Hydrogen Bonding between Flavin and Protein: A Resonance Raman Study†

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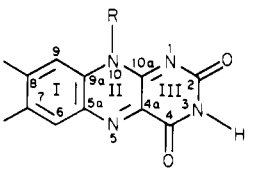
ABSTRACT: The effect of hydrogen bonding between flavins and solvent has been studied by resonance Raman (RR) spectroscopy. This has been accomplished by using several polar solvents which differ markedly in hydrogen-bonding ability. Included in this study are water (a good hydrogen-bond acceptor and donor), dimethyl sulfoxide (a weak hydrogen-bond acceptor) and acetonitrile (which is neither an acceptor nor donor of hydrogen bonds). Visible spectral studies on flavin species in Me_2SO are consistent with the picture of hydrogen bonding outlined above. There are several RR spectral changes between aqueous flavin species and flavin in a non-hydrogen-bonding solvent. Band II shifts to higher frequency, band IX disappears or shifts to lower frequency, band X shifts to lower frequency, and the intensities of bands III and IV are changed (see Figure 2 for band nomenclature). Bands X and IX shift upon deuteration in D_2O and therefore involve δN-H at N3 of the flavin (the only exchangeable position). The RR spectra of three flavoproteins have been examined. When compared to an aqueous solution of FAD, fatty acyl-CoA dehydrogenase shows a change in band IX consisting of the disappearance or shift to lower frequency of this band. This change is identical with the difference between the RR spectrum of riboflavin in 67% Me_2SO-33% H_2O when compared to that of an aqueous solution of riboflavin. The RR spectrum of FAD of fatty acyl-CoA oxidase differs from that of aqueous FAD by a shift of band II to higher frequency, the shift of band X to lower frequency, and the shift or disappearance of band IX. These changes are identical with those observed upon dissolving riboflavin in non-hydrogen-bonding solvent. In addition, band VI is split in the oxidase. The RR spectrum of the FAD of glutathione reductase is quite similar to that of aqueous FAD except that a new band is present between bands IX and X. Our conclusion from comparing our flavoprotein RR spectra to the RR spectra of free flavins in solvents of varying hydrogen-bonding ability is that FAD at the active site of glutathione reductase is strongly hydrogen bonded. On the other hand, FAD at the active site of yeast fatty acyl-CoA oxidase shows little evidence of hydrogen bonding, while FAD of fatty acyl-CoA dehydrogenase shows evidence for a hydrogen-bonding intermediate between that of glutathione reductase and fatty acyl-CoA oxidase. These RR investigations serve as the basis for a unique structure–function study of two enzymes, fatty acyl-CoA oxidase and dehydrogenase, with different activities for the same substrates.

Early investigators have reported the effect of organic solvents upon the visible spectrum of flavin derivatives in an attempt to define the solvent environment of flavin at the active site of flavoproteins (Kotaki et al., 1970). Later workers have interpreted the blue shift in the 370-nm band and the appearance of vibronic structure on the 450-nm band which accompanies dissolution of flavin species in organic solvent to result from decreased hydrogen bonding at N1, N5, the C=O at C2 and C4, and the hydrogen at N3 (Nishimoto et al., 1978; Yagi et al., 1980). Because of the variety of weak effects which influence electronic spectra, it is difficult to assign electronic spectral effects to hydrogen-bonding interactions between flavin and protein in a flavoprotein.

Vibrational spectroscopy has been used to study hydrogen-bonding interactions and is, in general, much more useful for such studies than is electronic spectroscopy. Since Raman spectroscopy is not nearly as sensitive to weak molecular perturbations (e.g., solvent effects) it should therefore supply the ideal tool for studying hydrogen bonding between flavin and protein in solution. For example, calculations on uracil carried out by using the MOCIC/MNDO technique suggest that a vibrational band at 1266 cm^{-1} with potential energy distributed as 21% C-N stretch, 37% C=O deformation, 21% N-H deformation, and 23% C-H deformation shifts upward to 1274 cm^{-1} when the solvent is changed from H_2O to D_2O (Bowman & Spiro, 1980). Notice that uracil constitutes ring III in the following flavin ring system and that the isotope shift is in the opposite direction to the normally expected change.



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This band is calculated to shift from 1266 to 1273 cm^{-1} upon addition of 20% to the N-H bending force constant. Such a change in bending force would be expected to result from hydrogen bonding at the N3 hydrogen. There is a vibrational band in the resonance Raman spectrum of flavin which occurs at approximately the same frequency as the band calculated for uracil (namely, 1256 cm^{-1}) and which shifts to higher frequency upon deuteration of flavin at N3 (Benecky et al., 1979; Dutta et al., 1980). This band might show H-bonding sensitivity similar to that of the analogous band in uracil. We report in this paper the solvent effects upon the resonance Raman (RR) spectrum of several flavin derivatives and the resonance Raman spectra of three FAD-containing flavoproteins: yeast fatty acyl-CoA oxidase, porcine liver fatty acyl-CoA dehydrogenase, and yeast glutathione reductase. We will use the data from the model system and the protein RR spectra to discuss hydrogen bonding in the flavoproteins.

Experimental Procedures

Lumiflavin (LF), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) were obtained from Sigma Chemical Co.; riboflavin was obtained from Aldrich Chemical Co. Tetraacetylriboflavin (TAR) was prepared by acetylation of riboflavin with acetic anhydride in glacial acetic acid by the method of McCormick (1970) and the product checked for complete acetylation by proton NMR. CoA and aceto-

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acetyl-CoA were obtained from P-L Biochemicals. Concentrations were measured by using the extinction coefficient for the adenine moeity, 15400 M^{-1} cm^{-1} at 259 nm.

UV–Visible Spectral Measurements. Absorption spectra were recorded on a Cary 17 UV–vis spectrophotometer, while absorbances at single wavelengths were determined on a Cary 16 spectrophotometer. For the determination of concentration, the following extinction coefficients were used: RF, FMN, and LF 12200 M^{-1} cm^{-1}, FAD, fatty acyl-CoA dehydrogenase, and glutathione reductase 11300 M^{-1} cm^{-1} at 450 nm, and yeast fatty acyl-CoA oxidase 15300 M^{-1} cm^{-1} at 450 nm (F. Frerman, personal communication).

