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Preferential interactions of urea with lysozyme and their linkage to protein denaturation

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Abstract

The interactions involved in the denaturation of lysozyme in the presence of urea were examined by thermal transition studies and measurements of preferential interactions of urea with the protein at pH 7.0, where it remains native up to 9.3 M urea, and at pH 2.0, where it undergoes a transition between 2.5 and 5.0 M urea. The destabilization of lysozyme by urea was found to follow the linear dependence on urea molar concentration, M_u, $\Delta G_u^o = \Delta G_w^o - 2.1\ M_u$, over the combined data, where ΔG_u^o and ΔG_w^o are the standard free energy changes of the $N \rightleftharpoons D$ reaction in urea and water, respectively. Combination with the measured preferential binding gave the result that the increment of preferential binding, $\delta\Gamma_{23} = \Gamma_{23}^D - \Gamma_{23}^N$, is also linear in M_u. A temperature dependence study of preferential interactions permitted the evaluation of the transfer enthalpy, $\Delta\bar{H}_{2,\mathrm{tr}}^o$, and entropy, $\Delta\bar{S}_{2,\mathrm{tr}}^o$ of lysozyme from water into urea in both the native and denatured states. These values were found to be consistent with the enthalpy and entropy of formation of inter urea hydrogen bonds (Schellman, 1955; Kauzmann, 1959), with estimated values of $\Delta\bar{H}_{2,\mathrm{tr}}^o$ = ca. −2.5 kcal mol^{-1} and $\Delta\bar{S}_{2,\mathrm{tr}}^o$ = ca. −7.0 e.u. per site. Analysis of the results led to the conclusion that the stabilization of the denatured form was predominantly by preferential binding to newly exposed peptide groups. Combination with the knowledge that stabilizing osmolytes act by preferential exclusion from peptide groups (Liu and Bolen, 1995) has led to the general conclusion that both the stabilization and destabilization of proteins by co-solvents are controlled predominantly by preferential interactions with peptide groups newly exposed on denaturation.

Keywords: Preferential interactions; Transfer free energy; Transfer enthalpy; Transfer entropy; Protein destabilization; Co-solvents

1. Introduction

It is with particular gratification that I (ST) welcome this opportunity to participate in the issue dedicated to Walter Kauzmann. I have always regarded Walter Kauzmann as one of my teachers, even though I was neither a student nor a postdoctoral in his laboratory. The learning came from remarks, comments and explanations that Walter Kauzmann would make at the early Gordon Conferences on proteins and at other meetings. One memorable occasion was early in my career when I became interested in protein denaturation and was confused. Walter Kauzmann invited me to come to Princeton, where he spent the day explain-

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ing denaturation to me. This set me off on a straight path from which I hope I have not deviated.

Urea and guanidine hydrochloride (Gdn·HCl) have been known as protein denaturants for close to a century. A characteristic feature is the requirement of high concentration, typically 8 M urea and 6 M Gdn·HCl. While it was accepted that the denaturation required some sort of interaction with the protein, little was understood about the nature of such interactions. Kauzmann and co-workers [1–5], in a set of pioneering papers on the quantitative macromolecular analysis of the process, concluded from the high order of the kinetics that the denaturation of a protein required the binding of a large number of urea molecules to the protein, with the replacement of interpeptide hydrogen bonds by urea–peptide hydrogen bonds playing an important role. The subsequent development of the Wyman linkage relation [6] and its combination with three-component thermodynamics [7,8] permitted to identify this binding as the change in preferential binding of the denaturant to the protein [9] in the Native $\rightleftharpoons$ Denatured equilibrium.

A general finding in studies of the urea and Gdn·HCl denaturations of proteins has been a slope of ca 10–20 additional molecules of denaturant bound to the protein at the mid-point of a Wyman transition plot [10,11]. This indicates that the preferential binding of the denaturant increases during the course of the denaturation. Hence, knowledge of preferential binding of denaturants to proteins in the two end states of the reaction should help in the elucidation of the role played by the denaturant in the unfolding of the protein.

Preferential interaction studies on several proteins have shown that urea could be preferentially bound to proteins, as is the case with β-lactoglobulin [12], as well as preferentially excluded from native proteins, as is the case with myoglobin [13]. Examination of the preferential interactions of a number of proteins with 8 M urea, the concentration normally used to denature proteins, gave low values of preferential binding [14], which reflect a shallow concentration dependence of the transfer free energy on urea concentration.

With the aim of obtaining a more detailed understanding of the interactions, which cause urea to destabilize proteins, we have carried out extensive preferential interaction measurements of urea to lysozyme at conditions where the protein remains native up to 9 M urea (pH 7.0), as well as conditions where it undergoes an unfolding transition (pH 2.0). A temperature dependence study of preferential interactions at pH 7.0 and thermal transition measurements at the two pH values permitted a detailed description of the interactions, which cause urea to stabilize the denatured form of the protein. It is the purpose of this paper to describe these results.

2. Experimental

2.1. Materials

Urea was purchased from BIO-RAD Laboratories and purified by the procedure developed by Prakash et al. [14]. 600 g of urea crystals were dissolved in 580 ml water at room temperature without heating, giving approximately 1 l of 9.5 M urea. 5–10 g of activated charcoal (carbon decolorizing from Fisher Scientific Co.) were added and stirred for 4 h. The solution was filtered through Whatman filter paper No. 1 to remove coarse charcoal particles and further filtered through a Millipore filter of 0.45-μm pore size to render the solution completely charcoal free. 0.81 g methylamine hydrochloride (final concentration-0.012 M) was added and the solution was stirred well for approximately 4 h, then 20–30 g BIO-RAD analytical-grade Mixed-bed resin, AG 501-x8(D), 20–50 mesh was added and the suspension was stirred for another 4 h. Finally, the solution was filtered through a Millipore filter of 0.45-μm pore size. The concentration of the urea solution was measured refractometrically (c (M) = $(n-1.33312)/0.008321$) [15]. The maximum storage period for the urea solution was three weeks at room temperature. Lysozyme was from Sigma. It was dissolved in distilled Poland Spring water, thoroughly dialyzed against it (for experiments at pH 2.0, 1 mM HCl was added), passed through a sintered glass filter and lyophilized. Glycine was the reagent grade product from Sigma. The molecularporous membrane tubing (23×100, M.W. cut-off 6000–8000) was purchased from Spectrum

Table 1
Characteristic parameters of urea solutions and absorbance values of lysozyme at pH 7.0 and (2.0)

Urea (M)	g_3 (g/g)	m_3 (mol/1 Kg H_2O)	ρ_o (g/ml) pH 7.0 (pH 2.0)	$\bar{\upsilon}_3$ (ml/g)	$e_{280\ nm}$ of lysozyme (dl/g.cm)		γ_3[a]	$\left(\frac{\partial \ln \gamma_3}{\partial m_3}\right)$[a]	$\left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2}$[b]
					pH 7.0	pH 2.0			
0			1.0007 (0.9992)		25.6	26.7			
0.5	0.0307	0.511	1.0085 (1.0073)	0.7311	25.8	26.8	0.9575	−0.0755	1096
1.0	0.0628	1.045	1.0155	0.7340	26.0	27.0	0.9256	−0.0690	517
1.5	0.0964	1.605	1.0245	0.7366	26.1	27.4	0.8944	−0.0635	326
1.75	0.1141	1.899	1.0280	0.7377			0.8788	−0.0610	279
2.0	0.1317	2.193	1.0315	0.7388	26.3	27.5	0.8639	−0.0585	232
2.5	0.1689	2.812	1.0390	0.7405	26.5	27.5	0.8342	−0.0540	176
3.0	0.2080	3.463	1.0465	0.7418	26.6	27.2	0.8057	−0.0497	139
3.5	0.2491	4.148	1.0540	0.7428	26.8	26.9	0.7783	−0.0455	114
3.75	0.2708	4.509	1.0578	0.7432			0.7649	−0.0435	104
4.0	0.2925	4.870	1.0615	0.7436	27.0	26.6	0.7523	−0.0415	95
4.5	0.3382	5.631	1.0695	0.7443	27.1	26.3	0.7277	−0.0377	81
5.0	0.3868	6.440	1.0767	0.7449	27.2	26.4	0.7047	−0.0342	71
5.5	0.4382	7.296	1.0842	0.7455	27.3	26.4	0.6835	−0.0310	62
6.0	0.4927	8.203	1.0918	0.7462	27.4	26.5	0.6641	−0.0287	54
6.5	0.5505	9.166	1.0995	0.7466	27.5	26.6	0.6466	−0.0257	49
7.0	0.6123	10.195	1.1070	0.7470	27.6	26.7	0.6309	−0.0235	43
7.5	0.6783	11.294	1.1145	0.7473	27.7	26.8	0.6169	−0.0215	39
7.75	0.7137	11.883	1.1183	0.7475			0.6104	−0.0208	37
8.0	0.7490	12.471	1.1220	0.7476	27.8	26.9	0.6044	−0.0200	35
8.5	0.8247	13.731	1.1295	0.7478	27.8	27.0	0.5929	−0.0183	32
9.0	0.9062	15.088	1.1370	0.7480	27.9	27.1	0.5815	−0.0170	29
9.5	0.9941	16.552	1.1445	0.7482	27.9	27.2	0.5689	−0.0158	26

[a] Calculated from the data of Scatchard et al. [23].
[b] cal – (mol of Urea)-^{2} in 1000 g H_2O.

and treated as follows. The membrane tubing was immersed in water overnight, 2 g sodium bicarbonate was added and boiled. After washing twice with water, 2 g EDTA (no Na^+) was added and boiled, washed twice with water, boiled in water and washed again. It was stored in 25% alcohol at 4 °C until use. Fisher standardized 1 N HCl solution was used for the pH adjustment of solutions. All solutions contained 0.02 M glycine at pH 2.0, or 0.02 M sodium phosphate at pH 7.0. The protein concentrations were determined by UV absorbance on a Perkin-Elmer UV/Vis lambda 3B spectrophotometer. The extinction coefficients for lysozyme were obtained by gravimetric measurements. The values of extinction coefficients in various concentration of urea are listed in Table 1. In all concentration determinations, the contribution of light scattering to absorptivity was corrected for by the method of Leach and Scheraga [16].

2.2. Thermodynamics of thermal denaturation

The change in protein absorbance as a function of temperature was followed on a Gilford Response II UV/Vis spectrophotometer at 301 nm. In all experiments, 1 mg/ml lysozyme was used. In order to avoid the formation of bubbles on heating, the solutions were degassed for 3–5 min. on a water pump. The temperature change steps were programmed so that the rate of heating was ~0.7 °C per min. The experiments at pH 7.0 were carried out at concentrations of urea above 4 M, because at lower urea concentrations the protein did not unfold completely, nor did it fully reverse

after heating. At both pH values, the cooling curves were mirror images of the heating curves, reaching equilibrium very rapidly at pH 2.0, while at pH 7.0 the reversal was slower. The isothermal transition at pH 2.0 was also followed by fluorescence with excitation at 280 nm and emission at 350 nm, using a Shimadzu RF 540 spectrofluorometer.

2.3. Preferential interactions

The preferential binding of urea to the protein was obtained from the apparent partial specific volumes of the protein measured with a Precision DMA-02 density meter (Anton Paar, Gratz). All measurements were made at 20 °C using previously published procedures [17,18]. At any solvent composition, density measurements were performed as a function of protein concentration, without and with prior dialysis against the solvent. These gave the exact co-solvent concentrations in the bulk solvent and in the solvent that had been equilibrated by interaction with the protein. The purpose of dialysis is to re-establish the chemical equilibrium disturbed by interaction with the protein, since the activity of urea must be brought to the state of identity inside and outside the bag (isopotential conditions). This can be accomplished only by changing its concentration inside the bag. Buffer solutions containing a given urea concentration were filtered through Whatman filter paper No. 1. Each preferential interaction point was obtained from the apparent partial specific volumes measured with dialysis against the solvent at the experimental temperature and without dialysis, giving, in turn, values at constant chemical potential, ϕ_2', and at constant molality, ϕ_2, of co-solvent. Each point required that two experiments be carried out. In the first, the protein solutions were dialyzed 24 h at the experimental temperature against 220 ml urea buffer solution with one change. In the parallel experiment, the protein solution was kept for 24 h at the same temperature without dialysis. Since the density measurements were carried out at 20 °C, both solutions were cooled to 20 °C for 2–3 h, with the one that had been dialyzed at high temperature transferred out of the dialysis bag prior to cooling. In constant molality experiments, for each point five samples (8–20 mg) of lysozyme were dried in small glass test tubes over phosphorus pentoxide at 40 °C for 2 days in a vacuum oven. The samples were cooled to room temperature; approximately 1 ml of mixed solvent was added (the amounts of protein (W_P) and the urea buffer (W_B) were determined gravimetrically and the protein concentration obtained as $c = \rho x W_P/(W_P + W_B)$). The tubes were sealed tightly immediately with Parafilm and left to stand overnight at the experimental temperature. When the samples had to be kept in a water bath, the tubes were sealed by 5 layers of Parafilm and 2 layers of Saran Wrap. Following this, the samples were equilibrated at 20 °C for 3–4 h prior to the densimetry measurements. In constant chemical potential measurements, i.e. at dialysis equilibrium with the co-solvent, each of five samples of protein (8–25 mg) was dissolved in approximately 1.1 ml of mixed solvent and transferred into a dialysis bag, i.e. the molecular-porous membrane tubing. The samples were dialyzed at the desired temperature for ca. 24 h against two 220 ml changes of co-solvent solution and then kept at 20 °C for 3–4 h in small tubes that have been sealed tightly to avoid evaporation. After each density measurement, the protein solution was diluted gravimetrically with solvent to a final optical density of approximately 0.7 (approx. 0.25 mg/ml) and the concentrations were measured spectrophotometrically with a Perkin Elmer Lambda 3B UV/Vis spectrophotometer. The individual points were determined in up to four independent experiments. The values were found to be highly reproducible both at high and low temperature.

