Hydrophobic Hydration Analysis on Amino Acid Solutions by the Microwave Dielectric Method

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Hydrophobic hydration was studied by the microwave dielectric method, which has been developed to separate the rotational relaxation of restrained water by hydrophobic side chains from the dielectric spectrum of an amino acid solution. Measurements were taken with a precision microwave network analyzer and a thermostated glass cell at 20.0 ± 0.01 °C with an open-end flat-surface coaxial probe. Examined amino acids were glycine, alanine, α-aminobutylic acid, norvaline, valine, norleucine, isoleucine, leucine, and phenylalanine. The present method assumes that the dielectric spectrum of an amino acid solution is approximated with that of a solute/water emulsion containing two kinds of spherical solutes having different Debye-type relaxation frequencies. We found that in straight alkyl side chains of tested amino acids, each $-CH_2-$ restrains three water molecules on average and its hydration shell has a relaxation frequency f_c around 5.5 GHz, while branched alkyl side chains restrain less than the straight ones and their hydration shells have a slightly lower f_c of 4.1−4.4 GHz. The f_c of the hydration shell of a phenylalanine side chain is 4.0 GHz and the phenyl group restrains nearly six water molecules, indicating a rather hydrophilic nature. The static dielectric constant of the hydrophobic hydration shell was found to be around 110, which is higher than that of bulk water.

1. Introduction

An apolar moiety in water is often considered to be surrounded by water molecules forming a rigid hydration cage based on the solvation heat analyses as summarized in ref 1. Goldammer and Hertz[2] studied the hydration behavior of nonelectrolytes by the ^{17}O NMR technique and found an absence of such a rigid cage and loosely bound hydration shell. Rossky and Karplus[3] carried out a molecular dynamics study on an aqueous solution of two apolar atomic solutes and concluded that the rotational mobility of water in the hydrophobic hydration shell decreases by a factor of 3 in going from the bulk to the hydration shell of one molecular layer of water.

Dielectric spectroscopic analyses were made by Salefran et al.[4] and Kaatze et al.[5] who studied amino acids with hydrophobic moieties in their main chains and demonstrated that the orientational relaxation of amino acids and the relaxation of hydrophobic hydration shells can be separately analyzed. However, their analyses were based on a linear combination of three Debye components. According to the dielectric emulsion theories by Wagner[6] and Hanai,[7] the relaxation spectrum of dispersed solutes appears as a modified profile from the intrinsic spectrum because of the nonlinearity.

We carried out a nonlinear analysis of hydrophobic amino acids based on Hanai's emulsion theory so that we are able to estimate the intrinsic relaxation frequency and the volume of hydrophobic hydration shell. The method was previously applied to measure protein hydrations and successfully distinguished weakly restrained water having a relaxation frequency around 4 GHz from strongly restrained water on proteins.[8]

In this analysis it is important to know how far a polar group affects water molecules that hydrate the other polar/apolar solutes. Kowall and Geiger[9] showed that the interaction between −COC− of crown ether and potassium ion in water acts within 0.5 nm in spacing. Pettitt and Karplus[10] showed that the interaction between >NH and >CH− acts within 0.3 nm in spacing. From these computational results the water molecules near a hydrophobic side chain of amino acids should be hardly affected by >NH or >C═O if they are apart from them more than 0.3 nm (the diameter of one water molecule). Therefore, it is possible to analyze the hydrophobic hydration separately in hydrophobic amino acid solutions. In this paper we show the method of dual-dispersion-model analysis for extracting the rotational relaxation component of water in hydrophobic hydration shells and the orientational motion of amino acid from the solution spectrum.

2. Methods

Our dual-dispersion-model analysis is based on the following assumptions (see Figure 1)

(1) The orientational relaxation of an amino acid molecule is approximated with a virtual dielectric sphere for each molecule having a single Debye relaxation function. We assume that the dielectric constant of the virtual sphere for the orientational polarization of the amino acid ϵ_r^* is given by

$$\epsilon_r^* = \epsilon_{r\infty} + \frac{\epsilon_{rs} - \epsilon_{r\infty}}{1 + j(f/f_r)} \tag{1}$$

where ϵ_{rs} is constant and f_r is the apparent orientational relaxation frequency depending on the side chain. ϵ_{rs} and the size of the sphere can be determined from the glycine spectrum, and $\epsilon_{r\infty}$ will be described later. Considering that the molar dielectric

