Polymer (Korea) Vol. 17, No. 3, pp 308－315 (1993)

Bovine Serum Albumin 수용액의 Proton전이와 초음파완화

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(1993년 3월 22일 접수)

Ultrasonic Relaxation Study of Proton Transfer and Conformational Change in Aqueous Solution of Bovine Serum Albumin

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(Received March 22, 1993)

요 약 : 소혈청 albumin(BSA) 수용액의 초음파흡수 mechanism을 규명하기 위해 pH 1.5～13.2에 대해 주파수 0.1～100MHz의 범위에 걸쳐 초음파 흡수측정을 행하였다. 측정방법은 plano-concave 공명법(0.1～2MHz), plano-plano 공명법(3～10MHz)과 Bragg 반사법(12～100MHz)을 사용하였다. 산측에는 2MHz와 200kHz 부근에 두개의 완화현상이 나타났고 2MHz의 완화현상은 carboxyl group의 proton전이에 의한 것으로, 200kHz는 BSA분자의 팽창에 의한 것임을 알았다. 알카리측에는 200k, 2와 15MHz 부근에 3개의 완화현상이 나타났고 2와 15MHz의 완화현상은 각각 phenolic와 amino group의 proton전이에 의한 것으로, 200kHz의 완화는 helix-coil 전이에 의한 것으로 임을 알았다.

Abstract : Ultrasonic absorption in bovine serum albumin(BSA) aqueous solutions(50 g/ℓ) has been measured at 20℃ over the frequency range 0.1～100 MHz in the pH range 1.5～13.2. Three different techniques were used : the plano-concave resonator, plano-plano resonator, and Bragg reflection methods. At acid pH's, excess absorption over that at 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 at 2 MHz resulted from the proton transfer reaction of carboxyl group. At alkaline pH's, the excess absorption was explained by triple relaxation. The relaxation at about 200 kHz was associated with a helix-coil transition, and the two relaxations at 2 and 15 MHz were attributed to the proton transfer reactions of phenolic and amino groups, respectively. The rate constants and volume changes associated with these processes were estimated.

INTRODUCTION

The widespread use of ultrasound in diagnostic and therapeutic medicine presents a need for a thorough understanding of the mechanisms of interaction of ultrasound and biological tissues. Nu-

merous studies have been directed toward the bulk ultrasonic properties of intact tissues.[1,2] These, and others, reveal the importance of proteins in the determination of ultrasonic absorption.[3,4] In the case of bovine serum albumin(BSA) specimens, for example, a number of ultrasonic works have been made on BSA to understand the mechanism of ultrasonic absorption in protein solutions.[5~10] 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. It appears to be established that significant contribution to the absorption peak is attributable to the proton transfer reactions. However, the additional absorption below pH 2 suggests the contribution of conformational changes. Barnes *et al.*[8~10] measured the absorption in the frequency range 60 kHz to 1 MHz using a spherical ultrasonic resonator. 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 reactions at carboxyl and amino groups, respectively. Further they reported that another maximum at 70 kHz for pH 4.2 could be ascribed to structural relaxation such as that arising from a helix-coil equilibrium. 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 theoretical relaxation curves.

The greatest difficulty to be encountered in measuring ultrasonic properties of protein solutions lies in the fact that the relaxation region extends to a very wide frequency range. The frequency range of previous experiments has been confined to two orders of magnitude at the widest, for example 1 to 100 MHz, because of experimental difficulties. In this situation, more extensive and precise ultrasonic study is needed as functions of frequency and pH to gain clearer insight into the relaxation mechanisms. This paper describes ultrasonic absorption measurement in BSA aqueous solutions over the frequency range 0.1~100 MHz in the pH range 1.5~13.2. The two excess relaxation peaks centered at about 200 kHz and 2 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 three relaxation peaks at about 200 kHz, 2 MHz, and 15 MHz observed in the alkaline region were attributed to a helix-coil equilibrium, to the proton transfer reaction of the phenolic group at the lysyl residue and to that of the amino group at the tyrosyl residue.

EXPERIMENT

The crystallized and lyopillized sample of bovine serum albumin(Sigma Chemical Co., A7638) 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 solutions of either HCl or NaOH. The final concentration was, therefore, a little lower than 50 g/*l*, but the uncertainty did not exceed 3%. The measurements were carried out at the pH's of 1.5, 2.1, 2.7, 3.5, 4.2, 5.0, 7.0, 10.6, 10.9, 11.3, 11.6, 12.3, and 13.2 at the temperature of 20℃. The temperature was controlled to within 0.1℃.