Enzyme Preparations. General fatty acyl-CoA dehydrogenase was purified as previously described (McKean et al., 1979) and stored at 77 K. Enzyme was allowed to thaw, was dialyzed overnight in 0.02 M Tris buffer, pH 8.5, and was filtered with a Millipore filter to remove particles which would scatter light. Enzyme solutions were concentrated for the RR experiment with a Schleicher & Schuell collodion bag apparatus. In the case of the experiments in D_2O, the enzyme was diluted with D_2O buffer and reconcentrated twice in D_2O after the first concentration in H_2O. RR spectral analysis of the 1254 cm^{-1} δN–H and 1139 and 1295 cm^{-1} δN–D spectral bands showed that residual H_2O was negligible in these experiments. pD was determined by applying the usual correction to the pH meter reading. The acetoacetyl-CoA complex of dehydrogenase was formed by addition of a small amount of a solution of concentrated acetoacetyl-CoA (about 15 mm) to the concentrated enzyme solution. Since the binding constant of acetoacetyl-CoA is large, K_d = 6 μM (McKean et al., 1979), addition of an excess of acetoacetyl-CoA ensured that enzyme was quantitatively complexed with acetoacetyl-CoA. Fatty acyl-CoA oxidase from yeast was prepared as previously described (Shimizu et al., 1979), except that buffers contained 0.1 mM EDTA and 0.1 mM phenylmethanesulfonyl fluoride, a serine protease inhibitor. Enzyme was stored at −20 °C in 0.5 M phosphate buffer, pH 6.7, with 23% glycerol. For Raman experiments the enzyme was dialyzed overnight in either Tris buffer, 0.02 M, pH 8.3, or 0.05 M phosphate buffer, pH 6.7. Concentration, substitution with D_2O solvent, and acetoacetyl-CoA complex formation were carried out as described above for fatty acyl-CoA dehydrogenase. Glutathione reductase (yeast type III) was obtained from Sigma (No. G-4751) as a purified suspension in ammonium sulfate solution. It was dialyzed overnight in 0.1 M potassium phosphate buffer, pH 7.6, containing 3 × 10^{-4} M EDTA and concentrated as described above. Polyacrylamide disc gel electrophoresis was carried out on a sample of the commercial glutathione reductase preparation. The electrophesis was carried out under nondenaturing conditions in Tris–glycine buffer, pH 8.8. One major band was observed in addition to a single minor impurity (<1%).

Laser Raman Experiments. Samples were contained in sealed capillary tubes, i.d. = 1 mm. Raman spectra were obtained with a Spex 1401 laser Raman spectrometer equipped with a cooled RCA photomultiplier tube. The excitation source was a Spectra-Physics 164 argon ion laser. Photomultiplier counts were amplified by a Pacific Precision Instruments Ad-6 amplifier/discriminator and counted by a 100-MHz counter module in a CAMAC crate interfaced to a PDP-11/34 computer. Raman spectral slits were 7 cm^{-1}. Positions of vibrational bands are reported relative to a calibration standard, the 981-cm^{-1} band of sulfate dissolved in water; band positions are reproducible within 1 cm^{-1}. However, low S/N often precluded this degree of accuracy. Excellent reproducibility of scanning was maintained by use of a servo-driven monochromator interfaced to the PDP-11/34 computer. Spectra were recorded and stored digitally as a collection of photocounts in channels 2 cm^{-1} apart; collection times were 2–6 s/channel. Spectra were scanned 3–10 times, the averages being stored.

Flavin RR spectra contain considerable fluorescence despite the presence of the protein matrix or of potassium iodide which was added as a fluorescence quenching agent in solutions of free flavins but not in solutions of flavoproteins. Therefore, spectra were taken in three ranges between 1050 and 1700 cm^{-1}, and three or more linear ramps, 100–700 cm^{-1}, were subtracted from the original data, such that the length of the ramps was much larger than the line widths of the Raman spectral bands, typically 15–40 cm^{-1}. Averaging of seven adjacent points by a parabolic function was performed on all spectra displayed here. By use of this method, good spectra were obtained even though the RR scattering constituted only 2% of the total photocounts measured. Since most of the riboflavin derivatives are very soluble in the solvent systems used, we maximized the ratio of photocounts due to RR relative to photocounts due to fluorescence by using high concentrations of flavin (12–22 mM) and exciting at 488 or 514.5 nm, well to the red side of the 450-nm flavin spectral absorption maximum. In the case of flavoproteins RR spectra were obtained at concentrations of 0.3–1.0 mM. Laser power was typically 0.1 W. A narrow band-pass optical filter was used to remove spurious frequencies for both 488- and 514.5-nm laser lines.

Solvent Effects. Small aliquots of FMN and FAD were added to H_2O, D_2O, or dimethyl sulfoxide (Me_2SO), all of which contained potassium iodide to quench fluorescence. Riboflavin was dissolved in these solvents by gentle heating. A second solvent system, acetonitrile with lithium iodide (quite soluble in acetonitrile), was used as a weaker H-bond acceptor than Me_2SO. Tetraacetylriboflavin is very soluble in all mixtures of water and acetonitrile in the presence of LiI. For experiments with dry solvents the Me_2SO or acetonitrile was dried over CaH_2 (acetonitrile) or BaO (Me_2SO) and distilled. Manipulations were carried out quickly with little air exposure, but with a hydroscopic solvent such as Me_2SO total dryness probably is not attainable by this technique. "Dry" deuterated samples contained 5–10% D_2O added to exchange protons on N3 with deuterons; lyophilization from D_2O solution does not leave all positions deuterated due to contamination by small amounts of residual water in solvents or atmospheric H_2O. Generally, centrifugation of the solutions or the capillary tubes was effective in removing particulates. Fluorescence quenching resulted from protein–flavin interaction; no KI was added to the enzyme solutions.

Laser-Induced Degradation of Samples. With both model and flavoprotein samples visible and UV spectra of flavin were monitored to detect any photo or thermal degradation. The UV–visible spectra of all species except lumiflavin did not change with laser irradiation. Lumiflavin showed irreproducible light-induced changes and the resonance Raman spectra shown for lumiflavin are of samples which showed no visible spectral change. Another indication that laser heating or photolysis was not causing chemical modification of the flavin was the stability of the RR spectra with time; none of the other samples showed RR spectral changes with increasing irradiation time. Thermal degradation of dehydrogenase or oxidase results in release of free FAD and precipitation of protein. We see no fluorescence background increase (an indication of release of FAD) even after 6-h irradiation of our flavoprotein

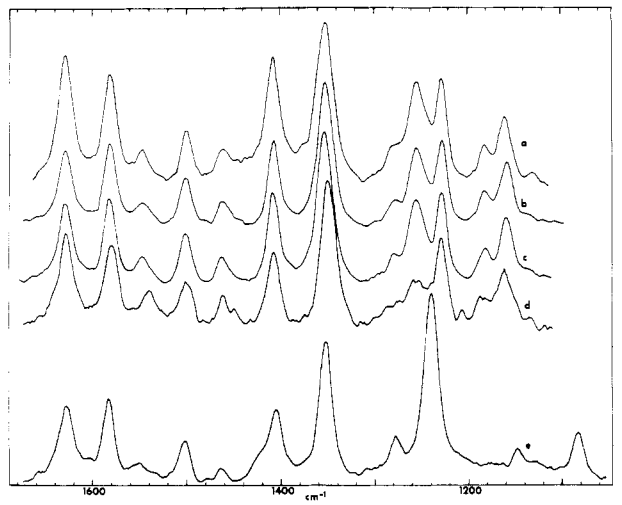

FIGURE 1: RR of flavin derivatives in aqueous solution. (a) 2 mM FAD in 0.02 M Tris buffer, pH 8.5, with 5 M KI; λ_0 = 488 nm. (b) 20 mM FMN in H_2O with 6 M KI; λ_0 = 514.5 nm. (c) 22 mM RF in H_2O with 6 M KI; λ_0 = 514.5 nm. (d) 19 and 38 mM TAR in H_2O (average of two experiments) with 6 M LiI; λ_0 = 514.5 nm. (e) 2 mM LF in H_2O with 10% Me_2SO–4 M KI; λ_0 = 488 nm.

samples. Finally, in order to assess photochemical damage to flavoprotein during laser irradiation, we have run kinetic assays as a function of irradiation time for the general dehydrogenase, fatty acyl-CoA oxidase, and glutathione reductase. The irradiation was carried out by defocusing the laser beam so as to cover ~1-mm sections of the capillary tube which contained the concentrated enzyme sample. Sections of the capillary tube were irradiated successively.

