *N,N'*-dimethylurea and ethylurea solutions the contributions between *N,N'*-dimethyl and ethyl groups on urea molecules and the nonpolar groups on the protein prevail.

Inspection of Table 3 shows that ΔH^0_{tr} and $T\Delta S^0_{tr}$ values are large in comparison with the values of

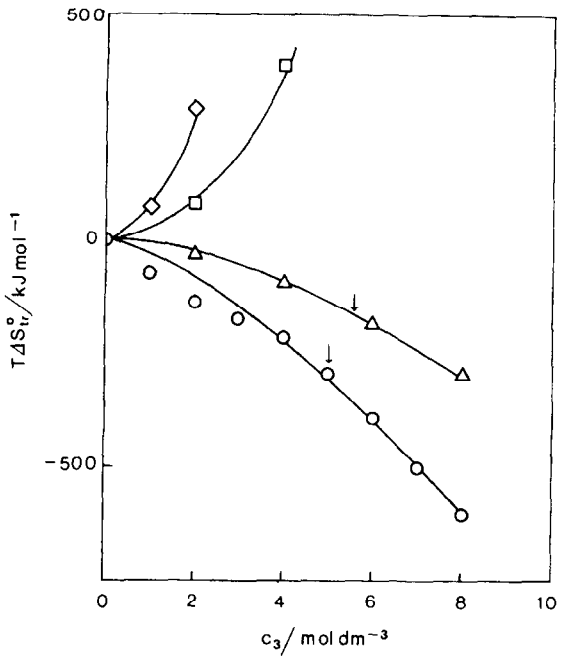

Fig. 3. The entropies of transfer (multiplied by temperature) of α-chymotrypsinogen A from water to aqueous urea and alkylurea solutions, $T\Delta S^0_{tr}$, at 25°C. (○) urea, (△) methylurea, (□) *N,N'*-dimethylurea and (◇) ethylurea. Arrows indicate the position of the half transition of α-ctg A in presence of different alkylurea solutions [28].

ΔG^0_{tr}. Thus this is another case of enthalpy–entropy compensation [40]. Thermodynamic data of transfer obtained for α-ctg A are comparable with those obtained for β-lg and HSA [1,2].

Makhatadze and Privalov have reported the results of an extensive study on the enthalpy of interaction of GuHCl and urea with several proteins in their native and denatured forms [41]. They have pointed out that protein unfolding by urea and GuHCl, which is associated with the increase of the number of binding sites, is a thermodynamically favourable process and is promoted by the increasing concentration of denaturant. They have also concluded that the calorimetrically observed binding cannot be ascribed to electrostatic or hydrophobic interactions, but mainly to hydrogen bonding and that the accompanying large negative entropic effects reflect the immobilisation of a denaturant on the protein surface and reduced flexibility of protein chains caused by bound denaturant molecules [41]. Multiple hydrogen bonding of urea with polypeptide groups has been recently shown by crystallographic studies of diketopiperazine crystals [42,43].

Recently it has been shown on the basis of solubility study of different cyclic dipeptides that urea's interaction with peptide group is strongly influenced by the alkylurea group next to it [44]. As has been mentioned above α-ctg A and β-lg were found to be preferentially hydrated at higher concentrations of *N,N'*-dimethylurea and ethylurea. Preferential exclusion of *N,N'*-dimethylurea and ethylurea from proteins seems to be due principally to the steric exclusion from the protein domain, although favourable interactions with protein surface residues, in particular nonpolar ones, may compete with exclusion as is the case of 2-methyl-2,4-pentadiol (MPD) [45]. The positive perturbation of chemical potentials of the proteins on addition of *N,N'*-dimethylurea and ethylurea at higher concentration indicates that the predominant interactions with *N,N'*-dimethylurea and ethylurea are repulsive in nature and that this repulsion became stronger with an increase in hydrophobic interactions. Such a thermodynamically unfavourable situation can be relieved either by a change in protein structure, i.e., denaturation, or by the formation of interprotein contacts that reduce the surface area of protein exposed to the solvent and hence reduce the interactions. This can be accomplished either by protein self-association or precipitation, i.e., by reduction of protein solubility [45].

