problem of ethanol abuse and its pathologic consequences.

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Resonance Raman Spectra for Flavin Derivatives Modified in the 8 Position†

Lawrence M. Schopfer,* Jeanne P. Haushalter, Michael Smith, Magdy Milad, and Michael D. Morris*

ABSTRACT: Spontaneous resonance Raman or resonance-enhanced AC-coupled inverse Raman spectra were obtained for 8-mercaptoriboflavin, 8-(dimethylamino)riboflavin, 8-hydroxyriboflavin, and 8-aminoriboflavin all free in solution. These Raman spectra were all similar to one another and markedly different from that of riboflavin. In addition, the Raman spectra of 8-mercaptoflavin bound to the apoproteins of old yellow enzyme, glucose oxidase, and L-lactate oxidase were determined. The Raman spectra of these protein-bound flavins were distinctly different from both those of the above 8-substituted riboflavins and that of riboflavin. An argument is presented in favor of assigning a quinonoid electronic structure to these protein-bound flavins.

On the basis of Raman spectroscopic studies, several investigators have recently proposed that the π-electronic structures of 8-methoxyriboflavin (Nishina et al., 1980), 8-aminoriboflavin (Nishina et al., 1980), 8-hydroxyriboflavin (Dutta et al., 1980), and 8-(methylamino)riboflavin (Dutta et al., 1980) all contain substantial contributions from a quinonoid form (see Figure 1). This conclusion was based primarily on the observation that the bands which occur at 1582 and 1547 cm^{-1} for riboflavin (bands II and III, see Table I) were markedly shifted in the Raman spectra of these modified riboflavins (Nishina et al., 1980; Dutta et al., 1980). These bands were previously assigned to carbon–nitrogen double-bond stretching modes in ring II, primarily based on isotopic substitution studies (Kitagawa et al., 1979) and normal mode analysis (Bowman & Spiro, 1981). Thus, a shift from a benzenoid to a quinonoid structure was expected to perturb these bands. Further support for this position comes from earlier work on 8-hydroxyflavins, which indicated that at neutral pH 8-hydroxyriboflavin was predominantly in the quinonoid form (Ghisla & Mayhew, 1976).

Recent studies on flavoproteins have employed modified flavins as probes of both structure and mechanism [see Massey & Hemmerich (1980) for a review]. A significant feature of this work has been the observation that the flavoprotein oxidases (as well as some other enzymes) stabilize the quinonoid form of the flavin (Massey et al., 1979). These findings provide important support for the thesis that the native flavin in flavoprotein oxidases stabilizes a negative charge in the N(1)–C(2α) locus during the course of normal reduction (Massey & Hemmerich, 1980; Massey et al., 1979; Ghisla & Massey, 1980).

The enzymological studies involving modified flavins were performed in large part with 8-mercaptoflavins. Therefore, we have sought to extend the Raman studies to include this

† From the Department of Biological Chemistry (L.M.S.) and the Department of Chemistry (M.D.M., J.P.H., M.S., and M.M.), The University of Michigan, Ann Arbor, Michigan 48109. *Received April 20, 1981.* This work was supported by U.S. Public Health Service Grant GM-11106 and National Science Foundation Grant CHE 79-15185.

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Table I: Resonance Raman Band Positions (cm^{-1})

band[a]	Rf[b]	8-N(CH_3)$_2$-Rf[c]	8-OH-Rf[d]	8-NH_2-Rf[e]	8-SH-Rf[f]	8-SH-FMN-Lact Ox[g]	8-SH-FAD-Glu Ox[h]	8-SH-FMN-OYE[i]
I	1631	1629	1635	1645	1620	1614	1606	1611
II	1582	1558	1560	1565	1548	1536	1530	1528
III	1547	1519	1508		1507	1504	1501	1500
IV	1501							
V	1462		1440	1480				
VI	1407	1405						
VII_a		1375	1365	1380	1360			
VII	1354							
VII_b		1335	1340	1330	1320	1345	1337	1343
VIII	1304							
IX	1281	1281	1285	1285		1292	1263	
X	1250	1251						
XI	1228	1212				1223	1206	1220
XII	1178							
XIII	1161					1161	1159	1168
		1133				1110		1111
						1092	1099	1087
XIV	1072					1063	1079	
						1043		1039
		1002				994	1008	1013

