Effect of Hydrophobicity of Amino Acids on the Structure of Water†

Makoto Ide, Yasushi Maeda, and Hiromi Kitano*

Department of Chemical and Biochemical Engineering, Toyama University, Toyama, 930 Japan

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The structure of water in various amino acid solutions was examined by Raman spectroscopy and ^{1}H NMR. Analyses of the relative intensity (*C* value) of the O−H stretching Raman band corresponding to an in-phase collective vibration of O−H oscillators that are connected by hydrogen bonds revealed that the structure of water in solutions of various amino acids (Gly, Ala, Ser, Val, Leu, Ile, His, Asn) at neutral pH did not differ from one another significantly, which shows that the structure of water is little affected by the side chains. Deionization of carboxylate or ammonium ions induced appearance of the effects of side chains on the structure of water around them. That is, the structure of water in aqueous solutions of amino acids with nonpolar side chain was enhanced, and the probability of hydrogen bonding between water molecules increased by forming water clathrate around the side chain. On the other hand, the structure of water in the solutions of amino acids with polar or charged side chain was destroyed. Moreover, the motion of water molecules around both hydrophilic and hydrophobic side chains was revealed to be restricted by spin−lattice relaxation time (T_1) measurements of hydrogen atoms in water molecules. Electrostatic interaction or hydrogen bonding between the amino acid and water molecule is responsible in the former case, and the formation of water clathrate around a hydrophobic moiety is important in the latter case. The deviations ($|C_x - C_w|$) of the values of *C* of amino acid solutions (C_x) from that of pure water (C_w) were well correlated with the solvent-accessible surface area of the side chain to water and increased in the order Leu > His > Ile > Val > Asn > Ser, Ala, Gly. The *C* values of amino acids, the physical meaning of which is clearer than those of indices obtained by thermodynamic methods, might be used as a novel index for relative hydrophobicities of amino acids.

Introduction

Proteins and nucleic acids have to keep specific three-dimensional structures to fulfill their functions, and interaction between water and these macromolecules largely relates to their structural stability in aqueous solutions. By X-ray crystallography,[1] NMR,[2] and other spectroscopic techniques, many investigators have studied the interaction between proteins and water to clarify the mechanism of formation of higher-order structure of proteins.[3] Hydrophobic interaction, which makes solvent-accessible surface area of nonpolar solutes to water minimal,[4] drives the folding of the secondary structure of polypeptides,[5] and therefore, hydrophobic properties of the amino acid side chains have been often studied mainly by thermodynamic methods. For example, indices of hydrophobicities of the side chains were determined by partition coefficients, K_d, of the amino acids from water to organic solvents,[6−9] and such macroscopic properties of amino acids have been effectively used to predict folding of proteins, especially of membrane proteins, from their amino acid sequences without crystallographic data.[10]

As for the hydration of protein molecules, most of experimental results came from the calorimetric method, which indicates amounts of unfrozen water even below 0 °C. These results, however, might not faithfully reflect the interaction between the solutes (proteins or amino acid side chains) and water molecules under natural conditions. Recently, many researchers have carried out computer simulations on hydration of proteins though the simulations were not necessarily supported by appropriate experimental results. On the other hand, investigations by infrared (IR) spectroscopy and Raman scattering, which potentially provide abundant information concerning the structure of hydration water and interaction between water and amino acid residues in proteins *in situ*, are not plentiful.

