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# Protein–Ligand Interactions in Lumazine Protein and in *Desulfovibrio* Flavodoxins from Resonance Coherent Anti-Stokes Raman Spectra†

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ABSTRACT: The resonance coherent anti-Stokes Raman technique was used to obtain vibrational spectra of flavin in flavodoxins from *Desulfovibrio gigas* and *Desulfovibrio vulgaris* and of the simpler 6,7-dimethyl-8-ribityllumazine chromophore in the blue fluorescence lumazine protein from the bioluminescent bacterium *Photobacterium phosphoreum*. In the region examined, 1100–1700 $cm^{-1}$, the Raman spectrum of the lumazine is less crowded than that of the flavin and this facilitates assignment of observed frequencies to particular vibrational modes. Certain modes are not affected by chromophore binding to the protein, but others are changed in frequency or intensity in a way that can be rationalized by expected interactions of the chromophore with the amino acid residues of the binding site. For example, a tentative assignment of change in hydrogen bonding at N(5) is suggested as the cause of the frequency shift for the chromophore in both flavodoxins (free–bound, 1582–1572 $cm^{-1}$) and for C(4)=O in glucose oxidase (1359–1364 $cm^{-1}$) and lumazine protein (1359–1362 $cm^{-1}$). Shifts of the C(2)–N(3) mode in $D_2O$ may arise from hydrogen-bonding changes at C(2)=O in lumazine protein (1284–1291 $cm^{-1}$), flavodoxin (1300–1280 $cm^{-1}$), and glucose oxidase (1297–1287 $cm^{-1}$). Bonding at N(3)–H may be the origin of changes in the frequency or intensity of the amide III mode in riboflavin binding protein and glucose oxidase. A stacking interaction is suggested for the change in a pyrimidine ring mode in FAD (1508 $cm^{-1}$) since this mode is found at 1504 $cm^{-1}$ in 30% $Me_2SO/H_2O$, where the adenine and pyrimidine are unstacked. The results clearly indicate different interactions in the binding sites of those proteins studied to date.

When a small molecule binds to a protein the specific groups involved in the interaction should be identifiable by changes in their vibrational frequencies. Recently there has been a great deal of interest in the use of laser Raman spectroscopy to provide this information, and the flavins and flavoproteins have received some study, among a number of other cases. It is well-known that redox and spectroscopic properties of flavins can be markedly changed by interactions in the protein binding site. It is of interest to see if these properties are reflected by changes in the vibrational spectrum of free and bound flavin.

Coherent anti-Stokes Raman spectroscopy (CARS),[1] a nonlinear optical technique, is generally best suited for such studies because resonance enhancement (Carreira et al., 1978; Chabay et al., 1976) allows the observation of Raman signals from even highly fluorescent samples in aqueous solution, at concentrations of millimolar or less. Additionally, the analysis of observed CARS line shapes and excitation profiles can yield important structural information about the geometry of the excited state chromophore (Carreira et al., 1977a,b).

Using CARS, Dutta et al. (1977, 1978) have obtained vibrational spectra from the flavin chromophore as it exists in flavin adenine dinucleotide (FAD), bound to riboflavin binding

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[1] Abbreviations used: CARS, coherent anti-Stokes Raman spectroscopy; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; LUM, 6,7-dimethyl-8-ribityllumazine; $Me_2SO$, dimethyl sulfoxide.

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protein and within the active site of the enzyme glucose oxidase. Resonance enhancement was observed for a number of bands associated with the isoalloxazine ring by using laser excitation within the two lowest energy electronic absorption bands of flavin. The observation of frequency shifts on exchange of each compound with $D_2O$ was employed to identify modes associated with the N(3)–H group, which has the only exchangeable hydrogen. Changes were also observed between free and bound flavin in riboflavin binding protein and glucose oxidase, and these were attributed to protein interactions at certain positions around the ring. Nishina et al. (1978) were able to record resonance Raman spectra of riboflavin and five of its derivatives when bound to riboflavin binding protein by taking advantage of the fact that their fluorescence was quenched on binding. Due to the high fluorescence of the free ligand, these authors could not measure the Raman spectra of the free flavins and so could make no conclusions about the effect of binding on the individual frequencies. However, they reported the first detailed assignment of frequencies to modes localized within the isoalloxazine ring.

Benecky et al. (1979) have used resonance Raman with KI to quench the intense fluorescence of unbound FMN, FAD, and the bridged molecule 7,8-dimethyl-1,10-ethyleneisoalloxazinium perchlorate. Spectra of several weakly fluorescent species were also reported by these authors, including FMN protonated at N(1), FMN semiquinone cation, the general fatty acyl-CoA dehydrogenase, and two "charge-transfer" complexes of fatty acyl-CoA dehydrogenase. Tentative assignments were made for several vibrational modes observed; however, the only significant changes in the spectra on binding to the enzyme were found in frequencies associated with the N(3)–H bending mode.

In this present work we report the resonance CARS spectra of *Desulfovibrio vulgaris* flavodoxin which has a known three-dimensional structure (Watenpaugh et al., 1973), of *Desulfovibrio gigas* flavodoxin, and of lumazine protein, the blue fluorescence protein recently isolated from the bioluminescent bacterium *Photobacterium phosphoreum* by Gast & Lee (1978) and shown to have 6,7-dimethyl-8-ribityllumazine (LUM) as its fluorescent prosthetic group (Koka & Lee, 1979). Since isoalloxazine is actually a benzalumazine derivative, the simpler ring structure of LUM provides a much less complex Raman spectrum (Figure 1) and comparison with flavin can be used to support assignments.

Also, a comparison of spectra between free and bound flavin and LUM reveals that certain modes are involved in the binding interaction. When the spectra are compared for the free and bound cases in $D_2O$, which is used primarily to identify modes coupled to N(3)–H, it is found that some modes not affected when the ligand is in free solution are affected when bound, indicating that the deuteration is changing the protein in some way.

It is well-known that FAD in aqueous solution is an intramolecular stacked complex (Sarma et al., 1968), and this is responsible for its low fluorescence efficiency in comparison to FMN. Intermolecular stacked dimers of FMN are also present (Gibson et al., 1962) at the high concentrations required for some Raman studies. In mixtures with dimethyl sulfoxide such complexes become unstacked and small changes in the Raman spectra are observed.