Timasheff has classified the preferentially hydrating solvent systems into two categories, those in which the preferential hydration is independent of solution conditions and those in which it is not. The first always stabilises protein structure while the second does not. In the first category the predominant interaction is that of cosolvent-exclusion, determined by solvent properties with the protein being essentially inert. In the second category interactions are determined to a major extent by the chemical nature of the protein surface and cosolvent molecules. This gives rise to a fine balance between exclusion and binding of cosolvents. The binding being dependent on the chemical nature of the surface of the protein in contact with solvent, can be enhanced on protein unfolding due to the exposure of additional hydrophobic sites and peptide bonds as well as to the

decrease in electrostatic free energy of the protein. Being hydrophobic in nature alkylureas interact with nonpolar regions of the protein when these become exposed upon denaturation and, as a consequence, stabilise the denatured form of protein.

In the previous studies of β-lg in the presence of alkylures [1,3] it has been found that the denaturing action of alkylureas increases as follows: methylurea < *N,N'*-dimethylurea < ethylurea. The increasing denaturing action has been shown to be correlated with increasing preferential hydration [1]. Similar behaviour was observed also for α-ctg A ([28], N. Poklar, unpublished data). From these observations as well as from the results obtained with some similar protein systems [46] one can conclude that the protein thermodynamic stability, its conformational changes and preferential binding are closely related. Velicelebi and Sturtevant [47] found that aliphatic alcohols play an important role in determining the thermodynamic stability of lysozyme. The most obvious effect of these alcohols was a significant decrease of the protein transition temperature with increasing alcohol concentration or increasing alkyl chain length. Authors suggested that alcohols destabilise proteins by weakening hydrophobic interactions between nonpolar residues as well as by perturbing the characteristic water structure around protein molecule. Similar results observed in the case of β-lg [3] and α-ctg A (N. Poklar, unpublished data) with alkylureas indicate that the differences in their denaturing ability may be explained in the same way.

4.3. Solvent exchange model

Schellman's exchange model [4–6] based on exchange of water and ligand molecules on protein molecules has been used to obtain the exchange constant for α-ctg A in the presence of 8 M urea. The problem involved in this calculation is the number of binding sites which depends on the concentration of denaturant and conformation of protein [7]. The total number of sites available to denaturant binding and the number of sites occupied by the ligands at given denaturant concentration can be obtained from careful microcalorimetric titration measurements followed by a Scatchard-type analysis as Makhatadze and Privalov have reported [41]. Unfortunately, this data are scarce. Another possibility to obtain the total number of bound molecules of denaturant and water is from known hydration values of protein constituent amino acids. In our calculation we have taken the value of B_1 0.305 g of water bonded to 1 g of α-ctg A in 8 M urea [48] and obtained for B_3 the value of 0.252 (Eq. 13), which means that there are 108 molecules of urea bound to one molecule of α-ctg A. By using Eq. 11 and assuming 108 binding sites, we obtain from a preferentially binding parameter, the practical exchange constant K'_{ex} for α-ctg A in 8 M urea, which is 1.63. In the case that we have preferential binding of component 3 to the protein molecule, $K'_{ex} > 1$; if $K'_{ex} < 1$ component 1 is preferentially bound to the protein and $K'_{ex} = 1$ when there is no preferential binding. From K'_{ex} and from literature values of activity of 8 M urea [31] the activity coefficients f_3 and f_1 and the real exchange equilibrium constant, K_{ex}, were calculated. Timasheff obtained the values for K_{ex} for lysozyme and ribonuclease in 8 M urea on a molal scale 0.036 m^{-1} [21]. Our value for α-ctg A in 8 M urea is 0.044 m^{-1}, which is based on the assumption that the number of binding sites is 108.

