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Ultrasonic Absorption Measurements of Bovine Serum Albumin Solutions in the Frequency Range 200 kHz to 3 MHz

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Abstract

Ultrasonic absorption and velocity spectra in bovine serum albumin (BSA) aqueous solutions have been measured at 20℃ over the frequency range 0.2-3 MHz in the pH range 1.5-13.2. The high-Q ultrasonic resonator and pulse-echo overlap methods were used. At acid pH's, excess absorption over that of pH 7 was explained by double relaxation. The pH dependences of the relaxation frequency and maximum absorption per wavelength, showed that the relaxation at about 200 kHz was related to the expansion of molecules and that about 3 MHz resulted from the proton transfer reaction of carboxyl group. At alkaline pH's, the excess absorption was explained by double relaxation. The relaxation at about 300 kHz was associated with a helix-coil transition, and that about 3 MHz was attributed to the proton transfer reaction of phenolic group. The rate constants and volume changes associated with these processes were estimated.

Keywords: *Relaxation Frequency, Maximum Absorption per Wavelength, Proton Transfer Reaction, Helix-Coil Transition*

I. Introduction

The mechanism of ultrasonic absorption in protein solutions has attracted considerable interest[1-4]. Because most protein solutions reveal broadband absorption spectra which cannot be described in terms of a single relaxation, the absorption measurement should be made over a very wide frequency range for quantitative discussion. To extend the frequency range below 1 MHz is especially desirable for this purpose.

Bovine serum albumin (BSA) is one of the typical and popular proteins, and a number of ultrasonic works have been made on BSA to understand the mechanism of ultrasonic absorption in protein solutions[5-6] Kessler and Dunn[5] first measured the absorption spectra up to 163 MHz over the pH range 2.3-11.8. The excess absorption below pH 4.3 and above pH 10 was attributed to conformational changes. Lang et al.[6], however, showed that proton transfer reactions at the acidic and alkaline side chains were responsible for the excess absorption peaks. Hussey and Edmonds[7] calculated the contribution of proton transfer reactions to absorption and obtained qualitative agreement with the experimental data. It appears to be established that significant contribution to the absorption peaks is attributable to the proton transfer reactions. However, the additional absorption below pH 2 and the velocity minimum at pH 4.1[5] suggest the contribution of conformational changes. Barnes et al measured the absorption in the frequency range 200 kHz to 1 MHz using a spherical ultrasonic resonator[8]. They showed that a maximum absorption per wavelength existed at 400 kHz in the acid region and at 3 MHz in the alkaline region, and ascribed those to the proton transfer reaction at carboxyl and amino groups, respectively. Their results are important to investigate the mechanism, but lack quantitative analysis. The absorption peaks they observed seem to be too narrow to be fitted to the

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theoretical relaxation curves.

This paper describes ultrasonic absorption in BSA aqueous solutions over the frequency range 0.2-3 MHz in the pH range 1.5-13.2. The two excess relaxation peaks centered at about 200 kHz and 3 MHz observed in the acid pH region were attributed to conformational changes and to the proton transfer reaction of carboxyl group at the glutamic acid and aspartic acid residues, respectively. The two relaxation peaks at about 300 kHz and 3 MHz observed in the alkaline region were attributed to a helix-coil equilibrium, to the proton transfer reaction of the phenolic group at the lysine residue.

II. Experiments

Detail of the experimental apparatus have been described elsewhere[2]. A standing wave established in the high-Q ultrasonic resonator method with cylindrical side walls is probed using the Raman-Nath light diffraction with an optical heterodyne detection. The bandwidth of the resonance spectrum gives the absorption coefficient of a sample liquid in the resonator. The high quality factor attained with this resonator made possible the reliable absorption measurements in the frequency range from 0.2-2 MHz. A pulse echo method was also used to measure absorption at 3 MHz with careful correction for diffraction.

The crystallized and lyophillized sample of bovine serum albumin (Sigma Chemical Co., A7906) was dissolved in distilled water of chromatography grade to make solutions with the concentration of 50 g/ℓ. The pH after the dissolution was 7.0 and was adjusted to the desired values using 1N solution of either HCl or NaOH. The measurements were carried out at the pH's of 1.5, 2.1, 2.7, 3.5, 4.2, 7.0, 10.6, 10.9, 11.3, 11.6, 12.3, and 13.2 at the temperature of 20℃. The temperature was controlled within 0.1℃.