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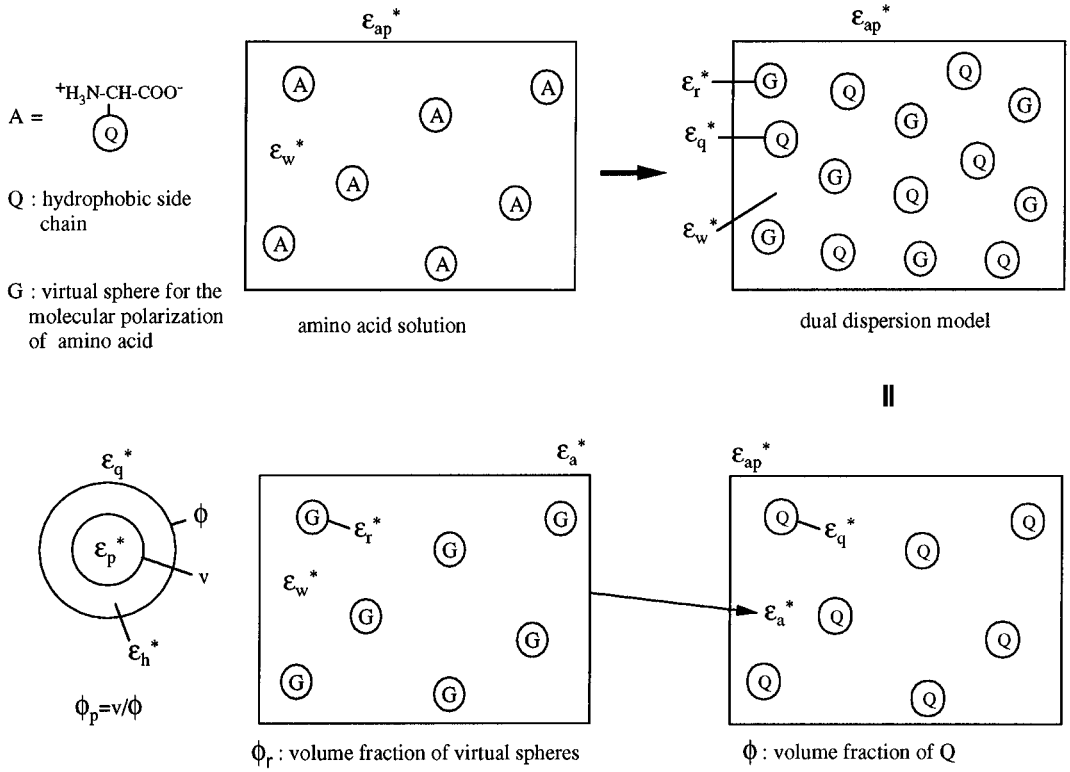

Figure 1. Dual dispersion model analysis. Assuming that the apparent complex dielectric constant of an amino acid solution is approximated with the dual dispersion model, the dielectric relaxation of the orientational motion of amino acid molecules and that of hydrated side chains are expressed by dielectric spheres G and Q in water, respectively (eqs 1−4).

increments of hydrophobic α-amino acids (δ (L/mol) = 23) are nearly independent of the side chains,[11,12] ϵ_{rs} and $\epsilon_{r\infty}$ are assumed to be independent of the side chains.

(2) A hydrophobic side chain is approximated with a free dielectric sphere having a spherical hydration shell. The hydrated sphere has a dielectric constant ϵ_q^* given by

$$\epsilon_q^* = \epsilon_{q\infty} + \frac{\epsilon_{qs} - \epsilon_{q\infty}}{1 + j(f/f_c)} \tag{2}$$

where we determine $\epsilon_{q\infty}$ based on the electronic polarization of atom groups.[12] f_c is the relaxation frequency of the hydrated solute. Since the dielectric constant of the core solute (a hydrophobic side chain) is constant, f_c becomes the relaxation frequency of the hydration shell according to eq 5. The dielectric constant of the hydration shell at the high-frequency limit is set to 5.6, the same as that of pure water.[13]

The apparent complex dielectric constant ϵ_{ap}^* of the dual disperse system of two kinds of spherical particles is expressed by the Hanai equation[7] with the dielectric constant ϵ_a^* of the solvent, which is a mixture of pure solvent and the first solutes, and that of the second dispersed solutes (hydrophobic side chains) ϵ_q^*:

$$\frac{\epsilon_{ap}^* - \epsilon_q^*}{\epsilon_a^* - \epsilon_q^*}\left(\frac{\epsilon_a^*}{\epsilon_{ap}^*}\right)^{1/3} = 1 - \phi \tag{3}$$

where ϕ is the volume fraction of the hydrated second solutes, i.e., hydrated hydrophobic side chains. The dielectric constant of solvent ϵ_a^* is expressed with ϵ_w^* and ϵ_r^* in the same manner:

$$\frac{\epsilon_a^* - \epsilon_r^*}{\epsilon_w^* - \epsilon_r^*}\left(\frac{\epsilon_w^*}{\epsilon_a^*}\right)^{1/3} = 1 - \phi_r \quad (\text{for } 0 < \phi_r < 1) \tag{4}$$

where ϵ_w^* is the dielectric constant of pure solvent (water) and ϕ_r is the volume fraction of the virtual dielectric spheres representing the orientational polarization of amino acid molecules in the solvent containing the first solutes.

When the second solute is a shelled sphere, ϵ_q^* is expressed with the dielectric constant of hydration shell ϵ_h^* and that of the core (a hydrophobic side chain) ϵ_p^*.[14]

$$\epsilon_q^* = \epsilon_h^* \frac{2(1 - \phi_p)\epsilon_h^* + (1 + 2\phi_p)\epsilon_p^*}{(2 + \phi_p)\epsilon_h^* + (1 - \phi_p)\epsilon_p^*} \quad (\text{for } 0 < \phi_p < 1) \tag{5}$$

