Flavins of NADPH-Cytochrome P-450 Reductase: Evidence for Structural Alteration of Flavins in Their One-Electron-Reduced Semiquinone States from Resonance Raman Spectroscopy†

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Received October 24, 1984

ABSTRACT: The mechanism of electron transfer from NADPH to cytochrome P-450 through FAD and FMN of the reductase is largely unknown. In this paper, we report the resonance Raman spectral properties of the oxidized and the semiquinonoid states of the flavins in the holoenzyme and the FMN-depleted forms, respectively, of detergent-solubilized rabbit liver microsomal NADPH-cytochrome P-450 reductase. The resonance Raman spectra of the oxidized forms [FAD; FMN] and [FAD; −] were essentially identical, indicating similar binding interactions of these flavins with the protein. To the contrary, the spectra of the semiquinonoid FADH· and FMNH· forms revealed significant spectral differences. Both O_2-unstable species, characterized as [FADH·; $FMNH_2$] and [FADH·; −] excited at 568.2 nm, have dominant spectral peaks at approximately 1611, 1539–1543, 1377, 1305, 1263, and 1226 cm^{-1}. However, in the O_2-stable [FAD; FMNH·] species, resonance Raman bands were located at 1611, 1532, 1388, 1304, 1268, and 1227 cm^{-1} when excited at the same wavelength. The ~10-cm^{-1} shifts of the 1532- and 1388-cm^{-1} bands suggest that the environments surrounding rings II and III of the isoalloxazines change upon reduction to semiquinonoid forms. It is proposed that N1 of FADH· (as a hydrogen-bond acceptor) and N5 of FMNH· (as donor) provide the distinguishing flavin–protein interactions in the semiquinonoid states. Furthermore, the resonance Raman spectra of the semiquinonoid species appear to be missing a number of bands assigned to ring I vibrations in the spectra of the oxidized flavins. This finding is consistent with the proposal that the three fused rings are less conjugated in the semiquinones and that the electron density is predominately localized in rings I and III.

NADPH-cytochrome P-450 reductase (EC 1.6.2.4) contains one molecule each of FAD and FMN per molecule of enzyme (Iyanagi & Mason, 1973). The enzyme transfers two reducing equivalents from NADPH[1] to cytochrome P-450 during the mixed-function oxidase catalytic cycle. It is known that the first transfer, from NADPH to FAD, involves a hydride ion with A-type stereospecificity (Sugiyama & Mason, 1984) and that the added reducing equivalents rapidly equilibrate themselves among the semiquinonoid and fully reduced states of the two flavins in accordance with the known potentials of the four one-electron redox couples involved (Iyanagi et al., 1974; Vermilion & Coon, 1978a; Oprian & Coon, 1982). However, the mechanisms of transfer of the hydride ion and electron equivalents are unknown. Attempts to define the spatial relationships between FAD and FMN in their various redox states by EPR spectroscopy (Iyanagi & Mason, 1973), by fluorescence quantum yield and emission anisotropy measurements (Blumberg et al., 1982), and by X-ray diffraction of enzyme crystals (Sugiyama et al., 1983) have not been decisive.

Resonance Raman spectroscopy has shown itself to be an additional powerful method for the study of the vibrational properties of the isoalloxazine rings of flavins both as free molecules and as cofactors. Although several studies of flavins and flavoproteins have utilized coherent anti-Stokes Raman scattering (Dutta et al., 1977; Irwin et al., 1980; Visser et al., 1983) to circumvent fluorescence problems, other investigators have successfully made spontaneous Raman scattering measurements after fluorescence quenching upon protein binding (Nishina et al., 1978) or addition of KI or metal complexation (Benecky et al., 1979, 1980). Recently, surface-enhanced Raman spectroscopy was shown to produce excellent spectra of the flavins of glucose oxidase and riboflavin binding protein with greatly reduced interference from fluorescence (Copeland et al., 1984). Assignments of resonance Raman spectra have been proposed by a number of workers from systematic isotopic substitutions (Kitagawa et al., 1979), chemical modifications (Nishina et al., 1980; Schopfer & Morris, 1980; Schmidt et al., 1982), and a normal-mode vibrational analysis (Bowman & Spiro, 1981). Although most of the effort toward the elucidation of the resonance-enhanced Raman spectra of fla-

† This work was supported by grants from the American Cancer Society (BC256) and the National Institutes of Health (GM 18865), U.S. Public Health Service.

