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Solvation of β-lactoglobulin in alkylurea solutions

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Abstract

Solvation of β-lactoglobulin in aqueous solutions of urea, methyl-, *N,N'*-dimethyl- and ethylurea was studied by density measurements. From the densities at constant chemical potential and constant molality, the preferential solvation parameters and the partial specific volumes of β-lactoglobulin in these solutions were determined. In urea and methylurea solutions urea is preferentially bound, whereas in *N,N'*-dimethyl- and ethylurea solutions at higher concentration water is preferentially bound. From preferential solvation data and partial specific volumes of protein Gibbs free energies of transfer from water to 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. In solvation of alkylureas the hydrophobic nature of alkyl groups is clearly reflected.

Keywords: β-Lactoglobulin; Urea; Alkylurea; Preferential solvation; Gibbs free energy

1. Introduction

In a previous paper the solvation of β-lactoglobulin in aqueous urea solutions was studied by means of equilibrium dialysis and differential refractometry [1]. It has been found that at all concentrations urea is preferentially bound. Gibbs free energies of transfer of β-lactoglobulin to urea solutions could be obtained from preferential solvation data, whereas the enthalpies of transfer to the same urea solutions were determined by calorimetric measurements [2]. The interactions of β-lactoglobulin with alkylureas, i.e. the transfer of the protein to alkylurea solutions, were studied by means of circular dichroism (CD) and calorimetry [3]. Analysis of the CD spectra revealed differences in conformational changes produced by urea and alkylureas, respectively. Also, the enthalpies of interaction of the alkylureas with β-lactoglobulin are distinctly different from those of area. The latter are all negative, i.e. exothermic, whereas the former, except at low concentrations, are positive, i.e. endothermic. Thus the difference in behaviour clearly reflects the presence of the hydrophobic moiety in alkylurea molecules.

In this publication the solvation of β-lactoglobulin in aqueous methyl-, *N,N'*-dimethyl- and ehtylurea solutions is examined. The results obtained by density measurements are presented. This method was used for studying the solvation of myoglobin in urea and alkylurea solutions [4,5].

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The results were quite unexpected: in urea solutions up to 8 M, water and not the denaturant was preferentially bound, whereas for all proteins examined thus far urea has been found to bind preferentially. Moreover, few data only were available for alkylureas. However, owing to aggregation and gelation of myoglobin at high alkylurea concentrations these data may not be fully correct, so that no ferm conclusions could be reached at. The peculiar behaviour observed, i.e. the preferential hydration of myoglobin, could be due to its high contents of ionic groups.

2. Theory

As usual, the following notation is assumed: water, the principal solvent, is component 1, the protein is component 2, and the added alkylurea is component 3. The preferential solvation parameter ξ_3 is obtained from densities at constant chemical potential and constant molality using the following equation [6,7]

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

where g_i is the concentration of component i in g/g of principal solvent, water; μ_i is the chemical potential of component i; ρ is the density of the solution in g/ml. The superscript $^{\circ}$ indicates infinite dilution of the protein. The two terms in the numerator were obtained from density data at constant chemical potential and constant molality (m), respectively. The term in the denominator is calculated from densities measured in the absence of protein.

There is another equation for calculating ξ_3 that involves the partial specific volumes determined at constant chemical potential and at constant molality [6,8]

$$\xi_3 = \rho_0\left(\Phi_2^0 - \Phi_2'^0\right)/\left(1 - \bar{v}_3\rho_0\right) \quad (2)$$

where ρ_0 is the density of the solvent in g/cm^3; Φ_2^0 and $\Phi_2'^0$ are the apparent partial specific volumes of the protein at constant molality and constant chemical potential extrapolated to zero protein concentration; and $\bar{v}_3$ the partial specific volume of component 3.

The apparent partial specific volumes Φ_2 were calculated from the densities of the solvent and protein solution at a given concentration, ρ_0 and ρ, by using the equation [9]

$$\Phi_2 = \frac{1}{\rho_0}\left(1 - \frac{\rho - \rho_0}{c_2}\right) \quad (3)$$

where c_2 is the protein concentration in g/ml.

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 from the relation

$$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (\mathrm{M}_2/\mathrm{M}_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (4)$$

where M_i denote the molar weights.

