Interpretation of the Doublet at 850 and 830 cm^{-1} in the Raman Spectra of Tyrosyl Residues in Proteins and Certain Model Compounds†

Mwindaace N. Siamwiza,‡ Richard C. Lord,* Michael C. Chen, Tadahisa Takamatsu, Issei Harada, Hiroatsu Matsuura, and Takehiko Shimanouchi*

ABSTRACT: The doublet at 850 and 830 cm^{-1} in the Raman spectra of proteins containing tyrosyl residues has been examined as to its origin and the relation of its components to the environment of the phenyl ring, the state of the phenolic hydroxyl group, and the conformation of the amino acid backbone. Raman spectral studies on numerous model molecules related to tyrosine, including certain deuterium derivatives, show that the doublet is due to Fermi resonance between the ring-breathing vibration and the overtone of an out-of-plane ring-bending vibration of the para-substituted benzenes. Further examination of the effects of pH and solvents on the Fermi doublet and of the crystallographic data demonstrates that the intensity ratio of the two components depends on changes in the relative frequencies of the two vibrations. These in turn are found to be sensitive to the nature of the hydrogen bonding of the phenolic hydroxyl group or its ionization, but much less so to the environment of the phenyl ring and the conformation of the amino acid backbone. By use of the relative intensities of the doublet in model systems where the phenolic hydroxyl group is strongly hydrogen-bonded, weakly hydrogen-bonded, free or ionized, the reported Raman intensities of the doublets observed in the Raman spectra of several proteins have been interpreted. The results are compared with those obtained by other techniques.

The state of tyrosyl residues in proteins has been investigated mainly by ultraviolet spectra (uv) and by chemical reactivity techniques (Kronman and Robbins, 1970). For example, the remarkable change in uv absorption that occurs on removal of the phenolic hydrogen is useful in determining titration curves and pK values for tyrosyl residues. A "normal" tyrosyl residue in a protein has been defined as one whose reactivities with appropriate reagents are those of the free amino acid and whose pK is about 10. An "abnormal" tyrosyl residue then is that which has reduced reactivities with these reagents or abnormally high pK values (higher than 11). In line with this definition, the normal state has been considered as a state exposed on the molecular surface, and the abnormal state as a state buried in the interior and thus inaccessible to reagents. Moreover, two kinds of buried states have been postulated: those in which the phenolic OH is hydrogen bonded to other parts of the protein, and those in which the surroundings of the aromatic ring are mainly hydrophobic.

It is difficult to distinguish between these abnormal states by uv spectra or chemical reactivity. However, there is at least the possibility of doing so by means of the Raman effect. In the course of studies to establish relationships between Raman spectra and the structure of proteins, the position and relative intensities of a closely spaced pair of Raman lines at 850 and 830 cm^{-1} due to tyrosyl residues have been noted to depend on the environments of the tyrosyl side chains. Differences in these lines observed in bovine serum albumin and β-lactoglobulin were attributed to differences in the hydrogen bonding of the phenolic hydroxyl group (Bellocq et al., 1972). Recently Yu and coworkers (1973) correlated the intensity ratios of these two lines and a third at 640 cm^{-1} with tyrosyl residues in two different environments, exposed and buried. However, systematic studies on the relationships between the intensity ratios and the environmental conditions have not been undertaken.

Since interpretation of the doublet in the Raman spectra of proteins is scarcely possible without a basic understanding of the Raman spectra of simple aromatic systems, the M.I.T. and Tokyo groups independently undertook investigations of the Raman spectra of tyrosine and related compounds. The purposes of these studies, as presented in this joint paper, were threefold. First, determination of the origin of the doublet at 850 and 830 cm^{-1}. Both the M.I.T. and Tokyo groups measured the Raman spectra of *p*-cresol and related para-substituted benzenes and examined the relative intensities of the doublet at 850 and 830 cm^{-1}. At M.I.T. about 20 model molecules including the deuterated derivatives of *p*-cresol were studied, while in Tokyo, an extensive experimental study was made particularly on *p*-cresol, in combination with a normal coordinate treatment on this molecule. On the basis of the results of these studies, both groups came to the same conclusion that the Raman doublet is due to Fermi resonance. Second, determination of the factors influencing the intensity ratio of the doublet, including the pH dependence by the Tokyo group and the solvent effect and effect of the conformation of the amino acid backbone by both M.I.T. and Tokyo groups. Third, interpretation of the Raman spectra of tyrosyl residues in pro-

† From the Spectroscopy Laboratory and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (M.N.S., R.C.L., and M.C.C.), and the Department of Chemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan (T.T., I.H., H.M., and T.S.). *Received June 30, 1975.* The research at M.I.T. was liberally supported by grants from the National Institutes of Health (GM 15310) and the National Science Foundation (GP 35677 and MPS 72-04979-A03).

‡ Present address: Department of Chemistry, University of Zambia, Lusaka, Zambia.