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[1] Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate in its reduced form; CARS, coherent anti-Stokes Raman scattering; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance; FAD, FADH·, and $FADH_2$, flavin adenine dinucleotide in its oxidized, half-reduced (semiquinone), and fully reduced forms, respectively; FMN, FMNH·, and $FMNH_2$, flavin mononucleotide in its oxidized, half-reduced, and fully reduced forms, respectively; NMR, nuclear magnetic resonance. Mixed redox states of the two-component flavins are shown as, e.g., [FADH·; $FMNH_2$] or for the FMN-depleted enzyme as [FADH·; −].

0006-2960/85/0424-3012$01.50/0

vins has been directed at the oxidized forms, spectral characterization of the semiquinonoid forms has also been reported (Dutta & Spiro, 1980; Nishina et al., 1980; Kitagawa et al., 1982; Benecky et al., 1983).

We have undertaken the present study to explore by resonance Raman spectroscopy the interactions of the two flavins with their surrounding binding sites in various redox states of the holoenzyme and FMN-depleted protein. Our results indicate that, whereas the oxidized forms of the two flavins give identical spectra suggestive of similar binding domains, the spectra of the FADH· vs. FMNH· semiquinonoid forms show distinct shifts that indicate differences in their interactions with their environments.

Experimental Procedures

Materials. Leupeptin (synthetic hemisulfate), pepstatin A, mixed adenosine 2′- and 3′-monophosphates (sodium salts from yeast), sodium deoxycholate, NADPH (tetrasodium salt, type X, >98%), cytochrome *c* (horse heart, type VI, >95%), glucose oxidase (type VII from *Aspergillus niger*), catalase (bovine liver), and Tris base (reagent grade) were obtained from Sigma. Emulgen 913 was the kind gift of the KAO Corp., Tokyo, Japan. Adenosine 2′,5′-diphosphate–Sepharose and DEAE-Sepharose were obtained from Pharmacia, hydroxylapatite (fast-flow dry powder) was from Calbiochem, and other reagents of best grade were from Baker.

Detergent-solubilized NADPH-cytochrome P-450 reductase was purified by the method of Iyanagi & Mason (1973) with modifications as follows. Liver microsomes were prepared in the presence of pepstatin and leupeptin (Masters & Okita, 1980) from phenobarbital-treated rabbits. The microsomes (8.5 g) were extracted with 1.2 L of 0.1 M Tris–acetate buffer, pH 7.6, containing 1 mM EDTA, 20% glycerol, 2.5% Emulgen 913, 0.5% sodium deoxycholate, and 0.075 M KCl at 0 °C for 30 min. The suspension was centrifuged at 10000*g* for 25 min. To this supernatant was added a net volume of 400 mL of DEAE-Sepharose CL-6B gel previously equilibrated with 0.1 M Tris–acetate buffer, pH 7.6, containing 1 mM EDTA, 20% glycerol, 0.1% Emulgen 913, and 75 mM KCl (buffer A). The gel suspension was gently stirred for 15 min, then collected on a Buchner funnel (17-cm diameter), and thoroughly washed with buffer A. The washed gel was packed into a 5 × 20 cm column from which the reductase was eluted with buffer A in which the [KCl] was 0.5 M. A dark brown fraction was collected (~100 mL), and 2 μM (final) FMN was added to the eluate to stabilize the enzyme. This solution was applied to a 1.7 × 15 cm column of 2′,5′-ADP–Sepharose equilibrated with 25 mM potassium phosphate, pH 7.0, containing 20% glycerol and 0.2% sodium deoxycholate (buffer B) according to the method of Yasukochi & Masters (1976). The column was washed with buffer B and then eluted with buffer B containing 2 mM 2′-AMP. The bright yellow fraction (~30 mL) was diluted 2.5-fold with 20% glycerol and applied to a calcium phosphate (hydroxylapatite) column previously equilibrated with 10 mM potassium phosphate, pH 7.0, containing 20% glycerol and 0.1% Emulgen 913. Approximately 30 mg of reductase was recovered from 8.5 g of microsomes (50% yield). The purified enzyme had a specific activity of 40 μmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$ at 25 °C. Judging from SDS gel electrophoresis, these preparations were >90% homogeneous.

