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

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ABSTRACT: The oxidized and semiquinone anion radical forms of flavin mononucleotide carried by flavocytochrome b_2 and L-lactate monooxygenase have been studied by resonance Raman (RR) spectroscopy. The RR spectra of their oxidized forms are compared with previously published RR data on various flavins and flavoproteins. Taking as a support available X-ray crystallographic data on flavoproteins, we have found correlations between the frequencies of RR bands II (1575−1588 cm^{-1}), III (1534−1557 cm^{-1}), and X (1244−1266 cm^{-1}) and the H-bonding environment and/or the structure of the flavin ring. The present RR data provide strong evidence that the electron density, the conformation, and the H-bonding environment of the oxidized flavin mononucleotide of flavocytochrome b_2 and L-lactate monooxygenase are different. As far as the anionic semiquinone form of flavoproteins is concerned, the behavior of two bands observed at 1280−1300 and 1320−1350 cm^{-1} suggests that they have vibrational origins similar to those of RR bands II and III of oxidized compounds. On this basis, the differences in conformation and H-bonding environment of the isoalloxazine ring, observed for the oxidized form of flavocytochrome b_2 and L-lactate monooxygenase, appear to be preserved upon one-electron reduction of the flavin. For both flavoproteins, the RR spectra of the semiquinone form are affected by pyruvate binding. The data are interpreted in the frame of a change in H-bonding interaction of the C4═O carbonyl group of the flavin without significant alteration of the isoalloxazine conformation. This modification in electrostatic interaction quantitatively accounts for the pyruvate-induced changes of the oxidized/semiquinone and semiquinone/reduced redox potentials of the flavoproteins. Considering the high homology in the flavin catalytic sites of flavocytochrome b_2 and L-lactate monooxygenase, the observed differences in H-bonding environment and conformation of the FMN ring are related to the different biological functions of the two flavoproteins.

Flavoproteins play important roles in a variety of biological functions involving electron transport, dehydrogenation reactions, activation of molecular oxygen, as well as photochemical processes (Bruice, 1980; Walsh, 1980; Müller, 1983; Ghisla & Massey, 1989; Mathews, 1991). In contrast with metalloproteins and nicotinamides, which assure purely mono- or bielectron transfers, respectively, the common cofactor of flavoproteins, i.e., the isoalloxazine ring of flavin mononucleotide (FMN)[1] or flavin adenine dinucleotide (FAD), can undergo both types of electron transfer. This property is linked to the chemical structure of this tricyclic ring, which makes accessible three oxidation states: the oxidized (Ox) or quinone state, the one-electron reduced or semiquinone (Sq) radical state, and the two-electron reduced (Red) or hydroquinone state (Müller, 1983). Flavoproteins can selectively use at least two of these three oxidation states depending on the protein−flavin interactions. The intermediate Sq state appears fundamental to the flavoprotein biochemistry since it can serve as an electron shuttle, play the role of an electron donor-acceptor, and/or couple one-electron and two-electron pathways in chains of electron transfer enzymes (Bruice, 1980). Moreover, the Sq radical state can be stabilized under a neutral and/or an anionic form(s) in the flavoproteins (Müller, 1983). The extreme importance of the Sq forms in the determination and regulation of the enzymatic activity of flavins requires the structural characterizations of these radicals. Unfortunately, in most of the cases, the reactivity of Sq flavins with oxygen and/or its intrinsic instability make(s) this species practically inacessible to structural determinations. The crystallographic

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[1] Abbreviations: RF, riboflavin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; LF, lumiflavin; Ox, oxidized form; Sq, semiquinone or one-electron reduced form; Red, dihydroquinone or two-electrons reduced form; DAOX, D-amino acid oxidase; FAOX, fatty acyl-CoA oxidase; FCB, flavocytochrome b_2; FD, flavodoxin; GluOX, glucose oxidase; GlyOX, glycolate oxidase; GR, glutathione reductase; LMO, L-lactate monooxygenase; MAOX, monoamine oxidase; OYE, old yellow enzyme; PHBH, *p*-hydroxybenzoate hydroxylase; cyt b_2, cytochrome b_2 core; Pyr, pyruvate; EDTA, ethylenediaminetetraacetic acid; CARS, coherent anti-Stokes Raman scattering; RR, resonance Raman.

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structures of a neutral Sq form have been solved at a high resolution only in the case of flavodoxins from *Clostridium MP* and *Desulfovibrio vulgaris*, flavoproteins in which the Sq is a stable species (Andersen et al., 1972; Smith et al., 1977; Watt et al., 1991).

In order to better understand the structure and function relationships of flavins carried by flavoproteins, we have undertaken a resonance Raman (RR) study on the Ox, anionic Sq, and Red forms of FMN of flavocytochrome b_2 (FCB) and L-lactate monooxygenase (LMO). Among the spectroscopies potentially able to produce structural information of flavins in flavoproteins, RR spectroscopy has already proved to be a valuable technique (Morris & Bienstock, 1986; McFarland, 1987). However, most of the RR studies have been focused on the Ox form of flavoproteins and flavin compounds with less attention being adressed to the Sq states, and particularly the anionic Sq flavins (Morris & Bienstock, 1986; McFarland, 1987).

In this work, FCB and LMO have been chosen because of the fact that, in spite of highly homologous flavin binding and catalytic sites, based on sequence identity (Choong & Massey, 1980; Giegel et al., 1990; Müh et al., 1994a,b,c), they exhibit different biological functions, i.e., a dehydrogenase-electron transferase activity and a dehydrogenase-oxidase-transferase activity, respectively. Yeast FCB [(L)-lactate:ferricytochrome-*c* oxidoreductase, EC 1.1.2.3) uses the Ox, Sq, and Red redox states *in vivo*. Its anionic Sq form appears to be a key intermediate in the catalytic cycle and enzyme regulation (Capeillère-Blandin et al., 1986; Tegoni et al., 1990). The X-ray data on FCB show two crystallographically distinguishable subunits in the same protein tetramer (Xia & Mathews, 1990): in one type of subunit of the crystallographic unit cell, FMN is thought to be fully reduced, while in the second type of subunit, a molecule of pyruvate (Pyr), the reaction product, is bound adjacent to the FMN, suggesting that the latter is in the Sq state (Xia & Mathews, 1990). Since each subunit has been refined independently, the three dimensional structure of FMN_{Sq} is accessible. However, due to the limited resolution (2.4 Å), the difference between Red and Sq, expected to be small, falls in the range of the experimental uncertainty. L-Lactate 2-monooxygenase (EC 1.13.12.4), formally refered to as lactate oxidase, is a monomeric enzyme that contains a single flavin per molecule. It catalyzes the oxidative decarboxylation of a variety of L-α-hydroxyacids and exhibits numerous common properties with FCB (Giegel et al., 1990; Ghisla & Massey, 1991). In particular, it uses L-lactate as a substrate and oxidizes it to Pyr under anaerobic conditions (Ghisla & Massey, 1991). As in the case of FCB, Pyr stabilizes a flavin anion radical by complex formation (Choong & Massey, 1980). The tridimensional structure of LMO has not been determined. However, its amino acid sequence shows a high degree of identity with that of FCB and that of glycolate oxidase (GlyOX), another oxidase for which a structure at 2.0 Å resolution is available (Lindqvist, 1989; Lindqvist et al., 1991). Moreover, recent biochemical studies on site-directed mutants of LMO have confirmed the strong homology of its catalytic site with that of FCB and GlyOX (Müh et al., 1994a,b,c).

Besides the expected similarity in the FMN binding and catalytic sites of FCB and LMO, important differences in the potentials of the Ox/Sq and Sq/Red redox couples as well as in the affinity for Pyr were observed (Stankovich & Fox, 1983; Tegoni et al., 1986,1990; Lockridge et al., 1972; Choong & Massey, 1980; Ghisla & Massey, 1991). This difference probably corresponds to subtle variations in the flavin structure, in the flavin−protein interactions and/or in the flavin−solvent interactions, parameters that likely modulate the biochemical activity of FMN in these two flavoproteins.

In this paper, we describe and analyze the RR spectra of the Ox and Sq forms of FCB and LMO. Our objective is to determine by this vibrational spectroscopy the extent of differences in the structure and the environment of the flavin in these flavoproteins. These differences will be discussed in relation to their similar protein folding and different biological activity.

EXPERIMENTAL PROCEDURES

Protein Purification and Sample Preparations. Hansenula anomala FCB was purified as described in Labeyrie et al. (1978) and Gervais et al. (1980). The resulting enzyme has a ratio A_{280}/A_{423} (reduced) = 0.5, is homogenous in SDS−PAGE, and has a standard molar activity of 1000 s^{-1} at 30 °C (Labeyrie et al., 1978). The enzyme was stored at 4 °C as a precipitate at 50% saturation ammonium sulfate, in Na/K_2 phosphate buffer 100 mM and sodium D,L-lactate 100 mM, pH 7.0. Prior to the Raman experiments, and in order to eliminate the ammonium sulfate and the sodium D,L-lactate, aliquots of the precipitated enzyme were solubilized in a minimum amount of Na/K_2 phosphate buffer 100 mM, pH 7.0, and filtered, at 4 °C, through a Sephadex G25 Fine column (10 × 1 cm) equilibrated in the same buffer. We have determined the concentration of the eluted enzyme on the dithionite-reduced form, using an extinction coefficient of 183 mM^{-1} cm^{-1} at 423 nm (Pajot & Groudinsky, 1970). The eluted enzyme was concentred by centrifugation in a Centricon 30 (4000*g* at 4 °C). The *Hansenula anomala* heme domain, cytochrome b_2 core (cyt b_2), was prepared according to Silvestrini et al. (1986).

The FCB samples (0.8−1.5 mM) were degassed in a Thunberg vessel by alternative vacuum pumping and argon saturation. Partial reduction of the enzyme in the presence of sodium Pyr (10 mM) was obtained by mixing the degassed enzyme with Pyr and with discrete amounts of concentrated sodium L-lactate (Tegoni et al., 1990). In the absence of Pyr, partial or full photoreductions of the FMN bound to the enzyme were obtained by the free flavin/EDTA/light system (Massey & Palmer, 1966) using two different methods: (i) a mixture of degassed enzyme, FMN (1.5−10 μM) and EDTA (1−2 mM), was irradiated with a 500 W lamp for 10 min periods, through an UV glass screen, and the Raman spectra were obtained with the sample subjected to magnetic stirring (Leondiadis et al., 1992); (ii) a mixture of degassed enzyme and EDTA (0.7−0.8 mM) was directly exposed to the laser beam. In the latter experiments, variable amounts of Sq form were accumulated in the samples depending on the illumination conditions. For this purpose, variable laser powers (5−50 mW) and focused or defocused (1−2 mm diameter) beams were used. In addition, the presence or the absence of magnetic stirring allowed us to get variable concentrations of Sq in the enzyme solution. Sets of RR spectra were accumulated under these different experimental conditions. In the experiments using pre-illumination steps, the Sq formation was followed indirectly

by checking the amount of reduced heme formed by visible absorption (Tegoni et al., 1986). At least three series of preillumination were necessary to obtain maximal levels of semiquinone. When the Sq state was directly formed by laser irradiation, the rates of heme and flavin reductions were directly controlled by RR spectroscopy using as a basis the RR and absorption data obtained for the preilluminated enzyme. The spectral contributions of the Ox and Sq form of FMN and of the oxidized and reduced form of heme were estimated through the intensities of the strongest RR band of each compound (at 1581, 1557, 1374, and 1362 cm^{-1}, respectively). For a given level of heme reduction, both methods gave identical RR spectra. The reversibility of the spectral changes (absorption and RR) was verified using the sample a second time after air oxidation. The full reduction of FCB was obtained by addition of sodium dithionite or of excess L-lactate under vacuum (Desbois et al., 1989).

To check for protein degradation, the L-lactate-ferricyanide reductase activity in Na/K_2 phosphate buffer (100 mM), L-lactate (10 mM), potassium ferricyanide (1 mM), pH 7.0, at 30 °C, and the amount of total and protein-bound FMN (Labeyrie et al., 1978; Gervais & Tegoni, 1980) were measured before and after the Raman experiments. During these experiments, the enzyme degradation was lower than 10%.

