Two Types of Protein Hydration Measured by Dielectric Dispersion in the Gigahertz Region, and Effects of Anesthetics

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Two types of hydration of bovine serum albumin, BSA, were found by microwave impedance dispersion according to Suzuki et al. (*J. Phys Chem.* **1996**, *100*, 7279). At 25.0 °C the number of the strongly bound water molecules, N_s, was 606 and that of the weakly bound water molecules, N_w, was 393 per one BSA molecule. Elevation of the temperature decreased N_s continuously, whereas it increased N_w discontinuously at 30 °C. Addition of an anesthetic halothane increased N_w at the expense of N_s. At halothane 2.5 mM, N_s decreased by 300, and N_w increased by about 350. The electrical conductance of BSA solution showed a minimum at about 1.0 mM halothane. The halothane concentration that showed the minimum electrical conductance coincided with the maximum in N_w. Because the volume fraction of BSA is less than 2.5%, the conductance change is attributable to the change of bulk ion concentrations. The initial decrease of conductance represents the suppression of ionization by halothane. The increase of conductance above 1.0 mM halothane indicates unfolding of the protein, exposing polar moieties to water, thus increasing the ion concentration.

Introduction

Thermal analysis, Fourier transform infrared spectroscopy (FTIR), vapor pressure analysis, impedance dispersion, etc. have been used to investigate the state of hydration of macromolecules in water. Recently, Suzuki et al.[1] reported that impedance dispersion in the microwave region can separate the weakly and strongly bound water molecules (N_w and N_s, respectively) to proteins. The impedance dispersion spectra of polymer solutions consist of at least three relaxation processes: β-dispersion due to the rotation of the polymer, δ-dispersion due to the water molecules bound to the polymer, and γ-dispersion of the unbound free water.

Theoretically, hydration volumes can be obtained from the difference between the polymer crystal volume and the total hydrated volume of the polymer estimated from the β-dispersion. However, β-dispersion is technically difficult to obtain because it appears at around 0.4 MHz[2] and overlaps the polarization effects on the electrode surface due to ionic conductance in water. In contrast, γ-dispersion is clearly observable at a frequency higher than 1.0 GHz and the hydration number can be estimated from the high-frequency limit of the impedance dispersion spectrum.[1] Wei et al.[3] reported that the hydration number can be obtained from the δ-dispersion where the relaxation frequency appears at 10 MHz to 1.0 GHz. Nevertheless, the hydration numbers obtained by γ- and δ-dispersion do not agree. Suzuki et al.[1] reported that the hydration number obtained from the γ-dispersion indicates the summation of the N_w and N_s, whereas the hydration number of the δ-dispersion indicates only the N_s. By comparing the results obtained by the $\gamma-$ and δ-dispersion, it is possible to estimate the N_w and N_s separately.

The present study estimates the N_w and N_s bound to bovine serum albumin from the microwave impedance dispersion according to Suzuki et al.[1] and attempts to clarify the effect of a volatile anesthetic halothane on the protein hydration.

Experimental Section

An open-end flat-surface coaxial probe was made from oxygen-free copper. The diameter of the external conductor was 2.2 mm, and that of the internal conductor was 0.51 mm. The probe was insulated by PTFE. The total length was 150 mm and the specific impedance was 50 Ω. The probe was inserted into aqueous solutions of BSA in a test cell (Pyrex glass 25 mL) and irradiated by microwaves of 0.2−20 GHz with an interval of 0.4 GHz. The microwave, reflected from the BSA solution, was conducted through the probe to a 3747A Vector Network analyzer (Wiltron, CA), and the reflectory index ρ_{ap} was measured. When the tip of the probe is open to air, the reflectory index is designated as ρ_0, and when short circuited by an indium sheet, it is designated as ρ_s. When the tip is in contact with the standard solution, it is designated as ρ_a. By combining these data with the above ρ_{ap}, Misra[4] showed that the following equation estimates the complex dielectric constant ϵ^*_{ap} of BSA solution.