Fatty acyl-CoA oxidase lost most of its activity when irradiated for 5 min with the 488-nm laser beam at 100-mW power. The kinetic assay was performed at 25 °C with 40 μM furylpropanoyl-CoA as the substrate. A solution of the irradiated enzyme subsequently diluted to a 1-mL volume nevertheless retained the characteristic features of its visible spectrum, particularly the maximum at 445 nm and a relative minimum at 392 nm (the free FAD values are 449 and 403 nm, respectively). This suggests that the chemical alteration did not involve the flavin; further quantitative studies are being carried out to determine exactly the mechanism of inactivation of this enzyme by laser irradiation. In contrast, neither the glutathione reductase nor fatty acyl-CoA dehydrogenase lost any activity after 5 min of irradiation with 100 mW of 488-mm laser light. This fact and the stability of the RR spectrum from 20 min to 6 h suggest that there is no photochemical degradation of these two flavoproteins.

Results

Figure 1 shows the RR spectra of FAD, FMN, riboflavin, tetraacetylriboflavin, and lumiflavin in aqueous solution with 6 M KI added to quench fluorescence. Notice that except for small intensity changes, the RR spectra of riboflavin, FMN, and FAD are identical for all 12 bands between 1100 and 1650 cm^{-1}. The RR spectrum of tetraacetylriboflavin differs significantly from the others in that bands IX and X are of lower intensity than the corresponding bands in the other spectra. For the numbering of the bands and a listing of the frequencies of these bands, see Table I.[1] Preliminary assignments of the bands are shown in Table II. The RR spectrum of lumiflavin

[1] Note that we have adopted the numbering system of Bowman & Spiro (1981) in spite of the fact that we see no apparent intensity at their band VIII.

Table I[a]

	XI_D	XIII	XII_H	IX_D	XI_H, XII_D	1240	X_H	IX_H	X_D	VII	VI	V	IV	III	II	I
TAR																
H_2O		1161	1184		1228		1255	br		1349	1408	1462	1500	1541	1580	1628
dry CH_3CN		1163	1184		1230		1253 w	v w		1349	1414	1465	1503	1545	1585	1629
D_2O	1144	1165		1209	1229				1292	1353	1408	1461	1499	1548	1581	1629
CH_3CN-5% D_2O	1137	1163	1182 sh		1230	br				1350	1408	1462	1502	1548	1585	1628
RF																
H_2O		1157	1182		1228		1256	1277		1353	1408	1462	1501	1547	1582	1629
$^2/_3Me_2SO$-$^1/_3H_2O$		1158	1183		1229		1253			1352	1407	1463	1501	1549	1583	1628
dry Me_2SO		1155	1181		1229		1248			1350	1405	1464	1502	1549	1583	1627
D_2O	1141	1163		1210	1232			1270 sh	1293	1352	1407	1462	1501	1549	1581	1629
Me_2SO-10% D_2O	1138	1162			1230				1257–1287 sh	1350	1405	1459	1500	1549	1583	1626
FMN, H_2O		1158	1183		1228		1256	1278		1353	1407	1462	1501	1547	1581	1628
FAD, H_2O		1160	1182		1228		1255	1280		1353	1408	1461	1500	1548	1582	1629
LF																
H_2O-10% Me_2SO	1084	1147				1240		1278		1353	1405	1465	1503	1551	1584	1628
Me_2SO-10% H_2O		1147				1238				1350	1404	1465	1504	1553	1584	1628
D_2O-10% Me_2SO	1078	1142					1268	1282	1306	1353	1405	1462	1503	1556	1583	1629

[a] Band labels as designated previously (Bowman & Spiro, 1981) are given for protonated RF derivatives; one pattern of shifts suggested in this study applicable to N3 deuterated RF derivatives is designated by labels with subscript D.

Table II: Raman Vibrational Band Assignments for Oxidized Riboflavin

band	obsvd freq (H_2O)	obsvd freq[d] (D_2O)	assignments[a]	ring
I	1629	1629	νC5a–C6, νC7–C8, νC8–C9, νC5a–C9a	I
II	1582	1581	νC4a–N5, νN10–C10a, νC10a–N1, νC4a–C10a	II, III
III	1547	1549	νC4a–N5, νC10a–N1	II, III
IV	1501	1501	δCH_3	I
V	1462	1462	νC6–C7, νC8–C9, νC9–C9a, δCH_3	I
VI	1408	1407	νN1–C2, νC5a–C6, νC8–C9, νC5a–C9a	I, III
VII	1353	1352	νN10–C10a, νC5a–C9a, δCH_3	I, II
IX	1277	1210[d]	δCH_3, δN3–H	III[b]
X	1256	1293[d]	νC9a–N10, δN3–H νC6–N1, δN3–H, δC2=O, δC4=O, δC5–H, δC6–H[c]	III[b]
XI	1228	1141[d]	νN3–C4, νC8–CH_3, δCH_3	I, III[b]
XII	1182	1232[d]	νN1–C2, νC2–N3, νN3–C4, νC4–C4a νC4a–C10a, νC7–CH_3, δCH_3	I, III[b]
XIII	1157	1163[d]	νC2–N3, νC4a–C4, νC4a–10a, νC7–CH_3, δC6–H	I, III[b]

[a] Modes previously calculated for lumiflavin are listed here (Bowman & Spiro, 1981) as assigned by these authors except as noted. [b] Modes in this region are not well understood. They show large N3–H, N3–D isotope shifts. The 1256-cm^{-1} band movement on deuteration is not correctly predicted. [c] Calculation for uracil (Bowman & Spiro, 1980) predicts an upward shift on deuteration for the 1236 cm^{-1} (observed), 1266-cm^{-1} (calculated) mode analogous to the flavin 1256-cm^{-1} mode. [d] We have assigned shifts of bands IX–XIII occurring in D_2O solution on the basis of arguments arising from our own experimental data, as explained in text.

is surprisingly different from those of the other derivatives even though the only structural alteration is the substitution of a methyl group (lumiflavin) for a ribityl group (riboflavin). This spectral difference has been observed and noted previously (Nishina et al., 1980). Since this alteration occurs outside the aromatic ring supporting the electronic transition resulting in resonance enhancement of the aromatic vibrational bands, one would predict that few changes would result in the RR spectrum as is the case with FAD, FMN, and riboflavin. However, the RR spectrum of lumiflavin is strikingly different in the 1100–1300-cm^{-1} region from that of the other flavin derivatives. Figure 1 and Table I show that the differences can be understood if bands X and XI of other flavin derivatives are replaced by a single band at 1240 cm^{-1} in lumiflavin.