[a] Arbitrary numbers to identify the bands of riboflavin for purposes of discussion. [b] Riboflavin bound to aporiboflavin binding protein taken from Schopfer & Morris (1981), used here for references. [c] 8-(Dimethylamino)riboflavin in the presence of 4 M potassium iodide. [d] 8-Hydroxyriboflavin in 0.1 M KP_i buffer, pH 7.3. [e] 8-Aminoriboflavin in 0.1 M KP_i buffer, pH 7.3. [f] 8-Mercaptoriboflavin in 0.1 M $NaPP_i$, pH 8.4, plus 3 M potassium iodide. [g] 8-Mercapto-FMN L-lactate oxidase, at neutral pH. [h] 8-Mercapto-FAD glucose oxidase, at neutral pH. [i] 8-Mercapto-FMN old yellow enzyme, at neutral pH.

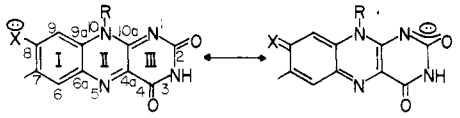

FIGURE 1: Structure of the isoalloxazine ring portion of flavin, indicating (1) the ring numbering scheme, (2) the atom numbering scheme, and (3) the canonical forms for the benzenoid and quinonoid mesomers. The X group represents any of a variety of substituents which are discussed in the text (not all of these substituents contain a free electron pair). The R group represents the remainder of the flavin, which varies depending on the level of flavin complexity (riboflavin, FMN, or FAD).

flavin. In addition, we have examined 8-(dimethylamino)-riboflavin (roseoflavin) and reexamined 8-hydroxy- and 8-aminoriboflavin. Spectra of the latter two flavins were obtained by using resonance-enhanced AC-coupled Raman techniques (Haushalter et al., 1980b; Haushalter & Morris, 1981).

8-Mercaptoflavin was studied under two distinct sets of conditions. First, we examined 8-mercaptoriboflavin free in solution, at neutral pH, where it was expected to be primarily in its thiolate form (Massey et al., 1979). Then we obtained Raman spectra for 8-mercaptoflavin bound to the apoproteins of either L-lactate oxidase, glucose oxidase, or old yellow enzyme. In this case, 8-mercaptoflavin was expected to exist primarily as its quinonoid mesomer (Massey et al., 1979).

The free solution Raman spectra of 8-hydroxy-, 8-amino-, 8-(dimethylamino)-, and 8-mercaptoriboflavin were all quite similar to one another, suggesting that all four contained similar π-electronic distributions. In the case of 8-hydroxy- and 8-aminoriboflavins, the spectra we obtained were similar to those previously published for these flavins (Nishina et al., 1980; Dutta et al., 1980). The Raman spectra of the protein-bound 8-mercaptoflavins were quite different from those of the 8-hydroxy type and from that of riboflavin. In all of the Raman spectra, bands II and III were shifted relative to their positions in the spectrum of riboflavin. We believe that these results indicate that the quinonoid structure is the dominant component in the π-electronic distribution of these protein-bound flavins, while it contributes much less to the π-electronic distribution of the free solution flavins such as 8-mercapto-, 8-hydroxy-, 8-amino-, and 8-(dimethylamino)-riboflavin.

Materials and Methods

The proteins and the corresponding apoproteins used in these experiments were prepared as previously described: L-lactate oxidase from *Mycobacterium smegmatis* (Sullivan et al., 1977; Choong et al., 1975), old yellow enzyme from brewer's bottom yeast (Abramovitz & Massey, 1976a,b), and glucose oxidase from *Aspergillus niger* (Swoboda & Massey, 1965; Swoboda, 1969). 8-Mercaptoflavins were prepared from the appropriate 8-chloroflavin by reaction with Na_2S as described (Moore et al., 1979). 8-Cl-FMN was prepared from 8-Cl-FAD by treatment with Naja naja venom (Sigma). 8-Cl-FAD was prepared from 8-chlororiboflavin by using the FAD synthetase complex, partially purified from *Brevibacterium ammoniagenes* as described (Spencer et al., 1976). The riboflavins were generous gifts: 8-chlororiboflavin from Dr. J. P. Lambooy, University of Maryland; 8-(dimethylamino)riboflavin from Dr. K. Matsui, Research Institute for Atomic Energy in Osaka, Japan; and both 8-hydroxyriboflavin and 8-aminoriboflavin from Dr. S. Ghisla, Universität Konstanz, West Germany. All other chemicals were of reagent grade and obtained commercially.