## Materials and Methods

Isolation and purification of lumazine protein was as described (Lee & Koka, 1978), and a sample of 6,7-dimethyl-8-ribityllumazine was a gift from Dr. H. C. S. Wood, University of Strathclyde. Flavodoxins from *D. gigas* and *D. vulgaris* were a gift of Dr. J. LeGall, CNRS, Marseille, and 2-thio-FMN was provided by Dr. F. Müller, Agricultural University, Wageningen. Both FAD and FMN were obtained from Sigma (St. Louis, MO), and all other chemicals were the best commercial grade. Solutions were made in a buffer of 50 mM sodium phosphate, pH 7.0 in $H_2O$ and pD 7.0 in $D_2O$. Concentrations were made in the range 1–2 mM (estimated) by using the following extinction coefficients: 10 300 $M^{-1}$ $cm^{-1}$ at 410 nm for lumazine (Maley & Plaut, 1959) and assuming the same for one lumazine bound in lumazine protein (Gast & Lee, 1978); 10 200 $M^{-1}$ $cm^{-1}$ at 456 nm for the flavodoxins (Dubourdieu et al., 1975); 12 200 $M^{-1}$ $cm^{-1}$ at 450 nm for FMN; 11 000 $M^{-1}$ $cm^{-1}$ for FAD at 450 nm; 20 800 $M^{-1}$ $cm^{-1}$ at 487 nm for 2-thio-FMN (Föry & Hemmerich, 1967). Isotopic exchange of proteins was performed by 50× dilution into 90–95% $D_2O$ for 24 h followed by reconcentration using a Micro-Pro DiCon dialysis concentrator (Bio-Molecular Dynamics, Beaverton, OR). Samples of about 10 μL in volume were contained in ordinary 1-mm glass capillaries or micropipets (Rogers et al., 1977), and it was sometimes necessary to clarify protein samples by centrifugation (20000*g*, 10 min) before taking spectra. Photochemical damage during collection was avoided by maintaining sample temperatures near 5 °C and using low laser powers.

CARS spectra were obtained by using the computerized instrument previously described (Goss, 1978) and also equipped with a 1-mm iris for increased spatial filtering of the anti-Stokes signal at $\omega_3$. Two JY-H10 monochromators and an RCA C31034 photomultiplier were utilized. Momentum matching was accurately computer controlled by crossing angle updates every 10 $cm^{-1}$. Laser powers at frequencies $\omega_1$ and $\omega_2$ were reduced to the minimum levels (10–20 μJ, rep. rate 10 pps) to avoid saturation effects.

The spectra presented represent the average of 15 pulses at each sampling point, interspaced by 2 $cm^{-1}$. Two or three complete scans were added, and the ensemble is presented. Only those weak bands which are reproducible in frequency (to ±2 $cm^{-1}$) and intensity from run to run were regarded as significant, and these are marked on the figures by their frequencies.

The dye lasers were calibrated by using a Spex 1401 monochromator. The measurement of the 992-$cm^{-1}$ line of benzene at several different pump wavelengths leads to an accuracy of ±3 $cm^{-1}$ in the determination of the frequencies in the range of interest. The precision of measurement between runs is ±1.5 $cm^{-1}$. Excitation was in the long wavelength electronic absorption of the lumazine or flavin chromophores; consult the figures for the appropriate number used.

## Results

*Vibrational Assignment.* The resonance CARS spectra of LUM and lumazine protein are in Figures 1 and 2. In these and subsequent figures, the spectrum in $H_2O$ is placed above that in $D_2O$ for ready comparison. Labels on some unshifted bands were omitted for clarity. The quality of the spectra is excellent, and band positions are repeatable to ±1.5 $cm^{-1}$. The close similarity of Figures 1 and 2 again confirms that the isolated chromophore characterized by Koka & Lee (1979) is identical with that bound to the protein. Table I lists the frequency values observed for LUM and lumazine protein and those for FMN, 2-thio-FMN, and flavodoxins (Figures 3–5). As a starting point for discussion, we list in the last column of Table I assignments which result from the studies of riboflavin binding protein by Nishina et al. (1978) as modified by the heavy-atom substitution study of Kitagawa et al. (1979) and the results of Benecky et al. (1979) for protonated FMN

Table I: Comparison of LUM, Lumazine Protein, FMN, 2-Thio-FMN, and Flavodoxin CARS Frequencies and Intensities[a]

| LUM | | lumazine protein | | FMN | | 2-thio-FMN, | *D. gigas* flavodoxin | | *D. vulgaris* flavodoxin, | assignment |
|---|---|---|---|---|---|---|---|---|---|---|
| $H_2O$ | $D_2O$ | $H_2O$ | $D_2O$ | $H_2O$ | $D_2O$ | $H_2O$ | $H_2O$ | $D_2O$ | $H_2O$ | to ring |
| | | | | 1629 s | 1628 s | 1630 m | 1624 s | 1625 s | 1626 s | I |
| 1584 s[b] | 1589 s | 1586 s | 1590 s | 1582 m | 1580 s | 1576 s | 1573 m | 1571 m | 1571 m | II, III |
| | | | | | | 1540 s | 1545 w | 1540 w | | |
| | | | | 1503 m | 1504 m | 1505 s | 1501 w | 1505 w | 1501 w | (III)[c] |
| | | | | 1412 s | 1414 s | 1415 m | 1407 s | 1407 s | 1407 s | III |
| 1359 s | 1360 s | 1362 s | 1368 s | 1355 s | 1357 s | 1362 s | 1352 s | 1355 s | 1354 s | II, III |
| 1238 m | 1284 s | 1242 s | 1291 m | 1261 m | 1300 m, br | 1256 w | 1251 w | 1280 m | 1254 m | III |
| 1264 s | 1265 s | 1260 s | 1262 m | 1233 m | 1243 m | 1232 w | 1227 m | 1229 m | 1228 s | II, III |
| | | | | 1217 w | 1215 m | | | | | |
| | | | | 1192 m | 1198 w | | | | 1185 m | (II, III) |
| | | | | 1170 m | 1179 w | | 1173 m | 1173 ms | | |
| | | | | 1151 w | 1150 ms | | 1163 vw | | 1164 m | (III) |
| | | | | 1128 w | 1127 w | | | 1136 m | | |

[a] All frequencies are in $cm^{-1}$ calibrated to the 992-$cm^{-1}$ line of benzene. [b] Strong = s, medium = m, weak = w, very = v, and broad = br. [c] Questionable assignments are designated in parentheses.

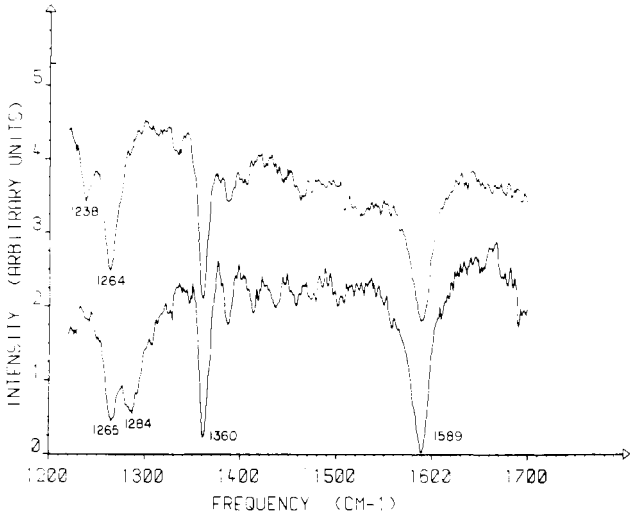



FIGURE 1: Resonance CARS spectra of 6,7-dimethyl-8-ribityllumazine in solutions of $H_2O$ (upper, 1.6 mM) and $D_2O$ (lower, 1.8 mM). Only the 1238-$cm^{-1}$ band is affected appreciably by deuteration at N(3). Spectra were obtained by using a 465-nm laser pump.

