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A Raman Study on the C(4)=O Stretching Mode of Flavins in Flavoenzymes: Hydrogen Bonding at the C(4)=O Moiety

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Raman spectroscopy was used to investigate the hydrogen bonding at the C(4)=O moiety of the isoalloxazine nucleus in a series of flavins and flavoproteins. Isotope effects of Raman bands confirmed that the band observed around 1,710 cm^{-1} is mainly derived from C(4)=O stretching vibrational mode. A linear correlation was observed between the frequency of C(4)=O stretching and the chemical shift of $^{13}C(4)$, suggesting that the data from both Raman and NMR spectroscopies reflect a common perturbation, *i.e.*, hydrogen bonding. The maximum difference of C(4)=O frequency among flavins and flavoproteins examined is 36 cm^{-1} [1,723 cm^{-1} for riboflavin-binding protein (Kim, M. and Carey, P.C. (1993) *J. Am. Chem. Soc.* 115, 7015-7016) and 1,687 cm^{-1} for the complex of medium-chain acyl-CoA dehydrogenase with acetoacetyl-CoA]; the maximum difference of 40-70 kJ/mol in the hydrogen bonding strength at the C(4)=O exists among flavoproteins. By use of an empirical linear correlation between the frequency of C=O stretching and the bond length of the C=O, it is estimated that the maximum difference in the bond length among flavoproteins treated here is *ca.* 0.017 Å. The hydrogen bonding at the C(4)=O in medium-chain and short-chain acyl-CoA dehydrogenases becomes stronger upon complexation with substrate analogs. Since the hydrogen bonding at the C(4)=O is expected to enhance the electron-accepting capacity of the N(5) position, substrate-binding itself probably raises the reactivity of flavin, through enhancing the hydrogen bonding.

Key words: carbonyl group, flavin, flavoprotein, hydrogen bond, Raman spectra.

Flavins are very versatile coenzymes, and flavoenzymes catalyze various oxidation-reduction reactions in living cells. The selection of a specific catalytic reaction from among various functions is accomplished by the interactive network of the isoalloxazine ring, apoprotein, and substrates. Hydrogen bonding is one of the most important interactions for this purpose. The isoalloxazine nucleus has five possible hydrogen-bonding sites, *i.e.*, N(1), C(2)=O, N(3)-H, C(4)=O, and N(5) (Fig. 1), and the hydrogen-bonding network is one of the most influential factors in fine-tuning the flavin reactivity, as demonstrated by molecular orbital calculations (*1-5*). In this paper, we report a Raman study, focusing on the hydrogen bonding at the C(4)=O moiety of the isoalloxazine ring.

The interactions between atomic groups, such as hydrogen bonding, affect the electronic distributions within bonds and the force constants of certain bonds. Thus, vibrational spectroscopy, which is very sensitive to the force constant, can not only detect protein-ligand interactions but also yield quantitative information about their nature and strength. An empirical linear correlation between the frequency of the C=O stretching vibration and the carbonyl bond length has been obtained (*6*), and the correlation between the decrease in the band frequency of C=O stretching vibration and the enthalpy of hydrogen bonding at the C=O group has been determined (*7-9*), indicating that the band frequency of C=O is decreased by hydrogen bonding at the carbonyl oxygen. Recently, Tonge and Carey (*8, 10, 11*) and Deng *et al.* (*12, 13*) estimated the polarization (due to hydrogen bonding) of a substrate's carbonyl group by means of vibrational spectroscopy and showed that the reaction rates are correlated with the extent of polarization in acyl-serine protease and lactate dehydrogenase. We also showed in the case of acyl-CoA dehydrogenases that the hydrogen bonding at the substrate C(1)=O moiety is important for enhancement of the reaction (*14*). Some investigations by using Raman spectroscopy have been carried out to study the hydrogen bonding of flavin (*15-17*). Recently, Kim and Carey (*18*) showed the C(4)=O stretching mode of riboflavin bound to riboflavin-binding protein appeared at a frequency 13 cm^{-1} higher than that of riboflavin in H_2O and concluded that the hydrogen bonding between the C(4)=O and the protein is less strong than that between the C(4)=O and water molecules for riboflavin free in aqueous solution.

NMR spectroscopy, which is sensitive to electronic distribution, is also a powerful method for the investigation of molecular interactions. It has been applied to many

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Abbreviations: DAO, D-amino acid oxidase; GO, glucose oxidase; IVD, isovaleryl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; OYE, old yellow enzyme; RBP, riboflavin-binding protein; SCAD, short-chain acyl-CoA dehydrogenase; TARF, riboflavin tetraacetate.

flavoproteins, and the studies provide information on the hydrogen-bonding network surrounding the flavin ring (*19* and references cited therein). The ^{13}C resonances of the carbonyl groups are sensitive to hydrogen bonding, which results in electron density modulation, and shift to lower field when the hydrogen bonding at the carbonyl oxygen becomes stronger and *vice versa*. The chemical shift values for the flavoproteins obtained to date are summarized in Ref. *19*; the chemical shift values for $^{13}C(4)$ cover a substantially wide range. X-Ray crystallographic studies supply more structural information, and prove the existence of hydrogen bonding and its qualitative strength, although the quantitative evaluation is now difficult due to the limitation of resolution in macromolecular studies.

In this study, we confirmed that the band around 1,710 cm^{-1} is mainly associated with C(4)=O stretching vibration, by using isotope-labeled flavins. We also measured the Raman spectra of different flavin species and flavoenzymes, whose chemical shifts for $^{13}C(4)$ have various values (*19*), and a linear correlation was observed between the band position of C(4)=O stretching mode and the chemical shift value for the $^{13}C(4)$. This suggests that both data reflect a common perturbation, *i.e.*, hydrogen bonding. Recently, the X-ray crystallographic structures of many flavoproteins have been clarified, and information about the hydrogen bonding around the isoalloxazine nucleus has been obtained. Thus, we also compared Raman results with the results from X-ray crystallography.