Properties of water observed by the thermodynamic methods contain information about the so-called *D-structure* of water, which is averaged by the rotational motions of water molecules.[11] Time resolutions of chemical shift and spin relaxation of NMR, X-ray analysis, dielectric relaxation, and light scattering method are not satisfactorily high either and provide nothing but information about the *D-structure*, whereas, IR spectroscopy, Raman scattering, and neutron scattering give knowledge about the *V-structure* of water, which contains information about orientational distribution of water molecules and is averaged only by vibration of atoms in water molecule. Therefore, these are important methods for studying the structure of water. The O−H stretching Raman band of water has been reported to consist of four or five Gaussian components, and the lowest wavenumber component was assigned to correlated *in-phase* stretching of O−H oscillators in hydrogen-bonded water clusters by Walrafen.[12] Green et al. have done the same assignment and called it a *collective band*.[13] The collective band whose relative intensity reflects the probability of water−water hydrogen bonding can be conveniently separated from the whole O−H stretching Raman band of water by using a polarization method. The procedure has been shown to be useful in examining the hydrogen-bonding structure of water, and we have been investigating the structure of water in aqueous polymer solutions and gels by using Raman spectroscopy, which revealed that the hydrophilicity of water-soluble polymers can be estimated from the relative intensity of the collective band in the hydration shells.[14]

In this report, we investigated the interaction between various amino acids and water molecules through analyses of the profiles

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* To whom correspondence should be addressed.

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of O−H stretching Raman band of water in their aqueous solutions and revealed how the structure of water around the amino acids is influenced by their side chains.

Experiment

Materials. Glycine (Gly), L-alanine (Ala), L-serine (Ser), L-valine (Val), L-leucine (Leu), L-isoleucine (Ile), L-threonine (Thr), L-histidine (His), and L-aspargine (Asn) were purchased from Nacalai Tesque, Kyoto, Japan, and were purified by recrystallization in water. Milli-Q grade water was used for a preparation of sample solutions.

Raman Spectroscopy. The Raman spectra of various amino acid solutions were recorded on a NR-1100 spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan; light source, argon laser 488.0 nm) with a band resolution of 5 cm^{-1}. For the polarization geometries $X(ZZ)Y$ (parallel position, $I_{||}$) and $X(ZX)$-Y (perpendicular position, $I_{\perp}$), a polarizer plate was rotated by exactly 90° in front of the slit, where X and Y are the directions of laser beam and observation, respectively. The electric vector of the laser beam was maintained in the vertical Z direction for both geometries. A polarization scrambler was used between the slit and the polarizer. The O−H stretching Raman band of water in various amino acid solutions was recorded in the region between 2500 and 4000 cm^{-1} by using a polarization method as exemplified in Figure 1.

To investigate the effect of amino acids on the structure of water, the intensity of the collective band (I_c) observed around 3250 cm^{-1} was separated from the spectra using eq 1

$$I_c = I_{||} - I_{\perp}/\varrho_{O-H} \quad (1)$$

where ϱ_{O-H} is the degree of depolarization. Since the intensities of Raman spectra are not absolute, the area of I_c was normalized as shown in eq 2.

$$C = \int I_c(w)\, dw / \int I_{||}(w)\, dw \quad (2)$$

where w is the Raman shift in cm^{-1}.

Spin−Lattice Relaxation Time, T_1, of Hydrogen Atom in Water. The spin−lattice relaxation time (T_1) of hydrogen atom of water in various amino acid solutions was measured with a 400 MHz FT-NMR apparatus (DX-400, JEOL, Tokyo, Japan). The T_1 values were measured by the inversion recovery method (180°−τ−90°) and were calculated by eq 3

$$M(\tau) = M_0[1 - 2\exp(-\tau/T_1)] \quad (3)$$

where τ is the recovery time and M_0 is the magnetization intensity at thermal equilibrium.

Results and Discussion

Collective Character of O−H Stretching Band. The O−H stretching vibration band in Raman spectra of water was a combination of two bands centered at about 3250 and 3400 cm^{-1} (Figure 1). The highly polarized lower frequency band has been assigned to the collective stretching of O−H oscillators. This assignment is consistent with many experimental results and theoretical calculations of a random network model, which is characterized by the fluctuation of defects in hydrogen bonds between water molecules.[15] There are two mechanisms that reduce the intensity of the collective band:[16] (1) When a stretching frequency of an O−H oscillator is significantly different from that of the O−H oscillator which is combined with it by hydrogen bonding, a decoupling of the O−H oscillators occurs. (2) When the hydrogen bond between the coupled O−H oscillators is broken by translational or rotational rearrangement of water molecules, a decoupling of the oscillators occurs. Reorientation of water molecule induced by interaction between water and solute molecule in aqueous solution increases or decreases the intensity of the collective band relative to that of pure water at the same temperature mainly by the second mechanism; in other words, the relative intensity of the collective band (C) reflects the probability of water−water hydrogen bonding in the solution.