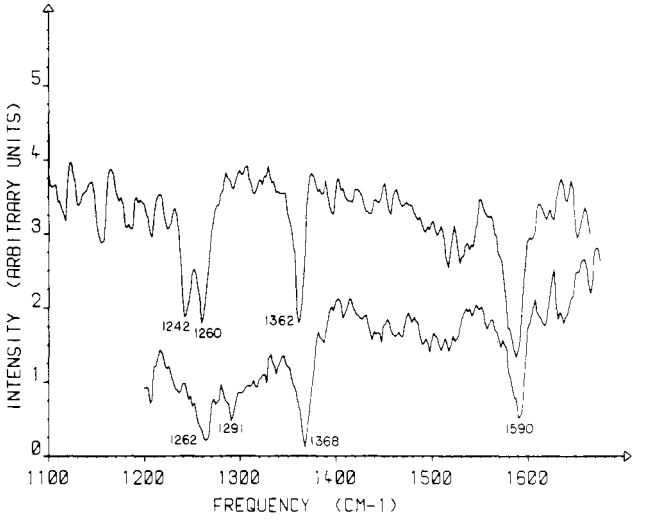
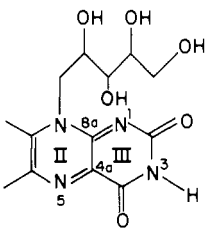
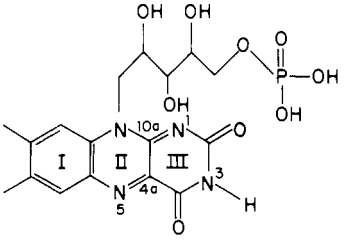



FIGURE 2: Spectra of lumazine protein in $H_2O$ (upper, 1.2 mM) and $D_2O$ (lower, 1.4 mM) are presented; the similarity to spectra of lumazine (see Figure 1) can be seen. The 1239-$cm^{-1}$ band has a much larger intensity in the protein because of structural differences in the excited state of the bound chromophore. Laser pump is 465 nm.

and bridged isoalloxazine cation. These papers should be consulted for details. Here we will show that some of these assignments need to be modified.



LUMAZINE (LUM)



FLAVIN MONONUCLEOTIDE (FMN)

Removal of benzene ring I from flavin to form LUM results in considerable simplification of the spectra. Only four intense bands remain in the range studied, 1700–1100 $cm^{-1}$. On the basis of heavy-atom labeling in riboflavin binding protein, Kitagawa et al. (1979) have assigned to ring III the lines corresponding to the FMN frequencies (Table I) at 1503, 1468, 1412, and 1192 $cm^{-1}$. The general absence of such bands from spectra of LUM and lumazine protein may or may not be significant since they have variable intensities in the flavins.

The highest frequency band in LUM is at 1584 $cm^{-1}$, and a prominent line of the same frequency is also found in flavins. Kitagawa has assigned this mode in flavin as a ring motion somewhat localized at C(4a)–N(5). Benecky et al. (1979) recently found a large frequency change in this vibration in N(1)-protonated FMN and N(1)–N(10)-bridged isoalloxazine cation. We observe a 9-$cm^{-1}$ decrease in this mode relative to FMN on substitution of C(2)=S for C(2)=O in 2-thio-FMN and somewhat smaller frequency increases on deuteration of LUM and lumazine protein (Table I). This evidence suggests the involvement of a larger portion of the ring III linkage in the motion: N(5)–C(4a)–C(8a)–N(1), with C(2)=O mixed in only to a small extent.

The next lower frequency in LUM appears at 1359 $cm^{-1}$. It is unaffected by deuteration and is seen at nearly the same place in FMN, 1356 $cm^{-1}$. This frequency is not radically different in N(1)-protonated FMN (1343 $cm^{-1}$), bridged isoalloxazine cation (1365 $cm^{-1}$), or 2-thio-FMN (1362 $cm^{-1}$). An assignment consistent with Kitagawa et al. (1979) is a ring III mode involving motions of C(4)–C(4a)–C(8a).

Below 1300 $cm^{-1}$ there are only two prominent bands in the LUM spectrum, 1264 $cm^{-1}$ and a weaker band at 1238 $cm^{-1}$. The former is unaffected by deuteration, but the latter band undergoes a remarkable increase in intensity and a large frequency shift to 1284 $cm^{-1}$ on exchange in $D_2O$. A similar phenomenon has been found in uracil on N(3) deuteration or

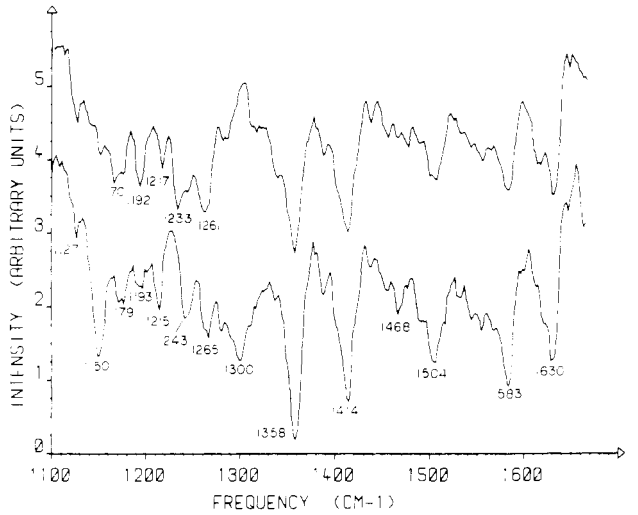



FIGURE 3: CARS spectra of FMN at 1 mM in $H_2O$ (upper) and $D_2O$ (lower) are presented. Negative Lorentzian line shapes are produced by excitation at 495 nm. The spectra are considerably more complex than those of the lumazines; two new bands are observed at 1300 and 1150 $cm^{-1}$ on deuterium exchange at N(3), and the bands at 1261 and 1233 $cm^{-1}$ appear weakened and are shifted to 1265 and 1243 $cm^{-1}$. A complex pattern of bands is observed below 1200 $cm^{-1}$.

