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The Partial Specific Volume of β-Lactoglobulin A in Aqueous Urea Solutions

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The partial specific volume of β-lactoglobulin A in 0.02 *M* NaCl — 0.01 *M* HCl containing different amounts of urea has been determined from density measurements. The partial specific volume first increases with urea concentration, reaches a maximum, decreases, reaches a minimum, and then increases again. In the interpretation of this behavior, the binding of urea to the protein and the imperfect atomic packing in native protein molecules have been assumed to be the dominant factors. From dilatometric experiments the differences between the partial molar volume of the protein in 0.02 *M* NaCl—0.01 *M* HCl with and without urea have been obtained. The values of the differences agree satisfactorily with those calculated from the partial specific volume. Furthermore, the volumes as well as their changes reflect the interaction of urea with the protein. Dilatometric experiments were also performed with the protein in 0.02 *M* NaCl to which urea was added. Comparison of the obtained results with those in 0.02 *M* NaCl—0.01 *M* HCl displays the fact that the partial specific volume is *p*H-dependent.

INTRODUCTION

In a previous paper[1] the partial specific volume of chymotrypsinogen A in aqueous urea solutions has been determined. It has been shown that its values as well as the differences of the partial specific volumes in water and urea solutions, respectively, depend on urea concentration. This reflects conformational changes brought about by the interaction of urea with the protein and the imperfect atomic packing in the native protein. This paper reports on a similar study of the partial specific volume of β-lactoglobulin A. The purpose of the study has been to find out if a behavior similar to that of chymotrypsinogen would be observed with β-lactoglobulin, another globular protein. Previous studies of this protein[2] have shown that with increasing urea concentration the native form is transformed within a relatively narrow concentration range into the denaturated form. As in the case of chymotrypsinogen, this transition should be reflected in the values of the partial specific volume. Moreover, since the interaction of proteins with urea is not limited to the concentration range where denaturation occurs, a complete picture can only be obtained by determining the values of the partial specific volume of the protein from zero urea concentration to urea concentrations near saturation.

As previously[1], the partial specific volumes were determined from densities whereas the relative partial specific volumes were measured directly using the dilatometric method.

EXPERIMENTAL

Protein and Reagents

β-Lactoglobulin A was obtained from Research Products Div.-Miles Laboratories, Inc. It was used without further purification. The urea used in this study was a Riedel-de-Haën product. Before use, it was recrystallized twice from 80% w/v ethanol at 50° C, air dried, heated at 40° C for 24 hrs, and finally dried to constant weight over phosphorous pentoxide. Other reagents were the best available commercial products.

Preparation of Solutions

Solutions for density measurements were prepared by weighing the solvent, an aqueous solution being 0.02 *M* in NaCl and 0.01 *M* in HCl, into tightly closing plastic weighing bottles, and then an appropriate amount of urea was added and dissolved. Afterwards, a weighed amount of the protein was added. It was equilibrated previously with an atmosphere of 60% relative humidity at 25° C which were the relative humidity and temperature of the room where all the weighings and density measurements were performed. The water content of the protein was accounted for. It was determined by drying the protein over phosphorous pentoxide in vacuum at 78° C over 48 hrs[3]. Reference solutions did not contain the protein.

Solutions for dilatometric measurements were prepared on the molar scale. The solvent in most experiments was 0.02 *M* NaCl—0.01 *M* HCl. However, a few experiments were also performed with 0.02 *M* NaCl as solvent. The temperature was 25° C.

Density and Volume Measurements

Density measurements were performed with the prototype of the digital precision densitometer DMA-02, now manufactured by Anton Paar K. G., Graz, Austria, according to the design of Kratky *et al.*[4]. The details have been given in the previous paper[1].

Volume changes accompanying the interaction of urea with the protein were measured in Linderstrøm-Lang dilatometers. Into one arm of each dilatometer 0.980 ml. of 0.02 *M* NaCl—0.01 *M* HCl (or 0.02 *M* NaCl) or 0.980 ml. of a 6.6% solution of β-lactoglobulin A in that solvent were pipetted, and into the other arm 4.00 ml. of urea solution was added. Four dilatometers were used in each run: Two contained identical protein solutions, two reference solutions. After thermal equilibration, readings were recorded at minute intervals until practically constant values were obtained which took 15 to 30 min. All other details have been described previously[1].

