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ACOUSTIC SPECTROSCOPY IN PROTEIN SOLUTIONS: HUMAN SERUM ALBUMIN

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This report presents the broadband acoustic study of aqueous solutions of human serum albumin (HSA), the most abundant protein in the circulatory system. Complicated attenuation spectra indicating the presence of relaxation processes in the whole frequency range extending from 200 kHz to 2 GHz have been found. Different relaxation functions were applied to analytically represent the spectra. Most consistent for the whole dataset at different pH, temperatures and concentrations was the function, consisting of the asymptotic high frequency attenuation and three relaxation contributions. The first of those located at 300 kHz was attributed to cooperative segmental motions of the protein chain. The second one found in the range of 1-5 MHz seems to be due to proton transfer reactions of the protein side-chain groups. And the third one with a maximum disposed between about 400 MHz and 4 GHz was interpreted in terms of solute-solvent interactions reflecting various hydration layers of HSA molecules

INTRODUCTION

During past decades a number of acoustic works have been made to understand the mechanism of sound attenuation in protein solutions. Acoustic spectroscopy is a powerful technique for examining the dynamic properties of liquid systems on a nanosecond to microsecond time scale [1]. It is generally accepted that processes associated with the hydration equilibrium, proton transfer reactions and conformational changes are directly observed in the relaxation part of sound absorption spectra of protein solutions. Although such investigations may shed more light on dynamic aspects of protein functions and be further employed in ultrasonic medicinal imaging, the systems studied and experimental conditions were not enough to clarify all aspects of the acoustic spectra. Serum albumin has been one of the frequently studied proteins and its static structure is now available for various species. An understanding of motions and conformational changes in this molecule is important, especially with respect to ligand binding, as serum albumin plays the central role in the delivery of drugs with the circulatory system. A number of studies have already been performed for bovine serum albumin solutions using acoustic relaxation method [2], and kinetic parameters of the mentioned processes were obtained. Less attention has been directed towards solutions of human serum albumin [3-5]. In this contribution we report on the first broadband acoustic spectra of human serum albumin (HSA) in aqueous solutions at different pH, temperature and concentration conditions. The most consistent relaxation profiles have been found and related to the underlying molecular mechanisms.

EXPERIMENTAL METHODS

Human serum albumin (Sigma, FRG, product A-1887) was dissolved in distilled water or in 0.1 mol/l sodium phosphate buffers at various pH. Mono-, di- and tri- substituted sodium phosphates, and orthophosphoric acid (Fluka or Merck, FRG, > 98%) were used for buffer solutions. The solutions of 10, 20 and 40 g/l HSA in water were investigated at 25 ^{0}C. The 40 g/l HSA in water solution was also measured at 15.6 and 35 ^{0}C. The solutions of 1, 2 and 10 g/l HSA in buffer, pH 7.4, were examined at 15.6 ^{0}C. The 2 g/l HSA were also studied in buffers at pH 1.8, 2.3, 11.9, and 12.3.

The sound velocity c_s and attenuation coefficient α were determined in the frequency f range 0.1 - 2000 MHz using resonator and pulse-modulated wave transmission methods [6]. The temperature of the samples was controlled to within ±0.05 ^{0}C. Additionally, the density ρ was measured pycnometrically for all solutions.

SOUND ATTENUATION SPECTRA IN HSA SOLUTIONS

Fig. 1 -4 show the representative results of the acoustic attenuation spectra for HSA solutions in water and in buffer at various concentrations, pH and temperatures. The data are displayed in the format excess-attenuation-per-wavelength

$$(\alpha\lambda)_{exc} = \alpha\lambda - Bf \qquad (1)$$

vs frequency. The asymptotic high-frequency part Bf with B independent of f has been subtracted from the total attenuation per wavelength $\alpha\lambda$ in order to accentuate the relaxation contributions to the spectra. Here $\lambda = c_s / f$ is the sonic wavelength.