We used three experimental techniques for measuring ultrasonic absorption to cover the wide frequency range 0.1~100 MHz : a plano-concave resonator in the range 0.1~2 MHz,[11] conventional plano-plano resonator in the range 3~10MHz,[12] Bragg reflection method in the range 12~100 MHz.[13]

The key apparatus of the present work is the plano-concave resonator that covers the lowest frequency range where the data have been scarce. The method is briefly described here. A block diagram of the new resonator method is shown in Fig.

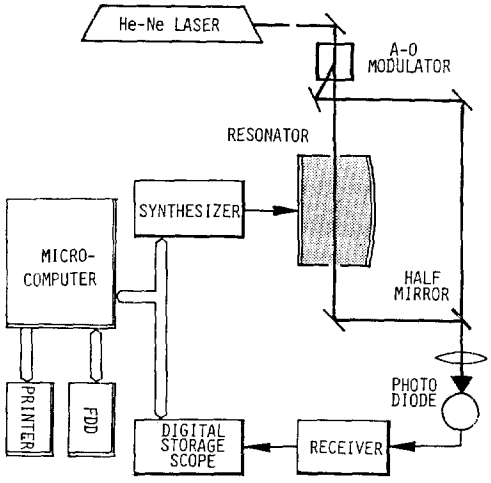

Fig. 1. Block diagram of the plano-concave resonator.

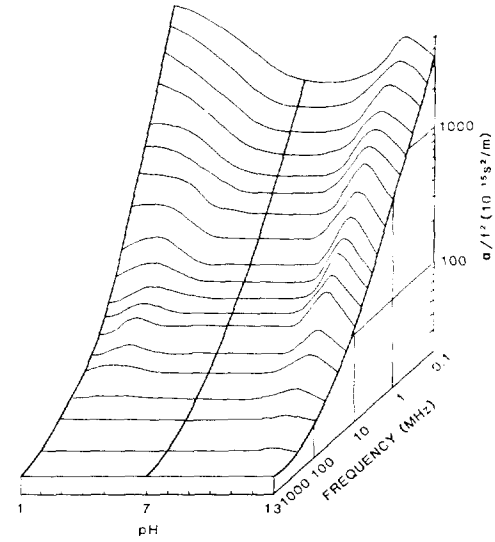

Fig. 2. Three-dimensional representation of absorption as functions of pH and frequency.

1. Standing waves are established in a cylindrical cavity that is composed of 2 MHz quartz transducer and a concave reflector. The diameter of the cavity is 56 mm and the sample volume required is 50 cm^3. Using the Raman-Nath light diffraction, a resonance spectrum is obtained with an optical heterodyne detection system. The bandwidth of one resonance curve gives absorption coefficient of the sample liquid. The high-quality factor attained with this resonator allowed the reliable absorption measurements in the frequency range below 1 MHz, where the conventional plano-plano resonator has poor accuracy. In the frequency range 3~10 MHz, we used the resonator of 28-mm diameter with a flat reflactor instead of the concave one.

RESULTS AND DISCUSSION

Analysis of the Absorption Spectra. Titration curves of ultrasonic absorption(α/f^2) obtained for various frequencies are shown in Fig. 2. In the acid region a peak is seen around 10 MHz, which can be attributed to proton transfer reaction. At frequencies below 1 MHz, another peak appears suggesting that another relaxation mechanism operates. In the alkaline region, the peak due to proton transfer reaction is prominent around 10 MHz, and this peak broadens at lower frequencies. Here, we asssume 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. 3 and 4 as a function of frequency for different pH's. At pH's in the acid region, double relaxation curves, represented by the solid lines, well fitted the excess absorption. The arrows indicate relaxation frequencies for the curve at pH 2.7. We designate the lower and higher frequency relaxations to be relaxation A_1 and A_2, respectively. At pH's in the alkaline region, triple relaxation curves, represented by the solid lines in Fig. 4, well fitted the excess absorption. The arrows indicate relaxation frequencies for the curve at pH 11.6. We designate the three relaxations to be the relaxation B_1, B_2, and B_3 in order of increasing frequency.