where $\phi_p = v/\phi$. v is the volume fraction of the core of the second solute in a solution calculated by $cM_w s_v/1000$. c, M_w, and s_v are the concentrations (mol/L), the molecular weight (g/mol), and the partial specific volume (L/kg) of the second solute core, respectively. According to a previous study,[15] we estimated the s_v's of hydrophobic side chains as 1.152 for $-CH_3$, 1.179 for $-CH_2CH_3$, 1.188 for $-CH_2CH_2CH_3$, 1.193 for $-CH_2CH_2CH_2CH_3$, 1.161 for $-CH(CH_3)_2$, 1.132 for $-CH_2CH(CH_3)_2$, 1.132 for $-CH(CH_3)CH_2CH_3$, and 0.860 for $-CH_2C_6H_5$, respectively. Equation 5 is used to obtain $\epsilon_{q\infty}$, the high-frequency limit ($f \rightarrow \infty$) of ϵ_q^*, where $\epsilon_h^* = \epsilon_{h\infty} = 5.6$ and $\epsilon_p^* = \epsilon_{p\infty} = 2$ were used at $f \rightarrow \infty$. Similarly, considering the virtual sphere in eq 1 as a shelled sphere, $\epsilon_{r\infty}$ equals 4.4 when the value of ϕ_r/c given below is used. It should be noted that $\epsilon_{r\infty}$ and $\epsilon_{q\infty}$ cause only a constant effect on ϵ_{ap}^*.

First we determine the parameters ϵ_{rs} and f_r in eq 1 by fitting eq 4 to the dielectric spectrum of a glycine solution as shown in Figure 2. We found that $\epsilon_{rs} = 980 \pm 70$ (SEM for seven experiments), $f_r = 0.77 \pm 0.08$ GHz, and $\phi_r/c = 0.1112 \pm 0.0043$. We adopt the values of $\epsilon_{r\infty} = 4.4$, $\epsilon_{rs} = 980$, and $\phi_r/c = 0.1112$ for the other amino acids. Second, when the initial value of ϕ is set, the dielectric spectrum of hydrated solute ϵ_q^* is calculated by eqs 3 and 4. Third, the obtained ϵ_q^* is fitted by eq 2 in the frequency range 0.50−8.73 GHz, by which we

determine f_c. Fourth, ϕ is iteratively adjusted in eqs 1−4 until $\epsilon_{q\infty}$ in eq 3 agrees with the value obtained by eq 5. Fifth, f_r is iteratively adjusted by repeating this procedure to minimize the following quantity:

$$Z = \sum_{f=f_i}^{f_f} \{(\epsilon(f)' - \epsilon_{sim}(f)')^2 + (\epsilon(f)'' - \epsilon_{sim}(f)'')^2\} \quad (6)$$

where f_i and f_f are 0.50 and 8.73 GHz, respectively. $\epsilon_{sim}^*(f)$ and $\epsilon^*(f)$ are the simulated spectrum ϵ_{ap}^* by eqs 1−4 and the spectrum of a tested solution, respectively.

Thus, we obtain ϕ, f_c, f_r and ϵ_{qs}. The number of restrained water molecules per solute molecule N_{H_2O} was then calculated by

$$N_{H_2O} = \frac{55.6(\phi - v)\rho_0}{c} \quad (7)$$

where ρ_0 is the density of a hydration shell, which is that of pure solvent (1 g/cm^3) as long as we use the partial specific volume s_v to calculate v by its definition.

3. Experiments

We examined glycine (purity > 99%), L-alanine (purity > 99%), D,L-aminobutylic acid (purity > 97%), D,L-norvaline (purity > 99%), L-valine (purity > 99%), D,L-norleucine (purity > 99%), L-leucine (purity > 99%), L-isoleucine (purity > 98%), and L-phenylalanine (purity > 99%), which were purchased from Wako Pure Chemical Ind., Ltd. and dissolved in pure water (by Milli-Q, 18 MΩ), noting that after drying at 105 °C for 4 h, each amino acid had a weight loss that was less than 0.5%.

The dielectric spectra were obtained with a microwave network analyzer, Hewlett-Packard 8720C, and an open-end flat-surface coaxial probe. To prevent the accumulation of microbubbles, the probe was fixed upward in a glass cell controlled at 20.0 ± 0.01 °C by a Neslab thermobath. The cell was a cylinder with a conical top for a stirrer space, with dimensions of 33 mm in inner diameter, 30 mm in maximum height, and 20 mL in volume. The solution temperature was monitored with a platinum-resistor thermosensor by the four-terminals method. The cell was filled with a sample solution degassed under reduced pressure. Microwaves in the frequency range 0.2−20 GHz were introduced to the cell through the probe. The calibration was done by a procedure consisting of an open-circuit in air and a short-circuit with mercury and by dipping in pure water at 20.0 ± 0.01 °C. The reflected waves were sampled by a network analyzer and converted to complex dielectric spectra with HP85070A software using the Nicolson−Ross method.[16] Since the oscillator of the network analyzer quickly loses its stability at high temperature (>53 °C), we kept the inside of the network analyzer below 50 °C by increasing air flow and suppressed the total drift of the dielectric constant within 0.027 for 0.50−8.73 GHz, which is one-fifth the usual case. In the frequency range 10−20 GHz there is a comparatively large system error caused by some reflections of microwaves within the test cell. It hides the phenomena underlying this frequency range. Therefore, we used the dielectric spectra of 32 frequency points from 0.50 to 8.73 GHz for the ϵ_q^* analysis. For each sample, 20 dielectric spectra were sequentially obtained every 10 s at 20.0 ± 0.01 °C and averaged.