III. Results and Discussion

3.1. Analysis of the absorption spectra

Fig. 1 shows titration curves of ultrasonic absorption(α/f^2) obtained for 0.3 and 2 MHz. At 2 MHz, the peaks are seen, which can be attributed to proton transfer reaction[6]. In the alkaline region, the peak due to proton transfer reaction is prominent. At 300 kHz, in the acid region, ultrasonic absorption increases with decreasing pH, which suggest that another relaxation mechanism operates. In the alkaline region, the width of the peak is wider than that at 2 MHz. Here, we assume that the titration curves are represented by the addition of pH-dependent excess absorption to pH-independent absorption. We calculated the excess absorption by subtracting the experimental values at pH 7. The excess absorption per wavelength,

$\mu = (\alpha\lambda)_{pH} - (\alpha\lambda)_{7.0}$,

is shown in Figs. 2 and 3 as a function of frequency for different pH's. The excess absorption per wavelength can be represented by the equation of double relaxation as follows[9];

$$\mu = 2\sum_{i=1}^{2} \mu_{mi} \frac{\frac{f}{f_{ri}}}{1+(\frac{f}{f_{ri}})^2} \qquad (1)$$

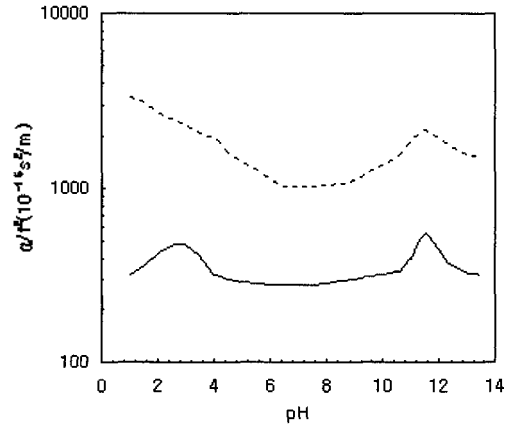

Fig. 1. zUltrasonic absorption titration of the bovine serum albumin solution at 20.0℃. The dash and dot lines represent the absorption of 2 and 0.3 MHz, respectively.

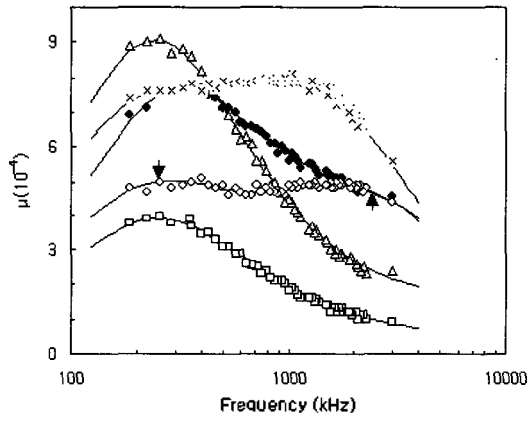

Fig. 2. Excess absorption per wavelength μ versus frequency at acid pH's. The solid lines represent double relaxation curves fitted to the data. The arrows indicate relaxation frequencies for the curve at pH 3.5. The symbols △, ●, ×, ○ and □ represent pH 1.5, 2.1, 2.7, 3.5 and 4.2, respectively.

Here μ_m denotes maximum absorption per wavelength at the relaxation frequency f_r, f the sound frequency. The solid lines in the figures represent the calculated ultrasonic relaxation spectra from Eq (1). At pH's in the acid region, double relaxation curves, represented by the solids lines, well fitted the excess absorption. The arrows indicate relaxation frequencies for the curve at pH 1.5. We designate the lower and higher frequency relaxations to be relaxation A1 and A2, respectively. At pH's in the alkaline region, double curves, represented by the solid lines in Fig. 3, well fitted the excess absorption. The arrows indicate relaxation frequencies for the curve at pH 11.6. We designate the double relaxations to be the relaxation B1 and B2, respectively.