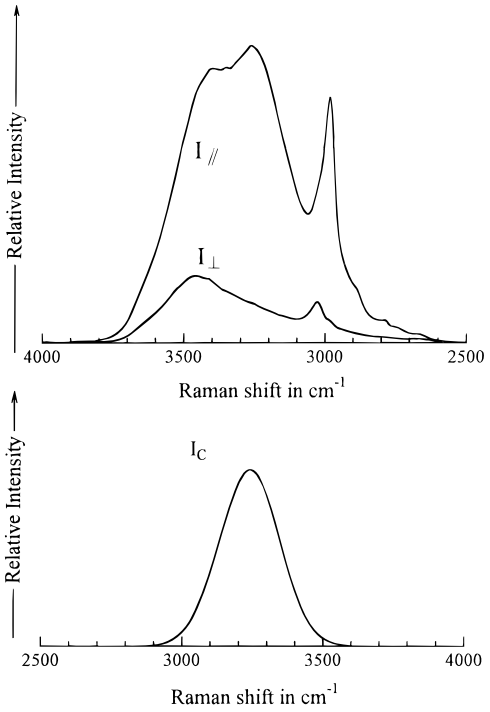

Figure 1. Raman spectra of aqueous glycine solution at parallel and perpendicular positions at 25 °C and $p_x = 0.01$ and separated collective band. Top: $I_{||}$, parallel component; $I_{\perp}$, perpendicular component. Bottom: I_c, the collective band.

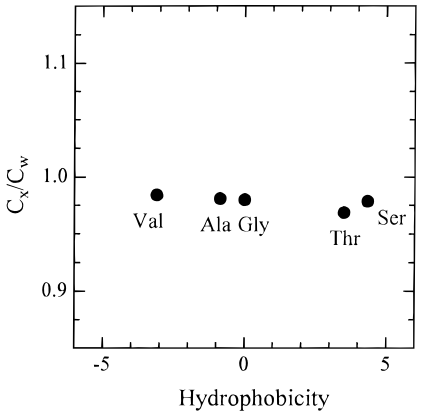

Figure 2. Relationship between hydrophobicity and the C_x/C_w values of various amino acid solutions at 25 °C, neutral pH, and $p_x = 0.01$.

Effects of Carboxyl and Amino Groups and Side Chains on the Structure of Water. Effects of amino acids on the structure of water were investigated at neutral pH where the amino acids exist as zwitterionic form. The C_x/C_w ($C_x = C$ value of amino acid solution; $C_w = C$ value of pure water) values for solutions of some amino acids with different side chains at molar ratio $p_x = 0.01$ are plotted as a function of hydrophobicity[6] in Figure 2.

We had expected that the structure of water in a solution of an amino acid depends on the nature of its side chain (hydrophilic or hydrophobic). However, the C_x/C_w values of amino

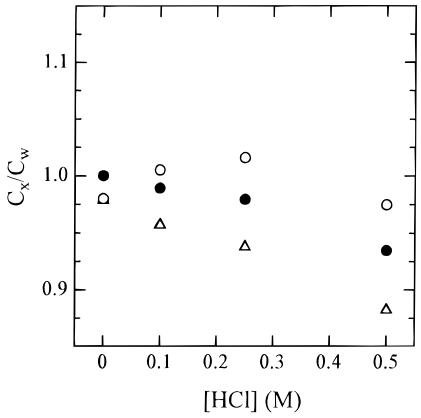

Figure 3. Relationship between concentration of HCl and the C_x/C_w value of Val, Ser, and HCl: (○) Val solutions, (△) Ser solutions, (●) HCl solutions. Degrees of neutralization of α-carboxylate groups are 0.01% ([HCl] = 0 M), 30.5% (0.1 M), and 80.3% (0.5 M) for Val and 0.02% (0 M), 28.5% (0.1 M), and 90.1% (0.5 M) for Ser.