$$\epsilon^*_{ap} = \epsilon^*_{ap}\frac{(\rho_0 - \rho_a)(\rho_s - \rho_a) + (\rho_0 - \rho_s)(\rho_a - \rho_{ap})}{(\rho_a - \rho_s)(\rho_0 - \rho_a)} \quad (1)$$

where ϵ^*_a is the complex dielectric constant of the standard solution. We used super-purified water with known dielectric constant ϵ^*_a as the standard solution.[5]

The electrical conductance was measured by platinum electrodes fixed at glass holder (cell constant 1.075 cm^{-1}) and a LCR-740 AC−Bridge (Linder, Tokyo, Japan) at 2.65 kHz and 25.00 ± 0.02 °C. To prevent microscopic solubilization of copper into water, the probe was plated with gold. Also the

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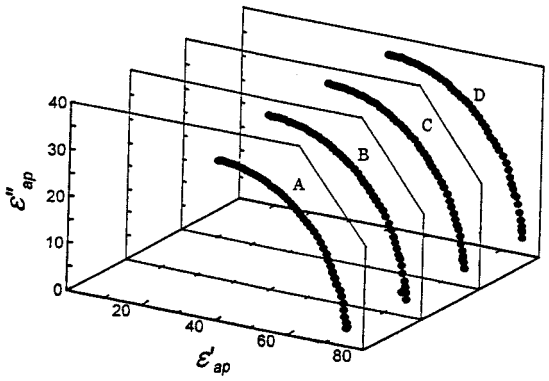

Figure 1. Cole−Cole plots for the complex dielectric constant ($\epsilon^*_{ap} = \epsilon'_{ap} - j\epsilon''_{ap}$) of the BSA solution. BSA concentration: 34 mg cm^{-3}. Halothane concentration: (A) 0, (B) 1.1 mM, (C) 2.2 mM, and (D) 4.5 mM. Frequency range: 0.2−20 GHz. Temperature: 25.00 ± 0.02 °C.

waveguide that connected the probe and the Vector Network analyzer was fixed securely to enhance the reproducibility of the experiment. The test cell containing BSA solution was placed in a constant-temperature water bath maintained at ±0.02 °C. The evaporation of the added anesthetics from the solution was prevented by closing the cell with a Teflon stopper.

The CD spectrum was measured by a JASCO J-600 (Tokyo, Japan) circular dichroism spectrophotometer. The experiment was performed at the sensitivity of 50 mdeg FS^{-1}, the time constant at 2 s, and the scan rate at 20 nm min^{-1}. Each sample was placed in a quartz cell with the light-path length 0.02 cm, and was scanned 50 times between 200−240 nm with 0.1 nm steps under the flow of nitrogen gas at 23 °C.

The fatty acid free BSA was obtained from Sigma (St. Louis, MO) and further purified by activated charcoal.[6] Water was purified by activated charcoal after treatment with ion-exchanger columns. The electrical conductance was below 0.07 μS cm^{-1}. Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) was obtained from Aldrich (Milwaukee, WI). Water was saturated by halothane vapor, and diluted with water to the required concentration. BSA was dissolved in water at 68 mg mL^{-1} and mixed with the halothane solutions at the equal volume ratio. We refrained from adding liquid halothane into the BSA solution to prevent formation of halothane micelles.[7]

Results and Discussion

1. Weakly Bound Water Molecules (N_w) and Strongly Bound Water Molecules (N_s). Figure 1 shows the Cole−Cole plot of the complex dielectric constant, ϵ^*_{ap}, consisting of the real part, ϵ'_{ap}, and the imaginary part, ϵ''_{ap}, of the BSA solution. By assuming that the dielectric dispersion of the BSA solution consists of the single dielectric relaxation of Debye, the curve-fitting procedure estimates the low-frequency limit values $(\epsilon'_{ap})_0$ of the δ-dispersion.