Figure 2 and Table I show the shifts on the RR spectrum of riboflavin upon deuteration at N3 as well as solvent effects on dissolution in Me_2SO. Previously, it had been suggested that a single strong vibrational band (X) shifted to higher frequency upon deuteration at N3 (Dutta et al., 1978, 1980; Bowman & Spiro, 1981). However, these data show that the spectral consequences of substitution are complex. We suggest here band assignments which are consistent with Me_2SO solvent effects on the bands' frequencies and intensities. The weak band IX (1277 cm^{-1}) disappears in Me_2SO solvent, while a band at 1210 cm^{-1} in the N3-deuterated species disappears in Me_2SO doped with D_2O. We suggest that this is the shifted band IX.[2] The following assignments for the remaining bands

[2] The band at 1205 cm^{-1} in D_2O solution of riboflavin which we have assigned as band IX has a line width of 12 cm^{-1}, which is much narrower than the 50-cm^{-1} line width of the D_2O solvent band which appears in this region; this precludes the possibility that the band is due to D_2O solvent.

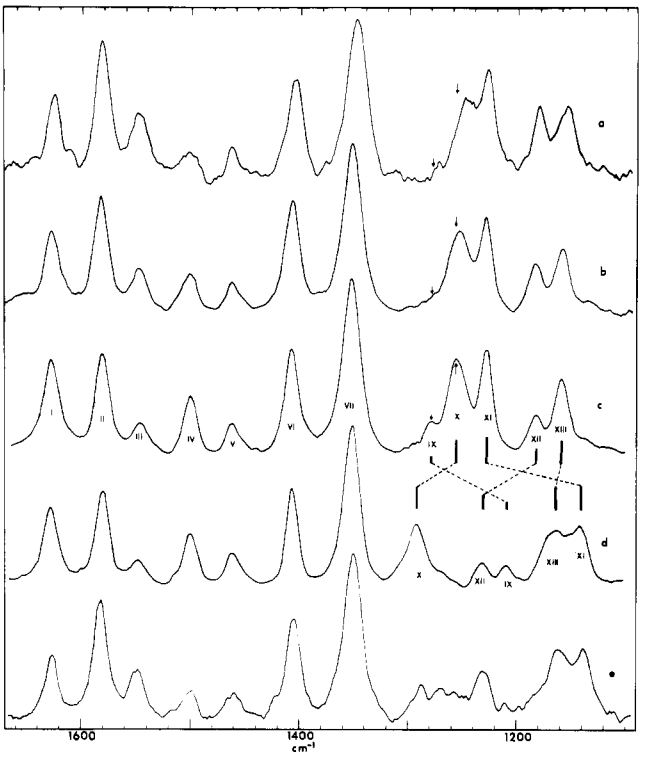

FIGURE 2: Solvent effects of RR of riboflavin (RF): (a–c) N3–H; (d, e) N3–D. λ_0 = 514.5 nm. (a) 14 mM RF in dry Me_2SO with ~2 M KI; (b) 18 mM RF in 3 M KI–67% Me_2SO–33% H_2O; (c) 22 mM RF in 6 M KI with H_2O; (d) 22 mM RF in 6 M KI with D_2O; (e) 22 mM RF in Me_2SO with 10% D_2O and ~2 M KI. Arrows in (a–c) mark positions of bands IX and X (100% H_2O) in order to illustrate the solvent shift in Me_2SO–H_2O mixtures. Bars and dotted lines show proposed D_2O solvent shifts for RR bands between 1100 and 1300 cm^{-1}. Notice that band IX disappears, broadens, or slides down in frequency in both D_2O and H_2O when the solvent is mostly Me_2SO.

XI–XIII are more speculative since solvent effects on these bands are small, but they are consistent with the requirement that the relative intensities of the bands not change appreciably on deuterium substitution. This relationship does not hold generally when significant changes in potential energy distribution occur in the normal modes of a vibrational band. In any case, it would seem to be worthwhile to consider such an assignment, since it is based on the simplest assumed relationship of intensities between the protonated and deuterated normal modes.

The data in Figure 2 indicate that band XI (1228 cm^{-1}) remains strong in Me_2SO, as does the weaker band XII (1182 cm^{-1}); in fact, the latter appears slightly enhanced in Me_2SO. We assign the strong band in D_2O at 1141 cm^{-1} to the shifted band XI, and the weaker band at 1232 cm^{-1} to band XII; this latter band also is enhanced slightly in Me_2SO doped with D_2O. Once more, the argument is somewhat tentative; it is not clear why band XII is not similarly enhanced in acetonitrile, when the 1230-cm^{-1} band in D_2O doped acetonitrile is. Finally, band XIII (1157 cm^{-1}) undergoes little solvent or deuterium shift. The D_2O shifts for lumiflavin are shown in Figure 3; notice that the single band at 1240 cm^{-1} splits into two or more bands in D_2O. This behavior is quite similar to the D_2O shifts of bands X and XI in riboflavin.

Figure 2 and Table I show additional solvent effects upon RF in Me_2SO. Me_2SO is a weak hydrogen bond acceptor with ϵ = 45; it cannot function as a hydrogen-bond donor. The shifts in the spectral bands upon dissolution in Me_2SO are (1) the disappearance of band IX, as mentioned above, (2) the shift in band X to lower frequency, and (3) the shift in band

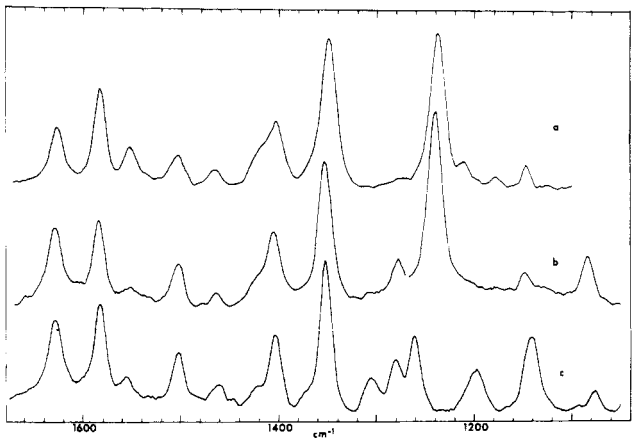

FIGURE 3: Solvent effect on RR spectra of lumiflavin. (a) 21 mM LF in Me_2SO with ~10% H_2O and ~2 M KI; notice the absence of the 1278-cm^{-1} band which we designate as band IX; λ_0 = 514.5 nm. (b) 1.9 mM LF in H_2O with 10% Me_2SO and 4 M KI, 488 nm. (c) ~3.7 mM LF in D_2O with 10% Me_2SO and 4 M KI; λ_0 = 488 nm.

