Resonance Raman Study of Flavins and the Flavoprotein Fatty Acyl Coenzyme A Dehydrogenase†

Michael Benecky, Ta Yuen Li, Jack Schmidt, Frank Frerman, Kenneth L. Watters, and James McFarland*

ABSTRACT: The resonance Raman (RR) spectra of FMN, FAD, FAD in D_2O, and 7,8-dimethyl-1,10-ethyleneisoalloxazinium perchlorate have been obtained by employing KI as a collisional fluorescence-quenching agent. The spectra are very similar to those obtained recently by using the CARS technique to eliminate fluorescence. Spectra have also been obtained for several species in which flavin is known to fluoresce only weakly. We report RR spectra of protonated FMN, FMN semiquinone cation, the general fatty acyl-CoA dehydrogenase, and two "charge-transfer" complexes of fatty acyl-CoA dehydrogenase. Tentative assignment of several vibrational bands can be made on the basis of our flavin spectra. RR spectra of fatty acyl-CoA and its complexes are consistent with the previous hypothesis that visible spectral shifts observed during formation of acetoacetyl-CoA and crotonyl-CoA complexes of fatty acyl-CoA dehydrogenase result from charge-transfer interactions in which the ground state is essentially nonbonding as opposed to interactions in which complete electron transfer occurs to form FAD semiquinone. The only significant change in the RR spectrum of FAD on binding to enzyme occurs in the 1250-cm^{-1} region of the spectrum, a region associated with δ_{N-H} of N-3. The position of this band in fatty acyl-CoA dehydrogenase and the other flavoproteins studied to date is discussed in terms of hydrogen bonding between flavin and protein.

Raman spectra of flavin coenzymes and their enzyme complexes are of interest since such spectra would supply vibrational information to complement the wealth of electronic spectral information which has been collected on these systems. Since fluorescence of many of these flavin species interferes with the detection of Raman scattered light, it has not been possible until recently to obtain Raman spectra of the highly fluorescent flavin species. Two groups of researchers have reported some success in this regard: a single band in the spectrum of flavin has been obtained by exciting with an ultraviolet laser (Tsuboi, 1976) and Spiro and co-workers have employed the coherent anti-Stokes Raman spectroscopy (CARS)[1] technique to obtain spectra of FAD bound to riboflavin-binding protein from egg white (Dutta et al., 1978), glucose oxidase, and other flavins (Dutta et al., 1977). In both of these studies the major spectral shift observed on binding FAD to protein was in the 1250-cm^{-1} region of the spectrum.

We have obtained RR spectra of fluorescent flavin species by adding 2 M KI as a collisional fluorescence-quenching agent. Early work on KI quenching of flavin fluorescence indicated that KI acts as a collisional quencher of the first kind (Weber, 1950). KI has previously been used as a fluorescence quencher to obtain Raman spectra of fluorescent species (Friedman & Hochstrasser, 1975). We report below the spectra of fluorescent flavin species where KI has been added to reduce fluorescence. Protonated flavin fluoresces only weakly; we have made use of this fact to obtain spectra of protonated FMN, of a charge-transfer complex between FMN and hydroquinone (Bear et al., 1970), and of the FMN cationic semiquinone. Flavin fluorescence is also quenched in many protein complexes; we have taken advantage of this property to obtain RR spectra of the general fatty acyl-CoA dehydrogenase, the first enzyme in the pathway for oxidative degradation of fatty acyl-CoA thioesters. During the consideration of this manuscript, another report of RR spectra of flavin derivatives has appeared (Nishina et al., 1978). This study also utilized the fluorescence-quenching properties of protein (riboflavin-binding protein) to obtain RR spectra of flavins.

Experimental Section

Materials. Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were obtained from Sigma. Flavin concentrations in solution were determined from their absorbance at 450 nm, where the nucleotides have molar extinction coefficients of 11 300 and 12 200 M^{-1} cm^{-1} for FAD and FMN, respectively. D_2O, riboflavin, *N,N*-dimethylformamide (DMF), and methanesulfonyl chloride were obtained from Aldrich. Periodic acid (H_5IO_6) and sodium borohydride ($NaBH_4$) were obtained from Fisher, while crotonyl-CoA and acetoacetyl-CoA were obtained from P-L Biochemicals. All other materials used were of reagent grade and were used without further purification.

UV–Vis Spectral Measurements. Absorption spectra were recorded on a Cary 17 UV–vis spectrophotometer. Optical densities at a single wavelength for flavin concentration measurements were determined on a Cary 16 spectrophotometer. Quartz cuvettes of a 1-cm path length were used.

