Biophysical Chemistry 24 (1986) 53–59
Elsevier

BPC 01054

INTERACTIONS OF MYOGLOBIN WITH UREA AND SOME ALKYLUREAS

I. SOLVATION IN UREA AND ALKYLUREA SOLUTIONS

Eva ŽEROVNIK and Savo LAPANJE

Department of Chemistry and Chemical Technology, Edvard Kardelj University, Ljubljana, Yugoslavia

Received 22nd July 1985
Revised manuscript received 14th February 1986
Accepted 28th February 1986

Key words: Myoglobin; Urea; Alkylurea; Preferential binding

The interactions of myoglobin with urea, methyl-, *N,N'*-dimethyl- and ethylurea in aqueous solutions were studied by density measurements. From the densities at constant chemical potential and constant molality, the partial specific volumes of myoglobin in these solutions as well as the extent of preferential binding of urea and alkylurea to myoglobin were determined. It has been found that water and not the denaturant is preferentially bound in urea solutions and alkylurea solutions up to 4 M so that the Gibbs free energy of myoglobin, i.e., its chemical potential in a denaturant solution, is larger than in water. This behavior of myoglobin is different from that of other globular proteins for which preferential binding of urea has been found. It appears that preferential hydration of myoglobin is due to its high content of ionic groups.

1. Introduction

In previous publications we reported on the interactions of several globular proteins with urea and alkylureas [1–4]. The methods used were circular dichroism (CD) and calorimetry. It was observed that the denaturing activity of individual alkylureas depends on protein structure. Considering the small number of proteins investigated, no attempt was undertaken to correlate the observed behavior of individual proteins with amino acid composition.

In this and the following paper [5] we report studies of the interactions of myoglobin with urea and methyl-, *N,N'*-dimethyl- and ethylurea.

By comparison with the proteins studied thus far, myoglobin possesses a higher fraction of ordered structure, i.e., about 79% α-helix, whereas the rest are β-turns and unordered [6]. The data refer to the crystalline protein but upon dissolution of the protein in water its structure appears to be by and large preserved. The effects of the alkylureas on myoglobin have already been studied by various methods, e.g., viscometry, spectrophotometry and optical rotation [7,8]. As with other globular proteins, the denaturing action of the alkylureas was found to increase with increasing hydrocarbon content and alkyl substitution of urea. A quantitative thermodynamic treatment of the effects of denaturants and salts on the temperature transition of proteins and polypeptides was extended to the analysis of isothermal denaturation using binding and Setchenow parameters based on the Gibbs free energy of transfer. Qualitative agreement of the experimental and calculated denaturation midpoints was observed [8].

To obtain a better insight into the nature of the interactions between myoglobin and the alkylureas, in addition to the two methods used previously, i.e., CD and calorimetry, in this study density measurements were also performed. From densities at constant chemical potential and constant molality it is possible to determine the extent of preferential binding to the protein of urea and

alkylureas [9–11]. The binding data can subsequently be used for calculation of the Gibbs free energy of transfer of the protein from aqueous solution to individual urea solutions [12]. Since the enthalpies of transfer are obtained by calorimetry directly, the entropies of transfer can also be calculated so that the main thermodynamic data for each transfer are available. From CD spectra conformational changes may be inferred.

This paper presents the results obtained by density measurements. The method was used by several investigators to study the preferential binding of urea to various proteins [13,14]. For all proteins examined thus far, urea has been found to bind preferentially. This behavior is expected considering the fact that urea interacts favorably with peptide groups and polar as well as nonpolar solutes [15–19]. Therefore, it was expected that myoglobin, the presence of the heme group notwithstanding, would behave similarly to the proteins studied previously. These expectations have not been fulfilled: in most urea and alkylurea solutions water binds preferentially which means that the transfer from water to urea solutions is thermodynamically unfavorable, i.e., the Gibbs free energy of transfer is positive. Thus, a new situation has been encountered in which solvation, i.e., changes in interaction, is directly involved, and an explanation for the cause of this different behavior is required.