MATERIALS AND METHODS

Acyl-CoA dehydrogenases were purified from bovine liver as described by Melde and Ghisla (*20*) and Davidson and Schultz (*21*) with some modifications. The concentrations of the enzymes were determined spectrophotometrically using $\varepsilon_{450}=14.9\ mM^{-1}\cdot cm^{-1}$ for SCAD (*22*), $\varepsilon_{448}=15.3\ mM^{-1}\cdot cm^{-1}$ for MCAD (*23*), and $\varepsilon_{446}=14.1\ mM^{-1}\cdot cm^{-1}$ for IVD (*23*). The dehydrogenases reconstituted with isotopically labeled FAD were prepared by the reported procedure (*24*). The holoenzyme and apoenzyme of porcine kidney DAO were purified according to Shiga *et al.* (*25, 26*) and Tojo *et al.* (*27*), respectively. The preparation of [4,10*a*-$^{13}C_2$]FAD and reconstitution of the labeled FAD to apoDAO were carried out following the procedures previously reported (*28*). Glucose oxidase (grade II) from *Aspergillus niger* was obtained from Boeringer Mannheim and used without further purification. The concentration of the enzyme was determined spectrophotometrically using $\varepsilon_{450}=15.2\ mM^{-1}\cdot cm^{-1}$ (*29*). The apoenzyme was prepared by the method of Mayer and Thorpe (*30*) with the following modification: the pH value of the resolving solution containing EDTA, ammonium sulfate, and KBr was decreased to 1.4 following the method of Swoboda (*31*). The reconstituted enzyme with isotopically labeled FAD or ADP was prepared by the method of Swoboda (*31, 32*). Old yellow enzyme (OYE) and the apoenzyme were purified from brewer's yeast (*33*). The concentration of the enzyme was determined spectrophotometrically using $\varepsilon_{462}=10.6\ mM^{-1}\cdot cm^{-1}$ (*33*).

Synthesis of ^{13}C- and ^{15}N-enriched riboflavin was essentially according to Yagi *et al.* (*34*). ^{13}C- and ^{15}N-enriched FADs were prepared according to the methods previously reported (*28*), and [4,10*a*-$^{13}C_2$]FMN was prepared by the same procedure for 8-fluoro-8-demethyl FMN (*35*). Riboflavin tetraacetate (TARF) and [4,10*a*-$^{13}C_2$]TARF were synthesized from non-labeled and labeled riboflavin, respectively, by the procedure reported previously (*36*). FAD and [4,10*a*-$^{13}C_2$]TARF were purified by C18 reverse-phase HPLC (*37*).

3-Ketoacyl-CoAs were synthesized as described elsewhere (*23*). CoA derivatives were quantified by using a molar absorption coefficient of $16\ mM^{-1}\cdot cm^{-1}$ at 260 nm (*38*). Chloroform-*d* (99.96 atom%) was obtained from Isotec., USA. D_2O (99.85 atom%) was obtained from the Commissariat a L'Energie Atomique (CEA), France. Other chemicals were of the highest grade available from commercial sources.

Raman spectra were obtained with a JASCO NR-1800 spectrometer with a He-Ne laser (NEC GLG 5900) as a light source, at room temperature (*ca.* 25°C). The wave number axis of the Raman spectra was calibrated with indene. Difference Raman spectra were obtained by a computerized subtraction of one spectrum from another. The two spectra were measured successively with an optical multichannel analyzer, within one or 2 h.

^{13}C-NMR spectra were measured in Wilmad 5-mm NMR tubes with a Varian UNITY Plus 500 spectrometer operating at 125.7 MHz under proton irradiation. The ^{13}C-chemical shift was scaled in ppm downfield relative to the methyl-carbon signal of external 3-(trimethylsilyl)propionate-d_4. ^{13}C-NMR spectra of SCAD reconstituted with [4,10*a*-$^{13}C_2$]FAD and the complex of the enzyme with acetoacetyl-CoA were measured at 25°C

RESULTS

Raman Spectra of Non-Labeled and Isotopically Labeled FADs Excited at 632.8 nm—Figure 2 shows the Raman spectra of non-labeled and isotopically labeled FADs in H_2O and D_2O excited at 632.8 nm. The spectra of non-labeled FAD are identical with those reported by Nishimura and Tsuboi (*39*), although they did not specifically mention the 1,711-cm^{-1} band. The 1,711-cm^{-1} band corresponds to the 1,714-cm^{-1} band of riboflavin reported by Kim and Carey (*18*). The 1,710-cm^{-1} and 1,685-cm^{-1} bands observed in the infrared absorption spectra of TARF in chloroform have been considered to be mainly associated with the C(4)=O and C(2)=O stretching mode of the isoalloxazine nucleus, respectively (*40*). The band around 1,710 cm^{-1}, which has been observed in the deep-UV resonance Raman spectra of free FAD and FMN, has been assigned in large part to the C(4)=O stretching mode (*41*). These assignments were based on the consideration that the C(2)=O bond, being conjugated with the C=N bonds (Fig. 1), should vibrate at

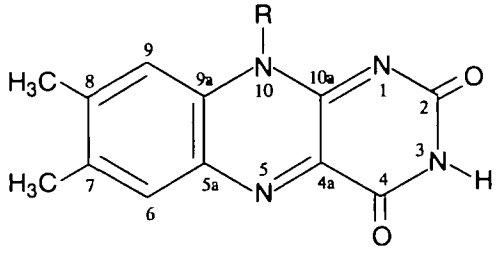

Fig. 1. **Numbering of flavin ring system.**

lower frequency than the C(4)=O bond (*40, 41*). These assignments are supported by a normal coordinate analysis of lumiflavin (*42, 43*) and infrared absorption spectra of isotope-labeled lumiflavins and riboflavin derivatives (*44*). However, the $^{13}C(4)$ labeled compound, which is important for the assignment of the vibrational mode involving the C(4) atom, has not been used. Thus, the present data of [4,10*a*-$^{13}C_2$]FAD are valuable for unequivocal assignment of the 1,711-cm^{-1} band.

