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Resonance Raman Study on Reduced Flavin in Purple Intermediate of Flavoenzyme: Use of [4-Carbonyl-^{18}O]-Enriched Flavin

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[4-Carbonyl-^{18}O]-enriched lumiflavin, riboflavin, and FMN were prepared by incubating each corresponding non-labeled flavin in 1 M Na^{18}OH ($H_2{}^{18}O$) at 25°C. [4-Carbonyl-^{18}O]FAD was prepared from the corresponding riboflavin by using FAD synthetase. Isotope effects by [4-carbonyl-^{18}O]-labeling confirmed that the 1,709-cm^{-1} band in the IR spectrum of lumiflavin and the 1,711-cm^{-1} band in the Raman spectrum of FAD are mainly derived from C(4)=O stretching vibrational mode. The 1,605-cm^{-1} Raman band of the anionic reduced flavin in the purple intermediate of D-amino acid oxidase (DAO) with D-proline or D-alanine does not shift in DAO reconstituted with [4-carbonyl-^{18}O]FAD, although it shifts with [4, 10*a*-$^{13}C_2$]- or [4*a*-^{13}C]FAD. Thus the band is mainly due to the C(4*a*)=C(10*a*) stretching vibrational mode and includes no contribution from C(4)=O stretching vibration. The band frequencies cover a fairly wide range (1,602-1,620 cm^{-1}) depending on the enzymes. The frequencies of the reduced flavin in the purple intermediates of the dehydrogenases (medium-chain acyl-CoA, short-chain acyl-CoA, and isovaleryl-CoA dehydrogenases) are higher than those of the oxidases (DAO and L-phenylalanine oxidase). This indicates that the C(4*a*)=C(10*a*) bond order of reduced flavin in the dehydrogenases with the low reactivity for molecular oxygen is stronger than that in the oxidases with high reactivity. Therefore, the band frequency of C(4*a*)=C(10*a*) stretching may serve as an indicator of the reactivity of flavoprotein with molecular oxygen. Furthermore, strong hydrogen bonding of flavin at the N(1) moiety with the hydroxyl group of Thr136 in MCAD is probably responsible for the strong bond of the C(4*a*)=C(10*a*) of reduced flavin in the dehydrogenase.

Key words: acyl-CoA dehydrogenase, D-amino acid oxidase, flavoenzyme, hydrogen bond, Raman spectra.

Flavin can exist in three redox states, *i.e.*, oxidized, semiquinone (one-electron reduced), and reduced (two-electron reduced) states (Fig. 1, Ref. *1*). Owing to this nature, flavin has unique properties, being able to function in either a one- or two-electron transfer process: other redox-related compounds, metaloproteins and nicotinamides, function only in a one-electron and two-electron transfer process, respectively.

Reduced flavin free in solution is efficiently reoxidized by molecular oxygen. This property is generally retained in flavoprotein oxidases, but the reoxidation of flavoprotein dehydrogenases by molecular oxygen is significantly suppressed (*2*). This difference in the reactivity with molecular oxygen is one of the bases for versatile functions of flavin. However, the mechanism underlying the difference between oxidase-type and dehydrogenase-type flavoproteins is not clear. Information about the difference in the structure of reduced flavin between the two types of flavoproteins is critical for understanding this mechanism. The reaction center of reduced flavin with molecular oxygen is at or near C(4*a*) (*2*) (Fig. 1), and π-electron transfer from reduced flavin should be an important process in reoxidation by molecular oxygen. Therefore, it is important to study the structural details of the C(4*a*)=C(10*a*) double bond of the reduced flavin in both types of flavoproteins by using Raman spectroscopy.

Raman spectroscopy has the potential to provide structural information on molecules, and during the past two decades this method has been extensively adopted in investigations of flavins and flavoproteins. Among the three redox states (Fig. 1), the oxidized state has been most frequently investigated, and many Raman bands have been observed. The observed bands have been assigned experimentally, *i.e.*, on the basis of the band shifts with chemically (*3-8*) and isotopically (*9-11*) substituted flavins, and also by normal mode analysis (*12-14*). Numerous Raman studies on flavosemiquinones have also been reported (*15-24*). Although normal mode analysis has not yet been done, isotope effects on the band around 1,615 cm^{-1} of neutral semiquinone (*25*) and on many bands of anionic semiquinone (*21*) have been reported, and qualitative assignments of the Raman bands have been made on the basis of isotope effects (*21*) and by comparison of resonance Raman (RR) spectra of semiquinone in different three ionic forms

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Abbreviations: DAO, D-amino acid oxidase; LUMO, lowest unoccupied molecular orbital; MCAD, medium-chain acyl-CoA dehydrogenase; RR spectrum, resonance Raman spectrum.

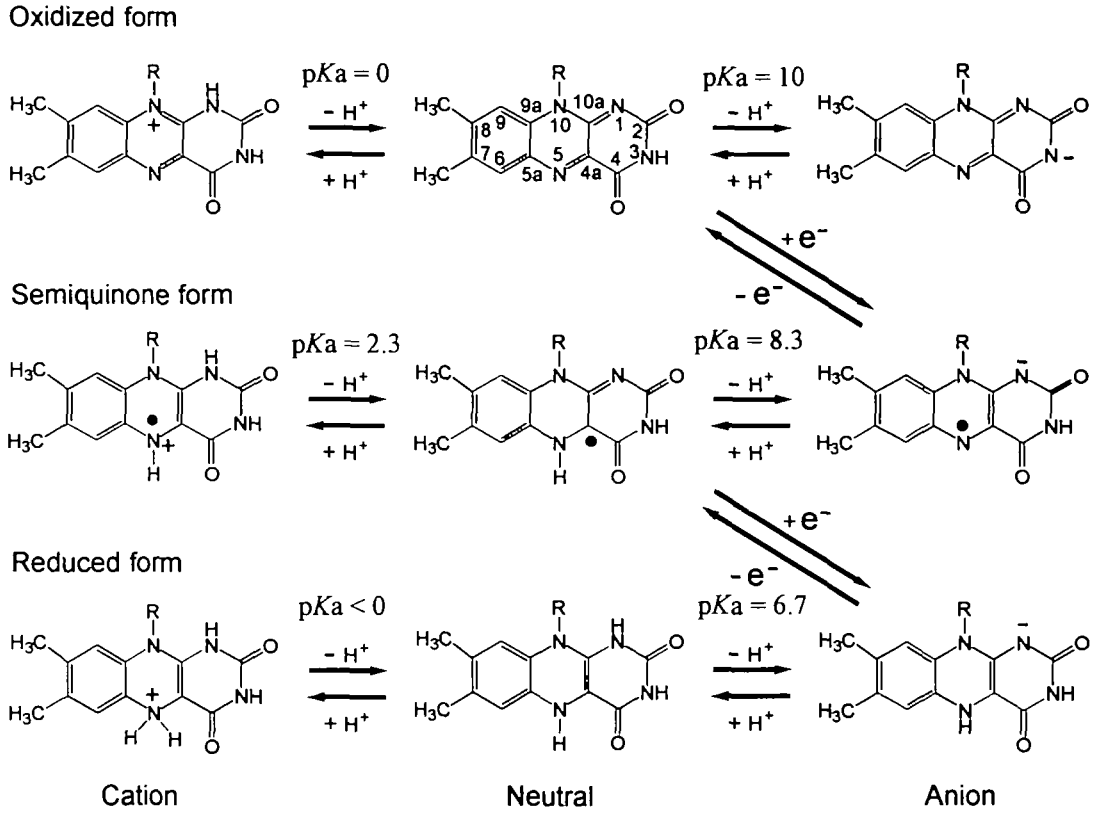

Fig. 1. **The structures of the cationic, neutral, and anionic flavin species in the three redox states.** Modified from Müller (*1*).