Wei et al.[3] showed that by combining the low-frequency limit values $(\epsilon'_a)_0$ and the high-frequency limit values $(\epsilon'_a)_\infty$ of the dielectric constant of the pure water (solvent), and the value of the BSA solution $(\epsilon'_{ap})_0$, the Φ_δ is obtained by the following equation.

$$\Phi_\delta = \frac{[(\epsilon'_a)_0 - (\epsilon'_{ap})_0][2(\epsilon'_a)_0 + (\epsilon'_a)_\infty]}{[2(\epsilon'_a)_0 + (\epsilon'_{ap})_0][(\epsilon'_a)_0 - (\epsilon'_a)_\infty]} \qquad (2)$$

Φ_δ is the volume fraction of the BSA molecule with the bound water in the BSA solution. By assigning 78.4 and 5.1 for $(\epsilon'_a)_0$ and $(\epsilon'_a)_\infty$, respectively, according to Kaatze,[5] we obtained the value for BSA in the absence halothane as $\Phi_\delta = 0.0306 \pm 0.0026$ ($n = 5$).

According to Wagner,[8] the next equation is obtained

$$\epsilon^*_{ap} = \epsilon^*_a \frac{2(1-\Phi)(\epsilon_a{}^*) + (1+2\Phi)(\epsilon_q{}^*)_D}{(2+\Phi)(\epsilon_a{}^*) + (1-\Phi)(\epsilon_q{}^*)_D} \qquad (3)$$

Here, ϵ^*_a, is the complex dielectric constant of pure water. Also, $(\epsilon^*_q)_D$ is the complex dielectric constant of hydrated BSA, and Φ is its volume fraction. By selecting the Φ value, $(\epsilon^*_q)_D$ can be calculated from eq 3. The high-frequency limits of $(\epsilon^*_q)_D$ for various values of Φ are estimated by curve-fitting according to the single Debye relaxation function. Zhang et al.[9] showed that the high-frequency limit of $(\epsilon^*_q)_D$ can be estimated as a function of Φ by assuming the dielectric constant of the hydrated water at the high-frequency limit is 5.1 of pure water[5] and that of BSA[10] is 2.5. The best-fit Φ value is determined when the high-frequency limit values, estimated by the above two procedures, are equal.[10] The best-fit Φ value of BSA in the absence of halothane was 0.0343 ± 0.0040 from five experiments.

The Φ_δ value calculated by eq 2 was smaller than Φ calculated by eq 3. Φ_δ is estimated from the low-frequency limit value in the gigahertz region, whereas Φ is estimated from the high-frequency limit. The difference between Φ and Φ_δ is attributable to the hydration modes: Φ is the volume fraction of BSA including weakly and strongly bound water molecules, whereas Φ_δ is the volume fraction including only strongly bound water molecules.[1]

The total number of water molecules, N_t, bound to one BSA molecule, and the number of strongly bound water molecules, N_s, were $55.6(\Phi - \Phi_B)\rho/c$ and $55.6(\Phi_\delta - \Phi_B)\rho/c$, respectively, where Φ_B is the volume fraction of BSA proper. Here, ρ is the density of water (g/cm^3) and c is the concentration (mol/L) of BSA. From the molecular weight of BSA ($M = 66000$), and the partial specific volume of BSA ($v = 0.733$ cm^3/g),[11] Φ_B is calculated as $cMv/1000$.

From the Φ and Φ_δ values, N_t, N_s, and N_w ($=N_t - N_s$) values were estimated to be 1004 ± 155, 606 ± 80, and 398 ± 75, respectively, at 25.00 ± 0.02 °C when the BSA concentration was 34 mg cm^{-3}. From the N_s value, the volume of strongly bound water molecules to 1.0 g BSA, W_s ($=N_s \times 18/66000$), was 0.17 g. By NMR, Oakes[12] reported that the water volume hydrating 1 g of BSA was 0.16 g. Theoretically, NMR reports the information on strongly bound water molecules; hence, the above agreement between the two indicates the propriety of N_s representing the strongly bound water. These results indicate that there are at least two binding sites for water on BSA.