Kinetic of the Proton Transfer Reactions. The two relaxations at acid pH have different pH de-

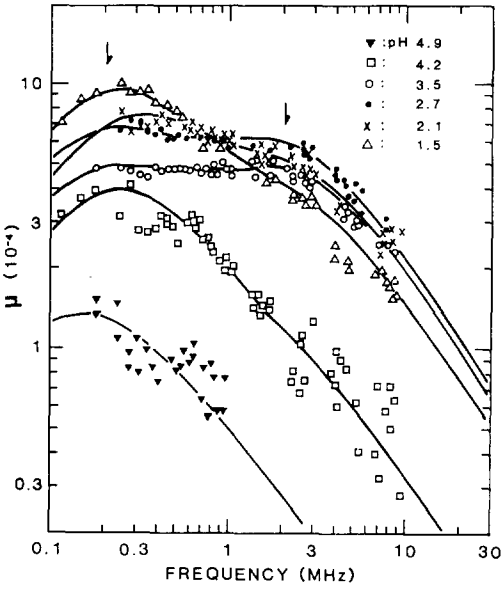

Fig. 3. Excess absorption per wavelength μ versus frequency at acid pHs. The solid lines represent double relaxation curves fitted to the data. The arrows indicate relaxation frequencies for the curve at pH 2.7.

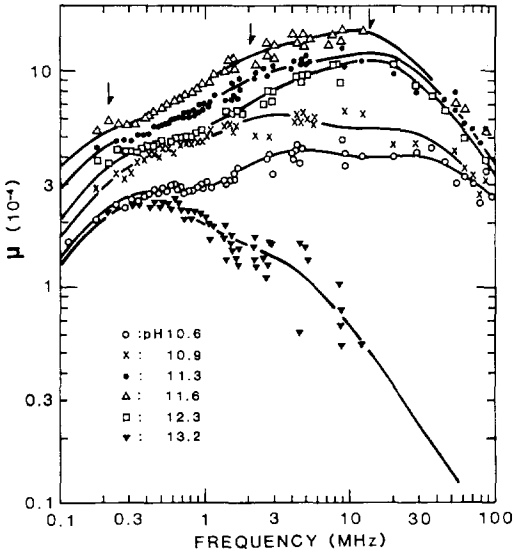

Fig. 4. Excess absorption per wavelength μ versus frequency at alkaline pHs. The solid lines represent triple-relaxation curves fitted to the data. The arrows indicate relaxation frequencies for the curve at pH 11.6.

pendence as can be seen in Fig. 3. The maximum absorption per wavelength μ_{max} of the relaxation A_1 increases with decreasing pH, while that of the relaxation A_2 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 minimum in relaxation frequency f_r vs pH curve.[14] We therefore assume that the mechanism of the relaxation A_2 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.[15] BSA molecules have been recognized to expand under acid conditions and finally to be denatured at extremely low pH.[16] This and the pH dependence of the maximum absorption per wavelength in Fig. 3 suggest that the relaxation A_1 is associated with some conformational changes. In the alkaline region, the relaxations B_2 and B_3 show pH dependences similar to that of the relaxation A_2. Thus the relaxations B_2 and B_3 are assumed to be due to proton transfer reactions. The relaxation B_1 may be related with conformational changes.

If a proton transfer reaction is express as

$$A+X \underset{k_b}{\overset{k_f}{\rightleftharpoons}} B \tag{1}$$

where X represents H^+ or OH^-, the relaxation frequency f_r and maximum absorption per wavelength μ_{max} are given by[14]

$$2\pi f_r = k_b\left(\frac{C_0K}{K+C_x}+C_x+K\right) \tag{2}$$

$$\mu_{max}=\frac{\pi\rho V_0^2}{2RT}(\Delta V)^2\left(\frac{KC_0C_x}{KC_0+(K+C_x)^2}\right) \tag{3}$$

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, ρ

the density. Equations (2) and (3) predict that f_r takes a minimum and μ_{max} takes a maximum at acid pH given by

$$pH_a = \frac{1}{2}(pK - \log C_0), \quad (4)$$

or at alkaline pH given by

$$pH_b = \frac{1}{2}(14 + pK + \log C_0), \quad (5)$$

where $pK = -\log K$. Figs. 5 and 6 show the pH dependences of the relaxation frequency and maximum absorption per wavelength, respectively, for the relaxations A_2, B_2 and B_3. The solid curves indicate the theoretical values calculated from Eqs. (2) and (3) with k_b, K, and ΔV as fitting parameters. The experimental values are in good agreement with the theoretical prediction. In the process of the fit, the assignment of relaxation B_2 and B_3 were made as follows. 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 in tyrosine (pK_a=9.6~10.0), and the guanidinium group in arginine(pK_a>12.5). Since the expected pK of the guanidinium group is out of the range that is interesting here, the contribution of the guanidinium group should be ruled out. Proton-transfer relaxations in amino acid solutions have been observed in lysine[17,18] and tyrosine.[17] 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, resonable that the lower-frequency relaxation B_2 is assigned to the phenolic group and the higher-frequency relaxation B_3 to the ε-amino 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 that 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. Since their measurements were limited up to 1 MHz, they might have overlooked the relaxation in the MHz region.