2. Theory

The following notation is assumed throughout the text: water, the principal solvent, is component 1, the protein is component 2, and the added denaturant component 3. The preferential interaction parameter ξ_3 is obtained from density data at constant chemical potential and at constant molality using the following equation [11,20]

$$\xi_3 = (\partial g_3/\partial g_2)^{\circ}_{T,\mu_1,\mu_3} = \frac{1}{1-\bar{v}_3 c_3} \cdot \frac{(\partial\rho/\partial c_2)_{T,\mu_1,\mu_3} - (\partial\rho/\partial c_2)_{T,P,m_3}}{(\partial\rho/\partial c_3)_{T,P,m_2}} \quad (1)$$

where g_i is the concentration of component i in g/g of principal solvent, water; μ_i the chemical potential of component i; ρ the density of the solution in g/ml; c_i the concentration of component i in g/ml; and $\bar{v}_3$ the partial specific volume of component 3 in ml/g. The superscript ° indicates infinite dilution of the protein. Positive values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ indicate preferential binding of denaturant to the protein; negative values signify preferential hydration. The two terms in the numerator are obtained from density data at constant chemical potential and constant molality, respectively. The protein concentration was determined spectrophotometrically. The term of the denominator, i.e., the density increment of the denaturant, is calculated from densities measured in the absence of protein.

Another equation for the calculation of ξ_3 involves the partial specific volumes determined at constant chemical potential and at constant molality [11,21]

$$\xi_3 = \rho_o(\Phi_2^o - \Phi'^o_2)/(1-\bar{v}_3\rho_o) \quad (2)$$

where ρ_o is the density of the solvent in g/cm^3; Φ_2^o and Φ'^o_2 the partial specific volumes of the protein measured under conditions of constant solvent molality and constant solvent chemical potential., respectively; and $\bar{v}_3$ the partial specific volume of component 3. The values of Φ_2^o and Φ'^o_2 used in the calculation of ξ_3 are obtained by extrapolation to zero protein concentration. The apparent partial specific volume Φ_2 was calculated from the densities of the solvent and protein solution at a given concentration, ρ_o and ρ, by using the standard equation [9]

$$\Phi_2 = \frac{1}{\rho_o}\left(1 - \frac{\rho-\rho_o}{c_2}\right) \quad (3)$$

The use of two different equations based on the same set of density data may appear redundant but is justified for the following reasons. In eq. 1 the density increments are obtained from the dependence of the density upon concentration which is linear for urea solutions, whereas for alkylurea solutions it is nonlinear. This means that the data at different concentrations are equivalent. From eqs. 2 and 3, on the other hand, we can infer that

the solvent density, i.e., a measured quantity, if of crucial importance.

The values of preferential binding of the principal solvent to the protein can be calculated by using the equation

$$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3} = -\frac{g_1}{g_3}\left(\frac{\partial g_3}{\delta g_2}\right)_{T,\mu_1,\mu_3} \quad (4)$$

The preferential interaction parameter on a molal basis $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, i.e., the number of moles of component 3 bound preferentially to 1 mole of component 2, is obtained by using the relation

$$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (M_2/M_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (5)$$

where M_i is the molecular weight of component i.

Then Gibbs free energies of transfer from water to aqueous denaturant solutions, reflecting all interactions, can be calculated by using the following relation [12]

$$\Delta G_{tr} = \mu_2^D - \mu_2^N = -RT\int_{(m_3=0)}^{(m_3)}\left(\frac{\partial m_3}{\partial m_2}\right)\mathrm{d}\ln a_3 \quad (6)$$

where the superscripts N and D refer to the native and denatured state, respectively, and a_3 is the activity of denaturant.

3. Experimental methods

Sperm whale myoglobin (primarily in the ferric form) was a product of Sigma (St. Louis, MO). Before use, it was extensively dialyzed against triply distilled water, and any suspended material was removed by filtration. The stock solutions obtain in this way were used in all measurements; their pH was about 6. Ultrapure urea was purchased from Schwarz-Mann (Orangeburg, NY). The alkylureas used were supplied by Fluka (Buchs, Switzerland) or Aldrich-Europe Division (Beerse, Belgium). Before use, they were recrystallized from hot methanol. Myoglobin concentrations were determined from absorbance measurements at 280 nm by using $E^{1\%w}_{1\,cm} = 18.0$. The value was obtained by the dry-weight method. Extinction coefficients for myoglobin in urea and alkylurea solutions were determined by adding dry denaturant to aqueous solutions of protein, followed by absorbance measurements. The values are assembled in table 1.

Table 1

Extinction coefficients of myoglobin in urea and alkylurea solutions at 280 nm

Denaturant	Concentration (M)	$E^{1\%w}$ (100 g/g cm)	$E^{1\%}$ (dl/g cm)
Urea	0	18.0	18.0
	2	18.2	17.7
	4	19.1	18.0
	6	19.6	18.0
	8	20.0	17.9
	9	18.9	16.7
Methylurea	4	19.0	18.2
	8	18.9	17.3
N,N'-Dimethyl-urea	4	19.7	19.0
	6.5	20.7	19.6
Ethylurea	4	20.2	19.5
	6	21.2	20.1

Protein solutions for density measurements at constant chemical potential were obtained by dialysis against individual solvents until equilibrium was reached, which took about 48 h. Isomolal protein solutions were prepared by adding dry urea or alkylurea to aqueous protein solutions. Protein concentrations were determined by measuring the absorbance at 280 nm using the extinction coefficients given in table 1.

