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Normal Mode Analysis of Lumiflavin and Interpretation of Resonance Raman Spectra of Flavoproteins†

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ABSTRACT: The normal modes of lumiflavin (10-methylisoalloxazine) are analyzed with a valence force field constructed with bond length–stretching force constant correlations and bending and interaction force constants transferred from small ring molecules. Observed resonance Raman (RR) bands of flavin are assigned to calculated modes on the basis of frequency and isotope shift matching. The normal mode patterns confirm previous inferences, based on selective effects of chemical substitutions, of localization to certain regions of the molecule. These results are used to interpret the observed variability of the prominent RR bands among different flavoproteins on the basis of protein–isoalloxazine interactions.

Resonance Raman (RR) spectroscopy is potentially useful for studying flavoprotein structure and dynamics. This technique allows in situ monitoring of vibrational modes of biological chromophores via the enhancement observed when the laser excitation source is tuned to the chromophore's electronic absorption band (Spiro & Loehr, 1975; Spiro & Stein, 1977). The flavin $\pi-\pi$ transitions (Sun et al., 1972; Eaton et al., 1975), occurring in the visible and near-ultraviolet region, are expected to provide enhancement of the in-plane modes of the isoalloxazine chromophore, whose structure is shown in Figure 1. Since this is the region of the coenzyme where the redox reactions involved in flavoenzyme reactivity are localized, the RR spectrum may reasonably be expected to probe biochemically significant structure changes.

The intense fluorescence exhibited by flavins has been a deterrent to RR studies. In 1977, however, flavin RR spectra were shown (Dutta et al., 1977) to be obtainable via coherent anti-Stokes Raman scattering (CARS), a technique for generating the Raman signal as a coherent beam of light, which can be filtered spatially from the isotropic fluorescence. In 1978 Nishina et al. showed that riboflavin binding protein (RBP) quenches the fluorescence of the bound riboflavin sufficiently to obtain good quality spontaneous RR spectra. These reports kindled interest in the field, and several additional flavin RR studies have appeared in the past two years (Dutta et al., 1978, 1980; Nishimura & Tsuboi, 1978; Kitagawa et al., 1979a,b; Beneckey et al., 1979; Dutta & Spiro, 1980; Schopfer & Morris, 1980; Benecky et al., 1980). Most of the available data are on oxidized flavin, although spectra of semiquinone forms (Dutta & Spiro, 1980) and of charge-transfer complexes (Kitagawa et al., 1976b) have also been obtained.

Attention has focused initially on the assignment of the observed RR bands to specific structural elements of the chromophore. A variety of chemically and isotopically substituted flavins have been examined, and qualitative interpretations of the resulting vibrational frequency shifts have been offered. These suggestions need to be extended, however, and to this end, we have carried out a normal mode analysis of the in-plant vibrations of lumiflavin, in which the ribose substituent at N_{10} is replaced by a methyl group (Figure 1). Bond length–stretching force constant correlations were used,

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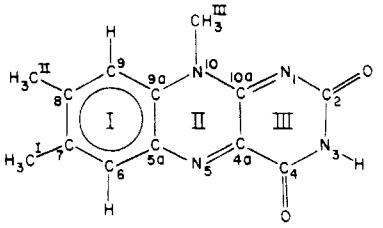

FIGURE 1: Structure and numbering for lumiflavin.

and bending and interaction force constants were transferred from smaller conjugated molecules to construct a resonable initial force field. The calculated frequencies and isotope shifts lead to satisfactory assignments for the flavin RR bands and provide insights into the observed chemical effects.

Methods

Structural Considerations (**G** *Matrix*). The **G** matrix (Wilson et al., 1955) was constructed from the X-ray data of Fritchie & Johnston (1975) on the lumiflavin-2,3-diol trihydrate complex. The C–H and N–H bond lengths were set to 1.10 and 1.03 Å to correct for the anomalously short bond distances observed in the X-ray structure (Halgren et al., 1971; Dixon et al., 1979). The methyl bond angles were assumed to be tetrahedral. All atoms except the methyl hydrogens are on the molecular plane of symmetry. The internal coordinate set contained all the valence bond stretching and planar valence angle bending displacements (Wilson, 1939). Standard symmetry coordinates were used for the methyl groups (King & Crawford, 1960), and difference coordinates were used for in-plane deformation of atoms attached to the ring.[1] This removed all local redundancies, leaving only cyclic redundancies associated with the fused three-ring system.