As has been mentioned before binding can be measured in two ways. The first involves true thermodynamic techniques e.g. dialysis equilibrium that measures the total preferential binding of the protein with solvent components at all sites on the surface of the protein molecule but gives no information on site occupancy by water and ligand, respectively. The second type of measurement involves non thermodynamic techniques which detect protein–ligand contacts reflected in the perturbation of the property, e.g. the unfolding on uptake of heat in calorimetric titration [41].

In conclusion, we can claim that the studies of preferential solvation of α-ctg A in solutions of urea and alkylureas have contributed to our understanding of this process. First, urea binds preferentially to protein, the extent of binding depends on denaturant concentration. Second, the thermodynamic quantities referring to solvation that have been determined from preferential solvation reflect various interactions accompanying the solvation process. However, for detailed precise studies of type and number of sites in protein solution, techniques such as calorimetric titration will have to be used.

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Appendix A

A short overview of Schellman theory [4–6] is given in this appendix:Suppose one exchangeable site on the surface of a macromolecule P [20,21]:

$$P(H_2O) + L \overset{K_{ex}}{\Leftrightarrow} PL + H_2O \tag{1a}$$

and

$$K_{ex} = \frac{[PL][H_2O]}{[P(H_2O)][L]} = \frac{a_{PL} \cdot a_{H_2O}}{a_{P(H_2O)} \cdot a_L} = \frac{a_{PL} \cdot a_1}{a_{P(H_2O)} \cdot a_3} \tag{2a}$$

where a_{H2O} (a_1) and a_L (a_3) are activity of water and ligand, respectively, K_{ex} is exchange equilibrium constant and is equal $K_{ex} = K_L/K_W$, where K_L and K_W are intrinsic ligand and water binding constants.

Binding polynomial Σ_b (or partition function) represents the sum of the activities of all the different macromolecule species found in the system relative to one reference species, such as the unliganded form [49]. The binding polynomial which is symmetric in components 1 and 3 and for one exchangeable site is:

$$\Sigma_b = \frac{[PL] + [P(H_2O)]}{[P]} = a_1 K_W + a_3 K_L = [a_1 + K_{ex} \cdot a_3] K_W. \tag{3a}$$

The chemical potential of the ligand or cosolvent is most conventionally represented in terms of the thermodynamic activity, a_3:

$$\mu_3 = \mu_3^0 + RT \ln a_3 \tag{4a}$$

and the chemical potential of macromolecule in the presence of the ligand is given by:

$$\mu_2 = \mu_2^0 + RT \ln x_2 + \Delta\mu_2^{int} \tag{5a}$$

where $\Delta\mu_2^{int}$ is the excess free energy of component 2 relative to an ideal solution in pure solvent component 1. Alternatively it is the transfer free energy of a mole of component 2 from an ideal solution in solvent 1 into the solvent mixture of components 1 and 3. $\Delta\mu_2^{int}$ represents interactions between macromolecules (which will be ignored by assuming high dilution) and interactions between component 2 and 3. $\Delta\mu_2^{int} = \Delta\mu_{tr}^0$ and it is given by [50,51]:

$$\Delta\mu_{tr}^0 = -RT \ln\Sigma_b. \tag{6a}$$

Thermodynamics provides a more general definition of binding which includes stoichiometric binding but extends to very small interactions which are too weak or subtle to be considered stoichiometrically. The thermodynamic measure of binding, called selective or preferential interaction is defined by thermodynamic formula mentioned before (Eq. 6) as:

$$\Gamma_{23} = \left(\frac{\partial m_3}{\partial m_2}\right)^0_{T,P,\mu_3} = -\left(\frac{\partial \mu_2}{\partial \mu_3}\right)^0_{T,P,m_2}. \tag{7a}$$

From Eqs. 4a, 5a, 6a and 7a it follows that:

$$\left(\frac{\partial m_3}{\partial m_2}\right)^{i,0}_{T,P,\mu_3} = -\frac{1}{RT}\left(\frac{\partial \Delta\mu_{tr}^0}{\partial \ln a_3}\right)_{T,P,m_2} = \left(\frac{\partial \ln\Sigma_b}{\partial \ln a_3}\right)_{T,P,m_2} = \left(\frac{\partial \ln[K_W(a_1 + K_{ex} \cdot a_3)]}{\partial \ln a_3}\right)_{T,P,m_2}. \tag{8a}$$

