The Size and Shape of Bovine Serum Albumin in Acidic Water–Dioxane Mixtures

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Abstract: The effect of dioxane on the conformation of bovine serum albumin in aqueous solutions at pH 2.0–2.2 and ionic strength 0.03 was studied by means of sedimentation and viscosity measurements. The results were interpreted in terms of the shape factor β and the end-to-end root mean square distance $\langle r^2 \rangle^{1/2}$. Dioxane enhances the expansion of the protein molecule in the acidic aqueous solution, but causes the decrease of its effective volume. These findings are consistent with the observations of the behavior of the protein in aqueous solutions containing other denaturing agents.

The molecular weight of bovine serum albumin (BSA) is shown to be constant in acid, base, as well as in urea and organic aqueous solutions.[1–3] Thus, the change of the hydrodynamic and thermodynamic properties of the protein with environment may reasonably be ascribed to the change of the size and shape of the molecule. In a previous publication[3] it was reported that while dioxane slightly affects the conformation of the molecule in the vicinity of the isoelectric point, it causes appreciable structural change in the acidic region. The change reaches a maximum at pH 2.0–2.2. Further studies[4,5] demonstrated that BSA in that pH range is no longer in compact form as it is in the pH range between 4.3 and 10.5 and that part of the polypeptide chain unravels, particularly in the presence of the organic molecule. The purpose of the present investigation is to ascertain the shape and size of the BSA molecule in water–dioxane mixtures near pH 2.0 at the ionic strength 0.03 by sedimentation and viscosity measurements. The experimental data are interpreted in terms of two parameters: β (the shape factor proposed by Scheraga and Mandelkern[6]) and $\langle r^2 \rangle^{1/2}$ (the end-to-end root mean square distance). Since each parameter requires a different model to formulate the theory, it is difficult to decide which model is applicable to the protein in dilute solutions. Our approach is to take each model as a limiting case of the conformation of the protein and assess its applicability to our system. The combination of the two parameters provides an overall picture of the change of the shape and size of the BSA molecule in water–dioxane mixtures.

Experimental Section

Materials. The protein used was Armour crystallized product without purification. The lot numbers were V68802 and A70011. Two methods were used for the determination of the moisture content in the samples, micro-Kjeldahl analysis and constant dry weight measurement. The 1,4 dioxane was purified by distilling over sodium. All the other chemicals were reagent grade and used without further purification. Glass-distilled water was used in all the experiments.

Preparation of Solutions. Each BSA solution was made up by dissolving the required amount of crystallized BSA in the specified water–dioxane mixture. The pH of the solution was adjusted to 2.2 using 1 *N* HCl in the same specified water–dioxane mixture and the ionic strength was adjusted to 0.03 by adding KCl. The HCl solution was standardized potentiometrically against Na_2CO_3. The concentration of dioxane was made in terms of v/v (the volume of dioxane in 100 ml of water–dioxane mixture). The solution was contained in a volumetric flask to prevent the dioxane from evaporating. The protein concentration of each solution was about 1.270 g/100 ml. The solvent mixture for dilution in the viscosity measurements was prepared in the same way except that there was no BSA present. Both the BSA solution and the solvent mixture were pressure filtered through sintered glass before use. The loss of BSA due to the filtration was determined spectrophotometrically, using $E_{1\text{ cm}}^{1\%} = 6.67$ at 279 mμ.[7] Sedimentation and viscosity measurements were usually performed within an hour after the solution was prepared. In such an acidic medium the solution was clear and could stand for more than a month in a cold room without precipitation.

Sedimentation Velocity. The sedimentation velocity measurements were performed in a Spinco Model E analytical ultracentrifuge, equipped with phase plate and rotor temperature indicator and control unit. The temperature was set at 20° and kept constant. The rotor speed was set at 56,100 rpm for each run and was checked regularly. Photographs were taken at 16-min intervals. Since acid might attack aluminum alloy cells, the cell used was equipped with a centerpiece of Kel-F.

Viscosity Measurements. Relative viscosities of BSA were determined at 25 ± 0.02°, using a Cannon-Ubbelohde No. 50 viscometer. The setup of the viscometer was exactly the same as described previously.[3]

Results and Discussion

The values of the sedimentation coefficients were corrected to the reference state (water at 25°) according to the following equation

$$S_{25,w} = S_{t,m} \frac{\eta_{t,m}}{\eta_{25,m}} \frac{\eta_{25,m}}{\eta_{25,w}} \frac{(1 - \bar{v}\rho)_{25,w}}{(1 - \bar{v}\rho)_{25,m}} \frac{(1 - \bar{v}\rho)_{25,m}}{(1 - \bar{v}\rho)_{t,m}} \quad (1)$$

where t is the temperature of the experiment, η the viscosity, $\bar{v}$ the partial specific volume, ρ the density, and the subscripts m and w refer to the solvent mixture and water, respectively.