2.4. Calculation of preferential interaction parameters

The apparent partial specific volume of a protein can be calculated from density measurements with the equation [19]:

$$\phi = 1/\rho_o \times [1 - (\rho - \rho_o)/c] \quad (1)$$

where ϕ is the apparent specific volume, ρ and ρ_o are the densities of the protein solution and

reference solvent, respectively, and c is the protein concentration in grams/ml. ϕ_2^o and $\phi_2'^o$ were determined at conditions at which the molalities of solvent components and their chemical potentials were, in turn, kept identical in the protein solution and in the reference solvent. Adopting the notation of Scatchard [20] and Stockmayer [8], component 1 = water, component 2 = protein, component 3 = additive (here urea), the preferential binding, $(\partial g_3/\partial g_2)_{T,\mu_1\mu_3}$ was obtained from [21]:

$$\xi_{23} = (\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = \rho_o(\phi_2^o - \phi_2'^o)/(1-\rho_o\bar{v}_3) \quad (2)$$

where g_i is the concentration of component i in grams per gram of water, T is the thermodynamic (Kelvin) temperature, μ_i is the chemical potential of component i, and $\bar{v}_3$ is the partial specific volume of component 3. The superscript o indicates extrapolation to zero protein concentration.[1] The pertinent values of ρ_o and $\bar{v}_3$ are listed in Table 1. The preferential binding parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, is a measure of the excess of component 3 in the immediate domain[2] of the protein with reference to its concentration in the bulk solvent. A positive value of this parameter means an excess of component 3, while a negative value indicates its deficiency in the domain, which means an excess of component 1, water. Within a negligible approximation, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ is equal to the binding measured by dialysis equilibrium and similar techniques, $(\partial g_3/\partial g_2)_{T,P,\mu_3}$ [22]. In molal units, preferential binding is expressed as:

$$\Gamma_{23} = (\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (M_2/M_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (3)$$

where m_i and M_i are the molality and molecular weight of component i. For non-ionizable molecules the preferential binding parameter is a direct expression of the perturbation of the chemical potentials of the co-solvent by the protein [7,23].

$$(\partial\mu_3/\partial m_2)_{T,P,m_3} = -(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}(\partial\mu_3/\partial m_3)_{T,P,m_2} \quad (4)$$

where $\mu_3 = RT\ln(m_3\gamma_3)$, R is the universal gas constant and γ_3 is the activity coefficient of the co-solvent, in the present case urea. The values of γ_3 and $(\partial\ln\gamma_3/\partial m_3)_{T,P,m_2}$ listed in Table 1 were calculated from the data of Scatchard et al. [24].

3. Results

3.1. Urea induced denaturation

The effect of urea on lysozyme stability was followed by thermal transition experiments as a function of urea concentration at pH 7.0 and 2.0 and by urea titration at a constant temperature, 20 °C. The results are presented in Fig. 1a and b. It is clear that at 20 °C pH 7.0, addition of urea does not unfold the protein up to 9 M denaturant. At pH 2.0, however, unfolding sets in at 2.5 M urea and the transition reaches a plateau at 5 M urea. The transition curve follows a symmetrical pattern with the midpoint at 3.7 M urea.

The midpoints of the thermal transition T_m as a function of urea concentration are presented in Table 2 and Fig. 1a. At pH 7.0, T_m could not be measured at urea concentrations below 4.5 M, because the protein did not unfold completely at the limit of heating (80 °C). At pH 2.0, the measurements had to be stopped at 2.5 M urea, because above that concentration the protein was already partly unfolded at the initial temperature in the heating experiments. It is evident that at both pH values urea exerts a destabilizing influence on the protein.

The thermodynamic parameters of the destabilizations were calculated from the transition curves

[1] The measured values of ϕ_2 and ϕ_2' were independent of protein concentration, whether the protein solution was heated or not. This indicates that the observed effects are not due to protein self-association or aggregation. In the case that the self-association is reversible, its effects should be eliminated by extrapolation to zero protein concentration. Random, non-specific irreversible aggregation (coagulation) has, in the past, generated poorly reproducible erratic values of the apparent partial specific volumes. Furthermore, heating at the high experimental temperature did not generate any measurable turbidity at 450 nm.

[2] By domain, we mean the volume around a protein molecule, in which it exercises a thermodynamic influence on solvent component molecules, water and urea in the present system. There is no implication of any well defined compartment, or shell, around the protein molecule that has any physical reality.

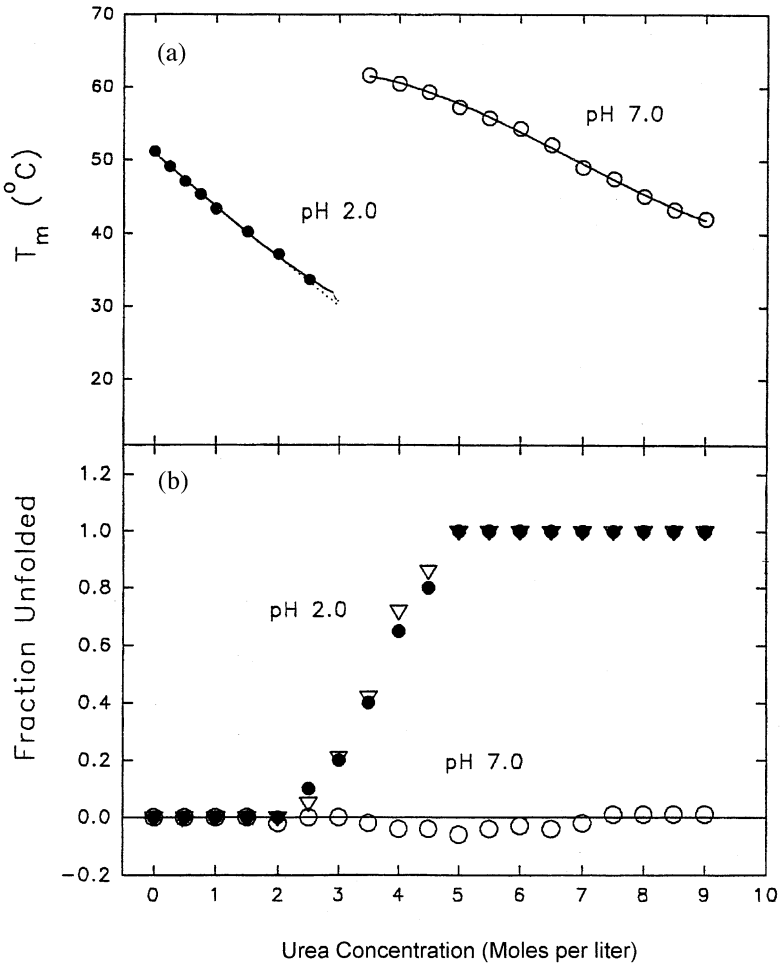


Fig. 1. Urea concentration and pH dependence of: (a) The transition midpoint temperature (T_m) of lysozyme denaturation, (○) pH 7.0, (●) pH 2.0; (b) The transition curve measured at 20 °C for lysozyme denaturation, (○) UV (301 nm), pH 7.0; (●) UV (301 nm), pH 2.0; (▽) Fluorescence (emission at 350 nm), pH 2.0.

in terms of a two state, N⇌D, transition. At each solvent composition and temperature the degree of conversion from the native to the unfolded state, α, was estimated from the UV absorbance by the method of Biltonen and Lumry [25], and the equilibrium was calculated from

$$K = \alpha/(1-\alpha) \tag{5}$$

At each solvent composition, the standard enthalpy change of unfolding was obtained by fitting the values of K to a truncated form of the integrated van't Hoff equation [26,27]

$$\begin{aligned} \mathrm{Ln}K &= a + b(1/T) + c\ln T \\ \Delta H^\circ &= R(cT - b) \\ \Delta C_P &= Rc \end{aligned} \tag{6}$$

where ΔC_P is the change in heat capacity at constant pressure.

The resulting values of ΔH° at T_m are listed in Table 2. At both pH values, the transition is characterized by a positive change in enthalpy, which decreases with increasing urea concentration

Table 2
Thermodynamic parameters of the thermal denaturation of lysozyme at pH 7.0 and pH 2.0 in urea-water systems

Urea (M)	T_m (°C)	$\Delta H°$ at T_m (kcal/mol)	$\Delta G°$ at 20 °C (kcal/mol)	$\Delta S°$ at 20 °C (e.u.)	ΔCp (cal-K^{-1}-mol^{-1})	$\delta(\Delta G°)$ at 20 °C (kcal-mol^{-1})[b]
pH 7.0						
4.5	59.4±2.0	93.6±5.0	10.9±0.5	278±20	28	−9.1
5.0	57.3	90.4	10.1	270	27	−9.9
5.5	55.9	87.7	9.5	263	26	−10.5
6.0	54.4	75.8	7.9	229	23	−12.1
6.5	52.2	64.9	6.3	198	20	−13.7
7.0	49.1	60.3	5.3	185	19	−14.7
7.5	47.5	53.9	4.5	167	17	−15.5
8.0	45.1	60.0	4.5	187	19	−15.5
8.5	43.3	59.6	4.3	187	19	−15.7
9.0	42.1	60.9	4.2	192	20	−15.8
pH 2.0						
0.0	51.2±0.2	81.4±2.0	7.7±0.5	249±20	25	0.0
0.25	49.1	81.4	7.2	251	25	−0.5
0.5	47.1	72.5	6.0	225	23	−1.7
0.75	45.3	69.8	5.4	218	22	−2.3
1.0	43.3	63.7	4.6	200	21	−3.1
1.5	40.3	57.6	3.6	183	19	−4.1
2.0	37.3	58.8	3.2	189	19	−4.5
2.5	33.8	50.1	2.1	163	17	−5.6
3.0			0.8[a]			−6.9
3.7			0.0[a]			−7.7
4.3			−0.8[a]			−8.5
4.5			−1.3[a]			−9.0

[a] Points taken from urea titration curve of Fig. 1b.
[b] Difference from value in H_2O obtained by extrapolation (see text).

in good agreement with the results of the calorimetric measurements of Makhatadze and Privalov [28]. The actual values are lower at pH 2.0 than at pH 7.0. Application of the small change in ΔC_P permitted the calculation of the changes in standard free energy change, ΔG^o, at 20 °C, and standard entropy change during the unfolding. At both pH values, ΔG^o values are decreasingly positive with urea concentration. At pH 2.0, $\Delta G^o = 0$ at 3.7 M urea and assumes negative values above that concentration. The ΔG^o values at 20 °C were plotted in terms of the well known linear dependence on urea concentration [10,11]:

$$\Delta G_u^o = \Delta G_w^o - m\ M_u \tag{7}$$

where ΔG_u^o is the standard free energy change at any given concentration of urea, M_u, in molar units, ΔG_w^o is its value in water and m is a characteristic constant. The results gave slopes of $m = 2.1$ kcal-mol^{-1}-M_u^{-1} at pH 7 (below 8 M urea) and 2.2 at pH 2.0. At the lower pH, the measured value of $\Delta G_w^o = 7.7$ kcal-mol^{-1} is in reasonable agreement with that calculated from the data of Green and Pace [10] at pH 2.9, $\Delta G_w^o = 6.0$ kcal-mol^{-1}. At pH 7.0, the extrapolated value of ΔG_w^o was 20 kcal-mol^{-1}. Therefore, lowering the pH from 7.0 to 2.0 decreased the protein stability by ca. 12.3 kcal-mol^{-1}.

The destabilization of the native structure by the denaturant can be expressed by $\delta\Delta G^\circ$, i.e. the lowering of the standard free energy of unfolding relative to that obtained in water at any urea concentration,

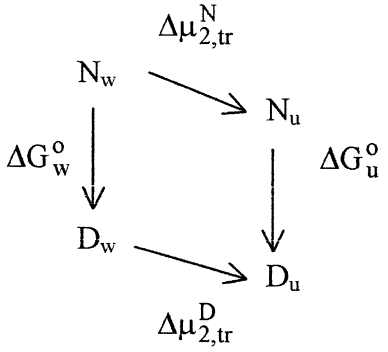
$$\delta\Delta G^\circ = \Delta G_u^o - \Delta G_w^o \tag{8}$$

The values calculated from the transition experiments are listed in the last column of Table 2. At pH 7.0, the ΔG_w^o was assigned the value, 20 kcal-mol^{-1}, obtained by extrapolation according to Eq. (7). At both pH values urea exerts a significant destabilizing action on the protein. The $\delta\Delta G^\circ$ values measured at the two pH values are plotted as a function of the molar concentration of urea in Fig. 2a. The two sets of points fall on the same straight line with a slope of 2.1 ± 0.2. This suggests that the destabilizing action of urea is independent of pH and follows the well-known linear dependence on the molar concentration of denaturant [10,11] over the range from pure water to 8 M urea.