Spontaneous resonance Raman spectra were taken with a Spex 1401 double spectrometer with a cooled C31034 photomultiplier and modular photon counting electronics. The spectral slit width was 5 cm^{-1}. The scattering source varied with the sample. A 632.8-nm helium–neon laser was used for the blue, quinonoid form of 8-mercaptoflavin (bound to either L-lactate oxidase, old yellow enzyme, or glucose oxidase). A Coherent Radiation Model CR-5 argon laser was used for the free solution spectra of both 8-(dimethylamino)riboflavin (488.0-nm line) and 8-mercaptoriboflavin (514.4-nm line). Laser power was 40–50 mW at the sample.

The instrumentation and experimental procedures for resonance-enhanced AC-coupled inverse Raman spectroscopy have been previously described (Haushalter et al., 1980a,b).

Resonance Raman spectra of 8-mercaptoriboflavin (0.4 mM) were taken in 0.1 M $NaPP_i$ buffer, pH 8.4, containing approximately 3 M potassium iodide. The spectra of 8-(dimethylamino)riboflavin (0.2 mM) were taken in water containing approximately 4 M potassium iodide. The potassium iodide was included to quench the intrinsic flavin fluorescence (Benecky et al., 1979). Spectra of 8-mercaptoflavin (0.2 mM) bound to protein (L-lactate oxidase, old yellow enzyme, or glucose oxidase) were taken at neutral pH. Samples were placed in melting point capillaries, which were flushed with nitrogen and then sealed. This process increased the stability of the flavins in the laser.

Resonance-enhanced AC-coupled inverse Raman studies on 8-hydroxyriboflavin and 8-aminoriboflavin were performed on 50 μM flavin in 0.1 M KP_i buffer, pH 7.3, held in 1-cm path-length spectrophotometer cells.

UV–visible absorbance spectra were taken with a scanning double-beam spectrophotometer, Cary 17 or 118. All spectra were taken at room temperature.

Results

Protein-Bound 8-Mercaptoflavins. When bound to the apoproteins of L-lactate oxidase, glucose oxidase, or old yellow enzyme, 8-mercaptoflavin exhibits a broad absorbance peak at 595–610 nm with a molar extinction coefficient of approximately 30000 M^{-1} cm^{-1} (Massey et al., 1979). These absorbance properties are characteristic of the quinonoid form of 8-mercaptoflavins (Massey et al., 1979). The samples were nonfluorescent and relatively stable in the laser. 8-Mercapto-FMN L-lactate oxidase was completely stable in the laser for periods of up to 3 h. 8-Mercapto-FMN old yellow enzyme and 8-mercapto-FAD glucose oxidase were less stable, losing 40% and 80% (respectively) of their starting Raman amplitude over the period of 1 h. 8-Mercapto-FMN luciferase, which also exhibits the blue absorbance spectrum (L. M. Schopfer and V. Massey unpublished experiments), was found to be so unstable in the laser that no spectra could be obtained.

The Raman spectrum of 8-mercapto-FMN L-lactate oxidase is shown in Figure 2B. Though the spectra for the other two enzymes were quite similar (see Table I), there were significant differences in the positions of all the major bands. These spectral differences can best be attributed to differences in the flavin–protein interactions present for each flavoprotein.

Compared to riboflavin, a model for the predominantly benzenoid structure, these spectra appear as a distinctly different group. Band I, at 1606–1614 cm^{-1}, is shifted about 25 cm^{-1} toward lower frequencies. Bands II and III are shifted even further toward lower frequencies (about 50 cm^{-1} each). There are no bands visible in the region between 1350 and 1500 cm^{-1}. Thus, the strong riboflavin band at 1407 cm^{-1} (band VI) is missing in this form of flavin. Band VII is shifted to lower frequencies (by 10–20 cm^{-1}) while band X in the 1250-cm^{-1} region is missing. The two other riboflavin bands in the 1200–1300-cm^{-1} region (IX and XI) are essentially unshifted, as are bands XIII at 1161 cm^{-1} and XIV at 1072 cm^{-1}. Band XII is missing while additional strong bands appear around 1090, 1040, and 1000 cm^{-1}. Though the Raman spectra of these protein-bound 8-mercaptoflavins have not yet been unambiguously interpreted, they reveal that Raman spectroscopy provides an extremely sensitive probe of the local protein environment around the flavin. Further studies of these systems are in progress.