The relation between $\Delta\mu_{tr}^0$ and Σ_b is derived in the literature [50]. In the case that sites are equal and independent, every site contributes to the free energy as follow:

$$\Delta\mu_{tr}^0 = -RT\ln\Sigma_b = -RT\ln[K_W(a_1 + K_{ex} \cdot a_3)]. \tag{9a}$$

Activities a_1 and a_3 are not independent of one another, but are related by the Gibbs–Duhem equation at constant T and P:

$$x_1 d\ln a_1 + x_2 d\ln a_2 + x_3 d\ln a_3 = 0 \tag{10a}$$

where x represents mole fraction. In the limit of very small concentration of macromolecule, x_2 $d\ln a_2$ will be negligible so that:

$$\left(\frac{\partial m_3}{\partial m_2}\right)^{i,0}_{T,P,\mu_3} = \frac{1}{a_1 + K_{ex} \cdot a_3}\left(-\frac{x_3}{x_1} \cdot a_1 + K_{ex} \cdot a_3\right). \tag{11a}$$

Activities can be eliminated by introducing activity coefficients on the mole fraction scale $a_i = f_i x_i$ and by introducing the new constant $K'_{ex} = K_{ex} f_3/f_1$, which depends on concentration as well as on T and P. The following expression is then obtained:

$$\left(\frac{\partial m_3}{\partial m_2}\right)^{i,0}_{T,P,\mu_3} = \frac{x_3(K'_{ex} - 1)}{x_1 + K'_{ex} \cdot x_3}. \quad (12a)$$

The relation between concentrations are independent of concentration units. It follows then that $x_3/x_1 = m_3/m_1$, where m_i is the molality of component i:

$$\left(\frac{\partial m_3}{\partial m_2}\right)^{i,0}_{T,P,\mu_3} = \frac{m_3(K'_{ex} - 1)}{m_1 + K'_{ex} \cdot m_3}. \quad (13a)$$