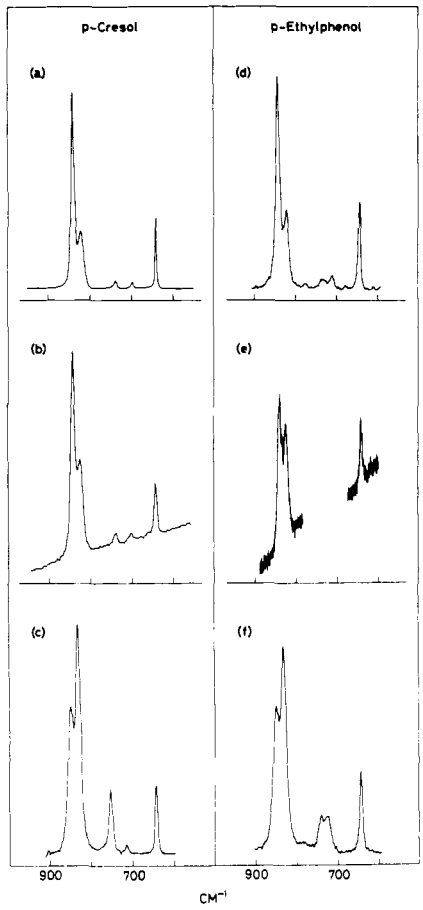

FIGURE 1: Raman spectra of *p*-cresol in the liquid state (a), in aqueous solution, pH 6.7 (b), and in alkaline solution with pH >13 (c), and of *p*-ethylphenol in the solid state (d), in aqueous solution, pH 6.8 (e), and in alkaline solution with pH >13 (f).

teins based on the results of these studies on model molecules.

Experimental Methods

At M.I.T. The following compounds were obtained commercially: L-tyrosine, L-tyrosine methyl ester, and *p*-cresol (gold label) from Aldrich Chemical Company, L-tyrosine hydrochloride, glycyl-L-tyrosine, and L-tyrosyl-L-tyrosine were from Sigma Chemical Company, *p*-methylanisole (reagent grade) and *p*-fluorotoluene (reagent grade) were from Eastman Chemicals, and *p*-xylene was from MCB Chemicals. *N,N*-Dimethyl-L-tyrosine methyl ester and *O*-methyl-*N,N*-dimethyl-L-tyrosine methyl ester were prepared by Dr. N. M. Relyea (Siamwiza, 1974). Deuterated derivatives of L-tyrosine and *p*-cresol were prepared by ring-proton exchange reactions and identified by means of proton magnetic resonance, infrared, and Raman spectroscopy.

The Raman spectra of L-tyrosine methyl ester, L-tyrosine hydrochloride, and L-tyrosyl-L-tyrosine as powders were obtained with a Jarrell-Ash 101 Raman spectrophotometer (spectral slit width, 7 cm^{-1}). The methyl and deuterated derivatives of tyrosine and *p*-cresol, and *p*-xylene and *p*-fluorotoluene were studied either as powders or as liquids with a Spex Ramalog 4 spectrophotometer (spectral slit width, 2–5 cm^{-1}). Both instruments were equipped with a Coherent Radiation 52G argon ion laser. The argon ion line at 488.0 nm at a power of about 70 mW was used as the exciting line. Solid samples were packed into a conical depression at the end of a stainless steel rod and scattered radiation was collected at ~90° to the incident beam. For solutions a "Kimax" melting-point capillary (1 mm i.d.) containing about 3 μl was used as a Raman cell.

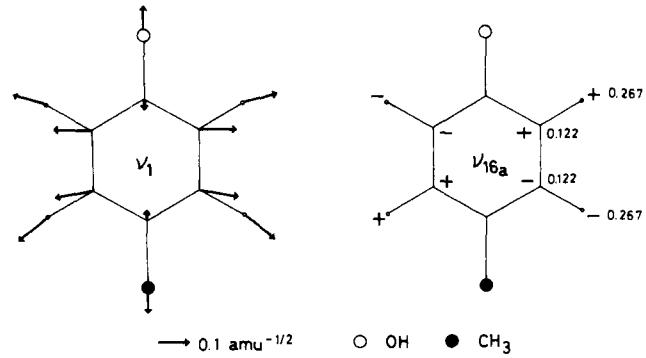

FIGURE 2: Vibrational modes of ν_1 and ν_{16a} for *p*-cresol [numbering of Wilson (1934)]. The vectors in ν_1 show quantitatively the relative atomic displacements on the mass-reduced scale given by the vector for 0.1 $amu^{-1/2}$. For ν_{16a} the displacements are perpendicular to the molecular plane (+ for upward motion, − for downward) and the relative magnitudes are shown numerically.

At the University of Tokyo. *p*-Cresol, *p*-ethylphenol, glycyl-L-tyrosine, and *N*-acetyl-L-tyrosine ethyl ester were purchased from Tokyo Chemical Industry Company, L-tyrosine was from Kyowa Hakko Company, and insulin was from Seikagaku Kogyo Company. Potassium *p*-cresoxide was obtained in the form of plates from 20% potassium hydroxide solution of *p*-cresol by addition of concentrated KOH solution. Glycyl-L-tyrosine hydrochloride was crystallized from aqueous solution as small needles. *N*-Acetyl-L-tyrosinemethylamide, kindly provided by Dr. Y. Koyama, was recrystallized from methanol-*n*-hexane solution. *p*-Cresol was purified by distillation, but the other compounds were used without further purification.

