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Characterization of Individual Tryptophan Side Chains in Proteins Using Raman Spectroscopy and Hydrogen-Deuterium Exchange Kinetics†

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ABSTRACT: Two Raman bands at 880 and 1360 cm^{-1} of tryptophan (Trp) side chains have been found useful in structural studies of the side chains in proteins. The frequency of the 880-cm^{-1} band reflects the strength of H bonding at the N_1H site of the indole ring: the lower the frequency is, the stronger the H bonding is. The intensity of the 1360-cm^{-1} band, on the other hand, is a marker of the hydrophobicity of the environment of the indole ring: particularly strong in hydrophobic environments. It is also demonstrated that a combination of stepwise deuteriation of the tryptophan side chains and difference spectrum techniques is useful to observe these marker bands due to each side chain separately. The states of six tryptophans in lysozyme revealed by this Raman spectroscopic method in solution are compared with those by X-ray diffraction in crystal. The Raman data on the outer four Trp's are consistent with the X-ray structure, whereas significant differences between solution and crystal are suggested for the strength of H bonding of the most and second most buried Trp's. Characterization of four Trp's in α-lactalbumin shows that the two outer Trp's are moderately H bonded to solvent water and closely surrounded by aliphatic side chains while the inner two are not H bonded nor closely surrounded by aliphatic side chains.

In order to understand the role of individual amino acid side chains in the function and structure of a protein, it is important to know the states of the side chains in addition to their locations in the protein. Raman spectroscopy has been a useful tool for such purposes. A vibrational mode localized at a side chain of a protein is often influenced by a specific environmental or structural factor (e.g., H bonding or conformation), and the corresponding Raman band reflects the state of the

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side chain directly, both in crystal and in solution. For instance, Raman bands at 1360 and 880 cm^{-1} due to tryptophanyl indole have been noted to depend on the environments. A sharp and strong band at 1360 cm^{-1} in the spectrum of aqueous α-lactalbumin becomes barely detectable on denaturation (Yu, 1974). Recently, we have assigned this band to the high-frequency component of a doublet due to Fermi resonance between one skeletal stretching fundamental (W7) and one or two combinations of the indole ring vibrations (Harada et al., 1986). The phenomenon observed in the spectrum of α-lactalbumin is interpreted as arising from a slight change in relative frequency of the fundamental and combination vibrations due to environmental changes, the slight change being amplified in the intensity ratio of the Fermi doublet. With 218-nm excitation, both components of the doublet are clearly seen in the resonance Raman spectrum of the protein. Denaturation causes the high-frequency component to decrease in intensity and the low-frequency one to increase (Rava & Spiro, 1985).

The intensity of the 880-cm^{-1} band also has been suggested to serve as a practical probe of the environment (Kitagawa et al., 1979). According to the normal coordinate analysis of skatole (3-methylindole), the 880-cm^{-1} band called W17 is a mixed mode of the benzene 12-like vibration and N_1H motion (Takeuchi & Harada, 1986), and the frequency of this band is expected to be sensitive to the environment.

In this work, we have made systematic studies on the relationships between these tryptophan Raman bands and the environmental conditions and have reached the conclusion that the intensity of 1360-cm^{-1} band is a marker of hydrophobicity of the environment (strong hydrophobic interaction between the tryptophanyl indole ring and other aliphatic groups) and the frequency of W17 is a marker of H bonding at N_1 of the indole ring.

The applicability of the two marker bands has been tested in two proteins, hen egg white lysozyme and bovine α-lactalbumin containing six and four tryptophan residues, respectively. In the Raman spectrum of such a protein that contains more than one tryptophan residues, each of the 1360-cm^{-1} and W17 bands is observed as an overlap of the bands due to all the tryptophan side chains. However, if the rates of hydrogen–deuterium exchange reaction at N_1 sites of these side chains are different from one another, they can be deuteriated in a stepwise manner in D_2O solution under suitable conditions, and it is possible to obtain information on each tryptophan side chain, separately. We have used a combination of stepwise deuteriation and spectral subtraction and succeeded in characterizing the states of the tryptophan side chains in the two proteins.

Materials and Methods

Skatole and 3-indoleacetic acid (IAA) were obtained from Nakarai Chemicals Co. and Wako Pure Chemical Industries, Ltd., respectively. *N*-Acetyl-L-tryptophan methyl ester (AcTrpME) and *N*-acetyl-DL-tryptophan methylamide (AcTrpMA) were prepared from L-tryptophan methyl ester (Sigma Chemical Co.) and DL-tryptophan (Nakarai Chemicals Co.), respectively, by ordinary methods and identified by elemental analysis and IR spectroscopy. AcTrpME, AcTrpMA, and IAA were crystallized from ethanol solution at 5 °C. L-Tryptophan hydrochloride (TrpHCl) was obtained in the form of needle-like crystals by dissolving L-tryptophan in concentrated hydrochloric acid and by allowing the solution to stand at 5 °C.