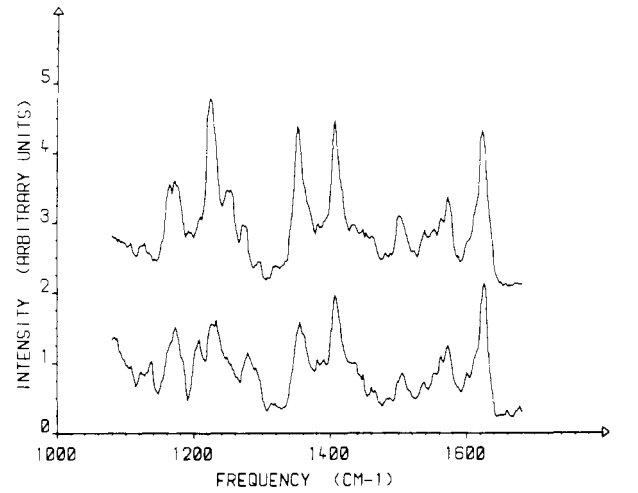



FIGURE 4: CARS spectra of *D. gigas* flavodoxin are given in $H_2O$ (upper, 1.6 mM) and in $D_2O$ (lower, 1.4 mM). The bands are observed positive at a laser pump of 480 nm. Heavy water again reveals two shifted bands at 1280 and 1136 $cm^{-1}$ as in FMN and FAD; however, below 1300 $cm^{-1}$ the spectra of flavodoxin appear much simpler. Significant changes in several vibrational frequencies are observed (1629–1624 and 1582–1573 $cm^{-1}$) between FMN and flavodoxin which suggest possible points of protein–prosthetic group interaction.

deprotonation (Nishimura et al., 1978). In light uracil, the 1266-$cm^{-1}$ band is most likely included in the broad structure centered at 1233 $cm^{-1}$. The deuterium-sensitive band can be assigned to the amide III mode of ring III, primarily due to C(2)–N(3) stretching. Strong coupling of this vibration to the in-plane N(3)–H bending would be diminished on deuteration at N(3), allowing the C–N mode to move to its natural frequency around 1295 $cm^{-1}$ (Nishina et al., 1978). No other bands were reproducibly observed in LUM or lumazine protein on deuteration, particularly the 1148-$cm^{-1}$ mode observed in deuterated uracil.

In this same region, 1300–1100 $cm^{-1}$, the flavin spectra (Figures 3–8) are much more complex than that of LUM. Here, the two bands which correspond in frequency to ones in LUM exhibit a somewhat different behavior on deuteration: that is a decrease in band intensity at 1233 $cm^{-1}$ and disappearance of the 1260-$cm^{-1}$ band, the appearance of medium-

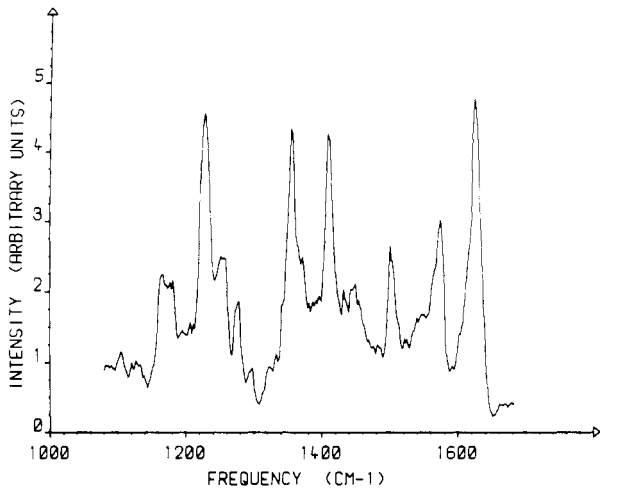



FIGURE 5: CARS spectrum of *D. vulgaris* flavodoxin (1.8 mM) revealing its apparent similarity to *D. gigas* flavodoxin. The spectra of these two flavodoxins imply that protein–chromophore interactions in *Desulfovibrio* are identical. Laser pump is 480 nm.

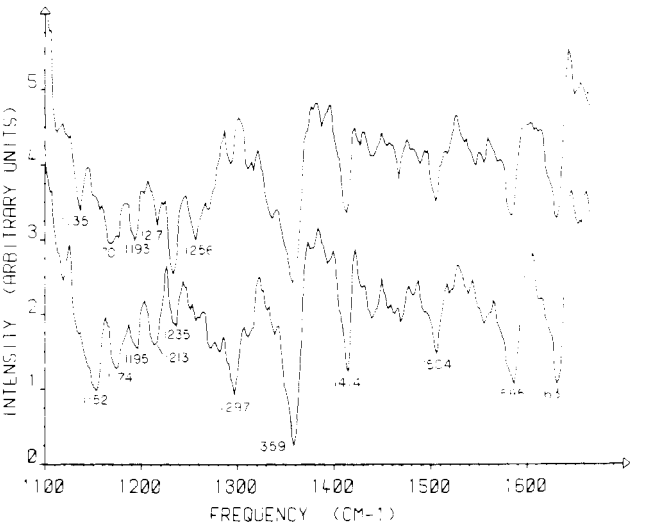



FIGURE 6: Effects of decreasing solvent polarity on the CARS spectra of FMN are illustrated. Spectra were recorded of FMN (1 mM) in solutions of 20% dimethyl sulfoxide ($Me_2SO$) with light (upper) and heavy (lower) water. The effects of deuteration are the same as those in pure solvent. Comparison of heavy-water species (Figures 3 and 6, lower) reveals only the disappearance of the 1243- and 1265-$cm^{-1}$ bands on addition of $Me_2SO$ and the appearance of the 1235-$cm^{-1}$ band. A similar trend is apparent in light-water spectra which may result in a sharpening of the 1233-$cm^{-1}$ band and a frequency shift to 1256 $cm^{-1}$ in the $H_2O/Me_2SO$ spectrum. Such spectral changes result from the dissociation of dimers of FMN by $Me_2SO$. Laser pump is 495 nm.

intensity features around 1300 and 1150 $cm^{-1}$, and the emergence of a weaker structure near 1240 $cm^{-1}$. Whether the latter is a dispersive band centered at 1235 $cm^{-1}$ or a negative peak centered at 1245 $cm^{-1}$ is uncertain. On consideration of each spectrum in turn, for aqueous FMN (Figure 3), the 1233-$cm^{-1}$ line diminishes and new bands are seen at 1300, 1265, 1243, and 1150 $cm^{-1}$ on going from $H_2O$ to $D_2O$. For FMN bound in flavodoxin (Figure 4), deuteration decreases the intensity of the 1236-$cm^{-1}$ band and new features appear at 1291 and 1148 $cm^{-1}$. For aqueous FAD similar behavior is also observed (Figure 7) and the effects of deuteration are most clearly seen. In this case the 1235- and 1261-$cm^{-1}$ bands are reduced and a structure remains at 1245 $cm^{-1}$. In aqueous $Me_2SO$ solutions of FMN and FAD (Figures 6 and 8) a similar commentary can be made; however, the positions and intensities at 1265 and 1245 $cm^{-1}$ appear