The Raman spectra were obtained with a JRS-S1 spectrophotometer with the 514.5-nm line of a Coherent Radiation CR3 argon ion laser as the exciting line (spectral slit width, 2–6 cm^{-1}). The alkaline solutions were prepared just before spectroscopic study. The pH of the solutions was adjusted with HCl and KOH and determined on a Hitachi-Horiba F-7 pH meter.

Results and Discussion

Assignment of the 850- and 830-cm^{-1} Raman Doublet. In discussing the Raman doublet at 850 and 830 cm^{-1}, it should be noted that this doublet has been observed for a large number of para-substituted benzenes (Green, 1970; Green et al., 1971; Jakobsen and Brewer, 1962; Jakobsen, 1965) (Figure 1), and that the doublet is associated with the benzene ring vibrations. If we assume C_{2v} symmetry for para-substituted benzenes, 30 benzene-like fundamentals are classified into ($11A_1 + 10B_2$) planar and ($3A_2 + 6B_1$) nonplanar modes. The selection rules for C_{2v} symmetry allow all fundamentals to be Raman active and all but A_2 fundamentals to be infrared active. This situation makes it difficult to pinpoint the origin of the Raman doublet.

The present study on *p*-cresol and related molecules shows that this Raman doublet is due to Fermi resonance between the symmetric ring-breathing fundamental ν_1 and the overtone $2\nu_{16a}$ of the nonplanar ring vibration of 413 cm^{-1} (Figure 2). The reasons for this interpretation are as follows.

Table I: Raman Doublets and ν_{16a} (cm^{-1}) in Various Para-Disubstituted Benzenes.

Molecule	Raman Doublet Frequency (Intensity)		ν_{16a}	$2\nu_{16a}$	Ref
p-Cresol (liquid)	842 (10)	823 (3)	413	826	*a,b*
p-Cresol-2,6-d_2 (liquid)	824 (10)	770 (0)	386	772	*a*
p-Cresol-d_4 (liquid)	791 (10)		362	724	*a*
Potassium-*p*-cresoxide (solid)	849 (4)	834 (10)	425	850	*b*
p-Ethylphenol (solid)	843 (10)	822 (4)	413	826	*b*
p-Methylanisole (liquid)	837 (3)	818 (10)	420	840	*a,b*
p-Xylene (liquid)	829 (10)	813 (3)	406 (ir)	812	*a*
p-Fluorotoluene (liquid)	844 (10)	826 (8)	425 (ir)	850	*a*
p-Aminotoluene (solid)	844 (10)	817 (3)	410	820	*c*
p-Dichlorobenzene (solid)	811 (1/2)	747 (10)	405	810	*c*
p-Dibromobenzene (solid)	809 (0)	709 (10)	402	804	*c*
L-Tyrosine (solid)	845 (3)	803 (10)	419 (ir)	838	*a*
L-Tyrosine-2,6-d_2 (solid)	824 (10)	772 (1)	376	752	*a*
L-Tyrosine·HCl (solid)	855 (10)	824 (4)	413 (ir)	826	*a*
L-Tyrosine methyl ester (solid)	843 (3)	829 (10)	423	846	*a*
N,N-Dimethyl-L-tyrosine methyl ester ($CHCl_3$)	849 (10)	827 (5)	417	834	*a*
O-Methyl-*N,N*-dimethyl-L-tyrosine methyl ester ($CHCl_3$)	845 (5)	826 (10)	419	838	*a*
L-Tyrosyl-L-tyrosine (solid)	850 (7)	820 (10)	423	846	*a*

[a] M.I.T. [b] The University of Tokyo. [c] Herz et al. (1947).

(1) In the Raman spectrum of *p*-cresol, the intensity ratio of the 842-cm^{-1} line to the 823-cm^{-1} line is 10:3. The former is the strongest in the entire Raman spectrum of liquid *p*-cresol. Polarization measurements show that both lines are highly polarized, so that both belong to the A_1 symmetry species. Therefore either both lines are A_1 fundamentals or one is an A_1 fundamental and the other is a combination vibration of A_1 symmetry. In the latter case, Fermi resonance must occur between the two vibrations, and the Raman intensity of the combination vibration is expected to increase as the result of coupling with the fundamental. Normal coordinate calculations indicate that only one A_1 benzene-like vibration (the ring-breathing vibration) is expected in the 800–900-cm^{-1} region, while among the possible combination frequencies in this region the overtone $2\nu_{16a}$ of the nonplanar ring vibration at 413 cm^{-1} (A_2) has the proper A_1 symmetry to interact with ν_1.