Lysozyme from hen egg white and α-lactalbumin from bovine milk were obtained from Sigma Chemical Co. The pH (pD) adjustments of aqueous protein were made with HCl (DCl) and NaOH (NaOD) solutions and determined on a Hitachi-Horiba M-7II pH meter. Lysozyme hydrochloride was crystallized from an aqueous solution of 4% protein and 5% NaCl at pH 4.7 according to the literature methods (Blake et al., 1965; Ataka et al., 1986).

Raman spectra were obtained with a multichannel detection system described previously (Harada et al., 1986). Typical experimental conditions were as follows: excitation wavelength, 488 nm; power, 200 mW; spectral slitwidth, 5 cm^{-1}; data accumulation, 300 s for protein solutions and 1500 s for skatole solutions; sample concentration, 7% for proteins and 50 mM for skatole; temperature, 22 °C. The wavenumber axis of the Raman spectra was calibrated for indene, and peak positions wre reproducible within 1 cm^{-1}. The reproducibility of relative peak intensities was better than 2%. The IR spectra of skatole solutions were obtained with a JASCO IR-810 IR spectrophotometer and a 0.05-mm KBr cell.

Stepwise Deuteriation at Indole N_1's in Proteins. The rates of the NH → ND exchange reaction at individual indole N_1 sites in lysozyme and α-lactalbumin depend upon pD and temperature. We monitored the reaction by the intensity of the 1382-cm^{-1} band of the N-deuteriated indole ring, which had been used in kinetic studies of NH → ND exchange in tryptophan side chains (Takesada et al., 1976; Peticolas, 1982). This band is useful because of the following reasons. (1) There is no overlapping strong band in the frequency region in the spectrum of the protein, in general. [The corresponding band at 1435 cm^{-1} in the normal (N_1H) compound is a mixed mode of the $N_1C_2C_3$ symmetric stretch and the N_1H bend and overlapped by the aliphatic CH bending bands.] (2) The intensity of the band is little affected by the environment, the mode being mainly the $N_1C_2C_3$ symmetric stretch [W6(ND)] (Takeuchi & Harada, 1986). It was possible to deuteriate most of the tryptophan side chains in the proteins separately. The difference spectra between the spectra at different steps of deuteriation were obtained by taking the 1010-cm^{-1} tryptophan band for lysozyme and the 1004-cm^{-1} phenylalanine band for α-lactalbumin as the internal intensity standard. The 1010-cm^{-1} band was used for lysozyme since the overlap of amide III′ and the 1004-cm^{-1} band was significant. The intensities of the 1382- and 1360-cm^{-1} bands were also measured against the internal intensity standard.

Results and Discussion

Relationship between the W17 Frequency and the H Bonding. (1) W17 Frequency in Model Systems. Skatole in solutions was chosen as a model system to find the relationship between the W17 frequency of the tryptophanyl indole ring and the environmental conditions. The W17 vibration of skatole appears at two different frequencies in Raman spectra of various solutions. In hexane, cyclohexane, and carbon disulfide, W17 appears at 882–883 cm^{-1}. In methanol–water (5:5), methanol, acetone, dimethyl sulfoxide, 1,4-dioxane, cyclohexanone, and acetonitrile, which are proton acceptors, it appears at 877–878 cm^{-1}. In a carbon disulfide solution with a small amount of dioxane mixed, there coexist skatole molecules H bonded with dioxane and those without H bonding. Therefore, a sharp peak at 3476 cm^{-1} arising from the free skatole NH stretching vibration and a broad band at 3346 cm^{-1} arising from the H-bonded skatole are observed in the infrared spectrum (Figure 1). When the fraction of dioxane increases, the former decreases and the latter increases in intensity. Concomitantly, the Raman W17 band at 883 cm^{-1} decreases in intensity, and the one at 878 cm^{-1} increases. These results indicate that the frequency shift of W17 is due to the H

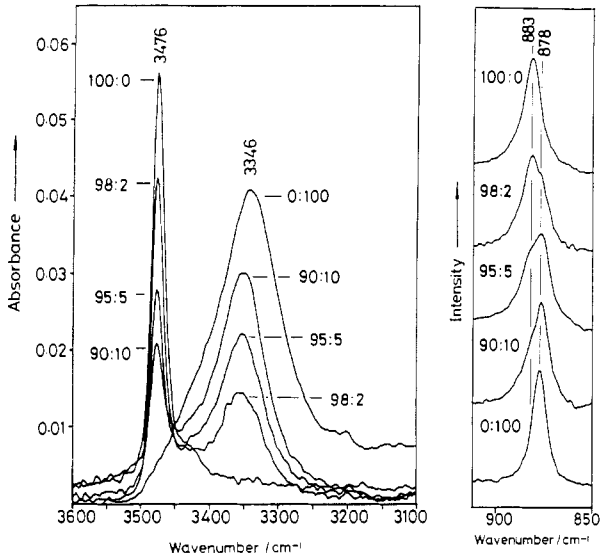

FIGURE 1: Infrared spectra (3600–3100 cm^{-1}) and Raman spectra (910–850 cm^{-1}) of skatole in CS_2 (100:0), in CS_2–dioxane mixtures of 98:2 (in volume), 95:5, and 90:10, and in dioxane (0:100). The concentration was 50 mM.

bonding, not due to the effect of other changes in the environment of the indole ring.