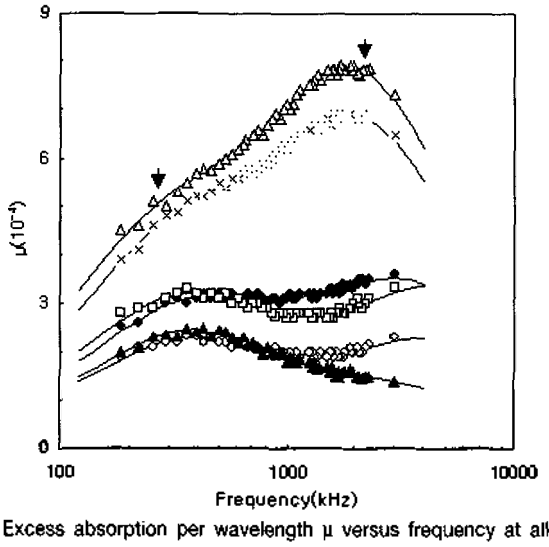

Fig. 3. Excess absorption per wavelength μ versus frequency at alkaline pH's. The solid lines represent double relaxation curves fitted to the data. The arrows indicate relaxation frequencies for the curve at pH 11.6. The symbols ○, ●, ×, △, □, and ▲ represent pH 10.6, 10.9, 11.3, 11.6, 12.3 and 13.2, respectively.

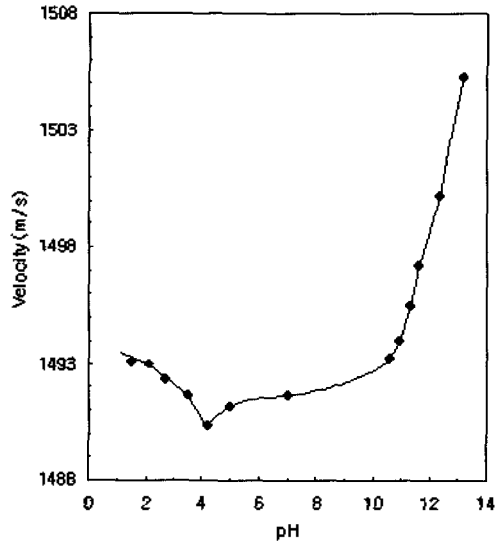

Fig. 4. Titration curve of ultrasonic velocity at 1 MHz in the bovine serum albumin solution with the concentration of 50g/ℓ. The solid line is drawn for a visual guide.

Figure 4 shows the titration curve of ultrasonic velocity measured at 1 MHz with the high-Q ultrasonic resonator method[9]. A minimum observed near pH 4 was also found by Kessler and Dunn[5]. They related this minimum to the conformational transformation between a compact rigid form of the molecule and an expandable form. The velocity measurements in HCl and NaOH solutions by Marks[10] indicate that the increase in velocity at lower pH's and the large increase at alkaline pH's are due to the effect of HCl or NaOH added to adjust pH.

3.2. Kinetics of the proton transfer reactions.

The two relaxations at an acid pH have different pH dependence as can be seen in Fig. 2. The maximum absorption per wavelength μ_{max} of the relaxation A1 increases with decreasing pH, while the relaxation A2 has a maximum at about pH 2.7. It is theoretically predicted that the relaxation caused by a proton transfer reaction should exhibit a maximum in μ_{max} vs pH curve and a minimum in relaxation frequency f_r vs pH curve.

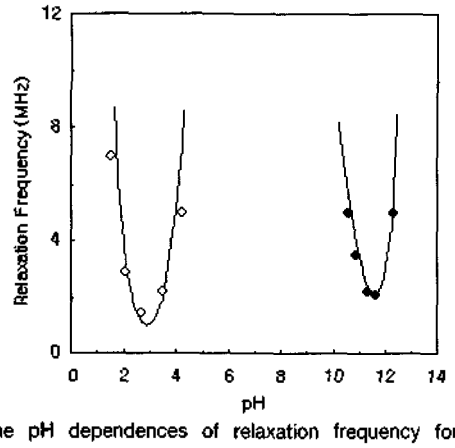

Fig. 5. The pH dependences of relaxation frequency for the proton transfer relaxations A2 and B2. The solid lines represent the theoretical curves calculated from Eq.(3).