(Fig. 1: anionic, neutral, and cationic forms) (*23*). Recently, C=O stretching bands of anionic semiquinone were observed and assigned (*24*). In contrast, no Raman spectrum of reduced flavin is available, except for those of reduced flavins in the purple intermediates of flavoproteins, where purple intermediates are charge-transfer complexes between the product from each substrate and the reduced flavin in the flavoproteins such as D-amino acid oxidase (DAO) (*25-28*), L-phenylalanine oxidase (*29*), medium-chain acyl-CoA dehydrogenase (MCAD) (*30, 31*), short-chain acyl-CoA dehydrogenase (*31*), and isovaleryl-CoA dehydrogenase (*31*). The Raman bands derived from the reduced flavin of the purple intermediates were observed around 1,610 cm^{-1} in all the purple intermediates: the frequencies cover a fairly wide range (1,602-1,620 cm^{-1}) depending on the enzymes. The 1,605-cm^{-1} band in the purple intermediate of DAO with D-proline shifted to 1,585 and 1,601 cm^{-1} for DAO reconstituted with [4,10a-$^{13}C_2$]-FAD and [4a-^{13}C]FAD (*27*), respectively. But the band scarcely shifted with [2-^{13}C]-, [5-^{15}N]-, or [1,3-$^{15}N_2$]FAD. Based on these isotope effects, we concluded that the bands around 1,610 cm^{-1} are associated with the C(10a)=C(4a)-C(4)=O region of reduced flavin in the purple intermediates (*27*). Although the bands must be a vibrational mode containing C(4a), it is not known how much C(4a)=C(10a) or C(4)=O stretching vibration contribute to the bands.

In this paper, we assigned the band around 1,610 cm^{-1} to a C(4a)=C(10a) stretching vibrational mode by measuring the RR spectra of purple intermediate of DAO reconstituted with [4-carbonyl-^{18}O]FAD or a 1:1 mixture of [4-^{13}C]- and [10a-^{13}C]FAD. Furthermore, we discuss the correlation between the oxygen reactivity of the reduced flavin in the flavoproteins and the bond structure of C(4a)=C(10a), aiming at a first step to resolving the different mechanisms involved in the reactions of oxidase- and dehydrogenase-type flavoproteins with oxygen.

MATERIALS AND METHODS

The holoenzyme and apoenzyme of porcine kidney DAO were purified as described by Shiga *et al.* (*32, 33*) and Tojo *et al.* (*34*), respectively. The reconstitution of isotope-enriched FAD to the apoenzyme was carried out following the procedure previously reported (*27*). The concentration of the reconstituted or the native enzyme was based on the molar absorption coefficient of 11,300 $M^{-1}\cdot cm^{-1}$ at 455 nm. The purple intermediate of DAO with D-proline was prepared by mixing DAO with the substrate (*26*). The purple intermediate with D-alanine was obtained by addition of pyruvate, ammonium sulfate, and D-alanine to the enzyme solution (*26*).

Potassium cyanide-^{13}C (99 atom%) and $H_2{}^{18}O$ (99.1 atom%) were purchased from Isotec, USA. Other chemicals were of the highest grade available from commercial sources and used without further purification. One molar $Na^{18}OH$ in $H_2{}^{18}O$ was prepared by the reaction of metallic sodium with $H_2{}^{18}O$.

A 1:1 mixture of [4-^{13}C]- and [10a-^{13}C]riboflavin was synthesized by the method of Tishler *et al.* (*35*). Due to the structural symmetry of the starting materials, separate synthesis of [4-^{13}C]- or [10a-^{13}C]riboflavin is impracticable. [1-^{13}C]Malonic acid was prepared from potassium

[^{13}C]cyanide following the procedure described elsewhere (*36*). Diethyl [1-^{13}C]malonate was synthesized by refluxing for 15 h a dichloroethane solution containing [1-^{13}C]-malonic acid, ethanol, and small amount of H_2SO_4. [4-^{13}C]-Barbituric acid was prepared from diethyl [1-^{13}C]malonate and urea as described elsewhere (*37*).

Isotope-labeled FADs and lumiflavins were prepared from the corresponding riboflavins as described previously (*27, 38*).

Visible absorption spectra were measured with Hitachi 220A and U-2000 spectrophotometers at 25°C. Raman spectra were obtained with a JASCO NR-1800 spectrometer (Japan Spectroscopic) 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 for indene. The RR spectra were measured in 50 mM sodium pyrophosphate buffer (pH 8.3). FT-IR spectra were measured in KBr tablets with a JEOL 6500W FT-IR spectrometer. EI and FAB mass spectra were measured on JEOL JMS-BU20 and JMS-DX303HF mass spectrometers, respectively.

RESULTS

Synthesis of [4-Carbonyl-^{18}O]-Enriched Flavin—[4-Carbonyl-^{18}O]-enriched lumiflavin was prepared by dissolving lumiflavin in 1 M Na^{18}OH in $H_2{}^{18}O$. Approximately 1 mg of lumiflavin was dissolved in 50 μl 1 M Na^{18}OH ($H_2{}^{18}O$) in a small tube with a cap and was allowed to stand at 25°C. Aliquots of 5 μl were removed at various times, and 0.7 μl of acetic acid was added to each aliquot to prevent further reaction. Each sample was extracted with CH_2Cl_2, the solution was dried with Na_2SO_4, and CH_2Cl_2 was removed with a stream of N_2 gas. The resulting ^{18}O-enriched lumiflavin was analyzed by mass spectroscopy. The mass spectrum of non-labeled lumiflavin has a molecular ion at m/z 256; the molecular weight of lumiflavin is 256.26. Depending on the incubation time, the ion at m/z 256 decreased and the ion at m/z 258 increased. But the molecular ion at m/z 260 was not significant, even after incubation for 5 d. This indicates that ^{18}O is exchanged at only one of the two oxygen atoms in lumiflavin. As described later in detail, the oxygen atom exchanged with ^{18}O under the conditions employed is the C(4)=O carbonyl oxygen.

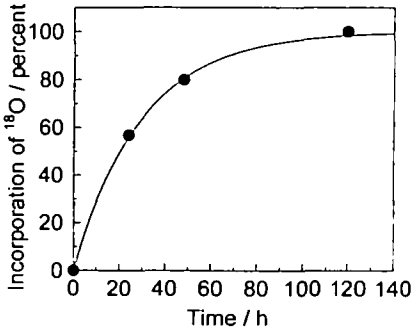

Fig. 2. **Time course of ^{18}O incorporation of lumiflavin in 1 M Na^{18}OH ($H_2{}^{18}O$) at 25°C.** The proportion of ^{18}O incorporation is plotted against incubation time. The curve shows the simulated time course for pseudo-first order kinetics with a reaction rate $k = 4.1 \times 10^{-2}$ h^{-1}.

Figure 2 shows the time dependence of the incorporation of ^{18}O. The ratio of [4-carbonyl-^{18}O]-labeled lumiflavin was obtained by $A_{258}/(A_{256}+A_{258})$, where A_{256} and A_{258} are the abundances of molecular ion at m/z 256 and 258, respectively. Approximately 17 h is needed for 50% incorporation.