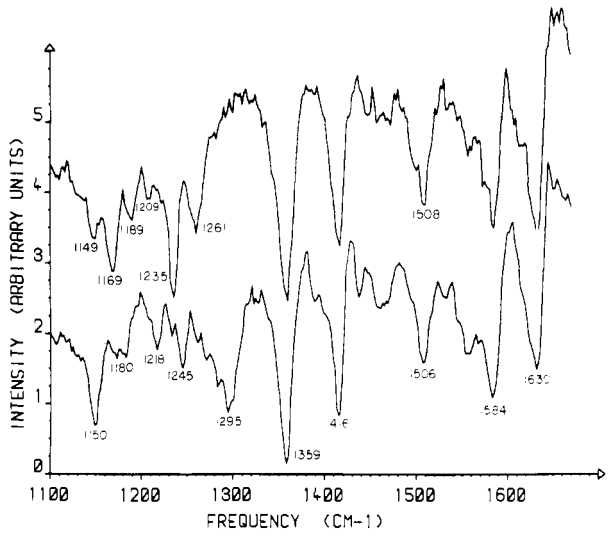



FIGURE 7: As in aqueous FMN, deuteration of FAD (1.5 mM) produces two new bands at 1295 and 1150 $cm^{-1}$ at the expense of the 1261- and 1235-$cm^{-1}$ intensities (upper, $H_2O$) and bands at 1245, 1218, and possibly 1265 $cm^{-1}$ remain. The general appearance of the 1235-$cm^{-1}$ band is most similar to that observed in the corresponding FMN–$Me_2SO$ spectrum. In contrast, obvious differences in band intensities, frequencies, and behavior on isotopic substitution exist in the 1200–1100-$cm^{-1}$ regions of aqueous FAD and FMN. These band positions and intensities reflect the presence of the stacked intramolecular flavin–adenine complex. Laser pump is 495 nm.

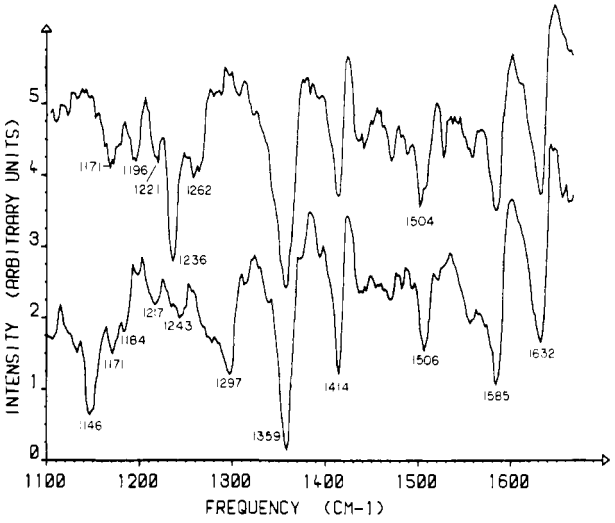



FIGURE 8: Addition of $Me_2SO$ to FAD solutions produces unstacking of the stacked intramolecular complex and consequent frequency shifts in several vibrational modes between 1200 and 1100 $cm^{-1}$. The CARS spectrum of FAD (1.5 mM) in 30% $Me_2SO$ with $H_2O$ (upper) is very similar to that obtained in aqueous FMN (or FMN–$Me_2SO$), having bands at 1221, 1196, and 1171 $cm^{-1}$. In $Me_2SO$–$D_2O$ (lower) weak bands are present at 1243, 1217, and 1171 $cm^{-1}$ and comparison with FAD in heavy water reveals only minor differences. Laser pump is 495 nm.

affected by the solvent change in deuterated FMN and may account for the sharpening of the 1233-$cm^{-1}$ band in FMN–$Me_2SO/H_2O$.

A comparison of uracil, LUM, and flavin results leads therefore to the conclusion that the 1238-$cm^{-1}$ band in LUM (1233 $cm^{-1}$ in uracil) corresponds to the 1261-$cm^{-1}$ flavin band, C(2)–N(3) stretch as shown in Table I. The 1265-$cm^{-1}$ mode in LUM (1266 $cm^{-1}$ in uracil) is due to the similarly unshifted mode at 1233 $cm^{-1}$ in flavin. An assignment consistent with the heavy-atom substitution results of Kitagawa et al. (1979) would be for the lumazine 1264-$cm^{-1}$ band to belong to a ring III motion not coupled to N(3)–H.

The highest frequency band of the FMN spectrum is at 1631 $cm^{-1}$. It is absent from the LUM spectrum so its assignment to the ring I benzene breathing mode by Nishina et al. (1978) is probably correct. This band is also present and unshifted in 2-thio-FMN (Table I). The absence of the C=O modes in the 1700–1600-$cm^{-1}$ region is puzzling; however, pure C=O motions could not be seen in uracil either (Nishimura et al., 1978).

Some caution needs to be given to the reporting of frequencies in resonance-enhanced CARS spectra since line shapes can influence the apparent peak position. The observed line shapes depend on the wavelength of the pump beam (this determines the value of the real and imaginary parts of the third-order susceptibility) and on the sample concentration which determines the extent of the Raman resonant and electronic nonresonant cross terms. The line shape (Carreira et al., 1978) at any one pump wavelength may be positive Lorentzian, negative Lorentzian, or dispersive. All the recorded spectra, including the "positive spectra", are somewhat dispersive. To see this the positive spectra can be turned upside down and compared to those spectra with "negative peaks".

The extent of peak position distortion was measured by using a line shape analysis that also accounts for Raman–Raman cross terms. This method will be published elsewhere.

*Spectra of Bound Chromophores.* On binding to protein in $H_2O$, the lowest frequency band of LUM undergoes a remarkable increase in intensity (Figures 1 and 2) and only a small frequency increase (1238–1242 $cm^{-1}$). The band of the highest frequency is not significantly affected (1584–1586 $cm^{-1}$). The other two bands exhibit small but significant shifts, 1359–1362 and 1264–1260 $cm^{-1}$. In $D_2O$ the situation is different. There is not such an obvious intensity change between the 1265- and 1284-$cm^{-1}$ (originating from the 1238-$cm^{-1}$ band) bands, and now the three bands have definite frequency shifts, 1360–1368, 1284–1291, and 1265–1262 $cm^{-1}$.