The stabilization or destabilization of a protein by a co-solvent (osmolyte) is caused by a difference in the interactions of the protein with the non-aqueous solvent component in the two end states of the N⇌D equilibrium [29]. When pure water is taken as reference state, this interaction is expressed by the transfer free energy of the protein from water to the co-solvent containing medium, in the present case urea, $\Delta\mu_{2,\mathrm{tr}}$. For a co-solvent to have an effect on protein stability relative to its stability in water, its interaction with the protein must be different in the native and denatured states. The stabilizing or destabilizing ability of the co-solvent is expressed by the change in transfer free energy during the course of the reaction:

$$\delta\Delta\mu_{2,\mathrm{tr}} = \Delta\mu_{2,\mathrm{tr}}^D - \Delta\mu_{2,\mathrm{tr}}^N \tag{9}$$

When, at any given concentration of co-solvents, $\delta\Delta\mu_{2,\mathrm{tr}}$ is negative, the interaction is thermodynamically more favorable with the denatured than the native state and there is destabilization, which leads to unfolding, i.e. denaturation. Positive $\delta\Delta\mu_{2,\mathrm{tr}}$ means stabilization of native protein structure. This is the observation with most osmolytes, such as sucrose, trehalose, sorbitol, TMAO, sarcosine [30 and references cited therein]. When $\Delta\mu_{2,\mathrm{tr}}^D = \Delta\mu_{2,\mathrm{tr}}^N$, the added co-solvent is inert. The change in transfer free energy during the unfolding being a measure of the thermodynamic effect of the co-solvent on the stability of the protein, it is equal to the change in the free energy of denaturation, i.e. $\delta\Delta\mu_{2,\mathrm{tr}} = \delta\Delta G^\circ$. This leads to the thermodynamic box [30,31]:



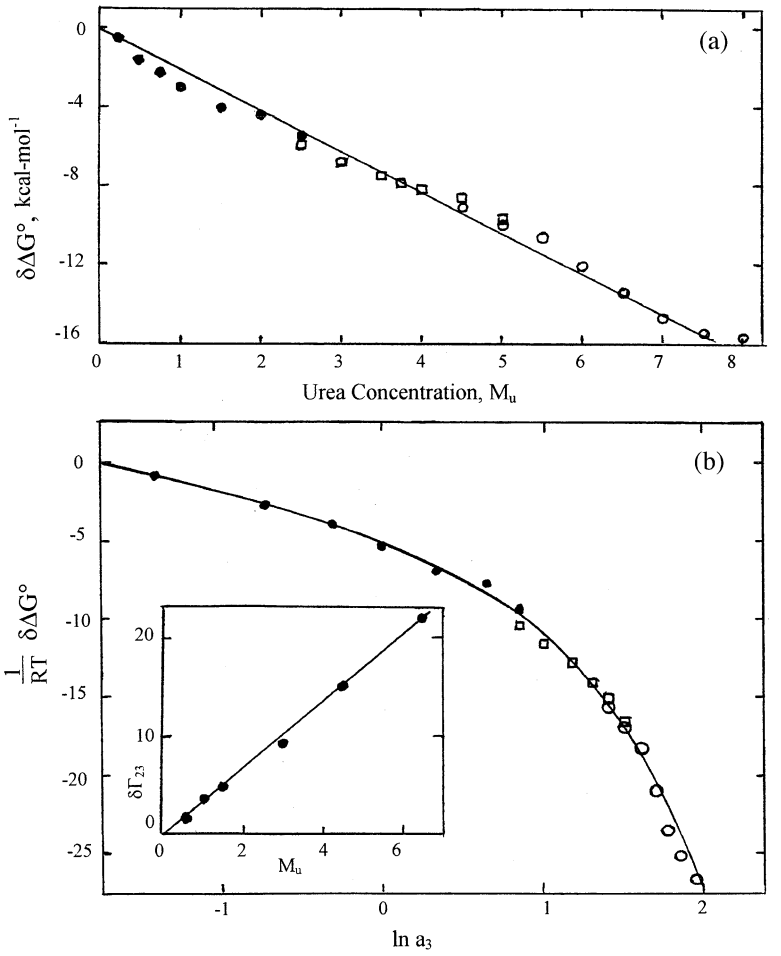


Fig. 2. (a) Dependence on urea molar concentration of the increment of standard free energy of lysozyme denaturation, $\delta\Delta G^\circ = \Delta G^o_u - \Delta G^o_w$; (b) Wyman plot of the standard free energy increment as a function of the molal activity of urea for the denaturation of lysozyme. For both figures: Solid circles (●): points obtained from thermal transition experiments at pH 2.0; open circles (○): points obtained from thermal transition experiments at pH 7.0; squares (□): point obtained from the isothermal urea titration of lysozyme at pH 2.0. Inset: Dependence of the preferential binding increment, $\delta\Gamma_{23}$, on the molar concentration of urea.

The equality of the two pathways of denaturation means that knowledge of $\delta\Delta G^\circ$ and the transfer free energy in either the native or denatured state permits the calculation of that parameter in the other state of the protein, because

$$\Delta\mu^N_{2,\mathrm{tr}} + \Delta G^o_u = \Delta G^o_w + \Delta\mu^D_{2,\mathrm{tr}} \tag{10}$$

When the reference state is the solvent of a given composition, m_3, the effect of the co-solvent on the denaturation equilibrium at that solvent composition is expressed by the difference between the preferential binding of the co-solvent to the protein in the two states, according to the standard Wyman linkage equation [6]:[3]

$$\frac{d\log K}{d\log a_3} = (\partial m_3/\partial m_2)^D_{\mu_3} - (\partial m_3/\partial m_2)^N_{\mu_3} = \delta(dm_3/dm_2)_{\mu_3} \tag{11}$$

[3] From here on we drop the subscripts *T*, *P*, because all the operations are carried out at the same temperature and pressure.

where a_3 is the activity of the co-solvent (urea), $a_3 = m_3\gamma_3$ and γ_3 is the activity coefficient of the co-solvent. The equilibrium constants as a function of urea concentration were calculated for the transition at pH 2.0 by using Eq. (5) (Fig. 1b) and the resulting values of K were plotted in terms of the Wyman equation. The slope of the plot between $\alpha = 0.25$ and 0.75 was found to be $\delta(dm_3/dm_2)_{\mu_3} = 8.20$. Therefore, at 3.7 M urea, which is the mid-point of the transition, the preferential binding of urea to lysozyme increases by 8.2 molecules of urea per molecule of protein. The values of K measured at pH 2.0 between 2.5 and 5.0 M urea were converted to ΔG_u^o, listed in Table 2. Subtraction of 7.7 kcal-mol^{-1} (the value of ΔG_w^o at pH 2.0) gave $\delta\Delta G^\circ$ measured by the isothermal urea titration. The resulting values are listed in Table 2 and plotted in Fig. 2a, where they fall on the same straight line as the two sets of $\delta\Delta G^\circ$ values obtained from the thermal transition experiments at pH 2.0 and 7.0. This indicates consistency between the three independent experiments.

Preferential binding is the expression in molecular terms of the mutual perturbations of the protein and co-solvent chemical potentials:

$$\left(\frac{\partial\mu_2}{\partial m_3}\right)_{m_2} = \left(\frac{\partial\mu_3}{\partial m_2}\right)_{m_3} = -\left(\frac{\partial m_3}{\partial m_2}\right)_{\mu_3}\left(\frac{\partial\mu_3}{\partial m_3}\right)_{m_2} \quad (12)$$

The chemical potential perturbation, being the gradient of the protein chemical potential with co-solvent concentration, its integration with respect to m_3 gives the transfer free energy [29,32–34]:

$$\Delta\mu_{2,\mathrm{tr}}^{m_3} = \int_o^{m_3}\left(\frac{\partial\mu_2}{\partial m_3}\right)_{m_2} dm_3 = -\int_o^{m_3}\left(\frac{\partial m_3}{\partial m_2}\right)_{\mu_3}\left(\frac{\partial\mu_3}{\partial m_3}\right)_{m_2} dm_3 \quad (13)$$

Wyman [6] expressed the linkage equation first in terms of free energy:

$$\frac{d\Delta G^\circ}{d\mu_3} = \delta\left(\frac{\partial\mu_2}{\partial m_3}\right)_{m_3}\Big/\left(\frac{\partial\mu_3}{\partial m_3}\right)_{m_2} = \delta\left(\frac{\partial\mu_2}{\partial\mu_3}\right)_{m_2} = -\delta\left(\frac{\partial m_3}{\partial m_2}\right)_{\mu_3} = -\delta\Gamma_{23} \quad (14)$$

where $\mu_3 = \mu_3^o + RT\ln a_3$. Therefore, the values of $(\delta\Delta G^\circ/RT)$ determined in the three experiments and listed in the last column of Table 2 were plotted as a function of ln a_3, as shown in Fig. 2b. The slope at each value of ln a_3 is equal to the difference between the mutual thermodynamic perturbations of protein and urea in the denatured and native states. It is evident that $\delta\Gamma_{23}$ increases with urea concentration and has values of, e.g. 2 at 0.6 M urea, 15 at 4.5 M urea and 22 at 6.5 M urea. This means that as the concentration of denaturant increases, the preferential interaction of urea with denatured protein becomes progressively larger than that with native lysozyme. A plot of $\delta\Gamma_{23}$ as a function of the molar concentration of urea, M_u, shown in the inset of Fig. 2b, resulted in the linear relationship

$$\Gamma_{23}^D = \Gamma_{23}^N + 3.3\ M_u \quad (15)$$

This linearity most probably reflects the fortuitous coincidence that, in the case of urea, the numerical values of molal activity, $a_3 = m_3\gamma_3$, are close to coincident with those of M_3, i.e. M_u, and the known fact that $(\Delta G_u^o - \Delta G_w^o)$ is a linear function of M_u.[4] Application of this empirical relation, $\delta\Gamma_{23} = 3.3\ M_u$, permits an easy calculation of the preferential interactions in one state of the protein from the values in the other state.

3.2. Preferential interactions

From the preceding discussion, it follows that knowledge of the preferential binding of urea to lysozyme as a function of the denaturant concentration in one state should permit to obtain the thermodynamic interaction parameters in the other state. To that end preferential interaction measurements were carried out at pH 7.0, where the protein is native up to 9.5 M urea and at pH 2.0, where it undergoes an unfolding transition between 2 and 5 M urea (Fig. 1b).

[4] The observation is $\delta\Gamma_{23} = bM_3 = d\Delta G^\circ/d\mu_3$. But $(\Delta G_u^o - \Delta G_w^o) = mM_3$ Eq. (7). Hence $m(dM_3/d\mu_3) = bM_3$, i.e. $(1/RT)(d\ \ln\ M_3/d\ \ln\ a_3) = b/m = \text{const}$, where b is the slope of the plot of $\delta\Gamma_{23}$ vs. M_3. Setting $(d\ \ln\ M_3/d\ \ln\ a_3) \cong 1$ gives $b = m/RT$. Introduction of the experimental value, $m = 2.1$, gives $b_{\mathrm{calc}} = 3.6$, which is very close to the measured value, 3.3.

Table 3
Partial specific volumes and preferential interaction parameters of lysozyme with urea solutions at 20 °C, pH 7.0

Urea (M)	ϕ_2^o (ml/g)	$\phi_2'^o$ (ml/g)	ξ_{23} (g/g)	ξ_{21} (g/g)	Γ_{23} (mol/mol)	Γ_{21} (mol/mol)	$\left(\frac{\partial\mu_2}{\partial m_3}\right)_{T,P,m_2}$ [a]
0	0.717±0.001	0.717±0.001					
0.5	0.718±0.001	0.714±0.001	+0.0154±0.004	−0.500±0.125	+3.67±0.9	−397±99	−4022±1006
1.0	0.718±0.001	0.711±0.001	+0.0279±0.004	−0.445±0.064	+6.64±1.0	−354±51	−3433±490
1.5	0.718±0.001	0.709±0.001	+0.0376±0.004	−0.390±0.038	+8.95±1.0	−310±30	−2918±324
2.0	0.718±0.001	0.707±0.002	+0.0477±0.007	−0.362±0.049	+11.36±1.5	−288±39	−2636±359
2.5	0.717±0.001	0.705±0.001	+0.0541±0.005	−0.320±0.027	+12.88±1.1	−254±21	−2268±189
3.0	0.717±0.001	0.705±0.001	+0.0562±0.005	−0.269±0.022	+13.37±1.1	−214±18	−1859±155
3.5	0.718±0.000	0.705±0.001	+0.0631±0.002	−0.253±0.010	+15.02±0.6	−201±8	−1712±66
4.0	0.719±0.001	0.705±0.001	+0.0706±0.005	−0.241±0.017	+16.80±1.2	−191±14	−1596±114
4.5	0.720±0.001	0.706±0.001	+0.0734±0.005	−0.217±0.016	+17.48±1.3	−173±12	−1415±101
5.0	0.719±0.001	0.705±0.001	+0.0762±0.005	−0.197±0.014	+18.14±1.3	−156±11	−1288±92
5.5	0.720±0.001	0.707±0.001	+0.0735±0.006	−0.168±0.013	+17.50±1.4	−133±10	−1085±84
6.0	0.719±0.001	0.707±0.001	+0.0707±0.006	−0.144±0.012	+16.83±1.4	−115±10	−909±76
6.5	0.720±0.000	0.708±0.001	+0.0737±0.003	−0.134±0.006	+17.54±0.7	−107±4	−859±36
7.0	0.720±0.001	0.709±0.001	+0.0704±0.006	−0.115±0.010	+16.76±1.5	−92±8	−721±66
7.5	0.720±0.001	0.709±0.001	+0.0733±0.007	−0.108±0.010	+17.47±1.6	−86±8	−681±62
8.0	0.720±0.001	0.710±0.001	+0.0696±0.007	−0.093±0.009	+16.58±1.7	−74±7	−580±58
8.5	0.721±0.001	0.712±0.001	+0.0654±0.007	−0.080±0.009	+15.58±1.7	−63±7	−499±55
9.0	0.721±0.001	0.711±0.001	+0.0760±0.008	−0.084±0.008	+18.10±1.8	−66±7	−525±53
9.5	0.717±0.001	0.708±0.001	+0.0717±0.008	−0.072±0.008	+17.09±1.9	−57±6	−445±49

[a] cal – (mol of urea)$^{-1}$ (mol of protein)$^{-1}$ in 1000 g H_2O.