Mycobacterium smegmatis LMO was obtained from Boehringer Mannheim, Inc. Prior to Raman measurements, the enzyme was dissolved in imidazole/HCl buffer (10 mM), pH 7.0, and filtered through a Sephadex G25 Fine column (8 × 0.5 cm) equilibrated in the same buffer. The concentration of the eluted enzyme was determined on the oxidized form using the extinction coefficients at 278 and 458 nm (113 and 11.06 mM^{-1} cm^{-1}, respectively) (Ghisla & Massey, 1991). The ratio A_{458}/A_{278} after molecular filtration was 0.098. The eluted enzyme was concentrated by centrifugation in a Centricon 30 (4000*g* at 4 °C). The absorption spectra of the Ox, Sq, and Red forms of the LMO preparations were not significantly different from those published previously (Choong & Massey, 1980). For the RR experiments, LMO samples (0.3−0.8 mM) were degassed as described above for FCB. Partial reduction of the enzyme was obtained by the photochemical methods (see above): the degassed enzyme was anaerobically mixed with FMN (40 μM) and EDTA (50 mM) [and sodium Pyr (16 mM), in some experiments] and irradiated. Total reduction of the enzyme was achieved by addition of L-lactate (50 mM) or of solid dithionite. The ratio A_{458}/A_{278} was not significantly changed after RR measurements.

In order to identify vibrational contributions from the N3H group of ring III of FMN_{Sq} as well as hydrogen bonds involving the chromophore and the protein or the solvent, H_2O/D_2O exchanges were performed on buffered solutions of FCB and LMO. The incubations in D_2O were limited both in relative amount, i.e., to D_2O:H_2O molar ratios ranging from 50:50 to 70:30, respectively, and in time exposure (up to 4 h). Indeed, preliminary experiments showed that higher relative concentrations of D_2O (75−90%) produced partial protein denaturations detected as changes in enzyme activity and/or in absorption and RR spectra. The degree of denaturation was found to be particularly elevated when the H/D exchanges were performed in the absence of Pyr. Under conditions of relatively low D_2O concentrations (50−70%), individual RR scans were recorded at various times (30 min−4 h) after D_2O mixing. They showed no measurable differences, indicating that the H/D exchange at the N3H FMN site of FCB and LMO is complete within 30 min.

Flavin mononucleotide, EDTA, and all the buffer components were obtained from Fluka, Sigma or Merck. D_2O (isotopic enrichment, 99.9%) was purchased from the Bureau des Isotopes Stables (Saclay, France).

Spectroscopy. UV−visible absorption spectra were recorded on a Beckman (DU7) and Perkin-Elmer (Model 555) spectrophotometers. Resonance Raman experiments were conducted using a Jobin-Yvon spectrometer (Ramanor HG2S-UV) and Ar (Coherent, Model Innova 100) and He/Cd (Liconix, Model 4050) lasers. The spectra were recorded at 20 ± 1 °C. The signal-to-noise ratios were improved by summation of individual spectra in a multichannel analyzer (Tracor Northern 1710). Spectral treatments (addition, subtraction, removing of broad and featureless fluorescence backgrounds) were made using the Spectra Calc software (Galactic Industries) (Othman et al., 1996). The precision of Raman band frequencies was 1−3 cm^{-1} depending on both the band intensity and the signal-to-noise ratio. The RR spectra of the FMN_{Ox} and FMN_{Sq} forms of FCB were obtained by a difference technique (Desbois et al., 1989; Tegoni & Desbois, 1991).

Potential Hydrogen Bonding. The examination of the flavins sites was performed with the molecular graphics and crystallographic package Turbo-Frodo (Roussel & Cambillau, 1991). Among numerous options, this program allows us to calculate the neighbors of an atom, at a defined distance (NEIG), and thus to select potential candidates for hydrogen bonding. Further criterion for choosing between these candidates comes from the observation of the relative orientation of the atoms suitable to form an hydrogen bond. The atomic coordinates of glucose oxidase (Hecht et al., 1993), glutathione reductase (Karplus & Schulz, 1987), *p*-hydroxybenzoate hydroxylase (Gatti et al., 1994), *Clostridium MP* and *D. vulgaris* flavodoxin (Smith et al., 1977; Watt et al., 1991), old yellow enzyme (Fox & Karplus, 1994), *S. cerevisiae* flavocytochrome b_2 (Xia & Mathews, 1990), and glycolate oxidase (Lindqvist, 1989) have been taken from the Protein Data Bank (entries: 1gal, 1gra, 1doc, 3fx2, 3fxn, 1oya, 1fcb, and 1gox).

RESULTS

Resonance Raman Spectra of Flavocytochrome b_2

Oxidized Flavocytochrome b_2. The RR spectra of oxidized FCB, excited at 441.6 nm, have been previously published (Desbois et al., 1989). This excitation wavelength is spectrally near the maxima of the Soret transition of oxidized b-type hemes (413 nm) and the first electronic transition of oxidized FMN (ca. 450 nm) and enhances the Raman vibrational modes arising from both chromophores (Desbois et al., 1989). Absorption spectra of oxidized flavins, however, exhibit a strong band in the 360−370 nm region (Sun et al., 1972). The presence of this electronic transition makes the observation of isoalloxazine RR modes accessible by using UV excitations (Nishimura & Tsuboi, 1978; Bowman & Spiro, 1980; Tegoni & Desbois, 1991). Oxidized FCB excited at 363.8 nm leads to the RR spectrum shown in Figure 1A (spectrum a). Comparing this spectrum with that of cyt b_2 (Figure 1A, spectrum b) clearly indicates that

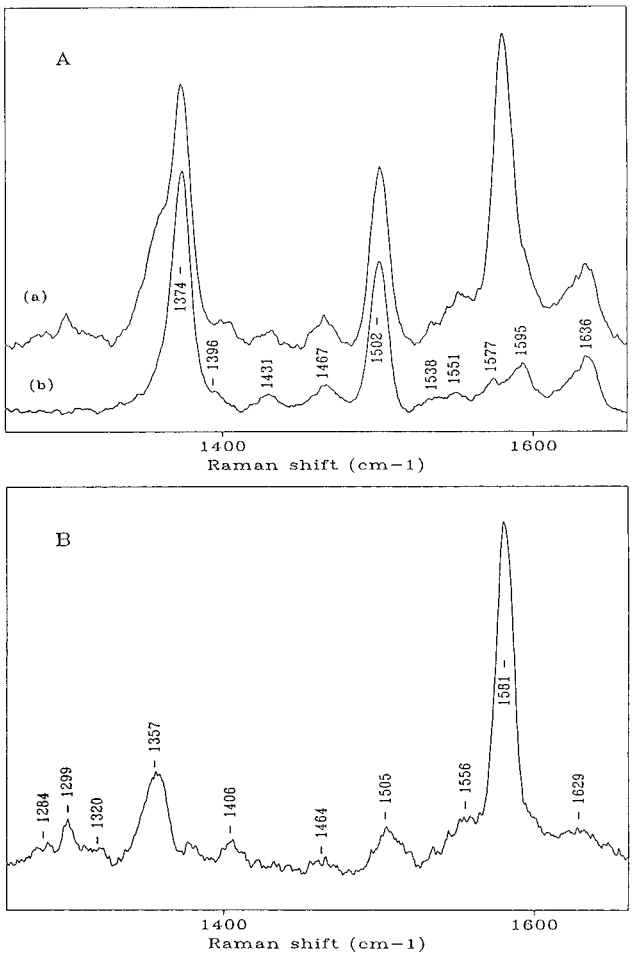

FIGURE 1: (A) High-frequency regions (1250−1650 cm^{-1}) of resonance Raman spectra of the oxidized form of flavocytochrome b_2 (a), and its cytochrome b_2 core (b), excited at 363.8 nm (experimental conditions, accumulation of 10 and 12 scans, respectively; [FCB], 0.9 mM; [cyt b_2], 0.9 mM; 100 mM phosphate buffer, pH 7.5). (B) Difference spectrum, (curve a − curve b) × 1.1

RR modes of both heme and FMN are observable for FCB. However, in the 363.8 nm excited spectrum, the 1581 cm^{-1} band becomes the major feature (Figure 1B), whereas in the spectrum of FMN_{Ox} excited at 441.6 nm, the 1360 cm^{-1} band dominates (Desbois et al., 1989). Similar changes in the RR activity of the high-frequency flavin modes have been detected in the spectra of free FMN and FAD, excited at 488 and 363.8 nm (Nishimura & Tsuboi, 1978). Despite these large intensity changes, the RR frequencies of FMN_{Ox} of FCB are in excellent agreement in the two sets of exciting conditions (Table 1).

Semiquinone Form of Flavocytochrome b_2. A preliminary report showed that RR modes of the FMN_{Sq} radical anion of FCB can be observed by the use of a 363.8 nm excitation (Tegoni & Desbois, 1991). The RR spectra of the photochemically reduced form of FCB, stabilized by Pyr complexation, is shown in Figure 2A. Its comparison with the RR spectra of the fully reduced enzyme or of the dithionite-reduced cyt b_2 clearly shows that the observed bands are attributable to either FMN_{Sq} or reduced heme b (Figure 2A) (Desbois & Tegoni, 1991). The absence of any RR signal arising from FMN_{Ox} and oxidized heme likely has its origin in the facts that (i) the Pyr binding to photoreduced FCB can stabilize up to 95% of the flavin in its Sq radical anion state and up to 85% of heme in its reduced form (Tegoni et al., 1986), and (ii) the excitation condition favors the activity of RR modes of both reduced heme and FMN_{Sq}. The general pattern of the RR spectrum of anionic FMN_{Sq} greatly resembles the spectra of the anionic FAD_{Sq} carried by glucose oxidase (GluOX) and monoamine oxidase (MAOX) (Yue et al., 1993).

The partial displacement of the buffer H_2O by D_2O induces positive or negative shifts on several RR bands (Figure 2B, spectra b and c). These spectral changes essentially affect the bands located in the 1580−1630, 1300−1400, and 1290−1310 cm^{-1} regions.

In the absence of Pyr, the maximal stabilization of the FMN_{Sq} in FCB represents 50 ± 5% of the total flavin and the enzyme contains about 30% of heme under the oxidized state (Tegoni et al., 1986). In our experiments using preillumination steps, the same rate of FMN_{Sq} and oxidized heme were obtained from absorption measurements (Tegoni et al., 1986). Under the conditions for maximal semiquinone level, the RR spectra obviously show contributions from reduced and oxidized hemes and oxidized FMN, as seen by the strong bands at 1362, 1374, and 1581 cm^{-1}, respectively (Figure 2C, spectrum a). Spectral treatments, sequentially removing the contributions of oxidized and reduced hemes and of FMN_{Ox}, systematically results in a spectrum weaker than, but exhibiting strong similarities with, that precedently obtained for the Sq form of the FCB−Pyr complex (Figure 2C, spectrum b, and Figure 2B, spectrum a, respectively). This residual spectrum is, therefore, attributed to that of FMN_{Sq} in Pyr-free FCB (Figure 2C, spectrum b). Although the signal-to-noise ratios of RR spectra of FMN_{Sq} in the presence and absence of Pyr largely differ, significant spectral changes are observed in the 1330−1380 and 1590−1630 cm^{-1} regions (Figure 2, panels B and C).

Fully Reduced Form of Flavocytochrome b_2. The RR spectrum of fully reduced FCB, formed by addition of sodium dithionite or of L-lactate, is not significantly different from that of dithionite-reduced cyt b_2 (Figure 2A, spectrum b, and spectrum not shown). These observations indicate no detectable Raman activity of dihydroflavin modes although the 363.8 nm excitation is close to a weak electronic transition of FMN_{Red} (Ghisla et al., 1974).

Resonance Raman Spectra of L-Lactate Monooxygenase

Oxidized Form of L-Lactate Monooxygenase. The high-frequency regions of RR spectra of oxidized LMO excited in the first (441.6 nm) and second (363.8 nm) electronic π-π* transitions of FMN are presented in Figure 3. Our frequencies determined from spontaneous RR spectroscopy are different with respect to those reported of a resonance coherent anti-Stokes Raman scattering (CARS) study (Visser et al., 1983). The maximal frequency dispersion between the CARS and RR spectra is from −8 to +8 cm^{-1}. These differences may arise from uncertainties in peak determination in the CARS spectra (Dutta et al., 1977; Irwin et al., 1980; Carreira & Antcliff, 1982).