The densities at 25°C were measured with a DMA-02 precision density meter (Anton Paar, Graz). The temperature of the cell compartment was controlled to ±0.10°C with a Heto circulating thermostat. The instrument was calibrated with air and water. Since all solutions must be free of large particles, protein stock solutions were filtered through Millipore filters (type HA, pore size 0.45 nm). For each urea and alkylurea solution more than three measurements at different protein concentration were made. After each series of measurements, the cell was washed and dried and the calibration constant redetermined.

4. Results and discussion

The preferential interaction parameters obtained by using eqs. 1 and 2 are given in tables 2

and 3, respectively. In fig. 1 the apparent specific volumes of myoglobin in urea solutions are plotted vs. protein concentrations.

In fig. 2 the values of the partial specific volumes of myoglobin obtained by extrapolation to zero protein concentration, are given as a function of urea concentration.

Examination of tables 2 and 3 reveals that with few exceptions the preferential interaction parameters ξ_3 in urea and alkylurea solutions are negative which indicates preferential interaction with water. One can also see that the two sets of values of the interaction parameters obtained from either eq. 1 or eq. 2 agree satisfactorily. As mentioned in section 1, for the proteins studied thus far only preferential interaction, i.e., positive values of ξ_3, has been observed in urea solutions.

For an interpretation of this difference in behavior, it is useful to make a detailed comparison between myoglobin and a protein that binds preferentially urea, and for which binding data exist over a wide concentration range. One such protein is β-lactoglobulin. This has been shown to bind urea at all concentrations, the extent of binding increasing with increasing urea concentration [22]. In an attempt to correlate the preferential interaction with the various characteristics related to the amino acid composition of the proteins binding preferentially urea, the average protein hydrophobicity, calculated according to the procedure of Bigelow [23], was shown to be of no significance [14]. On the basis of the estimated total binding of urea, it was concluded that one half of peptide groups and aromatic side chains should be involved. Such an interpretation does not appear to apply to myoglobin: the number of peptide groups is 151 in myoglobin and 162 in β-lactoglobulin. The numbers of aromatic side chains are 23 and 12, respectively, and the average hydrophobicity of myoglobin is not much less than that of β-lactoglobulin which is one of the most hydrophobic proteins. At this point, it is appropriate to emphasize the thermodynamic aspect of the difference in urea binding: $\Delta G_{tr} = \mu_2^U - \mu_2^{H_2O}$ is negative for β-lactoglobulin and positive for myoglobin (cf. eq. 6). We also know that in 6 M urea β-lactoglobulin is largely unfolded and in 8 M urea more or less completely unfolded, i.e., denatured. Myoglobin, on the other hand, as can be inferred from CD spectra (cf. the following paper [5]) is only slightly unfolded in 6 M urea and in 8 M urea still possesses about 20% α-helical structure. In 9 M urea no reproducible values for ξ_3 could be obtained, which is due to a high degree of myoglobin aggregation as observed by light scattering [24]. A detailed thermodynamic analysis of Gibbs free energy, calculated using eq. 6, enthalpy, and entropy values is made in the follow-

Table 2

Preferential interaction parameters of myoglobin in aqueous urea and alkylurea solutions at 25°C

Denaturant	Concentration (M)	$(\partial\rho/\partial c_2)_{T,\mu_1,\mu_3}$	$(\partial\rho/\partial c_2)_{T,P,m_3}$ ($\times 10^4$)	$(\partial\rho/\partial c_3)_{T,P,m_2}$	$(1-\bar{v}_3 c_3)^{-1}$	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$
Urea	2	2.330	2.331	2.576	1.0976	0.00 ± 0.01
	3	2.228	2.247	2.538	1.1545	-0.01 ± 0.02
	4	1.964	2.097	2.501	1.2178	-0.07 ± 0.02
	6	1.747	1.883	2.426	1.3702	-0.08 ± 0.02
	8	1.67	1.7405	2.350	1.5655	-0.05 ± 0.02
Methylurea	4	2.101	2.200	1.629	1.3304	-0.08 ± 0.03
	8	1.942	1.935	1.605	1.919	0.01 ± 0.02
N,N'-Dimethylurea	4	2.165	2.281	1.068	1.461	-0.16 ± 0.04
Ethylurea	4	2.234	2.284	1.033	1.459	-0.07 ± 0.03
	6	2.134	2.088	0.843	1.904	0.10 ± 0.04