Similar incorporation also occurs in the cases of both riboflavin and FMN, the incorporation being confirmed by mass spectroscopy. As FAD is easily hydrolyzed to FMN under the conditions, [4-carbonyl-^{18}O]FAD cannot be prepared directly from non-labeled FAD by the same procedure. Thus, the FAD was prepared from [4-carbonyl-^{18}O]riboflavin with FAD synthetase complex by the method previously reported (*27*).

FT-IR Spectra of Non-Labeled and Isotope-Labeled Lumiflavin—Infrared spectra of non-labeled and some isotope-labeled lumiflavins were previously observed (*39*), and the observed bands were assigned by normal mode analysis (*13*). Figure 3 shows the FT-IR spectrum of [^{18}O]-labeled lumiflavin prepared by incubation for 120 h as described in the foregoing section, together with those of non-labeled lumiflavin, [4,10a-$^{13}C_2$]lumiflavin, and the 1:1 mixture of [4-^{13}C]- and [10a-^{13}C]lumiflavin for compari-

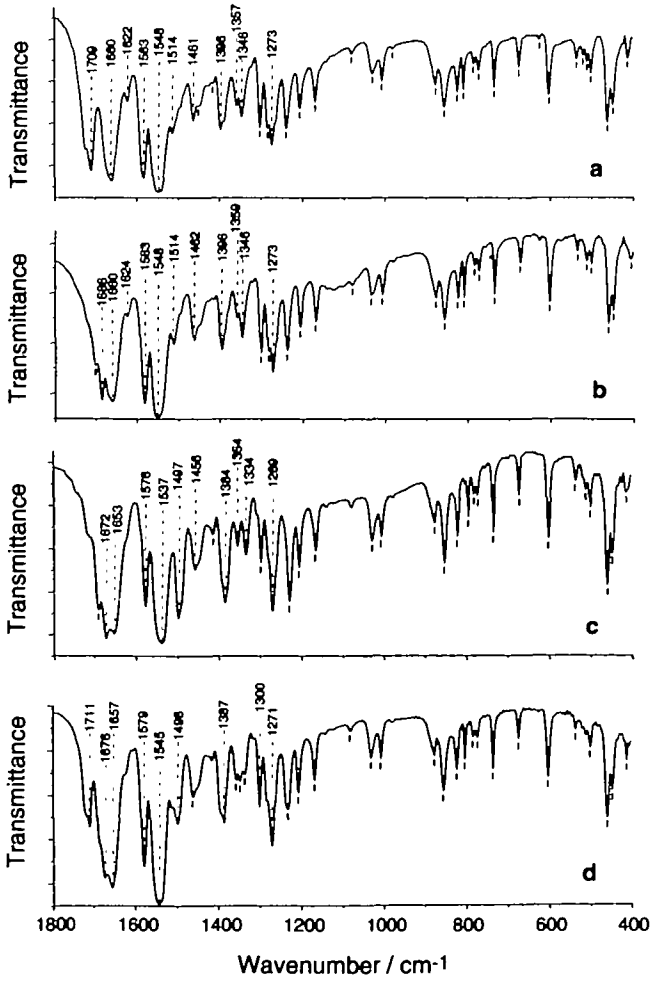

Fig. 3. **FT-IR spectra of (a) non-labeled lumiflavin, (b) [4-carbonyl-^{18}O]lumiflavin, (c) [4,10a-$^{13}C_2$]lumiflavin, (d) a 1:1 mixture of [4-^{13}C]- and [10a-^{13}C]lumiflavin.** The spectra were measured in KBr tablets.

son. These spectral patterns are identical with the previous results (*39*). It was pointed out that the doublet in the highest band may arise from Fermi resonance of the C(4)=O stretching vibration including the N(3)H deformation mode (*39*). Upon [^{18}O]-labeling, the 1,709-cm^{-1} band, which is the C(4)=O stretching vibration slightly coupled with C(2)=O stretching mode (*13*), downshifted by 23 cm^{-1}, while the 1,660-cm^{-1} band, which is mainly due to the C(2)=O stretching mode coupled with the N(3)-H bending and C(4)=O stretching mode (*13*), did not shift (Table I). This indicates that ^{18}O is located solely at C(4)=O carbonyl oxygen. The 1,709-cm^{-1} band was shifted to 1,672 cm^{-1} upon [4,10*a*-$^{13}C_2$]-labeling. In the FT-IR spectrum (Fig. 3) of the 1:1 mixture of [4-^{13}C]- and [10*a*-^{13}C]lumiflavin, the 1,709-cm^{-1} band splits into two bands: one remains at almost the same position and the other appears at 1,676 cm^{-1}. As the original 1,709-cm^{-1} band is from the C(4)=O stretching vibration mentioned above, the 1,672-cm^{-1} band is derived from [4-^{13}C]lumiflavin, and the remaining band at 1,711 cm^{-1} is derived from [10*a*-^{13}C]lumiflavin. The 1,660-cm^{-1} band downshifted by only 7 and 3 cm^{-1} in [4,10*a*-$^{13}C_2$]lumiflavin and the 1:1 mixture of [4-^{13}C]- and [10*a*-^{13}C]lumiflavin, respectively.

Raman Spectra of Non-Labeled and Isotope-Labeled FADs Excited at 632.8 nm—Previously we reported Raman spectra of non-labeled and some isotopically labeled FADs ([4,10*a*-$^{13}C_2$], [4*a*-^{13}C], [2-^{13}C], [5-^{15}N], and [1,3-$^{15}N_2$]) (*11*). Here, we measured Raman spectra of [4-carbonyl-^{18}O]FAD and the 1:1 mixture of [4-^{13}C]FAD and [10*a*-^{13}C]FAD in H_2O. The band positions of the spectra (Fig. 4, a, b, and c) in the 1,650-1,750 cm^{-1} region were determined from the difference spectrum (not shown) of non-labeled FAD minus [4-carbonyl-^{18}O]FAD or the 1:1 mixture of [4-^{13}C]- and [10*a*-^{13}C]FAD. The 1,711-cm^{-1} band, which is assigned to C(4)=O stretching vibrational mode of the isoalloxazine nucleus, is downshifted to 1,678 cm^{-1} with [4-carbonyl-^{18}O]FAD (Fig. 4 and Table I). This confirms that the FAD prepared is indeed labeled with ^{18}O: 4-carbonyl-^{18}O is maintained even after the preparation procedure for the labeled FAD from [4-carbonyl-^{18}O]-riboflavin. The large shift (33 cm^{-1}) supports that the band is mainly associated with the C(4)=O stretching vibrational mode (*11, 40*). No other bands appreciably shifted with [4-carbonyl-^{18}O]FAD. The 1,711-cm^{-1} band is downshifted to 1,660 cm^{-1} with [4,10*a*-$^{13}C_2$]FAD (*11*). As the FAD is labeled by ^{13}C at the two positions, it is difficult to distinguish the individual effect of single isotope labeling on the basis of the shift. In the Raman spectrum (Fig. 4) of the 1:1 mixture of [4-^{13}C]FAD and [10*a*-^{13}C]FAD, the 1,711-cm^{-1} band splits into two bands: one remains at the same position and the other appears at 1,668 cm^{-1}. This indicates that the shift of 1,711-cm^{-1} band with [4,10*a*-$^{13}C_2$]FAD is mainly due to [4-^{13}C]-labeling, and [10*a*-^{13}C]-labeling has practically no effect. This is consistent

TABLE I. **Isotopic frequency shifts of 1,710- and 1,660-cm^{-1} bands of FT-IR spectra of lumiflavin and 1,710-cm^{-1} band of Raman spectra of FAD.** In parentheses are the values of isotopic shifts (cm^{-1}).