Laser Raman Experiments. Samples were introduced in melting point capillaries under conditions listed in the spectral figure captions. Raman spectra were obtained by using a Spex 1401 lasar Raman spectrometer equipped with a cooled RCA photomultiplier tube. The excitation source for all reported spectra was the 488-nm line of a Coherent Radiation Model 3 argon ion laser. Spectral slit widths were ~4 cm^{-1} in all cases, and reported band positions are accurate to within ±5 cm^{-1}. Optimum flavin concentrations for Raman spectral analyses were 1–2 mM for FMN, FAD, and the bridged flavin model compound, while for fatty acyl CoA dehydrogenase the optimum flavin concentration was 0.25 mM. Samples were changed after each complete spectral scan to avoid the effects

† From the Department of Chemistry, University of Wisconsin—Milwaukee, Milwaukee, Wisconsin 53201. *Received November 7, 1978; revised manuscript received April 3, 1979.* This work was supported by National Institutes of Health Grants GM 21916 and AM 15527.

[1] Abbreviations used: FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; RR, resonance Raman; CoA, coenzyme A; CARS, coherent anti-Stokes Raman spectroscopy; DMF, dimethylformamide.

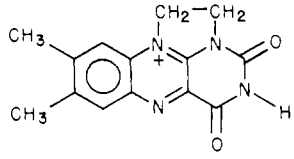
0006-2960/79/0418-3471$01.00/0

of photodecomposition. Except for the semiquinone cation, all samples gave reproducible spectra even after exposure for 30 min to ≈50 mW of laser power; under these conditions there was no discoloration of the sample. With the semiquinone cation, the solution contained in the capillary turned dark on laser irradiation; for this sample a spinning cell was employed to avoid photodecomposition.

Oxidized Flavins. A stock solution of 1 mM FMN or FAD in a 0.01 M Tris (pH 7) buffer was prepared. Solid KI was added to a final concentration of 2 M. KI at this concentration had no effect on the intensity or position of the 445-nm visible absorption maximum for oxidized flavin.

Deuterated Flavins. Deuterated FAD was prepared by adding solid FAD to D_2O to give a final flavin concentration of 1 mM. Again, KI was added to reach a final concentration of 2 M. No significant differences can be observed when comparing the RR spectra of deuterated FAD and deuterated FMN.

Synthesis of 7,8-Dimethyl-1,10-ethyleneisoalloxazinium Perchlorate. The bridged isoalloxazine I was synthesized in



I

three steps from riboflavin. The procedures of Fall & Petering (1956) were used to form 7,8-dimethyl-10-(β-formylethyl)-isoalloxazine from riboflavin, followed by the synthesis of 7,8-dimethyl-10-(β-hydroxyethyl)isoalloxazine. The final bridged compound was obtained from the β-hydroxyethyl compound by the method of Müller & Massey (1969) Spectral properties observed for the bridged compound were as follows: UV–vis (0.1 N sulfate buffer, pH 2) ϵ_{390} = 20 500, ϵ_{262} = 41 600, ϵ_{218} = 33 250 M^{-1} cm^{-1}; UV–vis (0.01 M phosphate buffer, pH 7) ϵ_{428} = 12 300, ϵ_{378} = 16 900, ϵ_{264} = 33 250, ϵ_{222} = 27 006 M^{-1} cm^{-1}; IR 3200, 3035 (ν_{N-H}), 1750, 1710 ($\nu_{C=O}$), 1100 ($\nu_{ClO_4^-}$) cm^{-1}. The product obtained displayed the pH behavior reported previously. The proton on N-3 ionized with a pK_a = 5.9; the visible λ_{max} at 390 nm in the protonated species shifted to 428 nm in the neutral species.

The RR spectrum of the bridged isoalloxazine was obtained following dissolution of 2.5 M KI at both pH 7 and 2.

Protonated Flavins. Protonated FMN was prepared by the dilution of a 10 mM FMN solution with 6 M HCl to a final concentration of 1 mM FMN. Prolonged exposure to laser light caused the production of a red color presumably due to photochemical reduction of FMN to FMN semiquinone cation. This hypothesis was confirmed by the fact that an EPR signal can be obtained from a capillary containing protonated FMN which has been intentionally exposed to the laser beam (power 1.2 W) for ~30 min. However, production of the semiquinone was not quantitative and resulted in no major changes in the Raman spectrum of protonated FMN.

FMN Semiquinone Cation. The red semiquinone cation was produced by incubation (5 min) of 5 mL of 2 mM protonated flavin with 100 mg of granular Zn metal. The reaction was stopped by transferring the solution to a sealed capillary tube; this transfer removed the flavin product from metallic Zn. The absorbance change at 503 nm was monitored at several flavin concentrations (0.1–1 mM), and the molar extinction for this species was determined to be 3250 M^{-1} cm^{-1}, which is consistent with a previous study (Beinert, 1956).

General Fatty Acyl-CoA Dehydrogenase. The enzyme preparation was carried out by the procedure of Hall & Kamin (1975). This procedure was modified by the addition of two DEAE-Sephadex anion-exchange chromatographic separations. Each of these chromatographic separations was carried out on DEAE-Sephadex columns equilibrated with 40 mM phosphate buffer (pH 7.4) containing 0.12 M KCl. Gradient elution of protein was accomplished by varying the ionic strength of the elution buffer from 0.12 to 0.26 M KCl. The purified protein was homogeneous as evidenced by disc gel electrophoresis at pH values of 4.4 and 8.0 and by $NaDodSO_4$ disc gel electrophoresis at pH 7.