BSA is composed of three domains, and each domain is a column of six long α-helix strands placed in antiparallel position.[13,14] According to this model, the following three sites are considered to be the possible binding sites for water molecules: (1) the main chain, containing strongly hydrophilic peptides, (2) hydrophilic side chains at the BSA surface, and (3) internal hydrophilic side chains that is not exposed to water. These hydrophilic side chains are buried inside due to the association among α-helix strands and domains.

1. The main peptide chain contains strongly hydrophilic moieties. Nevertheless, when these moieties are involved in intra- and intermolecular hydrogen bonding, they cannot be the binding sites for water molecules. When α-helices or β-struc-

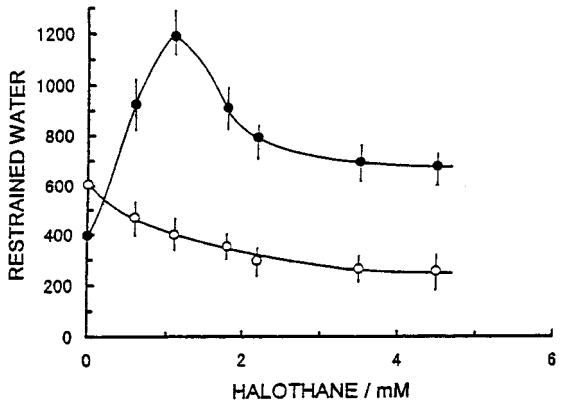

Figure 2. Effect of halothane on the hydration number: (○) strongly bound water, N_s; (●) weakly bound water, N_w. The error bars represents the results of at least three experiments. The BSA concentration was 34 mg cm^{-3}. Temperature: 25.00 ± 0.02 °C.

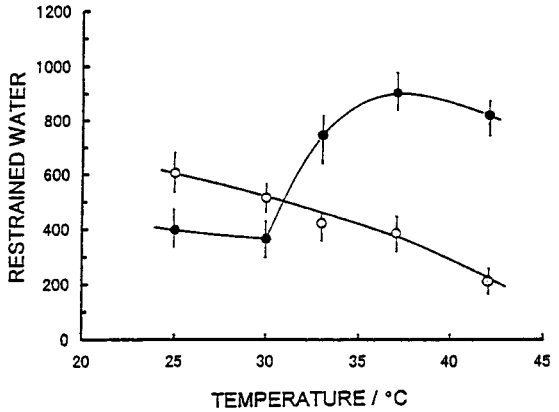

Figure 3. Effect of temperature on the hydration number: (○) strongly bound water, N_s; (●) weakly bound water, N_w. The experimental conditions were the same as Figure 2.

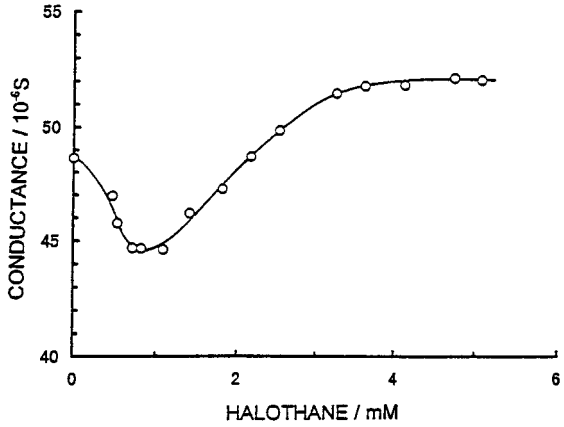

Figure 4. Effect of halothane on the electrical conductance of the BSA solution. The experimental conditions were the same as Figure 2.