In order to investigate the H-bonding dependence of the W17 frequency in more detail, the crystalline materials were chosen as a second model system. The crystals of L-tryptophan hydrochloride TrpHCl) and 3-indoleacetic acid (IAA) give a N_1H stretching frequency of 3390 cm^{-1}, which is similar to that of skatole in proton-acceptor solvent, indicating moderate H bonding. In these materials the N_1H group forms a NH···π type H bond to the indole ring of an adjacent molecule. The perpendicular distance between the nitrogen atom and the plane of the indole ring of the nearest molecule is calculated to be 3.36 Å in TrpHCl and 3.42 Å in IAA, respectively (Takigawa et al., 1966; Karle et al., 1964). In the Raman spectra of these molecules in the crystalline state, W17 appears around 877–878 cm^{-1}, which is also similar to that of skatole in proton-acceptor solvents.

The H bond is strong in crystalline *N*-acetyl-DL-tryptophan methylamide (AcTrpMA) and *N*-acetyl-L-tryptophan methyl ester (AcTrpME), where the proton acceptor is an acetyl oxygen of an adjacent molecule and the N···O bond distances are 2.99 and 2.86 Å, respectively (Harada & Iitaka, 1977 Cotrait & Barrans, 1974). The N_1H stretching frequencies in these crystals (3263 cm^{-1} in AcTrpMA and 3318 cm^{-1} in AcTrpME) also suggest strong H bonds, the one in AcTrpMA being rather stronger than that in AcTrpME. The apparent disagreement between the strength of these bonds and the bond lengths arises presumably form the difference in the N–H···O bond angle. Both of these H bonds are almost linear like many other strong intramolecular H bonds in organic crystals (Taylor & Kennard, 1984), and yet the bond angle in AcTrpMA, 168°, is closer to 180° than that in AcTrpME, 162°. The W17 frequencies are quite low in the Raman spectra, 871 and 873 cm^{-1}, respectively, in harmony with the low NH stretching frequencies.

Figure 2 shows the correlation between the frequencies of Raman W17 and the infrared N_1H stretching vibration in model systems. Apparently, the frequency of W17 reflects sensitively the strength of H bonding.

(2) W17 in Raman Spectra of Proteins. In the case of a protein containing only one tryptophan residue, the Raman frequency of W17 is an indicator of the strength of the H bond

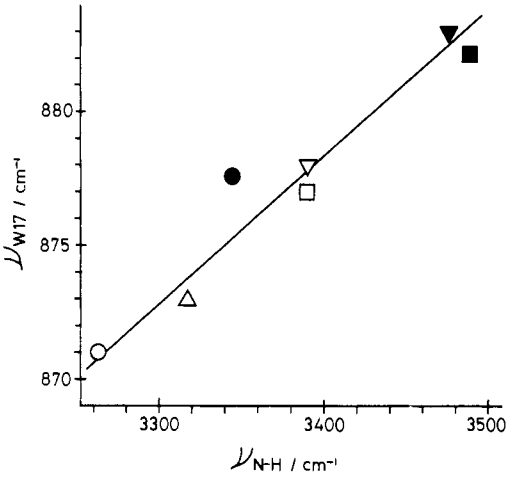

FIGURE 2: Correlation between the frequency of the Raman W17 band and that of the infrared N_1H stretching band in model systems: crystalline TrpHCl (▽), IAA (□), AcTrpME (△), and AcTrpMA (○) and skatole solutions in cyclohexane (■), CS_2 (▼), and dioxane (●).

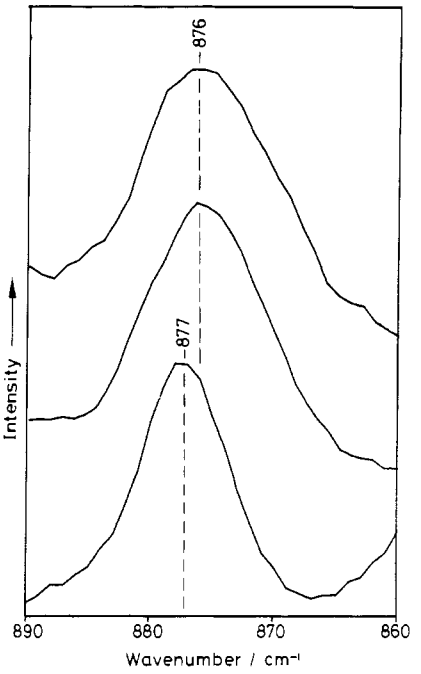

FIGURE 3: Raman spectra (W17 region) of lysozyme: (upper) native lysozyme in H_2O (7%, pH 5.5); (middle) crystalline lysozyme chloride grown at pH 4.7; (lower) denatured lysozyme in 8 M LiBr solution.