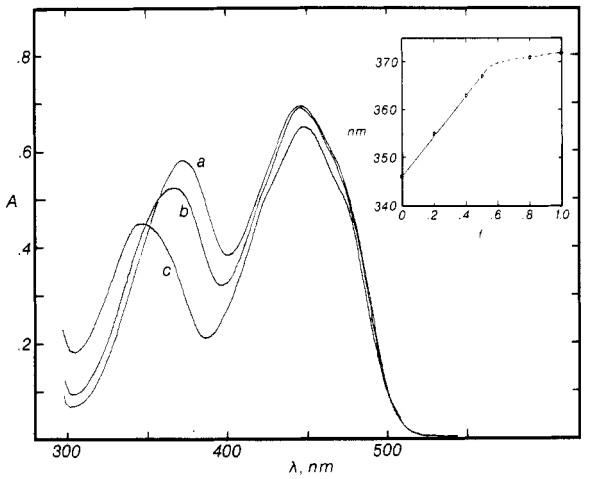

FIGURE 4: Solvent effect on visible spectrum of 0.06 mM FMN. (a) H_2O; (b) 50% Me_2SO–50% H_2O; (c) Me_2SO. The insert indicates the position of the relative maximum as a function of the aqueous fraction.

II to slightly higher frequency. In addition, there are intensity changes in bands III and IV which are not dependent upon excitation frequency. Corresponding changes take place in D_2O including (1) the disappearance of the 1210-cm^{-1} band, as mentioned above, (2) the shift of the 1290-cm^{-1} band to lower frequency, and (3) the shift in band II to higher frequency. The solvent effects shown here for riboflavin occur also in FMN and FAD. Figure 3 shows that the solvent effect on band IX in lumiflavin is similar to that in the other derivatives: in Me_2SO the intensity of this 1278-cm^{-1} band is greatly reduced.

It has been suggested that Me_2SO might cause unstacking of the isoalloxazines resulting in RR spectral changes (Irwin et al., 1980). In order to assess the spectral effects of stacking, we have studied the RR spectrum of FMN under stacking (>50 mM) and unstacking (<2 mM) conditions. We observe no significant RR spectral change under these two conditions.

Figure 4 shows the electronic spectrum of FMN in H_2O and Me_2SO. In Me_2SO there is a blue shift in the 370-nm band and resolution of vibronic fine structure in the 450-nm band. Theoretical calculations have shown that electronic spectral shifts such as these are similar to spectral shifts predicted for flavin species in a non-hydrogen-bonding environment. The calculations also indicate that hydrogen bonding at N1, N5,

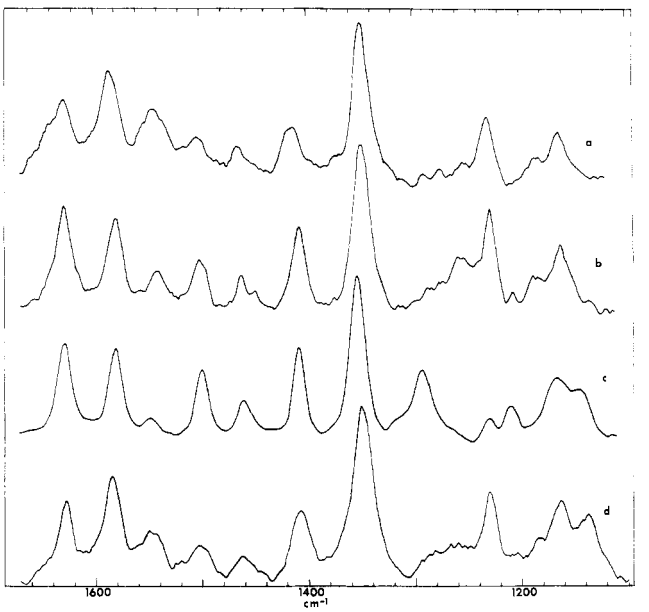

FIGURE 5: Solvent effects on RR spectrum of tetraacetylriboflavin (TAR); λ_0 = 514.5 nm. (a) 11 mM TAR in dried CH_3CN with 2 M LiI; (b) average of two experiments of 19 and 38 nM TAR in H_2O with 6 M LiI; (c) ~20 mM TAR in D_2O with 6 M LiI; (d) 37 mM TAR in dried CH_3CN with 5% D_2O and 2M LiI. Collapse of band IX and significant reduction in intensity of band X in CH_3CN are both evident.

and the C=O oxygens at C2 and C4 affects the electronic spectrum of the flavin species but that hydrogen bonding at N3 has little spectral effect (Nishimoto et al., 1978). It is not surprising that Me_2SO shows the electronic spectrum of flavin in a non-hydrogen-bonding solvent: there can be hydrogen bonding of flavin in Me_2SO only at N3 (a position producing no spectral effect) since the solvent can accept a hydrogen bond but is not a hydrogen-bond donor.

Figure 5 shows the spectra of tetraacetylriboflavin in acetonitrile and H_2O for both N3–H and N3–D species; tetraacetylriboflavin was used in these studies because it is more soluble in acetonitrile than is riboflavin. LiI is added as a fluorescence quenching agent because it is considerably more soluble in acetonitrile than is KI; also, LiI enhances the solubility of TAR in H_2O. The solvent effects in acetonitrile are similar to those in Me_2SO but are not identical: these effects include (1) the shift of band II to higher frequency in nonaqueous solvent, (2) the intensity reduction in both bands IX and X in nonaqueous solvent, and (3) the shift of band X to lower frequency; notice that the intensity of the 1255-cm^{-1} band is so low that it is impossible to determine the frequency precisely. Also notice the dramatic reduction in intensity of band X in N3–D tetraacetylriboflavin and that band IX in the N3–D species (1209 cm^{-1}) disappears in acetonitrile. Finally, there are intensity changes in bands I, II, III, and IV (again these intensity changes are not dependent on excitation frequency).

In conclusion, the following solvent effects have been observed on several flavin derivatives as water is replaced by solvents which result in weaker hydrogen bonding. For Me_2SO (1) disappearance of band IX, (2) small shift in band X to lower frequency, (3) small shift in band II to higher frequency, and (4) intensity changes in bands III and IV; no H bonding at N1, N5, C2=O or C4=O; possible H bonding at N3. For CH_3CN (1) disappearance of band IX, (2) intensity decrease or disappearance of band X, (3) larger shift in band II to higher frequency, and (4) intensity changes in bands III and IV; no H bonding at N1, N5, C2=O, C4=O, or N3–H.

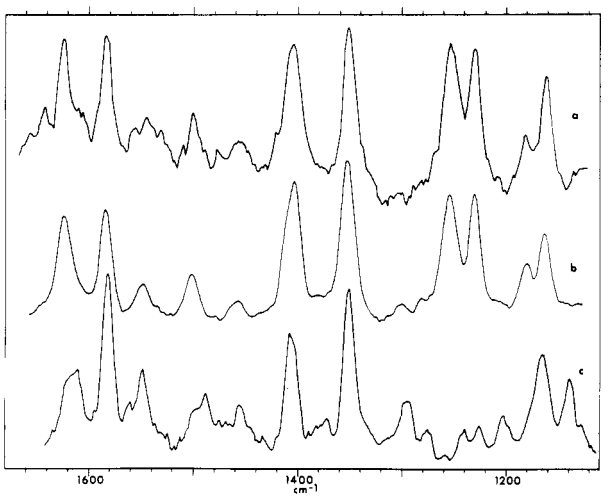

FIGURE 6: RR spectra of general fatty acyl-CoA dehydrogenase. (a) ~1 mM enzyme, free, in 0.05 M Tris, pH 8.4; λ_0 = 488 nm. (b) 1 mM enzyme complex with acetoacetyl-CoA in 0.05 M Tris, pH 8.4, λ_0 = 488 nm. (c) ~2.5 mM enzyme complex with acetoacetyl-CoA in D_2O buffer–0.05 M Tris, pD 8.4; λ_0 = 514.5 nm.