The excess attenuation in the albumin solutions extends over the whole frequency range of measurements, indicating the presence of several relaxation processes. Different theoretical relaxation functions were applied to analytically represent the spectra. It was found that the $(\alpha\lambda)_{exc}$ spectra of HSA solutions at neutral pH can be analytically represented by a sum of a Debye-type term with discrete relaxation time τ_1 and a term, representing asymmetric continuous distribution of relaxation times with a cutoff at small values τ_2. Choi et al. [2] were the first to propose the latter distribution function for the description of acoustic spectra of bovine serum albumin solutions. At low and high pH, however, an additional Debye-type term with discrete relaxation time τ' is required to fit the excess attenuation spectra of HSA solutions within the limits of experimental error (curve 2 in Fig.4). Applying such relaxation model the total attenuation per wavelength in all the studied solutions can be represented by

$$\alpha\lambda = \frac{A_1\omega\tau_1}{1+(\omega\tau_1)^2} + \frac{A'\omega\tau'}{1+(\omega\tau')^2} + \frac{A_2(\omega\tau_2)^\beta \sin\beta\Theta}{\left[1+(\omega\tau_2)^2\right]^{\frac{\beta}{2}}} + Bf\,, \qquad (2)$$

where $\omega = 2\pi f$, $\Theta = \arctan(1/\omega\tau_2)$, β is the parameter characterizing the width of the relaxation time distribution of term 2 ($0<\beta\leq 1$), A_1, A_2 and A' are relaxation amplitudes that express the volume and enthalpy effects as well as the concentrations of the species in the elementary equilibria. $A' = 0$ for all systems with almost neutral pH.

The solid lines in Fig.1 - 4 represent the fitted curves of Eq. (2). The values of the reciprocal relaxation times and relaxation amplitudes obtained from the fit of the measured spectra to theoretical expression (2) are plotted in Fig. 5 -8 as functions of pH, temperature and HSA concentration. These functions can be related to the following molecular mechanisms.

RELAXATION MECHANISMS

Conformational changes

Both relaxation times τ_1 and τ_2 are observed in all water and buffer solutions, and the corresponding amplitudes A_1 and A_2, respectively, increase with protein concentration. This increase is linear for the A_1 values. The corresponding relaxation time τ_1 is on the order of 400 ns and does not depend on protein concentration. Such behaviour suggests an intramolecular mechanism [1], likely a conformational change of protein molecule, for the low-frequency relaxation region (τ_1). Variation of temperature has practically no effect on the τ_1 and A_1 values, implying that internal rotation at these temperatures is characterized by a very small activation enthalpy. Nevertheless it may be controlled by high activation entropy. A high value of activa-