8-Mercaptoriboflavin (Free Solution Form). The UV–visible absorbance spectrum of 8-mercaptoriboflavin free in solution is vastly different from that of the bound form (Massey et al., 1979). The maximum at 523 nm (extinction

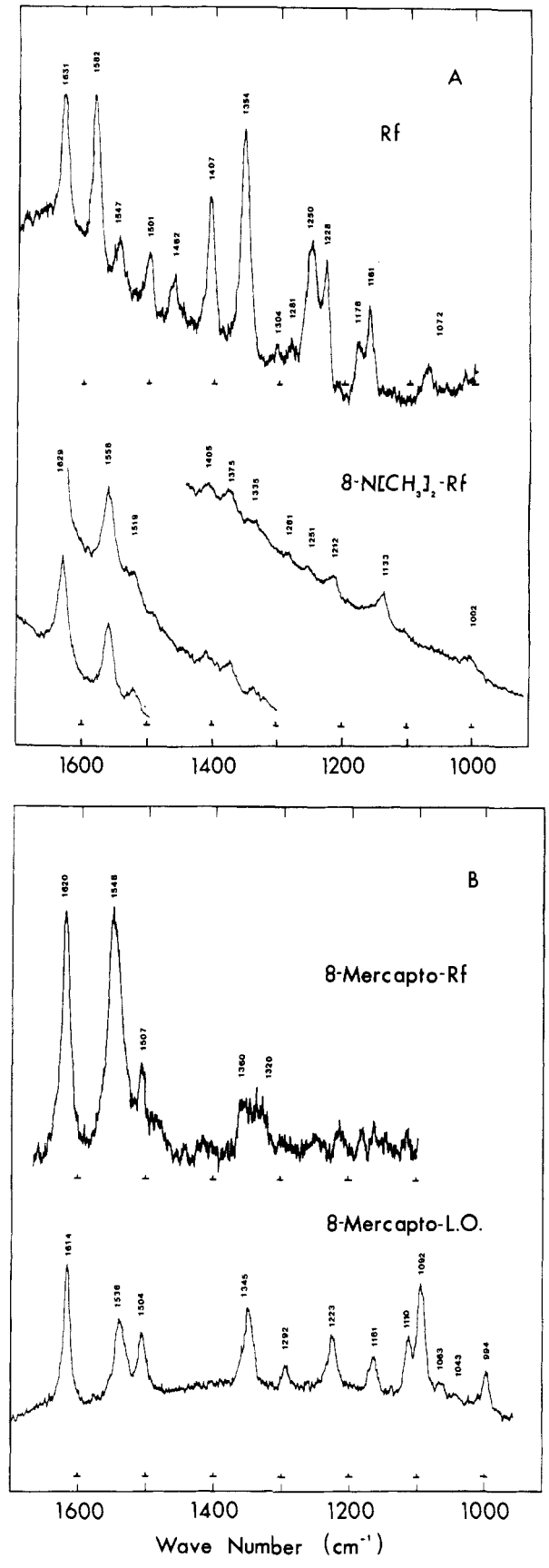

FIGURE 2: Resonance Raman spectra of four flavins. (A) Rf (riboflavin) data taken from Schopfer & Morris (1980) and shown here for comparison. 8-$N(CH_3)_2$-Rf [8-(dimethylamino)riboflavin] (0.2 mM) in water containing 4 M potassium iodide, excited at 488.0 nm. (B) 8-Mercapto-Rf (0.4 mM) in 0.1 M sodium pyrophosphate buffer pH 8.4, containing 3 M potassium iodide, excited at 514.5 nm. 8-Mercapto-LO (8-mercapto-FMN L-lactate oxidase) (0.2 mM) at neutral pH, excited at 632.8 nm.