The results are presented in Tables 3, (pH 7.0) and 4 (pH 2.0). The values of the apparent partial specific volumes at isomolal, ϕ_2^o, and isopotential, $\phi_2'^o$, conditions, extrapolated to zero protein concentration,[5] are listed in columns 2 and 3 of the Tables. The identity of ϕ_2^o and $\phi_2'^o$ in water indicates the absence of errors introduced by the handling of the dialyzed protein. The calculated preferential binding of urea is presented in columns 4 and 6 of the Tables in terms of grams urea per gram protein, ξ_{23}, and moles urea per mole protein, Γ_{23}. Preferential interaction can also be expressed symmetrically in terms of water molecules preferentially bound to the protein, ξ_{21} and Γ_{21}, i.e. of preferential hydration. These parameters are directly related to preferential binding of denaturant by [35,36]:

$$\xi_{21} = -(g_1/g_3)\xi_{23} \quad (16)$$

and

$$\Gamma_{21} = -(m_1/m_3)\Gamma_{23} \quad (17)$$

The corresponding values are listed in columns 5 and 7 of Table 3 and Table 4. At both pH values the experimentally measured preferential hydration is negative over the entire concentration range of urea.

The dependences of these parameters on urea concentration are shown in Fig. 3 in mass (gram) units and in Fig. 4 in molar units. As is evident from Fig. 3, the urea concentration attains a level of 50% mass fraction. Hence, the term co-solvent, as water and urea become equivalent in the solvent system [37,38]. Preferential binding at the two pH values follows strikingly different patterns. At pH 7.0, there is a monotone increase in ξ_{23} up to 0.35 g_3 (Fig. 3a), i.e. 4.5 M urea (Fig. 4), after which this parameter levels off at $\xi_{23} = 0.72 \pm 0.02$, i.e. $\Gamma_{23} = 17.0 \pm 0.5$. This is reflected by a corresponding monotone decrease in the preferential exclusion

[5] This eliminates protein-protein interactions and permits to treat the measurements as properties of a single protein molecule.

Table 4
Partial specific volumes and preferential interaction parameters of lysozyme with urea solutions at 20 °C, pH 2.0

Urea (M)	ϕ_2^o (ml/g)	$\phi_2'^o$ (ml/g)	ξ_{23} (g/g)	ξ_{21} (g/g)	Γ_{23} (mol/mol)	Γ_{21} (mol/mol)	$\left(\frac{\partial\mu_2}{\partial m_3}\right)_{T,P,m_2}$ [a]
0	0.718±0.001	0.718±0.001					
1.0	0.718±0.001	0.718±0.001					
1.5	0.713±0.001	0.712±0.002	+0.0042±0.006	−0.043±0.064	+1.00±1.5	−34±51	−326±489
1.75	0.714±0.001	0.711±0.001	+0.0128±0.004	−0.112±0.037	+3.04±1.0	−89±30	−848±283
2.0	0.714±0.001	0.709±0.002	+0.0217±0.007	−0.165±0.050	+5.16±1.6	−131±39	−1197±359
2.5	0.715±0.001	0.708±0.001	+0.0315±0.005	−0.187±0.027	+7.51±1.1	−149±21	−1322±189
3.0	0.717±0.001	0.708±0.001	+0.0421±0.005	−0.202±0.022	+10.02±1.1	−160±18	−1393±155
3.5	0.720±0.001	0.709±0.001	+0.0534±0.005	−0.215±0.020	+12.72±1.2	−171±16	−1450±132
3.75	0.718±0.001	0.706±0.001	+0.0594±0.005	−0.219±0.018	+14.13±1.2	−174±15	−1470±122
4.0	0.717±0.001	0.704±0.001	+0.0655±0.005	−0.224±0.017	+15.60±1.2	−178±14	−1482±114
4.5	0.716±0.001	0.701±0.002	+0.0787±0.008	−0.233±0.023	+18.73±1.9	−185±18	−1517±152
5.0	0.715±0.001	0.698±0.001	+0.0925±0.005	−0.239±0.014	+22.02±1.3	−190±11	−1563±92
5.5	0.715±0.001	0.697±0.001	+0.1018±0.006	−0.232±0.013	+24.24±1.4	−180±10	−1503±83
6.0	0.715±0.001	0.697±0.001	+0.1061±0.006	−0.215±0.012	+25.25±1.4	−170±9	−1364±76
6.5	0.714±0.001	0.696±0.001	+0.1105±0.006	−0.201±0.011	+26.31±1.5	−160±9	−1289±72
7.0	0.713±0.001	0.697±0.001	+0.1023±0.006	−0.167±0.010	+24.37±1.5	−133±8	−1048±66
7.5	0.715±0.001	0.701±0.001	+0.0934±0.007	−0.138±0.010	+22.23±1.6	−109±8	−867±62
7.75	0.715±0.001	0.703±0.001	+0.0818±0.007	−0.115±0.010	+19.47±1.6	−91±8	−720±60
8.0	0.716±0.001	0.705±0.001	+0.0766±0.007	−0.102±0.009	+18.23±1.7	−81±7	−638±58
8.5	0.715±0.001	0.704±0.001	+0.0800±0.007	−0.097±0.009	+19.04±1.7	−77±7	−609±55
9.0	0.716±0.001	0.706±0.001	+0.0760±0.008	−0.084±0.008	+18.10±1.8	−67±7	−525±53
9.5	0.715±0.001	0.705±0.001	+0.0797±0.008	−0.080±0.008	+18.96±1.9	−64±6	−493±49

[a] cal – (mol of urea)$^{-1}$ (mol of protein)$^{-1}$ in 1000 g H_2O.

of water.[6] At pH 2.0 the interaction pattern follows a much more complicated pattern. Following neutrality of interaction up to 1 M urea, preferential binding sets in with a steep dependence on the concentration of urea. It reaches a maximal value of $\Gamma_{23}=26.3$ at 6.5 M urea and then drops sharply until 7.75 M urea at which point it settles at a plateau. The observed similarity of the plateau Γ_{23} values to those measured at pH 7.0 must be regarded as a coincidence, because at pH 7.0 the protein is native at all urea concentrations, whereas at pH 2.0 it is native only below 2.5 M. Hence, the complex binding pattern between 5 and 8 M urea is a property of the denatured protein. As shown in Fig. 3b, this complex pattern is reflected in the preferential exclusion of water.

[6] This symmetrical behavior in the interactions of the protein with the ligand (urea) and water defines the epithet preferential [39,40]. Preferential binding of a solvent component means that the protein has greater affinity for that component than for the other one, even though both are entering into interactions with the protein.

As stated above, preferential binding of a ligand and, hence, preferential exclusion of water are representations in molecular terms of the mutual perturbations of the chemical potentials of protein and ligand, urea in the present case:

$$\Gamma_{23}=-(\partial\mu_3/\partial m_2)_{m_3}/(\partial\mu_3/\partial m_3)_{m_2} = -(m_3/m_1)\Gamma_{21} \quad (18)$$

As is evident from Eq. (18), the preferential exclusion of water, Γ_{21}, calculated from the preferential interaction experiments is not a property of water, but simply an expression in terms of water molecules of the perturbation of the chemical potential of the co-solvent by the protein when the last is introduced into the binary water–co-solvent system [30,34,39,40].

The chemical potential perturbation is presented in Fig. 5a, as $(\partial\mu_2/\partial m_3)_{m_2}=(\partial\mu_3/\partial m_2)_{m_3}$. At both pH values this parameter is negative, meaning that, at any given concentration of urea up to 9.3 M,

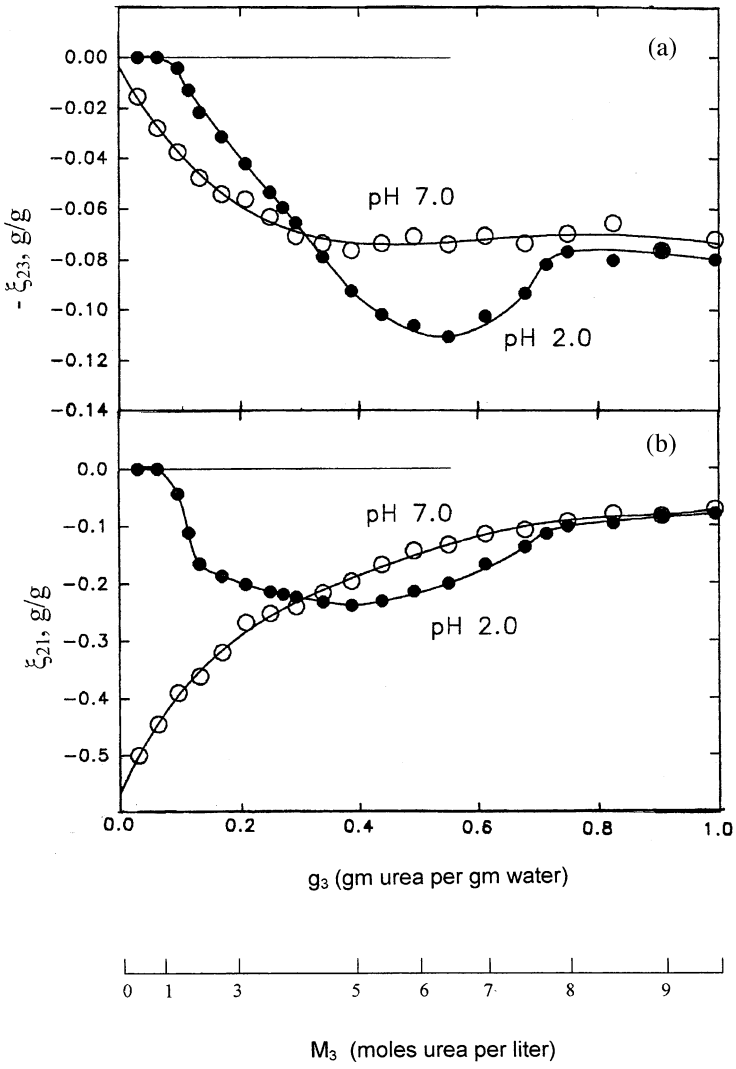


Fig. 3. Urea concentration dependence of: (a) the preferential binding of urea to lysozyme; (b) the preferential hydration of lysozyme. Both are expressed as grams of solvent component per gram of protein in one gram of water: (○) pH 7.0; (●) pH 2.0 (the molar concentration, M_3, is given for reference).

interaction between the protein and urea is thermodynamically favorable in the particular state (native or denatured) in which the protein is present under the experimental conditions. Integration under the curves of Fig. 5a Eq. (13) resulted in the transfer free energies, $\Delta\mu_{2,tr}$, relative to native protein in water as the reference state. The negative values of $\Delta\mu_{2,tr}$, shown in Fig. 5b, indicates that, at both pH values, the interaction with urea is more favorable than that with water in the folding state of the protein under the given conditions (pH and m_3). Comparison of Fig. 5b with Fig. 3a and Fig. 4 explains the complex pattern of interaction at pH 2.0. It is simply a reflection of the variation of the slope of the urea concentration dependence of $\Delta\mu_{2,tr}$ at pH 2.0, because Γ_{23} is a measure of the gradient of $\Delta\mu_{2,tr}$ with ligand concentration.

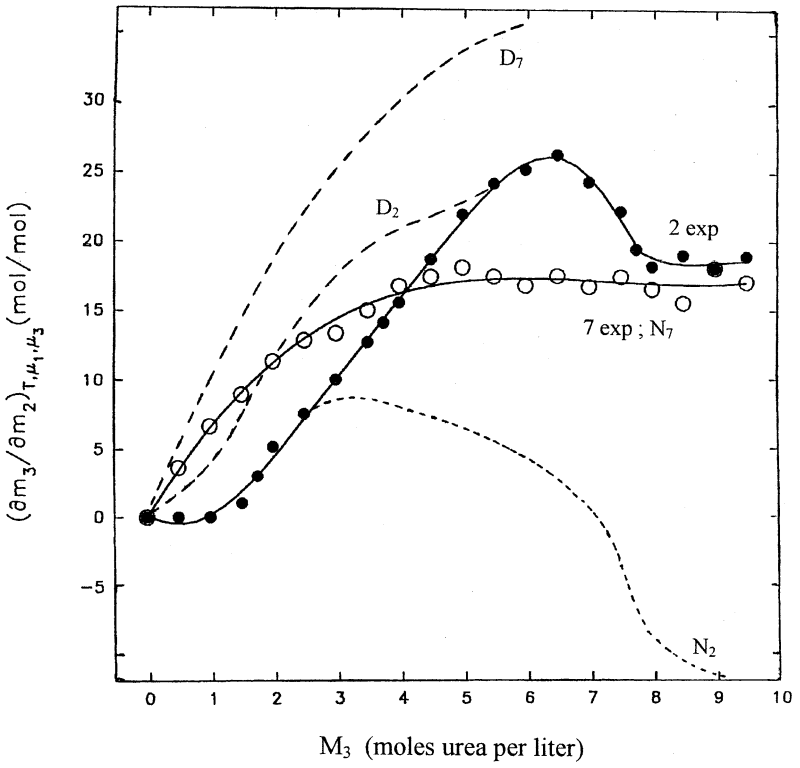


Fig. 4. Preferential binding of urea to lysozyme at pH 7.0 (○) and 2.0 (●) in molal units. The solid lines are the experimental results. The dashed lines (---) represent the calculated preferential bindings to denatured protein at pH 7.0 (D_7) and 2.0 (D_2). The dotted line is the calculated preferential binding to native protein at pH 2.0 (N_2); the preferential binding to native protein at pH 7.0 is identical with the experimental points. See text for details.