25 ^{0}C, HSA in water:
1 – 10 g/l, 2 –20 g/l, 3 – 40 g/l

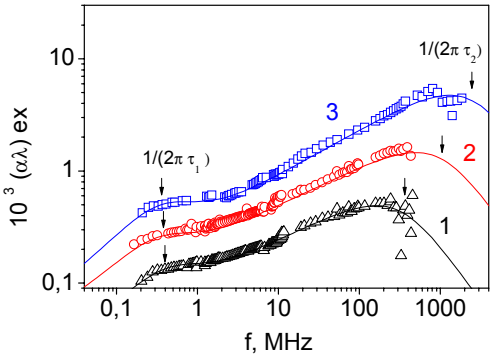

Fig.1

15.6 ^{0}C, HSA in buffer pH 7.4:
1 – 1 g/l, 2 – 2 g/l, 3 – 10 g/l

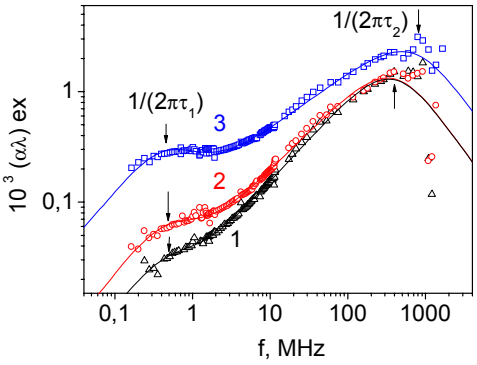

Fig.3

40 g/l HSA in water:
1 – 15.6 ^{0}C, 1 – 25 ^{0}C, 3 – 35 ^{0}C

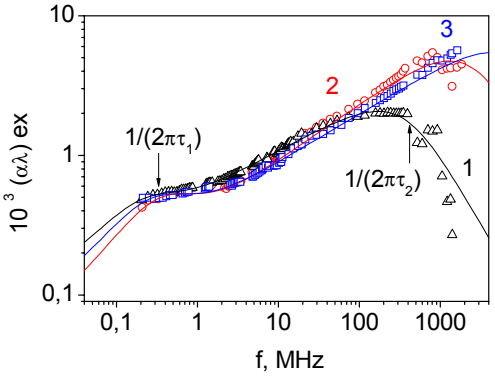

Fig.2

15.6 ^{0}C, 2 g/l HSA in buffer:
1 – pH 7.4, 2 – pH 11.9

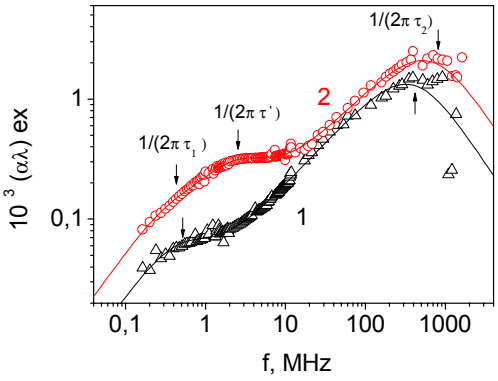

Fig.4

tion entropy is consistent with a relaxation process involving cooperative motions of several segments. Independency of the relaxation time and amplitude from temperature is usually attributed to cooperative conformational changes of the polymer chain backbone [7]. Hence these features allow for an assignment of the time τ_1 to segmental motions of albumin chain. The smaller relaxation rate $1/\tau_1$ at acid pH can thus be explained by a smaller mobility of rotating segments due to the loss in the helix structure that accompanies acid unfolding of the serum albumin molecule. At all that the amplitude A_1 is independent of pH.