	Non-labeled	^{13}C				^{15}N			^{18}O
		[4*a*-^{13}C]	[4,10*a*-$^{13}C_2$]	[2-^{13}C]	1:1 mixture of [4-^{13}C] and [10*a*-^{13}C]	[5-^{15}N]	[1,3-$^{15}N_2$]	[1,3,5-$^{15}N_3$]	[4-carbonyl-^{18}O]
Lumiflavin	1,708[a], 1,709	1,708[a](−1)	1,672(−37)	1,706[a](−3)	1,711(2) 1,676(−33)	1,708[a](−1)	1,707[a](−2)	1,707[a](−2)	1,686(−23)
	1,662[a], 1,660	1,664[a](4)	1,653(−7)	1,630[a](−30)	1,657(−3)	1,661[a](1)	1,656[a](−4)	1,651[a](−9)	1,660(0)
FAD	1,711[b]	1,710[b](−1)	1,660[b](−51)	1,708[b](−3)	1,711(0) 1,668(−43)	1,711[b](0)	1,708[b](−3)		1,678(−33)

[a]IR data from Abe and Kyogoku (*13*). [b]Raman data from Hazekawa *et al.* (*11*).

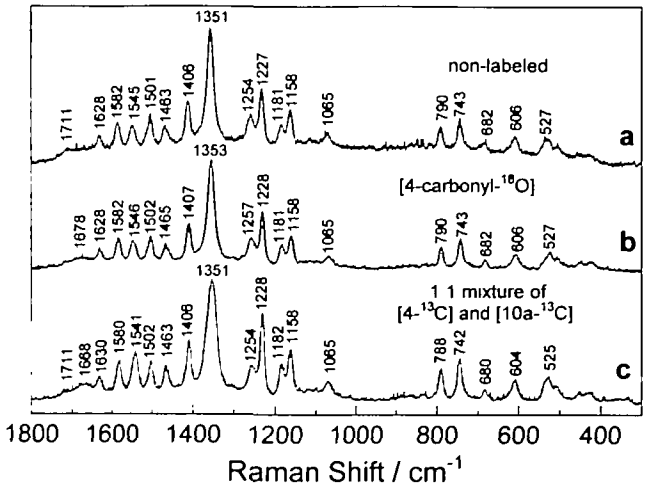

Fig. 4. **Raman spectra of (a) non-labeled FAD, (b) [4-carbonyl-^{18}O]FAD, (c) a 1:1 mixture of [4-^{13}C]- and [10*a*-^{13}C]FAD.** The spectra were observed in H_2O. The concentrations were: (a) 26 mM; (b) 30 mM; (c) 30 mM.

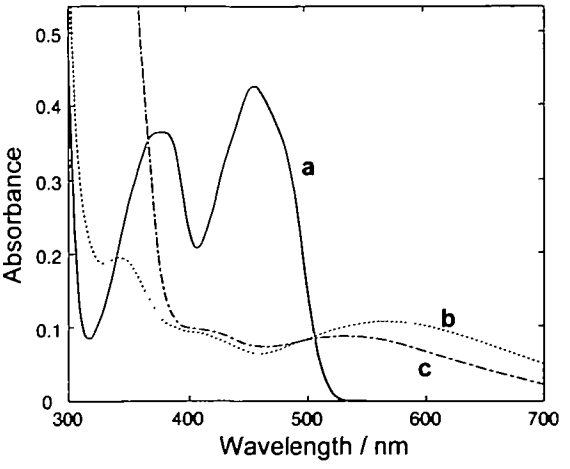

Fig. 5. **Absorption spectra of D-amino acid oxidase (DAO) and the purple intermediate of DAO with D-proline or D-alanine as a substrate.** The concentrations were: (a) DAO (55 μM); (b) DAO (55 μM), D-proline (91 mM); (c) DAO (55 μM), ammonium sulfate (0.16 M), sodium pyruvate (54.5 mM), D-alanine (91 mM).

TABLE II. **Isotopic frequency shifts of 1,606-cm^{-1} band of DAO purple intermediates.** In parentheses are the values of isotopic shifts (cm^{-1}).

Substrate	Non-labeled[a]	^{13}C				^{15}N		^{18}O
		[4a-^{13}C][a]	[4,10a-$^{13}C_2$][a]	[2-^{13}C][a]	1:1 mixture of [4a-^{13}C] and [10a-^{13}C]	[5-^{15}N][a]	[1,3-$^{15}N_2$][a]	[4-carbonyl-^{18}O]
D-Proline	1,605	1,601 (−4)	1,585 (−20)	1,606 (1)	1,605 (0) 1,585 (−20)	1,606 (1)	1,606 (1)	1,605 (0)
D-Alanine	1,606	1,602 (−4)	1,591 (−15)	1,605 (−1)	1,606 (0) 1,590 (−16)	1,607 (1)	1,607 (1)	1,606 (0)

[a]Resonance Raman data from Miura *et al.* (27).

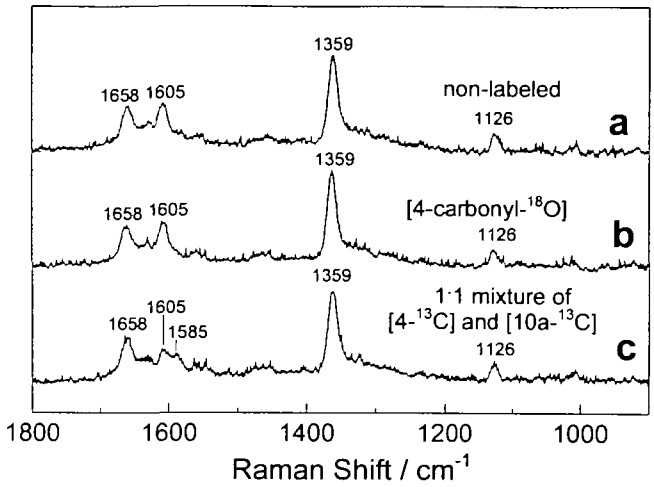

Fig. 6. **Resonance Raman spectra of the purple intermediate with D-proline and native D-amino acid oxidase (DAO) or reconstituted with FADs isotopically enriched in the flavin moiety.** Positions enriched in the flavin moiety are indicated in the figure. Concentrations were: (a) DAO (810 μM), D-proline (50 mM); (b) DAO (730 μM), D-proline (50 mM); (c) DAO (810 μM), D-proline (50 mM).

with the above conclusion from IR spectra.

Resonance Raman Spectra of DAO Purple Intermediate—Figure 5 shows visible absorption spectra of the purple intermediate produced in an anaerobic reaction of DAO with D-proline or D-alanine. Previously we showed by RR studies that the purple intermediate is a charge-transfer complex of reduced DAO with 2-imino acid derived from the substrate (*25*). This conclusion is based on the fact that the Raman bands of both the reduced flavin and the 2-imino acid are observed by excitation at 632.8 nm (*25-27*). Furthermore, the flavin is in the anionic reduced form as shown by NMR (*41*). The Raman band derived from the reduced flavin is observed at *ca.* 1,605 cm^{-1} and is downshifted by 15-20 cm^{-1} and 4 cm^{-1} upon [4,10a-$^{13}C_2$]- and [4a-^{13}C]-labeling, respectively (Table II). On the basis of the isotope effects, the band was tentatively assigned to the vibrational mode associated with the C(10a)=C(4a)-C(4)=O moiety of reduced flavin. However, exact assignment was not possible, because the individual effect of [4-^{13}C]- and [10a-^{13}C]-labeling was masked. That is, it was unknown how much C(4)=O stretching vibration contributes to the band. Therefore, we measured the RR spectra of the purple intermediate of DAO reconstituted with [4-carbonyl-^{18}O]-FAD or 1:1 mixture of [4-^{13}C]- and [10a-^{13}C]FAD to estimate the contribution of C(4)=O stretching to the 1,605-cm^{-1} band.