The 1,711-cm^{-1} band is downshifted to 1,698 cm^{-1} by deuteration of the isoalloxazine ring (Fig. 2B-a), which is identical with the result by Kim and Carey, indicating that the carbonyl stretching mode includes a contribution from N(3)H bending (*18*). The normal mode analysis has been carried out under the conditions where the band is not sensitive to the deuteration, and thus the calculation should be carried out by considering the deuterium effect as pointed out (*18*). The 1,711-cm^{-1} (H_2O) and 1,698-cm^{-1} (D_2O) bands are downshifted to 1,660 and 1,664 cm^{-1}, respectively, with [4,10*a*-$^{13}C_2$]FAD. However, both bands scarcely shift with other labeled FADs (Fig. 2A-c, e, Fig. 2B-c-f), except for slight shifts with [2-^{13}C]FAD and [1,3-$^{15}N_2$]FAD in H_2O (Fig. 2A-d, f). These results indicate that the 1,711-cm^{-1} band is mainly derived from the C(4)=O stretching vibrational mode. The 51-cm^{-1} downward shift in H_2O upon [4,10*a*-$^{13}C_2$] labeling of FAD exceeds that (38 cm^{-1}) predicted from the simple reduced mass calculation based on the assumption that the molecule is an isolated C=O molecule; this is probably related with the fact that the band includes a contribution from N(3)H bending. The 34-cm^{-1} downward shift in D_2O upon the labeling is consistent with the calculated result (38 cm^{-1}).

The 1,351-cm^{-1} (Fig. 2A-a) and 1,351-cm^{-1} (Fig. 2B-a) bands in H_2O and D_2O split into two bands with [4,10*a*-$^{13}C_2$] labeling (Figs. 2A-b, 2B-b). Müller *et al.* (*16*) showed that the band around 1,350 cm^{-1} consists of two bands in coherent anti-Stokes Raman spectra of TARF, and the corresponding band is normally broad and appears to have a shoulder or a doublet for several enzymes (*45-47*). The present results confirm the existence of two bands having isotope effects with different sensitivity.

The isotope effects for other bands are identical with the effects for resonance Raman bands of riboflavin bound to riboflavin-binding protein excited at 488.0 nm (*48*).

Band Positions Derived from the C(4)=O Moiety of Flavin Bound to Flavoproteins—To compare the positions of the band derived from the C(4)=O stretching mode of flavin bound to flavoproteins, we measured Raman spectra

