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Resonance Raman Spectra of Flavin Derivatives Containing Chemical Modifications in Positions 7 and 8 of the Isoalloxazine Ring†

Lawrence M. Schopfer* and Michael D. Morris

ABSTRACT: The resonance Raman spectra of riboflavin, 7,8-dichlororiboflavin, 8-chlororiboflavin, 8-bromoriboflavin, 8-(methylmercapto)riboflavin, 7-chlorolumiflavin, 8-norlumiflavin, 7,8-norlumiflavin, and 3-CH_2COOH-lumiflavin were measured in complex with riboflavin binding protein, which was used as a fluorescence quenching agent. Shifts in the positions of Raman bands in the vicinity of 1250, 1405, 1550, and 1585 cm^{-1} were observed in the spectra of many of these flavin derivatives. Comparable shifts were found in the IR spectra (solid KBr) of the uncomplexed flavins. The perturbed bands have been previously assigned to reasonably localized stretching modes in the isoalloxazine system, which are well removed from the 7 and 8 positions. Thus, a direct effect on these bands due to modification of the substituents at positions 7 and 8 is precluded. These observations have led us to conclude that these Raman bands are associated with highly delocalized aromatic framework vibrations.

For some time, evidence has been building which suggests that the different chemical reactivities of the various classes of flavoproteins are due, at least in part, to differences in the flavin–protein interactions for each class (Massey et al., 1969; Massey & Hemmerich, 1980). Direct evaluation of this suggestion via X-ray crystallography is not yet a viable option. Secondary approaches for evaluating flavin–protein interactions, e.g., circular dichroism, protein modification, dye probes, and solvent perturbation, have been used on some flavoenzymes (Blankenhorn, 1978; Mayhew, 1971; Edmondson & Tollin, 1971; Bright & Porter, 1975; Williams, 1975). However, these studies provide information primarily on the protein moieties involved. Information on the nature of the flavin involvement in these flavin–protein interactions is potentially available via ^{13}C NMR using enriched flavins (Yagi et al., 1976; Grande et al., 1977). However, this technique has not been exploited to any appreciable extent. The most extensive information on the role of the flavin in flavin–protein interactions to date has come from reconstitution studies employing flavin analogues and a series of apoflavoenzymes (Massey & Hemmerich, 1980).

Recent resonance Raman studies on flavins and flavoproteins (Dutta et al., 1977; Benecky et al., 1979; Nishina et al., 1978; Kitagawa et al., 1979) have established the feasibility of using resonance Raman spectroscopy to study the flavin and its interactions with proteins. The extensive vibrational information available through Raman spectroscopy and also the

† From the Department of Biological Chemistry (L.M.S.) and the Department of Chemistry (M.D.M.), The University of Michigan, Ann Arbor, Michigan 48109. *Received February 21, 1980.* This research was supported by U.S. Public Health Service Grant GM-11106.

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Table I: Raman and Infrared Band Positions in cm^{-1} [a]

Rf[b]		7,8-Cl_2-Rf		8-Cl-Rf		8-Br-Rf		7-Cl-Lf[c]		8-nor-Lf[d]		7,8-nor-Lf[d]		8-SCH_3-Rf		3-CH_2COOH-Lf	
Raman	IR	Raman	IR	Raman	IR	Raman	IR	Raman	IR	Raman	IR	Raman	IR	Raman	IR	Raman	IR
1631	1620	1608	1600	1621	1613	1618	1608	1616	1609		1621	1616	1613	1624	1615	1631	
1582	1576	1582	1574	1582	1576	1581	1577	1580	1577	1597	1586	1587	1585	1564	1558	1587	
1547		1552		1545		1545						1560		1533			
1501		1518		1510		1508								1505		1505	
1462		1472		1456				1488						1476			
1407	1401	1397	1388	1404	1399	1403	1395	1395	1388					1405	1398	1410	
1354	1346	1350	1348	1348	1347	1350	1340	1352	1348	1353	1347	1360	1351	1348	1348	1358	
1304				1296		1298											
1281																	
1250	1255	1260	1257	1247	1252	1247	1250	1266	1260	1233	1236	1232	1220	1246	1248	1226	
1228	1227	1187	1189	1205	1207	1207	1203	1232	1228					1194	1199	1206	
1178		1152		1175		1175										1190	
1161		1120		1154		1156								1150			
								1106									
1072		1084		1086		1075				1099		1097		1084		1094	
		1010						1019						1014			

[a] Raman band positions are normalized to the 981-cm^{-1} band of sulfate; IR band positions are normalized to polystyrene. [b] Rf stands for riboflavin. [c] Lf stands for lumiflavin. [d] The Raman spectra for these flavins exhibited low signal to noise ratios.