Force Field. The valence force field for lumiflavin was constructed by a scheme employed previously by Susi and Ard for calculations on several large heterocyclic molecules, including porphine (Susi & Ard, 1971, 1974, 1977). Diagonal stretching force constants were calculated from X-ray bond lengths by using bond length–force constant correlations previously reported for C–N (Susi & Ard, 1971) and C–O (Ladd et al., 1964) bonds. C–H stretch, N–H stretch, and symmetrized methyl force constants were taken from Susi & Ard (1974). Diagonal bending force constants were transferred from previous valence force field calculations on pyrimidines (Susi & Ard, 1971, 1974), pyrazine (Scrocco et al., 1965), and benzene (Duinker & Mills, 1968). Stretch–stretch and stretch–bend interaction constants were transferred from these same molecules. No bend–bend interactions were included. The **F** matrix was symmetrized according to the procedure given for the **G** matrix (vide supra). The resulting 65 force constant elements are given in Table I. The vibrational secular equation was solved by using previously reported computer programs (Schachtschneider, 1962).

Results

The 65 force field elements used in the calculation are given in Table I. Table II lists the calculated frequencies and potential energy distributions (contributions of >10%) for the 56 in-plane normal modes. Table III compares calculated and observed (for RBP) frequencies and ^{13}C and ^{15}N isotope shifts for the 13 RR bands, labeled I–XIII, detected above 1100 cm^{-1}. Figure 2 shows a representative RR spectrum, obtained by excitation in the long-wavelength absorption band of RBP.

[1] E.g., the symmetry coordinate for $\delta C(2)=O$ is $S = 1/(2)^{1/2}[\Delta d(O-C_2-N_1) - \Delta\alpha(O-C_2-N_3)]$.

Table I: Valency Force Constants for Lumiflavin[a]

(A) Diagonal Stretching Constants

$F_{N1-C2} = 6.351$	$F_{N10-C10a} = 6.946$
$F_{C2-N3} = 5.666$	$F_{C10a-N1} = 7.965$
$F_{N3-C4} = 6.351$	$F_{C4a-C10a} = 6.221$
$F_{C4-C4a} = 4.850$	$F_{C5a-C9a} = 6.947$
$F_{C4a-N5} = 7.965$	$F_{C2-O} = 10.217$
$F_{N5-C5a} = 6.351$	$F_{N3-H} = 5.397$
$F_{C5a-C6} = 6.694$	$F_{C4-O} = 11.223$
$F_{C6-C7} = 8.080$	$F_{C6-H} = 5.204$
$F_{C7-C8} = 6.452$	$F_{C7-CH_3} = 4.526$
$F_{C8-C9} = 7.212$	$F_{C8-CH_3} = 4.850$
$F_{C9-C9a} = 6.694$	$F_{C9-H} = 5.204$
$F_{C9a-N10} = 5.106$	$F_{N10-CH_3} = 3.809$

(B) Diagonal Bending Constants

	1.353		(ring II) 1.33		0.644
	1.421		(ring II) 1.18	δC=O δC–H, δN–H δC–CH_3 δN–CH_3	1.170 0.415 1.014 0.671
	1.062		(ring I) 1.10		

(C) Methyl Group Constants

	$C-CH_3$	$N-CH_3$
symmetric stretch	4.956	4.626
antisymmetric stretch	4.746	4.761
symmetric bend	0.569	0.603
antisymmetric bend	0.538	0.530
rock	0.663	0.852
symmetric bend, $X-CH_3$ stretch	−0.465	−0.320
$X-CH_3$ stretch, adjacent ring stretch	0.471	0.471

(D) Stretch–Stretch Interaction Constants

	ortho	meta	para
ring III	1.013[b]	−0.185	0.456
ring II	0.760	−0.110	0.280
ring I	0.531	−0.531	0.531

C=O stretch, ring stretch (adjacent) = 1.560

(E) Stretch–Bend Interaction Constants

C–N stretch, N–H bend	0.223	C=O stretch, C=O bend	0.857
C–C stretch, C–H bend	0.220	ring stretch, ring bend	
		ring III	0.295
		rings I and II	0.160

[a] Units: stretching, mdyn/Å; bending, mdyn Å/rad²; stretch-bend interactions, mdyn Å/rad². [b] This stretch-stretch interaction constant was also used for the following interactions: C_4-C_{4a}, $C_{4a}-N_5$; $N_{10}-C_{10a}$, $C_{10a}-N_1$; N_5-C_{5a}, $C_{5a}-C_6$; C_9-C_{9a}, $C_{9a}-N_{10}$.