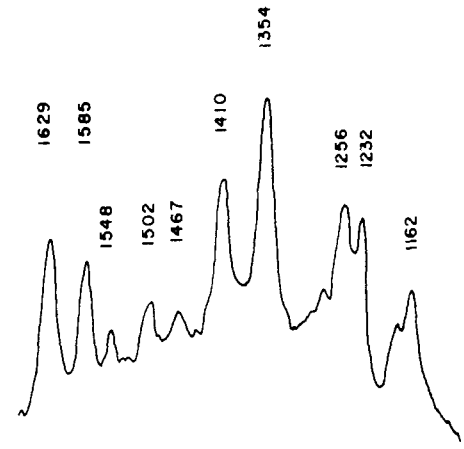


FIGURE 1: RR spectrum of flavin mononucleotide. (FMN) = 1.1 × 10^{-3} M; 0.01 M Tris, pH 7, buffer; 2 M KI.

Preparation of enzyme for Raman analysis was carried out by dialyzing enzyme against 40 mM phosphate buffer, pH 7.4, overnight at 4 °C. After dialysis, the dehydrogenase was concentrated to 0.2 mM [determined from FAD absorbance at 450 nm (ϵ = 11 300 M^{-1} cm^{-1})] by using a Schleicher and Schuell collodion protein concentrator. Complexes were prepared by adding the appropriate amounts of acetoacetyl-CoA and crotonyl-CoA to give a twofold excess over enzyme sites. Since the kinetically determined binding constants for these complexes, at pH 8, are K_i = 7 × 10^{-6} M for acetoacetyl-CoA and K_i = 1.6 × 10^{-5} M for crotonyl-CoA and the dissociation constants for acetoacetyl-CoA determined from spectrophotometric titration at pH 7.0 and 8.0 are K_D = 2.0 × 10^{-6} and 4.3 × 10^{-6} M, respectively (McKean et al., 1979); >95% of the enzyme is present as a charge-transfer complex.

Results and Discussion

We have examined the RR spectra of a number of chemically distinct flavin model compounds and the spectrum of a flavoprotein enzyme fatty acyl-CoA dehydrogenase. Our purpose in these studies is to evaluate the utility of RR spectroscopy in determining flavoprotein interactions.

Figure 1 shows the spectrum of FMN in 2 M KI; several bands are observed, including those at 1629, 1585, 1410, and 1354 cm^{-1} which are the strongest in the vibrational spectra. Also of interest are the medium intensity bands at 1256 and 1232 cm^{-1}. It is these six bands which are observed in the spectra of fatty acyl-CoA dehydrogenase and its complexes.

As can be seen in Figure 2 and Table I, the spectrum of FAD is quite similar to that of FMN; this indicates (as would be expected) that resonance enhancement is limited to the isoalloxazine ring which is thus responsible for all of the observed Raman intensity in these spectra. Our spectrum of FAD in 2 M KI corresponds closely to that obtained previously by CARS (Dutta et al., 1977), indicating that the addition of 2 M KI does not affect the RR spectrum. Previous infrared spectroscopic studies have indicated changes in the $\nu_{C=O}$ (1690

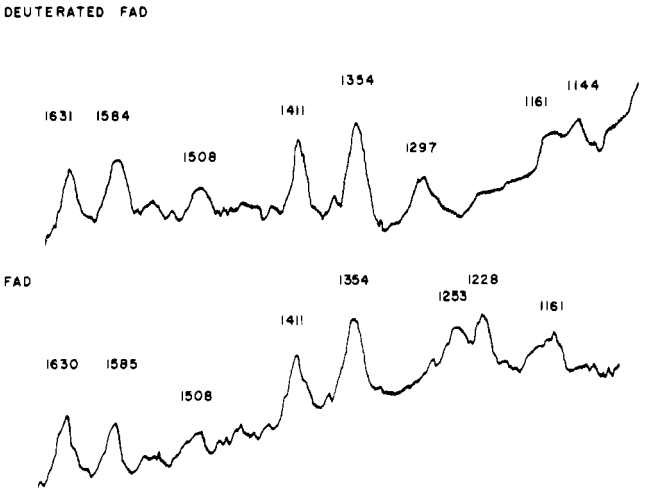


FIGURE 2: Upper: RR spectrum of FAD in D_2O. (FAD) = 2.3 × 10^{-3} M; 2 M KI (unbuffered, pH 7). Lower: RR spectrum of FAD in H_2O. (FAD) = 1.2 × 10^{-3} M; 2 M KI (unbuffered, pD 7).