Figure 6 and Table III show the spectra and vibrational frequencies of porcine liver fatty acyl-CoA dehydrogenase and the acetoacetyl-CoA (acacCoA) complex of the enzyme in H_2O and D_2O solutions; they may be compared to the RR spectrum of FAD (Figure 1). There are two major changes between the FAD coenzyme in aqueous solution and that bound to the enzyme: band IX has shifted to lower frequency (under bands X and XI) or has disappeared, and band I has shifted to lower frequency. There are no detectable differences between the RR spectrum of the acetoacetyl-CoA complex and the free enzyme except for a slight intensity enhancement in band II. However, upon excitation by longer wavelength laser radiation at the frequency of the charge transfer electronic band, the enhancement in the intensity of band II is considerable (Schmidt et al., 1981). The change in band IX observed for the dehydrogenase is similar to the change observed in 67% Me_2SO–33% H_2O solvent system (Figure 2): the band disappears or is shifted to lower frequency. Furthermore, the band at 1210 cm^{-1} observed in D_2O solutions of free FAD or riboflavin which we assign to band IX does not appear at 1210 cm^{-1}; however, there is a band at 1205 cm^{-1}, which could be the shifted 1210-cm^{-1} band. Once more, as in the model system discussed earlier, it is unlikely that this band is due to D_2O because the line width of the D_2O band is much greater than the line width of the observed band. The RR spectral effect upon dissolving flavin species in pure Me_2SO is larger than the effect of binding free flavin to the general dehydrogenase, including the total disappearance or shift in band IX, the shift to lower frequency of band X, and intensity changes discussed previously. Therefore, we conclude that hydrogen bonding between FAD and the dehydrogenase enzyme is weaker than that found in aqueous solution of flavins. Because band IX, unlike band X and band II, is not presently understood on the basis of a MOCIC/MNDO calculation on uracil or a valence force field calculation on flavin (Bowman & Spiro, 1980, 1981), we cannot comment on which part of the flavin molecule may be involved in this weakened hydrogen bonding, except to say that N3 must be involved. It is clear that band II is not shifted in the dehydrogenase when compared to aqueous solution, and on the basis of model studies we would conclude that N5 and N1 are hydrogen bonded much as they are in aqueous solution. Our picture of the hydrogen bonding of FAD in the dehydrogenase

Table III

enzyme	XI_D	XIII	XII_H	IX_D	XI_H, XII_D	1240	X_H	IX_H	X_D	VII	VI	VI	V	IV	III	II	I
gen DH free–H_2O		1162	1182		1231		1253			1352	1407		1454	1496	1544	1583	1624
acacCoA complex–H_2O		1163	1179		1230		1254			1352	1403		1457	1501	1548	1584	1623
acacCoA complex–D_2O	1139	1163		1205	1227	1240		1273	1295	1352	1407		1452	1490	1548	1582	1616
FA oxidase																	
pH 8 H_2O		1161	1182		1232		1251			1351	1404	1413	1464		1552	1586	1629
pH 8 acac complex		1160	1180		1231		1248			1351	1401	1409 sh		1499	1547	1586	1627
pH 6.7 H_2O		1161	1182		1233		1251			1351	1403	1412	1460	1498	1551	1586	1629
pD 6.7 D_2O	1137	1166			1217–1240 br				1290	1350		1409	1460	1502	1550	1584	1629
glutathione reductase																	
pH 7.6 H_2O		1155	1180		1224	1246	1261	1281		1353	1402					1580	1627
pD 7.7 D_2O	1138	1166		1209		1239			1289	1351	1407		1458	1497	1541	1580	1628

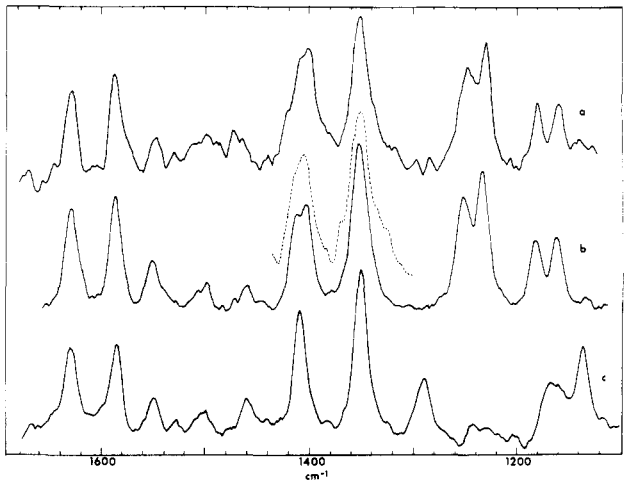

FIGURE 7: RR spectra of fatty acyl-CoA oxidase; λ_0 = 488 nm. (a) 0.5 mM enzyme complex with acetoacetyl-CoA in 0.02 M Tris, pH 8; dotted insert is 0.2 mM enzyme, free, in 0.02 M Tris, pH 8. (b) 0.4 mM enzyme in 0.05 M potassium phosphate buffer, pH 6.7. (c) 0.7 mM enzyme in 0.05 M potassium phosphate in buffer D_2O, pD 6.7.

is that N3, C2=O, and/or C4=O are still hydrogen bonded but more weakly than in aqueous solution and that N1 and N5 are hydrogen bonded at the coenzyme binding site of dehydrogenase.

Figure 7 and Table III show the resonance Raman spectrum and vibrational frequencies of yeast fatty acyl-CoA oxidase in H_2O and D_2O. Notice that the changes between FAD in water (Figure 1) and oxidase-bound FAD are much more substantial than the difference for dehydrogenase. Band II shifts to higher frequency, band IX shifts as noted above for dehydrogenase, and band X shifts to lower frequency. The RR spectrum of oxidase in D_2O solution (Figure 7c) shows very weak Raman scattering in the region 1200–1220 cm^{-1}, where we expect band IX to appear on the basis of our model studies; once more, band IX is either shifted or may be absent in the oxidase, as it is in the model systems in non-hydrogen-bonding solvent. A comparison with the model system tetraacetyl riboflavin in acetonitrile shows a very similar pattern of shifts. In addition to the spectral differences between FAD and FAD bound to oxidase mentioned above, in H_2O solutions band VI of oxidase splits into two partially resolved bands, at 1403 and 1412 cm^{-1}. We do not resolve two peaks in the solution of tetraacetylriboflavin; however, notice that Figure 5 shows a broadening of bands VI in the hydrogen bonding solvent acetonitrile. The results are similar for the acetoacetyl-CoA complex and free enzyme, and are pH independent within the pH range 6.7 < pH < 8.6, within the resolution of our RR spectra. However, in D_2O solution only one band at 1409 cm^{-1} is observed. On the basis of current understanding of the vibrational modes in flavins, we cannot explain the unusual isotope effect upon the splitting of band VI. Nor do our solvent studies offer any insight into the D_2O shift since the broadening of band VI mentioned above in acetonitrile is also present for the deuterated flavin in acetonitrile.