Semiquinone Form of L-Lactate Monooxygenase. Figure 4 displays the high-frequency regions of RR spectra of LMO in its anionic Sq form, in the presence and absence of Pyr (spectra a and b). The general features of RR spectra are analogous to those described above for FCB. The low signal-to-noise ratio observed in the absence of Pyr is ascribed to a lower resonance condition on the ca. 370 nm

Table 1: Frequencies (Wavenumbers) of the Bands Observed in the High-Frequency RR Spectra of Oxidized Flavin in Flavocytochrome b_2 (FCB), L-Lactate Monooxygenase (LMO), and D-Amino Acid Oxidase (DAOX)[a]

band[a]	mode[b]	FCB	LMO	DAOX[c]	assignment[d,e]	ring
I	ν_6	1629	1631	1627	ν(CC)	I
II	ν_8	1583	1588	1583	ν(CN), ν(CC)	II and III
III	ν_9	1557	1553	1547	ν(N5C4a), ν(N1-C10a), ν(C4a-C10a)	II and III
IV	ν_{10}	1503	1505	1498	ν(CC)	I
V	ν_{11}	1463	1464	1460	ν(CC)	I
	ν_{12}	1445	1448		ν(CC)	I
VI	ν_{14}	1407	1412	1406	ν(CC)	I
VII	ν_{15}	1359	1363	1354	ν(N10C10a)	II
VIII	ν_{16}	1301	1307		ν(N5C5a)	II
IX	ν_{17}	1284	1285			
X	ν_{18}	1257	1262		ν(N3C4), ν(N3C2)	III
XI	ν_{19}	1231	1234	1227	ν(N3C4)	II and III
XII	ν_{20}	1180	1183	1182		
XIII	ν_{21}	1165	1168	1161		
	ν_{22}	1131	1134			
		1085				
XIV	ν_{23}	1067	1071			

[a] Band numbering according to Bowman and Spiro (1981). [b] Mode numbering according to Abe and Kyogoku (1987). [c] Average of Raman frequencies of DAOX complexed with benzoate derivatives (Nishina et al., 1981). [d] Mode assignment from Bowman and Spiro (1981), Abe and Kyogoku (1987), and Lively and McFarland (1990). [e] See Figure 7 for the atom labeling and ring numbering of the isoalloxazine macrocycle.

absorption band, Pyr complexation to the Sq form of LMO producing a slight shift and a hypochromic effect on this electronic band (Choong & Massey, 1980).

The partial deuteration of the buffer induces spectral alterations similar to those precedently detected for the Sq form of FCB, i.e., in the 1300−1310, 1330−1390, 1440−1470, and 1580−1620 cm^{-1} regions (Figure 5).

Hydroquinone Form of L-Lactate Monooxygenase. Like in the case of the fully reduced form of FCB, we found no Raman activity of FMN_{Red} of the dithionite-reduced LMO, excited at 363.8 nm.

DISCUSSION

Oxidized FMN in Flavocytochrome b_2 and L-Lactate Monooxygenase

The assignment of RR bands is essential to the structural interpretation of observed spectral differences. On the basis of selective isotopic substitutions, as well as normal mode calculations on the isoalloxazine ring of flavins, numerous high-frequency bands of oxidized flavins have been tentatively assigned (Kitagawa et al., 1979a; Bowman & Spiro, 1981; Copeland & Spiro, 1986; Abe et al., 1986; Abe & Kyogoku, 1987; Lively & McFarland, 1990). None of the three normal mode analyses performed on oxidized lumiflavin (LF) completely described the observed frequencies (Bowman & Spiro, 1981; Abe & Kyogoku, 1987; Lively & McFarland, 1990). A large number of assignments of RR bands appear, however, to be in good agreement with experimental studies obtained for models compounds (Kitagawa et al., 1979a; Müller et al., 1983).

Raman Diagrams of Oxidized Flavins. Raman diagrams were previously used to visualize the apoprotein effect on the high-frequency in-plane modes of the isoalloxazine ring (bands I−XIII) (Desbois et al., 1989). The Raman diagram of oxidized LMO, redrawn from our present RR data, shows a remarkable similarity with that obtained for FCB (Figure 6). This similarity is related to the expected structural homology of the FMN binding sites of the two flavoproteins (Ghisla & Massey, 1991; Müh et al., 1994a,b,c). However, the frequency shifts detected for LMO are systematically higher by ca. 3 cm^{-1} than those found for FCB (Figure 6). They thus indicate an increased electron density at the flavin ring of LMO (Desbois et al., 1989). On the other hand, the most important difference in the Raman diagrams of FCB and LMO is seen for the relative positions corresponding to the frequency shifts of bands II, III, and X (Figure 6).

In particular, a homology in the profiles of the bands I−V regions of Raman diagrams of FCB, of various flavodoxins (FD) and of the FMN−luciferase complex was previously reported (Desbois et al., 1989). In these diagrams, the maximal positive shift corresponds to band II (Desbois et al., 1989) (Figure 6). On the other hand, the Raman diagrams drawn from RR data on D-amino acid oxidase (DAOX), fatty acyl-CoA oxidase (FAOX), and old yellow enzyme (OYE) (Kitagawa et al., 1979b; Schmidt et al., 1983; Nishina et al., 1980a,1981) exhibit a shape very similar to that obtained for LMO in the bands I−VII regions (Figure 6 and diagrams not shown). In the diagrams of these flavoproteins, band III exhibits a maximal positive shift in the bands I−V regions (Figure 6). Therefore, these Raman fingerprints distinguish a FCB/FD/luciferase family and an oxidase-like family (LMO/DAOX/FAOX/OYE) from the relative position of frequency shifts of bands II and III (Desbois et al., 1989; Figure 6). Selective isotopic substitutions on the isoalloxazine cycle of flavin indicate that these bands correspond to stretching modes of rings II and III (Kitagawa et al., 1979; Abe et al., 1986; Müller et al., 1983). Normal modes calculations have specified these assignments in proposing contributions from ν(CN) and ν(CC) modes of the N5═C4a−C10a═N1 diazabuta-1,3-diene fragment (Bowman & Spiro, 1981; Abe et al., 1986) (Table 1 and Figure 7). The difference in the electron distribution through the FMN ring of FCB and LMO therefore appears to be located in this flavin portion.

Relation between the H-Bonding State of the Flavin Rings II and III and the Frequencies of Raman Bands II and III. The number and strength of H bonds at the N1, C2O, C4O, and N5 acceptor sites and the N3H donor site of oxidized flavins are expected to influence the electron density and thus the strengths of the CC and CN bonds of rings II and III. For instance, H-bond formation at a pyridine-like

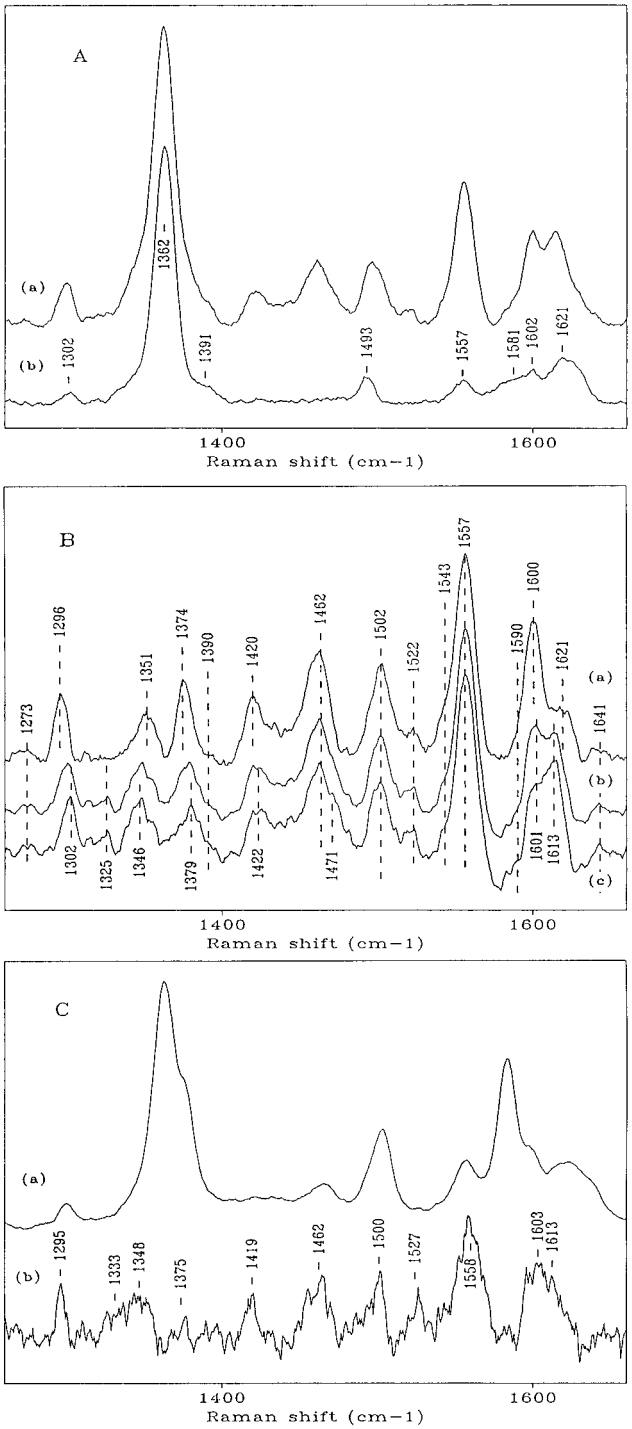

FIGURE 2: (A) High-frequency regions (1250−1650 cm^{-1}) of resonance Raman spectra of the semiquinone (a), and fully reduced (b) forms of flavocytochrome b_2, in the presence of pyruvate (experimental conditions, excitation: 363.8 nm; accumulation of 10 scans; [FCB], 0.6 mM in 100 mM phosphate buffer, pH 7.5; [Pyr], 10 mM; [L-lactate], 400 μM). (B) (a) Difference spectrum in H_2O (curve a − curve b) × 1.7. (b) Difference spectrum obtained for a D_2O/H_2O molar ratio of 72:28 (experimental conditions, see Figure 2A). (c) Computed spectrum of a hypothetical sample in 100% D_2O buffer solution ([curve b − 0.28(curve a)] × 1.39). The spectra are normalized on the intensity of the 1583 cm^{-1} band. (C) (a) High-frequency resonance Raman spectra (1250−1650 cm^{-1}) of flavocytochrome b_2, reduced by the FMN/EDTA/light system, in the absence of pyruvate (experimental conditions, excitation: 363.8 nm; accumulation of 30 scans; [FCB], 1 mM in 100 mM phosphate buffer, pH 7.5; [FMN], 1.5 μM; [EDTA], 1 mM). (b) Residual spectrum (×10) obtained after subtraction of RR contributions arising from oxidized and reduced heme and oxidized FMN (see text).

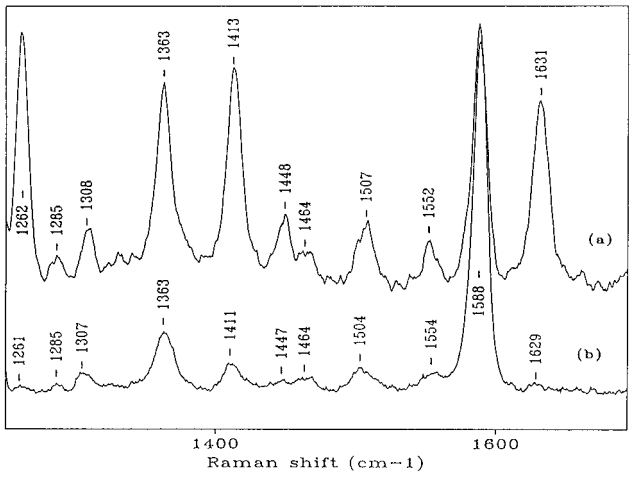

FIGURE 3: High-frequency regions (1250−1650 cm^{-1}) of resonance Raman spectra of the oxidized form of L-lactate monooxygenase, excited at 441.6 (a), and 363.8 (b) nm (experimental conditions, accumulation of 5 and 10 scans, respectively; [LMO], 0.8 mM in 10 mM imidazole buffer, pH 7.0).