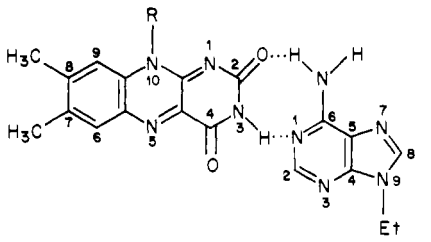
Table I: Raman Frequencies of Flavins and Flavoproteins[a]

FMN	FAD	FAD in D_2O	N-1 protonated FMN	1,10-bridged flavinium ion, N-3 protonated
		1144 m		
1162 m	1161 m	1161 m		
1182 sh	1182 sh			
1232 m	1228 m		1207 m	
1256 m	1253 m		1239 s	1236 m
1281 sh		1297 s	1290 m	1272 m
1354 s	1354 s	1354 s	1348 s	1368 s
1410 s	1411 s	1411 s		1412 m
1467 w			1454 w	
1502 w	1508 w	1508 w	1515 s	1513 s
1548 w			1549 m	
1585 s	1585 s	1584 s		
1629 s	1630 s	1631 s	1614 s	1626 s

N-3 ionized	FMN cationic semiquinone	uncomplexed enzyme[b]	dehydrogenase + AcAc-CoA	dehydrogenase + crotonyl-CoA
	1135 s			
			1162 w	
1187 m	1174 s			
	1204 w			
1241 m	1239 w	1234	1237 m	1237 m
1288 s	1271 s	1259	1262 m	1257 m
1365 s	1329 m	1350	1352 s	1350 s
1413 s		1407	1412 s	1407 s
1516 s				
			1587 m	1587 m
1629 s			1626 m	1627 m

[a] Relative intensity: s, strong; w, weak; m, medium; sh, shoulder. [b] Signal to noise ratio was not large enough to determine the relative intensities.

cm^{-1}) of C-2 of the isoalloxazine ring of 2′,3′,4′,5′-riboflavin tetraacetate in $CHCl_3$ on formation of a hydrogen-bonded complex with 9-ethyladenine (II) (Kyogoku & Yu, 1969). In addition, the N–H stretching region of 9-ethyladenine also reflects hydrogen-bond formation in the complex. X-ray crystallographic investigation has confirmed a structure similar to that shown for II for a complex of a riboflavin and an adenosine derivative; furthermore, it has been suggested that hydrogen-bond complex formation of the type shown for II occurs in FAD (Voet & Rich, 1970). We observe small frequency differences in the 1256–1232-cm^{-1} region of FMN



II

on formation of the presumed hydrogen-bonded structure in FAD. At the resolution of our spectra (≈4 cm^{-1}), we cannot be sure that these small frequency shifts are significant; however, since the vibrational modes at 1256 and 1232 cm^{-1} are associated with the N-3 position of the isoalloxazine ring (as discussed below) and probably include considerable N–H bending, such changes might be expected as the result of differences in intramolecular hydrogen bonding of solvent water molecules with FMN as compared with adenine hydrogen bonds to flavin rings in FAD. We also observe complex frequency shifts in the 1400–1100-cm^{-1} region upon deuteration of the exchangeable proton of FAD in D_2O; the two bands at 1228 and 1253 cm^{-1} disappear. New bands appear at 1297 and 1144 cm^{-1} in the deuterated flavin. The previous CARS study also indicated the appearance of the 1297-cm^{-1} band upon deuteration; the authors attributed this band to the isotopically shifted component of the two accidentally degenerate bands at 1359 cm^{-1} (Dutta et al., 1977). In any event, since only the N-3 proton is exchangeable in D_2O, it is clear that bands in the 1200–1300-cm^{-1} region are associated with vibrations involving N-3.

Spiro and co-workers have suggested that the bands in the 1200–1300-cm^{-1} region correspond to a ring mode localized in the N-3 region with admixture of N–H in-plane bending (Dutta et al., 1977). In order to further investigate the hypothesis that some of the vibrational bands in this region are associated with ionization of N-3, we had hoped to study FMN at pH values above the pK_a; however, the KI added as a quenching agent oxidizes to iodine at these pH values. In order to study the N-3 anionic form of flavin, we have prepared 7,8-dimethyl-1,10-ethyleneisoalloxazinium perchlorate (a compound which ionizes at N-3 with pK_a = 5.9). Figure 3 and Table I show the effect of ionization on the RR spectrum. As with deuteration, a change of ionization state has a major effect on the frequency of those bands in the 1230–1310-cm^{-1} region. Only the intensity of other bands in the spectrum changes upon ionization at N-3; these observations are in accord with the previously discussed assignment of bands in this region (Dutta et al., 1977).

The effect of protonation at N-1 is shown in Figure 4 and Table I for FMN in acidic solution (pH ~0). (Note that KI is not added to protonated species since they do not fluoresce.) Sizable frequency shifts are observed in bands in the 1200-cm^{-1} region and in the strong bands at 1630, 1584, and 1410 cm^{-1}; however, the band at 1354 cm^{-1} shifts only slightly upon protonation of FMN. Since it is possible to further protonate N-1 protonated cation to yield N-1,N-5 diprotonated flavin and since the λ_{max} for the diprotonated flavin is 460 nm while the monoprotonated flavin has λ_{max} at 390 nm and little 488-nm absorption (Land & Swallow, 1969), it seemed possible that our resonance Raman spectrum might represent a spectrum of the small amount of dication present in solution containing monocation. In order to assess this possibility, we attempted to obtain a spectrum of riboflavin dication in concentrated H_2SO_4; the spectrum we obtained was of very low quality with only two bands, at 1417 and 1510 cm^{-1},