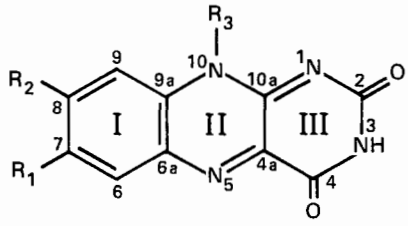

FIGURE 1: The structures of riboflavin ($R_1 = R_2 = CH_3$ and R_3 = ribityl) and lumiflavin ($R_1 = R_2 = R_3 = CH_3$).

ability to investigate selectively a chromophore in the midst of its protein environment (Spiro & Gaber, 1977) are two very attractive features of this technique. In addition, by employing flow techniques (Woodruff & Spiro, 1974), resonance Raman spectroscopy has the potential to be useful for investigating transient aspects of a reaction mechanism and the accompanying shifts in flavin–protein interactions which may occur.

Before the Raman spectra of flavoproteins can be interpreted, Raman active bands should be characterized with regard to the structural region of the flavin from which they derive. Several tentative assignments have already been reported (Dutta et al., 1977; Nichina et al., 1978; Kitagawa et al., 1979). In order to evaluate the band assignments, we have selected a series of flavins, variously modified in positions 7 and 8 of the isoalloxazine ring system (see Figure 1). Our expectations were that only Raman bands associated with the *o*-xylene subnucleus (ring I) of the isolloxazine would have been perturbed. However, we have found significant shifts occurring for bands previously assigned to the pyrazine (ring II) and uracil (ring III) subnuclei. It is our contention that these shifts in ring II and ring III bands indicate a significant aromatic framework vibrational contribution to the specific vibrations represented by these bands.

Materials and Methods

Materials. Riboflavin binding protein was prepared from hen egg white essentially according to the method of Rhodes et al. (1959). The first few steps were modified to include the dilution approach of Blankenhorn et al. (1975). Riboflavin was purchased from Sigma Chemical Co. 7,8-Dichlororiboflavin, 8-chlororiboflavin, and 8-bromoriboflavin were the generous gifts of Dr. John P. Lambooy from the University of Maryland. 7-Chlorolumiflavin, 8-norlumiflavin, 7,8-norlumiflavin, and 3-CH_2COOH-lumiflavin were the generous gifts of Dr. Peter Hemmerich from the University of Konstanz, West Germany. 8-(Methylmercapto)riboflavin was prepared as described by Moore et al. (1979). Flavin concentration was determined by titration with a standardized concentration of riboflavin binding protein. Progress of the titration was followed by monitoring changes in the UV–visible absorbance spectrum of the flavin with a Cary 118 double-beam, recording spectrophotometer.

Laser Raman Experiments. Raman spectra were taken with a Spex 1401 double spectrometer with a cooled C31034 photomultiplier and modular photon counting electronics. The scattering source was the 488.0-nm line of a Coherent Radiation Model CR-5 argon laser. Laser power was around 50 mW at the sample. The spectral slit width was around 5 cm^{-1}. The sample was held in a melting-point capillary tube at ambient temperature.

Native flavin fluorescence was quenched by binding the flavin to riboflavin protein (six- to eightfold excess of protein over flavin). Flavin was dissolved in water by being warmed and mixed with a stock solution of riboflavin binding protein (3.85 mM) in 0.1 M NaOAc buffer, pH 5.4, to a yield a final concentration of flavin of 0.15–0.4 mM. Lumiflavins, which could not be dissolved in sufficiently high concentrations to yield final concentrations of 0.15–0.4 mM directly, were mixed with riboflavin binding protein to yield more dilute solutions with the appropriate flavin–protein ratio. Then the complex was vacuum concentrated in a collodion bag (Sartorius Division, Brinkman Instruments Inc.). Raman band positions were measured relative to the 981-cm^{-1} line of SO_4^{2-} ($\sim$3%) which was included as an internal standard.