Figure 6 shows the RR spectra of the purple intermediates prepared with D-proline as a substrate and the DAO reconstituted with FAD, where designated positions of flavin are enriched. Similar results were obtained when D-alanine was used as a substrate (Table II). The 1,605-cm^{-1} band did not shift in the case of DAO reconstituted with [4-carbonyl-^{18}O]FAD in buffer solution in H_2O. These results indicate that the band is not sensitive to [4-carbonyl-^{18}O]-labeling, *i.e.*, this band includes no contribution from a C(4)=O stretching vibration. Furthermore, the band also did not shift or split in the case of non-labeled DAO in the buffer solution containing 50% $H_2{}^{18}O$ (not shown).

It is unlikely that 4-carbonyl oxygen of DAO-bound FAD is exchanged with ^{18}O of $H_2{}^{18}O$ since ^{18}O of 4-carbonyl oxygen of [4-carbonyl-^{18}O]isoalloxazine is retained during the synthetic procedure in buffer solution in $H_2{}^{16}O$. However, the possibility of the ^{18}O exchange between the bulk $H_2{}^{18}O$ and 4-carbonyl oxygen of DAO-bound FAD remains. In this case, the band should shift in 50% $H_2{}^{18}O$. The experimental result that the 1,605-cm^{-1} band did not shift in 50% $H_2{}^{18}O$ is consistent with the lack of C(4)=O contribution to the 1,605-cm^{-1} band.

The 1,605-cm^{-1} band splits into two bands at 1,605 and 1,585 cm^{-1} in the 1:1 mixture of [4-^{13}C]- and [10a-^{13}C]-FAD (Fig. 6 and Table II). This indicates that only one of the labels affects the band frequency. The band is downshifted by 4 cm^{-1} upon [4a-^{13}C]-labeling (*27*), but is not shifted upon [4-carbonyl-^{18}O]-labeling or [1,3-$^{15}N_2$]-labeling (Table II). Furthermore, the band frequency is too high to be associated with a single bond. Therefore, we conclude that the band is mainly due to the C(4a)=C(10a) stretching mode and that the 1,585-cm^{-1} band observed in the mixture is from the enzyme reconstituted with [10a-^{13}C]-FAD.

DISCUSSION

Resonance Raman Band and Oxygen Reactivity of Reduced Flavin in Purple Intermediates—We previously reported RR spectra of purple intermediates produced by the reaction with the substrate in some classes of flavoenzymes (*25-27, 29-31*), and confirmed that the purple

intermediates are charge-transfer complexes between reduced flavin and the product derived from the substrate. That the reduced flavin in the purple intermediate is in the anionic form has been confirmed only in DAO (*41*). Although it was shown by NMR (*42*) that the flavin in free reduced MCAD is also in the anionic form, the ionic form of reduced flavin in MCAD purple intermediate has not been unequivocally identified. The present report confirms, as described below, that all the reduced flavin in the purple intermediates discussed here is in the anionic form. The bands derived from the reduced flavin in the purple intermediates are commonly observed around 1,610 cm^{-1} (Table III), and these bands are considered to be C(4*a*)=C(10*a*) stretching vibration from analogy with DAO. The band frequencies cover a fairly wide range (1,602-1,620 cm^{-1}) depending on the enzymes examined. Interestingly, all the dehydrogenases show higher frequencies than the oxidases (Table III). This indicates that the C(4*a*)=C(10*a*) bond order of reduced flavin in the dehydrogenases is stronger than that in the oxidases. Therefore, the band frequency of C(4*a*)=C(10*a*) stretching may serve as an indicator of reactivity with molecular oxygen.

There is strong hydrogen bonding between N(1) nitrogen of reduced flavin and the hydroxyl group of Thr136 (2.55 Å) in MCAD-substrate complex (*43*). As Thr136 is conserved in short-chain acyl-CoA and isovaleryl-CoA dehydrogenases (*44*), similar strong hydrogen bonding is also expected in these two enzymes. On the other hand, DAO contains no such strong hydrogen bonding at N(1) nitrogen, although there are α-helix dipoles in the vicinity of N(1) to stabilize the negative charge in the anionic reduced flavin (*45, 46*). Thus, the strong hydrogen bonding at N(1) is probably responsible for the high frequency in the dehydrogenases.

The protonation of anionic reduced flavin at N(1) strengthens the C(4*a*)=C(10*a*) bond by breaking the electron-delocalization between C(4*a*)=C(10*a*) and N(1)-C(2)=O regions. In fact, the bond length of C(4*a*)=C(10*a*) of reduced flavin in the neutral form is shorter than that in the anionic reduced form, as shown by *ab initio* molecular orbital calculation for lumiflavin (*47*). The hydrogen bonding at N(1) partially modulates the structure of the anionic reduced flavin toward that of the neutral reduced flavin, and then the C(4*a*)=C(10*a*) bond becomes strong (Fig. 1). The reaction rate of neutral reduced flavin with molecular oxygen is smaller than that of anionic reduced flavin (*48*). Thus, the strong hydrogen bonding at N(1) may be at least partly responsible for the low reactivity of the dehydrogenases with molecular oxygen.

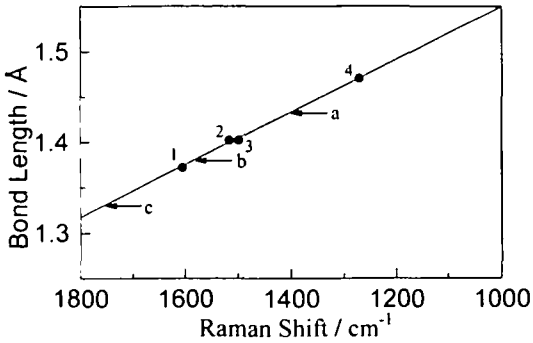

Fig. 7. **Correlation between Raman shift for the C(4*a*)-C(10*a*) stretching vibration of flavin and the chemical bond length calculated by the *ab initio* molecular orbital method.** Points: (1) the purple intermediate of DAO with D-alanine or D-proline (*27*); (2) the complex of anionic semiquinoid DAO with picolinate (*21*); (3) anionic semiquinoid riboflavin (*23*); (4) oxidized riboflavin in the neutral form (*13*). The bond lengths used here are those calculated for lumiflavin by the *ab initio* molecular orbital method (*47*). The line is expressed as (Bond length) (Å) = -2.839×10^{-4} (Raman shift) + 1.839. The arrows show the positions predicted from the calculated bond length (*47*): (a) neutral semiquinone; (b) cationic semiquinone; (c) neutral reduced form.