Gibbs free energies of transfer from water to aqueous denaturant solutions can be calculated by using the following relation [10]

$$\Delta G_{\mathrm{tr}} = \mu_2^{\mathrm{D}} - \mu_2^{\mathrm{N}} = -RT\int_{m_3=0}^{m_3}\left(\frac{\partial m_3}{\partial m_2}\right) \mathrm{d}\ln a_3 \quad (5)$$

where the superscripts $^{\mathrm{N}}$ and $^{\mathrm{D}}$ refer to the native and denatured state, respectively, and a_3 is the activity of denaturant.

3. Experimental methods

β-Lactoglobulin used was a three-times-over crystallized and lyophilized powder consisting of a mixture of A and B variants, supplied by Sigma Chemical Co. (St. Louis, MO). It was used without further purification. Ultra pure urea was a product of Kemika (Zagreb, Yugoslavia). The alkylureas used were supplied by Fluka (Buchs, Switzerland). Before used, they were recrystallized from hot methanol.

Protein was dissolved either in water (pH about 5.5) or in 0.1 M glycine/0.1 M HCl (pH 2.0). Its

concentration in water and buffer was determined by using $E_{1cm}^{1\%w} = 9.6$ at 278 nm.

Protein solutions for density measurements at constant chemical potential were obtained by dialysis against individual alkylurea solutions until equilibrium was reached, i.e. 48 h. Protein concentrations were determined from densities.

The densities at 25°C were measured with a DMA 620 precision density meter (Anton Paar, Graz). The temperature of the cell compartment was controlled to ±0.02°C with a Heto circulating thermostat. The instrument was calibrated with air and water. To remove possible dust particles, the solution were filtered through Millipore filters (type HA, pore size 0.45 μm). For each denaturant solution more than three measurements at different protein concentration were made.

4. Results and discussion

The preferential solvation parameters obtained by using eqs. (1) and (2) are given in Tables 1–4. Examination of Tables 1–4 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 they have been actually redetermined. In the first determination, as already mentioned, differential refractormetry was used to ascertain preferential solvation of β-lactoglobulin [2]. The two sets of data for urea agree within the limit of experimental error. Further examination of the tables reveals that in ethylurea solutions negative values of ξ_3 appear at concentrations above 4 *M*, whereas in *N,N'*-dimethylurea solutions the limit appears at concentrations above 2 *M*. In these solutions thus water is preferentially bound. From the tables one can also see that the two sets of values of ξ_3 obtained from either eq. (1) or eq. (2) agree satisfactorily.

From preferential solvation data the Gibbs free energy of transfer, ΔG_{tr}, can be calculated by graphical integration since a_3 as a function of (alkyl)urea concentration is known over a wide concentration range [11]. The results are given in Table 5. Examination of the values of ΔG_{tr} shows that for urea and methylurea solutions they are negative and relatively close. The influence of the methyl group is moderate. The values of ξ_3 based on eq. (2), cf. Tables 3 and 4, are essentially

Table 1

Preferential solvation parameters of β-lactoglobulin in aqueous urea and alkylurea solution, pH 5.5, at 25°C

Denaturant	Concentration (*M*)	$(\partial\rho/\partial g_2)_{T,\mu_1,\mu_3}$ (g/ml)	$(\partial\rho/\partial g_2)_{T,P,m_3}$ (g/ml)	$(\partial\rho/\partial g_3)_{T,P,m_2}$ (g/ml)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g protein)
Urea	0	0.2271	0.2271	–	–
	2	0.2178	0.2052	0.2136	0.06±0.02 [a]
	4	0.1964	0.1693	0.1682	0.16±0.02
	6	0.1611	0.1390	0.1278	0.17±0.03
	8	0.1123	0.0988	0.0948	0.14±0.04
Methylurea	2	0.2021	0.2014	0.1256	0.06±0.02
	4	0.1756	0.1648	0.0953	0.11±0.02
	6	0.1413	0.1319	0.0627	0.15±0.03
	8	0.0923	0.0882	0.0341	0.12±0.04
Ethylurea	2	0.2067	0.2026	0.0819	0.05±0.02
	4	0.1619	0.1601	0.0478	0.04±0.02
	5	0.1285	0.1287	0.0353	−0.01±0.01
	6	0.0947	0.0964	0.0238	−0.07±0.03
N,N'-Dimethylurea	2	0.1891	0.1859	0.0791	0.04±0.02
	4	0.1448	0.1450	0.0470	−0.00±0.01
	5	0.1338	0.1361	0.0386	−0.06±0.03
	6	0.1104	0.1131	0.0250	−0.11±0.03

[a] Error limits indicate the estimated absolute uncertainties.