Infrared Experiments. Infrared spectra of solid flavins (in KBr) were taken on a Perkin-Elmer Model 283 infrared spectrometer. All band positions were measured relative to polystyrene.

Results and Discussion

Raman spectra of eight flavins, each bound to riboflavin binding protein, have been obtained (Figure 2). The positions of the major Raman bands are tabulated in Table I. These flavins differ from one another by virtue of the groups attached to positions 7 and 8 of ring I. Both riboflavins and lumiflavins were employed. However, both of these classes of flavins contain the chromophoric isoalloxazine nucleus. Thus, their Raman spectra would be expected to be comparable (Dutta

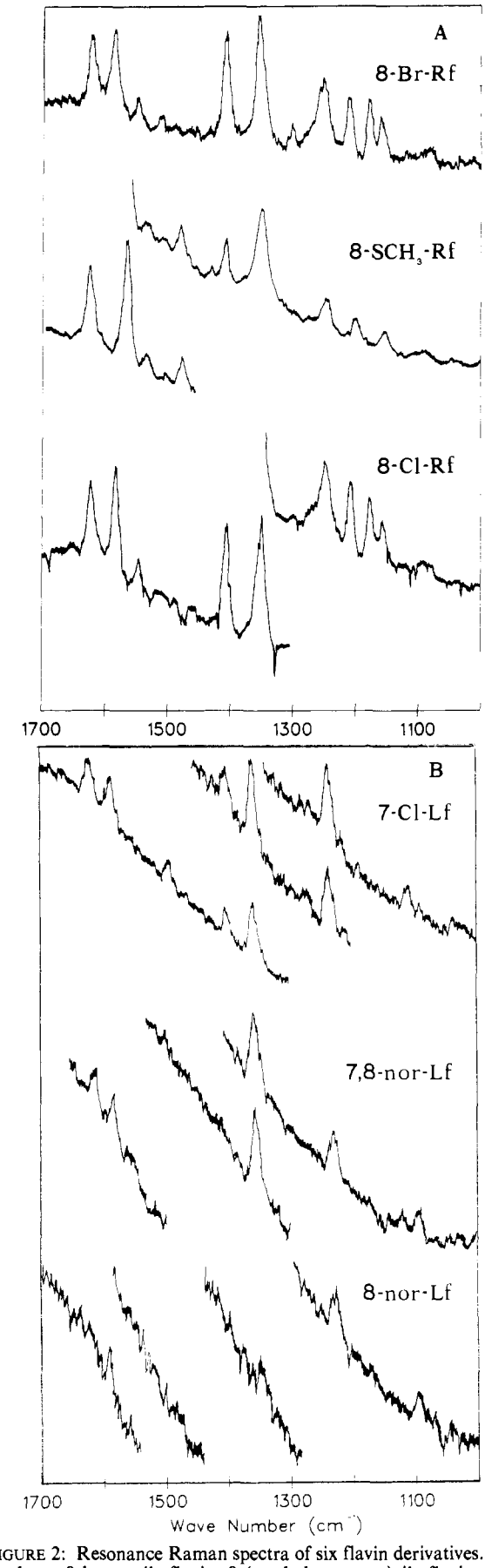

FIGURE 2: Resonance Raman spectra of six flavin derivatives. Panel A shows 8-bromoriboflavin, 8-(methylmercapto)riboflavin, and 8-chlororiboflavin. Panel B shows 7-chlorolumiflavin, 7,8-norlumiflavin, and 8-norlumiflavin. All spectra were calibrated to the 981-cm^{-1} line of SO_4^{2-}.

et al., 1978). This assumption was substantiated by comparing the Raman spectrum of 3-CH_2COOH-lumiflavin (Table I) to the published spectrum of 3-CH_2COOH-riboflavin (Nishina et al., 1978).