References

[1] N. Poklar and S. Lapanje, Biophys. Chem., 42 (1992) 283.
[2] Z. Kranjc and S. Lapanje, Int. J. Peptide Protein Res., 42 (1993) 320.
[3] N. Poklar, G. Vesnaver and S. Lapanje, Biophys. Chem., 43 (1993) 143.
[4] J.A. Schellman, Biopolymers, 26 (1987) 549.
[5] J.A. Schellman, Biophys. Chem., 37 (1990) 121.
[6] J.A. Schellman, Biophys. Chem., 45 (1993) 273.
[7] J.A. Schellman, Biopolymers, 34 (1994) 1015.
[8] J.C. Lee and S.N. Timasheff, Biochemistry, 13 (1974) 257.
[9] S.N. Timasheff and H. Inoue, J. Am. Chem. Soc., 90 (1968) 1890.
[10] T. Arakawa and S.N. Timasheff, Biochemistry, 23 (1984) 5924.
[11] H. Durschlag, Thermodynamic Data for Biochemistry and Biotechnology, Springer-Verlag, Berlin, 1973.
[12] E.F. Casassa, H. Eisenberg, Adv. Protein Chem., 19 (1964) 285.
[13] K. Gekko and S.N. Timasheff, Biochemistry, 20 (1981) 4667.
[14] V. Vlachy and S. Lapanje, Biopolymers, 17 (1978) 2041.
[15] K. Gekko and S. Koga, Biochim. Biophys. Acta, 786 (1984) 151.
[16] T. Arakawa, R. Bhat and S.N. Timasheff, Biochemistry, 29 (1990) 1914.
[17] D. Stigter, Adv. Protein Chem., 4 (1960) 400.
[18] J.A. Schellman, Biopolymers, 17 (1978) 1305.
[19] H. Eisenberg, Biological Macromolecules and Polyelectrolytes in Solution, Claredon Press, Oxford, 1976.
[20] S.N. Timasheff, Biochemistry, 31 (1992) 9857.
[21] S.N. Timasheff, Annu. Rev. Biophys. Biomol. Struct., 22 (1993) 6717.
[22] H. Inoue and S.N. Timasheff, Biopolymers, 11 (1972) 737.
[23] J.G. Kirkwood and R.J. Goldberg, J. Chem. Phys., 18 (1950) 54.
[24] H. Eisenberg, Biophys. Chem., 53 (1994) 57.
[25] F. Bonnete, G. Ebel, G. Zaccai and H. Eisenberg, J. Chem. Soc. Faraday Trans., 89 (1993) 2659.
[26] T.V. Chalikian, A.P. Sarvazyan and K.J. Breslauer, Biophys. Chem., 51 (1994) 89.
[27] J. Špan and S. Lapanje, Biochim. Biophys. Acta, 295 (1973) 371.
[28] N. Poklar, G. Vesnaver and S. Lapanje, J. Protein Chem., 13 (1994) 323.
[29] V. Prakash, C. Loucheux, S. Scheufele, M.J. Gorbunoff and S.T. Timasheff, Arch. Biochem. Biophys., 210 (1981) 455.
[30] E.P.K. Hade and C. Tanford, J. Am. Chem. Soc., 89 (1967) 5034.
[31] R.H. Stokes, Aust. J. Chem., 20 (1967) 2087.
[32] G. Barone, E. Pizzo and V. Volpe, J. Chem. Eng. Data, 21 (1976) 59.
[33] B.Y. Okamoto, R.H. Wood and P.T. Thomson, J. Chem. Soc., Faraday Trans. 1, 74 (1978) 1990.
[34] G. Barone, G. Gastrunova, P. Del. Vecchio and V. Elia, J. Chem. Soc. Faraday Trans., 1 84 (1988) 1919.
[35] G. Barone, P. Cacace, V. Elio and A. Cesaro, J. Chem. Soc. Faraday Trans. 1, 80 (1984) 2073.
[36] P.J. Cheek and T.H. Lilley, J. Chem. Soc. Faraday Trans. 1, 84 (1989) 1927.
[37] M.C. Chervenda and E.J. Toone, J. Am. Chem. Soc., 116 (1994) 10533.
[38] Y. Nozaki and C. Tanford, J. Biol. Chem., 238 (1963) 4074.
[39] S. Lapanje, M. Simič and A. Pavlič, Croat. Chim. Acta, 54 (1981) 481.
[40] R. Lumry and S. Rayender, Biopolymers, 9 (1970) 1125.
[41] G.I. Makhatadze and P.L. Privalov, J. Mol. Biol., 226 (1992) 491.
[42] M.M. Thayer, R.C. Haltiwanger, V.S. Allured, S.C. Gill and S.J. Gill, Biophys. Chem., 46 (1993) 165.
[43] A.H. Sijpkes, G. van de Kleut and S.C. Gill, Biophys. Chem., 46 (1993) 71.
[44] A.H. Sijpkes, G.J. van de Kleut and S.C. Gill, Biophys. Chem., 52 (1994) 75.
[45] T. Arakawa, R. Bhat and S.N. Timasheff, Biochemistry, 29 (1990) 1924.
[46] J.C. Lee and S.N. Timasheff, J. Biol. Chem., 256 (1981) 7193.
[47] G. Velicelebi and J.M. Sturtewant, Biochemistry, 18 (1979) 1180.
[48] V. Prakash and S.N. Timasheff, Methods Enzymol. 117 (1985) 53.
[49] J. Wayman and S.J. Gill, Binding and Linkage, Functional Chemistry of Biological Macromolecules, University Science Books, Mill Valley, 1990.
[50] J.A. Schellman, Biopolymers, 14 (1975) 999.
[51] J. Hermans and H. Scheraga, J. Am. Chem. Soc., 83 (1961) 3283.