Vibrational bands associated with C(4*a*)=C(10*a*) or C(4*a*)-C(10*a*) stretching have been also observed for an anionic flavosemiquinone or a neutral oxidized flavin. The 1,516-cm^{-1} band of anionic semiquinoid DAO complexed with picolinate (*21*) corresponds to the 1,498-cm^{-1} band of anionic semiquinoid riboflavin free in solution (*23*). The band has been assigned to a C(4*a*)=C(10*a*) stretching vibrational mode (*21, 23*). A band associated with a C(4*a*)-C(10*a*) stretching of oxidized flavin was observed at 1,272 cm^{-1} in the IR spectrum (*13*). Recently the structures of lumiflavin in different redox and protonation states (Fig. 1) have been investigated by *ab initio* molecular orbital calculation, and some geometrical parameters have been reported (*46*). According to the report, the bond lengths of C(4*a*)-C(10*a*) are 1.4706, 1.4023, and 1.3724 Å for oxidized, anionic semiquinoid, and anionic reduced forms, respectively. So, we examined the correlation between the frequency of an observed band of C(4*a*)-C(10*a*) stretching and the bond length calculated for the C(4*a*)-C(10*a*) of lumiflavin (Fig. 7). Namely, the band frequencies for C(4*a*)=C(10*a*) [or C(4*a*)-C(10*a*)] stretching of DAO purple intermediate (anionic reduced flavin) (*26*), the complex of anionic semiquinoid DAO with picolinate (*21*), anionic semiquinoid riboflavin (*23*), and oxidized riboflavin in the neutral form (*13*) were plotted against the calculated bond lengths (*47*) of C(4*a*)-C(10*a*) of lumiflavin in each redox and ionized state. In order to examine the relationship between the Raman frequency and the bond length, the value of the same molecule in the same conditions should inherently be used. However, although vibrational frequencies are affected by the molecular interactions, the shift is generally smaller than that in a reconstitution of a chemical bond, *e.g.*, from a double bond to a single bond. For example, the shift range of the band of C(4)=O stretching of oxidized flavin is within 30 cm^{-1} among various enzymes (*11*), and the difference in frequency of the band around 1,500 cm^{-1} of anionic flavosemiquinone is only 18

TABLE III. **Band positions of the 1,610-cm^{-1} band of purple intermediates of some flavoenzymes (cm^{-1}).**

Enzyme	Substrate	Raman frequency
DAO	D-Proline[a]	1,605
	D-Alanine[a]	1,606
	D-Lysine[b]	1,609
PAO[c]	D-Phenylalanine	1,602
MCAD[d]	Octanoyl-CoA	1,614
	Isovaleryl-CoA	1,620
SCAD[e]	Butyryl-CoA	1,615
IVD[f]	Isovaleryl-CoA	1,612

[a]After Nishina *et al.* (*26*). [b]After Nishina *et al.* (*52*). [c]L-Phenylalanine oxidase: datum from Suzuki *et al.* (*29*). [d]Medium-chain acyl-CoA dehydrogenase. [e]Short-chain acyl-CoA dehydrogenase. [f]Isovaleryl-CoA dehydrogenase. [d-f]Data from Nishina *et al.* (*30, 31*).

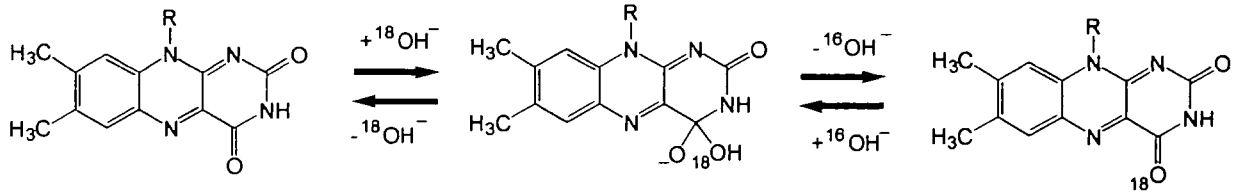

Fig. 8. **Simplified reaction coordinates of ^{18}O incorporation.**

cm^{-1} between riboflavin free in solution (1,498 cm^{-1}) (*23*) and FAD bound to DAO (1,516 cm^{-1}) (*21*). In other words, the frequency is not greatly different as long as the flavin is in the same redox and ionic states. Thus, to use the Raman frequency of the flavin bound to flavoprotein instead of lumiflavin may be allowed as an approximation. As shown in Fig. 7, the plots of the band frequencies of C(4*a*)-C(10*a*) stretching against the bond lengths of C(4*a*)=C(10*a*) gave a linear relationship with this approximation. By using this empirical linear relationship and the bond lengths calculated by *ab initio* MO method for lumiflavin (*47*), we can predict the band frequencies for a neutral flavosemiquinone (*ca.* 1,410 cm^{-1}), a cationic flavosemiquinone (*ca.* 1,580 cm^{-1}) , and a neutral reduced flavin (*ca.* 1,740 cm^{-1}). The bond lengths calculated for lumiflavin are 1.4316, 1.3794, and 1.3301 Å, respectively (*47*). The presumed frequency (1,740 cm^{-1}) for a neutral reduced flavin is much higher than the frequencies (1,602-1,620 cm^{-1}) observed in the purple intermediates of the flavoproteins treated here (Table III). Thus, we conclude that the reduced flavin in the purple intermediates is in the anionic form. The 1,507-cm^{-1} band of a cationic flavosemiquinone, which is assigned to contain a significant C(4*a*)-C(10*a*) stretching component (*23*), is substantially lower than the frequency expected (*ca.* 1,580 cm^{-1}). The reason for this large deviation is unknown at present. One possibility is that the 1,507-cm^{-1} band does not contain a large C(4*a*)-C(10*a*) stretching component. This can be checked by examining the isotope effects for the 1,507-cm^{-1} band of cationic flavosemiquinone. In addition, observation of Raman bands associated with C(4*a*)-C(10*a*) [or C(4*a*)=C(10*a*)] stretching mode of neutral flavosemiquinone or neutral reduced flavin should help to clarify the details of the relationship between the band positions and the bond length of C(4*a*)-C(10*a*).

Incorporation of ^{18}O into Flavin Ring by Exchange with $H_2{}^{18}O$ in Alkali—In this paper, we developed a simple method to introduce ^{18}O into the C(4)=O position of flavin. Band assignment is a very important step to clarify the details of the structure by vibration spectroscopy (Raman or infrared), and isotopically labeled compounds have been often used for the purpose. Thus, the present method developed for the labeled flavin is valuable for the investigation of flavins and flavoproteins, and the replacement mechanism of the C(4)=O by ^{18}O of $H_2{}^{18}O$ deserves discussion. It is known that several functional groups contain oxygen atoms that can undergo exchange with oxygen atoms in the bulk H_2O. For example, carboxylic acids can undergo either acid- or base-catalyzed exchange with $H_2{}^{18}O$ through the formation of an *ortho* acid intermediate (*49*). Extensive incorporation of ^{18}O at the 4-carbonyl group of the pyrimidine nucleus also results from exchange between $H_2{}^{18}O$ and nucleosides or base in 1 M HCl at 100°C (*50*). In nucleosides such as uridine, the incorporation of ^{18}O is limited to position C(4)=O. On the other hand, bases such as uracil with no sugar substituent at N(1) were found to undergo exchange at C(2), but the exchange is only minor (*50*). In the present case of flavin, extensive ^{18}O-incorporation in 1 M $Na^{18}OH$ ($H_2{}^{18}O$) occurs only at C(4)=O, not at C(2)=O. The exchange of carbonyl oxygen held between nitrogen atoms by ^{18}O may be generally difficult. As the pK_a value of oxidized flavin is 10, the N(3)H proton is almost dissociated in 1 M NaOH. But attack of OH^- on the 4-carbonyl carbon is difficult in the anionic form, due to the electrostatic repulsion for $N(3)^-$. Therefore, we consider that OH^- attacks the carbon of the neutral form, which is present in only a small amount. Incorporation of ^{18}O by exchange with $H_2{}^{18}O$ in $Na^{18}OH$ aqueous solution is presumed to proceed through equilibrium involving addition and elimination of a hydroxyl group (Fig. 8). In general, the lowest unoccupied molecular orbital (LUMO) is the most liable to accept an electron of a nucleophilic reagent. As the π orbital coefficient of LUMO for oxidized flavin at C(4) is larger than at C(2) (*51*), the C(4) is more acceptable position for attack by OH^-. Therefore, only the C(4)=O carbonyl may be susceptible to attack by OH^- under the conditions employed.