(2) If the doublet is due to Fermi resonance and ν_1 is higher than $2\nu_{16a}$, the upper component of the doublet is stronger, whereas if ν_1 is lower than $2\nu_{16a}$, the lower component is stronger. It can be shown[1] that in either case the intensity ratio is given approximately by $I_{2\nu_{16a}}/I_{\nu_1} \simeq (2W - \Delta)/(2W + \Delta)$ where $2W$ is the observed separation in cm^{-1} of the doublet and Δ the separation of the levels ν_1 and $2\nu_{16a}$ before Fermi resonance, both separations taken without algebraic sign. For example, in the Raman spectrum of potassium *p*-cresoxide, the intensity ratio of the 849-cm^{-1} line to the 834-cm^{-1} line is 4:10 (Table I). The reversal of the relative intensities compared to *p*-cresol is explained by the reversal of the relative positions of ν_1 and $2\nu_{16a}$ due to the upward frequency shift of ν_{16a} to 425 cm^{-1} in the cresoxide ion.

[1] The equation given here follows directly from eq 20-12 of Placzek (1934) if one assumes that $\partial^2\alpha/\partial q_{16a}^2$ is negligible compared to $\partial\alpha/\partial q_1$.

Table II: Frequencies (cm^{-1}) and Intensities of the Fermi Doublets of *p*-Methylanisole and *p*-Cresol.

State	Frequency (Intensity)			
	p-Methylanisole		*p*-Cresol	
Liquid	838 (3)	818 (10)	842 (10)	822 (3)
CCl_4 soln	836 (3)	818 (10)	842 (10)	822 (3)
Cyclohexane soln	836 (4)	818 (10)	845 (10)	824 (2)
Acetic acid soln	836 (3)	818 (10)	844 (10)	826 (5)
Triethylamine soln	836 (3)	818 (10)	848 (10)	828 (5)
Methanol soln	836 (3)	818 (10)	846 (10)	827 (4)

(3) The Raman spectra of deuterated derivatives of *p*-cresol also lend support to Fermi resonance as the origin of the doublet. In the spectrum of *p*-cresol-2,6-d_2, the strongest polarized line is observed as a singlet at 824 cm^{-1} which is assigned to the ν_1 fundamental vibration. The disappearance of the doublet in this deuterated molecule is explained by the removal of the interaction between ν_1 and $2\nu_{16a}$ as a result of the downward frequency shift of ν_{16a} to 386 cm^{-1}. A similar spectral feature is observed for *p*-cresol-d_4: the strongest polarized line is observed at 791 cm^{-1} as a singlet. The Fermi resonance is removed by the downward frequency shift of ν_{16a} to 362 cm^{-1} (Table I).

The doublet in the Raman spectra of other para-substituted benzenes can also be explained by a similar Fermi resonance (Table I). In the column "$2\nu_{16a}$" is the value of the overtone level calculated from the observed fundamental. In all cases but one (*p*-fluorotoluene) the calculated frequency of the overtone agrees approximately with that of the weaker component of the observed doublet. Moreover, in most cases, when the weaker component is lower in frequency than the stronger, its calculated frequency is higher than observed, while the reverse is true when the weaker component is higher in frequency than the stronger. This is precisely the behavior to be expected when the frequency of an overtone is affected by Fermi resonance.

Factors which affect the Ratio of Intensities of the Doublet. In order to investigate the intensity ratio of the Fermi doublet in more detail, those factors which may affect the frequencies of ν_1 and ν_{16a} in a significant way must be considered. These may be classified as follows: (1) change in the environment of the benzene ring; (2) state of the phenolic hydroxyl group (free, hydrogen bonded, or ionized); (3) conformation of the amino acid backbone.

(1) For the purpose of examining the effect of change in the environment of the benzene ring, the effect of different solvents on the doublet intensity was studied in *p*-methylanisole. Since this molecule is not a proton donor, hydrogen bonding is eliminated and only the effect of environmental change is reflected in the spectrum. The results listed in Table II show that no significant change in the relative intensity of the Raman doublet is produced by different solvent environments, whether polar or nonpolar.

(2) The effects of hydrogen bonding and ionization on the doublet intensity were examined for *p*-cresol and *p*-ethylphenol. In the spectrum of both *p*-cresol in the liquid state and *p*-ethylphenol in the solid state, the higher frequency peak is stronger (Figure 1). Under these conditions, the molecules are considered to be weakly hydrogen bonded. In aqueous solution the intensity of the lower frequency peak increases somewhat, but the higher frequency peak is still

Table III: Correlation among the Intensity Ratio of the Doublet, the Backbone Conformation, and the State of Hydrogen Bonding in L-Tyrosine and Its Derivatives.