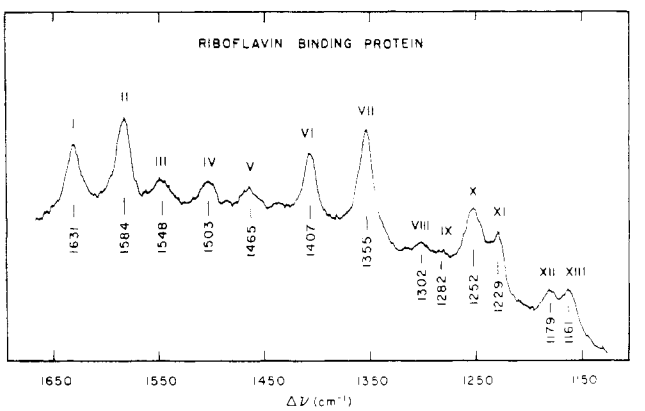

FIGURE 2: Resonance Raman spectrum of riboflavin (0.5 mM) bound to egg white riboflavin binding protein (~1 mM). Excitation wavelength = 488 nm, 100 mW. Spectral slit width = 10 cm^{-1}. Scan rate = 0.5 cm^{-1} s^{-1}.

Table II: Calculated Frequencies and Potential Energy Distribution (PED) for Lumiflavin