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REFERENCES

1. Müller, F. (1991) Free flavins: Syntheses, chemical and physical properties in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., ed.) Vol. I, pp. 1-71, CRC Press, Boca Raton, Florida
2. Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P.A., Howell, L.G., Mayhew, S.G., Matthews, R.G., and Foust, G.P. (1969) The reactivity of flavoproteins with sulfite. Possible relevance to the problem of oxygen reactivity. *J. Biol. Chem.* **244**, 3999-4006
3. Nishina, Y., Kitagawa, T., Shiga, K., Horiike, K., Matsumura, Y., Watari, H., and Yamano, T. (1978) Resonance Raman spectra of riboflavin and its derivatives in the bound state with egg riboflavin binding protein. *J. Biochem.* **84**, 925-932
4. Dutta, P.K., Spencer, R., Walsh, C., and Spiro, T.G. (1980) Resonance Raman and coherent anti-Stokes Raman scattering spectra of flavin derivatives. Vibrational assignments and the zwitterionic structure of 8-methylamino-riboflavin. *Biochim. Biophys. Acta* **623**, 77-83
5. Schopfer, L.M. and Morris, M.D. (1980) Resonance Raman spectra of flavin derivatives containing chemical modifications in positions 7 and 8 of the isoalloxazine ring. *Biochemistry* **19**, 4932-4935
6. Nishina, Y., Shiga, K., Horiike, K., Tojo, H., Kasai, S., Yanase, K., Matsui, K., Watari, H., and Yamano, T. (1980) Vibrational modes of flavin bound to riboflavin binding protein from egg white. Resonance Raman spectra of lumiflavin and 8-substituted riboflavin. *J. Biochem.* **88**, 403-409
7. Schopfer, L.M., Haushalter, J.P., Smith, M., Milad, M., and