coefficient of 30 000 M^{-1} cm^{-1}) (Massey et al., 1979) is not perturbed by the addition of potassium iodide up to 3 M. At neutral pH, this flavin is expected to exist primarily as the thiolate anion (Massey et al., 1979) i.e., benzenoid in form. The Raman spectrum (Figure 2B and Table I) is unlike that of either riboflavin (benzenoid form) or the protein-bound 8-mercaptoflavin. Bands I (1620 cm^{-1}) and II (1548 cm^{-1}) dominate the Raman spectrum. All other bands are quite weak. Band III appears at 1507 cm^{-1}. Thus, the positions of bands II and III are intermediate between those found in riboflavin and those found in the protein-bound 8-mercaptoflavins. Band VII appears as a minor broad peak (1320–1360 cm^{-1}) which probably represents more than one vibrational mode. The remaining bands are difficult to discriminate from the noise.

8-(Dimethylamino)riboflavin. The UV–visible absorbance spectrum of 8-(dimethylamino)riboflavin is blue shifted relative to that of 8-mercaptoriboflavin, with a maximum at 505 nm (extinction coefficient of 32 800 M^{-1} cm^{-1}) (Otani, 1976). Addition of potassium iodide (2 M) causes a shift in the absorbance maximum to 515 nm. This flavin was relatively stable in the laser, showing a 20% loss in amplitude during a 20-min scan. The Raman spectrum is quite similar to that of 8-mercaptoriboflavin free in solution. Bands I (1629 cm^{-1}) and II (1558 cm^{-1}) dominate the spectrum. However, the minor bands are relatively more prominent than was seen with 8-mercaptoriboflavin. Band VII appears to be split into VIIa (1375 cm^{-1}) and VIIb (1335 cm^{-1}). Band X at 1251 cm^{-1} is visible, but very small, as is band VI (1405 cm^{-1}).

8-Hydroxy- and 8-Aminoriboflavins. The UV–visible absorbance spectra of 8-hydroxyriboflavin (472 nm, extinction coefficient of 41 000 M^{-1} cm^{-1}) (Ghisla & Mayhew, 1976) and 8-aminoriboflavin (473 nm, extinction coefficient of 46 600 M^{-1} cm^{-1}) (Shiga et al., 1980) are different from that of riboflavin (445 nm, extinction coefficient of 12 500 M^{-1} cm^{-1}, 373 nm, extinction coefficient of 10 600 M^{-1} cm^{-1}) (Whitby, 1953) and those of the modified flavins described above. We were unable to quench the fluorescence of these flavins enough to obtain spontaneous resonance Raman spectra. However, we were able to obtain spectra by using resonance-enhanced AC-coupled inverse Raman spectroscopy (Haushalter et al., 1980b).

Figure 3 shows the spectra which were obtained. The bands are not Lorentzian. Vibrational frequencies were assigned by comparison of simulated bands to experimental bands. An analysis of the line shapes has been published elsewhere (Haushalter & Morris, 1981; Haushalter, 1980). The peak positions are listed in Table I. As with 8-mercaptoriboflavin and 8-(dimethylamino)riboflavin, bands I and II dominate both of these spectra. Band VII is again split while band VI is not visible. The Raman spectra for both 8-aminoriboflavin (bound to riboflavin binding protein) (Nishina et al., 1980) and 8-hydroxyriboflavin (using coherent anti-Stokes Raman scattering, CARS) (Dutta et al., 1980) reported earlier are reasonably similar to those obtained here.

Discussion

The Raman spectra of the protein-bound 8-mercaptoflavins are clearly different from that of riboflavin. It follows, therefore, that the π-electronic distribution of these flavins is unlike that of riboflavin, i.e., not predominantly benzenoid. These results are consistent with the previous assignment of the 8-mercaptoflavoprotein oxidases to a predominantly quinonoid electronic structure (Massey et al., 1979). The extent to which the quinonoid structure contributes to the overall π-electron distribution is however difficult to assess