ing paper where some other aspects of the urea action are also treated. Since there seems to be no apparent reason for the difference between myoglobin and β-lactoglobulin, it is clear that in the interpretation discussed above not all sites for urea binding have been considered. As is well known, charged groups on the surface of protein molecules, which are largely exposed, account for the major part of bound water [25]. It is also clear that the same groups bind urea molecules, and that ion-dipole interaction is involved [26]. Moreover, the number of water molecules bound to ionic groups is proportional to their number. Examination of the composition of the two proteins reveals that at neutral pH in myoglobin there are about 55 ionic groups per 152 residues and in β-lactoglobulin about 45 per 163 residues. In other words, the charge density in myoglobin is quite larger. Thus, in aqueous solution more water is bound to ionic groups in myoglobin than in β-lactoglobulin. Upon addition of ureas, the water molecules in the solvation shell of ionic groups are gradually displaced by urea molecules, the total amount of water molecules remaining bound to the groups being always larger for myoglobin. In accordance with this, preferential binding of urea first decreases, i.e., becomes more negative, with increasing urea concentration, reaches a minimum at 6 M and then increases.

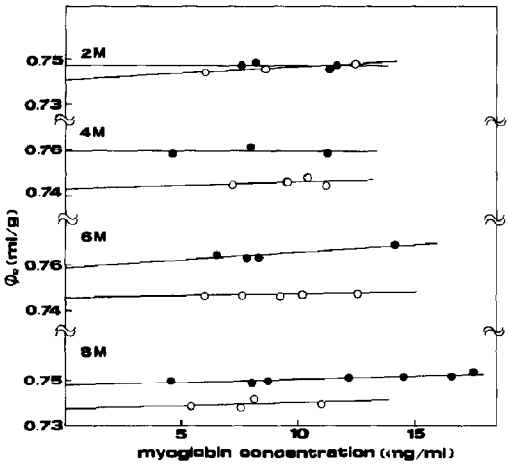

Fig. 1. Concentration dependence of the apparent partial specific volumes of myoglobin in urea solutions; isomolal (○) and isopotential (●) conditions.

In alkylurea solutions a similar mechanism of preferential interaction could be envisaged. However, in two instances, 8 M methylurea and 6 M ethylurea, positive values of binding have been observed. The first is very small (cf. tables 2 and 3) and the error involved is quite large. As for the second, it should be noted that myoglobin in 6 M ethylurea is already highly aggregated [24] so that

Table 3

Partial specific volumes and preferential interaction parameters of myoglobin in urea and alkylurea solutions

Denaturant	Concentration (M)	Φ_2' $c \to 0$ (ml/g)	Φ_2 $c \to 0$ (ml/g)	$(1-\bar{v}_3\rho_0)$	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)
Urea	2	0.747	0.740	0.2349	−0.03±0.02	0.23	−9
	3	0.749	0.744	0.2248	−0.02±0.02	0.10	−6
	4	0.758	0.743	0.2101	−0.07±0.02	0.24	−21
	6	0.759	0.746	0.1852	−0.07±0.02	0.14	−21
	8	0.747	0.739	0.1595	−0.05±0.02	0.07	−15
Methylurea	4	0.755	0.746	0.1231	−0.07±0.02	0.18	−17
	8	0.740	0.740	0.0848	0.01±0.02	−0.01	2
N,N'-Dimethyl-urea	4	0.757	0.746	0.0743	−0.15±0.04	0.27	−30
Ethylurea	4	0.749	0.744	0.0753	−0.07±0.03	0.14	−14
	6	0.746	0.751	0.0551	0.09±0.04	−0.09	18

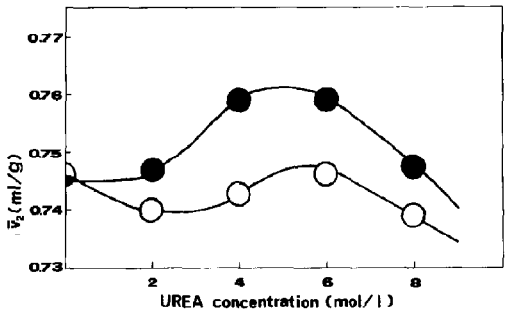

Fig. 2. The partial specific volumes of myoglobin as a function of urea concentration; isomolal (○) and isopotential (●) conditions.

concentration determination may be uncertain even after correction for light scattering. In *N,N'*-dimethylurea, measurements above 4 M were not feasible owing to gelation.