The RR spectrum for fatty acyl-CoA oxidase resembles the RR spectrum of flavin in polar non-hydrogen-bonding solvent. For example, the shift in band X to lower frequency and the disappearance of band IX are seen with RF in dry Me_2SO, the shift in band II is seen with TAR in acetonitrile, and the intensity changes in bands III and IV in enzyme bound FAD are seen in both tetraacetylriboflavin in acetonitrile and RF in dry Me_2SO. In addition, the splitting in band VI in oxidase

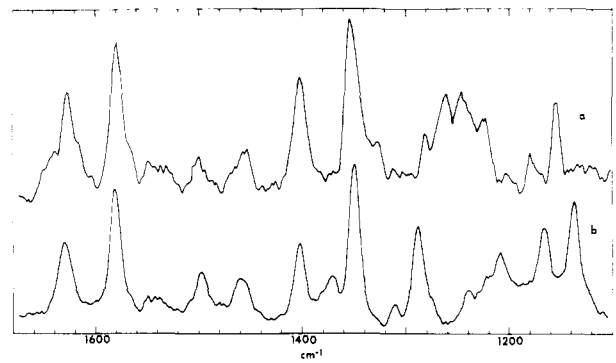

FIGURE 8: RR spectra of glutathione reductase from yeast; λ_0 = 514.5 nm. (a) 1.4 mM enzyme in H_2O with 0.1 M potassium phosphate, pH 7.6; (b) 1.2 mM enzyme in D_2O with 0.1 M potassium phosphate, pD 7.7.

is reflected in the broadening of band VI in acetonitrile. Since acetonitrile is neither a hydrogen-bond donor nor acceptor and since the visible spectrum of flavin in acetonitrile is consistent with no hydrogen bonding between flavin and solvent, this comparison would suggest that there is little or no hydrogen bonding between N1, N5, C2=O, C4=O, or N3-H of FAD and the protein of the oxidase enzyme.

Figure 8 and Table III show the RR spectrum of yeast glutathione reductase in H_2O and D_2O; once more, comparison of these spectra may be made with those of FAD in Figures 1 and 2. Notice that the RR spectrum for the enzyme is identical with that of aqueous FAD except for the addition of a new band between band X and XI. The three bands (X, the new band, and XI) are not well resolved so that their frequencies cannot be determined accurately. Band IX is present as in aqueous FAD and appears in D_2O solution as a moderately strong band at 1209 cm^{-1} superimposed on what appears to be a broad D_2O envelope in the 1200-cm^{-1} region (Figure 8b). Band II is unshifted from its frequency in aqueous FAD, while band X appears at a higher frequency than that of aqueous solutions of FAD. Furthermore, except for the addition of the new band at 1240 cm^{-1}, there is little relative intensity difference between FAD in aqueous solution and FAD bound to enzyme. On the basis of the model studies reported earlier, we conclude that glutathione reductase is hydrogen bonded much as in aqueous solutions of flavin, i.e., at N5, N1, C2=O, C4=O, and N3–H. We make that assertion on the basis of the unshifted band II and the presence of band IX which is only observed in hydrogen-bonding solvent. However, our model studies offer no explanation for the new band between bands X and XI.

Discussion

We have studied the RR spectra of flavin species in three polar solvents which differ in hydrogen-bonding ability. Because there are large electronic spectral shifts characteristic of changes in hydrogen bonding which result from changing these solvents, the solvent systems serve as models for hydrogen-bonding interaction between flavin and protein. Structure–function correlations based upon these spectral shifts and kinetic studies on the flavoproteins should permit evaluation of hydrogen-bond formation between protein and flavin and its effect upon activity. Me_2SO is a hydrogen-bond acceptor but not a hydrogen-bond donor, and acetonitrile is neither; both solvents have high dielectric constants (water, ϵ = 78; Me_2SO, ϵ = 45; CH_3CN, ϵ = 39). This makes these solvents ideal systems for investigation of hydrogen-bond interaction without the complication of large differences in solvent polarity.

The largest RR spectral change occurs in bands IX and X; there is also a small shift of band II and broadening in band VI in acetonitrile. In addition, there are a number of intensity changes noted above; these we have treated phenomenogically. In the following paragraphs, we will attempt to explain the frequency shifts which we observe.

Both bands IX and X must have some δN–H character since they shift on deuteration at N3. Table II shows the deuterium shifts which we assigned on the basis of the RR data for riboflavin in Figure 2. Notice that band X shifts in the manner suggested from MOCIC/MNDO calculations for the 1236-cm^{-1} band (calculated at 1266 cm^{-1}) of uracil as stated in the introduction (Bowman & Spiro, 1980). This band is predicted to shift to lower frequency upon decreasing hydrogen bonding at N3, C2=O, and C4=O in both the N3–H and N3–D species. In keeping with this suggestion, we find that the band X of flavin shifts to lower frequency in Me_2SO and acetonitrile. The data and the results of calculations discussed earlier would suggest that hydrogen bonding at C2=O, N3–H, and C4=O is weaker in Me_2SO and acetonitrile than in aqueous solution; however, the data cannot discriminate among the three possible hydrogen-bonding sites. In addition to the shift in band X, it appears that band IX also shifts to lower frequency and consequently underlies the bands X and XI in both acetonitrile and Me_2SO. Spiro and co-workers (Bowman & Spiro, 1981) have reported calculations on lumiflavin; these calculations predict two vibrational modes, ν_{28} and ν_{29} with δ N–H character in 1200-cm^{-1} region. However, neither of these bands shift to higher frequency on deuteration as predicted for the 1236-cm^{-1} band of uracil or observed for band X in flavin derivatives. We would tentatively suggest that these bands correlate with bands IX and XI in the flavin spectra. Either or both of these bands might be expected to shift to higher frequency upon an increase in δN–H due to hydrogen-bond formation. Conversely, less hydrogen bonding might be expected to result in a lowering in frequency. We observe either disappearance of band IX in non-hydrogen-bonding solvent or a shift to lower frequency. We believe the latter to be true, as can be seen in Figure 2a,b; notice in the N3–H spectra that the bands X and XI are less well resolved in Me_2SO. It is possible that this is the result of movement of band IX from 1277 to about 1240 cm^{-1}. A similar movement in D_2O would require the movement of a band at 1210 to 1160 cm^{-1}. Unfortunately, the presence of band XIII in the RR spectrum at 1160 cm^{-1} in D_2O precludes observation of the shifted band IX. Note that the proposed shift upon decreasing hydrogen bonding is much larger for band IX than for band X; this might be expected since the percent δN–H in the potential energy distribution for the predicted νC28 and νC29 in lumiflavin is 28% and 33%, respectively, whereas that predicted for the 1236-cm^{-1} band of uracil is only 12%. We propose that bands X and possibly XI also shift upon deuteration of flavin at N3 and therefore must involve δN–H. We have no indication that there is a solvent effect upon the frequencies of these bands.