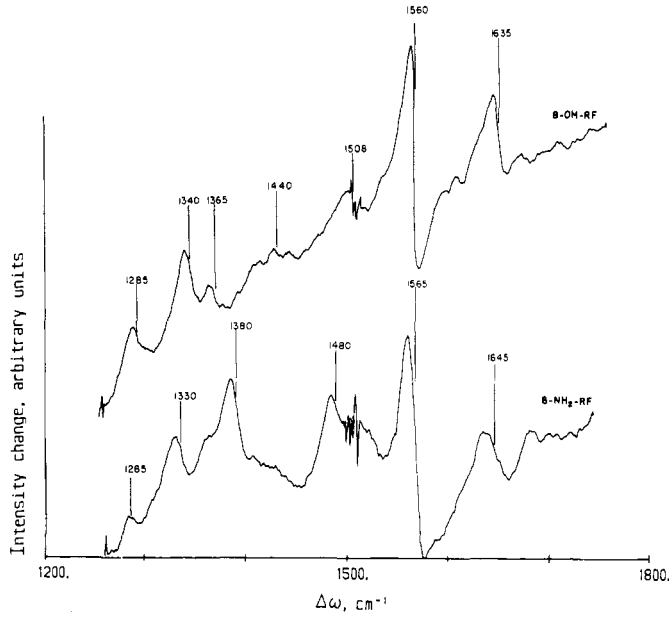

FIGURE 3: Resonance-enhanced AC-coupled inverse Raman spectra of 8-aminoriboflavin (8-NH_2-Rf) and 8-hydroxyriboflavin (8-OH-Rf). Both flavins were at a concentration of 50 μM in 0.1 M potassium phosphate buffer, pH 7.3. Samples were contained in a 1-cm pathlength spectral cell.

from the Raman data alone. Earlier workers have taken the extent to which band II shifts toward lower frequencies as a relative measure of quinonoid contribution (Nishina et al., 1980; Dutta et al., 1980). If this assessment is correct, the protein-bound 8-mercaptoflavins possess more quinonoid character than any flavin thus far described. In view of the fact that the quinonoid electronic distribution of 8-mercaptoflavins appears to be stabilized by the flavoproteins used in this study (Massey et al., 1979), it is reasonable to expect that the quinonoid electronic distribution is the major contributor in these cases. We believe that the Raman spectra reported above for 8-mercaptoflavin bound to the apoproteins of old yellow enzyme, glucose oxidase, and L-lactate oxidase are a good approximation of that of a fully quinonoid flavin structure (Figure 1). Though there is a significant amount of protein-dependent variation in the band positions, the following trends are present: bands I, II, III, and VII all appear to be shifted to lower frequencies; bands IV, V, VI, X, and XII are missing; bands IX, XI, XIII, and XIV appear to be essentially unshifted; and new bands have appeared at about 1110, 1090, 1040, and 1000 cm^{-1}. The protein-dependent variability in these spectra suggests that once proper band assignments have been made, Raman spectroscopy of the protein-bound 8-mercaptoflavin may provide important information on the nature of flavin–protein interactions.

The other flavins examined in this study, 8-hydroxyriboflavin, 8-aminoriboflavin, 8-(dimethylamino)riboflavin, and 8-mercaptoriboflavin (free in solution), possess Raman spectra which appear to be intermediate between those of riboflavin and 8-mercapto-FMN L-lactate oxidase. The π-electronic distribution of these flavins would thus be composed of significant contributions from both the benzenoid and quinonoid forms. These spectra are characterized by an apparent split in band VII, a shift in bands II and III to positions intermediate between those for riboflavin and 8-mercapto-FMN L-lactate oxidase, and a diminishing or total loss of bands IV, V, VI, and X [see also Dutta et al. (1980) and Nishina et al. (1980)]. Raman spectra of this general description are also seen for 8-(methylamino)riboflavin (Dutta et al., 1980), 8-methoxyriboflavin (Nishina et al., 1980), and 8-(methyl-