Appreciable changes in the frequencies of modes positively associated with ring I or II are apparent when FMN binds to *D. gigas* flavodoxin (Table I and Figures 3 and 4). Substantial changes are also observed in some of the bands assigned to ring III: 1582–1573, 1412–1407, 1261–1251, 1233–1227, 1192–1185 $cm^{-1}$. In contrast to the effect on the binding of LUM to lumazine protein, $D_2O$ makes no difference to most of these frequency shifts: 1580–1571, 1414–1407, 1243–1229 $cm^{-1}$. Identical frequency shifts were also observed in spectra of *D. vulgaris* flavodoxin (Figure 5), supporting the binding site homology of the two proteins (Watenpaugh et al., 1973).

*Spectra of Intramolecular Stacked Complexes.* The final set of observations pertains to the effect of stacking complexes on the flavin frequencies. In Table II we list the frequencies observed by the resonance Raman technique for FMN (Nishimura & Tsuboi, 1978) at a concentration of 60 mM, where it should be completely dimerized (Gibson et al., 1962). These can be compared with the CARS spectra taken at a much lower concentration, limited to a maximum of 2 mM because of photochemical problems. Nevertheless, the frequency positions agree quite well between the two techniques for FMN in $H_2O$ and $D_2O$. For FMN in the dimethyl sulfoxide mixture (Figure 6), where complete monomerization is expected, again no change in frequency of any band occurs. There is only an increase in intensity of the 1233-$cm^{-1}$ band relative to 1261 $cm^{-1}$, and this may be a result of decrease in solvent polarity.

Figures 7 and 8 are the CARS spectra of FAD in water and aqueous dimethyl sulfoxide. The fluorescence of FAD in water

Table II: Effects of Inter- and Intramolecular Interactions on the CARS Frequencies of FMN and FAD[a]

| FMN | | FMN (20% $Me_2SO$) | | FMN[b] | | FAD | | FAD (30% $Me_2SO$) | |
|---|---|---|---|---|---|---|---|---|---|
| $H_2O$ | $D_2O$ | $H_2O$ | $D_2O$ | $H_2O$ | $D_2O$ | $H_2O$ | $D_2O$ | $H_2O$ | $D_2O$ |
| 1631 m[c] | 1630 s | 1630 m | 1630 s | 1633 | 1628 | 1632 s | 1630 s | 1634 s | 1632 s |
| 1585 m | 1583 s | 1583 m | 1585 s | 1584 | 1584 | 1584 s | 1584 s | 1584 s | 1585 s |
| 1556 w | 1550 w | | 1550 w | 1551 | 1548 | | | | |
| 1503 m | 1504 m | 1505 m | 1506 m | 1503 | 1502 | 1508 m | 1506 m | 1504 m | 1506 m |
| | 1468 w | 1466 w | 1465 w | 1469 | 1468 | | | | |
| 1412 s | 1414 s | 1412 m | 1414 m | 1413 | 1411 | 1414 s | 1416 s | 1415 m | 1414 m |
| 1356 s | 1358 s | 1357 s | 1359 s | 1355 | 1351 | 1359 s | 1359 s | 1358 s | 1359 s |
| | 1300 m, br | | 1297 m, br | | 1300 | | 1295 m, br | | 1297 m, br |
| 1261 m | 1265 w | 1256 m | | 1261 | | 1261 m | | 1262 m | |
| 1233 m | 1243 w | 1233 ms | 1235 m | 1233 | 1230 | 1235 ms | 1245 w | 1236 ms | 1243 vw |
| 1217 w | 1215 m | 1217 w | 1213 m | | | 1209 w | 1218 w | 1221 w | 1217 vw |
| 1192 m | 1198 w | 1193 m | 1195 m | 1187 | | 1189 w | 1182 w, sh | 1196 m | 1182 sh |
| 1170 m | 1179 w | 1170 br, m | 1174 m | 1166 | 1172 | 1169 m | 1172 w | 1171 m | 1171 w |
| 1151 w | 1150 ms | | 1152 m | | 1148 | 1149 w | 1150 m | | 1146 m |
| 1128 w | 1127 w | 1135 w | 1118 w, sh | | | | | | |

[a] All frequencies are in $cm^{-1}$ calibrated to the 992-$cm^{-1}$ line of benzene. [b] Spontaneous Raman data by Nishimura & Tsuboi (1978). Excitation at 600 nm; concentration is 60 mM. [c] Strong = s, medium = m, weak = w, very = v, broad = br, and shoulder = sh.

is highly quenched; it is believed that this is due to intramolecular stacking of the adenine and isoalloxazine rings (Sarma et al., 1968). In the 30% dimethyl sulfoxide the fluorescence of this FAD sample was increased 40 times, approaching that of FMN itself. Table II shows that this change in $H_2O$ is accompanied by small frequency changes in ring III modes: 1508–1504, 1189–1196 $cm^{-1}$. Also, the 1149-$cm^{-1}$ band disappears and the 1169-$cm^{-1}$ line is weaker in the less polar solvent mixture. Hardly any changes at all are seen in the $D_2O$ case going into the dimethyl sulfoxide mixture.

## Discussion

Since the three-dimensional structure of flavodoxin is known in detail, it should be possible to correlate observed Raman frequency shifts with particular interactions between groups on the isoalloxazine ring and the amino acid residues at the protein binding site. The aim here is to use this information to interpret the spectra of lumazine protein and the other flavoproteins of unknown crystal structure.

The flavodoxins can be divided into two classes, represented by the structures of *Clostridium* MP (Burnett et al., 1974) and *D. vulgaris* (Watenpaugh et al., 1973); the X-ray crystal structure of a representative of each class is available. The classes differ mainly in the orientation and environment of the FMN within the protein. We have obtained vibrational spectra of two *Desulfovibrio* flavodoxins: *D. vulgaris* flavodoxin, which has a known crystal structure, and *D. gigas* flavodoxin. Our spectra do not reveal any difference in the FMN–protein interaction in these two flavodoxins. A more important assumption, however, is that the crystal and solution structures are the same.

In flavodoxins the phosphoribityl tail of FMN is buried within the protein with the ring I somewhat exposed to the solvent. In *D. vulgaris* it is proposed that several groups around the isoalloxazine ring hydrogen bond to peptide N–H or C=O groups from amino acid residues [see Figure 4 of Watenpaugh et al. (1973)]. There are two hydrogen bonds from C(2)=O to one residue, one from N(3)–H to another residue, and one from C(4)=O to an $H_2O$ molecule within the protein structure, which is itself held there by hydrogen bonding to other residues. Such an interaction as this last one would not be expected to differ greatly from that with $H_2O$ in free solution. There is also a possibility that N(5) might interact with an amino acid N–H, although the C(4)=O could also go to this same N–H instead.