Molecule	Raman Doublet (cm^{-1}) Frequency	(Intensity)	Conformation (deg) ψ^1	χ^1	χ^2	(Class)	State of Hydrogen Bonding of Phenolic OH Group (distance in Å) Acceptor Atom		Donor Group	
Glycyl-L-tyrosine·$2H_2O$	849 (10)	826 (5)	6	57	90	(A)	O in H_2O	(2.72[a])	NH_3^+	(2.98[a])
Glycyl-L-tyrosine·HCl	857 (10)	826 (4.5)	327	190	65	(B)	Cl^-	(3.04[b])	OH of COOH	(2.65[b])
L-Tyrosine·HCl	849 (10)	825 (4)	324	185	64	(B)	Cl^-	(3.05[c])	OH of COOH	(2.62[c])
L-Tyrosine	845 (3)	830 (10)	346	69	96	(A)	O in COO^-	(2.67[c])	NH_3^+	(2.88[c])
L-Tyrosine methyl ester	843 (3)	829 (10)	318	180	71	(B)	N in NH_2	(2.69[d])	NH_2	(3.27[d])

[a] Cotrait and Bideau (1974). [b] Smits and Wiebenga (1953). [c] Frey et al. (1973); Mostad et al. (1972). [d] The structure of L-tyrosine methyl ester was assumed to be the same as that of L-tyrosine ethyl ester determined by Pieret et al. (1970).

the stronger. When the pH of the solution is raised, the relative intensity of the lower frequency peak increases further and at pH greater than 13, the lower frequency peak is much stronger than the higher frequency peak. At these pH values, the intensity ratio of the doublet at 849 and 831 cm^{-1} in *p*-cresol is 6:10, and that of the doublet at 855 and 835 cm^{-1} in *p*-ethylphenol is 7:10 (Figure 1). Similar features are seen in the spectra of strong alkaline solutions of L-tyrosine and glycyl-L-tyrosine: the intensity ratio of the doublet at 850 and 829 cm^{-1} in L-tyrosine is 7:10, and that of the doublet at 856 and 829 cm^{-1} in glycyl-L-tyrosine is also 7:10.

The pH dependence of the intensity ratio of the doublet was investigated for *p*-cresol in more detail, although the full results are not given here. The titration curve gives a relationship between pH and the intensity ratio, with a drastic change in the ratio at pH 10–11. The p*K* value obtained from the titration curves is about 10.5, which is consistent with the results of previous studies (Kortüm et al., 1961; Yukawa and Ibata 1963). The ionization of the hydroxyl group is thus found to be related closely to the reversal of the relative intensities of the Raman doublet. The intensity reversal is probably due to the upward frequency shift of ν_{16a}, as in solid potassium *p*-cresoxide mentioned above, although direct observation of ν_{16a} is not possible because of its very weak intensity.

From the foregoing results it is concluded that the increase of $2\nu_{16a}$ as compared to ν_1 is related to the partial negative charge on the hydroxyl oxygen atom. In the extreme case of the cresoxide ion (Table I) this charge approaches nearly a full electron charge and the center of gravity of the doublet reaches the unusually high value of 842 cm^{-1}. In *p*-methylanisole, in which the *O*-methyl group is an electron donor to the oxygen atom, the intensity ratio is comparable, though the center of gravity is somewhat less (828 cm^{-1}). A similar effect of the methyl group is found in *O*-methyl-*N,N*-dimethyl-L-tyrosine methyl ester in chloroform solution, which shows a striking reversal of the intensity ratio observed for *N,N*-dimethyl-L-tyrosine methyl ester (Table I). In those solvents where hydrogen bonding is weak or nonexistent, regardless of their polarity, the intensity ratio in *p*-methylanisole is found to be constant (Table II), as mentioned earlier.

The question of the overall effect of hydrogen bonding is more complicated. A hydrogen bond formed by the hydroxyl hydrogen to a negative acceptor atom has the effect of introducing additional negative charge onto the hydroxyl oxygen. On the other hand, a hydrogen bond formed between an external hydrogen and the hydroxyl oxygen bleeds off negative charge from the oxygen. Hence the net effect of the two kinds of H-bonds when both are formed must be taken into account in assessing the effects of hydrogen-bonding solvents, of crystal structures with various H-bonds, and of other H-bonding environments. Fortunately the crystal structures of many of the solids listed in Table I are known and the geometric details of H-bonding therein can be specified.

We therefore consider the question of the net effect of hydrogen bonding in various model systems, beginning with *p*-cresol. In *p*-cresol solid (Bois, 1966) and presumably in the neat liquid the H-bonding is such that most OH groups act simultaneously as donors and acceptors. Since the effective charge transfer to the oxygen as a result of the hydroxyl's acting as a donor cancels almost precisely the transfer in the other direction when the oxygen is an acceptor, the oxygen atom in a free *p*-cresol molecule in a nonpolar solvent should have very nearly the same partial charge as a completely H-bonded molecule in the neat liquid or crystalline solid.

The results of the solvent effect on the doublet intensity of *p*-cresol are listed in Table II. The relative intensity of the lower frequency peak in the neat liquid, where H-bonding would be at a maximum, is the same as in nonpolar solutions. The intensity increase of the lower frequency peak in the polar solutions of the table is related to their capacity to act as acceptors of H-bonds. In glacial acetic acid and triethylamine, especially the latter, the *p*-cresol forms H-bonds to the solvent, thereby increasing the negative charge on the phenolic oxygen somewhat and thus raising the frequency of ν_{16a}. In methanol and in water, where the solvent acts as a donor as well as acceptor, the net effect is to produce little change from the neat liquid and thus the intensity ratio is not much different from that of nonpolar solvents.