acid solutions examined at neutral pH were only slightly smaller than that of pure water ($C_w/C_w = 1$) and were almost equal to one another, which suggests that the perturbation of the structure of water due to interaction between amino acids and water molecules localizes around common charged groups (ammonium and carboxylate groups) in all amino acid molecules examined.

Therefore, we investigated the effect of deionization of one of two charged groups in amino acids on the structure of water. In Figure 3, the C_x/C_w values for solutions of Val and Ser and that of HCl solution are plotted as a function of concentration of HCl ([HCl]). With an increase in [HCl], which raised the concentration of chloride ion and protonated carboxyl group ($-COO^- + H^+ \rightarrow -COOH$), the C_x/C_w value of Val increased as compared with that of HCl solution, whereas that of Ser decreased. The degree of protonation of the α-carboxylate group of Val would be approximately equal to that of Ser since pK_a values of the group in Val and Ser are similar (Val, 2.32; Ser, 2.21). Therefore, the different behaviors of C_x/C_w values for Val and Ser solutions must be caused by the difference in the nature of side chains, which are solely the distinct part of these two amino acids.

Two possible explanations for the appearance of influence of side chains on the structure of water at acidic or basic solutions are as follows: (a) Hydration shells of ammonium and carboxylate groups overlap that of side chains at neutral pH, because those charged groups strongly disrupt the structure of water up to a considerable distance. As a result, the interaction between the side chain and water molecules is hidden by the two ionic groups (Figure 4a). (b) The accessibility of water to side chains is largely reduced by a self-association of amino acid molecules due to electrostatic and/or hydrophobic interaction at neutral pH (Figure 4b).

After all, the structure of water in various amino acid solutions at neutral pH did not differ from each other significantly, which shows that the structure of water is little affected by the side chains, and the deionization of one charged group induces the appearance of the effect of side chains on the structure of water.

Relationship between Hydrophobicity of Side Chain and the Structure of Water. Next, we measured the *C* values for various amino acid solutions at acidic and basic conditions (in 0.5 M HCl and 0.5 M NaOH). The C_x/C_{HCl} ($C_{HCl} = C$ value of aqueous 0.5 M HCl solution) and C_x/C_{NaOH} ($C_{NaOH} = C$ value of aqueous 0.5 M NaOH solution) values of the amino acid solutions are compiled in Table 1 with several hydrophobicity values of the side chains reported in the literature. Hydrophobicity indices noted as "a" were calculated from the partition

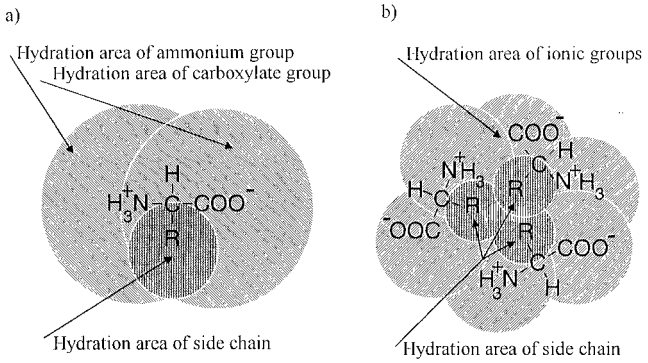

Figure 4. Schematic drawing of hydration shell. (a) Hydration shells of ammonium and carboxylate groups overlap that of side chains at neutral pH because those charged groups strongly disrupt the structure of water up to a considerable distance. (b) Accessibility of water to side chains is reduced by the self-association of amino acid molecules due to electrostatic and/or hydrophobic interaction at neutral pH.