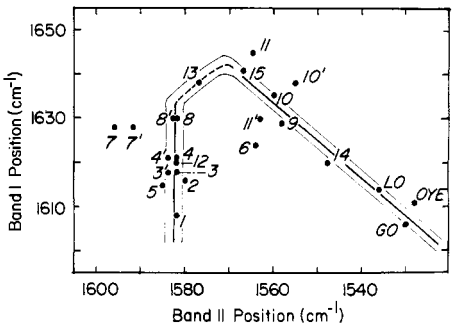

FIGURE 4: Plot of the position of band I vs. that of band II (two highest frequency bands) for a series of flavins modified at position 8. The flavins are the following: (1) 7,8-dichlororiboflavin, (2) 7-chlorolumiflavin, (3) 8-bromoriboflavin, (4) 8-chlororiboflavin, (5) 7,8-norlumiflavin, (6) 8-methylthioriboflavin, (7) 8-norlumiflavin, (8) riboflavin [taken from Schopfer & Morris (1980)], (9) 8-(dimethylamino)riboflavin, (10) 8-hydroxyriboflavin, (11) 8-aminoriboflavin, (14) 8-mercaptoriboflavin, (LO) 8-mercapto-FMN L-lactate oxidase, (OYE) 8-mercapto-FMN old yellow enzyme, (GO) 8-mercapto-FAD glucose oxidase (taken from the results in this report), (12) 8-iodoriboflavin, (4′) 8-chlororiboflavin, (3′) 8-bromoriboflavin, (7′) 8-norriboflavin, (11′) 8-aminoriboflavin, (13) 8-methoxyriboflavin, (8′) riboflavin [taken from Nishina et al. (1980)], (10′) 8-hydroxyriboflavin, (15) 8-(methylamino)riboflavin [taken from Dutta et al. (1980)].

thio)riboflavin (Schopfer & Morris, 1980).

In the preceding paragraphs, we indicated that the first three bands (highest frequency bands) in the Raman spectrum of each flavin correspond to the first three bands of riboflavin, even though the positions of these bands differ from flavin to flavin. We base these assignments upon the observation that the position of each band shifts in an orderly fashion from flavin to flavin. This can be illustrated by plotting band I vs. band II (Figure 4). The position of band II remains constant for the group of flavins on the left side of the figure (points 1–4, 7, 8, and 12), while the position of band I shifts from 1608 to 1631 cm^{-1}. The flavins in this group contain substituents which are less electron donating than a methyl group (halogens and protons). As the electron-donating capacity of the substituent approaches that of methyl (point 8, riboflavin), the position of band I moves toward higher frequency. Such behavior has been reported for disubstituted benzene (Green, 1970) and was used by Nishina et al. (1980) to assign band I to a ring I stretching mode. As the electron-donating capacity of the substituent exceeds that of methyl, band II begins to shift toward lower frequencies. Simultaneously, the shift in band I undergoes a transition which eventually causes it to move toward lower frequencies as well. For this group of "electron-donating" flavins, these two bands move in a relatively coordinated fashion which suggests a gradual transition between two states. Thus, we contend that there is a continuous shift in band II from 1582 to 1530 cm^{-1} as the benzenoid form changes to the quinonoid form. Band III moves in a parallel fashion, while band I shifts from about 1640 to 1610 cm^{-1}. We rely heavily upon this self-consistent body of data in order to support our assignment of the first three bands of the quinonoid spectra to bands I, II, and III of riboflavin. Taken out of this contextual framework, the Raman spectra of any individual flavin could be interpreted differently.

In addition to providing a basis for assigning the first three bands in the Raman spectrum of quinonoid flavins, the data in Figure 4 suggest that the shift from the benzenoid form to the quinonoid form involves a threshold transition. This threshold effect is illustrated more clearly in Figure 5, where the position of band II is plotted vs. the Hammett σ value (McDaniel & Brown, 1958) for each flavin. The position of

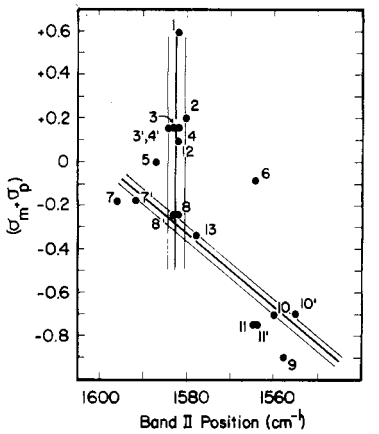

FIGURE 5: Hammett plot. The Hammett σ values for the 7- and 8-position substituents in a series of modified flavins were summed and plotted vs. the position of band II in the Raman spectrum for that flavin. Position 8 was assumed to be para and position 7 meta. σ values were taken from McDaniel & Brown (1958). The points are numbered in the same fashion as in Figure 4.

band II remains unchanged until the electron-donating capacity of the substituents exceeds that of riboflavin (point 8, Figure 5); then band II shifts toward lower frequencies. We wish to emphasize this threshold behavior in the dependence of the Raman spectrum on the electron-donating capacity of the 8-position substituent. Though the basis of this phenomenon is not yet clear, it is intriguing that the transition point should occur at riboflavin (the biologically active flavin). A similar threshold behavior appears in the UV–visible absorbance spectra of these 8-position modified flavins.