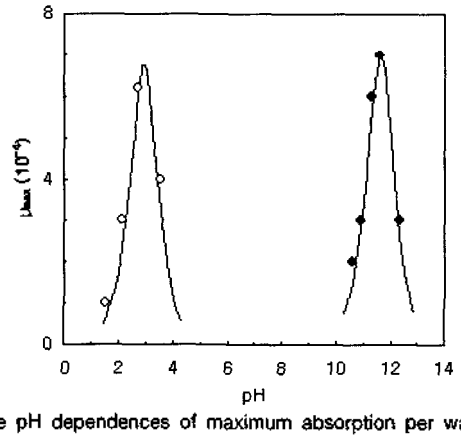

Fig. 6. The pH dependences of maximum absorption per wavelength for the proton transfer relaxations A2 and B2. The solid lines represent the theoretical curves calculated from Eq.(4).

Table 1. The obtained values of pK's, rate constants, and volume changes for the proton transfer reactions in BSA solutions.

	pK	$k_f(M^{-1}s^{-1})$	k_b	$\Delta V(cm^3/mol)$
BSA				
ASP/GLU (ω-carboxyl)	4.7	6.0×10^9	1.3×10^5	27.5
TYR (phenolic)	10.6	1.6×10^9	6.0×10^5	16.2
Amino acid				
L-lysine[13] (ε-amino)	10.6	1.3×10^{10}	4.7×10^6	22
L-tyrosine[13] (phenolic)	10.4	1.2×10^{10}	3.2×10^6	--

We therefore assume that the mechanism of the relaxation A2 is the proton transfer reaction of carboxyl groups that are involved in glutamic acid and aspartic acid residues. The quantity of these two residues that can participate in the proton transfer reaction is 99 mole per mole of BSA[11]. BSA molecules have been recognized to expand under acid conditions and finally to be denatured at an extremely low pH[12]. This and pH dependence of the maximum absorption per wavelength in Fig. 2 suggest that the relaxation A1 is associated with some conformational changes. In the alkaline region the relaxations B2 shows pH dependences similar to that of the relaxation A2. Thus the relaxations B2 is assumed to be due to proton transfer reactions. The relaxation B1 may be related with conformational changes.

If proton transfer reaction is expressed as

$$A + X \underset{k_b}{\overset{k_f}{\leftrightarrow}} B \qquad (2)$$

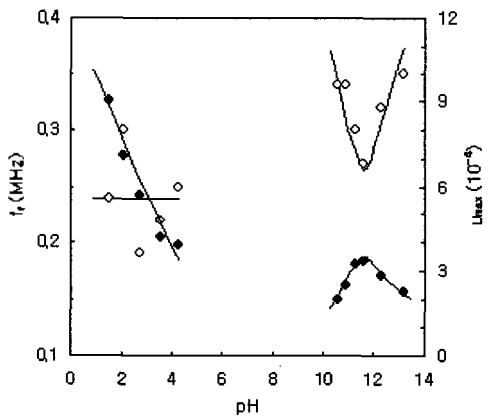

Fig. 7. The pH dependences of relaxation frequency(○) and maximum absorption per wavelength(●) for the relaxations A1 and B1. The solid lines are drawn for a visual guide.

where X represents H^+ or OH^-, the relaxation frequency f_r and maximum absorption per wavelength μ_{max} are given by

$$2\pi f_r = k_b\left(\frac{C_0}{K + C_x} + C_x + K\right), \qquad (3)$$

$$\mu_{max} = \frac{\pi\rho V_0^2}{2RT}(\Delta V)^2\left(\frac{K C_0 C_x}{K C_0 + (K + C_x)^2}\right). \qquad (4)$$