A better comparison between the transfer free energy and preferential interaction is afforded by the urea concentration dependence of the chemical potential perturbations, $(\partial\mu_2/\partial m_3)_{m_2}$, because the relation between $\Delta\mu_{2,\mathrm{tr}}$ and Γ_{23} is mediated by the non-ideality of the co-solvent, $(\partial\mu_3/\partial m_3)_{m_2}$. The values of this parameter, listed in the last column of Table 1, are a monotonely decreasing function of urea concentration. Comparison of the pH 2.0 $(\partial\mu_2/\partial m_3)_{m_2}$ and $\Delta\mu_{2,\mathrm{tr}}$ curves of Fig. 5a and b shows the close correspondence of their shapes. The steep decline of $(\partial\mu_2/\partial m_3)_{m_2}$ values at low concentrations reflect the increasingly negative slope of Fig. 5b, which reaches a maximal value at 5.0 M (6.5 m), at which point $(\partial\mu_2/\partial m_3)_{m_2}$ attains its apex. Above this solvent composition, the slope of the $\Delta\mu_{2,\mathrm{tr}}$ dependence decreases and attains a close to constant value above 8 M (12.5 m) urea. This is reflected in a decrease of the negative values of $(\partial\mu_2/\partial m_3)$, which become close to constant above 8 M urea. The far greater changes and sharp apex at 6.5 M urea in the preferential binding curve of Fig. 3b and Fig. 4 reflects division of the gradient of transfer free energy by $(\partial\mu_3/\partial m_3)_{m_2}$, which is a strongly declining function of M_3 (Table 1), e.g. it has values of 230 cal-mol^{-1} at 2 M urea, 95 at 4 M and 49 at 6.5 M.

The preferential binding measurements, when combined with the results of the denaturation experiments, make it possible to calculate the preferential binding of urea to denatured lysozyme by use of the relation that, at any urea concentration,

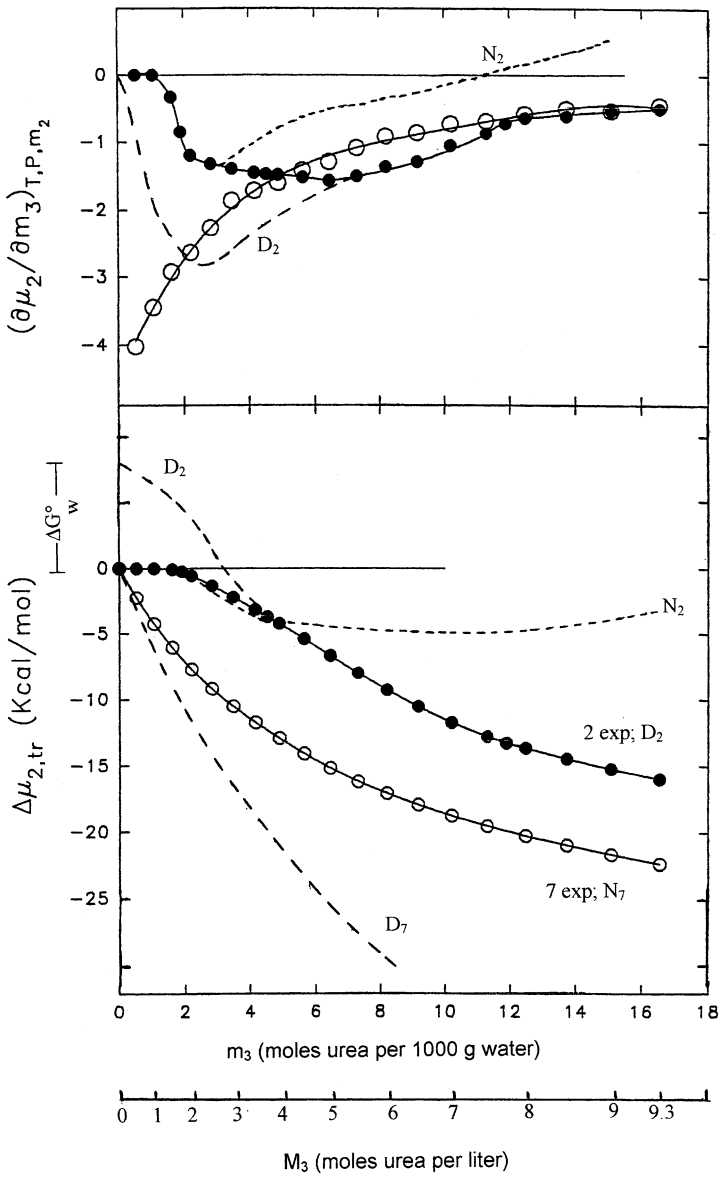


Fig. 5. (a) Variation with urea concentration of the perturbation of the chemical potential of lysozyme by urea, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$; (○) pH 7.0, the lysozyme is native over the entire urea concentration range (up to 9.3 M); (●) pH 2.0; the experimental curve is decomposed into contributions from native and denatured lysozyme. Note that in the transition region (2.5 to 5.0 M urea) both states of the protein contribute, as the experimental curve shifts from native protein (below 2.5 M urea) to denatured protein (above 5.0 M urea); this explains the flatness of the experimental curve. (b) Chemical potential change (transfer free energy) of lysozyme on transferring from water to an aqueous solution of urea. (○) pH 7.0; (●) pH 2.0. The dashed lines (---) are calculated for denatured protein at pH 7.0 (D_7) and pH 2.0 (D_2); the dotted line (….) is calculated for native protein at pH 2.0 (N_2). See text for details.

$$\Gamma_{23}^{D} = \Gamma_{23}^{N} + \delta\Gamma_{23} \quad (19)$$

At pH 7.0, the calculation is direct, because the protein is native up to the highest experimental concentration of urea. At pH 2.0, the situation is more complicated. Below the onset of denaturation at 2 M urea, the experimental values of Γ_{23} are for native protein and the values of Γ_{23}^{D} are obtained by addition of $\delta\Gamma_{23}$ to Γ_{23} (experimental). Above 5 M urea, the experimental values are for denatured protein. Hence Γ_{23}^{N} is obtained by subtraction of $\delta\Gamma_{23}$ from Γ_{23} (experimental). In the transition region, the experimentally measured preferential binding is the sum of contributions of both the native and denatured states, i.e.

$$\Gamma_{23}(\text{transition}) = \alpha\Gamma_{23}^{D} + (1-\alpha)\Gamma_{23}^{N} \quad (20)$$

where α is the degree of conversion in the $N \rightleftharpoons D$ equilibrium. Combination of Eq. (19) and Eq. (20) gives $\Gamma_{23}^{N} = \Gamma_{23}$ (transition)-$\alpha\delta\Gamma_{23}$ and $\Gamma_{23}^{D} = \Gamma_{23}$ (transition) $+ (1-\alpha)\delta\Gamma_{23}$

These calculations were carried out using values of $\delta\Gamma_{23}$ calculated with Eq. (15) and α from Fig. 1b. The results are shown in Fig. 4 by the dashed line for Γ_{23}^{D} at both pH values and the dotted line for Γ_{23}^{N} at pH 2.0. At pH 7.0, the preferential binding of urea to denatured protein increases monotonely over that to native protein. At pH 2.0, the pattern of interactions is complex. The preferential binding of the denatured protein is always positive: it increases in sigmoidal fashion up to a maximum at 6.5 M urea, after which it decreases and plateaus above 7.5 M urea. The preferential binding to the native protein follows a pattern that differs drastically from that measured at pH 7.0. The binding at first increases reaching a maximal value of 8 moles urea per mole protein at 3 M urea. It then follows a sigmoidal decrease and becomes negative above 7.5 M urea where it crosses the point of thermodynamic neutrality. Above that concentration urea is preferentially excluded, indicating that at pH 2.0, at high urea concentration, the native protein has a higher affinity for water than for urea, in the state of activity of the two solvent components at that solvent composition.

Preferential binding is an expression of the gradient of the transfer free energy of the protein from water to the solvent system of the given composition, $(\partial\Delta\mu_{2,\text{tr}}/\partial m_3)$. Urea concentration dependence curves of $\Delta\mu_{2,\text{tr}}$ were calculated at the two pH values for native and denatured protein. The integrated values of $(\partial\mu_2/\partial m_3)_{m_2}$ by Eq. (13) over the entire concentration range gave experimental transfer free energies $\Delta\mu_{2,\text{tr}}^{\text{exp}}$, as a function of M_3, without reference to the state of the protein (native or denatured). It must be emphasized that in this integration, the reference state is native protein in water, μ_w^N. In a case in which the protein undergoes a transition, below the transition the experimentally measured value is $\Delta\mu_{2,\text{tr}}^{\text{exp}} = \mu_{m_3}^{N} - \mu_w^N = \Delta\mu_{2,\text{tr}}^{N}$. When transition sets in, the denatured protein starts contributing. Above the transition, $\Delta\mu_{2,\text{tr}}^{\text{exp}}$ is an expression solely of the denatured state. By the nature of the operation, however, the quantity obtained is with reference to native protein in water, the zero point in Fig. 5a. True values of $\Delta\mu_{2,\text{tr}}^{D}$, however, must have denatured protein in water as reference state, μ_w^D. This imposes the requirement that, in the analysis of the integrated curve, the free energy of transition from native state in water to denatured state in water, $\mu_w^D - \mu_w^N = \Delta G_w^o$, must be taken into account properly. Then, if $\Delta\mu_{2,\text{tr}}^{D} = \mu_{m_3}^{D} - \mu_w^D$ is the transfer free energy of denatured protein with water as the reference state, the quantity calculated in the integration is $(\Delta\mu_{m_3}^{D} + \Delta G_w^o)$. From this it follows that

$$\Delta\mu_{2,\text{tr}}^{\text{exp}} = (1-\alpha)\ \Delta\mu_{2,\text{tr}}^{N} + \alpha(\Delta G_w^o + \Delta\mu_{2,\text{tr}}^{D}) \quad (21)$$

Then, with native protein in water as reference state,

$$\Delta\mu_{2,\text{tr}}^{N} = \Delta\mu_{2,\text{tr}}^{\text{exp}} - \alpha(\Delta G_w^o + \delta\Delta G^{\circ}) \quad (22)$$

and with denatured protein in water as reference state,

$$\Delta\mu_{2,\text{tr}}^{D} = \Delta\mu_{2,\text{tr}}^{\text{exp}} - \alpha\Delta G_w^o + (1-\alpha)\delta\Delta G^{\circ} \quad (23)$$

The values of $\Delta\mu_{2,\text{tr}}^{N}$ and $\Delta\mu_{2,\text{tr}}^{D}$ at pH 2.0 were calculated as a function of urea concentration with Eq. (22) and Eq. (23). The parameters used were: $\Delta G_w^o = 7.7$ kcal-mol^{-1}, $\delta\Delta G^{\circ} = -2.1\ M_u$, and α

values calculated from Fig. 1b. The results are presented in Fig. 5b, where the reference state is native protein in water, which is the starting experimental condition at which $\mu_2^N(\text{water})=0$. For denatured protein with the same reference state, $\mu_2^D(\text{water})=\mu_2^N(\text{water})+\Delta G_w^o$. Then, at any concentration of urea, m_3, the thermodynamic state of the denatured protein relative to the beginning of the process is

$$\mu_2^D=\Delta\mu_{2,\text{tr}}^N+\delta\Delta\mu_{2,\text{tr}}+\Delta G_w^o=\Delta\mu_{2,\text{tr}}^D+\Delta G_w^o \quad (24)$$

The resulting curves, plotted in Fig. 5b, can be compared now with the gradients of Fig. 5a. Remembering that $\Delta\mu_{2,\text{tr}}^D$ requires subtraction of $\Delta G_w^o=7.7$ kcal-mol^{-1} from the plot generated by integration shows that, in both states, the protein has a higher affinity for urea than for water over the entire concentration range. For native protein, $\Delta\mu_{2,\text{tr}}$ is a shallow function of urea concentration. Its shape, at first sigmoidal, then curving upward, is reflected by the variation of $(\partial\mu_2/\partial m_3)_{m_2}$, which at first decreases sharply and reaches a negative apex at 2.5 M urea, the point of the steepest slope of $\Delta\mu_{2,\text{tr}}^N$. At 7.5 M urea, $\Delta\mu_2^N$ reaches a shallow apex and reverses course, i.e. it assumes a positive slope. This is reflected by a change in the sign of $(\partial\mu_2/\partial m_3)_{m_2}$, as well as in the preferential interaction, Γ_{23}, shown on Fig. 4, which passes from positive, preferential binding, to negative, preferential exclusion, values. The concentration dependence of $\Delta\mu_{2,\text{tr}}^D$, similarly assumes progressively more negative values with a sigmoidal dependence on urea concentration, which gives rise to the observed gradient of Fig. 5a. Comparison of Fig. 5a with Fig. 4 shows clearly that the origin of the complex binding isotherms is found in the urea concentration dependence of the transfer free energies, with proper introduction of the gradient of urea non-ideality.