The density data were calculated by the equation

$$\rho = \rho_0 + \frac{1 - \bar{v}\rho_0}{100} C$$

(1) W. F. Harrington, P. Johnson, and R. H. Ottewill, *Biochem. J.*, **62**, 569 (1956).

(2) H. Neurath and A. M. Saum (*J. Biol. Chem.*, **128**, 347 (1939)) studied the denaturation of horse serum albumin in concentrated urea solutions and found that the molecular weights of native and denatured serum albumins are the same. Since there is no basic difference in properties between bovine and horse serum albumins, it is believed that the molecular weight of BSA likewise remains the same in the urea solutions.

(3) K. E. Van Holde and S. F. Sun, *J. Amer. Chem. Soc.*, **84**, 66 (1962).

(4) S. F. Sun, *Arch. Biochem. Biophys.*, **129**, 411 (1969).

(5) S. F. Sun, *Biochim. Biophys. Acta*, **181**, 473 (1969).

(6) H. A. Scheraga and L. Mandelkern, *J. Amer. Chem. Soc.*, **75**, 179 (1953).

(7) M. D. Sterman, Ph.D. Thesis, Purdue University, 1955.

where ρ_0 is the density of the solvent and C is the concentration of the solution in g/100 ml. The density of solvent data ρ_0 and the value of the ratio $\eta_{t,m}/\eta_{25,m}$ were obtained from Geddes.[8] The value of $\eta_{25,m}/\eta_{25,w}$ was determined experimentally. The value of $\bar{v}$ was taken to be 0.734 for BSA in water–dioxane mixtures.

For a three-component system (water (1), protein (2), dioxane (3)), eq 1 in general is not valid, for it neglects the correction terms for flow interactions. On the basis of the thermodynamics of irreversible processes, an equation[9] has been derived for a three-component system

$$S_2 = \frac{L_{22}(1 - \bar{v}_2\rho)}{C_2}\left(1 + \frac{L_{23}(1 - \bar{v}_3\rho)}{L_{22}(1 - \bar{v}_2\rho)}\right)$$

where L_{22} and L_{23} are the phenomenological coefficients relating flows to driving forces and ρ is the solution density. However, using water–dioxane mixture as solvent, the correction term $L_{23}(1 - \bar{v}_3\rho)/L_{22}(1 - \bar{v}_2\rho)$ is small and, for the first approximation, it is negligible. This is because the partial specific volume of dioxane in water is close to unity,[8] and the quantity $(1 - \bar{v}_3\rho)/(1 - \bar{v}_2\rho)$ is not more than 0.15. (For example, in 40% dioxane and at 25°, $\bar{v}_2$, = 0.734, $\bar{v}_3$ = 0.969, ρ = 1.0099, the ratio = 0.12.) The ratio L_{23}/L_{22} is usually less than unity. Therefore, the first term $L_{22}(1 - \bar{v}_2\rho)/C_2$ which varies with solvent environment and temperature is sufficient to treat our data and is accounted for by eq 1.[3]

The experimental results are listed in Table I. The pH value of the BSA solution in the presence of dioxane was corrected according to the equation[10]

$$-\log [H^+] = R + \log U_H$$

where R is the pH-meter reading and U_H is a conversion term which is a function of solvent composition. The corrected pH values are listed in column 2 of Table I.

Table I. Measurements on BSA in Acidic Water–Dioxane Mixtures at μ = 0.03

% dioxane	pH	$S_{25,w} \times 10^{13}$	$[\eta]$	$100\eta_0$[a]
0	2.20	2.25 ± 0.02	0.170 ± 0.003	0.893
15	2.12	2.09 ± 0.01	0.198 ± 0.005	1.190
30	2.12	1.98 ± 0.02	0.231 ± 0.005	1.502
40	2.12	2.06 ± 0.01	0.253 ± 0.005	1.705

[a] Reference 8.

Experiments were carried out for the determination of sedimentation coefficient as a function of the concentration of protein solution. It was found that for BSA in water–dioxane mixtures, whether at the isoelectric point as reported in the previous publication[3] or in the acidic region as reported in this communication, there is little change in the value of sedimentation coefficient with concentration. Therefore, the S values are not extrapolated to zero concentration.

The sedimentation data in the acidic region between 0 and 30% of dioxane can be described by a linear equation

$$S_{25,w} = -0.009P_D + 2.24$$

where P_D is the per cent of dioxane in the mixture. The negative slope indicates the change in conformation, *i.e.*, the increase in the radius of gyration or the increase in the end-to-end distance. The increase in intrinsic viscosity is parallel to the decrease in sedimentation coefficient as the concentration of dioxane increases in the solvent medium.

To interpret the data of BSA in acidic water–dioxane mixtures, we consider two different but related models for the conformation of the molecule.