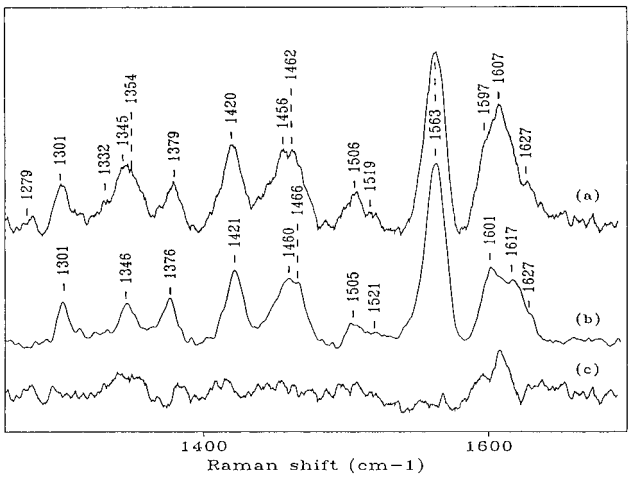

FIGURE 4: High-frequency regions (1250−1650 cm^{-1}) of resonance Raman spectra of the anionic semiquinone form of lactate monooxygenase in the absence (a), and presence (b) of pyruvate; (c) difference spectrum (curve a − curve b) (experimental conditions, excitation, 363.8 nm; accumulation of 12 and 8 scans, respectively; [LMO], 0.8 mM in 10 mM imidazole buffer, pH 7.0; [EDTA], 10 mM; [FMN], 40 μM; [Pyr], 13 mM).

nitrogen (N1 or N5) is expected to decrease the electron density of the adjacent C═N bonds (McFarland, 1987). Moreover, a strong H bond at the C2O group can withdraw electron density from the N1 atom. Similarly, a H-bond at the C4O group can withdraw electron density from the C4a atom. Therefore, the electron density of the three bonds constituting the N5═C4a−C10a═N1 diazabutadiene group can be affected by H bonding at the N1, N5, C2O, and C4O sites. Considering the similar assignments proposed for bands II and III (Bowman & Spiro, 1981; Abe & Kyogoku, 1987), one would expect that the hydrogen-bonding patterns at these sites could influence the frequencies of modes involving the N1, N5, O2, and O4 atoms. In an attempt to measure the overall H-bonding interaction at these atoms, we have first checked how the frequency of band II could be related to that of band III for a series of oxidized flavoproteins and flavin compounds, and secondly, compared the sensitivities of bands II and III to H bonding at rings II and III of the flavins.

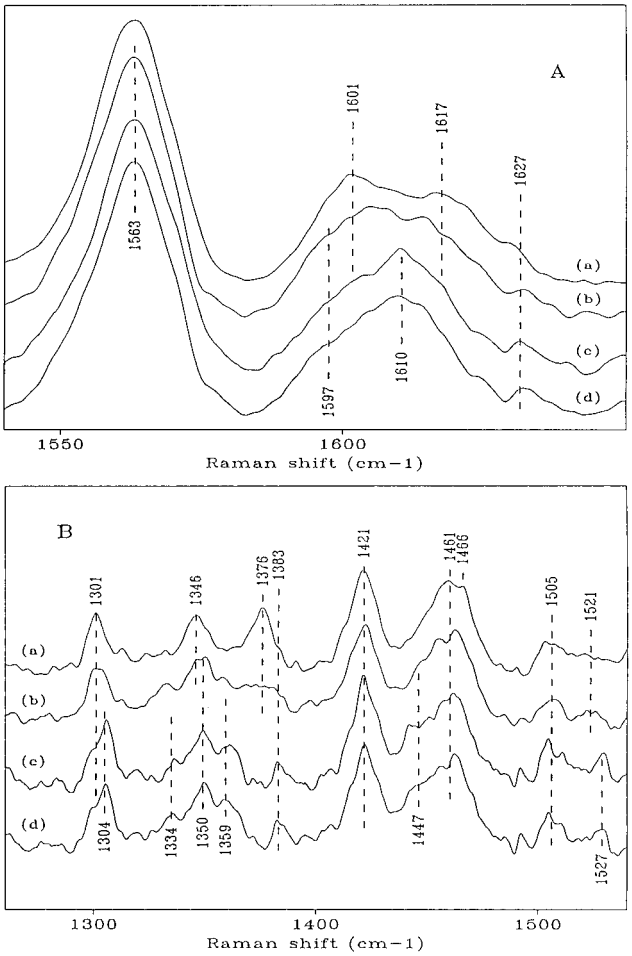

FIGURE 5: Regions 1550−1650 (A) and 1270−1530 (B) cm^{-1} of resonance Raman spectra of the anionic semiquinone form of the lactate monooxygenase−pyruvate complex (a) in H_2O, (b) in a D_2O/H_2O mixture of 50:50, (c) in a D_2O/H_2O mixture of 70:30, and (d) computed spectrum for a 100% D_2O (experimental conditions, excitation, 363.8 nm; accumulations of eight scans; [LMO], 0.3 mM in 10 mM imidazole buffer, pH 7.0; [EDTA], 50 mM; [FMN], 40 μM; [Pyr], 16 mM).

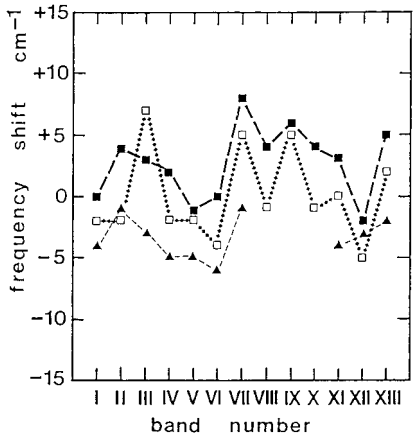

FIGURE 6: Raman diagrams of frequency shifts of high-frequency modes [bands I−XIII according to the numbering of Bowman and Spiro (1981)] of oxidized flavins induced by their binding to flavocytochrome b_2 (open squares), L-lactate monooxygenase (solid squares) and D-amino acid oxidase (solid triangles). The Raman data on the flavoproteins are listed in Table 1, those on the flavins in water are from Desbois et al. (1989).

Considering the whole Raman data set on bands II and III, there is little direct evidence for a relationship between their frequencies (Figure 8). Nevertheless, the comparisons

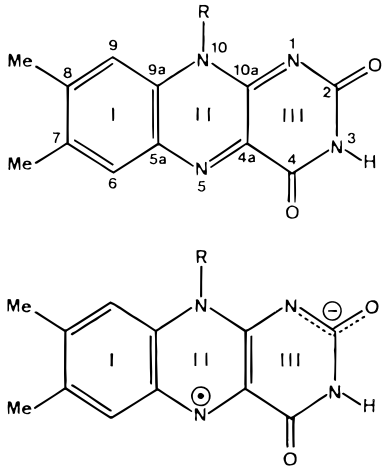

FIGURE 7: Chemical structures of the isoalloxazine ring in its oxidized (upper) and anionic semiquinone (lower) forms.

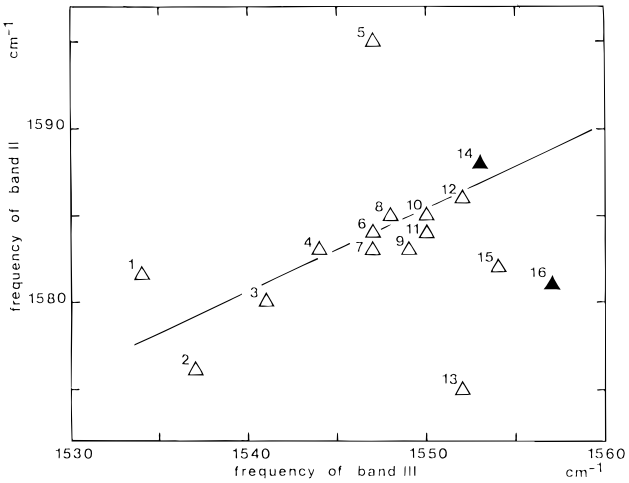

FIGURE 8: Plot of the observed band II frequency versus band III frequency of oxidized flavins and flavoproteins. The numbers in the figure correspond to the following flavoproteins: 1, glucose oxidase; 2, NADPH-cytochrome P450 reductase; 3, glutathione reductase; 4, general fatty acyl-CoA dehydrogenase; 5, *p*-hydroxybenzoate hydroxylase; 6, riboflavin binding protein; 7, D-amino acid oxidase; 8, old yellow enzyme; 9, medium-chain acyl-CoA dehydrogenase; 10, riboflavin, flavin mononucleotide and flavin adenine mononucleotide; 11, butyryl CoA dehydrogenase; 12, fatty acyl-CoA oxidase; 13, flavodoxin; 14, L-lactate monooxygenase; 15, luciferase; 16, flavocytochrome b_2. The Raman data are from Dutta et al. (1977, 1978), Nishimura and Tsuboi (1978), Nishina et al. (1978, 1980a,b,c, 1981, 1992), Kitagawa et al. (1979a,b), Benecky et al. (1979), Irwin et al. (1980), Bowman and Spiro (1981), Williamson et al. (1982), Visser et al. (1983), Schmidt et al. (1983), Sugiyama et al. (1985), Copeland and Spiro (1986), Bienstock et al. (1986), Desbois et al. (1989) and Kim and Carey (1993). The solid line corresponds to points 1−4, 6−12, and 14 and is fit to a straight line of equation $\nu_{II} = 0.477\nu_{III} + 846$, with a correlation coefficient of 0.89. The filled triangles show the data presently obtained on FCB and LMO.

of the vibrational and crystallographic data shed light on the sensitivities of bands II and III to electrostatic effects on the isoalloxazine ring. From X-ray crystallographic studies, the plausible H-bonding patterns between the flavin and the protein and/or the solvent are available for glucose oxidase (GluOX), glutathione reductase (GR), *p*-hydroxybenzoate hydroxylase (PHBH), old yellow enzyme (OYE), FD, and FCB (Karplus & Schulz, 1987; Schreuder et al., 1989; Xia & Mathews, 1990; Watt et al., 1991; Hecht et al., 1993; Fox & Karplus, 1994) (Tables 2 and 3). For these six flavoproteins, also investigated by Raman spectroscopy, band III

Table 2: Potential Hydrogen-Bonding Interactions (≥3.4 Å) at the Flavin Rings II and III of Glucose Oxidase (GluOX), Glutathione Reductase (GR), *p*-Hydroxybenzoate Hydroxylase (PHBH), *Clostridium MP* (CMP) and *D. vulgaris* (DV) flavodoxin (FD), and Old Yellow Enzyme (OYE)[a]

	isoalloxazine site				
flavoprotein	N1	O2	N3H	O4	N5
GluOX		M561 N (2.9)	T110 O (3.2)	T110 O (3.3) T110 OG1 (3.3) W745 (3.3)	
GR		T339 N (3.15) W497 (3.0)	W760 (3.3)	K66 NZ (2.75) W500 (3.3)	K66 NZ (3.1)
PHBH	L299 N (2.9)	L299 N (2.9) N300 N (3.0) N300 ND2 (2.9)	V47 O (3.0)	G46 N (3.05) V47 N (3.0)	
FD CMP	G89 N (2.9)	G89 N (2.9) G91 N (3.1)	E59 OE1 (2.8)	E59 N (2.9)	
FD DV	D95 N (3.2)	D95 N (3.1) C102 N (2.7)	Y100 O (3.0)	D62 N (3.2) W6 (2.8)	
OYE	N194 ND2 (3.3) R243 NH1 (2.9)	Q114 NE2 (3.1) H191 NE2 (3.3) R243 NH1 (2.8)	Q114 OE1 (2.8)	T37 OG1 (2.8) G72 N (3.3)	T37 N (2.9)

[a] The numbers in the parentheses are the H-bond distances in angstroms (see Experimental Procedures for references).