no.	cm^{-1}	PED (%)[a] and isotopic frequency shifts[b]	no.	cm^{-1}	PED (%)[a] and isotopic frequency shifts[b]
ν_1	3166	$\nu^{s}CH_3^{III}$ (46),[c] $\nu^{as}CH_3^{III}$ (*29*), $\nu^{as}CH_3^{III}$ (16) [0,0,0,0,0,0]	ν_{29}	1258	νC_{9a}-N_{10} (*15*), δN_3H (33) [0,1,4,1,6,7]
ν_2	3131	νN_3-H (*99*) [0,0,0,0,8,8]	ν_{30}	1204	νN_3-C_4 (*20*), νC_8-CH_3 (19), $\delta^{rock}CH_3^{III}$ (*14*) [0,2,6,0,3,3]
ν_3	3104	νC_9-H (98) [0,0,0,0,0,0]	ν_{31}	1195	νN_3-C_4 (17), νC_8-CH_3 (10), νN_{10}-CH_3 (11) [1,1,13,0,4,4]
ν_4	3096	νC_6-H (99) [0,0,0,0,0,0]			
ν_5	2987	$\nu^{as}CH_3^{II}$ (100) [0,0,0,0,0,0]	ν_{32}	1147	νN_1-C_2 (*12*), νC_2-N_3 (16), νN_3-C_4 (*17*), νC_4-C_{4a} (14), νC_{4a}-C_{10a} (*12*), νC_7-CH_3 (*16*), $\delta^{rock}CH_3^{III}$ (12) [4,3,9,1,5,6]
ν_6	2955	$\nu^{s}CH_3^{I}$ (54), $\nu^{as}CH_3^{I}$ (*43*) [0,0,0,0,0,0]			
ν_7	2939	$\nu^{as}CH_3^{III}$ (*63*), $\nu^{s}CH_3^{III}$ (*33*) [0,0,0,0,0,0]			
ν_8	2934	$\nu^{s}CH_3^{II}$ (*100*) [0,0,0,0,0,0]	ν_{33}	1124	νC_2-N_3 (11), νC_{4a}-C_4 (20), νC_{4a}-C_{10a} (*11*), νC_7-CH_3 (14), δC_6H (20) [4,8,13,0,3,3]
ν_9	2686	$\nu^{as}CH_3^{I}$ (*40*), $\nu^{s}CH_3^{I}$ (*35*), $\nu^{s}CH_3^{III}$ (17) [0,0,0,0,0,0]	ν_{34}	1108	$\delta^{rock}CH_3^{III}$ (*29*), δC_6-H (*39*) [0,5,11,0,1,1]
ν_{10}	1783	νC_6-C_7 (*33*), νC_9-C_{9a} (*17*), νC_{9a}-C_6 (11) [0,0,0,0,0,0]	ν_{35}	1024	νN_{10}-CH_3 (*11*), $\delta^{rock}CH_3^{II}$ (*56*) [0,0,1,1,1,2]
ν_{11}	1731	νC_{5a}-C_6 (10), νC_7-C_8 (18), νC_8-C_9 (13) νC_{5a}-C_{9a} (17) [0,3,5,1,0,2]	ν_{36}	1004	νN_1-C_2 (*11*), νC_2-N_3 (*11*), $\delta^{rock}CH_3^{II}$ (*24*), [1,1,3,1,5,6]
ν_{12}	1713	νC_4=O (*56*) [3,3,43,1,1,2]	ν_{37}	996	νC_2-N_3 (20), νN_{10}-CH_3 (22) [1,1,5,1,14,15]
ν_{13}	1645	νC_2= (50), νC_{10a}=N_1 (18) [15,4,43,1,5,6]	ν_{38}	912	$\delta^{rock}CH_3^{I}$ (*37*) [0,1,3,1,2,3]
ν_{14}	1615	νC_4=O (*28*), νC_2=O (28), νC_{10a}=N (*15*) [23,5,37,2,1,3]	ν_{39}	892	$\delta^{rock}CH_3^{I}$ (35)
			ν_{40}	868	νC_{4a}-C_{10a} (10), δringIII (11),[d] δringII (17)
ν_{15}	1563	νC_7-C_8 (*16*), νC_{5a}-C_6 (15) [4,1,13,3,2,4]	ν_{41}	758	νC_7-C_8 (21), νC_7-CH_3 (14), δringI (13)
ν_{16}	1511	νC_{4a}-N_5 (15), νN_{10}-C_{10a} (18), νC_{10a}-N_1 (*12*), νC_{4a}-C_{10a} (*13*) [1,11,26,5,3,8]	ν_{42}	722	δC_4=O (*10*), δringIII (21), δringII (12), δringI (20)
ν_{17}	1451	νC_{4a}-N_5 (*28*), νC_{10a}-N_1 (14) [6,11,16,5,7,12]	ν_{43}	673	δC_2=O (16), δC_4=O (17), δringI (12)
ν_{18}	1435	$\delta^{as}CH_3^{II}$ (87) [0,0,1,0,0,0]	ν_{44}	609	δringIII (16), δringII (12), δringI (13)
ν_{19}	1433	$\delta^{as}CH_3^{I}$ (*85*) [0,1,7,0,0,1]	ν_{45}	575	δC_7-CH_3 (*15*), δC_8-CH_3 (*17*), δringIII (19), δringI (17)
ν_{20}	1424	$\delta^{as}CH_3^{III}$ (*72*), $\delta^{s}CH_3^{III}$ (*10*) [1,1,6,0,1,1]			
ν_{21}	1407	νC_6-C_7 (*14*), νC_8-C_9 (*13*), νC_9-C_{9a} (10), $\delta^{as}CH_3^{III}$ (10) [0,2,3,2,0,2]	ν_{46}	528	δringII (29), δringI (11)
			ν_{47}	506	δringIII (55), δringI (11)
ν_{22}	1394	νN_1-C_2 (12), νC_{5a}-C_6 (*17*), νC_8-C_9 (23), νC_{5a}-C_{9a} (19) [4,3,7,2,3,4]	ν_{48}	444	δringIII (36), δringI (21)
			ν_{49}	424	δC_2=O (29), δringI (19)
ν_{23}	1386	$\delta^{s}CH_3^{III}$ (56), $\delta^{as}CH_3^{III}$ (*11*) [2,0,6,0,1,2]	ν_{50}	387	δC_4=O (*19*), δringI (22)
ν_{24}	1379	νN_{10}-C_{10a} (22), νC_{5a}-C_{9a} (12), $\delta^{s}CH_3^{III}$ (*19*) [9,2,25,0,4,4]	ν_{51}	320	δC_7-CH_3 (25), δC_8-CH_3 (*17*), δC_9-H (*32*)
			ν_{52}	300	δC_8-CH_3 (25), δC_9-H (*52*)
ν_{25}	1344	νN_5-C_{5a} (14), $\delta^{s}CH_3^{II}$ (*73*) [0,1,1,2,0,2]	ν_{53}	296	δC_7-CH_3 (14), δN_{10}-CH_3 (27), δringII (20)
ν_{26}	1316	νN_5-C_{5a} (*18*), $\delta^{s}CH_3^{II}$ (*24*) [0,2,3,5,0,5]	ν_{54}	255	δringIII (10), δringII (23), δringI (13)
ν_{27}	1281	$\delta^{s}CH_3^{I}$ (*59*) [0,1,2,0,1,1]	ν_{55}	189	δN_{10}-CH_3 (*26*), δringIII (10), δringII (35), δringI (13)
ν_{28}	1269	$\delta^{s}CH_3^{I}$ (*24*), δN_3H (*28*) [1,4,6,0,4,5]			
			ν_{56}	109	δringIII (36), δringII (16), δringI (20)

[a] Only contributions ≥10% are included. Italicized percent contributions indicate that the corresponding eigenvector element is negative. [b] The numbers in brackets are isotopic frequency shifts for 2-^{13}C, 4a-^{13}C, 2,4,4a,10a-^{13}C, 5-^{15}N, 1,3-^{15}N, and 1,3,5-^{15}N. [c] Methyl groups are numbered as shown in Figure 1. s and as superscripts refer to symmetric and antisymmetric coordinates as described by King & Crawford (1960). [d] δringI, δringII, and δringIII represent the sum of contributions from all internal angles for that ring; rings are numbered as shown in Figure 1.