RESULTS

As has been mentioned above, the solvent used in most experiments was a 0.02 *M* NaCl—0.01 *M* HCl solution to which urea was added. The *p*H of protein solutions after mixing was measured and found to be between 2.5 and 3.2 depending on the urea concentration. At these *p*H's the protein exists as a monomer[2] and changes of *p*H within this range do not result in conformational changes. In 0.02 *M* NaCl, where the *p*H is around 6.0, the protein may be partially associated which makes the interpretation of the observed results more difficult.

Density Measurements

The densities of solutions of β-lactoglobulin in 0.02 *M* NaCl—0.01 *M* HCl — urea are given in Table I. From the densities the apparent specific volume of the solute, Φ_v, can be obtained by use of the relation

$$\Phi_v = \frac{1}{w_2}\left(\frac{1}{\varrho} - \frac{1 - w_2}{\varrho_0}\right) \qquad (1)$$

where w_2 is the weight fraction of solute, ϱ the density of solution, and ϱ_0 the density of solvent. If the specific volume of the solute is concentration

independent, the apparent specific volume equals the partial specific volume, $\bar{v}_2$. Within the concentration range studied, *i. e.*, up to 4% protein concentration, such behavior has been observed, *cf.* Table I. Each series of experiments included five protein and one reference solution. The error involved in these determinations has been estimated at ± 0.003 ml/g[1] and therefore concentration dependence cannot be completely ruled out. The molecular weight of β-lactoglobulin A was taken to be 17.800.

TABLE I

Densities, Partial Specific, and Partial Molal Volumes of β-Lactoglobulin A (M. W. = 17,800) in 0.02 M NaCl—0.01 M HCl-Urea Solutions at 25° C

Solvent	w_2 [mg. LG/$g._{sol}$]	ρ [g./ml.]	$\bar{v}_2$ [ml./g.]
0.02 *M* NaCl—	0.000	0.998120	
0.01 *M* HCl	3.619	0.999022	0.752
	7.212	0.999924	0.751
	10.778	1.000814	0.752
	17.831	1.002580	0.752
	35.040	1.006920	0.752
			Mean 0.752 (13,386 ml./mole)
2.20 *m* urea (2.00 *M* at $w_2 = 0$) 0.02 *M* NaCl— 0.01 *M* HCl	3.198 to 31.081		Mean 0.753 (13,403 ml./mole)
4.89 *m* urea (4.00 *M* at $w_2 = 0$) 0.02 *M* NaCl— 0.01 *M* HCl	2.801 to 27.317		Mean 0.752 (13,386 ml./mole)
8.22 *m* urea (5.99 *M* at $w_2 = 0$) 0.02 *M* NaCl— 0.01 *M* HCl	2.427 to 23.745		Mean 0.745 (13,261 ml./mole)
12.63 *m* urea (8.03 *M* at $w_2 = 0$) 0.02 *M* NaCl— 0.01 *M* HCl	2.062 to 20.244		Mean 0.744 (13,243 ml./mole)
16.56 *m* urea (9.44 *M* at $w_2 = 0$) 0.02 *M* NaCl— 0.01 *M* HCl	1.819 to 17.895		Mean 0.745 (13,261 ml./mole)

Dilatometric Measurements

The volume changes observed upon mixing 0.980 ml of 0.02 *M* NaCl—0.01 *M* HCl (0.02 *M* NaCl) or 6.6% β-lactoglobulin in this solvent with 4.00 ml of appropriate urea solution are summarized in Table II. The procedure used in the analysis of data is the same as described previously[1]. The terms ΔV_1 and ΔV_2 in Table II represent the volume changes produced upon mixing 0.980 ml of 0.02 *M* NaCl—0.01 *M* HCl (0.02 *M* NaCl) or 6.6% protein in that solvent with 4.00 ml of urea in the same solvent which results in urea solutions of definite concentration. The term $(\overline{V}_{2u} - \overline{V}_2)$ is the difference between the partial molar volume in a given urea solution and the same quantity in 0.02 *M* NaCl—0.01 *M* HCl (0.02 *M* NaCl). As previously[1], the values of $(\overline{V}_{2u} - \overline{V}_2)$ have been obtained from the following relation

$$\Delta V_2 = \Delta V_{(v-\phi)} + n_2 (\overline{V}_{2u} - \overline{V}_2) \qquad (2)$$

where the term $\Delta V_{(v-\phi)}$ represents the volume change when $(v - \Phi)$ ml of solvent were added to 4.00 ml of urea solution, and n_2 the number of moles of protein. The quantity $(v - \Phi)$ is the difference between the volume of protein solution, 0.980 ml, and the protein's displacement volume, Φ, which is equal to the product of the protein weight, g_2, and its partial specific volume, $\overline{v}_2$. The value we have used for $\overline{v}_2$ is 0.752, *cf.* Table I.