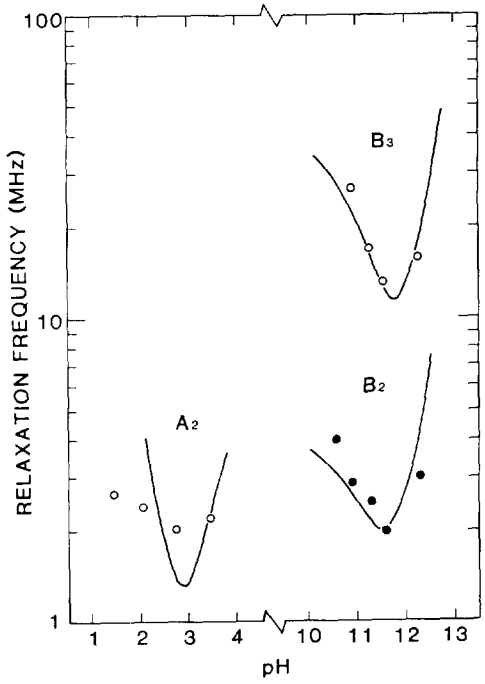

Fig. 5. The pH dependences of relaxation frequency for the proton transfer relaxations A_2, B_2 and B_3. The solid lines represent the theoretical curves calculated from Eq.(2).

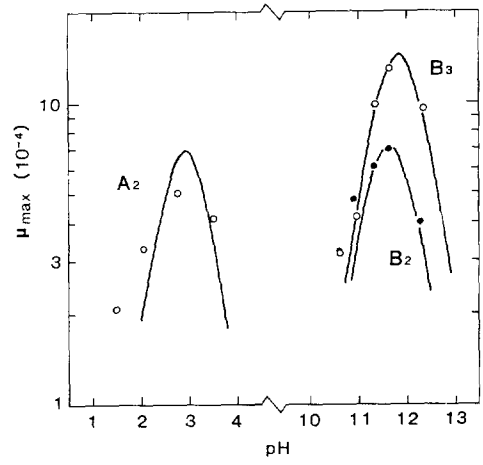

Fig. 6. The pH dependences of maximum absorption per wavelength for the proton-transfer relaxation A_2, B_2 and B_3. The solid lines represent the theoretical curves calculated from Eq.(3).

Table 1. The Obtained Values of pK's, Rate Constants, and Volume Changes for the Proton Transfer Reactions in BSA Solutions. Here, n Represents the Quantity of Titratable Residue Per 1 Mol of BSA. The Values in Polypeptide and Amino Acid Solutions are Listed for Comparison

	pK	k_f ($M^{-1}s^{-1}$)	k_b (s^{-1})	ΔV (cm^3/mol)	n (mol/mol BSA)
BSA					
ASP/GLU(ω-carboxyl)	4.7	4.9×10^9	1.0×10^5	27.5	99
LYS(ε-amino)	11.0	5.7×10^9	5.1×10^6	19	57
TYR(phenolic)	11.0	1.0×10^9	1.1×10^6	18	19
Polypeptide					
Poly-lysine[a](ε-amino)	10.2	7.4×10^9	1.3×10^6	24	
Amino acid					
L-lysine[b](ε-amino)	10.6	1.3×10^{10}	4.7×10^6	22	
lysine[c]		2.4×10^{10}		20	
L-tyrosine[b](phenolic)	10.4	1.2×10^{10}	3.2×10^6	···	

[a] Reference 19
[b] Reference 17
[c] Reference 18

The rate constants, pK's and the volume changes obtained from the fit are summarized in Table 1. The titratable quantity of the amino acid residues is also listed in Table 1. The rate constants k_f obtained are about one order of magnitude smaller than those in 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 by Zana and Tondre.[19] They dicussed the comparison of the rate constant between amino acid and polypeptide. The difference in the diffusion coefficient of the reacting species and steric effects may explain the smaller rate constant in polypeptide and protein. It should be noted that the proton transfer reactions of amino and phenolic groups in BSA may interact with each other since the time ranges of both reactions are close. This interaction may result in some modification of the rate constants. Dilatometric measurements have shown that the volume changes accompanying the proton transfer reactions of carboxyl group and amino group in protein are about 11 cm^3/mol and 18 cm^3/mol, respectively.[20] The agreement with the present results is good for the amino group, though poor for the carboxyl group. The present result of ΔV for the amino group is in agreement with that in amino acid and polypeptide by ultrasonic studies.[17,19] 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 8~50 cm^3/mol in some carboxylic acids.[21,22]