25 ^{0}C, HSA in water: 1 – conformational change, 2 - hydration

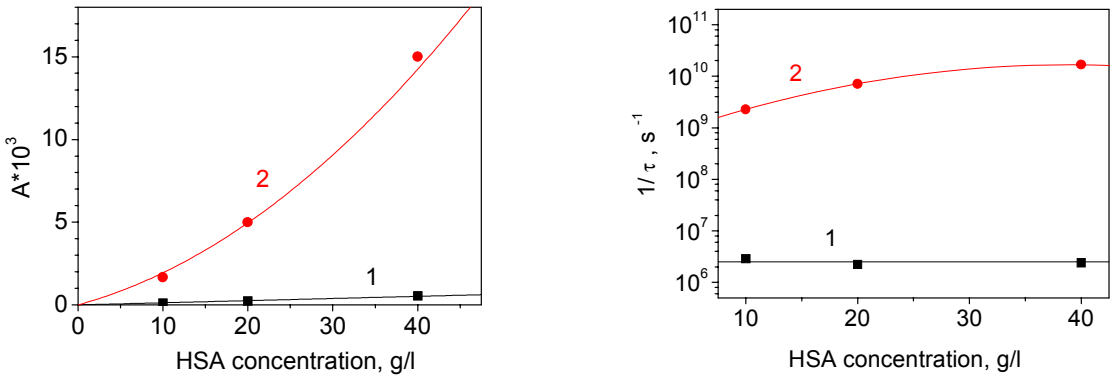

Fig.5

40 g/l HSA in water: 1 – conformational change, 2 – hydration

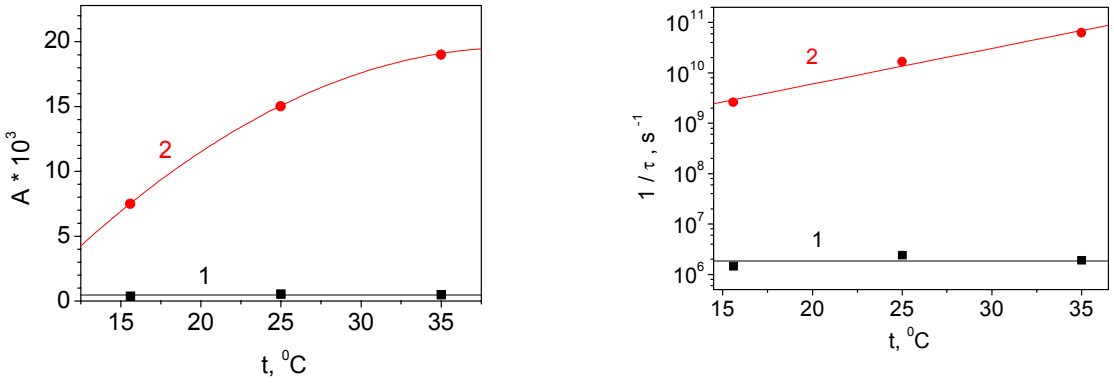

Fig.6

Hydration equilibria

For the high-frequency term (τ_2) of excess attenuation the range of the distribution of the relaxation times is in conformity with that of residence times 10 $^{-6}$ - 10 $^{-11}$ s of hydrated water molecules reported in NMR studies of the HSA solutions [8]. The relaxation contribution is larger at higher frequencies. This result is consistent with a significantly larger amount of water molecules in the outer hydration layers, with comparatively small residence times as compared to the inner layers. Hence the relaxation function profile may be interpreted in terms of solute-solvent interactions, reflecting various hydration layers of protein molecule [2]. Relaxation behaviour agrees with this model also with respect to the following points. The limiting relaxation rate $1/\tau_2$ exhibits a concentration dependence and an Ahrrenius temperature dependence, as characteristic for bimolecular processes. The amplitude A_2 decreases with the reduction of HSA concentration and adopts zero value in water, while in buffer solutions it tends to a finite value

at zero protein concentration. Such difference evidences the effect of relaxation processes of pure buffers. Assuming hydration mechanism, the increase of the $1/\tau_2$ value at shifts of the pH towards smaller and higher values may be explained by an extension of the outer hydration layer when native protein transforms to its acid and alkaline unfolded states. This treatment of ultrasonic relaxation terms is in conformity with the presence of the similar minimum on the pH dependence of relaxation amplitude A_2 as well as with its temperature behaviour.

15.6 ^{0}C, HSA in buffer pH 7.4: 1 – conformational change, 2 – hydration

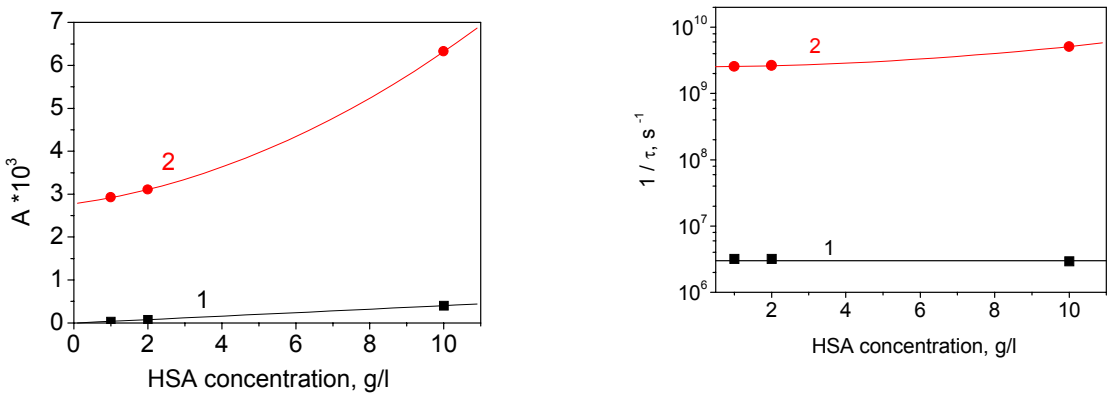

Fig.7

15.6 ^{0}C, 2 g/l HSA in buffer: 1 – conformational change, 2 – hydration, 3 – proton transfer