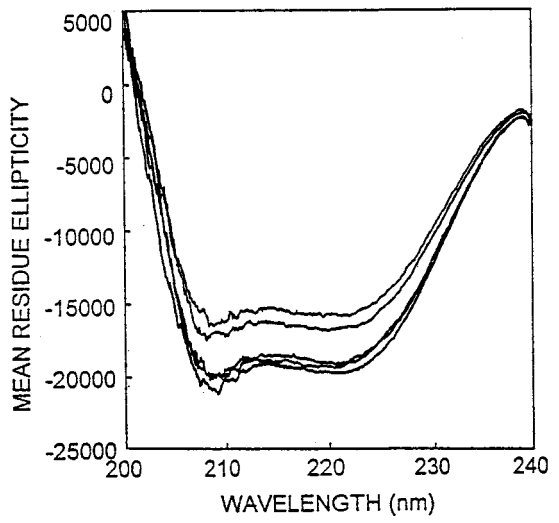

Figure 5. CD spectra of BSA 1.5 mg/mL in 168 mM NaCl solution at 23 °C. The lines are, from the bottom, control and halothane 11.1, 11.6, 14.9, and 16.9 mM. Halothane did not induce measurable changes when its concentrations were below this level.

tures are disjoined and form random structures, they become the hydration sites.

2. The hydrophilic side chains at the BSA surface are those with (a) charged moieties (lysine, arginine, glutamine, etc., type A), and (b) neutral moieties (serine, threonine, tyrosine, etc., type B). The charge−dipole (ion−water) interaction energies are stronger than the dipole−dipole (uncharge−water) interaction energies.[15] It is reasonable to assume that water molecules would interact with the type A side chains stronger than those with the type B side chains. The strongly (N_s) and weakly (N_w) bound water molecules, estimated by the microwave dielectric dispersion, correlate with the water molecules that interact with the type A and type B side chains, respectively. The ratio of the amino acids of BSA between the type A and type B side chains is about 179:120 (1.4:1).[16] The present data on N_s and N_w are 606:398 (1.5:1), and agree with the side-chain ratio. The correlation supports the two-site model.

According to this model, 3−4 water molecules are bound to each side chain. These hydration numbers appear to be reasonable when compared with the hydration number 4−6 of ions in water.[17] These agreements indicate that the hydrophilic side chains at the BSA surface are the binding sites of strongly and weakly bound water molecules.

2. Effect of Volatile Anesthetic Halothane on the Hydration. From the complex dielectric constant in the presence of halothane (Figure 1), the effect of halothane on the hydration of BSA was estimated (Figure 2). The increase of the halothane concentration decreased the strongly bound water molecules (N_s) to about a half of the control. In contrast, the weakly bound water molecules (N_w) were increased to exceed N_s. We[18−20] have shown that volatile anesthetics interact with lipid membranes and suppress the dissociation of the polar sites. When halothane is added to the aqueous solution of BSA, dissociation of the polar side chain is suppressed and the number of the undissociated side chains would increase. The two-site model predicts that type A and B represent N_s and N_w, respectively. Then the suppression of the dissociation of side chains decreases N_s and increases N_w.

Figure 2 shows that halothane >2.5 mM decreases N_s by about 300 and increases N_w by about 350. These numbers do not contradict the anesthetic action expected by the two-site model. At lower halothane concentration, however, N_s decreased gradually but N_w increased steeply and showed a maximum. This result does not agree with the idea that anesthetics suppress ionization. Figure 2 indicates that the hydration effect of halothane differs between the low and high concentration ranges.

In addition to the suppression of ionization, halothane also can break the hydrogen bonds.[21] This ability would weaken the attractive forces (a part of it is electrostatic) among side chains that maintain the protein conformation. Circular dichroism spectrum showed that halothane at low concentrations does not induce conformational change of the main chain (Figure 5). Halothane did not show observable change until its concentration

exceeded 11 mM. Halothane concentrations that induced clear changes in the BSA structure grossly exceeded the clinical concentrations (0.2−0.3 mM). The anesthetic did not induce significant conformational change of the main chain. Fourier transform infrared spectroscopy[22] showed that 1.5 M ethanol was required to show a change in the main peptide chain of firefly luciferase, whereas the anesthetizing concentration is about 0.19 M in tadpoles. Therefore, halothane at low concentrations is not powerful enough to break the hydrogen bonds among the BSA main peptide chain.