TABLE 1: Relative Hydrophobicities and C_x/C_{HCl} and C_x/C_{NaOH} Values for Various Amino Acids

	hydrophobicity				
amino acid	side-chain analogue[a]	amino acid[b]	*N*-acetylamide[c]	C_x/C_{HCl},[d] C_x/C_{NaOH}[e]	T_1[f]
Asn	7.58	0.2	0.60	0.931/0.953	2.87
His	5.60	−0.5	−0.13	0.905/0.939	2.81
Ser	4.34	0.3	0.04	0.959/0.972	3.06
Gly	0	0	0	0.987/0.976	3.22
Ala	−0.87	−0.5	−0.31	0.986/0.990	2.87
Val	−3.10	−1.5	−1.22	1.06/1.04	2.85
Ile	−3.98	−1.8	−1.80	1.09/1.09	2.66
Leu	−3.98	−1.8	−1.70	1.11/1.08	2.72

[a] Reference 6. [b] References 7 and 8. [c] Reference 9. [d] [HCl] = 0.5 M. [e] [NaOH] = 0.5 M. [f] T_1 values of hydrogen atom of water in various amino acid solutions at acidic condition ([HCl] = 0.5 M), $p_x = 0.01$ and 25 °C.

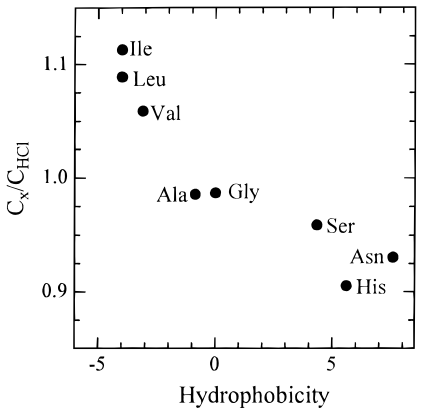

Figure 5. Relationship between hydrophobicity and the C_x/C_w values of amino acid solutions at acidic conditions ([HCl] = 0.5 M), 25 °C, and $p_x = 0.01$.

coefficient, K_d, of the model compound for each amino acid side chain (the backbone was replaced by hydrogen atom (H−R, R = side chain)) from water to cyclohexane,[6] "b" from the partition coefficient of *N*-acetylamino acids between water and octanol,[7,8] and "c" from the K_d of amino acids between water and hexane.[9] Among them, the values estimated by Radzicka and Wolfenden ("a") have the best correlation with the C_x/C_{HCl} and the C_x/C_{NaOH} values. Hereafter, we will use hydrophobicity reported by Radzicka and Wolfenden as a reference unless indicated otherwise.

Figure 5 shows that the C_x/C_{HCl} values of various amino acids in the 0.5 M HCl solution at 25 °C, and $p_x = 0.01$ increased

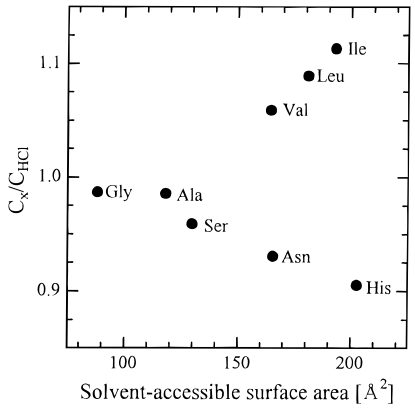

Figure 6. Relationship between C_x/C_{HCl} and the solvent-accessible surface area of side chain to water.