of this side chain. In contrast, the W17 band of a protein with more than one tryptophan residues is an overlap of individual W17's and affords a rough estimate on the distribution of the strength of H bonds of such side chains. Raman spectra of hen egg white lysozyme, which contains six tryptophan residues, are shown in Figure 3. In H_2O solution, the W17 band (an overlap of six W17's) that peaked at 876 cm^{-1} is not symmetric and is broader than that of skatole in various solvents (the FWHM being 12.2 cm^{-1} for lysozyme solution and 7.5–8.5 cm^{-1} for some skatole solutions). The shape of this band indicates that the strengths of H bonds are different at six tryptophan side chains. In particular, the shoulder at the low-frequency side, 871–872 cm^{-1}, indicates that at least one tryptophan side chain takes part in a very strong H bond. Lysozyme is denatured in concentrated LiBr solution (Lord & Mendelsohn, 1972), and the W17 band at 877 cm^{-1} is symmetric and sharp (FWHM, 7.8 cm^{-1}). This drastic change suggests that all the tryptophan side chains are exposed on the

molecular surface and moderately H bonded to solvent water in the denatured structure. The W17 band of lysozyme in the crystalline state is slightly more symmetric and sharper (FWHM, 11.2 cm^{-1}) than that in solution, indicating a difference in the state of tryptophan side chains between solution and crystal.

The 1360-cm^{-1} Band as a Hydrophobicity Marker. As is stated in introduction, the 1360-cm^{-1} band is the upper component of the 1360–1340-cm^{-1} Fermi doublet (Harada et al., 1986). There are three major observations with respect to relations between the intensity ratio of the doublet (or the intensity of 1360-cm^{-1} band) and the environment of indole ring, including a result of this work. (1) The 1360-cm^{-1} band in the spectra of some proteins decreases in intensity on denaturation or molecular unfolding (Chen et al., 1973, 1974; Lord & Mendelsohn, 1972; Yu, 1974; Rava & Spiro, 1985; Lafaut & Dael, 1986). (2) The upper component of the doublet in indole is strong in hydrophobic solvent. The intensity ratio, $R = I$(high-frequency peak)/I(low-frequency peak), is 0.65–0.93 in hydrophilic solvents (methanol–water mixture, methanol, acetonitrile, dioxane, dimethylformamide, dimethyl sulfoxide, cyclohexanone, etc.), 1.11 in benzene and toluene, and 1.23–1.32 in saturated hydrocarbons, hexane and cyclohexane (Harada et al., 1986). (3) As will be described below, there are some tryptophan side chains, in lysozyme and α-lactalbumin, which have a large intensity at 1360 cm^{-1} in spite of being exposed on the molecular surface. In contrast, there are some tryptophan side chains which have a weak intensity at 1360 cm^{-1} in spite of being buried in the interior (this work).

Yu interpreted the first observation as due to changes in the environments of the "buried" tryptophan residues and surmised that the indole rings accessible to water are not expected to contribute to the distinctly sharp feature at 1360 cm^{-1} (Yu, 1974). This interpretation is consistent with the second observation but not sufficient to explain all the observations, consistently. The third observation indicates that the intensity of 1360-cm^{-1} band is not correlated with the degree of exposure estimated in the crystal structure. We speculate that the intensity ratio of the 1360–1340-cm^{-1} doublet is not a marker of the accessibility by the solvent water, but a marker of hydrophobicity of the environment. To be exact, aliphatic side chains that surround the indole ring may primarily affect the intensity ratio of the doublet. In the case where a tryptophan side chain is partially but closely surrounded by other aliphatic groups and the N_1H group is exposed on the molecular surface, the 1360-cm^{-1} band due to this side chain is expected to be strong.

In the Raman spectra of proteins with visible excitation, the peak intensity at 1360 cm^{-1} is used instead of the intensity ratio of the doublet, because the 1340-cm^{-1} peak is usually overlapped by CH bending bands of aliphatic side chains.