The Relaxations Due to Conformational Changes. We have suggested in the preceding section that the relaxations A_1 and B_1 were associated with some conformational changes. Fig. 7 shows the pH dependences of the relaxation frequency and maximum absorption per wavelength for the relaxations A_1 and B_1. The relaxation frequency for the relaxation A_1 is almost constant, while that for the relaxation B_1 exhibits a minimum at about pH 11.6. The maximum absorption per wavelength for the relaxation A_1 increases with decreasing pH, while that for the relaxation B_1 exhibits a maximum at about pH 11.6. Thus the pH dependences of f_r and μ_{max} are rather different between the relaxations A_1 and B_1, though the relaxation frequencies lie in the 200~300 kHz for both the relaxations. Tanford *et al.*[23] showes that BSA molecules expand in the ranges pH<4.3 and

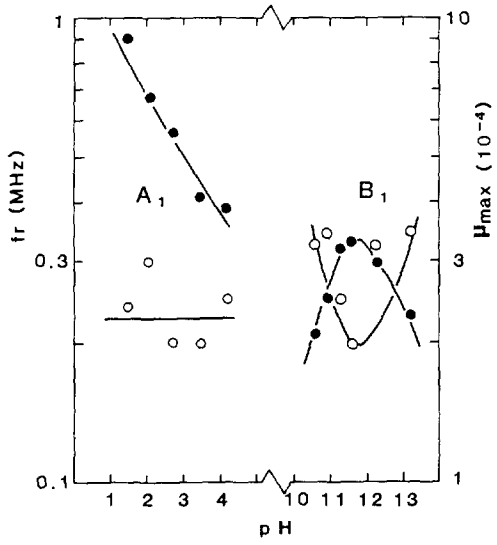

Fig. 7. The pH dependences of relaxation frequency (○) and maximum absorption per wavelength(●) for the relaxations A_1 and B_1. The solid lines are drawn for a visual guide.

pH>10.5. As the pH is reduced below 3.6, the molecules take the F form, which is proposed by Aoki and Foster,[24] as a result of further expansion and unfolding. These observations and the present results in Fig. 7 indicate that the relaxation A_1 is closely related to the expansion of molecules. Aggregation of BSA molecules has been observed at extreme pH of both acid and alkaline regions.[25] It is unlikely, however, that the aggregation is responsible for the relaxation A_1 and B_1. 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 B_1, a probable mechanism is the perturbation of an equilibrium of helix-coil transition. Schwartz[26] 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. On the basis of the Schwartz theory, Inoue[27] attributed the relaxation observed around 200 kHz in sodium poly-α D-glutamate solutions to the helix-coil transition, and obtained the rate constant for helical growth to be $k_F=4.4\times10^7 s^{-1}$ and the volume change associated with the transition to be $\Delta V=1.2$-cm^3/mol residue. Barkesdale and Stuehr[28] also reported $k_F=(8\pm5)\times10^7 s^{-1}$ and $\Delta V=0.9\sim1.4$ cm^3/mol residue for the helix-coil transition in poly-L-glutamic acid solutions. Assuming that the helix-coil transition is responsible for the relaxation B_1, we can estimate the values of k_F and ΔV from the peak values of f_r and μ_{max} using the equiations given by Schwartz and $\sigma=0.5\times10^{-3}$, a measure of the degree of difficulty of nucleation. Thus we obtained $k_F=6.3\times10^7 s^{-1}$ and $\Delta V=0.39$-cm^3/mol residue. The value of k_F agrees well with those obtained by Inoue and Barkesdale *et al.* The value of the volume difference per mole of residue is about one third compared with those by Inoue and Barkesdale *et al.* This disagreement is explainable if one considers that not all residues suffer the helix-coil transition in BSA though all residues suffer the transition in poly-L-glutamic acid. In calculating ΔV, we used the number of all the residues of BSA as the concentration of participating residues. This concentration should be smaller in reality.

CONCLUSION

We have demonstrated that broadband absorption measurements over three orders of magnitude are essential to understanding relaxation phenomena occuring at various time scales in biomolecular solutions. However, these measurements are not enough to clarify the whole relaxation mechanisms. Especially the accurate technique for measuring absorption lower than 100 kHz with small sample volume 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. Conformational changes induced by pH change may vary the degree of hydration, which negates this assumption. We believe, however, that this does not significantly change our conclusions though some

minute corrections should be needed in the rate constants and volume changes estimated.

Acknowledgment : This paper was supported in part by NON DIRECTED RESEARCH FUND, Korea Research Foundation, 1992.

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