The thermodynamic analysis leading to Eq. (2) in a three-component system has been given by Katz and Ferris[5] who assumed that the mixing of hydrated protein with either water or urea solutions is an athermal process. They also found that, within the limits of experimental error, this assumption is justified. Since the concentration of NaCl and HCl in our solvent is relatively small, Eq. (2) can be applied in the present case as well.

In our case $\Delta V_{(1-\phi)}$ can be calculated from the relation

$$\Delta V_{(1-\phi)} = \Delta V_1 \left(1 - \frac{4}{5} \Phi\right) \qquad (3)$$

derived from the equation relating the apparent molar volume of urea with concentration[1]. For each urea concentration six parallel experiments were performed. The experimental error was estimated at ± 20 ml./mole which essentially agrees with the value obtained by a detailed analysis[5].

The differences $(\overline{V}_{2u} - \overline{V}_2)$ in 0.02 *M* NaCl—0.01 *M* HCl can be obtained by substracting the partial molar volume of the protein in that solvent from the partial molar volume in 0.02 *M* NaCl—0.01 *M* HCl-urea. We are aware of the fact that the use of the term »molar« in a four- or five-component system is quite ambiguous. However, considering the fact that $\overline{V}_{2u}$ changes very little with urea concentration and that the final protein concentration in dilatometric experiments is relatively small, we can, as previously[1], expect that the values of $(\overline{V}_{2u} - \overline{V}_2)$ obtained in this way should compare favorably with those found in dilatometric experiments.

In Fig. 1 the differences $(\overline{V}_{2u} - \overline{V}_2)$ obtained by the two methods are plotted as a function of urea concentration. For comparison, a plot of $(\overline{V}_{2u} - \overline{V}_2)$ for protein solutions in 0.02 *M* NaCl has also been included.

TABLE II

Volume Change at 25° C as a Function of Urea Concentration

ΔV_1 refers to the addition of 0.980 ml. of solvent to 4.00 ml of urea solution; ΔV_2 refers to the addition of 0.980 ml. of 6.6% β-lactoglobulin A solution to 4.00 ml. of urea solution. In the last row the values of ΔV_1 and ΔV_2, respectively, refer to the addition of 0.980 ml. of 8 *M* urea and 0.980 ml. of 6.6% β-lactoglobulin A in 8 *M* urea to appropriate urea solution.

Equil. urea conc. [mole/l.]	$-\Delta V_1$ [μ l.]	$-\Delta V_2$ [μ l.]	$(\bar{V}_{2u}-\bar{V}_2)^a$ [ml./mole]
A.	Solvent: 0.02 *M* NaCl—0.01 *M* HCl		
2.0	0.65 ± 0.03	0.52 ± 0.03	29 ± 20
4.0	2.21	2.00	34
5.8	4.21	4.53	— 134
7.0	5.91	6.24	— 154
8.0	7.24	7.43	— 129
9.6	0.43	0.13	78
B.	Solvent: 0.02 *M* NaCl		
2.0	0.64	0.46	43
6.0	4.47	4.01	80
8.0	7.30	7.22	— 55
9.6	0.25	0.15	22

[a]In the calculations the partial specific volume of β-lactoglobulin with no urea present was taken to be 0.752 ml./g, cf. Table I, and the molecular weight 17,800.