Table III: Assignments and Isotopic Frequency Shifts for Resonance-Enhanced Modes

band[a] label	mode no.	obsd	calcd	isotopic frequency shifts[b] 2-^{13}C	4a-^{13}C	2,4,4a,10a-^{13}C	5-^{15}N	1,3-^{15}N	1,3,5-^{15}N
I	11	1631	1731	2 (0)	1 (3)	2 (5)	1 (1)	1 (0)	2 (2)
II	16	1584	1510	3 (1)	8 (11)	13 (26)	4 (5)	1 (3)	5 (8)
III	17	1548	1451	6 (6)	5 (11)	21 (16)	3 (5)	2 (7)	4 (12)
IV	20	1503	1424	3 (1)	2 (1)	7 (6)	1 (0)	2 (1)	4 (1)
V	21	1465	1407	5 (0)	6 (2)	8 (3)	1 (2)	3 (0)	6 (2)
VI	22	1407	1394	3 (4)	5 (3)	12 (7)	1 (2)	4 (3)	6 (4)
VII	24	1355	1379	4 (9)	3 (2)	15 (25)	0 (0)	1 (4)	1 (4)
VIII	25	1302	1334	0 (0)	2 (1)	5 (1)	0 (2)	0 (0)	2 (2)
IX	27	1281	1281	0 (0)	2 (1)	? (2)	0 (0)	0 (1)	0 (1)
X[c]		1252		14 (–)	0 (–)	17 (–)	0 (–)	10 (–)	10 (–)
XI	30	1229	1204	0 (0)	3 (2)	4 (6)	0 (0)	2 (3)	2 (3)
XII	32	1179	1147	3 (4)	4 (3)	8 (9)	2 (1)	4 (5)	6 (6)
XIII	33	1161	1124	1 (4)	2 (8)	5 (13)	1 (0)	1 (3)	2 (3)

[a] See Figure 2 for band assignments. [b] Observed isotopic frequency shifts according to Kitagawa et al. (1979). Calculated shifts are given in parentheses. [c] This band is not assigned to a calculated mode; see text for details.

A number of low-frequency RR bands have also been reported, but there is no clear basis at present for assigning them to the numerous low-frequency deformation modes that are calculated.

A total of 25 frequencies are calculated to fall between 1100 and 1800 cm^{-1}. The assignment of 13 of these to the observed RR bands is based on the general frequency order, a detailed match of the observed isotope shifts (modes other than those given in Table III have much more discrepant shifts), and chemical considerations, as discussed below. The disparity between observed and calculated frequencies is as high as 100 cm^{-1}, and some of the calculated isotope shifts are significantly in error. Many of these discrepancies could have been removed by judicious adjustment of the interaction force constants. However, refinement of the force field would be premature because of the incompleteness of the vibrational data. Only

a minority of the isoalloxazine normal modes have been observed at all, and the isotope data are restricted to substitutions in a limited region of the molecule.

Assignments

C–H and N–H Stretching. The three bonds connecting H to ring atoms, N_3–H, C_9–H and C_6–H, are calculated to give rise to relatively pure modes clustered around 3100 cm^{-1}. Six methyl C–H stretches, arising from symmetric and asymmetric stretches[2] on each methyl group, are calculated over a range of frequencies from 3166 to 2686 cm^{-1}. These modes are not expected to be resonance enhanced, since they are not coupled to the π electronic system, and have not been reported in RR spectra.

Band I (1631 cm^{-1}). The two frequencies calculated just below the H stretching modes, ν_{10} = 1783 and ν_{11} = 1713 cm^{-1}, are modes of the xylene portion, ring I, of the isoalloxazine skeleton, both involving in-phase stretching of bonds para to one another. (Note that the potential energy contributions in Table II are italicized when the internal coordinate displacements are opposite in phase to those which are not underlined.) This pair is readily correlated with the degenerate (E_{2g}) benzene mode at 1596 cm^{-1}, Wilson vibrations 8a and 8b (Wilson, 1934). The two components split in *o*-xylene to 8a = 1607 and 8b = 1585 cm^{-1}, but in 2,3,5,6-tetrasubstituted benzene derivatives, 8b > 8a, and both frequencies are somewhat higher than in *o*-xylene (Varsanyi, 1969). Flavin band I, at 1631 cm^{-1}, has been shown to shift down upon 7,8-dichloro substitution, by Nishina et al. (1978), who associated it with the 1607-cm^{-1} *o*-xylene mode. We assign it to ν_{11} (8a), calculated at 1713 cm^{-1}. The 100-cm^{-1} discrepancy is attributable to the likelihood that the transferred benzene para stretch–stretch interaction constant, which would specifically influence this para stretching mode, is too high for the tetrasubstituted benzene ring of flavin. The 8b counterpart, ν_{10}, is either not enhanced in the RR spectrum or is not resolved from 8a; the separation between these modes in other tetrasubstituted benzene derivatives is often small (Varsanyi, 1969).