Table 3: Potential Hydrogen-Bonding Interactions (≥3.4 Å) at the FMN Rings II and III of Flavocytochrome b_2 (FCB) in the Presence and Absence of Pyruvate (Pyr), Glycolate Oxidase (GlyOX), and Lactate Monooxygenase (LMO)[a]

	isoalloxazine site				
flavoprotein	N1	O2	N3H	O4	N5
FCB (−Pyr)	K349 NZ (2.9)	K349 NZ (2.6) T280 OG1 (2.8)	Q252 NE2 (3.1)	S228 OG1 (2.7) W664 (2.8)	A198 N (3.2) W664 (3.0)
FCB (+Pyr)	K349 NZ (2.8)	K349 NZ (2.8) T280 OG1 (2.8)	Q252 NE2 (3.0)	S228 OG1 (2.8)	A198 N (3.0)
GlyOX	K230 NZ (3.0)	T155 OG1 (2.7) K230 NZ (2.8)	T155 OG1 (3.2) W394 (3.3)	Y129 OH (2.8) W394 (2.7)	W394 (3.0)
LMO	K266	T178 K266	T178	Y124 or S127 or Y152	

[a] The numbers in the parentheses are the H-bond distances in angstroms. References for X-ray data are given in Experimental Procedures; sequence data are from Ghisla and Massey (1991).

exhibits a low frequency for GluOX (1534 cm^{-1}), a medium frequency for GR, PHBH, and OYE (1541, 1547 and 1548 cm^{-1}, respectively), and a high frequency for FD and FCB (1552 and 1557 cm^{-1}, respectively) (Visser et al., 1983; Dutta et al., 1978; Schmidt et al., 1983; Bienstock et al., 1986; Kitagawa et al., 1979b; Nishina et al., 1980a, 1981; Desbois et al., 1989). A gradual increase in band III frequency is roughly correlated to an increased H-bonding interaction at the N1/N5 sites, except for FD. Indeed, GluOX has no H bond at N1 and N5 while the total number of plausible H bonds at these sites is 1 for GR and PHBH and 3 for OYE and FCB (Tables 2 and 3). In the case of GR and PHBH, the different band III frequency appears to be related to a weaker H bond for GR (Table 2). On the other hand, the behavior of band II is much more difficult to understand, since the band II frequency is practically the same for GR and FCB (1580−1581 cm^{-1}) (Schmidt et al., 1983; Desbois et al., 1989; this work) while the H-bonding patterns at N1/N5 largely differs (Tables 2 and 3). A symmetric situation occurs for PHBH and FD with a 20 cm^{-1} variation for band II (1595 and 1575 cm^{-1}, respectively) (Bienstock et al., 1986; Visser et al., 1983) and nearly equivalent H-bonding states at N1/N5 (Table 2). Therefore, the frequency of band III is roughly sensitive to the H-bonding state at the isoalloxazine N1/N5 sites. As far as band II is concerned, we need to invoke other factors to account for its behavior.

Out-of-Plane Distortions of the Isoalloxazine Ring. The fact that different portions of the isoalloxazine macrocycle might be not coplanar has been rarely considered, despite the fact that this ring is nonaromatic (Dixson et al., 1979; Hall et al., 1987). However, a number of recent NMR and X-ray diffraction studies give strong evidence for distortions from planarity of the three-rings system. Two main out-of-plane isoalloxazine deformations have been identified, i.e., a butterfly bending along the virtual N5-N10 axis, and a propeller twisting around the longest axis passing through the centers of the C7−C8, C5a−C9a, C4a−C10a, and C2−N3 bonds. Among the flavoproteins imposing a chromophore distortion, the FAD of PHBH adopts a propeller twisted conformation (Schreuder et al., 1989). In the case of FD, deformations around the N10 atom of FMN are suggested for FD from *Azotobacter vinelandii* and *Clostridium* MP (Vervoort et al., 1986a; Laudenbach et al., 1987), but the bending or twisting that occurs in the crystal structure of oxidized FD from *D. vulgaris* seems to be within the experimental error of the atomic positions (Watt et al., 1991). An NMR investigation of the bacterial FMN−luciferase complex indicates that the N10 atom is significantly placed out of the FMN molecular plane (Vervoort et al., 1986b). As far as the FAD structure of GluOX and GR is concerned, its isoalloxazine ring adopts a slightly bent and twisted conformation, similar in amplitude to that found for protein-

free LF (Wang & Fritchie, 1973; Karplus & Schulz, 1987; Hecht et al., 1993). No geometric distortion has been reported for the FMN ring of OYE (Fox & Karplus, 1994).

Returning to the RR data on oxidized flavoproteins, we precedently found no clear relationship between the frequencies of bands II and III (Figure 8). However, now excluding the flavoproteins for which a marked isoalloxazine deformation has been reported (i.e., FMN−luciferase and PHBH) or is suspected (FD), a linear correlation becomes evident between the frequencies of bands II and III of protein-free flavins (RF, FMN, FAD), riboflavin binding protein, oxidase-like flavoproteins (DAOX, FAOX, OYE, LMO), and reductases (NADPH-cytochrome P450 reductase, GR) (Figure 8). The data point corresponding to oxidized FCB, however, strongly deviates from this linear relationship, indicating some distortions of the FMN_{Ox} rings. The three-dimensional structure of FMN_{Sq} and FMN_{Red} in FCB shows a marked butterfly bending and a slight twist, with dihedral angles C9-N10-N5-C4 and N1-N10-N5-C6 of 169−171° and 173−176°, respectively (Xia & Mathews, 1990). The deviation from planarity observed in the structure of the FMN_{Ox}−sulfite complex is even more pronounced (Tegoni & Cambillau, 1994). The RR data strongly suggest that this isoalloxazine deformation also occurs in the uncomplexed Ox state of FMN in FCB.

Figure 9, panels A and B, shows that the frequencies of bands II and III are sensitive to both the H-bonding state of the N1/N5 flavin sites and out-of-plane distortions of the flavin ring. For the planar isoalloxazine structures (GluOX, GR, and OYE), these frequencies are essentially correlated with the number of H bonds at N1/N5 (Figure 9, panels A and B). The band III frequencies observed for the distorted flavin structures of FD, PHBH, and FCB appear to constitute another relationship with the H-bonding state of N1/N5 (Figure 9B).

In conclusion, the comparison of RR data with the three-dimensional structures of several flavoproteins demonstrates that the frequencies of bands II and III are linearly correlated when the isoalloxazine ring is close to planarity. This is due to the fact that the frequencies of both bands are sensitive to the H-bonding environment of the N1/N5 sites. When the isoalloxazine ring is markedly distorted, the overall extent of H bonding at N1/N5 is more difficult to predict even from the band III frequency. In this case, the frequencies of bands II and III become sensitive to the out-of-plane distortion of the flavin ring.

Assignments and Sensitivities of Raman Bands II and III of Oxidized Flavins. Band III was assigned to a mode involving ν(CN) and ν(CC) of the diazabuta-1,3-diene group (Bowman & Spiro, 1981; Abe & Kyogoku, 1987; Lively & McFarland, 1990). Its trend to increase its frequency upon increasing H-bond strength at the N1/N5 sites is in agreement with this assignment, but this effect reflects an overall sensitivity expected for the ν(C4aC10a) mode and not for the ν(C4aN5) and ν(C10aN1) modes. Indeed, the strength of the C4a−N5 (or C10a−N1) bond would be decreased as the result of H bonding at N5 (or N1) and, consequently, the frequency of the ν(C4aN5) [or ν(C10aN1)] mode would be decreased (McFarland, 1987). To reconcile the sensitivity of band III with its assignment, one may notice that the normal mode calculations made on this band (ν_9) underestimate the mass effects at the C2, C4, C4a and C10a positions and overestimate the mass effects at the N1, N3, and N5 positions, so that the contribution of the ν(C4aC10a) mode seems to be underestimated relative to that of the ν(C4aN5) [or ν(C10aN1)] mode (Bowman & Spiro, 1981; Abe & Kyogoku, 1987; Lively & McFarland, 1990).

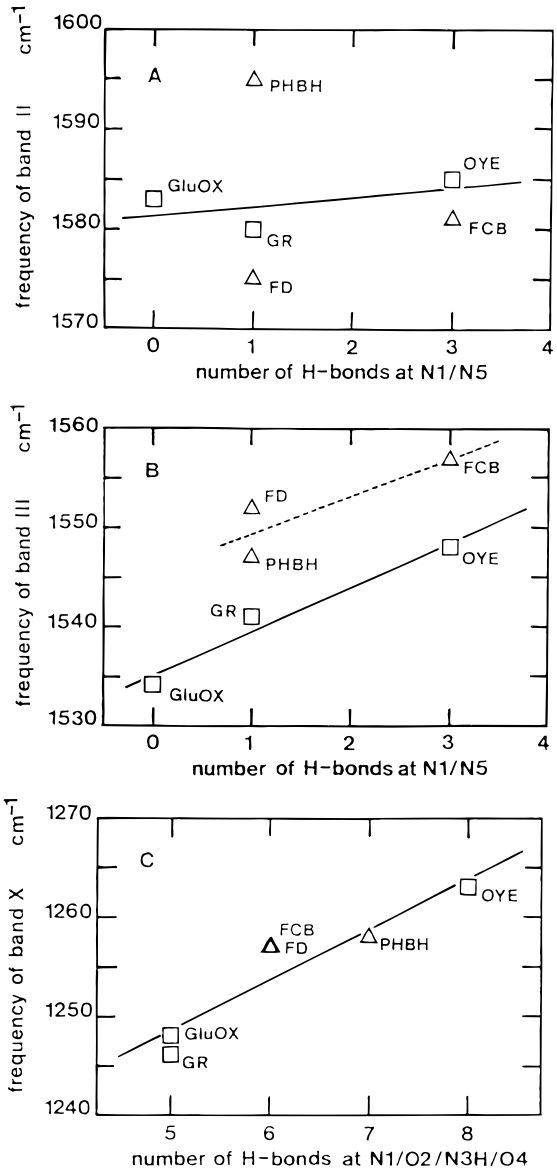

FIGURE 9: Plots of the band II (A) and band III (B) frequency as a function of the total number of H-bonds at the flavin N1/N5 sites of oxidized glucose oxidase (GluOX), glutathione oxidase (GR), *p*-hydroxybenzoate hydroxylase (PHBH), *D. vulgaris* flavodoxin (FD), old yellow enzyme (OYE), and of flavocytochrome b_2 (FCB). (C) Plot of the band X frequency as a function of the total number of H-bonds at the flavin N1/O2/N3H/O4 sites of the same flavoproteins. The electrostatic interactions are listed in Tables 2 and 3. The Raman data are from Dutta et al. (1978), Visser et al. (1983), Schmidt et al. (1983), Bienstock et al. (1986), Kitagawa et al. (1979b), Nishina et al. (1980a,1981), and Desbois et al. (1989). The squares and triangles correspond to nearly planar and distorted flavin rings, respectively.

Band II (ν_8) corresponds to a large mixing of the ν(C4aN5), ν(C4aC10a), ν(N1C10a), ν(N10C10a), and ν(C4C4a) modes (Bowman & Spiro, 1981 Abe & Kyogoku, 1987; Lively & McFarland, 1990). The sensitivity of the band II frequency to out-of-plane deformations of the isoalloxazine macrocycle is thus in agreement with a collective ring II−ring III mode.

H-Bonding Environment of Ring III. Information concerning the environment of the flavin ring III is expected to

be provided by band X. This band involves the stretching modes of the C4N3 and C2N3 bonds coupled with a bending mode of the N3H group (Abe & Kyogoku, 1987; Lively & McFarland, 1990). As previously proposed by Lively and McFarland (1990), it exhibits a rough sensitivity to the H-bonding state at the N3H site since an increase in H-bonding interaction produces an increase in the band X frequency. Indeed, the H bond formed by the N3H group appears weak in GR and GluOX with a N3−O (acceptor) distance of 3.2−3.3 Å (Table 2). The band X frequency was detected at 1248 cm^{-1} for GluOX (Visser et al., 1983) and assigned to a band at 1261 cm^{-1} for GR (Schmidt et al., 1983). However, another band observed at 1246 cm^{-1} and exhibiting an intensity equal to that of the 1261 cm^{-1} band was observed in the band X region of RR spectra of GR (Schmidt et al., 1983). Considering that (i) the band X frequency of deuterated GR is unambiguously assigned to a strong band at 1289 cm^{-1} (Schmidt et al., 1983) and (ii) the deuteration of various flavins and flavoproteins downshifts band X by 41−44 cm^{-1} (Nishina et al., 1978; Kitagawa et al., 1979; Benecky at al., 1979; Schmidt et al., 1983), band X of normal GR can be assigned to the 1246 cm^{-1} band and not to the 1261 cm^{-1} band. The apparent frequency shift upon solvent deuteration is indeed 43 cm^{-1} for the former band while it is 28 cm^{-1} only for the latter band. Therefore, the 1246 cm^{-1} band of GR as well as the 1248 cm^{-1} band of GluOX is in agreement with a weak H bond at N3H (Table 2). A moderate H bond at this site is expected for PHBH, FCB, and *D. vulgaris* FD (N3−acceptor distance of 3.0−3.1 Å) (Tables 2 and 3). The band X frequency of these flavoproteins is detected at 1257−1258 cm^{-1} (Visser et al., 1983; Bienstock et al., 1986; Desbois et al., 1989), i.e., at a frequency intermediate between that of GR or GluOX (1246−1248 cm^{-1}) and that of OYE (1263 cm^{-1}) (Dutta et al., 1978; Schmidt et al., 1983; Visser et al., 1983; Kitagawa et al., 1979b; Nishina et al., 1980a, 1981). Among the flavoproteins examined (Tables 2 and 3), this is precisely OYE in which the strongest H bond at N3H is expected. Beside this sensitivity of band X to the H-bonding strength at N3H, Figure 9C shows that the band X frequency is linearly correlated with the total number of H bonds at ring III (N1/O2/N3H/O4 sites). All these observations therefore establish that band X can be used either as an indicator of H-bond strength at the N3H site or as a marker of the degree of H-bonding state of ring III. From the inspection of Tables 2 and 3, these two parameters exhibit some convergence since the higher the H-bonding state at ring III (number and strength of H bonds), the stronger the H bond at N3H.