The attractive forces among domains and α-helices are weaker than the forces in the main chain.[13,14] Halothane may partially break the weak attractive forces and may open the domain structure. Though there is no experimental evidence, there is a possibility that this may form microscopic crevices among domains and α-helices. When water molecules are trapped in the cavity by the capillary force, they contribute to the steep increase of N_w. When the halothane concentration is further increased, the protein unfolds and exposes internal hydrophobic residues. The trapped water molecules, which exceed the hydration number of side chains, will be released as free water molecules. This process may be the cause of the decrease of the N_w following the maximum in Figure 2.

Suzuki et al.[1] reported that water molecules bound to the hydrophobic parts of amino acids represent N_w. Their study, however, dealt with the aqueous solution of amino acids. There is a major difference in hydration of the hydrophobic part of a single amino acid in water and the amino acids in a macromolecule. BSA is composed of 582 residues. In water, BSA forms a higher order structure, exposing hydrophilic parts to water and hydrophobic parts are mostly enclosed inside with little interaction with the aqueous phase. With micelles and lipid bilayers, we[23] have shown that anesthetics interact selectively with the interfacial polar area. This is because anesthetics are dipolar molecules. Accordingly, we maintain that the primary action site of halothane is the surface polar sites.

The total hydration number of BSA (N_w plus N_s) showed a temperature dependence (Figure 3). Temperature elevation monotonically decreased N_s. However, N_w discontinuously and steeply increased at about 35 °C. The three-dimensional structure of BSA in water is not rigid. The side chains and peptide chains are always fluctuating and are in a dynamic state. By the temperature dependency of partial specific volume of BSA, Iqbal and Verrall[23] showed that the fluctuation becomes larger with temperature elevation and induced microscopic unfolding of the protein. If it is allowed to assume that the small unfolding opens small spaces among subdomains and α-helices, similar to the halothane-induced structural relaxation, the sudden increase in N_w (Figure 3) may be attributable to the increase of the trapped weakly bound water molecules in Figure 2. The temperature-induced decrease of intramolecular attractive force, and the anesthetic-induced detachment of hydrogen bonding, loosen the three-dimensional structure and may invite water molecules into the created empty space in the protein.

3. Effect of Halothane on the Electrical Conductivity of BSA Solution. The electrical conductivity of BSA solution responded to halothane addition with a minimum (Figure 4). BSA molecules in water stay practically stationary under the alternating current because they are macromolecules. The volume fraction of BSA in water is smaller than 2.5%. Therefore, the surface electrical conductance on the BSA molecules can be ignored.[24] Then, Figure 4 represents the change in the bulk conductance caused by the ions in the solvent in equilibrium with BSA. There is a strong similarity between Figures 4 and 2. The correlation implies that halothane affects the polar side chains. The suppressing effect of halothane on ionization of polar side chains decreases the ionic concentration in the aqueous phase, which is in equilibrium with BSA. Therefore, the electrical conductance of BSA solution is decreased by halothane. The decrease of electrical conductance by halothane up to about 1 mM is caused by this mechanism.

Above 1 mM, however, halothane increased the electrical conductance (Figure 4). As discussed earlier about the hydrogen-bond breaking activity of halothane, it relaxes BSA structure and exposes internal polar side chains to water. The increase of polar moieties at the protein−water interface increases the number of ions in water, resulting in an increase of electrical conductivity. Because of the combined effect, the minimum in the bulk electrical conductance is formed.

The present study revealed that halothane does not alter the total hydration number of BSA significantly at clinical concentrations (about 0.3 mM) but reverses the ratio between N_s and N_w and doubles the number of the weakly bound water molecules. This change in the hydration is not the result of a large conformational change involving the main chain. It is a result of microscopic conformational change involving the side chains. At present, the role of weakly bound water on the protein function is unclear. However, it is established that proteins are active only when they are hydrated. The water molecule of the bound protein has a fundamental role in the function of the protein. It is not difficult to imagine that the water molecules bound at the inner surface of ion channels affects ionic current. The increase of less rigidly bound N_w by anesthetics is expected to influence the characteristics of the interacting ions. It is highly probable that anesthetic effect on hydration of macromolecules is related to anesthetic action mechanisms.

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