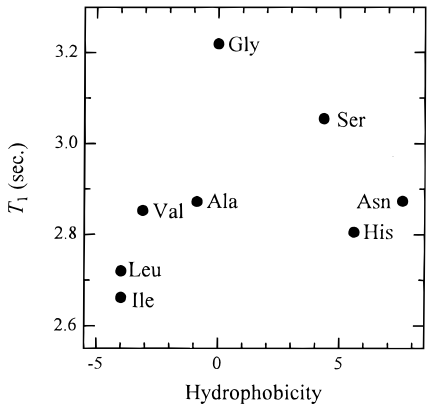

Figure 7. Relationship between hydrophobicity and T_1 values of water hydrogen atom in amino acid solutions at 25 °C, $p_x = 0.01$, and acidic conditions ([HCl] = 0.5 M).

with an increase in the hydrophobicity values.[6] Investigations on the structure of water in many kinds of solutions of electrolytes, organic compounds, and polymer materials with various hydrophobicities by the same analytical method used here revealed that more hydrophilic solutes which may destroy the structure of water in solutions reduce the *C* value to a larger extent. As mentioned in the previous section, reduction of the *C* value means a breakdown of long-range vibrational coupling of O−H oscillators induced by cleavage of hydrogen bond between water molecules. On the other hand, hydrophobic solutes that may enhance the structure of water raise the *C* value.[17−19] Accordingly, hydrophilic amino acids destroy the structure of water in solutions and hydrophobic amino acids enhance it.

Hydration of proteins has been often simulated by molecular dynamics with various models of water molecule such as ST2 and SPC water, which provides quantitative information about the structure and physical properties of water in pure water and aqueous solutions. For example, a simulation about the hydration of melittin predicted the average number of water−water hydrogen bonds in the first and second hydration shells of charged, polar, and nonpolar residues.[20] Almost the same result was obtained in the simulation of hydration of synthetic macromolecules.[21] The number of water−water hydrogen bonds was shown to decrease in the order nonpolar > polar > charged residue. According to their prediction, the numbers of water−water hydrogen bonds around the side chain of polar or charged amino acids (Ser, Asn, and His) may be lower than those of nonpolar ones (Ala, Leu, Ile, and Val). The C_x/C_{HCl} values, which are closely related to the degree of hydrogen bonding between water molecules, in the solution of amino acids with nonpolar side chains were larger than those of amino acids with polar or charged side chain in a similar manner to the simulation by Kitao et al.[20] Our experimental results provide a strong experimental support for those simulations.

Relationship between Solvent-Accessible Surface Area of the Side Chain and the Structure of Water. There is a possibility that the C_x/C_{HCl} values of amino acids are related to the size of the side chain, because the size of hydration shell would expand with an increase in solvent-accessible surface area (abbreviated as A_{SF} hereafter) of the side chain. In Figure 6, the C_x/C_{HCl} values are plotted against the A_{SF} value[22] of the side chains to water. With an increase in the A_{SF} value, the C_x/C_{HCl} values of nonpolar amino acids increased (Ala < Val < Ile < Leu), and those of amino acid with polar or charged side chain decreased (Ser > Asn > His), which shows that the effects of the side chain on the structure of water become more significant with an increase in the A_{SF} value of the side chain. As for the hydration of hydrophobic amino acids, the hydrophobicity closely relates with the A_{SF} value. Investigation of the structure of water in solutions of various amino acids by Hechte et al. with infrared spectroscopy[23] revealed that the size of water clathrate around hydrophobic side chain increases in the order Gly < Ala < Val, Ile, Leu and that the size of clathrate has a good correlation with the size of the alkyl side chain, which is not inconsistent with our results.

In the case of monosaccharides, geometrical arrangements of hydroxyl groups play a significant role in determining the structure of water around the monosaccharide because the chair conformation of pyranoses fits the so-called tridimide structure of water:[24] sugars with few equatorial OH groups break the structure of water, and sugars with many equatorial OH groups make structure of water stable. On the contrary, the structure of water in amino acid solutions has a good correlation with the A_{SF} value of the side chains, and the geometrical effects may play a minor role in the hydration of amino acids.