Procedure To Investigate the State of Individual Tryptophan Side Chains. In order to investigate the state of individual tryptophan side chains in proteins which have more than one tryptophan residue, stepwise deuteriation at the N_1 sites is useful. Deuteriation at the N_1 site of one tryptophan side chain causes a downward frequency shift of its W17 to 860 cm^{-1}, so that W17 due to each tryptophan side chain disappears, one by one, from the 880-cm^{-1} region as the stepwise deuteriation proceeds. It is possible to discriminate the frequency of W17 due to each tryptophan side chain by means of the difference spectral technique. This combined method enables us to evaluate the intensity of 1360-cm^{-1} band due to each tryptophan side chain, because this band also shifts down

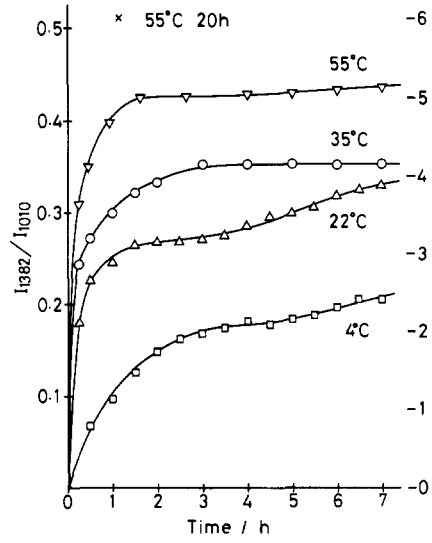

FIGURE 4: Intensity of W6(ND) in lysozyme relative to that of the 1010-cm^{-1} band (I_{1382}/I_{1010}) as a function of temperature and time after dissolution in D_2O (pD 5.5).

on deuteriation and disappears from the 1360-cm^{-1} region, the shifted band being observed at 1352 cm^{-1} in L-tryptophan and undetectable in proteins due to overlapping of CH bending bands of aliphatic side chains. The detailed methods of the stepwise deuteriation and spectral subtraction are described under Materials and Methods.

(1) State of Tryptophan Side Chains in Lysozyme. In 8 M LiBr–D_2O solution, lysozyme takes a denatured structure, and all the indole N_1H hydrogens of the tryptophan side chains are replaced by deuterium. Lysozyme in the native form with six deuteriated tryptophan side chains is then obtained by excluding LiBr on dialysis of the solution in D_2O. In the Raman spectrum of this solution, the peak intensity of the 1382-cm^{-1} band, the indicator of deuteriation of tryptophanyl indole (Takesada et al., 1976; Peticolas, 1982), is 0.51 (versus that of the 1010-cm^{-1} tryptophan band), indicating that one deuteriated tryptophan side chain has an intensity of 0.085. Figure 4 shows the time course of the intensity increase of the 1382-cm^{-1} peak in the Raman spectra of lysozyme in D_2O at various temperatures. This figure shows that the stepwise deuteriation of all the tryptophan side chains except the two that have very rapid H → D exchange rates is practicable. The first two tryptophan side chains [to be termed Trp(1) and Trp(2)] are deuteriated within 4 h even at 4 °C, that is, just above the freezing point. The third one [Trp(3)] is deuteriated 2 h after dissolution at 22 °C, the forth one [Trp(4)] 4 h at 35 °C, the fifth one [Trp(5)] 2 h at 55 °C or 100 h at 35 °C, and the last one [Trp(6)] 20 h at 55 °C. The complete deuteriation at the final step has been verified by a 500-MHz NMR spectrum.

Raman spectra of these stepwisely deuteriated lysozymes are shown in Figure 5. A shoulder at 1355 cm^{-1} in the spectrum of fully deuteriated lysozyme (bottom one) may be assigned to W7(ND) of deuteriated tryptophan side chains or a CH bending mode of aliphatic side chains. We tentatively assume the latter assignment because the intensity of this shoulder does not parallel that of the 1382-cm^{-1} tryptophanyl bands in the fully deuteriated α-lactalbumin which contains only four tryptophan side chains (the spectrum of α-lactalbumin will be shown later). A decrease in intensity of the 1360-cm^{-1} band at each step of deuteriation corresponds to the intensity due to the tryptophan side chain deuteriated at that step. W17 due to each tryptophan side chain is clearly observed in the difference spectrum (Figure 5b). The results

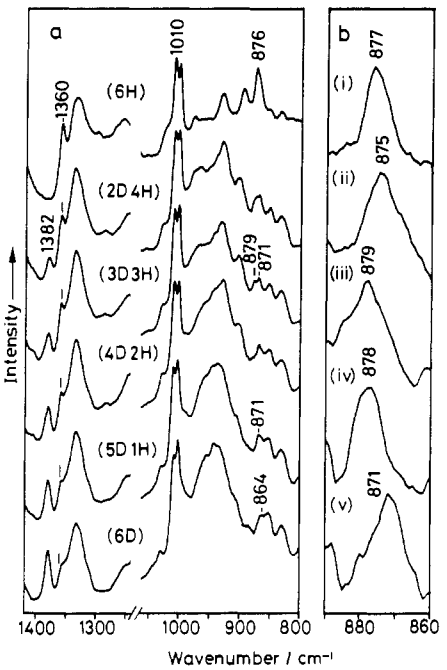

FIGURE 5: Raman spectra of stepwisely deuteriated lysozyme (a) and the difference spectra in the W17 region (b). (a) 6H, aqueous solution of lysozyme; 2D4H, two tryptophan side chains are deuteriated; 3D3H, three are deuteriated; 4D2H, four are deuteriated; 5D1H, five are deuteriated; 6D, all the tryptophan side chains are deuteriated. The spectra except for 6H were measured in D_2O solution. (b) (i) 6H − 2D4H; (ii) 2D4H − 3D3H; (iii) 3D3H − 4D2H; (iv) 4D2H − 5D1H; (v) 5D1H − 6D.