3.3. Thermodynamic parameters of preferential binding

The measurement of the transfer free energies of the native and denatured protein from water into urea solutions has given the destabilizing ability of the co-solvent, $\delta\Delta\mu_{2,\text{tr}}$. It should be recalled that preferential binding is a measure of the variation with co-solvent concentration of the partial molal free energy, $\overline{G}_2=\mu_2=(\partial G_2/\partial m_2)_{m_3}$. This, in turn, is related to the partial molal enthalpy, $\overline{H}_2=(\partial H_2/\partial m_2)_{m_3}$ and partial molal entropy, $\overline{S}_2=(\partial S_2/\partial m_2)_{m_3}$, by

$$\overline{H}_2=\overline{G}_2+T\overline{S}_2 \quad (25a)$$

and

$$\Delta\mu_{2,\text{tr}}=\Delta\overline{H}_{2,\text{tr}}-T\Delta\overline{S}_{2,\text{tr}} \quad (25b)$$

where $\Delta\overline{H}_{2,\text{tr}}$ and $\Delta\overline{S}_{2,\text{tr}}$ are the transfer enthalpy and transfer entropy of the protein from water into the urea solution.

Knowledge of $\Delta\overline{H}_{2,\text{tr}}$ and $\Delta\overline{S}_{2,\text{tr}}$ as a function of urea concentration should lead to a full thermodynamic description of the interaction of urea with the protein. To this end, preferential binding measurements were carried out at pH 7.0 as a function of temperature at four urea concentrations. The results are given inTable 5. Within the scattering of the points, it seems that $(\partial\mu_2/\partial m_3)_{m_2}$ is essentially independent of temperature up to 37 °C. The values become more negative at the higher temperatures at 6.0 and 8.0 M urea where there is incipient unfolding. This means that, within experimental error, $\Delta\mu_{2,\text{tr}}^N$ is also independent of temperature for native lysozyme. Therefore, the transfer entropy, $\Delta\overline{S}_{2,\text{tr}}=-(d\Delta\overline{G}_{2,tr}/\text{d}T)=0$, $\Delta C_{P,\text{tr}}\cong 0$ and $\Delta\overline{H}_{2,\text{tr}}^N$ is equal to $\Delta\overline{G}_{2,\text{tr}}^N$.

Combination of these values with the denaturation data of Table 2 permits the calculation of the transfer enthalpy of the denatured protein because, similarly to the free energy box, it is possible to construct an enthalpy box:

$$\Delta\overline{H}_{2,\text{tr}}^D=\Delta\overline{H}_{2,\text{tr}}^N+\delta\Delta H^\circ \quad (26)$$

where $\delta\Delta H^\circ=\Delta H_u^o-\Delta H_w^o$. Plots of the ΔH° values of Table 2 as a function of urea molarity resulted in straight lines at both pH values within the scatter of the points with identical slopes:

$$\Delta H_u^o-\Delta H_w^o=\delta\Delta H^\circ=-12.5M_u \quad (27)$$

Extrapolation of the data to zero urea concentra-

Table 5
Temperature dependence of the partial specific volumes and preferential interaction parameters of lysozyme with urea solutions at pH 7.0

Temp (°C)	ϕ_2^o (ml/g)	$\phi_2^{'o}$ (ml/g)	ξ_{23} (g/g)	ξ_{21} (g/g)	Γ_{23} (mol/mol)	Γ_{21} (mol/mol)	$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$ [a,b]
3.0 M Urea							
5	0.717±0.001	0.704±0.001	+0.0609±0.005	−0.291±0.022	14.48±1.1	−232±18	−1911±147
20	0.717±0.001	0.705±0.001	+0.0562±0.005	−0.269±0.022	13.37±1.1	−214±18	−1859±155
30	0.719±0.001	0.707±0.001	+0.0562±0.005	−0.269±0.022	13.37±1.1	−214±18	−1912±159
37	0.718±0.001	0.707±0.001	+0.0515±0.005	−0.247±0.022	12.26±1.1	−196±18	−1802±164
45	0.720±0.001	0.708±0.001	+0.0562±0.005	−0.269±0.022	13.37±1.1	−214±18	−2005±167
4.5 M Urea							
5	0.720±0.001	0.706±0.001	+0.0734±0.005	−0.217±0.016	17.48±1.3	−173±12	−1346±96
20	0.720±0.001	0.706±0.001	+0.0734±0.005	−0.217±0.016	17.48±1.3	−173±12	−1415±101
30	0.721±0.001	0.707±0.001	+0.0734±0.005	−0.217±0.016	17.48±1.3	−173±12	−1468±104
37	0.722±0.001	0.708±0.003	+0.0734±0.010	−0.217±0.031	17.48±2.5	−173±25	−1503±215
45	0.723±0.001	0.706±0.002	+0.0891±0.008	−0.264±0.023	21.23±1.9	−210±19	−1868±165
6.0 M Urea							
5	0.720±0.002	0.708±0.001	+0.0707±0.009	−0.144±0.018	16.83±2.1	−115±14	−858±107
20	0.719±0.001	0.707±0.001	+0.0707±0.006	−0.144±0.012	16.83±1.4	−115±10	−909±76
30	0.722±0.001	0.710±0.001	+0.0707±0.006	−0.144±0.012	16.83±1.4	−115±10	−942±79
37	0.722±0.001	0.710±0.001	+0.0707±0.006	−0.144±0.012	16.83±1.4	−115±10	−959±80
45	0.722±0.001	0.704±0.002	+0.1061±0.009	−0.216±0.018	25.25±2.1	−173±14	−1489±124
8.0 M Urea							
5	0.720±0.001	0.710±0.002	+0.0696±0.010	−0.093±0.014	16.58±2.5	−74±11	−547±82
20	0.720±0.001	0.710±0.001	+0.0696±0.007	−0.093±0.009	16.58±1.7	−74±7	−580±58
30	0.720±0.002	0.711±0.001	+0.0626±0.010	−0.084±0.014	14.92±2.5	−67±11	−537±90
40	0.719±0.002	0.703±0.001	+0.1114±0.010	−0.149±0.014	26.53±2.5	−118±11	−1008±95
50	0.715±0.002	0.682±0.002	+0.2297±0.014	−0.307±0.019	54.71±3.3	−244±15	−2134±129

[a] cal – (mol of urea)$^{-1}$ (mol of protein)$^{-1}$ in 1000 g H_2O.

[b] values of $\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$:

for 3 M urea solution: 132, 139, 143, 147 and 150 cal – (mol of Urea)$^{-2}$ in 1000 g H_2O at 5, 20, 30, 37 and 45 °C, respectively.
for 4.5 M urea solution: 77, 81, 84, 86 and 88 cal – (mol of Urea)$^{-2}$ in 1000 g H_2O at 5, 20, 30, 37 and 45 °C, respectively.
for 6.0 M urea solution: 51, 54, 56, 57 and 59 cal – (mol of Urea)$^{-2}$ in 1000 g H_2O at 5, 20, 30, 37 and 45 °C, respectively.
for 8.0 M urea solution: 33, 35, 36, 38 and 39 cal – (mol of Urea)$^{-2}$ in 1000 g H_2O at 5, 20, 30, 40 and 50 °C, respectively.

Table 6
Thermodynamic parameters of the urea destabilization of lysozyme at pH 7.0

Urea conc. M	$\Delta\overline{H}^N_{2,tr}$ kcal-mol^{-1}	$\Delta\overline{H}^D_{2,tr}$ kcal-mol^{-1}	$\Delta\overline{S}^D_{2,tr}$ e.u.	$\delta\Gamma_{23}$ mol-mol^{-1}	$\theta_3\delta n$ mol-mol^{-1}
3	−10.3	−48	−105	9.9	16.7
4.5	−13.9	−70	−161	14.9	23.1
6	−16.8	−92	−211	19.8	29.5
8	−19.7	−120	−282	26.4	37.6

tion gave $\Delta H_w^o = 150$ kcal/mol at pH 7.0. Application of Eq. (27) resulted in the values $\Delta H_{2,\mathrm{tr}}^D \Rightarrow$ listed in column 3 of Table 6. Finally, combination of these values with $\Delta\mu_{2,\mathrm{tr}}^D$ of Fig. 4 led to the $\Delta S_{2,\mathrm{tr}}^D$ values listed in column 4 of Table 6. These calculation show that the transfer of denatured lysozyme from water to urea is accompanied by enthalpy and entropy changes which are both increasingly negative with urea concentration, whereas transfer of the native protein is characterized by a much shallower negative enthalpy and close to zero entropy. Because of the paucity of temperature dependence data, however, the actual numbers listed in Table 6 should not be regarded as the intrinsic values of the parameters, but rather as indications of the order of magnitude of the thermodynamic parameters and the trends of their variations with denaturant concentration.

4. Discussion

4.1. Preferential binding is the manifestation of many weak interactions of the ligand with the protein

The preferential binding results demonstrate a complicated pattern of interactions. At pH 7, the immediate domain[2] of the protein is richer in urea than the bulk solvent both in the native and denatured states. In molecular terms, this means that, when averaged over its entire surface, the protein prefers to be in non-thermodynamically neutral contacts with urea rather than with water. This preference increases as the protein structure becomes loosened during denaturation. At pH 2.0 this holds true only for the denatured protein; for the native protein, preference of contacts shifts from urea to water at 7.5 M urea, above which urea is preferentially excluded. This, however, does not lead to stabilization of structure, as is the case with polyols and methyl amines [30], because, contrary to the situation with these stabilizing osmolytes which are excluded even more strongly from the unfolded form of the protein [41], urea remains preferentially bound to the denatured protein over the entire experimental range (9.5 M). In the context of preferential interactions, reference to protein–urea or protein–water contacts is simply descriptive. It does not imply any kinds of specific interaction at sites or complexation of solvent component molecules to the protein molecule. In fact, in order to interact a urea or water molecule does not need to be in contact with the protein surface, because any perturbations of the rotational or translational motions of a solvent component molecule by the protein gives an increment of interaction free energy, $\Delta\mu_{2,\mathrm{tr}}^{(i)}$, which is reflected in preferential binding whether positive or negative [33,39,40].

The surface of a protein molecule may be regarded as a mosaic of loci[7] of various chemical natures, -ionized, polar, non-polar, strongly hydrophobic, etc. Each locus exerts its idiosyncratic thermodynamic influence on neighboring water and co-solvent molecules, whether these make contacts with the protein surface or not. At each locus, *i*, this can be expressed by preferential binding, $\Gamma_{23}^{(i)}$, which may be positive, negative or neutral. The experimentally measured value is the sum of all such interactions, $\Gamma_{23} = \sum \Gamma_{23}^{(i)}$. At any locus, $P^{(i)}$, occupation by urea means displacement of water. Hence, the binding process means exchange with water molecules:

$$P^{(i)}\cdot(\mathrm{H_2O})_n + U \rightleftharpoons P^{(i)} \cdot U + n\mathrm{H_2O};\ K_{\mathrm{ex}} \qquad (28)$$

Therefore, the measured binding constant, K_b, is an exchange constant for the reaction of Eq. (28), $K_b = K_u/K_w = K_{\mathrm{ex}}$, where K_u and K_w are the binding constants of urea and water for the hypothetical equilibria of urea and water molecules with a dry site on a protein, $P^{(i)}$, in aqueous medium [39,40]:

$$P^{(i)}(\mathrm{dry}) + U \rightleftharpoons P^{(i)}\cdot U; \quad K_u \qquad (29)$$

$$P^{(i)}(\mathrm{dry}) + W \rightleftharpoons P^{(i)}\cdot W; \quad K_w \qquad (30)$$

From this, it follows that the measured standard free energy of binding is the difference between

[7] In this context, the term locus does not refer to a physically defined site on the protein surface. Locus means an element of volume that can contain a molecule of co-solvent or the corresponding number of water molecules. To be an interacting locus, the volume element does not need to make contact with the protein surface. The same qualifications apply to our usage of the term reacting site.

the free energies of binding of urea and water to dry protein in aqueous medium. Summed over the entire protein molecule, this gives [42]:

$$\Delta G^{o}_{\mathrm{ex}} = \Delta G^{o}_{u} - \Delta G^{o}_{w} \tag{31}$$

This exposition leads to the realization that $\Delta G^{o}_{\mathrm{ex}}$is the standard free energy change of transferring the protein from pure water to a urea solution of a given composition. Therefore,

$$\Delta G^{o}_{\mathrm{ex}} = \Delta \mu_{2,\mathrm{tr}} \tag{32}$$

The requirement of high concentrations of co-solvents, 1–9 M in the case or urea, to generate a measurable interaction and exert an effect on protein structure, signifies that the individual interactions are very weak. Typically, exchange equilibrium constants are of the order of 0.02–0.07 m^{-1} and the corresponding free energies of interaction per site are $\sim|0.05–0.1|$ kcal-mol^{-1} [37,43,44]. Therefore, a minute shift in the relative affinities of water and urea for some loci on the protein surface can lead to a shift in the measured preferential interactions. This is the most probable explanation of the marked difference between the preferential binding to native protein measured at pH 7.0 and 2.0. The experimentally measured binding and free energy values are summations of hundreds of very weak equilibria at individual sites, which explains the requirement of very high ligand concentration.