4. Results

Table 1 shows the apparent orientational relaxation frequency f_r shown for each amino acid. The larger amino acids have

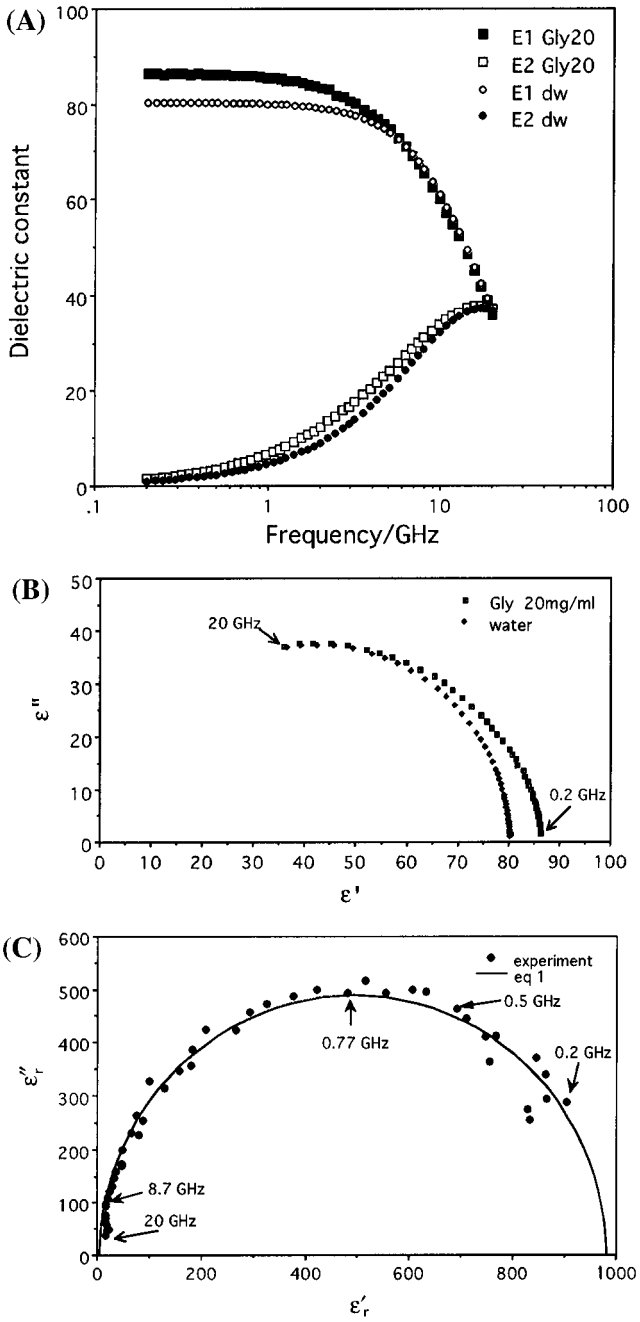

Figure 2. Dielectric analysis of glycine spectrum: (A) Dielectric spectra of glycine solution (Gly 20:20 mg/mL, pH = 6.1) and pure water (dw); (B) Cole−Cole plots corresponding to part A; (C) Cole−Cole plot of the dielectric constant ϵ_r^* of the virtual sphere for a glycine molecule derived from eq 4.

lower orientational relaxation frequencies. Here, we must note that these frequencies do not directly represent the intrinsic relaxation frequency f_p of the molecular polarizability of amino acids. The f_p can be estimated with the following considerations of fundamental electromagnetism. The dipole moment **p** of the virtual sphere of radius a in water in a uniform electric field $\mathbf{E}_0$ is given by

$$\mathbf{p} = \alpha^* \mathbf{E}_0 \quad (8)$$

$$\alpha^* = 4\pi\epsilon_0 a^3 \frac{\epsilon_r^* - \epsilon_w^*}{\epsilon_r^* + 2\epsilon_w^*} \approx \alpha_0 + \frac{\alpha_1}{1 + j(f/f_p)} \quad (9)$$

where

TABLE 1: Dielectric Properties of Amino Acid Solutions Measured by the Present Method[a]

amino acid	concentration (mg/mL)	SAS area (Å^2)	f_r (GHz)	f_p (GHz)	f_c (GHz)	N_{H_2O}	δ (L/mol)	ϵ_{hs}
Gly	20	226.7	0.77 ± 0.08	4.07			22.60 ± 0.20	
Ala	20	249.5	0.48 ± 0.05	2.67	(4.7 ± 0.3)	(1.75 ± 0.28)	22.72 ± 0.23	(250 ± 12)
ABA	20	275.5	0.36 ± 0.03	2.10	5.3 ± 0.3	6.80 ± 1.14	22.53 ± 0.23	117 ± 5
n-Val	20	306.0	0.29 ± 0.03	1.75	5.6 ± 0.3	10.61 ± 1.70	21.70 ± 0.22	108 ± 5
n-Leu	10	325.4	0.25 ± 0.03	1.55	5.6 ± 0.3	11.80 ± 3.5	20.43 ± 0.21	106 ± 10
Val	20	286.8	0.30 ± 0.03	1.80	4.1 ± 0.3	7.37 ± 1.18	22.42 ± 0.22	120 ± 5
Leu	20	317.6	0.26 ± 0.03	1.60	4.4 ± 0.3	8.53 ± 1.36	20.52 ± 0.20	110 ± 5
Ile	20	311.6	0.24 ± 0.02	1.50	4.1 ± 0.3	8.60 ± 1.38	21.87 ± 0.23	123 ± 5
Phe	20	351.2	0.21 ± 0.02	1.36	4.0 ± 0.3	7.51 ± 1.20	19.42 ± 0.20	103 ± 5