Morris, M.D. (1981) Resonance Raman spectra for flavin derivatives modified in the 8 position. *Biochemistry* **20**, 6734-6739
8. Schmidt, J., Lee, M.-Y., and McFarland, J.T. (1982) Resonance Raman studies on 8-mercaptoriboflavin. *Arch. Biochem. Biophys.* **215**, 22-27
9. Kitagawa, T., Nishina, Y., Kyogoku, Y., Yamano, T., Ohishi, N., Takai-Suzuki, A., and Yagi, K. (1979) Resonance Raman spectra of carbon-13- and nitrogen-15-labeled riboflavin bound to egg-white flavoprotein. *Biochemistry* **18**, 1804-1808
10. Müller, F., Vervoort, J., Lee, J., Horowitz, M., and Carreira, L.A. (1983) Coherent anti-Stokes Raman spectra of isoalloxazines. *J. Raman Spectrosc.* **14**, 106-117
11. Hazekawa, I., Nishina, Y., Sato, K., Shichiri, M., Miura, R., and Shiga, K. (1997) A Raman study on the C(4)=O stretching mode of flavins in flavoenzymes: Hydrogen bonding at the C(4)=O moiety. *J. Biochem.* **121**, 1147-1154
12. Bowman, W.D. and Spiro, T.G. (1981) Normal mode analysis of lumiflavin and interpretation of resonance Raman spectra of flavoproteins. *Biochemistry* **20**, 3313-3318
13. Abe, M. and Kyogoku, Y. (1987) Vibrational analysis of flavin derivatives: Normal coordinate treatments of lumiflavin. *Spectrochim. Acta* **43A**, 1027-1037
14. Lively, C.R. and McFarland, J.T. (1990) Assignment and the effect of hydrogen bonding on the vibrational normal modes of flavins and flavoproteins. *J. Phys. Chem.* **94**, 3980-3994
15. Dutta, P.K. and Spiro, T.G. (1980) Resonance coherent anti-Stokes Raman scattering spectra of oxidized and semiquinone forms of *Clostridium MP* flavodoxin. *Biochemistry* **19**, 1590-1593
16. Nishina, Y., Shiga, K., Horiike, K., Tojo, H., Kasai, S., Matsui, K., Watari, H., and Yamano, T. (1980) Resonance Raman spectra of semiquinone forms of flavins bound to riboflavin binding protein. *J. Biochem.* **88**, 411-416
17. Kitagawa, T., Sakamoto, H., Sugiyama, T., and Yamano, T. (1982) Formation of the semiquinone form in the anaerobic reduction of adrenodoxin reductase by NADPH. Resonance Raman, EPR, and optical spectroscopic evidence. *J. Biol. Chem.* **257**, 12075-12080
18. Benecky, M.J., Copeland, R.A., and Spiro, T.G. (1983) Resonance Raman spectra of flavin semiquinones stabilized by N5 methylation. *Biochim. Biophys. Acta* **760**, 163-168
19. Sugiyama, T., Nisimoto, Y., Mason, H.S., and Loehr, T.M. (1985) Flavins of NADPH-cytochrome P-450 reductase: Evidence for structural alteration of flavins in their one-electron-reduced semiquinone states from resonance Raman spectroscopy. *Biochemistry* **24**, 3012-3019
20. Xu, J., Birke, R.L., and Lombardi, J.R. (1987) Surface-enhanced Raman spectroscopy from flavins adsorbed on a silver electrode: Observation of the unstable semiquinone intermediate. *J. Am. Chem. Soc.* **109**, 5645-5649
21. Nishina, Y., Tojo, H., and Shiga, K. (1988) Resonance Raman spectra of anionic semiquinoid form of a flavoenzyme, D-amino acid oxidase. *J. Biochem.* **104**, 227-231
22. Yue, K.T., Bhattacharyya, A.K., Zhelyaskov, V.R., and Edmondson, D.E. (1993) Resonance Raman spectroscopic evidence for an anionic flavin semiquinone in bovine liver monoamine oxidase. *Arch. Biochem. Biophys.* **300**, 178-185
23. Su, Y. and Tripathi, G.N.R. (1994) Time-resolved resonance Raman observation of protein-free riboflavin semiquinone radicals. *J. Am. Chem. Soc.* **116**, 4405-4407
24. Tegoni, M., Gervais, M., and Desbois, A. (1997) Resonance Raman study on the oxidized and anionic semiquinone forms of flavocytochrome b_2 and L-lactate monooxygenase. Influence of the structure and environment of the isoalloxazine ring on the flavin function. *Biochemistry* **36**, 8932-8946
25. Nishina, Y., Shiga, K., Miura, R., Tojo, H., Ohta, M., Miyake, Y., Yamano, T., and Watari, H. (1983) On the structures of flavoprotein D-amino acid oxidase purple intermediates. A resonance Raman study. *J. Biochem.* **94**, 1979-1990
26. Nishina, Y., Shiga, K., Watari, H., Miura, R., Miyake, Y., Tojo, H., and Yamano, T. (1982) Resonance Raman study on the purple intermediates of the flavoenzyme D-amino acid oxidase. *Biochem. Biophys. Res. Commun.* **106**, 818-822
27. Miura, R., Nishina, Y., Ohta, M., Tojo, H., Shiga, K., Watari, H., Yamano, T., and Miyake, Y. (1983) Resonance Raman study on the flavin in the purple intermediates of D-amino acid oxidase. *Biochem. Biophys. Res. Commun.* **111**, 588-594
28. Kitagawa, T. (1985) Resonance Raman study on enzyme-substrate interactions of heme- and flavoenzymes in *Biomolecules, Electronic Aspects* (Nagata, C. *et al.*, eds.) pp. 205-223, Japan Scientific Societies Press, Tokyo/Elsevier, Amsterdam
29. Suzuki, H., Koyama, H., Nishina, Y., Sato, K., and Shiga, K. (1991) A resonance Raman study on a reaction intermediate of *Pseudomonas* L-phenylalanine oxidase (deaminating and decarboxylating). *J. Biochem.* **110**, 169-172
30. Nishina, Y., Sato, K., Shiga, K., Fujii, S., Kuroda, K., and Miura, R. (1992) Resonance Raman study on complexes of medium-chain acyl-CoA dehydrogenase. *J. Biochem.* **111**, 699-706
31. Nishina, Y., Sato, K., Hazekawa, I., and Shiga, K. (1995) Structural modulation of 2-enoyl-CoA bound to reduced acyl-CoA dehydrogenases: A resonance Raman study of a catalytic intermediate. *J. Biochem.* **117**, 800-808
32. Shiga, K., Nishina, Y., Horiike, K., Tojo, H., Watari, H., and Yamano, T. (1980) On the existence of quasi D-amino acid oxidase in hog kidney extract. *Med. J. Osaka Univ.* **30**, 71-78
33. Shiga, K., Nishina, Y., Horiike, K., Tojo, H., Yamano, T., and Watari, H. (1982) A new material for flavoprotein researches: Purification and properties of hog kidney quasi D-amino acid oxidase in *Flavins and Flavoproteins* (Massey, V. and Williams, C.H., Jr., eds.) pp. 188-191, Elsevier North Holland, New York
34. Tojo, H., Horiike, K., Shiga, K., Nishina, Y., Miura, R., Watari, H., and Yamano, T. (1982) Thermodynamic characterization of hog kidney D-amino acid oxidase apoenzyme in concentrated guanidine hydrochloride solution. Preferential interaction with the solvent components and the molecular weight of monomer unit. *J. Biochem.* **92**, 1741-1752
35. Tishler, M., Pfister, K. 3rd, Babson, R.D., Ladenburg, K., and Fleming, A. (1947) The reaction between *o*-aminoazo compounds and barbituric acid. A new synthesis of riboflavin. *J. Am. Chem. Soc.* **69**, 1487-1491
36. Weiner, N. (1943) Malonic acid in *Organic Synthesis*. Collective Vol. 2, pp. 376-378, John Wiley & Sons, New York, London
37. Juri, N., Kubo, Y., Kasai, S., Otani, S., Kusunose, M., and Matsui, K. (1987) Formation of roseoflavin from 8-amino- and 8-methylamino-8-demethyl-D-riboflavin. *J. Biochem.* **101**, 705-711
38. Suzuki, A.T., Ohishi, N., and Yagi, K. (1979) Simple procedure for the separation of lumiflavine and lumichrome from photolysates of riboflavine. *J. Chromatogr.* **169**, 459-461
39. Abe, M., Kyogoku, Y., Kitagawa, T., Kawano, K., Ohishi, N., Takai-Suzuki, A., and Yagi, K. (1986) Infrared spectra and molecular association of lumiflavin and riboflavin derivatives. *Spectrochim. Acta* **42A**, 1059-1068
40. Kim, M. and Carey, P.C. (1993) Observation of a carbonyl feature for riboflavin bound to riboflavin-binding protein in the red-excited Raman spectrum. *J. Am. Chem. Soc.* **115**, 7015-7016
41. Miura, R. and Miyake, Y. (1987) ^{13}C-NMR studies on the reaction intermediates of porcine kidney D-amino acid oxidase reconstituted with ^{13}C-enriched flavin adenine dinucleotide. *J. Biochem.* **102**, 1345-1354
42. Miura, R., Nishina, Y., Sato, K., Fujii, S., Kuroda, K., and Shiga, K. (1993) ^{13}C- and ^{15}N-NMR studies on medium-chain acyl-CoA dehydrogenase reconstituted with ^{13}C- and ^{15}N-enriched flavin adenine dinucleotide. *J. Biochem.* **113**, 106-113
43. Kim, J.-J.P., Wang, M., and Paschke, R. (1993) Crystal structure of medium-chain acyl-CoA dehydrogenase from pig liver mitochondria with and without substrate. *Proc. Natl. Acad. Sci. USA* **90**, 7523-7527
44. Matsubara, Y., Indo, Y., Naito, E., Ozasa, H., Glassberg, R., Vockley, J., Ikeda, Y., Kraus, J., and Tanaka, K. (1989) Molecular cloning and nucleotide sequence of cDNAs encoding the precursors of rat long chain acyl-CoA, short chain acyl-CoA, and isovaleryl-CoA dehydrogenases. Sequence homology of four enzymes of the acyl-CoA dehydrogenase family. *J. Biol. Chem.*

264, 16321-16331
45. Mizutani, H., Miyahara, I., Hirotsu, K., Nishina, Y., Shiga, K., Setoyama, C., and Miura, R. (1996) Three-dimensional structure of porcine kidney D-amino acid oxidase at 3 Å resolution. *J. Biochem.* **120**, 14-17
46. Mattevi, A., Vanoni, M.A., Tonone, F., Rizzi, M., Teplyakov, A., Coda, A., Bolognesi, M., and Curti, B. (1996) Crystal structure of D-amino acid oxidase: A case of active site mirror-image convergent evolution with flavochytochrome b_2. *Proc. Natl. Acad. Sci. USA* **93**, 7496-7506
47. Zheng, Y.-J. and Ornstein, R.L. (1996) A theoretical study of the structures of flavin in different oxidation and protonation states. *J. Am. Chem. Soc.* **118**, 9402-9408
48. Eberlein, G. and Bruice, T.C. (1983) The chemistry of a 1,5-diblocked flavin. 2. Proton and electron transfer steps in the reaction of dihydroflavins with oxygen. *J. Am. Chem. Soc.* **105**, 6685-6697
49. Murphy, R.C. and Clay, K.L. (1990) Preparation of labeled molecules by exchange with oxygen-18 water in *Methods in Enzymology*, Vol. 193, pp. 338-348, Academic Press, London
50. Puzo, G., Schram, K.H., and McCloskey, J.A. (1977) Incorporation of oxygen-18 into nucleotides and bases. *Nucleic Acids Res.* **4**, 2075-2081
51. Yagi, K. (1987) Hydrogen bonding in flavoproteins. *Chem. Scripta* **27A**, 27-30
52. Nishina, Y., Miura, R., Tojo, H., Miyake, Y., Watari, H., and Shiga, K. (1986) A resonance Raman study on the structures of complexes of flavoprotein D-amino acid oxidase. *J. Biochem.* **99**, 329-337