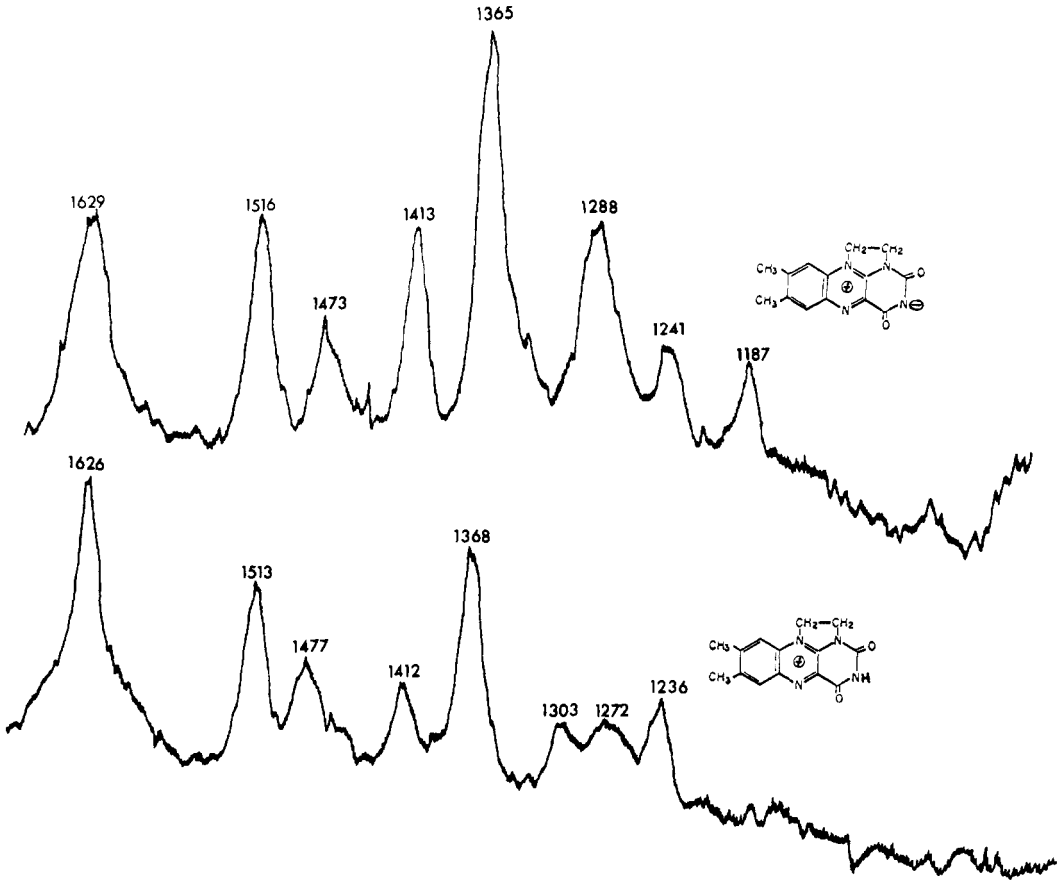


FIGURE 3: RR spectra of bridged isoalloxazine. Upper: ionized form; pH 7.0. Lower: protonated form; pH 2.0.

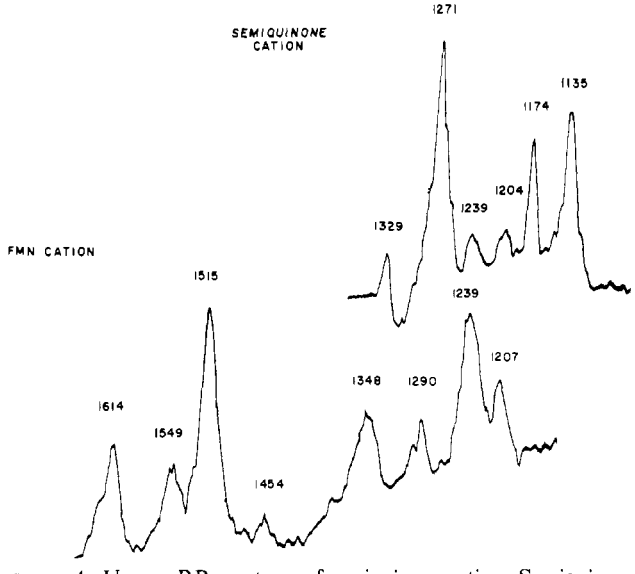


FIGURE 4: Upper: RR spectrum of semiquinone cation. Semiquinone was produced by the reduction with Zn of a 2.5 mM protonated FMN solution. Lower: RR spectrum of protonated FMN. (FMN) = 1×10^{-3} M.

identifiable in the spectrum. These bands are not the strongest bands in our monocation spectrum, so we conclude that the spectrum in Figure 4 represents the N-1 protonated monocation. Addition of the ethylene bridge, resulting in production of a positive charge on the nitrogen involved in a C=N, produces changes in the RR spectrum shown in Figure 3 and Table I. Since protonation of N-1 (Figure 4 and Table I) similarly generates a positive charge on the nitrogen involved in a C=N, we might expect that bands of FMN which change with bridge formation and with protonation might include vibrational bands containing $\nu_{C=N}$. Figures 3 and 4 and Table I indicate that one band showing a large frequency change on protonation and bridging is the 1585-cm^{-1} band. We, therefore, suggest that the 1585-cm^{-1} band has significant C=N stretching character. Lord & Thomas (1967) have previously assigned bands in the 1500–1650-cm^{-1} region of adenine derivatives to combinations of C=C and C=N stretching modes.