Table 2

Preferential solvation parameters of β-lactoglobulin in aqeuous urea and alkylurea solutions, pH 2.0, at 25°C

Denaturant	Concentration (M)	$(\partial\rho/\partial g_2)_{T,\mu_1,\mu_3}$ (g/ml)	$(\partial\rho/\partial g_2)_{T,P,m_3}$ (g/ml)	$(\partial\rho/\partial g_3)_{T,P,m_2}$ (g/ml)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g protein)
Urea	0	0.2316	0.2361	–	–
	2	0.2211	0.2148	0.2100	0.03 ± 0.02 [a]
	4	0.1813	0.1696	0.1674	0.07 ± 0.02
	6	0.1734	0.1556	0.1329	0.13 ± 0.03
	8	0.1381	0.1219	0.0943	0.17 ± 0.04
Methylurea	2	0.2173	0.2136	0.1245	0.03 ± 0.02
	4	0.1754	0.1692	0.0883	0.07 ± 0.02
	6	0.1360	0.1303	0.0591	0.10 ± 0.03
	8	0.1115	0.1065	0.0366	0.14 ± 0.04
Ethylurea	2	0.2131	0.2108	0.0765	0.03 ± 0.02
	4	0.1629	0.1632	0.0520	−0.01 ± 0.02
	5	0.1544	0.1557	0.0428	−0.03 ± 0.03
	6	0.1123	0.1143	0.0255	−0.08 ± 0.04
N,N'-Dimethylurea	2	0.2094	0.2080	0.0775	0.02 ± 0.02
	4	0.1574	0.1600	0.0517	−0.05 ± 0.03
	5	0.1360	0.1395	0.0349	−0.10 ± 0.03
	6	0.1242	0.1271	0.0242	−0.12 ± 0.03

[a] Error limits indicate the estimated absolute uncertainties.

identical with those obtained from eq. (1) as they should be. In ethylurea solutions the values of ΔG_{tr} become distinctly smaller, i.e. less negative, whereas in *N,N'*-dimethylurea positive values of ΔG_{tr} are found. Comparison of the two sets of data at pH 5.5 and 2.0, Table 5, reveals that

Table 3

Partial specific volumes and preferential solvation parameters of β-lactoglobulin in aqueous urea and alkylurea solutions, pH 5.5, at 25°C

Denaturant	Concentration (M)	Φ_2' $m_2 \to 0$ (ml/g)	Φ_2 $m_2 \to 0$ (ml/g)	$(1-\bar{v}_3\rho_0)$	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g protein)
Urea	0	0.750	0.750	–	–
	2	0.737	0.751	0.2383	0.05 ± 0.02 [a]
	4	0.717	0.748	0.2114	0.17
	6	0.716	0.745	0.1852	0.18
	8	0.719	0.745	0.1596	0.18
Methylurea	2	0.740	0.749	0.1439	0.06
	4	0.735	0.747	0.1240	0.10
	6	0.730	0.744	0.1023	0.15
	8	0.736	0.744	0.0791	0.11
Ethylurea	2	0.743	0.747	0.0955	0.04
	4	0.743	0.746	0.0763	0.04
	5	0.745	0.745	0.0673	0.00
	6	0.747	0.744	0.0555	−0.05
N,N'-Dimethylurea	2	0.743	0.747	0.0887	0.04
	4	0.745	0.746	0.0752	−0.01
	5	0.750	0.745	0.0647	−0.08
	6	0.752	0.744	0.0569	−0.15

[a] Error limit indicates the estimated absolute uncertainty.

Table 4

Partial specific volumes and preferential solvation parameters of β-lactoglobulin in aqueous urea and alkylurea solutions, pH 2.0, at 25°C

Denaturant	Concentration (M)	Φ_2' $m_1 \to 0$ (ml/g)	Φ_2 $m_2 \to 0$ (ml/g)	$(1-\bar{v}_3\rho_0)$	$(\partial g_3/\partial g_2)_{T,\mu_1,gm_3}$ (g/g protein)
Urea	0	0.754	0.752	–	–
	2	0.746	0.753	0.2341	0.03 ± 0.02 [a]
	4	0.737	0.752	0.2080	0.08
	6	0.721	0.745	0.1850	0.14
	8	0.718	0.744	0.1567	0.19
Methylurea	2	0.746	0.750	0.1402	0.03
	4	0.741	0.749	0.1207	0.07
	6	0.738	0.748	0.0997	0.11
	8	0.735	0.746	0.0791	0.16
Ethylurea	2	0.741	0.744	0.0924	0.03
	4	0.742	0.743	0.0748	0.01
	5	0.745	0.743	0.0648	−0.03
	6	0.746	0.742	0.0545	−0.08
N,N'-Dimethylurea	2	0.742	0.743	0.0869	0.02
	4	0.746	0.742	0.0718	−0.05
	5	0.746	0.740	0.0631	−0.10
	6	0.748	0.739	0.0543	−0.12

[a] Error limit indicates the estimated absolute uncertainty.

generally the (negative) values of ΔG_{tr} are larger for neutral solutions which is in accord with expectation. Around pH 2, owing to hydrogen bonding, the native structure is more stable compared to the structure at pH 5.5 [12].