Band II in flavin RR spectra involves νC=N as indicated both by the frequency of the mode and the isotope shift upon ^{15}N substitution at N5 (Kitagawa et al., 1979). Calculations indicate a potential energy distribution of νC4a–N5 (15%), νN10–C10a (18%), νC10a–N1 (12%), νC4a–C10a (13%) (Bowman & Spiro, 1981). As hydrogen bonding at N1 and N5 of the flavin is reduced, an increase in force constant for νC4a–N5 and νC10a–N1 would be expected, resulting in an increased frequency of band II. Indeed, we do observe a small but significant increase in the frequency of this band in acetonitrile. The observed shift in band II in Me_2SO is quite small and we cannot be sure that the frequency of band II is increased in this solvent. These observations are consistent with the expectation that Me_2SO and acetonitrile would not function as hydrogen-bond donors to N1 or N5 whereas water would.

It is interesting to compare our flavoprotein spectra with those reported by other groups. Three groups have reported the RR spectrum of riboflavin bound to riboflavin binding protein (Kitagawa et al., 1979; Dutta et al., 1978; Schopfer et al., 1981). These studies indicate that there is a decrease in the intensity of band IX on binding to protein. The reported spectrum is quite similar to that of FAD bound to fatty acyl-CoA dehydrogenase except that the shift in band I observed for the dehydrogenase is not observed for riboflavin binding protein. Flavodoxin's RR spectrum has also been reported by two groups (Dutta & Spiro, 1980; Irwin et al., 1980). The spectrum of FMN bound to flavodoxin shows a prominent band IX, typical of strong hydrogen bonding at N3–H. Band X is in the same position as in aqueous flavin, indicating normal hydrogen bonds at C2=O, C4=O, and N3–H [see Irwin et al. (1980) for a discussion of this point), corresponding to X-ray structural studies on flavodoxin which show that there are hydrogen bonds at these positions [Burnett et al., 1974 (*Clostridium* MP); Watenpaugh et al., 1973 (*Desulfovibrio vulgaris*)]. Both Raman papers report shifts in band II to lower frequency upon enzyme complexation. If, as suggested by our studies, removal of hydrogen bonding results in an increase in the frequency of band II, this decrease in frequency might indicate stronger hydrogen bonding on the protein surface than in aqueous solutions. Further, if band II does represent νC=N5 and νC=N1 as suggested (Bowman & Spiro, 1981), the data indicate strong hydrogen bonding at N1 or N5. The X-ray study of *Clostridium* flavodoxin indicates hydrogen bonding of the flavin at N1, C2=O, and N3–H but not at C4=O or N5 (Burnett et al., 1974). A recent CARS study of the *Clostridium* enzyme reports a relatively low frequency, 1577 cm^{-1}, for band II (Dutta & Spiro, 1980), in accord with strong hydrogen bonding at N1. An X-ray study of *D. vulgaris* flavodoxin (Watenpaugh et al., 1973) reports hydrogen bonding at C2=O, N3–H, C4=O, N5, and possibly N1. A CARS study on this protein indicates a still lower frequency of band II, 1571 cm^{-1} (Irwin et al., 1980), commensurate with the hypothesis that hydrogen bonding at both N1 and N5 would lower the Raman frequency of this band to a larger degree than the band II in the *Clostridium* enzyme where only a single nitrogen atom N1 is hydrogen bonded.

In summary, the RR spectrum in the 1200-cm^{-1} region, involving N3–H motion, is consistent with the following hydrogen-bond patterns for a series of flavoproteins: for glutathione reductase and flavodoxin, strong hydrogen bonds as in aqueous solution; for riboflavin binding protein and fatty acyl-CoA dehydrogenase, weaker hydrogen bonding as in 67% Me_2SO–33% H_2O–3 M KI; for fatty acyl-CoA oxidase, no hydrogen bonding as in acetonitrile. As mentioned previously, X-ray structure studies on flavodoxin support this viewpoint. The other protein above that has been subjected to X-ray analysis is glutathione reductase. We have studied the yeast enzyme, while the X-ray studies were carried out on the erythrocyte enzyme. The X-ray studies for the erythrocyte protein suggests hydrogen bonding at the N1 and N3 positions on flavin (Pai & Schultz, 1982). Thus, these studies are also consistent with the conclusion reached on the basis of RR spectra.

If the RR conclusions are valid, a particularly intriguing structure–function argument can be analyzed. The yeast fatty acyl-CoA oxidase shows characteristics of a typical oxidase enzyme, in that the protein stabilizes a red anionic semiquinone and reacts rapidly with O_2 in its reduced form (Coudron & Frerman, 1982). In addition, the enzyme stabilizes no product charge transfer complex with substrate. On the other hand, the porcine liver fatty acyl-CoA dehydrogenase reacts with analogous substrates in a different manner. Protein stabilizes a blue neutral semiquinone, the product charge transfer complex is stabilized, and O_2 reacts with reduced flavin very slowly (at the rate of breakup of the charge transfer complex) (McFarland et al., 1982). It is interesting that the RR data suggest that the degree of hydrogen bonding at N3–H, C2=O, and C4=O, and perhaps at N1 and N5 as well, is quite different in the two enzymes. There is a recent proposal that the general difference between oxidases and dehydrogenases can be understood on the basis of a different mode of hydrogen bonding of the FAD semiquinones in these two classes of enzymes. The proposal is that oxidases show strong hydrogen bonding at N1 and dehydrogenases at N5 and that these two specific hydrogen bonds stabilize the anion and neutral forms of semiquinone, respectively (Massay et al., 1979). Our RR studies on the oxidized form of fatty acyl-CoA oxidase do not support a structural generality for oxidase enzymes since the reported spectrum of glucose oxidase (Dutta et al., 1977, 1978) is quite different than that for fatty acyl-CoA oxidase. In fact, it has been suggested that FAD in glucose oxidase exists to some extent in the quinoid form (Schopfer et al., 1981). None of the spectral changes which support this conclusion in glucose oxidase are observed in fatty acyl-CoA oxidase.

The results from our RR studies of fatty acyl-CoA dehydrogenase and oxidase indicates that the very different hydrogen-bonding environment in the two proteins should present a unique opportunity for studying the structure–function relationship in these proteins. We are currently carrying out kinetic studies on the two enzymes to permit the functional comparison to be completed.

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Registry No. LF, 1088-56-8; FMN, 146-17-8; FAD, 146-14-5; TAR, 752-13-6; fatty acyl-CoA oxidase, 61116-22-1; fatty acyl-CoA dehydrogenase, 9027-65-0; glutathione reductase, 9001-48-3; riboflavin, 83-88-5.

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