The charge-transfer complex between protonated FMN (acceptor) and hydroquinone (donor) was studied by adding 0.01 and 0.1 M hydroquinone. (At the higher concentration, hydroquinone contributes some spectral bands.) Under these conditions, complexation of the flavin is about 58 and 88%, respectively; however, no changes in the frequency of spectral bands were observed. This is in keeping with the usual situation for charge-transfer complexes in which these complexes show significant bonding changes only in the excited electronic state (Jencks, 1969). The observed Raman active vibrations are, of course, ground-state vibrations, and this state is relatively unaffected. Therefore, the usual situation is that charge-transfer complex formation does not affect the frequency of Raman bands since this is a function of the electronic ground state of the molecule; however, the intensity of RR bands may be affected since intensity is a function of the electronic excited state (Carey et al., 1973).

Having established that a charge-transfer complex had the same RR spectrum as protonated FMN, we attempted to study the effect of oxidation-state change upon the RR spectrum. The cationic semiquinone was formed by reducing protonated FMN with Zn metal (Beinert, 1956). The extent of reaction was determined by observation of the visible spectral change at 503 nm; we observed the visible spectral change reported previously (Beinert, 1956). We also observed an EPR spectrum for this reduction product. The spectrum of the red

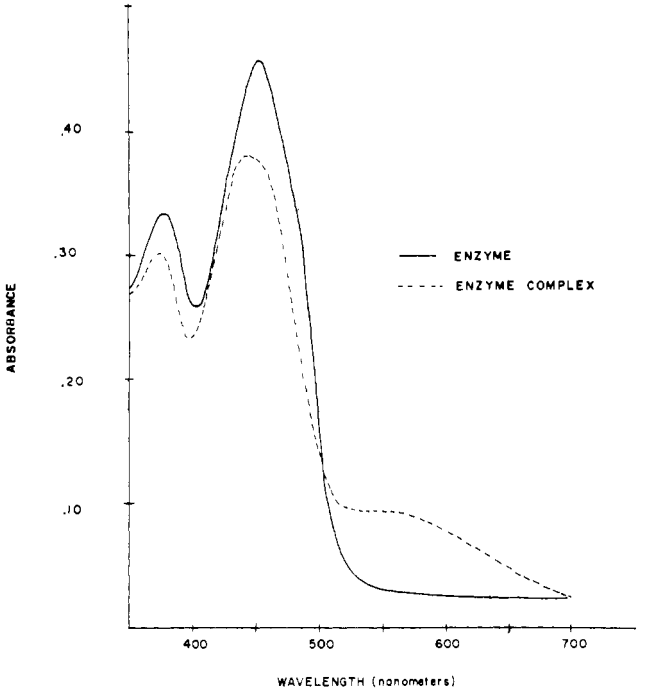


FIGURE 5: Visible (electronic) spectrum of "general" fatty acyl-CoA dehydrogenase [4.1 × 10^{-5} M (—)] and the acetoacetyl-CoA complex [acetoacetyl-CoA concentration 80 μM (- - -)].

semiquinone is shown in Figure 4 and Table I. Large spectral changes are observed upon reduction: these include the appearance of strong bands at 1135 and 1174 cm^{-1} and the disappearance or loss of almost all intensity for bands at 1614 and 1515 cm^{-1}. We have not been able to observe any significant Raman scattering above 1329 cm^{-1} for the red semiquinone.

The visible spectrum of fatty acyl-CoA dehydrogenase and its acetoacetyl-CoA complex is shown in Figure 5; both the free enzyme and the complex show significant absorbance at 488 nm, and neither fluoresces strongly. The RR spectrum of the general fatty acyl-CoA dehydrogenase from porcine liver mitochondria is shown in Figure 6 and Table I. Although the spectrum is weak and only four bands can be seen, it appears that the bound flavin spectrum is nearly identical with that of free FAD except for the shift of the 1253- and 1228-cm^{-1} bands of FAD to 1259 and 1234 cm^{-1} in the protein FAD complex. The spectrum of the acetoacetyl-CoA complex of the enzyme is of considerably better quality, presumably due to the red visible spectral shift, toward 488 nm, caused by complexation, which enhances the resonance effect. Again, there is little spectral change noted between FAD and the enzyme complex with the exception of the 1253- and 1228-cm^{-1} bands of FAD which shift to 1262 and 1237 cm^{-1} in the FAD enzyme–acetoacetyl-CoA complex. The only sizable intensity changes upon complex formation between enzyme and acetoacetyl-CoA are the lower intensity of the 1630- and 1585-cm^{-1} bands in free enzyme.