C=O and C=N Stretching. The next calculated frequency, ν_{12} = 1713 cm^{-1}, is mainly C_4=O_9 stretching, while the following pair, ν_{13} = 1645 and ν_{14} = 1615 cm^{-1}, are a combination of C=O (mostly C_2=O) and C_{10a}=N_1 stretching. These are identifiable with three strong infrared bands seen at 1727, 1663, and 1651 cm^{-1} in a Ag^+ complex of flavin mononucleotide (FMN) (Benecky et al., 1980); in FMN itself the latter two are unresolved (Benecky et al., 1980; Nagy et al., 1979). A similar pattern is found in uracil (Susi & Ard, 1971; Bowman & Spiro, 1980), with infrared bands at 1716, 1664, and 1611 cm^{-1}, except that the ring double bond is C=C, which is conjugated with C_4=O instead of C_2=O. These modes are not enhanced via resonance with the lowest energy π–π^* transition, either in uracil (Nishimura et al., 1978) or in flavin.

Bands II and III (1584 and 1548 cm^{-1}). The next mode, ν_{15} = 1563 cm^{-1}, involves mostly ring I stretching coordinates and is correlated with mode 19 of benzene (Varsanyi, 1969). It does not seem to be resonance enhanced. There is a strong flavin mode at 1584 cm^{-1}, band II, but we assign it to ν_{16}, calculated at 1511 cm^{-1}, because of its large isotope shifts for $^{15}N_5$ and $^{13}C_{4a}$ substitution (Table III, Kitagawa et al., 1979a). ν_{16} has a major component of C_{4a}=N_5 stretching, and also of C_{4a}–C_{10} and C_{10a}–N_{10} stretching, in alternating phase. In this it resembles the pyrazine mode at 1584 cm^{-1} (Lord et al., 1957). This correspondence has been noted by Dutta & Spiro (1980), along with the remarkable parallel in the upshift on pyrazine protonation (1612 cm^{-1}) and protonated flavin semiquinone formation (1611 cm^{-1}). Moreover, the 1584- and 1611-cm^{-1} modes of oxidized flavin and semiquinone are the only bands that are observed to be enhanced in the second π–π^* transition of these two species (Dutta & Spiro, 1980). It appears that the molecular distortion in the second π–π^* state (Eaton et al., 1975) specifically involves a large displacement along the ν_{16} normal mode.

This mode also involves a significant contribution of C_{10a}=N_1 stretching, out of phase with C_{4a}=N_5. Thus it strongly involves the N_5=C_{4a}–C_{10a}=N_1 conjugation pathway. This accounts for the large downshift of band II when the conjugation pathway is altered by substitution, at position 8, of electron-donor groups which tend to stabilize the quinoid resonance structure shown in Figure 3, e.g., CH_3HN (1567 cm^{-1}) or O^- (1555 cm^{-1}) (Dutta et al., 1980), and CH_3S (1564 cm^{-1}) (Schopfer & Morris, 1980), or by substitution of CH for N_3 (1569 cm^{-1}) (Dutta et al., 1980).

Band III, 1548 cm^{-1}, is a low-intensity neighbor of band II, with a similar isotope shift pattern (Table III). It is assigned to ν_{17}, calculated at 1451 cm^{-1}, which also involves out-of-phase stretching of C_{4a}=N_5 and C_{10a}=N_1. The omission of an interaction force constant between these two stretches accounts for the low frequencies calculated for bands II and III.

Bands IV, V, and VI (1503, 1465, and 1407 cm^{-1}). The next three calculated modes, ν_{18}–ν_{20}, are primarily CH_3 deformations, with small contributions from ring coordinates. ν_{21} is primarily a ring I mode, while ν_{22}, at 1394 cm^{-1}, has important contributions from both ring I and ring III. For this reason, and because of the isotope shift pattern, we assign ν_{22} to the relatively strong band VI, at 1407 cm^{-1}, which shifts down on chemical substitutions both on ring I (7,8-dichloro; Nishina et al., 1978) and on ring III (deprotonated N_3 or 1- and 3-deaza; Dutta et al., 1980). The weak bands IV and V, at 1503 and 1465 cm^{-1}, are assigned to ν_{20} and ν_{21} calculated at 1424 and 1407 cm^{-1}. ν_{18} and ν_{19} are less likely candidates, since they are nearly pure deformation modes of 8- and 7-CH_3 groups, respectively, while bands IV and V are reported (Schopfer & Morris, 1980) still to be present upon 7,8-dichloro substitution.