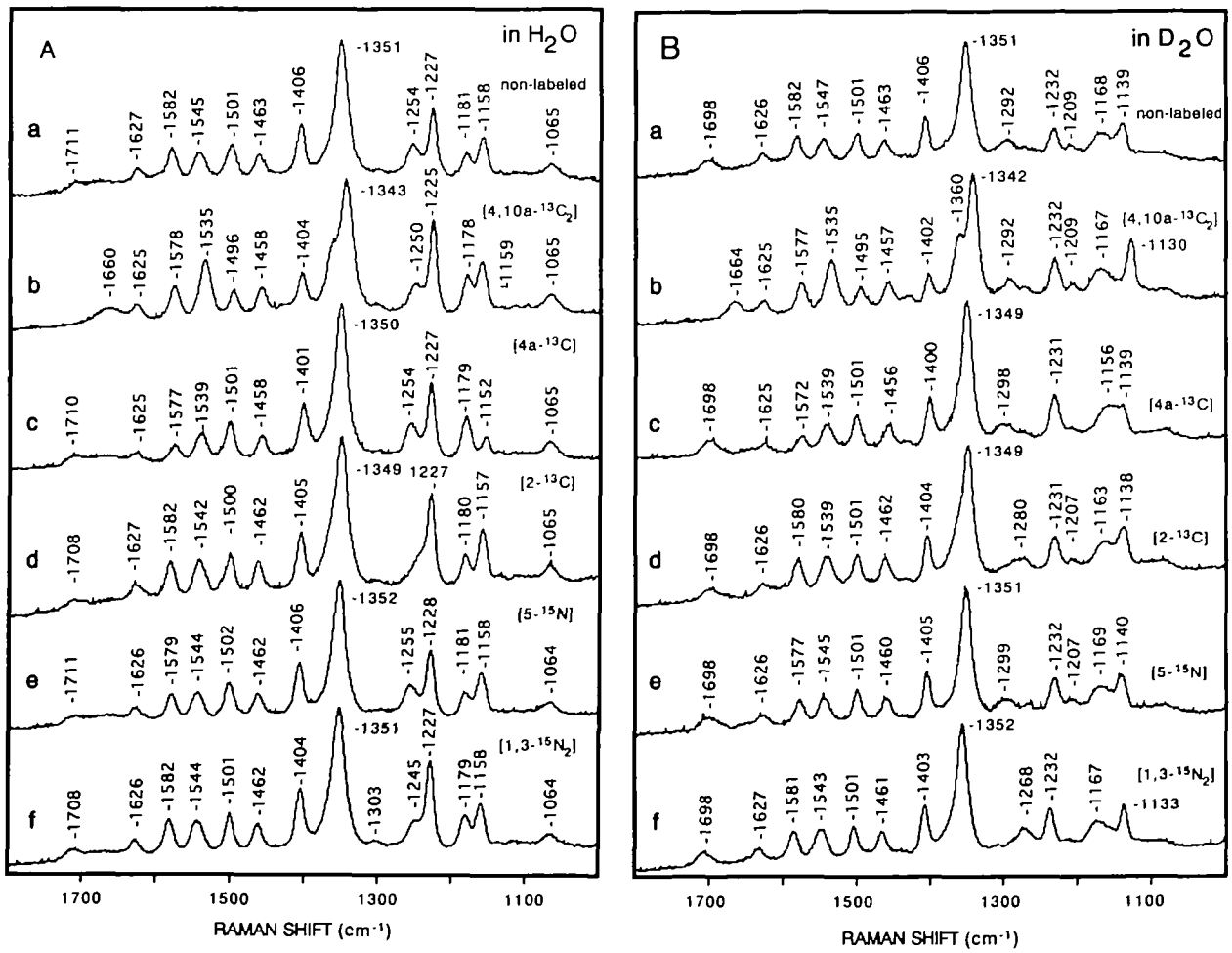

Fig. 2. **Raman spectra of non-labeled and isotopically labeled FADs excited at 632.8 nm.** The spectra were observed in 50 mM Tris buffer [H_2O (A), D_2O (B)], pH or pD 7.5. The concentrations were: (A) a, 53 mM; b, 31 mM; c, 45 mM; d, 34 mM; e, 32 mM; f, 47 mM; (B) a, 34 mM; b, 30 mM; c, 21 mM; d, 32 mM; e, 26 mM; f, 37 mM.

of various flavoproteins in the frequency region of carbonyl stretching vibration. Figure 3 shows the Raman spectra of bovine liver SCAD and of SCAD complexed with acetoacetyl-CoA with excitation at 632.8 nm, which is within the broad absorption band derived from the charge-transfer interaction between the ligand and flavin. The bands around 1,585 and 1,550 cm^{-1} are associated with the C(4*a*)=N(5) region of oxidized flavin (*23*). The 1,643-cm^{-1} band (Fig. 3-e) of SCAD-acetoacetyl-CoA complex is derived from acetoacetyl-CoA (*23*). The broad band around 1,656 cm^{-1} (Fig. 3-a) contains the band from the protein (amide I mode), and the band is also observed in the apoprotein of SCAD (not shown). The 1,700-cm^{-1} band (Fig. 3-a) shifted to 1,647 cm^{-1} upon [4,10*a*-$^{13}C_2$] labeling and to 1,689 cm^{-1} in D_2O (Fig. 3-c, d). The band shifts were of comparable magnitude to those in the case of FAD (Fig. 2). Thus, we assigned the 1,700-cm^{-1} band to the C(4)=O stretching vibration. The spectra of SCAD-acetoacetyl-CoA complexes were obtained in high S/N ratio; the complex has a charge-transfer absorption band around 632.8 nm, and some Raman bands are resonance-enhanced. The 1,693-cm^{-1} band disappears with [4,10*a*-$^{13}C_2$]FAD reconstituted (Fig. 3-f). The band is probably concealed behind the 1,643-cm^{-1} band of acetoacetyl-CoA; the band is expected to shift to 1,642 cm^{-1} if the magnitude of the shift is the same as the shift (51 cm^{-1}) for the band of FAD in H_2O (Fig. 2). The band shifts to 1,685 cm^{-1} in D_2O. Thus, the band is assigned to the C(4)=O stretching mode and is lower by 7 cm^{-1}, than that for uncomplexed SCAD.

Figure 4 shows the Raman spectra of MCAD and IVD. By subtraction of the spectrum of apo-MCAD, the 1,694-cm^{-1} band of MCAD becomes distinct (Fig. 4-b), and the band shifts to 1,653 cm^{-1} with [4,10*a*-$^{13}C_2$]FAD reconstituted.