Structure and H-Bonding Environment of Oxidized Flavin Mononucleotide in L-Lactate Monooxygenase. Unlike FCB, the FMN ring in LMO appears nearly planar considering that this flavoprotein obeys the linear correlation of Figure 8. No out-of-plane distortion of the FMN ring has been reported in the structure of GlyOX for which the FMN binding site exhibits structural homologies with that of FCB and sequence homologies with that of LMO (Table 3) (Lindqvist, 1989; Lindqvist et al., 1991; Ghisla & Massey, 1991). Thus, the isoalloxazine conformation in LMO is nearly planar like in GlyOX.

Table 3 shows that the N1/N5 sites of FMN are significantly less H bonded in GlyOX than in FCB (two H bonds versus three H bonds) (Lindqvist, 1989; Lindqvist et al., 1991). The band III frequency of LMO (1553 cm^{-1}) suggests that these sites are globally strongly H bonded (Table 1, Figures 8 and 9B). A total number of four H bonds at the N1/N5 sites is a reasonable estimate for the nearly planar flavin ring in LMO (Figure 9B). The band III frequency therefore shows that the overall H bonding at N1/N5 is stronger for LMO than for GlyOX (Table 3).

Band X of FCB and LMO is located at 1257 and 1262 cm^{-1}, respectively (Table 1). These frequencies are indicative of a stronger H-bonding interaction at the flavin ring III (Figure 9C) and/or at its N3H site (Tables 2 and 3) in LMO than in FCB. The crystal structure of GlyOX shows that N3H and ring III can form two and seven H bonds, respectively (Table 3). Considering the large homology in the flavin binding site of GlyOX and LMO (Table 3), a similar H-bonding pattern at N3H (one strong or two weak H bonds) and/or at ring III (ca. eight H bonds) is plausible for LMO (Figure 9C). The possible small difference in the patterns of GlyOX and LMO could correspond to a different ring III interaction with water molecule(s) (Table 3).

In conclusion, the environment of N3H and/or of ring III as well as the FMN conformation appears to be similar in LMO and GlyOX. On the contrary, their N1/N5−protein interaction differs. The FMN of FCB are nonplanar. The H-bonding environment of N3H and/or ring III is weaker in FCB than in LMO.

Mode Assignments of the Anionic Semiquinone Form of Flavins

The one-electron reduction of flavins modifies the electron conjugation of the isoalloxazine ring. In particular, the C−C bond and the two C−N bonds of the N5═C4a−C10a═N1 moiety change their bond order upon reduction (Figure 7). The frequencies of the stretching modes involving the C4a−N5 and C10a−N1 bonds are expected to be lowered in the RR spectra of the anionic Sq forms since these bonds lose their double bond character upon flavin reduction. On the contrary, the frequency of the modes involving the C4a−C10a stretch is expected to increase its frequency in relation to the increased electron density of the C4a−C10a bond (Figure 7). Moreover, a delocalization of the negative charge carried by the N1 atom of the Sq anion radical may strenghen the C2−N1 bond and weaken the C2═O bond according to the following mesomeric forms: $N1^{-}-C2{=}O \leftrightarrow N1{=}C2-O^{-}$ (Figure 7). Therefore, the frequencies of the stretching modes involving significant contributions from the C5a−N5, N5−C4a, C4a−C10a, C10a−N1, N1−C2, and C2−O bonds are likely the most affected by the one-electron reduction of the isoalloxazine ring. These modes are expected to be significantly coupled, as in the case of oxidized flavins.

No vibrational analysis on a flavin Sq has been performed yet. However, isotopic data on a FAD_{Sq} anion stabilized by DAOX are available (Nishina et al., 1988). Assignments have been proposed for several high-frequency RR bands on the basis of (i) observed frequency shifts caused by selective isotopic substitutions at the C4a, C10a, C2, N1, N3, and/or N5 isoalloxazine atom(s) of DAOX (Nishina et al., 1988) and (ii) comparisons of RR spectra of the protein-free RF radicals (Su & Tripathi, 1994).

Modes Involving CC Bonds. The RR spectra of neutral Sq exhibit a strong band at 1611−1617 cm^{-1} (Dutta & Spiro, 1980; Nishina et al., 1980; Kitagawa et al., 1982; Sugiyama

Table 4: Frequencies (Wavenumbers) of the Bands Observed in the High-Frequency RR Spectra of Anionic Flavin Semiquinone of Flavocytochrome b_2 (FCB), L-Lactate Monooxygenase (LMO), D-Amino Acid Oxidase (DAOX), Glucose Oxidase (GluOX), and Protein-Free Riboflavin (RF)[a]

FCB		LMO						
−Pyr	+Pyr	−Pyr	+Pyr	DAOX	GluOX	RF	assignment[a]	ring
	1641	1627	1627		1623			
1613	1621	1607	1617				ν(C4O)	III
1603	1600	1597	1601	1602	1578	1610	ν(C5aC9a)	I and II
1558	1557	1563	1563	1555	1553	1558		I and II
1527	1522	1519	1521					
1500	1502	1506	1505	1516	1514	1498	ν(C4aC10a)	II and III
1462	1462	1462	1460	1448	1451	1460	ν(N1C2), ν(C4aC10a)	II and III
		1456	1466					
1419	1420	1420	1421	1422		1425	ν(CN), ν(CC)	II and III
1375	1374	1379	1376	1361				
		1354						
1348	1351	1345	1346	1331	1328	1334	ν(N5C4a)	II
1333		1334						
1295	1296	1301	1301	1292	1288	1292	ν(N5C4a)	II
	1273	1279			1270			
	1250	1261	1262					

[a] Mode assignment from Nishina et al. (1988), Su and Tripathi (1994), and this work.

et al., 1985; Xu et al., 1987; Su & Tripathi, 1994). In the spectra of Sq anions, an analogous band is observed at a lower frequency (1590−1610 cm^{-1}). The 1602 cm^{-1} band observed in the RR spectra of the Sq anion of DAOX was assigned to a ν(CC) mode of ring I taking into account its insensitivity to various isotopic labelings in rings II and III (Nishina et al., 1988). The systematic decrease in frequency of this band upon N5 protonation of RF_{Sq} specifies this ν(CC) assignment in the ring I−ring II junction [ν(C5aC9a)] (Su & Tripathi, 1994). In the RR spectra of the Sq anion of FCB and LMO, the homologous band is attributed at 1600−1603 and 1597−1601 cm^{-1}, respectively (Table 4).

The 1555−1558 cm^{-1} band of the anionic Sq form of DAOX and RF has been assigned to a ν(CC) mode centered on ring II, most likely involving a significant contribution from ν(C4aC10a) (Su & Tripathi, 1994). The corresponding band is observed at 1563 and 1557−1558 cm^{-1} in the spectra of the Sq anion of LMO and FCB, respectively (Table 4).

Modes Involving CN Bonds. Various RR bands sensitive to $^{14}N \rightarrow {}^{15}N$ substitution at the N1, N3, and/or N5 position(s) of the FAD_{Sq} anion of DAOX have been observed in the 1290−1430 cm^{-1} regions (Nishina et al., 1988). A 1292 cm^{-1} band was assigned to a mode involving ν(C4aN5) on the basis of its marked sensitivity to isotopic substitutions on the C4a and N5 atoms, respectively (Nishina et al., 1988). Its frequency is observed at ca. 1290 cm^{-1} in the RR spectra of the anionic Sq form of GluOX, MAOX, and of protein-free RF (Yue et al., 1993; Su & Tripathi, 1994). It is increased at 1295 and 1301 cm^{-1} in the spectra of the Sq form of FCB and LMO, respectively (Figures 2 and 4, Table 4). These bands exhibit significant shifts (+3−6 cm^{-1}) upon H/D exchange (Figures 2B and 5A). These upshifts suggest a changed coupling of the ν(C4N5) mode upon deuteration of the H-bonding partner(s) of the N5 atom.

The 1300−1400 cm^{-1} regions of RR spectra of FCB and LMO are complex (Figures 2, panels B and C, and 4). In the spectra of the FCB−Pyr complex, the H/D exchange shifts the 1351 cm^{-1} band by 5 cm^{-1} and apparently split the 1374 cm^{-1} band into two components at 1325 and 1379 cm^{-1} (Figure 2B). Similar spectral alterations occur in the 1330−1390 cm^{-1} regions of RR spectra of the LMO−Pyr complex (Figure 5B). From these sensitivities, one can suggest that the 1346−1351 and 1374−1376 cm^{-1} bands involve significant contribution from the ν(C2N3) and ν(C4N3) modes of ring III.

Modes Involving Flavin Carbonyl Groups. The ν(C4═O) stretching mode of oxidized flavins is Raman-active when either an ultraviolet (218 nm) or a red (647 nm) excitation is used (Copeland & Spiro, 1986; Kim & Carey, 1993). It was observed in the 1710−1725 cm^{-1} regions of RR spectra of FMN, FAD, and RF. On the other hand, the ν(C2═O) mode was detected at 1647−1663 cm^{-1} in the infrared spectra of LF and RF (Abe et al., 1986; Copeland & Spiro, 1986). The frequencies of these two ν(C═O) modes are downshifted by 13−14 cm^{-1} upon deuteration of the N3H group of ring III (Abe et al. 1986; Copeland & Spiro, 1986; Kim & Carey, 1993). An increase in relative intensity of the ν(C4═O) mode is also observed upon N3H deuteration (Kim & Carey, 1993). All these spectral changes are attributed to a coupling of the C═O stretch with a NH bending mode (Abe et al., 1987; Copeland & Spiro, 1986). As far as the Sq forms of flavins are concerned, no ν(C═O) stretching mode has been assigned. The RR spectra of the Sq form of the FCB−Pyr complex, however, exhibit a 1621 cm^{-1} band which is downshifted at 1613 cm^{-1} and increases its relative intensity upon H/D exchange (Figure 2B). Another band hidden by the ν(CC) mode at ca. 1600 cm^{-1} is apparently downshifted at 1590 cm^{-1} upon solvent deuteration (Figure 2B). On the basis of the 8−10 cm^{-1} shifts, the 1600 and 1621 cm^{-1} bands are assigned to stretching modes involving the C═O bonds of FMN_{Sq}. Similarly, the 1601 and 1617 cm^{-1} bands seen in the RR spectra of the LMO−Pyr complex are also assignable to ν(C═O)-involving modes since they exhibit downshifts of 4−7 cm^{-1} upon H/D substitution (Figure 5A). Considering the electron distribution in the anionic Sq form of flavins (Figure 7), the C2═O and C4═O groups are expected to have different bond orders. Due to a partial charge delocalization through the N1−C2═O group, the C2═O bond would be weaker than the C4═O bond (Figure 7). This relative bond order of the C═O bonds is also observed for the oxidized flavins, the C2═O carbonyl group being

conjugated with the N1═C10a−C4a═N5 system (Abe et al., 1986; Copeland & Spiro, 1986). Therefore, the bands observed at 1600−1601 and 1617−1621 cm^{-1} in the RR spectra of the FMN_{Sq} of FCB and LMO are assigned to ν(C═O) stretching modes involving primarily the C2═O and C4═O bond, respectively. The ca. 50−100 cm^{-1} downshifts of the ν(C═O) modes observed upon one-electron reduction of the flavin are associated with the decrease in π electron density at ring III. This decreased bond order is similar to that observed for the C═O bonds of *p*-benzoquinone and its radical anion (Tripathi, 1981). Using an empirical relation between ν(C═O) and the C═O bond length (Horvath et al., 1987), we can evaluate from the frequency shifts of the ν(C═O) modes the increases in bond length accompanying the one-electron reduction of FMN. This calculation estimates a shortening of the C2═O and C4═O bonds by 0.026 and 0.046 Å, respectively, upon flavin reduction. On the other hand, the decreased sensitivities of the ν(CO) modes of FMN_{Sq} to the H/D substitution (4−10 cm^{-1}), relative to the 13−14 cm^{-1} shifts observed for the oxidized flavins, may be attributed to a decreased and different coupling of the C═O stretching modes with the N3H bending modes. Finally, the enhancement of the FMN_{Sq} ν(C═O) modes by the 363.8 nm excitation could be associated with the large redshifts of the flavin electronic transitions observed upon one-electron reduction of the isoalloxazine ring (Eaton et al., 1975; Morris & Bienstock, 1986; McFarland, 1987).