For a complete thermodynamic analysis of transfer to (alkyl)urea solutions, two other functions, i.e. ΔH and ΔS are needed. Since the enthalpies of transfer have been determined at pH 2 we can use the data to calculate the en-

Table 5

Thermodynamic quantities (in kJ/mol) of transfer of β-lactoglobulin from water to aqueous (alkyl)urea solutions of 25°C.

Denaturant	Concentration (M)	ΔG_{tr} (pH 5.5)	ΔG_{tr} (pH 2.0)	ΔH_{tr} [a] (pH 2.0)	$T\Delta S_{tr}$ (pH 2.0)
Urea	2	−15 ± 5 [b]	−10 ± 5	−130 ± 20	−120
	4	−75 ± 20	−30 ± 20	−220 ± 20	−190
	6	−120 ± 30	−60 ± 30	−455 ± 30	−395
	8	−165 ± 40	−100 ± 40	−680 ± 30	−580
Methylurea	2	−10 ± 5	−5 ± 5	−23 ± 4	−18
	4	−50 ± 10	−25 ± 10	−23 ± 4	2
	6	−80 ± 20	−50 ± 20	20 ± 4	70
	8	−115 ± 30	−80 ± 30	35 ± 4	115
Ethylurea	2	−6 ± 3	−7 ± 3	16 ± 4	23
	4	−20 ± 5	−10 ± 5	133 ± 10	143
	5	−23 ± 8	−9 ± 5	–	–
	6	−20 ± 10	−5 ± 3	176 ± 15	178
N,N'-Dimethylurea	2	−5 ± 3	−5 ± 3	42 ± 5	47
	4	−12 ± 4	−2 ± 3	280 ± 15	282
	5	−8 ± 3	8 ± 4	–	–
	6	3 ± 3	20 ± 10	780 ± 20	760

[a] Data from Ref. [3].

[b] Error limits indicate the estimated absolute uncertainties.

tropies of transfer [3]. All the values of thermodynamic functions are assembled in Table 5.

Before starting with the discussion on thermodynamic data it is appropriate to recall briefly the results of CD studies [3]. In urea solutions below 5 *M* only small changes of the mean residue ellipticity $[\theta]$ are observed. At higher concentrations, however, they become less negative which reflects increasing unfolding. The common feature of all spectra in methylurea solutions is the small change of $[\theta]$, so that in this respect methylurea is a less efficient denaturant than urea. The values of $[\theta]$ of β-lactoglobulin in *N,N'*-dimethylurea solutions become more and more negative with increasing denaturant concentration. By considering the protein structure, the negative shifting of $[\theta]$ can be explained in terms of a rearrangement of part of β-sheet into α-helix [3]. β-Lactoglobulin behaves similarly in ethylurea solution, but the values of $[\theta]$ are less negative. Based on the findings, one can conclude that only in concentrated urea solutions the denatured form is close to a random coil, whereas methylurea is a weaker denaturant. *N,N'*-Dimethylurea and ethylurea bring about larger conformational changes and are thus strong denaturants by definition, although they do not lead to random coils [13].

Returning now to examination of Table 5 one sees that at pH 2.0 the values of ΔH_{tr} and $T\Delta S_{tr}$ for methylurea solutions differ considerably from those for urea solutions, although the values of ΔG_{tr} are relatively close. The values of ΔH_{tr} are negative and rather large. They contain contributions from processes accompanying the transfer. The first is solvation changes involving the ionic groups on the surface of protein molecules. The process is exothermic and the contribution large. The second contribution is due to unfolding and is endothermic. Actually this is the stabilization enthalpy ΔH_D^0 at 25°C, 121 kJ/mol [14]. The third contribution, due to interactions with peptide groups, is exothermic, the values appear to be smaller than those from ionic solvation changes [15]. The overall values of ΔH_{tr} are negative and increase with increasing urea concentration. As one can infer from Table 5, the introduction of a methyl group in the urea molecule produces quite large changes in ΔH_{tr} for the transfer water–methylurea solution. Nonpolar, e.g., $-CH_3$ and $-C_2H_5$, groups give rise to hydrophobic interactions with nonpolar groups on the surface of protein molecules. The effect is endothermic and appears to be predominant at high concentrations since interactions with the ionic groups are still negative but considerably smaller [15]. The overall effect is exothermic up to about 4 *M* and then it changes the sign, i.e., becomes endothermic. In *N,N'*-dimethylurea and ethylurea the values of ΔH_{tr} are possitive at all concentrations and increase with increasing denaturant concentration. The hydrophobic effect is predominant.