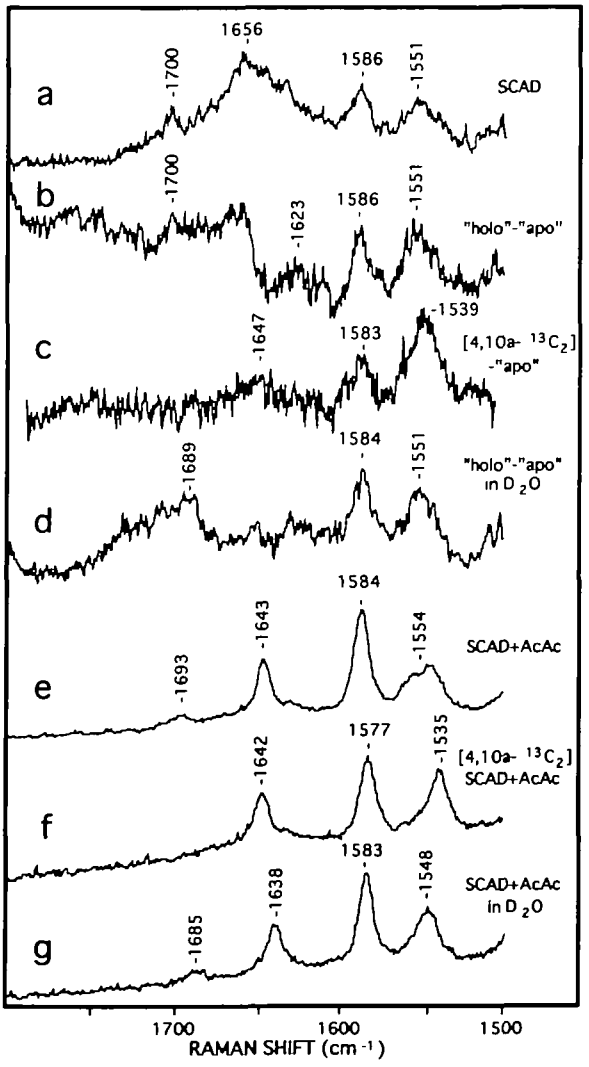

Fig. 3. **Raman spectra of SCAD and the complex of SCAD with acetoacetyl-CoA excited at 632.8 nm.** The spectra were observed in 50 mM potassium phosphate buffer containing 0.3 mM EDTA, pH 7.6 (a-c, e, f) or pD 7.6 (d, g). (a) SCAD (1.5 mM); (b) the difference spectrum of SCAD (2.0 mM) minus apoSCAD (*ca.* 2 mM); (c) the difference spectrum of SCAD (1.5 mM) reconstituted with [4,10*a*-$^{13}C_2$]FAD minus apoSCAD (*ca.* 1.5 mM); (d) the difference spectrum of SCAD (2.0 mM) minus apoSCAD (*ca.* 2 mM); (e) the complex of SCAD (1.2 mM) with acetoacetyl-CoA (1.4 mM); (f) the complex between SCAD (1.1 mM) reconstituted with [4,10*a*-$^{13}C_2$]FAD and acetoacetyl-CoA (1.4 mM); (g) the complex of SCAD (1.4 mM) with acetoacetyl-CoA (1.5 mM).

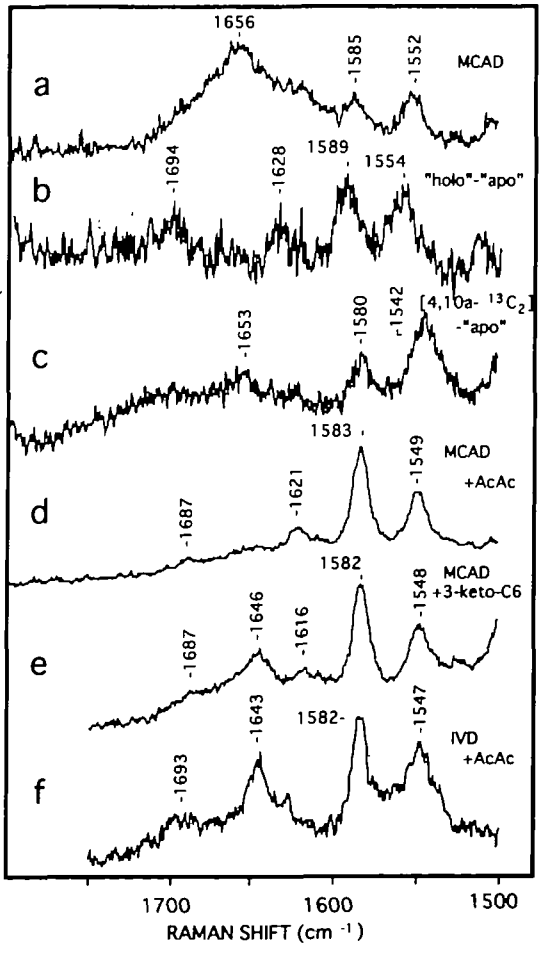

Fig. 4. **Raman spectra of MCAD and IVD excited at 632.8 nm.** The spectra were observed in 50 mM potassium phosphate buffer containing 0.3 mM EDTA, pH 7.6. (a) MCAD (1.8 mM); (b) difference spectrum of MCAD (1.7 mM) minus apoMCAD (*ca.* 1.7 mM); (c) difference spectrum of MCAD (0.9 mM) reconstituted with [4,10*a*-$^{13}C_2$]FAD minus apoMCAD (*ca.* 0.9 mM); (d) the complex of MCAD (1.6 mM) with acetoacetyl-CoA (1.7 mM); (e) the complex of MCAD (2.0 mM) with 3-ketohexanoyl-CoA (2.3 mM); (f) the complex of IVD (0.45 mM) with acetoacetyl-CoA (1.6 mM).