In the crystalline materials, the effects of H-bonding can fortunately be discussed somewhat more precisely because of available crystallographic data. The intensity ratios of the doublet and the H-bonds to and from the phenolic hydroxyl group in solid L-tyrosine and some of its derivatives are shown in Table III. For glycyl-L-tyrosine·$2H_2O$, glycyl-L-tyrosine·HCl, and L-tyrosine·HCl, the intensity of the higher frequency peak is stronger, while for L-tyrosine and L-tyrosine methyl ester, the lower frequency peak is much stronger. In glycyl-L-tyrosine·$2H_2O$, glycyl-L-tyrosine·HCl, and L-tyrosine·HCl the phenolic OH forms hydrogen bonds to the oxygen atom of the water molecule and to the chloride ions, respectively. The bond to the former is only of moderate strength (O–H···O distance of 2.72 Å) while the latter, although probably stronger because of the negative charge on Cl^-, is definitely counterbalanced by much

Table IV: Correlation of the Classification of Tyrosyl Residues in Proteins by Raman Spectra and by Other Methods.

Raman Spectra		Other Methods	
Intensity Ratio $I(850)/I(830)$	State of Phenolic Hydroxyl Group	"Buried" Tyrosyl Residue (pK > 11, Decreased Reactivity)	"Exposed" Tyrosyl Residue (pK ~ 10, Normal Reactivity)
10:4 [L-tyrosine·HCl (solid)]	Acceptor of strong H-bonds	(Acceptor of strong H-bonds)	
10:8 (e.g., glycyl-L-tyrosine aq soln, pH 3)[a]	Donor and acceptor of moderate H-bonds (N)	Moderate-to-weak H-bonds (A)	Moderate-to-weak H-bonds (N)
3:10 (L-tyrosine solid)	Donor of strong H-bonds to CO_2^- (H)	Donor of strong H-bonds (A)	
7:10 (L-tyrosine, aq soln pH 12)	Ionized (I)	Ionized (I) clearly distinguished by uv absorption	

[a] The range of values for the ratio in various tyrosyl model compounds in aqueous solution is 10:10–10:7.

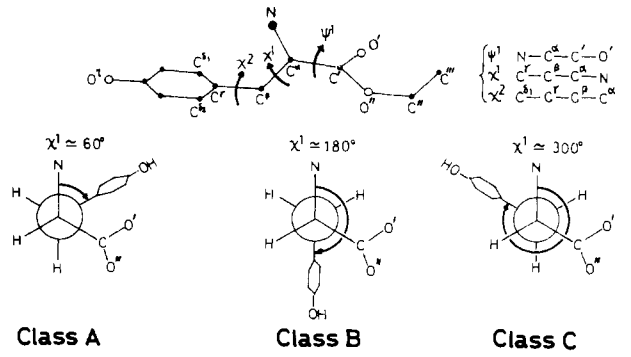

FIGURE 3: Conformations of tyrosyl residues.

stronger H-bonds from the carboxyl group to the phenolic oxygen (OH···O of 2.62 and 2.65 Å in L-tyrosine·HCl and glycyl-L-tyrosine·HCl, respectively). Thus the effective negative charge on the phenolic oxygen in these solids is expected to be rather similar to or less than that in *p*-cresol itself and the intensity ratio of the doublet reflects this.

In sharp contrast, the H-bonds in crystalline L-tyrosine and L-tyrosine methyl ester, where the proton acceptors are CO_2^- and NH_2 and the bond distances OH···O and OH···N are 2.67 and 2.69 Å, are evidently quite strong. Moreover, the donor H-bonds to the phenolic oxygen are of moderate strength, being formed by the NH_3^+ and NH_2 groups (N-H···O at 2.88 and 3.27 Å, respectively). The partial negative charge on the phenolic oxygen is then expected to be substantially increased, leading to an increase in ν_{16a} and an intensity enhancement of the lower component of the doublet, as in cresoxide ion. This effect may also be increased by a downward shift in ν_1, as shown by the fact that the relative intensity of the lower component is even higher than in cresoxide ion.

(3) It is essential to examine the effects of the conformation of the amino acid backbone, in order to evaluate the extent to which it may be responsible for the intensity reversal. The backbone structures have been divided into three major classes, A, B, and C, in accordance with the three staggered conformations about the C^α–C^β bond (Cody et al. 1973) as defined in Figure 3. The backbone structures of the classes A, B, and C have the approximate χ^1 values of 60, 180, and 300°, respectively. Table III shows the correlation between the intensity ratio of the Raman doublet and the backbone conformation of L-tyrosine and its derivatives, for which both Raman and crystallographic data are available. This table shows that the conformational difference between classes A and B has no significant influence on the intensity ratio of the doublet.