[a] Gly, L-glycine; Ala, L-alanine; ABA, D,L-α-amino-*n*-butylic acid; *n*-Val, D,L-norvaline; *n*-Leu, D,L-norleucine; Val, L-valine; Leu, L-leucine; Ile, L-isoleucine; Phe, L-phenylalanine. SAS area: solvent accessible surface area calculated with an oxygen radius of 1.4 Å, a nitrogen radius of 1.55 Å, and a carbon radius of 2.0 Å. f_r: the apparent rotational relaxation frequency of the amino acid molecule in eq 1. The deviations indicate the errors due to $\epsilon_{rs} = 980 \pm 70$. f_p: the rotational relaxation frequency of the amino acid molecule calculated from f_r by eqs 9 and 10. f_c: the relaxation frequency of hydrophobic hydration shell. N_{H_2O}: number of water molecules restrained by the side chain calculated by eq 7. δ: the molar dielectric increment $\delta = (\epsilon - \epsilon_1)/c$, where ϵ and ϵ_1 are the dielectric constants at the low-frequency limit of solution and pure solvent, respectively. c is the molar concentration. The errors come from the purity uncertainty of 1%. ϵ_{hs}: the dielectric constant of the hydrophobic hydration shell at the low-frequency limit. Parentheses mean that the data are shown only for reference.

$$\epsilon_w^* = 5.6 + \frac{74.8}{1 + j(f/17)} \quad \text{and} \quad \epsilon_r^* = 4.4 + \frac{980 - 4.4}{1 + j(f/f_r)} \tag{10}$$

The f_p of glycine is numerically obtained as 4.1 for $f_r = 0.77$ in GHz. Similarly, we obtained f_p's for the other amino acids, which are shown in Table 1.

Figure 3 shows the dielectric spectra of a leucine solution and pure water (Figure 3A) and the Cole−Cole plots of the complex dielectric constant ϵ_q^* (Figure 3B) of a hydrated side chain of leucine by eqs 1−5. Figure 4 shows the results of ϵ_q^* for different amino acids. In our analysis, the Debye fitting was made in the frequency range 0.50−8.73 GHz because of the high *S*/*N* values. The relaxation frequency f_c of the hydrophobic hydration shell and the relaxation frequency f_p of amino acid molecular polarizability are shown in Figure 5. The f_c's for the straight alkyl chains were around 5.5 GHz, while for the branched alkyl chains the f_c's were 4.1−4.4 GHz, apparently lower than those of straight chains. The number of water molecules restrained on the side chains is given in Figure 6. The linear regression line shows that, on average, three water molecules are restrained on each carbon. For the branched alkyl chains the water molecules are slightly less restrained than for straight chains. Although the solvent accessible surface (SAS) area of an alkyl chain, say, butane, depends on its conformation, the discrepancy between trans and gauche is only 4.4% over the total SAS area of 207 Å^2 (trans). The SAS area of a $-CH_2-$ group is 35−40 Å^2 using the van der Waals radius of carbon of 2.0 Å and a water radius of 1.4 Å as shown in Table 1. The occupation area of one water molecule on the SAS is approximately 9 Å^2. Thus, the average number of water on the SAS of $-CH_2-$ is 4.

The side chain of a phenylalanine restrains 7.5 water molecules with an f_c of 4.0 GHz. We found that a phenyl group restrains approximately 6 water molecules (simply by 7.51(Phe) − 1.75(Ala)), which is about half the value calculated from the SAS area and in good correspondence with the number of π electrons showing its hydrophilic nature.

For each amino acid, ϵ_{qs} was found in the range 80−110. According to eq 5, the dielectric constant of the hydrophobic hydration shell ϵ_{hs} at $f \rightarrow 0$ is 103−125 except for alanine (ϵ_{hs} = 250; see Table 1). This indicates that the water molecules in the hydrophobic hydration shell are more polarizable than the bulk water. The results are summarized in Table 1.