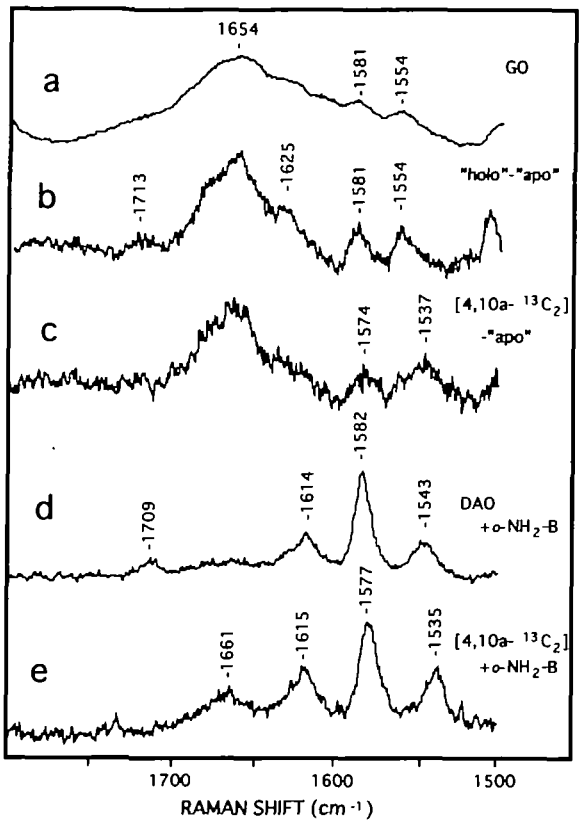

Fig. 5. **Raman spectra of GO and DAO excited at 632.8 nm.** The spectra were observed in 50 mM potassium phosphate buffer, pH 6.0 (a-c) and 50 mM Tris-HCl buffer, pH 7.5 (d, e). (a) GO (2 mM); (b) difference spectrum of GO (2.0 mM) minus ADP-bound apoGO (*ca.* 2 mM); (c) difference spectrum of GO (1.9 mM) reconstituted with [4,10*a*-$^{13}C_2$]FAD minus apoGO (*ca.* 2.0 mM); (d) the complex of DAO (630 μM) with *o*-aminobenzoate (2.0 mM); (e) the complex between DAO (560 μM) reconstituted with [4,10*a*-$^{13}C_2$]FAD and *o*-aminobenzoate (2.0 mM).

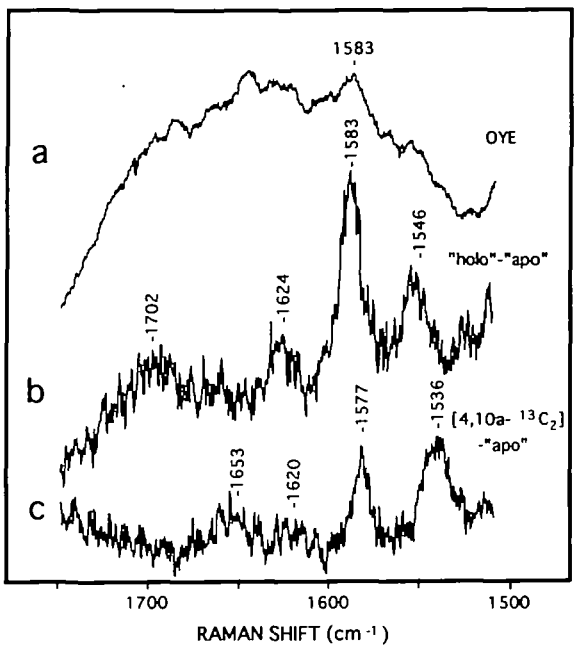

Fig. 6. **Raman spectra of OYE excited at 632.8 nm.** The spectra were observed in 50 mM potassium phosphate buffer, pH 8.0. (a) OYE (2.2 mM); (b) difference spectrum of OYE (2.2 mM) minus apoOYE (*ca.* 2 mM); (c) difference spectrum of OYE (2.0 mM) reconstituted with [4,10*a*-$^{13}C_2$]FAD minus apoOYE (*ca.* 2 mM).

Thus, the 1,694-cm^{-1} band is assigned to the C(4)=O stretching vibration. In the complexes of MCAD with acetoacetyl-CoA and 3-ketohexanoyl-CoA, both the corresponding bands appeared at 1,687 cm^{-1} (Fig. 4-d, e); the band is also lower by 7 cm^{-1}, than that for free enzyme. The band derived from the C(4)=O moiety was observed at 1,693 cm^{-1} in the IVD-acetoacetyl-CoA complex (Fig. 4-f).

Figure 5 shows the Raman spectra of GO and the complex of DAO with *o*-aminobenzoate. It has been shown that the apoenzyme of GO exists in two forms of a loose flexible coil structure, and binding of ADP or FAD alters the conformation of the protein and stabilizes its tertiary structure (*32*). Therefore, we used the Raman spectrum of the apo-GO complexed with ADP to obtain the difference spectra. The broad band remained in the difference spectra (Fig. 5-b, c); the conformation of the apoenzyme complexed with ADP may be different from that of the holoenzyme. However, a band appeared at 1,713 cm^{-1}, and the band disappeared with [4,10*a*-$^{13}C_2$]FAD reconstituted (Fig. 5-b, c). The band is probably concealed behind the broad band around 1,650 cm^{-1}; the band is expected to shift to 1,662 cm^{-1} if the magnitude of the shift is the same as the shift (51 cm^{-1}) for the band of FAD in H_2O (Fig. 2). Thus, the 1,713-cm^{-1} band is assigned to the C(4)=O stretching vibration. The 1,709-cm^{-1} band of the complex of DAO with *o*-aminobenzoate shifted to 1,661 cm^{-1} upon [4,10*a*-$^{13}C_2$]FAD reconstitution, thus the band is assigned to C(4)=O stretching (Fig. 5-d, e).

Figure 6 shows the Raman spectra of OYE. The 1,702-cm^{-1} band shifts to 1,653 cm^{-1} upon [4,10*a*-$^{13}C_2$]-FAD reconstitution, indicating that it is derived from C(4)=O stretching.

Table I summarizes the band frequency of C(4)=O stretching vibrational mode for flavins and flavoproteins examined to date; the variation of the frequency value of C(4)=O stretching mode among flavoproteins is greater than 30 cm^{-1}, and the variation reflects the difference of the environment around the isoalloxazine ring. The C(4)=O bands of FMN and TARF were also confirmed by using [4,10*a*-$^{13}C_2$]-labeled compounds (not shown).