(1) Effective Hydrodynamic Ellipsoid. In this connection the shape factor β is calculated by the Scheraga–Mandelkern equation

$$\beta = \frac{NS[\eta]^{1/3}\eta_0}{M^{2/3}(1 - \bar{v}\rho)} \quad (2)$$

where N is the Avogadro's number, $[\eta]$ the intrinsic viscosity of the solution, η_0 the viscosity of the solvent, and M the molecular weight of the solute. The molecular weight of BSA was taken to be 66,500 in all the calculations. Like eq 1, eq 2 is valid for a two-component system. Since dioxane–water mixtures provide an exceptionally advantageous case in that the partial specific volume of dioxane in water is near unity as discussed before, we assumed that the effect of mixed solvent on $\bar{v}$ is negligible and that eq 2 is sufficient to interpret our data for the first approximation.

(2) Flexible Chain-Like Form. Calculations are made to investigate the effect of dioxane on the root mean square distance from one end to the other, $\langle r^2\rangle^{1/2}$. The equation used for calculation was derived by Flory and Fox[11]

$$\langle r^2\rangle^{1/2} = (M[\eta]/\Phi)^{1/3}$$

where Φ is a shape factor which is independent of the solvent. Thus the $\langle r^2\rangle^{1/2}$ value is related to one hydrodynamic quantity only, $[\eta]$, instead of two. The value of Φ for the polystyrene was estimated by Flory and Fox[12] to be 3.6×10^{21} (according to the theory of Kirkwood and Riseman[13]), or 2.1×10^{21} (on the basis of the light-scattering data obtained by Zimm and coworkers[14]). Wasserman[15] used the value of 2.6×10^{21} for myosin in aqueous dilute solutions. We chose the value 2.6×10^{21} for our calculations.

The calculated shape and size parameters are listed in Table II.

Table II. Calculated Values of Shape and Size Parameters of BSA

% Dioxane	$\beta \times 10^{-6}$	$\langle r^2\rangle^{1/2}$, A
0	1.51	163
15	2.04	171
30	2.66	181
40	3.30	186

The value of β is doubled from 0 to 40%, an evidence of the increase in axial ratio as the environment

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(10) L. G. Van Utiters and C. G. Haas, *J. Amer. Chem. Soc.*, **75**, 451 (1953).
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of the protein molecule changes. The disturbing fact is the low values of β for BSA in 0% as well as in 15% dioxane solutions. The values of β for BSA in aqueous solutions at pH range 4.3–10.5 were also found low in other laboratories,[16,17] but our value for BSA at pH near 2.0 is even lower than those hitherto reported. The interpretation of the low value of β ($<2.12 \times 10^6$) is somewhat controversial.[18] In view of the models recently proposed by some investigators,[19–22] it seems that the discrepancy may arise from a lack of internal rigidity of the protein molecule.[23] The protein molecule in the native state is not in the form of a perfect prolate ellipsoid. As the pH of the solution decreases, the molecule departs further from the ellipsoidal conformation.

On the other hand, it is highly possible that the presence of dioxane affects the electrostatic force which is already off-balanced due to the removal of negative charge on the surface of the molecule. This results in an adjustment of conformation. The β values indicate that in the acidic water–dioxane mixture the BSA molecule adapts toward the form of prolate ellipsoid.

It is well known that the value of β is related to that of ν, another shape factor, in such a way that as β increases, ν increases exponentially. For example, for $\beta = 2.66 \times 10^6$, $\nu \cong 44$; and for $\beta = 3.30 \times 10^6$, $\nu \cong 980$. If we compare the change in ν with the change in $[\eta]$, we find that the increase in ν is much faster than in $[\eta]$ as the concentration of dioxane increases. A simple calculation using the following equation[18]

$$[\eta] = \left(\frac{N}{100}\right)\left(\frac{V_e}{M}\right)\nu$$

where N is the Avogadro's number and M is the molecular weight, shows that the effective volume V_e decreases as the concentration of dioxane increases. These findings are consistent with the observations of the behavior of BSA in acid as well as other solutions containing a denaturing agent.[24,25]

In the calculations of the end-to-end root mean square distance, it is assumed that the protein molecule exists in the form of flexible chains which is capable of changes in conformation under appropriate environments. The justification for using this model is that in the acidic region at least 35% of the polypeptide of BSA molecule unravels and the internal structure of the molecule becomes less rigid. The increase in the end-to-end distance of BSA molecule from 0 to 40% dioxane concentrations seems to be within a reasonable range of the extension of the molecule as expected. The value of the most extended form, *i.e.*, BSA in 40% dioxane concentration, 187 Å, is, however, low in comparison with the value 300 Å that Luzzati, *et al.*,[21] obtained from X-ray scattering measurements.

The above observations lead us to conclude that the internal structure of BSA in acidic aqueous solutions is basically not rigid. The presence of dioxane increases the axial ratio a/b of the protein. Although dioxane enhances the expansion of the molecule, it causes the decrease in the effective volume of the molecule. The effect of dioxane on the conformation of BSA molecule in acidic aqueous solutions seems to be similar to that of the other denaturing agents.[25]

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