Band VII (1355 cm^{-1}). The next mode, ν_{23}, is again composed mainly of CH_3 deformation. We assign the strong band VII, at 1359 cm^{-1}, to ν_{24}, calculated at 1379 cm^{-1}, which is mainly in-phase stretching of the N_{10}–C_{10a} and C_{9a}–C_{5a} bonds of ring II. Because of strong downshifts (12–18 cm^{-1}) upon CH substitution for N_1, N_3, or N_5, this band had been suggested to involve the N_5–C_{4a}–C_{10a}–N_1–C_2 system (Dutta et al., 1980), but this assignment is inconsistent with the very small ^{15}N shifts. The deaza shifts must involve alterations in the conjugation pathway.

Bands VIII and IX (1302 and 1281 cm^{-1}). These weak bands are assigned to ν_{25} and ν_{27}, at 1334 and 1281 cm^{-1}, involving 7- and 8-CH_3 deformations primarily. They are not seen in 7,8-dichlororiboflavin (Nishina et al., 1978). The intervening ν_{26}, at 1316 cm^{-1}, which is similar in composition to ν_{27} is apparently not observed.

Band X (1252 cm^{-1}). This band has been the focus of much attention because of its strong upshift, to ~1295 cm^{-1}, upon N_3 deuteration (Nishina et al., 1978; Dutta et al., 1978). A similar mode is observed in uracil at 1236 cm^{-1}, shifting to 1258 cm^{-1} upon N_1–N_3 dideuteration (Susi & Ard, 1971). This effect has been explained (Nishina et al., 1978) as re-

[2] The 2-fold degeneracy of the asymmetric stretches is preserved via the CH_3 symmetry coordinates, despite the fact that the molecule as a whole lacks the 3-fold symmetry of the methyl group.

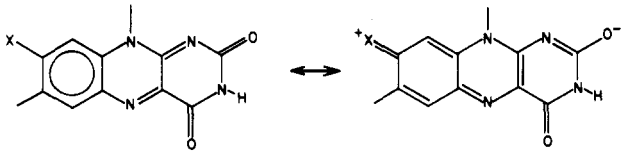

FIGURE 3: Quinoid resonance structure for 8-substituted isoalloxazines.

sulting from an interaction of a C–N_3 stretching mode with an unobserved N_3–H bending mode, whose frequency is expected to be somewhat higher; deuteration would greatly lower this frequency and relieve the interaction, allowing the 1252-cm^{-1} mode to rise. Our calculation produced two modes, ν_{28} and ν_{29}, at 1269 and 1258 cm^{-1}, with substantial N_3H bending contributions. But neither one is calculated to shift up on deuteration; nor do they give the large $^{13}C_2$ isotope shift observed for band X (Table III). Likewise, standard valence force field calculations on uracil fail to show a deuteration upshift (Nishimura, 1978; Susi & Ard, 1971). A molecular orbital constrained force field did give an upshift of the uracil 1236-cm^{-1} mode (Bowman & Spiro, 1980). It was then found to have only a small contribution from NH bending, but a large contribution from C=O bending, which, however, decreased on deuteration, leaving a relatively pure C–N stretching mode at a higher frequency. A similar change in normal mode composition is likely to account for the deuteration upshift of the flavin 1252-cm^{-1} mode. A much more sophisticated calculation will be needed to explore the nature of this important band, which is believed to be responsive to H bonding at the heteroatoms of ring III (Bowman & Spiro, 1980).

Band XI (1229 cm^{-1}). This band is assigned to ν_{30}, calculated at 1204 cm^{-1}. The mode contains a substantial contribution from C_8–CH_3 stretching, accounting for its large 7,8-dichloro shift (Nishina et al., 1978), but also from N_3–C_4 stretching, which might explain its 12-cm^{-1} shift upon Ag^+ complexation (Benecky et al., 1980), believed to involve C_4=O and N_5.

Bands XII and XIII (1179 and 1161 cm^{-1}). Band XII is assigned to ν_{32} (1147 cm^{-1}), which contains a substantial C_7–CH_3 stretching contribution, rather than to ν_{31}, which contains a similar C_8–CH_3 contribution, because it is unaffected by 8-Cl or 8-Br substitution but decreases 11 cm^{-1} upon 7,8-dichloro substitution (Schopfer & Morris, 1980). ν_{32} also has bond alternate ring III character, analogous to the 1217-cm^{-1} mode of uracil (Bowman & Spiro, 1980).

The same 7,8 substitution pattern is shown by band XIII, at 1161 cm^{-1} (Schopfer & Morris, 1980). It is assigned to ν_{33} (1124 cm^{-1}), which also contains a C_7–CH_3 stretching contribution, as well as substantial ring III character.