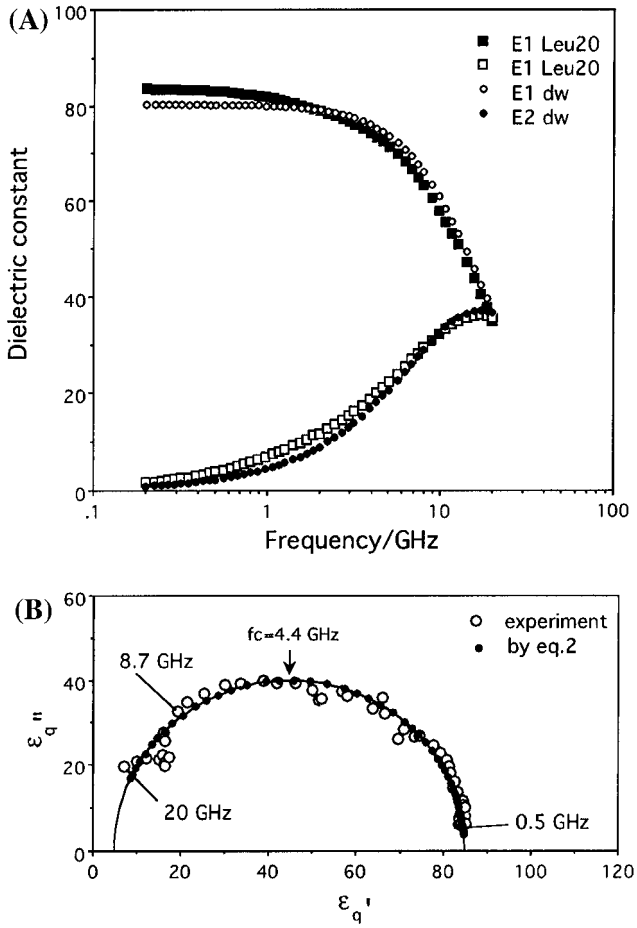

Figure 3. Dielectric analysis of an amino acid solution: (A) Dielectric spectra of a leucine solution (Leu 20:20 mg/mL, pH = 6.0) and pure water (dw); (B) Cole−Cole plot of the dielectric constant ϵ_q^* of the hydrated side chain of leucine derived from eqs 1−5.

5. Errors and Discussion

The absolute error of the measured value N_{H_2O} possibly resulted from the stability of the network analyzer, $\epsilon_{q\infty}$, and the shape of the solute. $\epsilon_{q\infty}$ has a maximum possible uncertainty of 0.5, which causes a 4.5% error in the volume of the hydration shell V_{hs} according to eq 3. The single Debye fitting on ϵ_q^* may have caused underestimation of V_{hs} because any relaxations higher than 10 GHz, if there were indeed any, were neglected. Therefore, in this study the measured amount of restrained water corresponds to that of a hydration shell having a relaxation

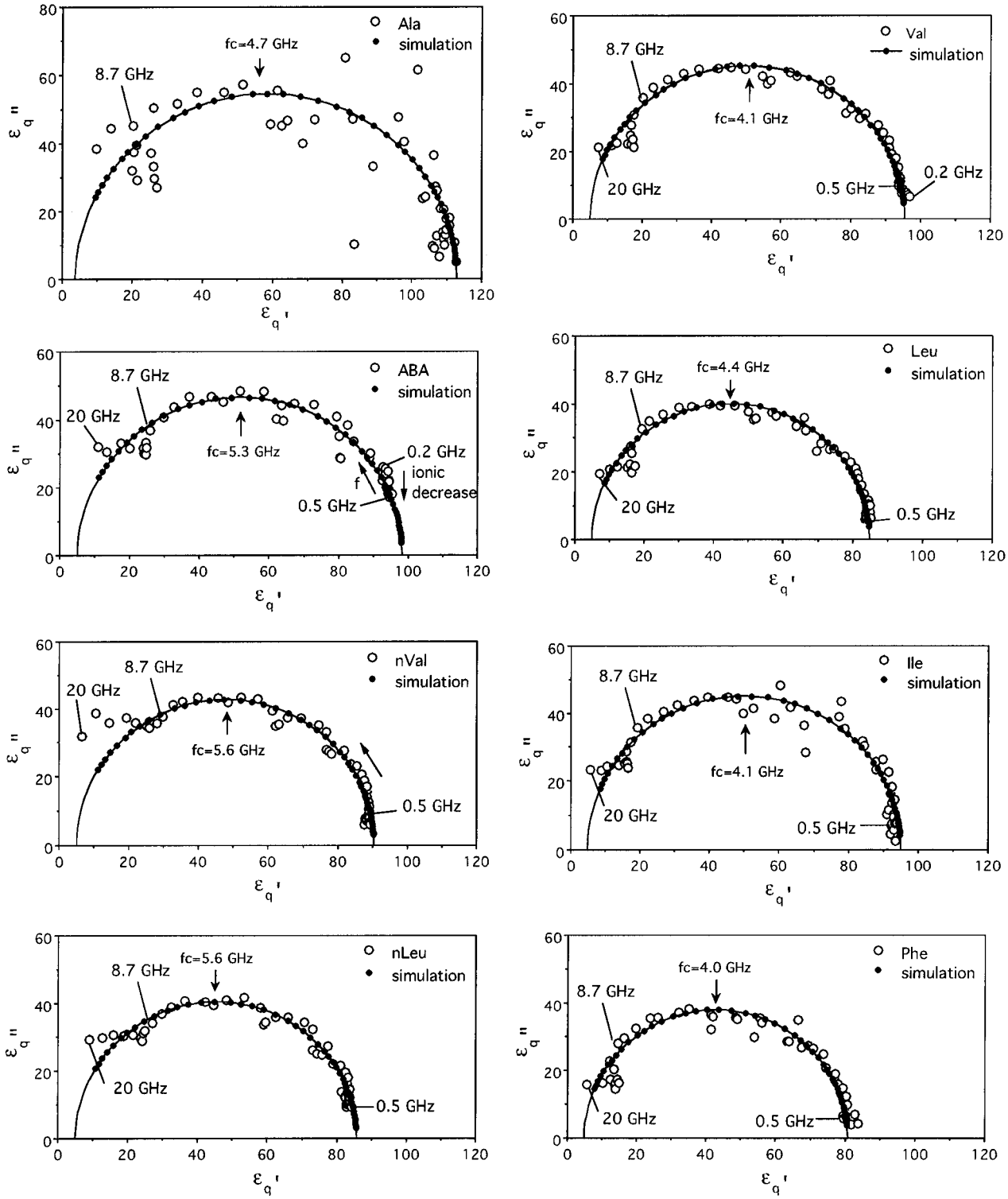

Figure 4. Cole−Cole plots of the dielectric constant ϵ_q* of the hydrated side chain of amino acids derived from eqs 3 and 4: Ala, alanine (pH = 6.5); ABA, α-aminobutylic acid (pH = 6.0); *n*-Val, norvaline (pH = 6.1); *n*-Leu, norleucine (pH = 6.0); Val, valine (pH = 6.0); Leu, leucine (pH = 6.0); Ile, isoleucine (pH = 6.8); Phe, phenylalanine (pH = 6.3). Simulation lines were calculated with eq 2.