Table I: Frequencies of W17 and Intensities of the 1360-cm^{-1} Band of Tryptophan Side Chains in Lysozyme

	W17 (cm^{-1})	I_{1360}[a]	assignment[b]
Trp(1) and -(2)	877	0.92[c]	Trp-62 and -63
Trp(3)	875	0.49	Trp-108
Trp(4)	879	0.34	Trp-123
Trp(5)	878	0.85	Trp-111
Trp(6)	871	1.00	Trp-28

[a] Normalized to the intensity of Trp(6). [b] Wedin et al. (1982). [c] Value per one tryptophan.

on the W17 frequency and the 1360-cm^{-1} intensity are shown in Table I.

According to an NMR study, Trp(1) and Trp(2), deuteriated at the first step, have been assigned to Trp-62 and Trp-63 (Wedin et al., 1982). These two give a symmetric W17 band at 877 cm^{-1}, indicating moderate H bondings with water molecules on the protein surface or with the other part of the protein in the interior. Quite rapid exchange rates of these hydrogens support the former H bonding, which is also expected from the X-ray crystal structure. The strong 1360-cm^{-1} band, on the other hand, suggests that the indole rings of these side chains are closely surrounded by aliphatic side chains. The crystal structure of lysozyme shows that Trp-62 and Trp-63 are exposed to the active site cleft and do not take part in intramolecular H bonding (Blake et al., 1981). Figure 6 shows the distribution of carbon atoms around each tryptophan side chain in the crystal of lysozyme. This figure shows that the side chain of Trp-62 is closely surrounded by the methylene chain of Arg-61 and the side chain of Trp-63 by Ile-98.

Trp(3) has been assigned to Trp-108 (Wedin et al., 1982). The frequency of W17, 875 cm^{-1}, indicates that the H bonding is slightly stronger than that to solvent water. The weak 1360-cm^{-1} band indicates that the environment is less hydrophobic than those of Trp-62 and Trp-63. In the crystal structure the N_1H of Trp-108 takes part in an angular H

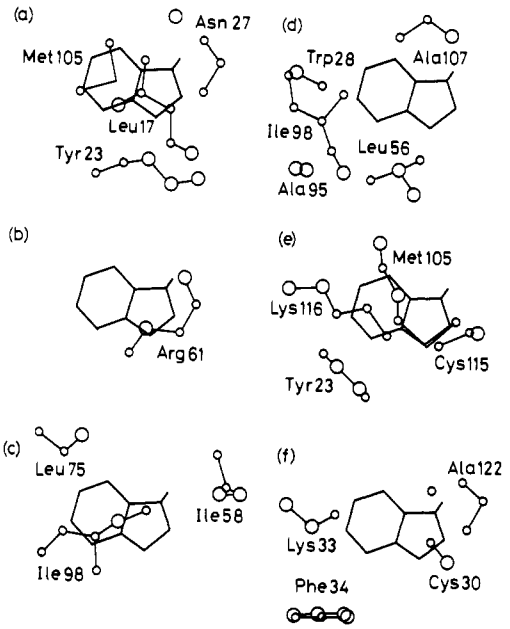

FIGURE 6: Environments of tryptophan side chains of lysozyme in the X-ray crystallographic structure on the basis of the atomic coordinates of "2LYZ" in the Brookhaven Protein Data Bank (Diamond et al., 1971). The circle indicates a carbon atom, and the radius stands for the distance from the plane of indole ring: large, ≤3.5 Å; small, 3.5–5.0 Å. (a) Trp-28, (b) Trp-62, (c) Trp-63, (d) Trp-108, (e) Trp-111, and (f) Trp-123.

Table II: Intramolecular Hydrogen Bondings of Tryptophan Side Chains in Lysozyme in the Crystalline State[a]

	H acceptor	distance, N···O (Å)	angle, N–H···O (deg)
Trp-28	main-chain amide O		
	Leu-17	2.8	102
	Tyr-23	3.4	125
Trp-108	main-chain amide O		
	Leu-56	2.7	128
Trp-111	side-chain amide O		
	Asn-27	2.7	170

[a] Diamond et al. (1971).

bonding to an amide oxygen of the main chain at Leu-56 (Table II), and hydrophobic interaction between the indole ring of Trp-108 and other side chains is considered to be weak (Figure 6).