The complex of acetoacetyl-CoA and fatty acyl-CoA dehydrogenase may be important in regulation of enzyme activity since acetoacetyl-CoA (a ketone body precursor) is a tight-binding competitive inhibitor of binding of fatty acyl-CoA substrates (McKean et al., 1979). This acetoacetyl-CoA complex has been postulated to be a charge-transfer complex (Massey & Ghisla, 1974). This suggestion is based upon the similarity between visible spectral changes produced upon complex formation between acetoacetyl-CoA and fatty acyl-CoA dehydrogenase and those spectral changes observed with phenols and Old Yellow Enzyme. The latter system is well characterized, and the spectral changes have been shown to occur due to charge-transfer complexation (Abramovitz & Massey, 1976). Charge-transfer theory suggests that the wave function of the complex is

$$\Psi_n(D,A) = a\Psi_0(D,A) + b\Psi_1(D^+,A^-)$$

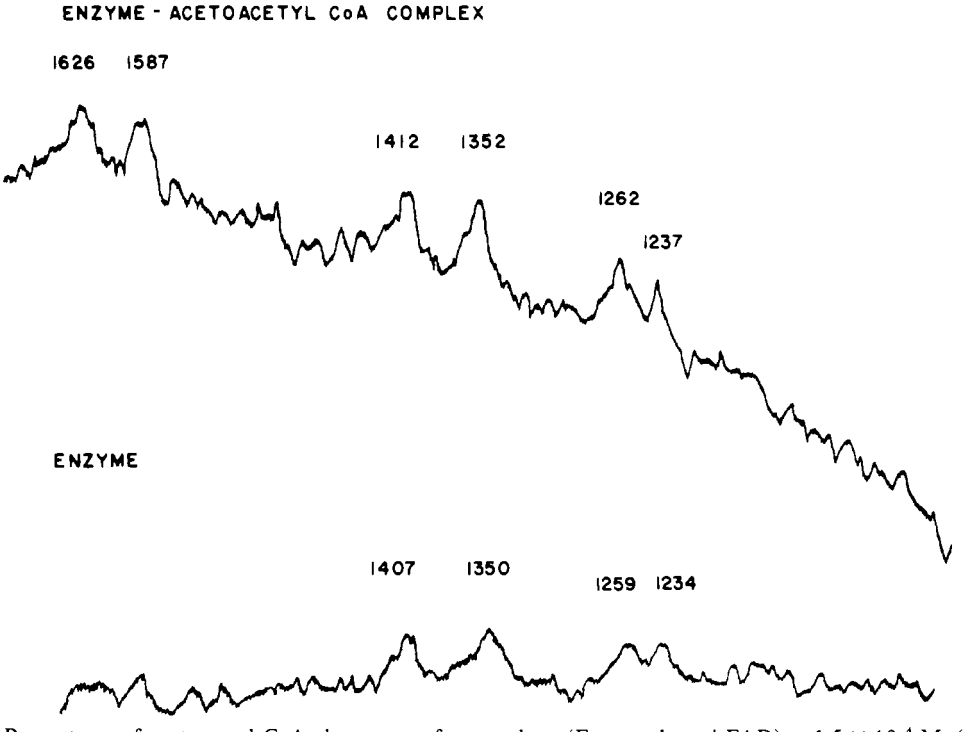


FIGURE 6: Upper: RR spectrum of acetoacetyl-CoA charge-transfer complex. (Enzyme-bound FAD) = 1.5 × 10^{-4} M; (acetoacetyl-CoA) = 3.0 × 10^{-4} M; pH 7.4, 40 mM phosphate buffer. Lower: RR spectrum of fatty acyl-CoA dehydrogenase. (Bound FAD) = 2.2 × 10^{-4} M; pH 7.4, 40 mM phosphate buffer.

Table II

	frequency (cm^{-1})		
	FAD[b]	protein-FAD	$\Delta\nu$ (cm^{-1})
riboflavin-binding protein[a]	1231 1260	1229 1250	−2 −10
glucose oxidase	1231 1260	1230	−1 (1260-cm^{-1} band missing)
fatty acyl-CoA dehydrogenase	1228 1253	1234 1259	+6 +6
AcAc-CoA complex	1228 1253	1237 1262	+9 +9
crotonyl-CoA complex	1228 1253	1237 1257	+9 +4

[a] Dutta et al. (1978). [b] The frequencies of these bands in this report differ slightly from those of Spiro and co-workers (Dutta et al., 1978); this is presumably due to instrument calibration factors. Bands reported in this study are 7–8 cm^{-1} lower in frequency, on the average, than those reported by Spiro.

where D is the charge-transfer donor and A the acceptor (Matsunaga, 1964). Most complexes are characterized by $a \gg b$ for the electronic ground state. Increased contribution of b to the equation for the first electronic excited state results in a visible charge-transfer band. However, there also are complexes in which the dative bonding characterized by the b term makes an important contribution to ground-state structure. This results in the observation of semiquinone in the ground-state vibrational spectrum (Matsunaga, 1964). Our RR spectra are consistent with the designation in which $a \gg b$ (no ground-state dative bonding) for the acetoacetyl-CoA–enzyme complex, since we see no spectral changes diagnostic of full electron transfer to flavin to form a semiquinone.