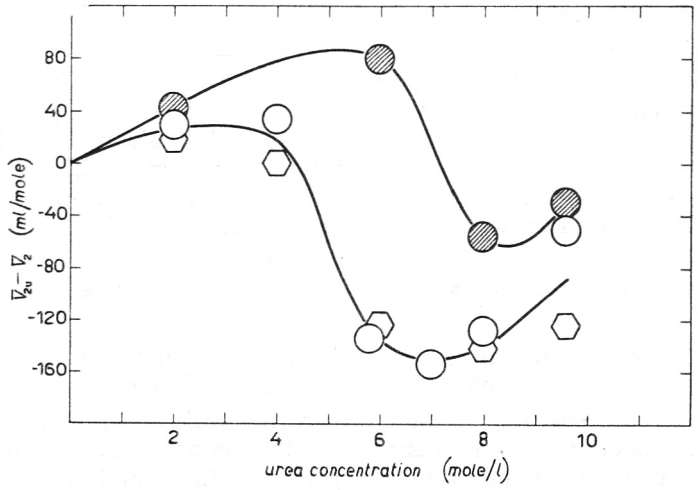

Fig. 1. Plots of the differences of the partial molar volumes of β-lactoglobulin A in urea solutions and 0.02 *M* NaCl—0,01 *M* or 0.02 *M* NaCl. ●: dilatometric measurements in 0.02 *M* NaCl; ○: dilatometric measurements in 0.02 *M* NaCl—0.01 *M* NaCl; *hexagons:* density measurements in 0.02 *M* NaCl—0.01 *M* HCl.

DISCUSSION

The shape of the curves in Fig. 1 is similar to that observed with chymotrypsinogen A[1]. Therefore an interpretation along similar lines appears to be justified.

The partial specific volume of a protein in solution is considered to be the sum of the constitutive volume, the voids due to imperfect atomic packing in protein molecules, and the volume of solvent incorporated into (or bound to) protein molecules[6]. We can assume that the constitutive volume is constant and therefore the volume changes observed are attributed only to the filling up of the voids during unfolding and to interactions of solvent with the protein.

The initial increase of the value of $(\overline{V}_{2u}—\overline{V}_2)$ can thus be attributed to the gradual competitive displacement of water molecules in hydration sheaths of ionic groups on the surface of protein molecules with urea molecules, which causes a reversal of the electrostriction effect. When the protein begins to unfold, *i. e.*, at urea concenration somewhere above 3 *M*, this increase is counteracted by gradual filling up of the hollows in protein molecules with solvent. The value of $(\overline{V}_{2u}—\overline{V}_2)$, after reaching a maximum, begins to decrease. When unfolding is completed, *i. e.*, when the protein is denaturated, the value of $(\overline{V}_{2u}—\overline{V}_2)$ reaches a minimum. The urea concentration is then about 7 *M*. Afterwards the value increases again which reflects additional binding of urea. The results of binding studies which are now in progress in our laboratory tend to support such an interpretation. This does not mean that other effects, *e. g.*, hydrophobic interactions, might not be operative. However, their contribution appears to be much smaller[1].

In contrast to chymotrypsinogen[1], the values of $(\overline{V}_{2u}—\overline{V}_2)$ are from 4.5 *M* urea on negative, the highest negative value being observed in 7 *M* urea. However, it is to be remembered, that the differences are to an unknown extent due to different solvation.

The reader will note that the curve for $(\overline{V}_{2u}—\overline{V}_2)$ in 0.02 *M* NaCl—0.01 *M* HCl has been drawn by considering the results from density as well as dilatometric measurements. In this way we have given equal weight to both sets of results. Inspection of the position of single points with respect to the curve, *cf.* Fig. 1, reveals that if two curves were drawn — one for each set of results — their course, except at high urea concentrations, would be nearly identical. Furthermore, considering the fact that the differences of $(\overline{V}_{2u}—\overline{V}_2)$ at single urea concentrations obtained by the two methods are smaller than the experimental errors involved, this procedure appears to be justified.

Having shown that the section of the curve between maximum and minimum encompasses the concentration range of urea where unfolding, *i. e.*, denaturation, proceeds and within which the values of $(\overline{V}_{2u}—\overline{V}_2)$ change relatively considerably, it is instructive to compare the volume changes with the changes of some other property during unfolding. For such a comparison the data of Pace and Tanford[2] who also studied the urea denaturation of β-lactoglobulin A under conditions very similar to ours are very appropriate. They measured the dependence of optical rotation on urea concentration. From their Fig. 1 it can be inferred that unfolding begins somewhere above 3 *M* urea and is concluded at 7 *M* urea which is in agreement with our findings. This is not surprising, since if the change of a property reflects only unfolding or if unfolding is the dominant factor contributing to that change, such a behavior is to be expected. Namely,

both properties, volume and optical rotation, depend on the extent of unfolding.