DISCUSSION

The band frequency of C=O stretching vibration decreases on formation of hydrogen bonding (*7-9*). Kim and Carey suggested that the observed high frequency for C(4)=O stretching vibration of riboflavin bound to RBP is due to a reduction in hydrogen-bonding strength at the carbonyl oxygen, compared to riboflavin free in solution (*18*). NMR studies are very useful for investigation of intermolecular interactions. That the carbonyl 4-^{13}C resonance of flavin is sensitive to hydrogen bonding at oxygen was initially indicated by Moonen *et al.* (*49*). However, since chemical shift is also sensitive to other factors, *e.g.*, ring current, changes in chemical shift cannot be attributed unequivocally to hydrogen-bonding modulation. It is very valuable to compare the chemical shift with a different parameter (in

TABLE I. **C(4)=O Raman frequencies and $^{13}C(4)$-chemical shifts of flavins free in solution and bound to flavoproteins.**

	Raman frequency for C(4)=O	Chemical shift for ^{13}C
FAD	1,711	164.8[m]
FMN (10 mM)	1,713	164.2[n]
TARF[a] in $CDCl_3$	1,716	161.4[n]
TARF in $CDCl_3$ (10% methanol)	1,713	163.0[n]
SCAD[b]	1,700	167.0
SCAD+AcAc-CoA[c]	1,693	167.7
MCAD[d]	1,694	166.8[o]
MCAD+AcAc-CoA[e]	1,687	167.0[o]
MCAD+3-keto-C16[f]	1,687	
IVD+AcAc-CoA[g]	1,693	
GO[h]	1,713	164.1[o]
DAO+o-NH_2-B[i]	1,709	163.5[m]
OYE[j]	1,702	165.8[o]
RBP[k]	1,723[l]	163.6[o]

[a]Riboflavin tetraacetate. [b]Short-chain acyl-CoA dehydrogenase. [c]Complex of SCAD with acetoacetyl-CoA. [d]Medium-chain acyl-CoA dehydrogenase. [e]Complex of MCAD with acetoacetyl-CoA. [f]Complex of MCAD with 3-ketohexanoyl-CoA. [g]Complex of isovaleryl-CoA dehydrogenase with acetoacetyl-CoA. [h]Glucose oxidase. [i]Complex of D-amino acid oxidase with o-aminobenzoate. [j]Old yellow enzyme. [k]Riboflavin binding protein. [l]Raman datum from Kim and Carey (*18*). [m]NMR data from Miura and Miyake (*58*). [n]NMR data from Moonen *et al.* (*49*). [o]NMR data summarized in Miura *et al.* (*19*).

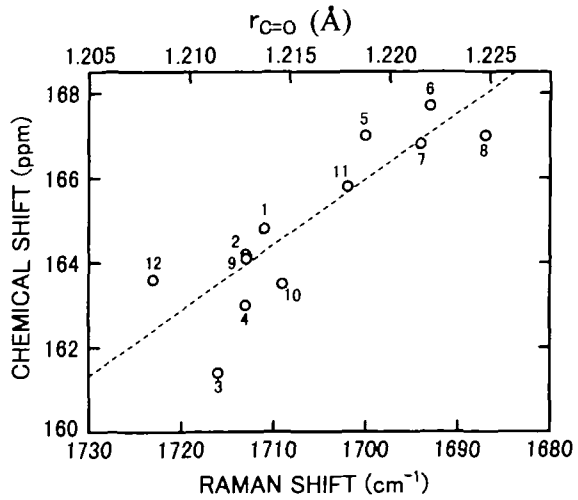

Fig. 7. **Correlation between Raman shift for the C(4)=O stretching vibration and the chemical shift of $^{13}C(4)$.** The data in Table I were used. Points: (1) FAD; (2) FMN (10 mM); (3) TARF in $CDCl_3$; (4) TARF in $CDCl_3$ containing 10% methanol; (5) SCAD; (6) the complex of SCAD with acetoacetyl-CoA; (7) MCAD; (8) the complex of MCAD with acetoacetyl-CoA; (9) glucose oxidase; (10) the complex of D-amino acid oxidase with o-aminobenzoate; (11) old yellow enzyme; (12) riboflavin binding protein. The upper abscissa scale, $r_{C=O}$, shows the carbonyl bond length calculated from the frequency of C=O stretching. The line shown is a linear regression fit to all the data points with the correlation coefficient of 0.864. The line is expressed as (Chemical shift) (ppm) $= -0.155$ (Raman shift) (cm^{-1}) $+ 429.48$.

this case, Raman frequency) affected by hydrogen bonding. Therefore, we examined the correlation between the Raman frequency and the chemical shift. Table I summarizes the Raman data and NMR data of flavins and flavoproteins. As shown in Fig. 7, the Raman frequency for the C(4)=O stretching vibration correlates well with the chemical shift of $^{13}C(4)$, indicating that a decrease of vibrational frequency by 10 cm^{-1} is associated with an increase of chemical shift by 1.55 ppm. This high correlation strongly suggests that the data from both spectroscopies reflect a common perturbation, *i.e.*, hydrogen bonding. Linear correlations also obtain between the frequency of the C=O band and ΔH (the enthalpy of hydrogen bonding), the details of which will be described later, and between the chemical shift and π-electron density (*50*). Therefore, π-electron density at C(4) may change linearly with the strength of hydrogen bonding at C(4)=O.