Though we feel that it is reasonable to suggest that the Raman spectral changes described above represent a shift in the π-electron distribution from a predominantly benzenoid to a predominantly quinonoid structure, the Raman evidence is not definitive. Furthermore, there are some apparent inconsistencies which need to be considered.

The most obvious inconsistency centers on the comparison of 8-hydroxyriboflavin (at neutral pH) and 8-mercapto-FMN bound to L-lactate oxidase. On the basis of extensive studies of the UV–visible absorbance spectra of model compounds, both of these flavins have been reported to exist predominantly in a quinonoid form (Massey et al., 1979; Ghisla & Mayhew, 1976). It would be expected, therefore, that their Raman spectra would be quite similar. This expectation is not borne out in fact. Such an inconsistency suggests either that one of these flavins is not as extensively quinonoid as was previously suggested or that the Raman spectrum for the quinonoid form is highly dependent upon environment. We have attempted, unsuccessfully, to obtain the Raman spectrum of 8-hydroxy-FMN L-lactate oxidase in an effort to resolve this problem. At this time, our hypothesis is that 8-hydroxyriboflavin (free in solution, at neutral pH) contains less quinonoid character than was previously believed.

A similar problem develops when one tries to predict the electron distribution of free 8-mercaptoriboflavin from both UV–visible absorbance and Raman spectral data. The absorbance spectrum of free 8-mercaptoriboflavin is considerably different from that of the 8-mercapto quinonoid form (protein bound). Therefore, the electron distribution of the free flavin would appear to be predominantly benzenoid. However, the Raman band positions for free 8-mercaptoriboflavin (bands I–III) are closer to those of the quinonoid form than to those of the benzenoid form. From this, the electron distribution for the free flavin would seem to be mainly quinonoid. We can offer no solution to this apparent dilemma. However, it

is important to note that the band positions in the Raman spectra need not be linearly related to the fraction of quinonoid character in the electronic distribution. We have used the shift in position of bands I–III as a qualitative indication of the relative amount of quinonoid character contributing to the electronic distribution of a given flavin. That is to say that free 8-mercaptoriboflavin (point 14, Figure 4) appears to have a larger quinonoid component than does 8-(dimethylamino)-riboflavin (point 9, figure 4). We do not intend to suggest that 8-mercaptoriboflavin is 70% quinonoid simply because the position of band II for this flavin is about 70% of the distance between that for riboflavin and that for the quinonoid form.

8-(Methylthio)riboflavin offers another problem. The Raman spectrum of this flavin possesses the features of a partially quinonoid form (Schopfer & Morris, 1980), as does its absorbance spectrum. However, the methylthio substituent is more electron withdrawing than is a methyl group (McDaniel & Brown, 1958). Thus, 8-(methylthio)riboflavin would be expected to appear benzenoid.

Although the relationships between Raman band positions and quinonoid character are not fully worked out, enough is known to reach some conclusions about systems in which the shifts are quite small. In an early CARS paper, a 10-cm^{-1} decrease in the position of band I was noted as one went from free FAD to riboflavin binding protein to glucose oxidase (Dutta et al., 1977, 1978). The position of band II was identical in free FAD and riboflavin binding protein but decreased 6 cm^{-1} in glucose oxidase. In addition, only one band was observed at 1357 cm^{-1} for free FAD and riboflavin binding protein, while two bands were observed (1345 and 1364 cm^{-1}) for glucose oxidase. The shifts in the glucose oxidase system are all in the same direction as those found for flavins which appear to be partially or fully quinonoid. These observations suggest that the native FAD in glucose oxidase assumes a slightly quinonoid structure. Such a change is consistent with the presence of a positively charged amino acid residue adjacent to the N(1)–C(2α) position in ring III of the flavin.

In order to shed more light on some of these questions, a more direct measure of the flavin's quinonoid character would be useful. We are currently pursuing the possibility of performing natural-abundance ^{13}C NMR in order to establish more clearly the electronic distribution of the free flavins.

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