The second curve in Fig. 1 was obtained from the values of $(\overline{V}_{2u}-\overline{V}_2)$ at *p*H's around 6.0. The curve is distinctly different from the first and clearly displays the importance of *p*H in unfolding: The positions of maximum and minimum have been shifted towards higher urea concentrations which reflects a higher stability of the native form near the isoelectric point. The form of the curve, on the other hand, is very similar to that for low *p*H. Furthermore, it is to be noted that the measurements of density as well as of volume changes were limited to relatively short times. It is well possible, however, that in the transition region, 3 *M*—7 *M* urea, the equilibrium state was not reached[7] and the values of $(\overline{V}_{2u}-\overline{V}_2)$ obtained correspond to metastable states.

As has been mentioned above, a similar behavior has been observed with bovine serum albumin[5] and chymotrypsinogen A[1]. One would be tempted now to make the generalization that all globular proteins behave similarly. The differences between single proteins are due to different composition and structure. However, the recent paper of Katz and Denis[8] in which the results of a similar study of myoglobin and apomyoglobin are given suggests some precaution. Namely, it has been shown that myoglobin behaves very much differently. However, it is quite possible that the anomaly observed is associated with the presence of the heme group in myoglobin.

Let us, finally, say a few words about the absolute values of the partial specific volume. The value in 0.02 *M* NaCl—0.01 *M* HCl, 0.752, is very close to that reported in the literature, 0.751[9]. The value does not depend much on urea concentration which results in small values of $(\overline{V}_{2u}-\overline{V}_2)$.

In conclusion, the statement can be made that in protein solutions containing urea volume changes are indicative of unfolding brought about by urea as well as its interactions with proteins. Unfortunately, the changes are very small so that even with the most sensitive methods for volume measurements the error involved is relatively large. Therefore for denaturation studies other methods, *e. g.* optical ones, are more useful. However, the general picture obtained is in complete agreement with that reached by other methods.

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REFERENCES

1. J. Škerjanc, V. Doleček, and S. Lapanje, *Eur. J. Biochem.* **17** (1970) 164.
2. N. C. Pace and C. Tanford, *Biochemistry* **7** (1968) 198.
3. M. J. Hunter, *J. Phys. Chem.* **70** (1966) 3285.
4. O. Kratky, H. Leopold, and H. Stabinger, *Z. angew. Phys.* **27** (1969) 273.
5. S. Katz and T. G. Ferris, *Biochemistry* **5** (1966) 3246.
6. W. Kauzmann, *Adv. Protein Chem.* **14** (1959) 1.

7. L. K. Christensen, *Compt. rend. trav. lab. Carlsberg Sér. chim.* **28** (1952) 37.
8. S. Katz and J. Denis, *Biochim. Biophys. Acta* **207** (1970) 331.
9. K. O. Pedersen, *Biochem. J.* **30** (1936) 961.

IZVLEČEK

Parcialni specifični volumen β-laktoglobulina A v vodnih raztopinah sečnine

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Iz merjenih gostot je bil izračunan parcialni specifični volumen β-laktoglobulina A v 0.02 *M* NaCl—0.01 *M* HCl, ki je vsebovala različne množine sečnine. Parcialni specifični volumen najprej narašča s koncentracijo sečnine, doseže maksimum, pada, doseže minimum in nato spet narašča. To obnašanje je moč razločiti z vezanjem sečnine na protein in nepopolnim skladom atomov v nativnem proteinu. Iz dilatometričnih poskusov so bile dobljene razlike med parcialnim molskim volumnom proteina v 0.02 *M* NaCl—0.01 *M* HCl z in brez sečnine. Vrednosti teh razlik se zadovoljivo ujemajo s tistimi, ki so bile izračunane iz parcialnega molskega volumna. Nekaj dilatometričnih poskusov je bilo tudi narejenih s proteinom v 0.02 *M* NaCl. Dobljeni rezultati pokažejo ob primerjavi z rezultati v 0.02 *M* NaCl—0.01 *M* HCl važnost *p*H za potek denaturacije s sečnino.

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