Here, k_f and k_b are the forward and backward rate constants, respectively, $K(=k_b/k_f)$ is the equilibrium constant, C_0 the concentration of the relevant residue, C_x the concentration of H^+ or OH^-, ΔV the volume change associated with the reaction, V_0 the velocity at low-frequency limit, and ρthe density. Equations (3) and (4) predict that fr takes a minimum and μ_{max} takes a maximum at acid pH given by

$$pHa = \frac{1}{2}(pK - \log C_0), \qquad (5)$$

or at alkaline pH given by

$$pHb = \frac{1}{2}(14 + pK + \log C_0), \qquad (6)$$

where pK = - logK. Figures 5 and 6 show the pH dependences of the relaxation frequency and maximum absorption per wavelength, for the relaxations A2, and B2, respectively. The solid curves indicate the theoretical values calculated from Eqs.(3) and (4) with k_b, K, and ΔV as fitting parameters. The experimental values are in good agreement with the theoretical prediction. Possible groups at which proton transfer reactions would occur in the alkaline region are the amino group in lysine (pKa = 10.0-10.4), the phenolic hydroxyl group in tyrosine (pKa = 9.6-10.0), and the guanidinium group in arginine (pKa> 12.5)[13]. Since the expected pK of the guanidinium group is out of the range that is interesting here, the contribution of the guanidinium group should therefore be ruled out. Proton-transfer relaxations in amino acid solutions have been observed in lysine and tyrosine[14]. The relaxation frequency of the ε-amino group in lysine is higher than that of the phenolic group in tyrosine by a factor of about three. It is, therefore, reasonable that the relaxation B2 is assigned to the phenolic group. Barnes et al[8] reported that the absorption peak arising from the proton transfer reaction of carboxyl group was observed at pH 3.2 around 400 kHz. This is one order of magnitude lower than the relaxation

frequency in the present investigation. The absorption peak they observed seems to be too narrow to be fitted to the theoretical relaxation curve. The rate constants, pK's and the volume change obtained from the fit are summarized in Table I. The titratable quantity of the amino acid residues is also listed in Table I. The rate constants k_f obtained are about one order of magnitude smaller than those in the amino acid solutions. The k_f for lysine in BSA is comparable with $k_f=7.35\times10^9 M^{-1}s^{-1}$ in polylysine measured in ref.[6]. The present result of ΔV for the amino group is in agreement with that in amino acid by ultrasonic study[14]. There have been no ultrasonic studies on the volume change for the ω-carboxyl group at the glutamic (or aspartic) acid residue and for the phenolic group. The present value of $\Delta V=27.5 cm^3/mol$ for the carboxyl group seems to be reasonable compared with ΔV determined ultrasonically to be 8-50cm^3/mol in some carboxylic acids[15].

We have suggested in the preceding section that the relaxations A1 and B1 were associated with some conformational changes. Figure 7 shows the pH dependences of the relaxation frequency and maximum absorption per wavelength for the relaxation A1 and B1. The relaxation frequencies for the relaxation A1 is almost constant, while that for the relaxation B1 exhibits a minimum at about pH 11.6. The maximum absorption per wavelength for the relaxation A1 increases with decreasing pH, while that for the relaxation B1 exhibits a maximum at about pH 11.6. Thus the pH dependences of f_r and μ_{max} are rather different between the relaxations A1 and B1, though the relaxation frequencies lie in the 200-400 kHz for both the relaxations. Tanford et al showed that BSA molecules expand in the ranges pH<4.3 and pH>10.5[16]. The present results in Fig 7 indicate that the relaxation A1 is closely related to the expansion of molecules. The velocity minimum found around pH 4 in Fig. 4 may be explained by the velocity decrease due to the expansion and the following increase due to the addition of HCl. Aggregation of BSA molecules has been observed at extreme pH of both acid and alkaline regions[17]. It is unlikely, however, that the aggregation is responsible for the relaxations A1 and B1. If the aggregation was responsible, the pH dependences of f_r and μ_{max} in the alkaline region should show similar behavior as those in the acid region. As for the relaxation B1, a probable mechanism is the perturbation of an equilibrium of helix-coil transition. Schwartz[18] developed the kinetics of a helix-coil transition and predicted that the relaxation time and relaxation amplitude should exhibit a maximum at a certain pH, that is the midpoint of the transition.

IV. Conclusion

Broadband absorption measurements are essential to understanding relaxation phenomena occurring at various time scales in biomolecular solutions. However, measurements are not enough to clarify the whole relaxation mechanism. Especially the accurate technique for measuring absorption higher than 10-100 MHz should be explored. A problem underlying the present analysis may be the assumption that the absorption at acid and alkaline pH's is represented as a simple summation of the value at neutral pH and its excess value. We believe, however, that this does not significantly change our conclusions though some minute corrections would be needed in the rate constants and volume changes estimated.

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