4.2. Measured Γ_{23} is the balance between a variety of general and specific interactions

In a series of important papers, Schellman [37,38,43,45,46] has analyzed the thermodynamics of preferential interactions in terms of the exchange concept and has described the strong influence of water and co-solvent non-ideality on the shape of the measured binding isotherms, such as shown in Fig. 3 and Fig. 4. Treating explicitly a one-to-one exchange at identical independent sites, Schellman has derived the relation between preferential binding, Γ_{23}, and the exchange constant, K_{ex}:

$$\Gamma_{23} = \frac{(K_{\mathrm{ex}}(f_3/f_1) - (1/m_1))m_3}{1 + K_{\mathrm{ex}}(f_3/f_1)} \tag{33}$$

where f_3 and f_1 are the mole fraction activity coefficients of urea and water. This relation permits to estimate the contribution of non-ideality of the solvent system to the shape of the preferential binding isotherm. This was calculated for the results at pH 7.0, using activity coefficient data of Scatchard et al. [24]. First, it must be noted that, in the water-urea system, water remains close to ideal ($f_1 = 1.022$ in 8 M urea). Urea, however, displays a strong non-ideality; at 8 M, $f_3 = 0.722$. Two binding isotherms were calculated for the simple exchange model. The first, which we will call *real*, was in terms of the full Eq. (33). In the second, which we will call *ideal*, the activity coefficient term, f_3/f_1, was neglected. The exchange constant used was that derived by Makhatadze and Privalov [28] from their description of their calorimetric study of lysozyme-urea interaction in terms of the binding at n identical independent sites, $K_{\mathrm{ex}} = 0.067$ m^{-1} at 20 °C. The ratio of the two isotherms, $R = \Gamma_{23}$ (ideal)$/\Gamma_{23}$ (real), is a measure of the contribution of solvent non-ideality to the shape of the binding isotherm. For aqueous urea this ratio reaches a value of 1.42 at 8 M urea. The Γ_{23} values measured at pH 7.0 were multiplied by this ratio, R. The results of the calculation, plotted as the dashed line in Fig. 6, clearly shows the major contribution, which non-ideality of solvent components can make to preferential binding. At this point, it must be stressed that the calculation was made for a very simple demonstration model which, as pointed out by Schellman, does not conform to any real case of protein–co-solvent interactions. First, in the real situation the sites are not independent. There must be cooperativity between interactions at neighboring sites, as well as between individual water and co-solvent molecules, both of which are in complex dynamic interactions in the bulk solvent and in the domain of the protein. Second, the exchange is not one-to-one, because co-solvent molecules are invariably larger than water. In the case of urea, the volume of one ligand molecule corresponds to essentially 2.5 waters. As pointed out by Kauzmann in 1948 (as quoted by Schachman

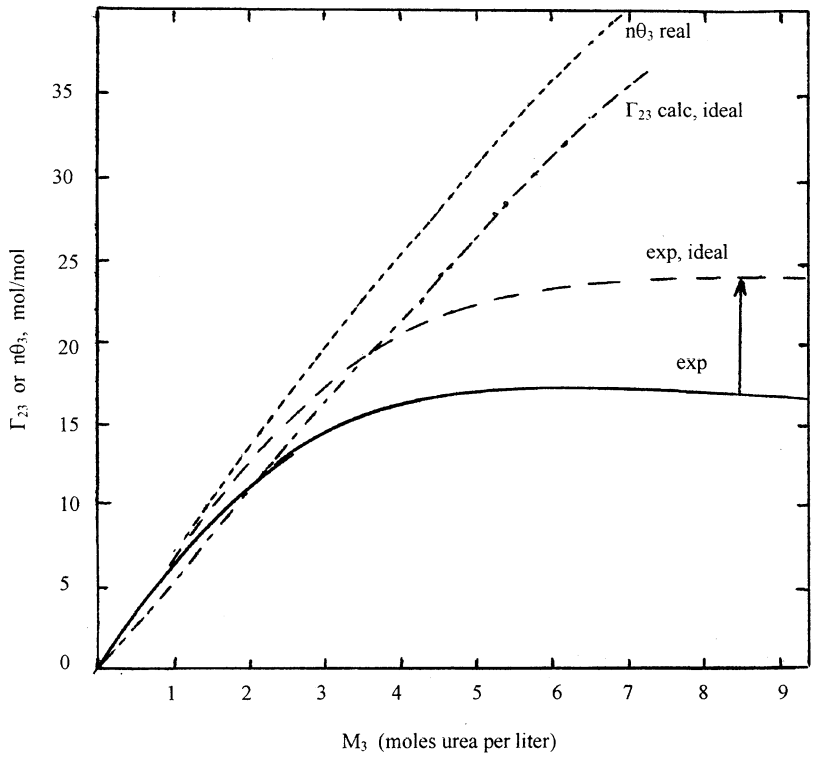


Fig. 6. Effects of urea non-ideality and site heterogeneity on the preferential binding of urea to lysozyme at pH 7.0, where the protein remains native over the entire concentration range of the denaturant (up to 9.3 M urea). Solid line: experimental curve (from Fig. 4). Dashed line: experimental curve that has been modified by subtracting the contribution of urea non-ideality; the difference between the dashed and solid lines represents the contribution of urea non-ideality to the preferential binding. Dot-dash line: Preferential binding calculated for n identical sites with a single binding constant and neglect of non-ideality. Dotted line: site occupancy by urea for n identical sites, $n\theta_3$ real, calculated with a binding constant corrected for non-ideality, $K_{ex}(f_3/f_1)$. See text for details.

and Lauffer [47]), the bulkiness of co-solvent molecules creates around a protein molecule a zone that is impenetrable to co-solvent, the thickness of which is determined by the distance of closest approach between protein and ligand molecules. This region can be penetrated by the smaller water molecules. Hence, it is enriched in water relative to bulk solvent. In preferential interaction measurements this manifests itself as a contribution to the preferential hydration of the protein [48,49]. In a recent analysis, Schellman [50] has pointed out that the relation between the measured preferential binding, Γ_{23}, and solvation at sites must take into account explicitly the excluded volume, because the measured thermodynamic interaction, Γ_{23}, represents the balance between the non-specific excluded volume, which always makes a negative contribution, and binding interactions specific to protein site–co-solvent pairs, which can be positive or negative. The parameters Γ_{23}, $\delta\Gamma_{23}$, $\Delta\mu_{2,tr}$, $\delta\Delta\mu_{2,tr}$ are true thermodynamic numbers: they sum all the various general and specific interactions. These parameters may be decomposed heuristically into contributions from ligand binding and ligand excluding events [51]. The present illustrative calculation of the contribution of solvent non-ideality to measure preferential binding, being relative (a ratio) rather than absolute, can serve to indicate the magnitude of that particular effect.

The complexity of the lysozyme–urea preferential interactions can be brought out further by comparing the present experimental results, corrected for non-ideality, with the results of the calorimetric study analyzed in terms of a simple Scatchard plot for n identical independent sites [28]. As pointed out by Schellman [45,46], such a Scatchard analysis gives only a description of the data in terms of an apparent dependence of site occupancy on urea concentration, and not a measure of the thermodynamic binding, Γ_{23}, because it neglects exchange with water, and uses only the first term of Eq. (33), when it is decomposed into site occupancies by urea and by water [37]:

$$\Gamma_{23}=\frac{K''m_3}{1+K''m_3}-\frac{m_3/m_1}{1+K''m_3}=\theta_3-\frac{m_3}{m_1}\theta_1 \qquad (34)$$

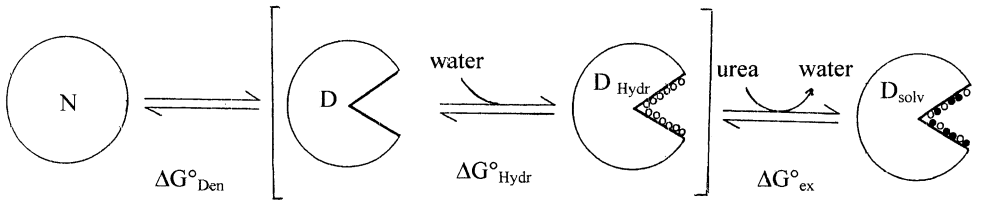
where $K''=K_{ex}(f_3/f_1)$, and θ_3 and θ_1 are the relative occupancies of the site by urea and water as a time average; $(\theta_1+\theta_3=1)$. To carry out the comparison, a binding isotherm was calculated for native lysozyme for the independent identical site model with neglect of the non-ideality in Eq. (33). The parameters used were those of the calorimetric study [28], $K=0.067\ m^{-1}$; $n=119$, where n is the total number of interacting sites. This is plotted as the dot-dash line of Fig. 6. Comparison with the experimental data adjusted for non-ideality (dashed line of Fig. 6) shows clearly that the isotherm calculated for the simple independent site model deviates drastically from the experimental measurements. The experimental curve flattens above 5 M urea, whereas that calculated for the simple model continues to rise. This deviation reflects the complexity of the interactions over the highly heterogeneous surface of the native protein, over which some individual loci preferentially bind urea with different affinities, others preferentially exclude it and some are indifferent to whether contact is with water or urea. This heterogeneity is greatly reduced in the differential binding, $\delta\Gamma_{23}$, which concerns only sites newly exposed on denaturation.

4.3. Thermodynamics of urea interactions with the protein during denaturation

The thermodynamic parameters reported in this paper are in general agreement with published data [10,11,28]. Extrapolation to pure aqueous medium gives values of $\Delta H^\circ=150$ kcal-mol^{-1} at pH 7.0 and 81.5 kcal-mol^{-1} at pH 2.0 and $\Delta S^\circ=460$ e.u. at pH 7.0 and 250 e.u. at pH 2.0, in agreement with the calorimetric values [28], while the concentration dependences of the enthalpy, -12.5 kcal-mol^{-1}-M_3^{-1}, and entropy, -36.5 e.u.-M_3^{-1}, are identical with those calculated from the calorimetric data, -13 kcal-mol^{-1}-M_3^{-1} for ΔH° and -35 e.u.-M_3^{-1} for ΔS°. Comparison of the thermodynamic parameters shows that a lowering of the pH from 7.0 to 2.0 destabilizes the protein by -12.3 kcal-mol^{-1} of free energy. This value of $\Delta G_w^o(2.0)-\Delta G_w^o(7.0)=\delta\Delta G_w^o(2.0-7.0)$ is in agreement with the known destabilization of globular proteins at low pH [52–54], as the average net protonic charge of lysozyme changes from $+7.5$ at pH 7.0 to $+17.5$ at pH 2.0 [55].[8]

The principal concern of the current study, however, is the effect of urea on the denaturation reaction. This means that we must deal with the stabilization of the denatured form by interaction with urea. We will approach the problem on the basis of the thermodynamic box (Eq. (10)). The effect of urea on denaturation is expressed by $\Delta G_u^o-\Delta G_w^o=\delta\Delta G^\circ$, which is equal to $\Delta\mu_{2,tr}^D-\Delta\mu_{2,tr}^N=\delta\Delta\mu_{2,tr}$. This analysis involves two assumptions: (1) That the conformational state of the native protein remains the same whether it interacts with urea or not; (2) That the necessary unfolding, or loosening, incurred in the $N\rightleftharpoons D$ reaction, takes place on denaturation in water and that all the new loci for interaction with urea created by denaturation become already available in water and are fully hydrated. This is depicted schematically in Fig. 7, in which the process is decomposed into three hypothetical steps: in the first step, the protein unfolds opening to the outside regions which were buried within the native struc-

[8] The electrostatic free energies, ΔG^e, of the native protein at the two pH values were calculated using the hydrated impenetrable sphere model with the charge smeared over the surface and Debye-Hückel screening [56]. The resulting values were: $\Delta G^e=3.8$ kcal-mol^{-1} at pH 7 and 20.8 kcal-mol^{-1} at pH 2. The difference, $\delta\Delta G^e=17$ kcal-mol^{-1}, seems consistent with $\delta\Delta G_w^o(2.0–7.0)$, in view of the simple model used and the neglect of any binding of anions to the protein at the acid pH.



$$\Delta G^\circ_{Den} + \Delta G^\circ_{Hydr} = \Delta G^\circ_{w} \quad ; \quad \Delta G^\circ_{u} = \Delta G^\circ_{w} + \Delta G^\circ_{ex}$$

Fig. 7. Schematic representation of the process of protein denaturation in the presence of urea. The process is decomposed into three hypothetical steps: (1) Denaturation with the opening in a dry state to the outside of regions, which are buried in the interior of the native structure; (2) Hydration of the new exposed surface; (3) Exchange of water of hydration for urea at the new exposed surface; (○): sites occupied by water; (●): sites occupied by urea.

ture; in the second step, this new surface becomes hydrated; and, in the last step, the water molecules on some of the sites are replaced with urea molecules. In the present study, we are dealing solely with the urea–water exchange, i.e. the last reaction in the scheme of Fig. 7. Therefore, the quantities, which interest us are $\delta\Delta G^\circ(w \rightarrow u)$, $\delta\Gamma_{23}(w \rightarrow u)$ and $\delta\Delta H^\circ(w \rightarrow u)$. The experimental results have shown that the dependence of $\delta\Delta G^\circ$ on the molar concentration can be represented by a unique straight line characterized by a slope of -2.1 ± 0.2 kcal-mol^{-1}-M_3^{-1}, for the measurements both at pH 7.0 and pH 2.0. The same is true of the variation of $\delta\Gamma_{23}$.[9] Examination of the urea concentration dependence of the standard enthalpy of denaturation (ΔH° of Table 2) again shows that values of this parameter decrease with an identical slope at pH 2.0 and 7.0 as a function of molar concentration of urea, $\Delta H^o_u = \Delta H^o_w - 12.5\ M_u$, in good agreement with the calorimetric data of Makhatadze and Privalov [28]. These observations seem to support the validity of the assumptions, at least as a first approximation within the uncertainty of the data, and to confirm that we are dealing with a single phenomenon in the various experiments. The essential constancy of the native conformation of the protein seems supported by the observation of a lack of any significant spectrophotometric variation on addition of urea up to 9 M at pH 7.0. The second assumption, that of the availability to solvent of the additional urea interacting loci already on denaturation in water, seems validated by the simplicity of the variations of $\delta\Delta G^\circ$, $\delta\Delta H^\circ$ and $\delta\Gamma_{23}$. This does not mean, however, that the denatured protein conformations are identical in water and concentrated urea. In a thoughtful examination of the states of denatured proteins in different environments, Pace and Shaw [57] observe that 'the denatured state ensemble that exists under physiological conditions is thermodynamically equivalent to the ensembles that exist after thermal or urea denaturation, even though they do not appear to be structurally equivalent.'