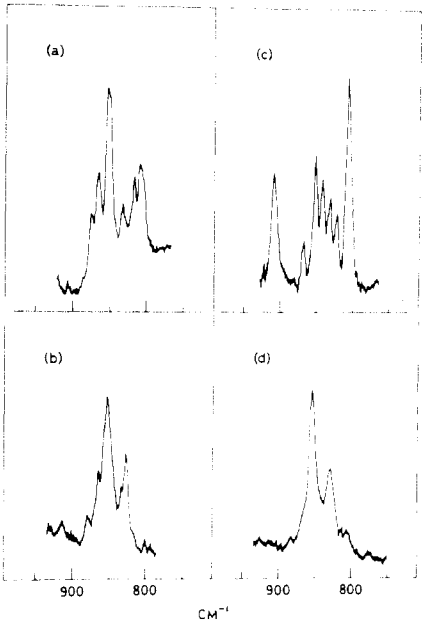

FIGURE 4: Raman spectra of *N*-acetyl-L-tyrosine ethyl ester in the solid state (a) and in methanol solution (b), and of *N*-acetyl-L-tyrosinemethylamide in the solid state (c) and in methanol solution (d).

The class C molecules, *N*-acetyl-L-tyrosinemethylamide and *N*-acetyl-L-tyrosine ethyl ester, in the solid state show complicated Raman spectra in the 800–900-cm^{-1} region and the doublets are not identified definitely. On the other hand, methanol solutions of these molecules exhibit the doublets clearly (Koyama, 1974; Matsuura et al., 1975) (Figure 4) and with the expected intensity ratio (compare *p*-cresol in methanol, Table II). Such features have not yet been reported in the spectra of proteins, which suggests that the factors which complicate the Raman spectra of the class C molecules in this frequency region are absent in the protein molecules so far studied.

Thus we conclude from the above experimental data of model molecules that the intensity ratio of the doublet is related primarily to the state of the phenolic hydroxyl group, rather than to the environment of the benzene ring or to the conformation of the amino acid backbone. In particular, the effects of ionization of the phenolic hydroxyl group and of strong hydrogen bonding by the hydroxyl hydrogen to negatively charged acceptors and by strong proton donors to the hydroxyl oxygen are significant. When these effects lead to a net increase of partial negative charge on the oxygen atom, they raise ν_{16a} and thereby produce an intensity enhancement of the lower frequency component, which takes

on more of the character of the fundamental ν_1. A decrease of negative charge has the opposite effect.

Interpretation of the Raman Spectra of Tyrosyl Residues in Proteins. As was indicated in the introduction, there is the possibility of determining the state of tyrosyl residues in proteins by means of the intensities of the Raman doublet. From the preceding discussion of the spectra of model systems, we may suppose that the tyrosyl residues in proteins lie somewhere in the range from those in which the phenolic oxygen is the acceptor atom in a strong H-bond for which the proton donor is very acidic hydrogen (intensity ratio 10:4 as in L-tyrosine·HCl solid), to those in which the phenolic hydroxyl is the proton donor in a strong H-bond to a very negative acceptor such as a carboxylate ion (intensity ratio 3:10, as in L-tyrosine solid). There is also the ionized state, which exists only at high pH (pH ≥12) with the ratio 7:10. If a tyrosyl residue is on the surface of a protein in aqueous solution, the state of the phenolic OH will be that of a simultaneous acceptor and donor of moderate to weak H-bonds, that is, it will fall in the middle of the above range. Let us define such a surface ("exposed") tyrosine as a "normal" tyrosine and note that the doublet intensity ratio will be about 10:8, though the actual ratio may vary from 9:10 (aqueous L-tyrosine at pH 0.3) to 10:7 (aqueous glycyl-L-tyrosine, pH 2.5).

Tyrosines buried within the protein in hydrophobic regions may in principle also fall within the same range. However, it seems rather unlikely that the phenolic oxygen will be the acceptor of a strong H-bond from a highly acidic group, i.e., the usual range of the buried tyrosines will probably be from "normal" to those forming strong H-bonds with negative acceptors. Thus the range of the $I(850)$: $I(830)$ ratio should be from about 10:7 to about 3:10 in the extreme case of strong H-bonding. A correlation of the expected behavior of this ratio in the Raman effect of proteins with behavior inferred from uv spectra and chemical reactivity is suggested in Table IV.

By means of this correlation, the intensity ratio as observed in the Raman spectra of proteins can be used to estimate the distribution of tyrosyl residues among the various states. Since the ratio is only a single number, it is clearly not possible in principle to do so for more than one tyrosine group. However, when the number of tyrosines per protein molecule is small and the value of the ratio is clearly on the side of either "normal" or "strongly H-bonded," the corresponding conclusion that all the tyrosines are of one kind or the other may be drawn. Examples of this kind of inference are shown in Table V, where the results for five globular proteins of low molecular weight, containing from one to six tyrosyl residues, are displayed. In Table V the intensity ratio of 10:8 (as in aqueous L-tyrosine and glycyl-L-tyrosine) is adopted for normal tyrosine (N) and 5:10 (as in erabutoxin *a* aqueous solution) for strongly H-bonded (H) tyrosine in the classification of tyrosyl residues from Raman data. For comparative purposes the conclusions drawn from other data are given in the last column.