Semiquinone Anion in Flavocytochrome b_2 and L-Lactate Monooxygenase

Table 4 lists the high-frequency RR bands of the FMN_{Sq} anion of FCB and LMO, in the absence and presence of Pyr. For comparison, the RR data previously obtained for the flavin Sq anion of DAOX, RF, and GluOX have been included (Nishina et al., 1988; Yue et al., 1993; Su & Tripathi, 1994).

Structure and Environment of the Flavin Mononucleotide Semiquinone in Flavocytochrome b_2 and L-Lactate Monooxygenase. For the oxidized derivatives, we have previously shown that the frequencies of bands II and III, involving ν(CN) and ν(CC) modes of the diazabuta-1,3-diene junction, are informative with respect to the H-bonding state at the N1 and N5 sites as well as the isoalloxazine structure. In the RR spectra of the anionic Sq derivatives, homologous RR bands would be those observed in the 1290−1310 and 1330−1350 cm^{-1} regions (Table 4). In addition to their sensitivities to the isotope substitutions at the N5 and C4a positions (Nishina et al., 1988), their decreased frequencies by ca. 250 cm^{-1} are indeed in agreement with the decreased bond order of the N5−C4a and C10a−N1 bonds upon flavin reduction (Figure 7). Moreover, we have observed a gradual increase in the band III frequency for the following oxidized compounds, i.e., GluOX < DAOX < RF < LMO < FCB (Figure 8). As far as the 1325−1355 cm^{-1} band of the anionic Sq derivatives is concerned, the same order in relative frequency is observed for GluOX (1328 cm^{-1}), DAOX (1331 cm^{-1}), RF (1334 cm^{-1}), LMO (1345 cm^{-1}), and FCB (1348 cm^{-1}) (Table 4). Finally, the plot of the frequency of the 1285−1305 cm^{-1} band versus that of the 1325−1355 cm^{-1} band gives a relationship very similar to that obtained with the frequencies of bands II and III of oxidized flavin compounds (Figures 8 and 10). The frequencies of the

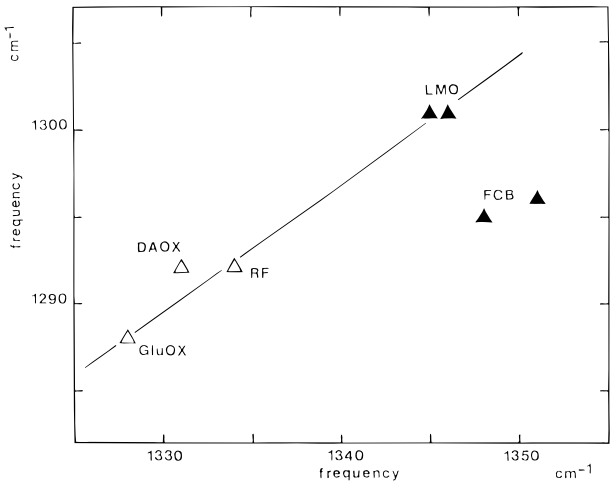

FIGURE 10: Plot of the frequency of the 1285−1300 cm^{-1} band as a function of that of the 1325−1350 cm^{-1} band of the anionic semiquinone form of riboflavin (RF), glucose oxidase (GluOX), D-amino acid oxidase (DAOX), L-lactate monooxygenase (LMO) and flavocytochrome b_2 (FCB). All the Raman data are listed in Table 4. The straight line corresponds to RF, GluOX, DAOX, and LMO and is fit with the equation $y = 0.73x + 319$ (correlation coefficient, 0.98). The filled triangles show the data presently obtained on FCB (±Pyr) and LMO (±Pyr).

1285−1305 and 1325−1355 cm^{-1} bands are linearly correlated for GluOX, DAOX, RF, and LMO while the data points obtained for FCB significantly deviate from the straight line (Figure 10). All these observations suggest that the two bands observed in the 1290−1330 cm^{-1} regions of RR spectra of anionic Sq flavins have similar vibrational origins and contain information similar to those of bands II and III of oxidized flavins. The comparison of Figures 8 and 10 strongly indicates that the differences in H bonding at the N1/N5 sites as well as in isoalloxazine conformation observed for FMN_{Ox} of LMO and FCB are preserved upon one-electron reduction of the flavin ring.

The ν(C4═O) mode of the Sq form of LMO is systematically observed at a lower frequency than that of FCB, either in the absence or in the presence of Pyr (1607 and 1617 cm^{-1} versus 1613 and 1621 cm^{-1}) (Table 4). This shift indicates that the H-bonding interaction of the flavin C4═O group with the protein or the solvent is stronger in LMO than in FCB. Whatever the association state of Pyr with FCB, the hydroxyl group of S228 is the plausible H-bond donor to the C4═O site (Xia & Mathews, 1990) (Table 3). In the sequence of LMO, P123 is the amino acid corresponding to S228 (Ghisla & Massey, 1991). The side chain of this residue cannot be a H donor to C4═O; however, the tyrosine residue (Y124) immediately following P123 could be a good H-bond donor through its hydroxyl group. In the same region, a Ser residue is conserved in the sequences of LMO (S127), FCB (S232), and GlyOX (S106) (Ghisla & Massey, 1991). On the other hand, the tridimensional structure of GlyOX indicates that the hydroxyl group of a Tyr residue is the plausible H-bond donor to the C4═O flavin group (Linqvist, 1989; Lindqvist et al., 1991). This residue is conserved in the sequences of LMO (Y152) and FCB (Y254). Therefore, the sequence comparisons suggest that the side chain of Y124, S127, or Y152 of LMO might be the H-bond donor to the C4═O group. The hydroxyl group of a Tyr residue being a better donor than that of a Ser residue (Jeffrey & Saenger, 1994) and the decreased fre-

quency of the ν(C4═O) mode of LMO rather suggests that a Tyr residue would be the H-bonding partner of C4═O.

Pyruvate Effect on the Anionic Semiquinone Form of Flavocytochrome b_2 and L-Lactate Monooxygenase. The presence of Pyr alters the redox potentials of FCB and LMO. When Pyr is added to FCB, the potential of the Ox/Sq couple is increased from −18 to +71 mV while that of the Sq/Red couple is decreased from −60 to −133 mV (Tegoni et al., 1986). This positive or negative 80 (±10) mV shift is essentially determined by the strong complex formation between Pyr and the Sq form of FCB (Tegoni et al., 1990). A similar phenomenon likely occurs for LMO. The redox potentials of the Ox/Sq and Sq/Red couples are respectively −67 and −231 mV, in the absence of Pyr (Stankovich & Fox, 1983). The tight binding of Pyr to the Sq form is expected to increase the potential separation with the Ox/Sq couple raised to approximately +80 mV and the Sq/Red couple lowered to approximately −370 mV (Ghisla & Massey, 1991).

Several factors may be invoked to account for these changes, i.e., (i) differences in flavin−protein interaction inducing changes in the electrostatic charge and/or in the polarity of the flavin environment and (ii) differences in the conformation of the flavin ring system. The Pyr binding to the anionic Sq form of LMO and FCB affects RR bands in the 1300−1380 and 1595−1620 cm^{-1} regions (Figures 2, panels B and C, and 4). Since these bands are also sensitive to the H_2O/D_2O substitution (Figures 2B and 5), one can conclude that the Pyr ligand affects polar sites of the flavin ring in influencing the protein environment around N5 and/or ring III and/or in changing the solvation state of the flavin ring. On the other hand, the modes involving major contributions from rings I or II show no significant shift upon Pyr binding (Table 4). This observation indicates that the Pyr ligand has no significant influence on the conformation of the flavin Sq ring. The most important spectral change concerns an upshift of 8−10 cm^{-1} for the mode essentially involving the C4═O bond (Figures 2 and 4, Table 4). This increase in frequency is indicative of a less H-bonded carbonyl group when Pyr is bound to the Sq form of flavoproteins. The crystal structure of FCB shows that the Pyr site is in a plane parallel to the flavin ring and involves the hydroxyl groups of Y143 and Y254, the imidazole group of H373 and the guanidine group of R376 (Xia & Mathews, 1990). In the absence of Pyr, two water molecules occupy this site (Xia & Mathews, 1990). These solvent molecules are in correct position to interact with the N5 and O4 atoms of FMN_{Sq}. The modes involving the N5 atom exhibit no significant shift upon Pyr binding (Table 4), suggesting that the H-bonding state of N5 is equivalent in the absence or presence of Pyr. However, the increased frequency of the ν(C4═O) mode observed upon Pyr binding is indicative of a strengthening of the C4═O bond and thus of a decreased H bonding at this group. This effect may originate from either (i) a decreased H-bonding interaction between the C4═O group and the hydroxyl group of Ser 228 or (ii) the exclusion of the two water molecules out of the Pyr site (Xia & Mathews, 1990; Balme & Lederer, 1994).

The free energy change (ΔG), associated with the Pyr binding to the Sq form of flavoproteins, can be obtained by the relation $\Delta G = -0.023n\Delta E$, where n is the number of electrons transfered and ΔE the change in redox potential. With $n = 1$ and $\Delta E = 80$ (±10) mV corresponding to the Ox/Sq and Sq/Red couples of FCB, the absolute value of ΔG spans from 1.6 to 2.1 kcal mol^{-1}. On the other hand, several correlations between the carbonyl vibrational frequencies and the interaction energies of the carbonyl groups have been determined. Using the relationship of Zadorozhnyi and Ishchenko (1965), we can estimate the variation in H-bonding energy of the C4═O group associated with the Pyr binding. From the 8 (±3) and 10 (±2) cm^{-1} upshifts of the ν(C4═O) mode of FCB and LMO, a change in hydrogen bond energy of 1.2 (±0.5) and 1.6 (±0.3) kcal mol^{-1} is calculated (Zadorozhnyi & Ishchenko, 1965). These energy calculations are probably slightly underestimated if one assumes that the ν(C═O) modes are rarely pure (Colthup et al., 1975). Considering that the conformation of the FMN_{Sq} of FCB and LMO is not significantly affected by the Pyr binding (Table 4), a large part of the energy of redox changes is therefore associated with a modification of the electrostatic interactions of the C4═O flavin group.

Structure−Function Relationships of Flavin Mononucleotide carried by Flavocytochrome b_2 and L-Lactate Monooxygenase

Despite the predicted structural similarities of the FMN and catalytic sites of FCB and LMO, these two flavoproteins exhibit functional differences (Ghisla & Massey, 1991; Müh et al., 1994a,b,c). The apoproteins can influence the biological activity of the flavin at three levels: (i) in selecting the H-bonding states of the polar atoms of the flavin rings, (ii) in molding the conformation of the flavin, and (iii) in making accessible to the solvent the flavin sites usable for the flavoprotein function(s). The RR spectra of FCB and LMO show that the two proteins have different interactions with the flavin ring of FMN_{Ox} and FMN_{Sq}. Under these two oxidation states, the RR spectral differences have been interpreted in terms of differences in both conformation and H-bonding environment of the flavin ring. These differences have consequences on the electron density through the isoalloxazine ring (Figure 6). On the other hand, the flavin macrocycle of FCB, in its Ox and Sq states, is found significantly distorted with respect to that of LMO, under the same oxidation states. This difference in the planarity of the flavin ring can modulate different electron densities and thus different chemical reactivities of the N5, C4, C4a, and C10a atoms which constitute key sites in the biochemistry of flavoenzymes (Bruice, 1980; Walsh, 1980; Müller, 1983; Ghisla & Massey, 1989).

The different structure and environment of the flavin ring may originate from slightly different FMN orientations in the proteins as previously observed in FCB and GlyOX (Lindqvist et al., 1991). Considering that both GlyOX and LMO exhibit an oxidase activity and likely possess a high identity in their protein structure (Ghisla & Massey, 1991; Müh et al., 1994a,b,c), one would be tempted to assume that their flavin environment is very similar. The comparison of the X-ray data on GlyOX with the present RR data on LMO strongly indicates that at least the N1/N5−protein interaction is different in the two flavoproteins.

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REFERENCES

Abe, M., & Kyogoku, Y. (1987) *Spectrochim. Acta 43A*, 1027−1037.

Abe, M., Kyogoku, Y., Kitagawa, T., Kawano, K., Ohishi, N., Takai-Susuki, A., & Yagi, K. (1986) *Spectrochim. Acta 42A*, 1059−1068.