The partial specific volumes of myoglobin at constant chemical potential and constant molality show the usual dependence on urea concentration [27]. The increase is due to the replacement of water in the solvation shell with urea which is accompanied by an increase in volume. When unfolding begins the voids in the proteins become filled with solvent leading to a decrease in volume.

Preferential hydration has also been encountered in solutions of sugars, e.g., glucose, maltose and sucrose, which has been interpreted in terms of surface Gibbs free energy perturbation since the sugars increase the surface tension of water [28]. Considering the fact that the surface tension of urea solutions is only slightly larger than that of water, whereas that of alkylurea solutions is quite smaller, an interpretation based on surface tension changes does not make sense. Studies of diols, on the other hand, revealed that the solvent component interaction strongly depends on the nature of proteins so that preferential hydration or dehydration has been found with individual proteins [29].

In summary, it can be stated that the interaction of urea with myoglobulin reflects its composition. Owing to a high charge density at neutral pH, up to high urea concentrations, water is preferentially bound. This is reflected in increased stability towards urea, i.e., in a higher chemical potential of myoglobin. For the interaction of myoglobin with alkylureas in 4 M solutions which is also characterized by positive hydration the same interpretation is applicable.

Acknowledgement

This study was supported by the Slovene Research Community.

References

1 A. Pavlič and S. Lapanje, Biochim. Biophys. Acta 669 (1981) 60.
2 S. Lapanje, M. Simič and A. Pavlič, Croat. Chem. Acta 54 (1982) 481.
3 S. Lapanje and Z. Kranjc, Biochim. Biophys. Acta 705 (1982) 111.
4 S. Lapanje, N. Bezmalinović and E. Žerovnik, Vestn. Slov. Kem. Drus. 31 (1984) 277.
5 S. Lapanje, E. Žerovnik and Z. Kranjc, Biophys. Chem., submitted for publication.
6 J.C. Kendrew, H.C. Watson, B.E. Standberg, D.C. Philips and J.C. Shore, Nature 190 (1961) 666.
7 T.T. Herskovits and H. Jaillet, Science 163 (1969) 282.
8 T.T. Herskovits, H. Jaillet and B. Gadekbeku, J. Biol. Chem. 245 (1970) 4544.
9 E.F. Casassa and E. Eisenberg, Adv. Protein Chem. 19 (1964) 287.
10 G. Cohen and H. Eisenberg, Biopolymers 6 (1968) 1077.
11 J.C. Lee and S.N. Timasheff, Biochemistry 13 (1974) 257.
12 V. Vlachy and S. Lapanje, Biopolymers 17 (1978) 2041.
13 S. Lapanje, B. Črešnar and V. Vlachy, Vestn. Slov. Kem. Drus. 25 (1978) 257.
14 V. Prakash, C. Loucheux, S. Scheufele, M.J. Gorbunoff and S.N. Timasheff, Arch. Biochem. Biophys. 210 (1981) 455.
15 D.R. Robinson and W.P. Jencks, J. Am. Chem. Soc. 87 (1965) 2462.
16 C. Tanford, Adv. Protein Chem. 24 (1970) 2.
17 M. Roseman and W.P. Jencks, J. Am. Chem. Soc. 97 (1975) 631.
18 S. Lapanje, Physicochemical aspects of protein denaturation, ch. 6 (Wiley-Interscience, New York, 1978).
19 P.K. Nandi and D.R. Robinson, Biochemistry 23 (1984) 6661.
20 S.N. Timasheff and H. Inoue, Biochemistry 7 (1968) 2501.
21 T. Arakawa and S.N. Timasheff, Biochemistry 23 (1984) 5924.
22 J. Špan and S. Lapanje, Biochim. Biophys. Acta 295 (1973) 371.
23 C.C. Bigelow, J. Theor. Biol. 16 (1967) 187.
24 S. Lapanje, T. Takagi and K. Kameyama, paper in preparation

25 H.B. Bull and K. Breese, Arch. Biochem. Biophys. 128 (1968) 488.
26 J. Špan, S. Lenarčič and S. Lapanje, Biochim. Biophys. Acta 359 (1974) 311.
27 S. Lapanje, J. Škerjanc and V. Doleček, Croat. Chem. Acta 43 (1971) 65.
28 T. Arakawa and S.N. Timasheff, Biochemistry 21 (1982) 6536.
29 K. Gekko and S. Koga, Biochim. Biophys. Acta 786 (1984) 151.