Table I also lists the vibrational bands of a second charge-transfer complex of enzyme with unsaturated fatty acyl-CoA (crotonyl-CoA). Again, the only significant change is in the 1250-cm^{-1} band; the crotonyl-CoA complex spectrum shows a 1257-cm^{-1} band which is closer to the 1253-cm^{-1} band of FAD than this band either in unliganded enzyme or in the acetoacetyl-CoA complex.

The shifts that we observe during complexation of FAD to enzyme or enzyme complexes are in the same spectral region as the largest shifts observed by Spiro & co-workers (Dutta et al., 1978). Namely, the 1250-cm^{-1} region associated with a δ_{N-H} vibrational mode at N-3 of the flavin ring. Table II compares the RR spectral changes for the three flavoproteins studied to date.

Since N-3 is involved in hydrogen bonding in flavin systems, as discussed previously in this paper, it seems likely that the molecular basis of the small shifts observed on binding FAD to protein is hydrogen-bonding changes between FAD in aqueous solvent and FAD bound at the enzyme active site. Barring any changes in the percentage of δ_{N-H} represented in the normal coordinate composition of the 1200-cm^{-1} bands, the increase in frequency on FAD binding to fatty acyl-CoA dehydrogenase would represent a stronger hydrogen-bonding environment on the enzyme surface than that in aqueous solutions of FAD. Conversely, glucose oxidase and riboflavin-binding protein would represent a weaker hydrogen-bonding environment than that found between adenine and flavin rings of FAD in water. However, such changes must be small perturbations since all three proteins have visible spectra characteristic of an aqueous environment as opposed to visible spectra characteristic of a non-hydrogen-bonding solvent such as CCl_4 (Kotnki et al., 1970). Conclusive understanding of the small spectral shift in the 1200-cm^{-1} bands of flavin must await determination of a spectrum of flavin in nonaqueous solvent and at an enzyme active site yielding a visible spectrum typical of a nonaqueous environment.

In summary, we have obtained RR spectra of protonated, deuterated, and partially reduced flavin compounds. In each case the RR spectrum of flavin changes substantially during these chemical modifications. On the other hand, charge-transfer complexation does not modify the spectrum. An RR spectrum of fatty acyl-CoA dehydrogenase is quite similar to that of FAD, showing no significant covalent binding effects upon enzyme complexation of flavin; the only significant spectral change is associated with a region of the spectrum which would respond to changes in the hydrogen-bonding environment. Furthermore, formation of complexes of acetoacetyl-CoA and of crotonyl-CoA with the general fatty acyl-CoA dehydrogenase enzyme shows only very minor spectral changes of the flavin, in keeping with the postulated charge-transfer nature of the bonding between the enzyme and these competitive inhibitors.

References

Abramovitz, A. S., & Massey, V. (1976) *J. Biol. Chem. 251*, 5327.

Bear, C., Waters, J., & Waters, T. (1970) *J. Chem. Soc. D 20*, 702.

Beinert, H. (1956) *J. Am. Chem. Soc. 78*, 5323.

Carey, P. R., Froese, A., & Schneider, H. (1973) *Biochemistry 12*, 2198.

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

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

Fall, H., & Petering, H. (1956) *J. Am. Chem. Soc. 78*, 377.

Friedman, J. M., & Hochstrasser, R. M. (1975) *Chem. Phys. Lett. 33*, 225.

Hall, C., & Kamin, H. (1975) *J. Biol. Chem. 250*, 3476.

Jencks, W. (1969) *Catalysis in Chemistry and Enzymology*, pp 437–462, McGraw-Hill, New York.

Kotnki, A., Naoi, M., & Yagi, K. (1970) *J. Biochem. (Tokyo) 68*, 287.

Kyogoku, Y., & Yu, B. (1969) *Bull. Chem. Soc. Jpn. 42*, 1387.

Land, E. J., & Swallow, A. J. (1969) *Biochemistry 8*, 2117.

Lord, R. C., & Thomas, J. (1967) *Spectrochim. Acta, Part A, 23*, 2551.

Massey, V., & Ghisla, S. (1974) *Ann. N.Y. Acad. Sci. 227*, 446.

Matsunaga, Y. (1964) *J. Chem. Phys. 41*, 1609.

McKean, M. C., Frerman, F. E., & Mielke, D. M. (1979) *J. Biol. Chem. 254*, 2730.

Müller, F., & Massey, V. (1969) *J. Biol. Chem. 244*, 4007.

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

Tsuboi, M. (1976) *Proc. Int. Conf. Raman Spectrosc., 5th*, 135–143.

Voet, D., & Rich, A. (1970) in *Flavins and Flavoproteins* (Kamin, H., Ed.) pp 23–33, University Park Press, Baltimore, MD.

Weber, G. (1950) *Biochem. J. 47*, 114.