Discussion

The present calculation must be considered only a starting approximation to the analysis of the flavin normal modes. It nevertheless does provide a framework for understanding the selective effects observed for chemical substitutions. Although the internal coordinates mix extensively, as expected, they do segregate themselves into identifiable regions of the molecule in certain modes. Attention naturally focuses on the most prominent RR bands, I, II, VI, VII, and X, which are readily identified and monitored.

Band I is clearly localized on ring I, as suggested by Nishina et al. (1978). It shifts up when position 8 is substituted with an electron-donating group which shifts the resonance structure toward the quinoid form (Figure 3), e.g., 1641 and 1638 cm^{-1} for 8-CH_3NH and 8-O^- (Dutta et al., 1980). Schopfer & Morris (1980) report that the band I frequency shows an inverse linear correlation with Hammet σ values for the 7,8 substituents. The slight upshift in band I for flavin adenine dinucleotide (FAD) in water (1635 cm^{-1}) has been suggested (Dutta & Spiro, 1980) to result from stabilization of the 8-H^+CH_2= resonance form responsible for the appreciable 8-CH_3 H–D exchange rate (Bullock & Jardetzky, 1965).

Band II at 1584 cm^{-1} is localized mainly on ring II, as has previously been inferred (Kitagawa et al., 1979a; Dutta et al., 1980), but it also involves C_{10a}=N_1 and can be described as an out-of-phase stretching of the conjugated double bonds N_5=C_{4a} and C_{10a}=N_1. It decreases strongly as the resonance structure shifts to the quinoid form (1587 cm^{-1} for 8-CH_3NH and 1555 cm^{-1} for 8-O^-; Dutta et al., 1980). This is opposite to the response of band I, and on the bais of the above discussion, band II would be expected to shift down in water, as 8-H^+CH_2= is stabilized. However, the frequency is the same in water as in RBP, 1584 cm^{-1} (Dutta et al., 1978), and decreases to 1577 cm^{-1} in flavodoxin (Dutta & Spiro, 1980) and in glucose oxidase (Dutta et al., 1977). There appear to be protein influences which run counter to the 8-H^+CH_2= effect.

One possibility is a charge-transfer interaction, since flavodoxin is known (Smith et al., 1977) to have an indole ring (Trp-90) stacked against the isoalloxazine. Such an interaction is not expected to alter ground-state frequencies appreciably, however, although the RR intensity might be further enhanced. This effect is seen in charge-transfer complexes of phenols with old yellow enzyme (Kitagawa et al., 1979b; Nishina et al., 1980), which show marked intensification of band II, but only small frequency shifts (1583–1588 cm^{-1}). It is notable that the band II relative intensity is not greatly different for FAD in water, RBP, flavodoxin, or glucose oxidase (Dutta et al., 1978, 1980). Thus, the indole interaction appears not to influence the RR spectrum.

H bonding to either N_5 or N_1 might be expected to lower the band II frequency since these heteroatoms are at either end of the conjugated double bond system. Flavodoxin has a N_5 H bond in the semiquinone but not in the oxidized form (Smith et al., 1977). There is a potential H-bond donor (Gly-89 NH) in the vicinity of N_1 and C_2=O; H bonding in this region might be responsible for the lowered band II frequency in flavodoxin and glucose oxidase.

This interaction might also explain the lowered band VI frequency in flavodoxin (1409 cm^{-1}) relative to FAD in H_2O (1416 cm^{-1}) since this mode contains a substantial contribution from N_1–C_2 stretching. However, RBP shows the same frequency (1407 cm^{-1}) while that of glucose oxidase is even lower (1404 cm^{-1}); the shifts in bands II and VI are not parallel, and somewhat different influences appear to be at play in the three proteins so far studied.

This is even more apparent for bands VII and X. RBP and flavodoxin have essentially the same frequency as FAD in H_2O for band VII (1357, 1355, and 1359 cm^{-1}), which is also localized mainly on ring II but not on the double bond system. The glucose oxidase frequency, however, is higher, 1364 cm^{-1} (and there is a neighboring band, 1345 cm^{-1}, as yet unidentified, which is unique to glucose oxidase). Band X, which clearly involves the ring III region around N_3, and is believed to be sensitive to H bonding from N_3H, and possibly also to C_2=O and C_4=O (Bowman & Spiro, 1980), shows the greatest variability among the proteins. Flavodoxin shows essentially the same band X frequency (1257 cm^{-1}) as FAD in water (1260 cm^{-1}) while that of RBP (1252 cm^{-1}) is 8 cm^{-1} lower. In glucose oxidase this band is *missing*, but its upshifted counterpart in D_2O is observed, at 1287 cm^{-1}, 6 cm^{-1} lower than that of RBP and 10 cm^{-1} lower than that of FAD in H_2O.

Thus the H-bond pattern appears to appreciably different for the these proteins.

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