The entropy term, $T\Delta S_{tr}$, cf. Table 5, is mainly due to hydrophobic interactions between nonpolar groups in protein molecules and the alkyl gropus of ureas [16]. The effect, compared to ΔG_{tr}, is large, with a positive ΔS. Another smaller contribution to ΔS_{tr} stems from dipole–dipole interactions between urea molecules–peptide groups and also from hydrogen bonding [17]; i.e. ΔS is negative. The overall effect for alkylureas is thus positive ΔS. For urea solutions, on the other hand, the second contribution to $T\Delta S_{tr}$ is predominant, cf. Table 5. The values of $T\Delta S_{tr}$ are rather negative and large, increasing with increasing urea solutions. However, the values of $T\Delta S_{tr}$ for alkylurea solutions—except for low concentrations of methylurea—are positive, the absolute values being much smaller than those for urea. This is typical of hydrophobic interactions [16].

There is another feature that is characteristic of the thermodynamic data in Table 5. The values of ΔH and $T\Delta S$ are generally large compared to the values of ΔG. This appears to be another case of enthalpy–entropy compensation [18]. Moreover, with a few exceptions at high concentrations of *N,N'*-dimethylurea and ethylurea the values of ΔG_{tr} are negative.

The apparent partial specific volumes of β-lactoglobulin, Φ_2^0 and $\Phi_2'^0$, needed for calculation of ξ from eq. (2) are given in Tables 3 and 4 and are plotted in Figs. 1 and 2. The volumes refer to infinite dilution of protein. They were determined at two pH's, 2.0 and 5.5. The two sets of values are relatively close. We will first consider the values of Φ_2^0 and $\Phi_2'^0$ in urea solutions. The former decrease slightly with increasing urea con-

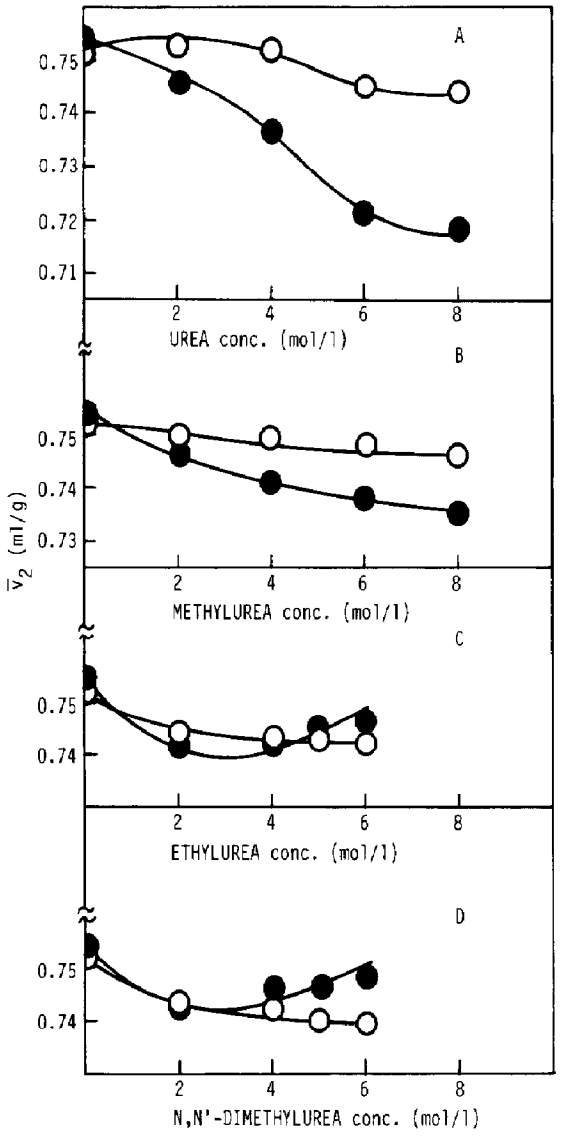

Fig. 1. The partial specific volumes of β-lactoglobulin at infinite dilution as a function of denaturant concentration: (○) isomolal; (●) isopotential. (A) Urea, (B) methylurea, (C) ethylurea, and (D) *N,N'*-dimethylurea; pH 2.0.