These hydrogen-bonding changes that occur when FMN binds to flavodoxin would be expected to result in frequency changes of modes associated with the following: N(5), 1582 $cm^{-1}$ (for FMN in Table I); stacking, 1503 $cm^{-1}$; C(4)=O, 1355 $cm^{-1}$; C(2)=O, 1300 $cm^{-1}$ in $D_2O$; N(3)–H bending, 1261 $cm^{-1}$. The reverse inference can be made with less confidence; that is, that a certain frequency change is indicative of a particular interaction. Nevertheless, as predicted from the X-ray structure of flavodoxin, there is a change in the ring I mode of FMN on binding, 1629 (free)–1624 $cm^{-1}$ (bound), and an expected change in the ring II mode (1582–1573 $cm^{-1}$) is also observed. If the assignment is correct and in view of the above reservations, the suggestion can be tentatively advanced that N(5) is a binding position in flavodoxin. A small rotation of flavin from its position in the crystal state would allow the amino group of residue 62 to bind to C(4)=O instead of N(5). The observed lack of shift in frequency of the mode associated with motion of C(4)–C(4a)–C(10a) would suggest that there is no such change in the nature of hydrogen bonding at C(4)=O going from the free state to the protein-bound state.

The mode at 1261 $cm^{-1}$ in light-water spectra, which is deuterium sensitive by its coupling to N(3)–H bending, is changed by the binding of FMN to flavodoxin. This would result if the binding provided a change in the environment about N(3)–H. In $D_2O$ spectra, the 1300-$cm^{-1}$ mode in FMN is lowered in flavodoxin to 1280 $cm^{-1}$. When deuterated, this vibration is primarily composed of C(2)–N(3) stretching with very little N(3)–D bending mixed in. Indeed, ionization at N(3) or N(3)-methylation produces similar frequencies (Benecky et al., 1979; Nishina et al., 1978). Thus, this frequency is likely to be particularly sensitive to changes at C(2)=O when deuterated; its lowering from 1300 to 1280 $cm^{-1}$ in flavodoxin would result if binding occurred at this site. Below 1200 $cm^{-1}$, other modes associated with ring III also seem to be affected.

Flavodoxin is completely nonfluorescent, and in *D. vulgaris* it has been proposed that this quenching is due to a stacking interaction of the isoalloxazine ring and tyrosine residue 98. In the case of FAD, unstacking of the adenine and flavin rings by dissolving it in 30% $Me_2SO$/water is accompanied by a shift of a band from 1508 $cm^{-1}$ in water (Table II) to 1504 $cm^{-1}$. For FMN this band is found at exactly the same frequency in $H_2O$ (Table I, 1503 $cm^{-1}$) or in 20% $Me_2SO$ (Table II, 1505 $cm^{-1}$). In both *D. vulgaris* and *D. gigas* flavodoxin it is at the same frequency as that in FMN. The mode is not influenced in the same way by stacking in flavodoxin as in FAD.

Table III: Comparison of Observed Vibrational Frequencies for FAD, Glucose Oxidase, and Riboflavin Binding Protein[a]

| FAD[b] | | FAD[c] | | glucose oxidase[b] | | riboflavin binding protein[b] | | riboflavin binding protein[d] | |
|---|---|---|---|---|---|---|---|---|---|
| $H_2O$ | $D_2O$ | $H_2O$ | $D_2O$ | $H_2O$ | $D_2O$ | $H_2O$ | $D_2O$ | $H_2O$ | $D_2O$ |
| 1635 s[e] | 1634 s | 1633 m | 1628 m | 1626 | 1627 | 1631 s | 1629 ms | 1631 ms | 1631 ms |
| 1584 s | 1584 s | 1584 m | 1584 m | 1578 | 1578 | 1584 s | 1581 ms | 1583 ms | 1585 ms |
| | | 1551 m | 1548 m | | | 1545 w | 1545 vw | 1546 w | 1548 w |
| 1507 m | 1507 m | 1504 m | 1502 m | 1501 | 1502 | 1504 m | (1505 vw?) | 1500 w | 1496 w |
| | | 1468 m | 1469 m | | | 1460 w | 1460 w | 1463 w | 1466 w |
| 1416 s | 1416 s | 1412 ms | 1410 ms | 1404 | 1408 | 1410 s | 1409 s | 1407 s | 1407 ms |
| 1359 s | 1357 s | 1356 s | 1351 s | 1364 | 1364 | 1357 s | 1354 s | 1355 s | 1352 s |
| - | - | - | - | 1345 | 1348 | - | - | - | - |
| | 1297 m | | 1300 m | - | 1287 | - | 1293 w | - | 1295 m |
| 1260 m | | 1261 m | - | ? | | 1250 w | - | 1251 m | - |
| 1231 w | 1237 w | 1234 ms | 1233 m | 1230 | ? | 1229 w | 1231 w | 1229 m | 1234 m |
| | 1209 w | - | - | | | - | - | - | 1211 w |
| 1185 | 1179 w | 1190 m | 1171 m | 1182 | 1169 | 1180 w | (1180 vw?) | 1179 m | 1179 m |
| 1164 w | 1144 w | 1166 m | 1147 m | 1158 | 1137 | 1161 w | 1140 w | 1161 m | 1139 m |

[a] All frequencies are in $cm^{-1}$ calibrated (except for glucose oxidase data) to the benzene 992-$cm^{-1}$ line. [b] After Dutta et al. (1978). CARS excitation $\omega_1$ = 480 nm. [c] Spontaneous Raman frequencies after Nishimura & Tsuboi (1978). Excitation at 600 nm. [d] Resonance Raman data from Nishina et al. (1978). Excitation at 488 nm. [e] Strong = s, medium = m, weak = w, very = v, broad = br, and dispersive = d.

Changes in the Raman spectrum of LUM on binding in lumazine protein are different from those of FMN in flavodoxin. The frequency shifts are not as pronounced, suggesting that the lumazine ring is more exposed to the solvent than the isoalloxazine ring is in flavodoxin. The strong fluorescence of lumazine protein (Gast & Lee, 1978) is consistent with this model in which lumazine is attached to the protein via the ribityl tail, rather analogous to flavodoxin (Visser & Lee, 1980).

When LUM binds to lumazine protein, there is a striking intensity increase in the 1238-$cm^{-1}$ C(2)–N(3) band, with no significant change in its frequency. This must mean that N(3)–H interacts with the protein in the electronic excited state but not in the ground state. Changes in the electronic ground-state vibrational coordinate would involve frequency shifts. Alternatively, it is seen that mixing in $Me_2SO$ in the case of FMN does the same thing to this band (Table II) so that some solvation difference in the excited state may be responsible.