frequency of less than 10 GHz. The standard deviation of measured dielectric constants by the network analyzer was 0.027 from 0.50 to 8.73 GHz. The error of the average value for the 20 sequentially measured dielectric constants in this frequency range decreased to 0.010. From this, eq 3 gives the error of V_{hs} of 3% for a 20 mg/mL solute solution. It is inversely proportional to the solute concentration. The long time drift of the network analyzer was 0.06/h on average. Since it took 30 min to exchange sample solutions, we should take into account the long time drift, which caused a 15% error of V_{hs}. Taken together, the total absolute error of N_{H_2O}, E_{rt} in %, becomes $E_{rt} = (4.5^2 + 15^2)^{0.5} = 16$. Hence, N_{H_2O} has 16% of the absolute error for a 20 mg/mL amino acid solution.

The errors of ϵ_{qs} and the rotational relaxation frequency of the amino acid f_r depend on the long time drift of the network analyzer. The standard error of the mean of ϵ_{qs} for glycine was 70 for seven independent experiments. This error causes a 10% error in f_r. The f_c error of 0.3 GHz comes from the frequency step and the drift of the network analyzer. These are involved in the error of N_{H_2O}.

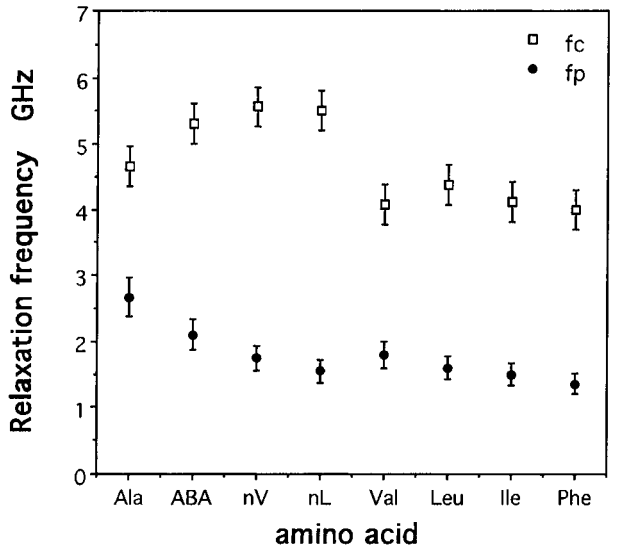

Figure 5. Relaxation frequencies of water in the hydrophobic hydration shell (f_c) and of amino acid molecular polarizability using eqs 8−10 (f_p).

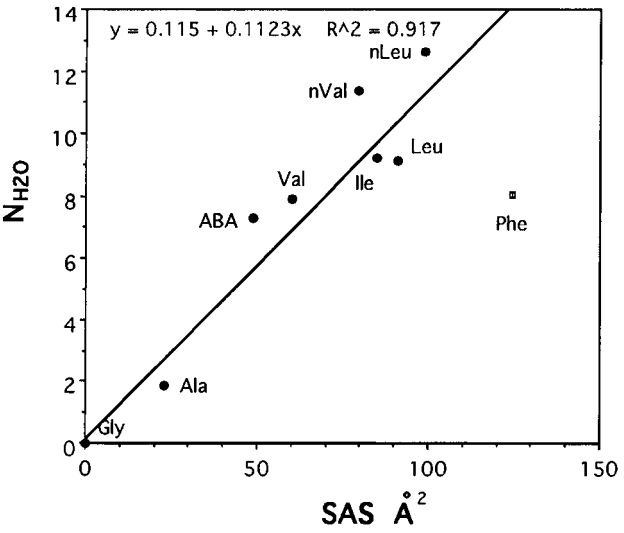

Figure 6. Number of restrained water molecules on each hydrophobic side chain as a function of the SAS by eq 7. The least-squares linear regression line was obtained except for Phe.

According to our measurement of the dielectric spectra of NaCl aqueous solutions up to 0.1 M, any relaxation was not detected in the gigahertz region. Those spectra are of linear ionic conduction. The Cole−Cole plot of the glycine relaxation (Figure 2C) extracted from the solution spectrum showed α almost equal to 0 in the empirical Cole−Cole equation,

$$\epsilon^*(\omega) = \epsilon_\infty + (\epsilon_s - \epsilon_\infty)/(1 + (j\omega\tau)^{1-\alpha})$$

which means that it is approximated with a single Debye relaxation process. If the water molecules in the hydration shell of charged groups have a relaxation frequency in the range 10^8−10^{10} Hz, α of glycine cannot be 0. From this result we conclude that the relaxation frequency of the hydration shell of charged groups must be much lower than 10^8 Hz. The water molecules around polar groups seem to be tightly restrained and move together with the amino acid molecule. Then by assuming that the hydration properties of polar groups of α-amino acid cores except for glycine are similar to those of glycine, we are able to separate the dielectric property of hydrated side chain from the amino acid orientational motion.