Table V indicates that the number of moderately hydrogen-bonded tyrosines and of strongly hydrogen-bonded tyrosines obtained from the Raman spectrum is in good agreement with those of the "normal" tyrosines and "abnormal" tyrosines, respectively, obtained by other methods. This fact suggests that the abnormal tyrosines are mainly in the strongly hydrogen-bonded state in the interior of proteins. The structural changes of proteins caused by decreasing pH, heating, lyophilization, and other denaturing agents commonly increase the intensity of the higher frequency component of the doublet. This intensity change is thus related to the disruption of the strong hydrogen bonds of the tyrosyl residues. The intensity change in the RNase A solution by alteration in pH (from 8.9 to 1.7) (Yu et al., 1972a) indicates that one of the three strong hydrogen bonds is disrupted by partial structural change. Study of the effect of low pH in RNase A solution by means of ultraviolet spectra also shows that one of the abnormal tyrosines becomes normal at pH below 2.0 (Bigelow and Ottensen, 1959).

We have measured the Raman spectrum of insulin in strongly alkaline solution (35 mg/ml, 1 *M* KOH), where all four tyrosines in insulin are found to be ionized by the ultraviolet spectrum (Inada, 1961). The intensity ratio of the doublet at 855 and 834 cm^{-1} is 8:10, which is close to that for ionized model molecules.

Table V: Classification of Tyrosyl Residues in Certain Proteins.

Protein (No. of Tyrosyl Residues)	State of Sample	Observed Doublet Ratio	Classification of Tyrosyl Residues: Raman	Classification of Tyrosyl Residues: Other Methods[y]
Lysozyme (3)	Crystals	10:9[a]	3 N or 2 N + 1 H	X-ray (2 N + 1 A)[b]
	Aq, pH 4.5	10:10[a]	2 N + 1 H	Uv (2 N + 1 A,[c] 1 N + 2 A[d]), I_2 (2 N + 1 A),[e] CyF (2 N + 1 A),[f] TNM (3 N)[g]
Ribonuclease A (6)	Powder	10:9[h]	4 N + 2 H or 5 N + 1 H	
	Aq, pH 5.0 and 8.9	8:10[h]	3 N + 3 H	Uv (3 N + 3 A),[i] I_2 (3 N + 3 A,[j] 4 N + 2 A[k]), CyF (3 N + 3 A,[l] 2 N + 4 A[m]), AI (3 N + 2 A),[n] TNM (3 N + 3 A)[g]
	Aq, pH 1.7	10:10[h]	4 N + 2 H	Uv (4 N + 2 A)[o]
Insulin (4)	Crystals	10:8[p]	4 N	
	Aq, pH >13	8:10[q]	4 I or 3 I + 1 N	Uv (4 I)[c]
	Aq, pH 2.4 and 8.3	10:8[p]	4 N	Uv (3 N + 1 A),[c] I_2 (4 N),[r,s] CyF (2 N + 2 A),[o,t] AI (4 N),[n] TNM (2 N + 2 A)[g]
Erabutoxin a (1)	Aq, pH 7	5:10[u]	1 H	Uv (1 A),[v] I_2 (1 A)[w]
Cobramine B (3)	Powder	5:10[x]	3 H	
	Aq, pH 7, 30°C	6:10[x]	3 H or 2 H + 1 N	
	Aq, 85°C	10:8[x]	3 N	

[a] Yu and Jo (1973). [b] Blake et al. (1967); and the Kendrew wire model of lysozyme in the Department of Biology, M. I. T. [c] Inada (1961). [d] Tojo et al. (1966). [e] Covelli and Wolff (1966); Wolff and Covelli (1966). [f] Kurihara et al. (1963). [g] Sokolovsky et al. (1966). [h] Yu et al. (1972a). [i] Tanford et al. (1955). [j] Woody et al. (1966). [k] Cha and Scheraga (1963). [l] Gorbunoff (1967). [m] Takenaka et al. (1967). [n] Riordan et al. (1965). [o] Bigelow and Ottensen (1959). [p] Yu et al. (1972b). [q] Present study. [r] Gruen et al. (1959). [s] Springell (1962a,b). [t] Aoyama et al. (1965). [u] Harada (1974). [v] Sato (1971). [w] Sato and Tamiya (1970). [x] Yu et al. (1973). [y] Abbreviations used are: uv, ultraviolet spectrum; I_2, reactivity with iodine; CyF, reactivity with cyanuric fluoride; AI, reactivity with *N*-acetylimidazole; TNM, reactivity with tetranitromethane.

The above experimental data support our interpretation that the intensity ratio of the Raman doublet depends mainly on the state of the hydroxyl group, and that the standard intensity ratios, adopted in this study, for the normal and the strongly hydrogen-bonded states are reasonable. Nevertheless, the actual intensity ratio of each tyrosyl residue in proteins may be somewhat different from the standard values because of other factors neglected in the present discussion. As one can see in Table V, these standard values seem to be practically useful for studying tyrosyl residues in proteins, so long as the neglected factors have little effect on the intensity ratio and the number of tyrosyl residues is not large.

The present study indicates the importance of Raman spectroscopy for the investigation of the state of tyrosyl residues and of the role of the hydrogen bonding of the phenolic hydroxyl in the environment of these residues in proteins.

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