Trp(4) has been assigned to Trp-123 (Wedin et al., 1982). The frequency of W17, 879 cm^{-1}, and the intensity of the 1360-cm^{-1} band of this side chain indicate no strong H bonding nor hydrophobic environment. In the crystal, the N_1H group does not take part in an intramolecular H bonding but possibly in a weak H bonding to a water molecule incorporated into the protein (N···O distance of 3.9 Å). Figure 6 shows that the indole ring is not closely surrounded by other aliphatic side chains.

The Raman data on Trp-62, -63, -108, and -123 described above are consistent with the X-ray structure (Diamond et al., 1971). Hence, it is seen that the state of these side chains in the crystal is retained in the solution substantially. However, the states of Trp(5) and Trp(6) estimated from the Raman spectra in solution are not fully consistent with the X-ray structure. Trp(5) and Trp(6) have been assigned to Trp-111 and Trp-28, respectively (Wedin et al., 1982). According to the X-ray structure, the N_1H group of Trp-111 forms a linear H bond to the amide oxygen of the Asn-27 side chain, and this H bond is as strong as that in the crystal of AcTrpMA

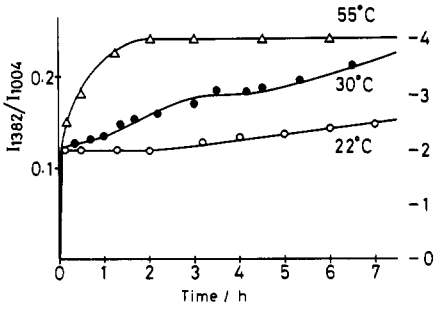

FIGURE 7: Intensity of W6(ND) in α-lactalbumin relative to that of the phenylalanyl 1004-cm^{-1} band (I_{1382}/I_{1004}) as a function of temperature and time after dissolution in D_2O (pD 6.8).

(Table II). However, the W17 frequency, 878 cm^{-1}, indicates that Trp(5) is involved in a moderate H bonding. In sharp contrast to this, the N_1H group of Trp-28 forms two H bonds to amide oxygens of the main chain at Leu-17 and Tyr-23 in the crystalline state (Table II). Both of them are angular and as weak as the H bond between Trp-108 and Leu-56. The W17 frequency (871 cm^{-1}), however, suggests a strong H bonding. The inconsistency is thought to originate in the difference between the structures in the crystal and in solution.

Crystals of lysozyme with only one deuteriated tryptophan side chain (5H1D) were obtained by dissolving the fully deuteriated lysozyme (6D) in an aqueous solution of NaCl (3.5%) and by keeping the solution at pH 4.7 and 5 °C for 0.5 day. Attempts to obtain the other crystals of partially deuteriated lysozyme failed because of rapid D → H exchange and the difficulties in crystallization from D_2O solutions. The Raman spectra of crystalline 5H1D as well as crystalline 6H lysozyme have been measured. The difference Raman spectrum between lysozyme 6H and 5H1D shows that in the crystalline state Trp(6) gives W17 at a frequency of 875 cm^{-1}, which is in the range expected from the X-ray structure and 4 cm^{-1} higher than that in solution. Therefore, the intramolecular H bonding of the N_1H group of Trp-28 must be much stronger in solution than in the crystal.

Figure 6 shows that the indole rings of Trp-28 and Trp-111 are closely surrounded by other aliphatic side chains in the crystalline state. The large intensities of 1360-cm^{-1} band due to these side chains suggest that the hydrophobic environments remain in the solution.

(2) State of Tryptophan Side Chains in α-Lactalbumin. Lactose synthetase is separated into two protein components, A and B. B protein, the smaller one, has been identified as α-lactalbumin (Brodbeck et al., 1967) and considered as an "on–off" switch for lactose synthesis (Hill & Brew, 1975). Bovine α-lactalbumin has extensive sequence homologies with egg white lysozyme and has four tryptophan residues at sequence positions 26, 60, 104, and 118, corresponding to 28, 63, 108, and 123 in lysozyme, respectively (Brew et al., 1967). Trp-62 and Trp-111 are replaced by isoleucine and histidine in α-lactalbumin, respectively. Henceforth, the numbering of the amino acid residues in egg white lysozyme are adopted to avoid confusion.