The Raman spectra found for both riboflavin and 7,8-dichlororiboflavin are in excellent agreement with those previously published (Nishina et al., 1978). The remaining spectra are, to a first approximation, consistent with the band assignments already published. However, a closer inspection of these spectra revealed significant perturbations in bands which had previously been assigned to ring II and ring III, i.e., bands in the vicinity of 1585, 1550, 1405, and 1250 cm^{-1}.

More specifically, both the 1582- and the weaker 1547-cm^{-1} bands of riboflavin have been assigned to C=N stretching in the central pyrazine ring, ring II (Nishina et al., 1978; Kitagawa et al., 1979). These assignments were based, in part, on the failure of these bands to be influenced by substitution of chloride for the methyl groups at positions 7 and 8 (Nishina et al., 1978). We have found that these bands were not perturbed when halogens were substituted into the 7 and/or 8 positions (Table I). However, 8-(methylmercapto)riboflavin showed a large red shift in both bands (shifting them to 1564 and 1533 cm^{-1}, respectively). It should be noted that the UV–visible absorbance spectrum of 8-(methylmercapto)riboflavin (single peak at 475 nm, extinction 32.1 mM^{-1} cm^{-1}) is considerably different from that of the more traditional two-peak spectrum (peaks at around 445 nm, extinction 11–14 mM^{-1} cm^{-1}, and around 350 nm, extinction 8–10 mM^{-1} cm^{-1}) of the other flavins in this study. Consequently, it was not surprising that the relative amplitudes of the Raman bands were different from those seen with the other flavins (see Figure 2). However, a change in the electronic spectrum does not necessarily cause a shift in the positions of the Raman active bands. On the other hand, both 7,8-norlumiflavin and 8-norlumiflavin caused a significant blue shift in the 1582-cm^{-1} band (shifting it to 1587 and 1597 cm^{-1}, respectively).

The 1407-cm^{-1} band of riboflavin has been assigned to ring III (Nishina et al., 1978; Kitagawa et al., 1979). Consistent with this assignment, the position of this band was generally unaffected by alterations in the 8 position. However, there was a significant red shift when the 7 position was occupied by a chloro residue, as in 7,8-dichlororiboflavin (1397 cm^{-1}) and 7-chlorolumiflavin (1395 cm^{-1}).

The 1250-cm^{-1} band of riboflavin, previously assigned to ring III (Dutta et al., 1977; Nishina et al., 1978; Kitagawa et al., 1979), was also sensitive to a chloro substituent in the 7 position. A blue shift was found for both 7,8-dichlororiboflavin (1260 cm^{-1}) and 7-chlorolumiflavin (1266 cm^{-1}). In addition, this band was red shifted by both 7,8-norlumiflavin (1232 cm^{-1}) and 8-norlumiflavin (1233 cm^{-1}). The possibility exists that the 1232-cm^{-1} bands of 7,8-norlumiflavin and 8-norlumiflavin could be correlated with the 1228-cm^{-1} band of riboflavin. However, this would leave no observable band to correlate with the 1250-cm^{-1} band of riboflavin. We believe that our correlation is reasonable but must be considered tentative.

Since the flavins in this study were all bound to riboflavin binding protein, the unexpected shifts in ring II and ring III modes could have been due to selected flavin–protein interactions. This possibility was tested by comparing the Raman spectrum of each flavin to its crystalline (KBr) IR spectrum. In most instances, a corresponding IR band could be found for each of the major Raman bands (Table I). For any given flavin, the exact position (wavenumber) of each band in the Raman spectrum was different from the position of the cor-