centration and they are close to those obtained previously [19]. By and large they reflect unfolding of β-lactoglobulin. The values of $\Phi_2'^0$ are generally more negative than those of Φ_2^0. They reflect unfolding as well as preferential solvation [20]. The behaviour of β-lactoglobulin in methylurea solutions is similar to that in urea. In ethylurea and *N,N'*-dimethylurea the values of Φ_2^0 also slightly decrease with denaturant concentration which apparently reflects partial β-sheet to α-helix transition. The values of $\Phi_2'^0$, on the other hand, pass through a shallow minimum and increase around 4 *M* above the values of Φ_2^0, which may reflect preferential hydration.

Summing up, we may state that the presence of alkyl groups in urea molecules is reflected in solvation processes that accompany the transfer of β-lactoglobulin from water to aqueous solutions of alkylurea. From preferential solvation data Gibbs free energies of transfer could be

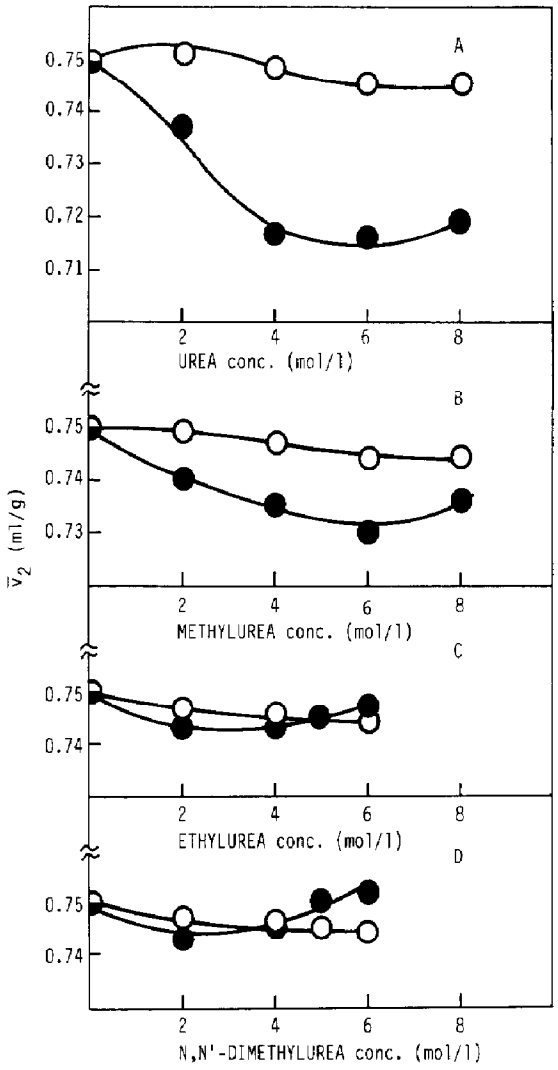

Fig. 2. The partial specific volumes of β-lactoglobulin at infinite dilution as a function of denaturant concentration: (○) isomolal; (●) isopotential. (A) Urea, (B) methylurea, (C) ethylurea; and (D) *N,N'*-dimethylurea; pH 5.5.

calculated. They clearly reflect the presence of hydrophobic groups. The same holds true for the enthalpies and entropies of transfer. The former were obtained by calorimetry [3]. Comparison of transfer data to urea solutions with those to alkylurea solutions reveals negative values of ΔH_{tr} and ΔS_{tr} for urea solutions, and positive values of ΔH_{tr} and ΔS_{tr}. However, owing to enthalpy–entropy compensation the values of ΔG_{tr} are small but still negative.

However, the thermodynamic quantities do not give any insight into conformational changes accompanying the transfer. They have to be obtained by other methods, e.g. CD. In this respect the denaturing actions of urea and *N,N'*-dimethylurea on β-lactoglobulin are a good example. As we have already mentioned, in both cases large conformational changes are produced so that we deal with denaturation [12]. However, the final product in the first case is a random coil, whereas in the second at high concentrations we still have a largely ordered structure. Therefore when studying solvation parallel conformational studies are always indicated. Only thus a statement regarding the final state can be made.

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