Andersen, R. D., Apgar, P. A., Burnett, R. M., Darling, G. D., Lequesne, M. E., Mayhew, S. G., & Ludwig, M. L. (1972) *Proc. Nat. Acad. Sci. U.S.A. 69*, 3189−3191.

Balme, A., & Lederer, F. (1994) *Protein Sci. 3*, 109−117.

Benecky, M., Li, T. Y., Schmidt, J., Frerman, F., Watters, K. L., & McFarland, J. (1979) *Biochemistry 18*, 3471−3476.

Bienstock, R. J., Schopfer, L. M., & Morris, M. D. (1986) *J. Am. Chem. Soc. 108*, 1833−1838.

Bowman, W. D., & Spiro, T. G. (1980) *J. Chem. Phys. 73*, 5482−5492.

Bowman, W. D., & Spiro, T. G. (1981) *Biochemistry 20*, 3313−3318.

Bruice, T. C. (1980) *Acc. Chem. Res. 13*, 256−262.

Capeillère-Blandin, C., Barber, M. J., & Bray, R. C. (1986) *Biochem. J. 238*, 745−756.

Carreira, L. A., & Antcliff, R. A. (1982) in *Advances in Laser Spectroscopy* (Garetz, B. A., Ed.) pp 21−30, Heyden, London.

Choong, Y. S., & Massey, V. (1980) *J. Biol. Chem. 255*, 8672−8677.

Colthup, N. B., Daly, L. H., & Wiberley, S. E. (1975) *Introduction to Infrared and Raman Spectroscopy*, 2nd ed., pp 278−310, Academic Press, New York.

Copeland, R. A., & Spiro, T. G. (1986) *J. Phys. Chem. 90*, 6648−6654.

Desbois, A., Tegoni, M., Gervais, M., & Lutz, M. (1989) *Biochemistry 28*, 8011−8022.

Dixon, D. A., Lindner, D. L., Branchaud, B., & Lipscomb, W. N. (1979) *Biochemistry 18*, 5770−5775.

Dutta, P. K., & Spiro, T. G. (1980) *Biochemistry 19*, 1590−1593.

Dutta, P. K., Nestor, J. R., & Spiro, T. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4146−4149.

Dutta, P. K., Nestor, J. R., & Spiro, T. G. (1978) *Biochem. Biophys. Res. Commun. 83*, 209−216.

Eaton, W. A., Hofrichter, J., Makinen, M. W., Andersen, R. D., & Ludwig, M. L. (1975) *Biochemistry 14*, 2146−2151.

Fox, K. M., & Karplus, P. A. (1994) *Structure 2*, 1089−1105.

Gatti, D. L., Palfey, B. A., Lah, M. S., Entsch, B., Massey, V., Ballou, D. P., & Ludwig, M. L. (1994) *Science 266*, 110−114.

Gervais, M., & Tegoni, M. (1980) *Eur. J. Biochem. 111*, 357−367.

Gervais, M., Labeyrie, F., Risler, Y., & Vergnes, O. (1980) *Eur. J. Biochem. 111*, 17−31.

Ghisla, S., & Massey, V. (1989) *Eur. J. Biochem. 181*, 1−17.

Ghisla, S., & Massey, V. (1991) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) vol. II, chapter 8, pp 243−289, CRC Press, Boca Raton.

Ghisla, S., Massey, V., Lhoste, J.-M., & Mayhew, S. G. (1974) *Biochemistry 13*, 589−597.

Giegel, D. A., Williams, C. H., Jr, & Massey, V. (1990) *J. Biol. Chem. 265*, 6626−6632.

Hall, L. H., Bowers, M. L., & Durfor, C. N. (1987) *Biochemistry 26*, 7401−7409.

Hecht, H. J., Kalisz, H. M., Hendle, J., Schmid, R. D., & Schomburg, D. (1993) *J. Mol. Biol. 229*, 153−172.

Horvath, G., Illényi, J., Pusztay, L., & Simon, K. (1987) *Acta Chim. Hung. 124*, 819−822.

Irvin, R. M., Visser, A. J. W. G., Lee, J., & Carreira, L. A. (1980) *Biochemistry 19*, 4639−4646.

Jeffrey, G. A., & Saenger, W. (1994) in *Hydrogen Bonding in Biological Structures*, pp 309−411, Springer-Verlag, Berlin.

Karplus, P. A., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 701−729.

Kim, M., & Carey, P. R. (1993) *J. Am. Chem. Soc. 115*, 7015−7016.

Kitagawa, T., Nishina, Y., Kyogoku, Y., Yamano, T., Ohishi, N., Takai-Suzuki, A., & Yagi, K (1979a) *Biochemistry 9*, 1804−1808.

Kitagawa, T., Nishina, Y., Shiga, K., Watari, H., Matsumura, Y., and Yamano, T. (1979b) *J. Am. Chem. Soc. 101*, 3376−3378.

Kitagawa, T., Sakamoto, H., Sugiyama, T., & Yamano, T. (1982) *J. Biol. Chem. 257*, 12075−12080.

Labeyrie, F., Baudras, A., & Lederer, F. (1978) *Methods Enzymol. 53*, 238−256.

Laudenbach, D., Straus, N. A., Pattridge, K. A., & Ludwig, M. (1987) in *Flavins and Flavoproteins* (McCormick, D. B., & Edmondson, D., Eds.) pp 249−260, Walter de Gruyter, Berlin.

Leondiadis, L., Momenteau, M., & Desbois, A. (1992) *Inorg. Chem. 31*, 4691−4696.

Lindqvist, Y. (1989) *J. Mol. Biol. 209*, 151−166.

Lindqvist, Y., Brändén, C.-I., Mathews, F. S., & Lederer, F. (1991) *J. Biol. Chem. 266*, 3198−3207.

Lively, C. R., & McFarland, J. T. (1990) *J. Chem. Phys. 94*, 3980−3994.

Lockridge, O., Massey, V., & Sullivan, P. A. (1972) *J. Biol. Chem. 247*, 8097−8106.

Massey, V., & Palmer, G. (1966) *Biochemistry 5*, 3181−3189.

Mathews, F. S. (1991) *Curr. Opin. Struct. Biol. 1*, 954−967.

McFarland, J. T. (1987) in *Biological Applications of Raman Spectroscopy. Resonance Raman Spectra of Polyenes and Aromatics* (Spiro, T. G., Ed.) vol. 2, pp 211−302, J. Wiley and Sons, New York.

Morris, M. D., & Bienstock, R. J. (1986) in *Spectroscopy of Biological Systems* (Clark, R. J. H., & Hester, R. E., Eds.) pp 395−442, J. Wiley and Sons, New York.

Müh, U., Massey, V., & Williams, C. H. (1994a) *J. Biol. Chem. 269*, 7982−7988.

Müh, U., Williams, C. H., & Massey, V. (1994b) *J. Biol. Chem. 269*, 7989−7993.

Müh, U., Williams, C. H., & Massey, V. (1994c) *J. Biol. Chem. 269*, 7994−8000.

Müller, F. (1983) *Top. Curr. Chem. 108*, 71−107.

Müller, F., Vervoort, J., Lee, J., Horowitz, M., and Carreira, L. A. (1983) *J. Raman Spectrosc. 14*, 106−117.

Nishimura, Y., & Tsuboi, M. (1978) *Chem. Phys. Lett. 59*, 210−213.

Nishina, Y., Kitagawa, T., Shiga, K., Horiike, K., Matsumura, Y., Watari, H., & Yamano, T. (1978) *J. Biochem. 84*, 925−932.

Nishina, Y., Kitagawa, T., Shiga, K., Watari, H., & Yamano, T. (1980a) *J. Biochem. 87*, 831−839.

Nishina, Y., Shiga, K., Horiike, K., Kasai, S., Yanase, K., Matsui, K., Watari, H., & Yamano, T. (1980b) *J. Biochem. 88*, 403−409.

Nishina, Y., Shiga, K., Horiike, K., Tojo, H., Kasai, S., Matsui, K., Watari, H., & Yamano, T. (1980c) *J. Biochem. 88*, 411−416.

Nishina, Y., Shiga, K., Tojo, H., Miura, R., Watari, H., & Yamano, T. (1981) *J. Biochem. 90*, 1515−1520.

Nishina, Y., Tojo, H., & Shiga, K. (1988) *J. Biochem. 104*, 227−231.

Nishina, Y., Sato, K., Shiga, K., Fujii, S., Kuroda, K., & Miura, R. (1992) *J. Biochem. 111*, 699−706.

Othman, S., Richaud, P., Verméglio, A., & Desbois, A. (1996) *Biochemistry 35*, 9224−9234.

Pajot, P., & Groudinsky, O. (1970) *Eur. J. Biochem. 12*, 158−164.

Roussel, A., & Cambillau, C. (1991) in *Silicon Graphics Geometry Partners Directory* (Silicon Graphics, Ed.), p 86, Silicon Graphics, Mountain View, CA.

Schmidt, J., Coudron, P., Thompson, A. W., Watters, K. L., & McFarland, J. T. (1983) *Biochemistry 22*, 76−84.

Schreuder, H. A., Prick, P. A., Wierenga, R. K., Vriend, G., Wilson, K. S., Hol, W. G. J., & Drenth, J. (1989) *J. Mol. Biol. 208*, 679−696.

Schulz, G. E., Schirmer, R. H., & Pai, E. F. (1982) *J. Mol. Biol. 160*, 287−308.

Silvestrini, M. C., Brunori, M., Tegoni, M., Gervais, M., & Labeyrie, F. (1986) *Eur. J. Biochem. 161*, 465−472.

Smith, W. W., Burnett, R. M., Darling, G. D., & Ludwig, M. L. (1977) *J. Mol. Biol. 117*, 195−225.

Smith, W. W., Pattridge, K. A., & Ludwig, M. L. (1983) *J. Mol. Biol. 165*, 737−755.

Stankovich, M., & Fox, B. (1983) *Biochemistry 22*, 4466−4472.

Su, Y., & Tripathi, G. N. R. (1994) *J. Am. Chem. Soc. 116*, 4405−4407.

Sugiyama, T., Nisimoto, Y., Mason, H. S., & Loehr, T. M. (1985) *Biochemistry 24*, 3012−3019.
Sun, M., Moore, T. A., & Song, P.-S. (1972) *J. Am. Chem. Soc. 94*, 1730−1740.
Tegoni, M., & Cambillau, C. (1994) *Protein Sci. 3*, 303−313.
Tegoni, M., & Desbois, A. (1991) in *Spectroscopy of Biological Molecules* (Hester, R. E., & Girling, R. B., Eds.) pp 273−274, Royal Society of Chemistry, Cambridge.
Tegoni, M., Janot, J. M., & Labeyrie, F. (1986) *Eur. J. Biochem. 155*, 491−503.
Tegoni, M., Janot, J. M., & Labeyrie, F. (1990) *Eur. J. Biochem. 190*, 329−342.
Tripathi, G. N. R. (1981) *J. Chem. Phys. 74*, 6044−6049.
Vervoort, J., Müller, F., Mayhew, S. G., van den Berg, W. A. M., Moonen, C. T. W., & Bacher, A. X. (1986a) *Biochemistry 25*, 6789−6799.
Vervoort, J., Müller, F., O'Kane, D. J., Lee, J., & Bacher, A. (1986b) *Biochemistry 25*, 8067−8075.
Visser, A. J. W. G., Vervoort, J., O'Kane, D. J., Lee, J., & Carreira, L. A. (1983) *Eur. J. Biochem. 131*, 639−645.
Walsh, C. (1980) *Acc. Chem. Res. 13*, 148−155.
Wang, B. M., & Fritchie, C. J. (1973) *Acta Crystallogr. 29B*, 2040−2045.
Watt, W., Tulinski, A., Swenson, R. P., & Watenpaugh, K. D. (1991) *J. Mol. Biol. 218*, 195−208.
Williamson, G., Engel, P. C., Nishina, Y., & Shiga, K. (1982) *FEBS Lett. 138*, 29−32.
Xia, Z. X., & Mathews, F. S. (1990) *J. Mol. Biol. 212*, 837−863.
Xu, J., Birke, R. L., & Lombardi, J. R. (1987) *J. Am. Chem. Soc. 109*, 5645−5649.
Yue, K. T., Bhattacharyya, A. K., Zhelyaskov, V. R., & Edmonson, D. E. (1993) *Arch. Biochem. Biophys. 300*, 178−185.
Zadorozhnyi, B. A., & Ishchenko, I. K. (1965) *Opt. Spectrosc.* (Engl. Transl.) *19*, 306−308.

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