A striking characteristic of the effect of urea on the denatured state of the protein is the linearity in urea molarity of the thermodynamic parameters of the interaction, $\delta\Delta G^\circ$, $\delta\Delta H^\circ$, $\delta\Delta S^\circ$ and $\delta\Gamma_{23}$. As already noted in the Results section, the linearity of $\delta\Gamma_{23}$ can be explained by the coincidence of numerical values of urea molarity and molal activity, and the linearity of $\delta\Delta G^\circ$. In their examination of the meaning of the slope m in the relation $\delta\Delta G^\circ = mM_u$, Pace and co-workers [57,58] have found that the values of m vary linearly with

[9] One should remark that $\delta\Gamma_{23}$, which is the Wyman slope, is a continuous function of urea concentration. Usually it is reported at the mid-point of a transition. Its general definition, however, as the gradient of $\delta\Delta G^\circ$ with denaturant activity ($\ln a_3$), makes it possible to measure the change in preferential binding during denaturation from thermal transition information over a broad concentration range of denaturant under conditions at which no detectable transitions occur at room temperature.

the change in accessible surface area on unfolding. Hence, $\delta\Delta G^\circ$ can be expected to follow a similar monotone relationship. Elaborating on this finding, Courtenay et al. [59] have proposed that $\delta\Gamma_{23}$ is close to proportional to denaturant concentration on the basis of their conclusion that water occupancy (second term of Eq. (34)) is proportional to the accessible surface. The observation of Pace and co-workers [57,58] suggests strongly that the urea-water exchange is a thermodynamically homogeneous process over the newly exposed surface, i.e. that the exchangeable sites are thermodynamically similar, if not equivalent. This is supported by the linearity of $\delta\Delta H^\circ$. Schellman and Gassner [45] have shown that the enthalpy of binding is a response to the formation of protein site-ligand contacts, i.e. the first term of Eq. (34). Linearity of $\delta\Delta H^\circ$ in molar urea concentration suggests that these contacts are not highly heterogeneous; hence, an increase in urea concentration results in a monotonely increasing occupancy of available exchange sites. This is manifested by the observed negative increase in transfer free energy in linear fashion, i.e. relative to water as reference state the environment becomes progressively more favorable to the stability of the denatured state of the protein. The change in preferential binding, $\delta\Gamma_{23}$, however, is a derived quantity. It is a measure of the gradient of the transfer free energy with urea activity, with the solvent of the given composition as reference state. Therefore, knowledge of $\delta\Gamma_{23}$ at any given concentration of urea gives information neither on the thermodynamic interactions nor on the value of this parameter at any other solvent composition [38]. In the case of urea, $M_3 \approx a_3$. Therefore, if $\delta\Delta G^\circ$ and $\delta\Delta H^\circ$ are linear in M_3, $\delta\Gamma_{23}$ must also be linear in urea molarity.

4.4. What are the sites of urea interaction during denaturation?

At this point it seems of interest to reflect on the types of interactions of urea with the protein, which can generate the measured thermodynamic parameters. In the case of native lysozyme, the protein surface consists of a mosaic of a variety of chemical groups related to amino acid residues and peptide groups. These interact with urea in highly diverse ways, so that the measured transfer parameters are summations of favorable and unfavorable interactions of various affinities. Therefore, the low negative values of $\Delta H^N_{2,\mathrm{tr}}$ and a near zero $\Delta S^N_{2,\mathrm{tr}}$ reflect cancellations of positive and negative values for various amino acid residues that interact, some predominantly via hydrophobic forces, others by the formation of hydrogen bonds or by electrostatic effects or other polar interactions [60].

In the case of $\Delta H^D_{2,\mathrm{tr}}$, $\Delta S^D_{2,\mathrm{tr}}$, $\delta\Delta G^\circ$ and $\delta\Gamma_{23}$, we are dealing with the interactions of urea solutions with groups newly exposed to solvent. Subtraction of the transfer parameters for native protein eliminates the complex contributions of the native protein surface. Therefore, the measured differential values listed in Table 6 are strictly for urea-water exchange at newly exposed loci. These loci consist predominantly of peptide groups and non-polar residues. Interactions of these groups, hydrogen bonding in the case of peptide, hydrophobic in the case of non-polar, provide the stabilization of the compact native globular structure [61]. We are dealing, however, with destabilization and, by the nature of our measurements, we are concerned strictly with those groups that interact with urea, i.e. undergo the exchange reaction depicted in the last step of the scheme of Fig. 7. It is true that, on unfolding, other groups will also enter into interactions with water, as depicted in the second step of the scheme of Fig. 7. But, since they do not participate in the last exchange step, which is our particular concern, they need not be considered in the analysis. Urea is known to have a strong affinity for peptide groups [62–64]. This interaction has been attributed to the formation of hydrogen bonds [62] that replace peptide group-water hydrogen bonds in the already unfolded protein. Transfer free energy measurements [63,64] have shown that aromatic amino acid side chains are also good candidates for binding urea molecules. Both types of groups can be expected, then, to contribute to $\delta\Delta G^\circ$. The relative number of peptide and aromatic residue groups in the structure of the protein relegates the major contribution to peptide groups. The three tyrosine residues titrate normally in the native structure [55], which indicates at least partial exposure to solvent. Fur-

thermore, it is difficult to assess the contribution of the urea-water exchange at aromatic groups to the measured transfer enthalpy. Transfer of these groups to an aqueous medium is exothermic [60], but the heat of transfer from water to aqueous urea is not known. Schellman [65] has measured the thermodynamic parameters of inter urea hydrogen bond formation in aqueous medium. Kauzmann, in his classical article that for the first time presented systematically the thermodynamic contributions of various types of groups to protein stability, carried out a more detailed analysis of the data [60], which led to the estimations of $\Delta H \sim -1.5$ kcal-mol^{-1} and of $\Delta S \sim -3.3$ e.u. Normalization of the thermodynamic parameters of Table 6 with respect to $\delta\bar{\Gamma}_{23}$ gives $\delta\Delta\bar{H}_{2,tr}/\delta\Gamma_{23} = -3.9$ kcal-mol^{-1}, $\delta\Delta\bar{S}_{2,tr}/\delta\Gamma_{23} = -11.3$ e.u. While these values are consistent with the formation of hydrogen bonds, they are considerably higher than those deduced for urea dimerization. This may be due simply to the fact that urea-peptide and inter urea hydrogen bonds are highly different. Furthermore, the quantity used for normalization is an expression of the change in free energy over the entire ensemble of interacting loci, n. This contains contributions both from sites occupied by urea and by water as described by Eq. (34) for the simple independent site model. The absorption or emission of heat, however, mechanistically is caused by the formation of protein site-ligand contacts [45]. These are expressed by the first term of Eq. (34). The second term, which reflects occupancy of sites by water does not contribute to ΔH°, because for these sites all the contacts remain the same, whether co-solvent molecules are or are not bound to the protein at other sites. Similar considerations apply to the entropy change. Therefore, it seems more appropriate to carry out the normalization relative to occupation of sites by urea, i.e. $\theta_3 n$. To this end, the change in site occupancy during denaturation was calculated for a model based on the assumption that lysozyme denaturation generates 100 additional identical sites (δn) for interaction with urea [28]. Application of Eq. (33) with the parameter, $K''_b = K_b(f_3/f_1)$, where $K_b = 0.067$ m^{-1}, gave the numbers presented in column 6 of Table 6 as $\theta_3 \delta n$. Combination with the values of $\delta\Delta\bar{H}_{2,tr}$ and $\delta\Delta\bar{S}_{2,tr}$ resulted in a transfer enthalpy of ~ -2.5 kcal-mol^{-1} per site and a transfer entropy of ~ -7 e.u. per site.[10] While these values must be regarded strictly as descriptive, they nevertheless are consistent with those deduced by Schellman [65] and Kauzmann [60] for the dimerization of urea.

4.5. Peptide groups play a key role in protein stabilization and destabilization by co-solvents

We can conclude, therefore, that preferential binding measurements, when carried out over a broad concentration range of the co-solvent, can give a full thermodynamic description of the interactions of a denaturant with the unfolded (denatured) form of the protein. This is the reaction, which stabilizes the denatured form of the protein. Interactions with the native form are the necessary baseline, but they do not give any information on the destabilizing or stabilizing action of a co-solvent. It is interesting that, as seen in Fig. 4 and Fig. 5, all the complexities of the preferential binding isotherms are located in the interactions with the native state. They are translated into the isotherms of the denatured form. Subtraction of parameters for native protein from those for denatured protein gives smooth, in the case of urea linear functions. This supports the concept that the destabilizing action of urea lies in its interactions with very similar sites which appear to be primarily peptide groups with some contribution from aromatic residues.

The preceding analysis leads to the realizations that peptide group interactions with co-solvent molecules play a major role both in the destabilization and stabilization of globular proteins by co-solvents. Bolen and co-workers [66,67] have shown that the stabilizing action of preferentially excluded osmolytes is exercised through unfavorable interactions with peptide groups newly uncov-

[10] It must be cautioned that the quantities $\delta\Delta\bar{H}_{2,tr}/\theta_3 n$ and $\delta\Delta\bar{S}_{2,tr}/\theta_3 n$ are not thermodynamic quantities, because the site occupancies are not thermodynamic quantities [38]; $\delta\Delta\bar{H}_{2,tr}$ and $\delta\Delta\bar{S}_{2,tr}$, on the other hand, are true thermodynamic quantities.

ered during denaturation. In this case, the measured interaction is that of preferential exclusion of co-solvent rather than preferential binding. It is noteworthy that the corresponding values of $\delta\Delta G^{\circ}$ and $\delta\Delta\mu_{2,\mathrm{tr}}$, while opposite in sign, are of equal magnitude to those obtained with urea. For example, 0.9 M trehalose stabilizes ribonuclease A by 2.5 kcal-mol^{-1} against heat denaturation [41]; the destabilization of lysozyme by 1 M urea is -3.1 kcal-mol^{-1} (Table 2). This observation brings into focus again the fact that preferential binding and preferential exclusion are equivalent symmetrical phenomena [39,40] about a point of thermodynamic indifference (neutrality, inertness) [43]. Whether a co-solvent is preferentially bound or excluded is a reflection strictly of the magnitude of the exchange constant, K_{ex}. In the Schellman simple model, if $K_{\mathrm{ex}} > 0.18$ m^{-1}, the co-solvent is bound; if $K_{\mathrm{ex}} < 0.18$ m^{-1}, it is excluded.

As stated above, the measured values of the free energy of exchange per site are extremely weak, $\sim|0.05-0.1|$ kcal-mol^{-1}. Therefore, it takes only a small difference, ~ 0.1 kcal-mol^{-1}, between the affinities of two ligands (such as urea and trehalose) for a site on a protein molecule relative to the affinity of that site for water to make one preferentially excluded, $\Delta G^{o}_{\mathrm{ex}} > 0$, and the other preferentially bound, $\Delta G^{o}_{\mathrm{ex}} < 0$. Yet, the functional consequence is major: the first ligand is a structure stabilizer, the second ligand is a structure destabilizer, i.e. a denaturant. It is this small difference in their affinities for peptide groups, which locates these ligands on the two sides of the point of neutrality (inertness) [40]. Peptide groups, being the predominant entity newly exposed to solvent during protein unfolding to a disordered structure are, therefore, the primary targets for the action of urea and preferentially excluded osmolytes.[11] It is, therefore, the sign and cumulative strength of the interaction of the co-solvents with newly exposed peptide groups, which determines predominantly their stabilizing or destabilizing action. Interactions with other groups play a lesser role, while interactions with the native compact form are marginal to the stabilization/destabilization process.

[11] In a similar way, the transition of native globular protein to a helical denatured state in non-polar solvents (e.g. ethanol) is the consequence of the sum of the favorable interactions of non-polar residues with the co-solvent, which promotes their exposure to solvent and of the highly unfavorable interactions of peptide groups with such co-solvents which induces them to form inter-peptide hydrogen bonds that result in a helical structure [64].

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