An empirical linear relationship between the frequency of C=O stretching and the bond length of the C=O has been derived from X-ray crystallographic and IR spectroscopic measurements on the same crystals of a large series of compounds (*6*). The relationship is expressed as ($r_{C=O}$) (Å) $= 2.00988 - 0.0004653$ ($\nu_{C=O}$)(cm^{-1}), where $r_{C=O}$ and $\nu_{C=O}$ are the bond length and the vibrational frequency of the C=O (*6*), respectively. Using this empirical relationship, we estimated the bond length to be in the range of 1.208-1.225 Å, as shown in Fig. 7. The bond lengths of C(4)=O of lumiflavin in two forms of lumiflavin-hydroquinone-hydrobromide crystal have been determined to be 1.211 and 1.216 Å (*51*). Thus, the relation between bond length and vibrational frequency in the flavin molecule is probably not far removed from the empirical relationship. The 36-cm^{-1} difference in vibrational frequency between RBP and MCAD-acetoacetyl-CoA complex is the maximum difference in vibrational frequency observed in Fig. 7; this deviation is equivalent to a change in the bond length of *ca.* 0.017 Å. An extension in length of 0.017 Å represents about 8% of the bond length change expected on conversion from a C=O double bond to a C-O single bond; the typical bond lengths of C=O and C-O are 1.22 Å (*6*) and 1.44 Å (*52*), respectively. This is an important quantitation since it provides data with which to investigate quantitatively the modulation of flavin reactivity induced by hydrogen bonding.

Linear correlations between the frequency of carbonyl-stretching vibration and ΔH upon formation of a hydrogen bond between a carbonyl group of a ketone or an ester and a hydrogen donor (phenol, ethanol, *etc.*) in CCl_4 have been shown (*7*). The changes in ΔH per frequency shift of 1 cm^{-1} are as follows: acetone (1.96 kJ/mol), acetophenone (1.28 kJ/mol), and benzophenone (1.49 kJ/mol) (*7*). The value differs depending on the chemical nature of the ketone or ester; this may depend on the resonance interaction by substituent groups bonded to C=O bond (*7*). It is difficult to measure ΔH upon formation of a hydrogen bond at C(4)=O oxygen of the isoalloxazine ring in non-hydrogen-bonding solvents, because the flavin ring has another carbonyl group, C(2)=O. Thus, we roughly estimated the difference of the enthalpy change induced by hydrogen bonding in RBP and MCAD-acetoacetyl-CoA complex ($\Delta\Delta H$). The value of $\Delta\Delta H$ corresponds to the difference in the Raman shift (36 cm^{-1}) in Fig. 7. The difference in the frequency of 36 cm^{-1} in flavin C(4)=O stretch (Fig. 7) is equivalent to $\Delta\Delta H = 71$ kJ/mol (from the case of acetone, *i.e.*, 1.96×36) and $\Delta\Delta H = 46$ kJ/mol (from the case of acetophenone, *i.e.*, 1.28×36). The value of 71 or 46 kJ/mol is the maximum value of $\Delta\Delta H$ in Fig. 7. Whichever the case may be, the strength of hydrogen bonding varies from one flavoprotein to another.

X-Ray crystallographic structures of various flavoproteins have now been determined and the details of active sites have been clarified. The presence of hydrogen bonding at the C(4)=O moiety of the isoalloxazine nucleus has been examined and its strength has been qualitatively discussed, although a quantitative discussion would be difficult. Thus, it is valuable to compare the present Raman data with the X-ray crystallographic data. The acyl-CoA dehydrogenases show lower frequencies than the other flavoproteins studied here (Table I), which indicates stronger hydrogen bonding in the dehydrogenases. The structure of medium-chain acyl-CoA dehydrogenase has been determined at 2.4 Å resolution by X-ray crystallography, indicating the existence of hydrogen bonding between the C(4)=O oxygen of flavin ring and the amide hydrogen of Thr-168 (*53*). The existence of a strong hydrogen bond between C(4)=O and apoMCAD was also suggested by ^{13}C NMR (*19*). Acyl-CoA dehydrogenases constitute a superfamily (*54*). The present results suggest that the strong hydrogen bonding is conserved within the superfamily; the strong hydrogen bonding is important for the enzyme reaction. In the case of OYE, which is expected to have a relatively strong hydrogen bond from the Raman data, the C(4)=O moiety of FMN is hydrogen bonded to Thr-37 (2.8 Å) and Gly-72 (3.3 Å) (*55*). The three-dimensional structure of DAO-*o*-aminobenzoate complex showed that the hydrogen-bonding interaction at C(2)=O is strong, while that at C(4)=O is weak (*56*). The present Raman results also show that the hydrogen bonding at C(4)=O is weak.

The hydrogen bonding at the C(4)=O in MCAD and SCAD becomes stronger upon complexation with substrate analogs; the band frequencies of the C(4)=O are lower in the complexes (Table I). The hydrogen bonding at C(4)=O lowers the energy of LUMO and increases the atomic orbital coefficient at the N(5) in LUMO of oxidized flavin (*4, 5*). The reductive half-reaction of these dehydrogenases is initiated by removal of the pro-*R* α-hydrogen as a proton by an active site base and the β-hydride transfer to the N(5) locus of the isoalloxazine ring in a concerted manner (*57*). Therefore, the enhancement of the hydrogen bonding at C(4)=O is advantageous for the reaction; substrate-binding itself probably raises the reactivity of flavin, through enhancing the hydrogen bonding. The present Raman study thus provides a molecular insight into the modulation of the reactivity of the flavin molecule by the binding of ligands. In contrast, the hydrogen bonding is extremely weak in RBP; which is consistent with the fact that RBP has no enzymatic activity.

X-Ray crystallographic, NMR, and Raman studies are valuable in investigating the hydrogen bonding at C(4)=O of the isoalloxazine ring. The predicted strength of hydrogen bonding is qualitatively consistent among these three methods. The three methods provide complementary information: X-ray crystallographic analysis shows what the hydrogen bond donor is, NMR gives information on the electron density at C(4), and Raman spectroscopy gives quantitative information about the hydrogen bonding in relation to the bond order. Thus, many more flavoproteins should be investigated by these methods in order to clarify the details of the environment of the flavin ring.

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