Dependence of T_1 of Hydrogen Atom of Water on Hydrophobicity of Side Chain. To reveal the relationship between the structure of water around amino acids as shown by the probability of water−water hydrogen bonding measured by Raman spectroscopy with mobility of hydration water of amino acids, we measured relaxation time of ^{1}H of water in solution.

In general, the observed T_1 of ^{1}H of water molecule can be expressed as

$$1/T_1 = B/T_{1B} + H/T_{1H} \quad (4)$$

where T_{1B} and T_{1H} are the spin−lattice relaxation times, and *B* and *H* are the relative proportions of bulk and hydration water.[25] The T_1 was observed as a single mode, and the two phases of water could not be detected as separate signals by ^{1}H NMR, because of the rapid exchange between bulk water and hydration water.[26]

The T_1 values of hydrogen atom of water in various amino acid solutions at 25 °C, $p_x = 0.01$, and acidic conditions are compiled in Table 1 and plotted against the hydrophobicity values in Figure 7. The T_1 value showed the highest value when the index of hydrophobicity was zero, and the solutions of more hydrophobic or hydrophilic amino acids had lower T_1 values.

The T_1 of ^{1}H of water reflects the motion of water molecules and closely relates with the correlation time (τ_c) of rotational motion of water molecule. In general, the T_1 vs τ_c curve has a minimum, the position of which depends on the strength of magnetic field. Since the data in this report were measured with a 400 MHz NMR apparatus, the minimum of T_1 is 10^{-3} s at $\tau_c = 4 \times 10^{-10}$ s.[27] The τ_c of water molecule in solution of

low molecular weight electrolytes was reported to be about 10^{-12} s.[28] The measurements in this work were performed under extreme narrowing conditions (these conditions are satisfied when τ_c is shorter than 4×10^{-10} s when the resonance frequency is 400 MHz), and therefore the T_1 value should decrease linearly with an increase in τ_c.

Figure 7 shows that thermal motions of hydration water molecules around both hydrophilic and hydrophobic side chains are restricted, though the reason of restriction for hydrophobic side chains should differ from that for hydrophilic ones as expected from the Raman spectroscopic data (Figure 4). As for hydrophobic hydration, it was previously reported by Bagno et al. that alkyl groups cause a decrease in T_1 of ^{17}O in water molecule because of the restriction on the thermal motion of water molecules and that the number of hydrating water increased with an increase in the size of the alkyl group.[29] Our experimental result that the T_1 of water proton decreased with an increase in the size of the alkyl group in the order Ala > Val > Leu > Ile is consistent with the result by Bagno et al. Raman spectroscopic results indicated that the probability of water−water hydrogen bonds in the hydration shells of hydrophobic amino acids is higher than that of bulk water and that water clathrate is formed around the side chain. Enhancement of water−water hydrogen bonding may be an important factor that causes the restriction of water around hydrophobic side chains. The values of T_1 of water hydrogen in aqueous solutions of hydrophobic amino acids were lower than that of Gly, and extents of difference increased with A_{SF}. The result also suggests that the size of water clathrate around a more hydrophobic side chain is larger than that of a less hydrophobic one. As for the hydrophilic amino acids, restriction in the thermal motion of water is due to direct interaction between water and hydrophilic moieties of amino acid molecules such as hydrogen bonding and ion-dipole interaction, which also cause a break in water−water hydrogen bonding.

Conclusion

The structure of water and thermal motion of water molecule were investigated by Raman spectroscopy and ^1H NMR. The structure of water in solutions of various amino acids did not depend on the nature of side chains at neutral pH, but did at acidic and basic conditions. The degree of hydrogen bonding between water molecules was revealed to increase around hydrophobic side chains and decrease around hydrophilic side chains. In both cases thermal motion of water molecules was restricted though the mechanisms of the restriction are different, that is, the formation of water clathrate in the former case and the attractive interaction between the side chain and water in the latter case. Both C and T_1 values of amino acid solutions were correlated with the solvent-accessible surface area of the side chain to water. The effect of side chain on the structure of water was enhanced with an increase in the area.

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