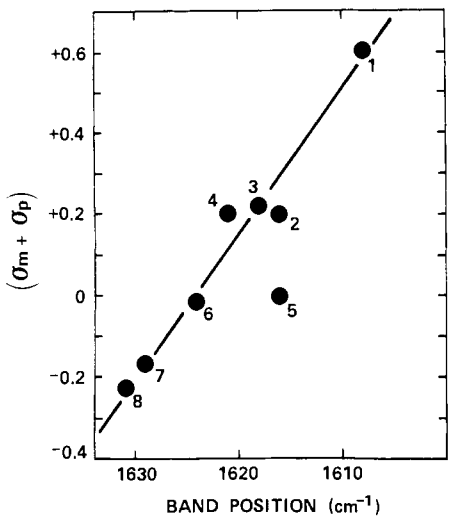

FIGURE 3: Correlation of the Hammett σ values (summed) with the position of the highest energy Raman active stretching vibration for a series of flavins modified in positions 7 and 8. σ values were taken from McDaniel & Brown (1958). It was assumed that position 7 was para and position 8 was meta for this plot. Reversing this assumption gave essentially the same values. The flavins employed were (1) 7,8-dichlororiboflavin, (2) 7-chlorolumiflavin, (3) 8-bromoriboflavin, (4) 8-chlororiboflavin, (5) 7,8-norlumiflavin, (6) 8-(methylmercapto)riboflavin, (7) 8-norlumiflavin, and (8) riboflavin.

responding band in the IR. This finding was not unexpected, since the environment of the flavin in the IR experiments (crystalline solid) was very different from that in the Raman experiments [protein induced hydrophobic domain; see Blankenhorn (1979)]. Nonetheless, the difference in band position (IR vs. Raman) for each band was essentially constant from flavin to flavin. That is, the 1631-cm^{-1} Raman band of riboflavin appeared at 1620 cm^{-1} in the IR. This band was red shifted 7–10 cm^{-1} (relative to its position in the Raman spectrum) in the IR spectrum of all the flavins investigated. Similarly, the 1582-cm^{-1} Raman band of riboflavin appeared at 1576 cm^{-1} in the IR. This band was red shifted ~6 cm^{-1} in the IR of all the flavins. Thus, the large (18-cm^{-1}) red shift seen for this band in the Raman spectrum of 8-(methylmercapto)riboflavin was accompanied by a similar shift for the corresponding band in the IR spectrum. Similar comparisons between IR and Raman spectra exist for the 1407- and 1354-cm^{-1} Raman bands of riboflavin. For the 1250-cm^{-1} Raman band of riboflavin, assignment of a corresponding IR band was complicated by the complexity of the IR spectrum in this region. Thus, the IR assignments for this band in Table I are tentative. These results argue strongly that flavin–protein interactions are not likely to be responsible for the unexpected shifts seen in the positions of the Raman bands. It follows then that the observed differences in band positions are most probably due to the intrinsic molecular properties of each flavin. The structural alterations which produced these band shifts are well removed from the loci assigned to be the primary source of the vibrational motion. Thus a significant contribution from delocalized modes of the condensed ring system appears to exist in each of these otherwise localized vibrations. A significant aromatic framework contribution is not surprising in a structure so highly conjugated as the isoalloxazine nucleus.

That the Raman bands of riboflavin at 1250 and 1407 cm^{-1} were moved in the Raman spectrum of 7,8-dichlororiboflavin was first reported by Nishina et al. (1978). These authors attributed the shifts to differences between the electronic states of riboflavin and 7,8-dichlororiboflavin. The explanation is still incomplete since the Raman and infrared spectra probe only the vibrational states of the ground electronic state.

The Raman band in the 1630-cm^{-1} region has been assigned to C=C stretching in ring I (Nishina et al., 1978; Kitagawa et al., 1979). By analogy with *o*-xylene, replacing the methyl groups with electron-donating substituents would be expected to cause this band to shift to the red. Our results are consistent with this expectation (Table I). A plot of the sum of the Hammett σ values ($\sigma_m + \sigma_p$) for each flavin vs. this band position is shown in Figure 3.

As a result of these studies, a major note of caution must be sounded for all those who intend to use Raman spectra to probe flavin–protein interactions. Reliance upon band shifts to pinpoint the portion of the flavin interacting with the protein is not totally defensible. We have clearly shown that modifications in one portion of the isoalloxazine ring system can cause band shifts throughout the system. It is suggested that before a change in Raman band position be translated into a flavin–protein interaction, a careful study of relevant model flavins be made.

Acknowledgments

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