Binding of LUM in lumazine protein does not shift the N(5)–C(4a)–C(8a)–N(1) mode significantly (1584–1586 $cm^{-1}$) and only slightly affects the C(4)–C(4a)–C(8a) mode (1359–1362 $cm^{-1}$). In $D_2O$, and in contrast to flavodoxin, the shift of this latter mode is greater (1360–1368 $cm^{-1}$) and a "subtle change" in protein conformation at the binding site, caused by differences in interaction (N–D to $D_2O$ vs. N–H to $H_2O$), may be responsible. In any case there are no frequency changes that would implicate N(5), C(4)=O, or N(3)–H being involved in the binding interaction in lumazine protein. As in flavodoxin, a change in the C(2)–N(3) mode is observed in $D_2O$ (1284–1291 $cm^{-1}$) but not in $H_2O$ (1238–1239 $cm^{-1}$) and this would result if there was hydrogen-bond involvement at C(2)=O. These results support the suggestion of Visser & Lee (1980) that the ribityl portion of the molecule provides most of the interaction necessary for protein binding.

Table III lists the published Raman frequencies in the range of present interest for flavins and flavoproteins. The resonance Raman data for FMN in Table II can also be included in this list. Bearing in mind that the resonance Raman technique will enhance lines to a different extent than the CARS method and the use of excitation at 480 nm gave rise to positive line shapes in the work of Dutta et al. (1978), the agreement in frequency values in the three tables is very satisfactory for the bands that are of medium or strong intensity above 1200 $cm^{-1}$. Our reported vibrational frequencies for FMN (Table I) are on the average within ±1.0 $cm^{-1}$ of the average of all published Raman and CARS frequencies (Table III) common to FMN and FAD. Below 1200 $cm^{-1}$ the numbers, relative intensities, and overlap of line shapes make results more difficult to analyze and interpret. Although some changes are evident, only above 1200 $cm^{-1}$ can we make direct intercomparison of the flavins and flavoproteins.

Binding of FAD in glucose oxidase (Table III) is accompanied by a number of new effects. Clear decreases of frequency in both the ring I (1635–1626 $cm^{-1}$) and N(5)–C(4a)–C(10a)–N(1) modes (1584–1578 $cm^{-1}$) were observed by Dutta et al. (1978). The lowering of the 1507-$cm^{-1}$ band in FAD to 1501 $cm^{-1}$ in glucose oxidase may be an indication of unstacking, although this is not accompanied by a fluorescence increase. In ring III, there is a change from 1416 to 1404 $cm^{-1}$ and the 1359-$cm^{-1}$ C(4)–C(4a)–C(10a) mode in FAD is split into two roughly equal components at 1364 and 1345 $cm^{-1}$. Since two FAD's are bound in this enzyme, such a splitting would result if their environments at C(4)=O are very different. No other splittings were reported. In $D_2O$, the C(2)–N(3) frequency was found lowered in comparison to FAD, 1297–1287 $cm^{-1}$, and this would result if there was binding at C(2)=O. In light-water spectra of glucose oxidase, the N(3)–H-coupled mode normally at 1260 $cm^{-1}$ in FAD could not be located by Dutta et al. (1978), consistent with strong hydrogen bonding at N(3).

From this information it can be suggested that in glucose oxidase the isoalloxazine ring lies tightly bound in two different sites which are both well within the protein. A resultant shift in $\pi$-electron distribution alters the ring I frequency, and the other changes are likely the result of C(2)=O, C(4)=O, N(5), and N(3)–H interactions.

Riboflavin bound in riboflavin binding protein exhibits few vibrational shifts. Although solubility has prevented the direct observation of the CARS spectrum of free riboflavin, it is expected to be the same as that of FMN (Table I). In spectra reported for riboflavin binding protein there are no effects at ring I (1631–1631 $cm^{-1}$) or N(5) (1585–1583 $cm^{-1}$). No stacking interactions appear (1503–1504 $cm^{-1}$), although again the bound flavin is nonfluorescent. There is no effect on the mode 1356–1357 $cm^{-1}$ which should reflect changes in the environment of C(4)=O, in contrast to the clear effects in other flavoproteins and lumazine protein. The C(2)–N(3) mode is shifted, 1300–1293 $cm^{-1}$ in $D_2O$, which could result

from the involvement of C(2)=O in the binding, while the shift of the 1261-$cm^{-1}$ mode to 1250 $cm^{-1}$ suggests that N(3)–H is strongly involved also. In contrast to flavodoxin, these results suggest an appreciable difference in bonding with only the N(3)–H and C(2)=O sites actively taking part.

We have shown here by using the CARS technique to obtain vibrational spectra of free and protein-bound chromophores that the frequencies of some bands do not change while others shift by a small amount. While the technique allows these differences to be measured with high precision so that they are experimentally significant, the valid criticism can be made as to whether such small shifts can be confidently attributed to the ring–protein interactions we have suggested. It is encouraging to find that the bands that shift are assigned to modes that involve atoms which logically participate in the ring–protein interactions, viz., C(2)=O, C(4)=O, N(5), N(3)–H, stacking, and ring I effects. The question cannot be answered one way or the other until many more instances are collected, particularly for those other flavoproteins of known three-dimensional structure.

The case of strongest binding is seen in glucose oxidase which binds isoalloxazine in two different protein sites and the frequency changes would be explained by attachment at C(2)=O, C(4)=O, N(5), and N(3)–H. Here, flavin is found most deeply buried in protein and the ring I frequency is affected. *Desulfovibrio* flavodoxin is observed to bind flavin at C(2)=O and N(5) only, and an effect on the benzene ring can also be seen. Agreement with the predictions from the X-ray structure of Watenpaugh et al. (1973) is good. Spectra of riboflavin binding protein suggest that the flavin ring is bound by interactions at N(3)–H and C(2)=O only. Binding of the ribityl tail by protein is most likely responsible for its protein affinity. Only in glucose oxidase and riboflavin binding protein were frequency changes observed that implicate N(3)–H as a binding position.

Interactions in lumazine protein are found to be the most subtle. In this case, only minor changes in frequencies involving C(4)=O and C(2)=O are observed, indicating that the lumazine chromophore lies at the surface of the protein and is bound primarily by its ribityl chain. The dramatic intensity change of the frequency coupled to N(3)–H upon binding to protein suggests, in addition, that the nature of this binding site is modified upon electronic excitation of the chromophore. This and the exposure of the LUM moiety on the surface of the protein may be relevant to the function of lumazine protein in the bioluminescence of *P. phosphoreum*. In this reaction the LUM becomes electronically excited in an association complex with luciferase; preliminary Raman evidence suggests that such a complex exists.

Additional study using CARS to obtain vibrational data on the effects of substitution and heavy-atom labeling in lumazine and flavins would be most desirable in order to further interpret spectral changes and provide information for normal coordinate analysis of lumazine. Instrumental modifications to enable acquisition of spectra down to lower frequency ranges would be helpful. Also, an improved picture of protein–prosthetic group interaction will be available by using CARS to obtain spectra of both of the two flavodoxins of known crystal structure.

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