The stepwise deuteriation was made in a way similar to the case of lysozyme. All labile hydrogens were replaced by deuterium in D_2O solution at pD 2 where α-lactalbumin takes a denatured structure. By an increase of pD to a neutral point, α-lactalbumin in the native structure with four deuteriated tryptophan side chains was obtained. In the Raman spectrum of this compound, the peak intensity of W6(ND) against that of the 1004-cm^{-1} band due to phenylalanine is 0.24, indicating that one deuteriated tryptophan side chain has an intensity of 0.06. Figure 7 shows the time course of intensity increase

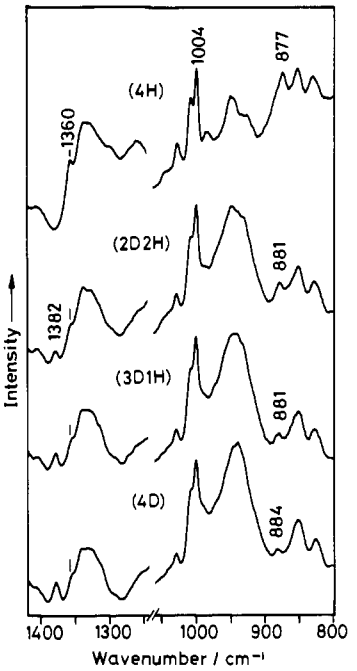

FIGURE 8: Raman spectra of stepwisely deuteriated α-lactalbumin: 4H, aqueous solution of α-lactalbumin; 2D2H, two tryptophan side chains are deuteriated; 3D1H, three are deuteriated; 4D, all the tryptophan side chains are deuteriated.

Table III: Frequencies of W17 and Intensities of the 1360-cm^{-1} Band of Tryptophan Side Chains in α-Lactalbumin

	W17 (cm^{-1})	I_{1360}[a]
Trp(1) and -(2)	877	1.00[b]
Trp(3)	880	0.21
Trp(4)	881	0.14

[a] Normalized to the average intensity of Trp(1) and -(2). [b] Value per one tryptophan.

of W6(ND) in the Raman spectra of α-lactalbumin in D_2O solution at various temperatures. Two of the four tryptophan side chains have a very rapid H → D exchange rate. This is similar to the case of lysozyme and consistent with a previous result (Takesada et al., 1976). Raman spectra of stepwisely deuteriated α-lactalbumin are shown in Figure 8. These results on the 1360- and 880-cm^{-1} bands are summarized in Table III.

The two rapidly deuteriated tryptophan side chains, Trp(1) and (2), are exposed on the molecular surface and moderately H bonded to solvent water, in spite of the hydrophobic environment. This state is similar to those of Trp-62 and Trp-63 in lysozyme. Slowly deuteriated Trp(3) and Trp(4) take part in no strong H bonding, and their environments are less hydrophobic than those of Trp(1) and Trp(2). This state is similar to that of Trp-123 in lysozyme.

Many studies, for example, by model building (Browne et al., 1969), optical rotatory dispersion, circular dichroism, infrared (Cowburn et al., 1970), and Raman spectroscopy (Yu, 1974) have shown close conformational similarities between lysozyme and α-lactalbumin. Accordingly, it may be reasonable to assign one of the exposed two Trp [Trp(1) or Trp(2)] to Trp-63 and Trp(3) to Trp-123, on the basis of environmental similarities. This assignment minimizes the difference between the two proteins in the state of the tryptophan side chains. On the other hand, it is certain that the states of Trp-28 and Trp-108 are much different between these two proteins. The very strong intramolecular H bonding of Trp-28 and the moderate one of Trp-108 in lysozyme solution are both absent in α-lactalbumin. These results suggest that

the states of some side chains are largely different between the two proteins in spite of the conformational similarities in the main chain structure.

Conclusions

The Raman band around 880 cm^{-1} due to the tryptophan side chain, W17, is a marker of H bonding; the strength of H bonding at the tryptophan N_1H site can be determined from its frequency. If the N_1H group is free of H bonding, W17 appears at 883–882 cm^{-1}. If the N_1H group forms a very strong H bond (e.g., crystalline AcTrpMA or Trp-28 of lysozyme in solution), W17 shifts down to 871 cm^{-1}.

The tryptophan doublet at 1360 and 1340 cm^{-1} is a marker of hydrophobicity of the environment. Hydrophobic interactions between an indole ring of the tryptophan residue and the surrounding aliphatic groups cause the 1360-cm^{-1} peak to increase in intensity and the 1340-cm^{-1} one to decrease. Thus, the intensity of the 1360-cm^{-1} peak is useful to estimate the hydrophobicity of the environment.

The states of individual tryptophan side chains in lysozyme and α-lactalbumin have been characterized by a combination of Raman spectroscopy and hydrogen–deuterium exchange kinetics. The results are summarized as follows: (1) the states of Trp-62, -63, -108, and -123 of lysozyme in solution are identical with those in the crystal; (2) the strengths of H bonding of Trp-28 and -111 of lysozyme are different between solution and crystal; (3) the states of two tryptophan side chains, Trp-28 and -108, in α-lactalbumin are largely deviated from those in lysozyme.

The present method will be applicable to investigation of the structure of various proteins in solution and, in certain cases, in the solid state.

Registry No. Trp, 73-22-3; H_2, 1333-74-0; lysozyme, 9001-63-2.

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