The shape of the dipole solutes may cause another uncertainty. The rotational motion of spherical dipoles in a viscous fluid in an ac field is represented by a single Debye relaxation equation. When the dipole shape is ellipsoid or more complicated, it has two or more characteristic times of relaxation. In this case we can adopt the Cole−Cole equation rather than the Debye equation for the amino acid−dipole relaxation. If α is not zero and the Cole−Cole plot of the amino acid−dipole relaxation has a lower maximum than that of Debye's, then the Cole−Cole plot of the relaxation of the hydrated side chain must have a high maximum to fit the spectrum. Already we found a good fitting with two Debye processes. It means that if α for the amino acid−dipole relaxation is not zero, the maximum height of the Cole−Cole plot of the hydrated side chain must be higher than that of the Debye's half circle to fit the spectrum experimentally obtained. This is not acceptable. Therefore, using the Cole−Cole equation does not improve the fit in our case.

The shape of side chains may be better approximated by a prolate spheroid than by the spherical model adopted in this study. We have compared the results between the spherical model and the prolate spheroid model[17] with an axial ratio of 3. The discrepancy was 4% in the N_{H_2O} for solutes of the same volume fraction. It should not cause any essential changes in the results.

We must comment on the validity of our method, which assumes no interference between rotational motion of amino acids and that of water molecules in the hydration shell of a side chain. We simply estimate the validity by comparing the results with respect to the size of side chains. Since amino acids with longer side chains have lower rotational relaxation frequencies, the interference should become less for those with a short side chain. We obtained similar values of ϵ_{hs} from 103 to 123 for *n*-Leu, *n*-Val, Leu, Ile, ABA, and Phe. The values of f_c for *n*-Leu, *n*-Val, and ABA were about the same, 5.5 GHz. However for Ala ϵ_{hs} was evaluated as 250 and f_c was 4.7 GHz so that the methyl group as a side chain is not large enough to be analyzed by our method. It is consistent with the interaction distance between polar and apolar atoms shown by computational chemistry.

We assumed that ϵ_{rs} is independent of the size of the side chain and equated to the value of glycine. As long as we see the correlations between δ and f_c or between δ and ϵ_{hs} in Table 1, for example, those of *n*-Val, *n*-Leu, Val, and Leu, the assumption did not cause any systematic changes in f_c and ϵ_{hs}.

6. Conclusion

We analyzed the rotational relaxation frequencies of amino acids and the water molecules in the hydrophobic hydration shell around their side chains simultaneously. By assuming a single Debye relaxation for the hydration shell on the hydrophobic side chains and taking into account the high-frequency limit of the dielectric constant of the solute, we found that on average, three water molecules are restrained for each $-CH_2-$ with a relaxation frequency of 5.5 GHz for straight alkyl side chains, with slightly less hydration for a branched alkyl side chain with a relaxation frequency in the range 4.1−4.4 GHz. Thus, most of the water molecule access to an apolar moiety of alkyl groups is restrained. On the other hand, a phenyl group restrains only six water molecules, indicating a rather hydrophilic nature. The static dielectric constant of the hydrophobic hydration shell is found to be around 110, suggesting that the water molecules in the shell are subjected to low thermal fluctuations as if they were cooled without freezing.

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References and Notes

(1) Tanford, C. *The hydrophobic effect*; John Wiley & Sons: New York, 1980.
(2) Goldammer, E. v.; Hertz, H. G. *J. Phys. Chem.* **1970**, *74*, 3734.
(3) Rossky, P. J.; Karplus, M. *J. Am. Chem. Soc.* **1979**, *101*, 1913−1937.
(4) Salefran, J. L.; Delbos, G.; Marzat, C; Bottreau, A. M. *Adv. Mol. Relax. Interact. Processes* **1977**, *10*, 35.
(5) Kaatze, U.; Bieler, H.; Pottel, R. *J. Mol. Liq.* **1985**, *30*, 101.
(6) Wagner, K. W. *Arch. Elektrotech.* **1914**, *2*, 371.
(7) Hanai, T. *Kolloid Z.* **1960**, *171*, 23.
(8) Suzuki, M.; Shigematsu, J.; Kodama, T. *J. Phys. Chem.* **1996**, *17*, 7279.
(9) Kowall, T.; Geiger, A. *J. Phys. Chem.* **1995**, *99*, 5240.
(10) Pettitt, B. M.; Karplus, M. *J. Phys. Chem.* **1988**, *92*, 3994.
(11) Wyman, J.; McMeekin, T. L. *J. Am. Chem. Soc.* **1933**, *55*, 908.
(12) Pethig, R. *Dielectric and Electronic Properties of Biological Materials*; John Wiley & Sons: New York, 1979.
(13) Kaatze, U. *J. Chem. Eng. Data* **1989**, *34*, 371.
(14) Zhang, H. Z.; Sekine, K.; Hanai, T.; Koizumi, N. *Colloid Polym. Sci.* **1983**, *261*, 381.
(15) Millero, F. J.; Surdo, A. L.; Shin, C. *J. Phys. Chem.* **1978**, *82*, 784.
(16) Misra, D. *IEEE Trans. Microwave Theory Tech.* **1987**, *35*, 925.
(17) Asami, K.; Hanai, T.; Koizumi, N. *Biophys. J.* **1980**, *31*, 215.