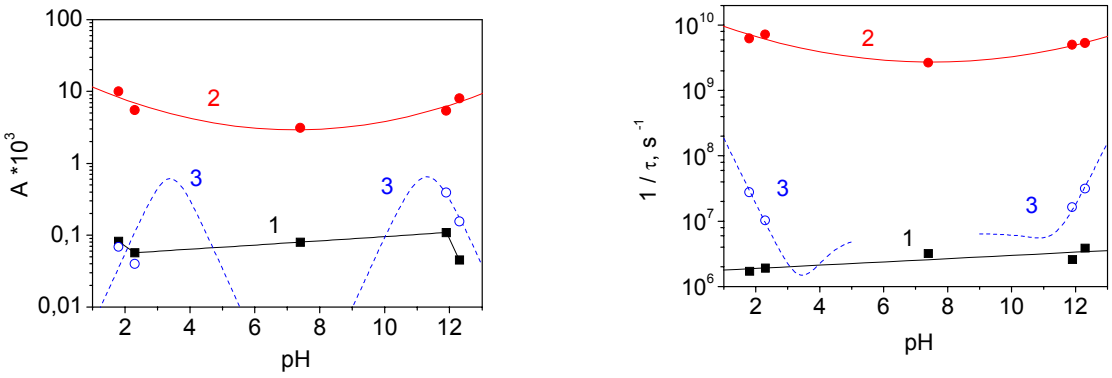

Fig.8

Proton transfer reactions

The intermediate relaxation term of excess attenuation with the relaxation time τ' ranging from 30 to 100 ns exhibits a strong dependence on pH. There are at least two possible reasons for this behaviour of the excess attenuation in protein solutions: proton transfer

reactions at ionizable groups on the protein and charge induced helix-coil transition [2, 9-11]. Both mechanisms suggest the existence of sharp maxima on the pH dependencies of relaxation time and amplitude, located close to the pK of a particular ionizable group or at the midpoint of the helix-coil transition, respectively. For the alternative mechanism, however, unreasonable parameter values follow from the fit of experimental data to theoretical model functions [9] both in an acid and alkaline pH range. Hence the relaxation region with characteristic time τ' is not likely due to a helix–coil transition.

If one considers the possibility of the proton transfer reaction

$$-R + X \underset{k'}{\overset{k}{\rightleftharpoons}} -R' \tag{3}$$

to cause the relaxation region τ', the relaxation rate and amplitude are given by [1]

$$1/\tau' = \frac{k'}{K}\left(\frac{C_R K}{K + C_X} + C_X + K\right), \tag{4}$$

$$A' = \frac{\pi \rho c_s^{\,2}}{RT}(\Delta V)^2\left(\frac{K C_R C_X}{K C_R + (K + C_X)^2}\right). \tag{5}$$

In Eqs. (3) - (5) X represents H^+ or OH^- for acid or base conditions, respectively, -R and -R′ are different ionization states of the same protein residue, C_X is the concentration of H^+ or OH^-, C_R is the concentration of the relevant residue, k and k' are the rate constants, $K=k'/k$ is the equilibrium constant, ΔV is the isentropic volume change associated with the reaction. Suggesting that, at acid pH, all residues of aspartic and glutamic acid in HSA molecule contribute to the relaxation, the values of pK=4.3, ΔV=35 cm^3/mol and k' = $1 \cdot 10^5$ s^{-1} were obtained for this model. For alkaline pH the values of pK=11, ΔV=20 cm^3/mol and $k' = 1.6 \cdot 10^6$ s^{-1} follow implying that proton transfer, occurring at all residues of lysine, tyrosine and arginine of HSA, contribute to the relaxation. All parameter values sufficiently correspond to those obtained for proton transfer reactions in solutions of bovine serum albumin [2]. The dashed lines in Fig. 8 indicate the theoretical values of the relaxation rate and amplitude calculated from Eqs. (4) and (5), respectively, with the parameters given above. The experimental values of $1/\tau'$ and A' are in good agreement with theoretical prediction. Thus, the relaxation region τ' for the HSA solutions at acid and alkaline pH can be attributed to proton exchange occurring between relevant ionizable side-chain groups of the protein and the solvent.

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