FMN-depleted reductase was prepared according to the method of Vermilion & Coon (1978b) as modified by Iyanagi et al. (1981). This enzyme has <3.5% of the original cytochrome *c* reductase activity and 8% of the original FMN content. Both the detergent-solubilized and the FMN-depleted proteins were highly concentrated by ultracentrifugation just prior to resonance Raman investigations. The concentrations of native and FMN-depleted enzymes were determined spectrophotometrically with the extinction coefficients 21.4 and 9.7 mM^{-1} cm^{-1} at 454 nm, respectively (Oprian & Coon, 1982).

Spectral Measurements. Stepwise anaerobic reduction of the reductase was carried out in a Thunberg cell. A mixture of 27 μL of enzyme (~0.20–0.87 mM), 0.5 μL of 0.4 mM glucose oxidase, 0.2 μL of 0.2 mM catalase, and 3 μL of 1.0 M D-glucose in 0.1 M Tris–acetate, pH 7.6, containing 0.1% Triton N-101 and 20% glycerol, was placed in the bottom of the vessel, and NADPH solution was in a separate compartment. After thorough degassing with Ar, the solutions were mixed and then sealed in a glass capillary (90 mm × 1.5 mm i.d.) that was used for both electronic absorption and resonance Raman spectral measurements. Absorption spectra were recorded on a Perkin-Elmer 559A spectrophotometer, employing a ~1-mm mask for the capillary cell. The resonance Raman spectra were obtained on a modified Jarrell-Ash 25-300 instrument described previously (Loehr et al., 1979; Sjöberg et al., 1982) using Spectra-Physics 164-01 (Kr) and 164-05 (Ar) lasers and an Ortec 9302 amplifier/discriminator. Signal-to-noise enhancement was carried out by both repetitive scanning and computer smoothing. Raman spectra of oxidized samples were typically obtained on frozen solutions at ~77 K whereas the spectra of semiquinonoid samples were obtained on solutions at ~2 °C. Sample integrity was monitored spectrophotometrically after laser exposure with no apparent degradation.

Results

Absorption Spectra of NADPH-Cytochrome P-450 Reductase. The electronic absorption spectra of native and FMN-depleted reductase in their oxidized and partially reduced states are shown in Figure 1. The spectral features of the three redox states of FMN and FAD have been reported previously by Iyanagi & Mason (1973), Iyanagi et al. (1974), Vermilion & Coon (1978a,b), Yasukochi et al. (1979), and Oprian & Coon (1982) and were confirmed in the present study. The oxidized flavins have intense bands at ~380 and 450 nm with only negligible absorbance above 540 nm. The major changes in the electronic spectrum upon removal of the loosely associated FMN group are a loss of optical density, corresponding to half of the absorbing species, and a decrease in the ratio of the 450/380-nm bands (Figure 1, A1 vs. B1).

Partial reduction of the enzyme leads to a decrease in the absorbance in the 300–500-nm region that is reminiscent of the spectral change upon loss of one of the oxidized flavins and the development of new bands centered around 600 nm. The spectrum of the aerobically reduced native enzyme (Figure 1, A2) has a new maximum at ~590 nm *with a prominent shoulder at ~630 nm*; the predominant chemical species include oxidized FAD and the FMN semiquinone, [FAD; FMNH·]. However, when the reduction with excess NADPH is performed anaerobically, the optical spectrum characteristic of the [FADH·; $FMNH_2$] semiquinone (O_2-unstable form) appears as noted by the almost complete loss of the 450-nm band (oxidized flavins) and the shoulder at 630 nm owing to FMNH· (Figure 1, A3). The anaerobic reduction of the FMN-depleted protein with excess NADPH gives rise to very similar spectral changes as observed for the corresponding native enzyme preparation; the reduced optical density (Figure 1, B2) is due to the nearly complete absence of any FMN-containing species; this system may be abbreviated as the [FADH·; −] semiquinone.

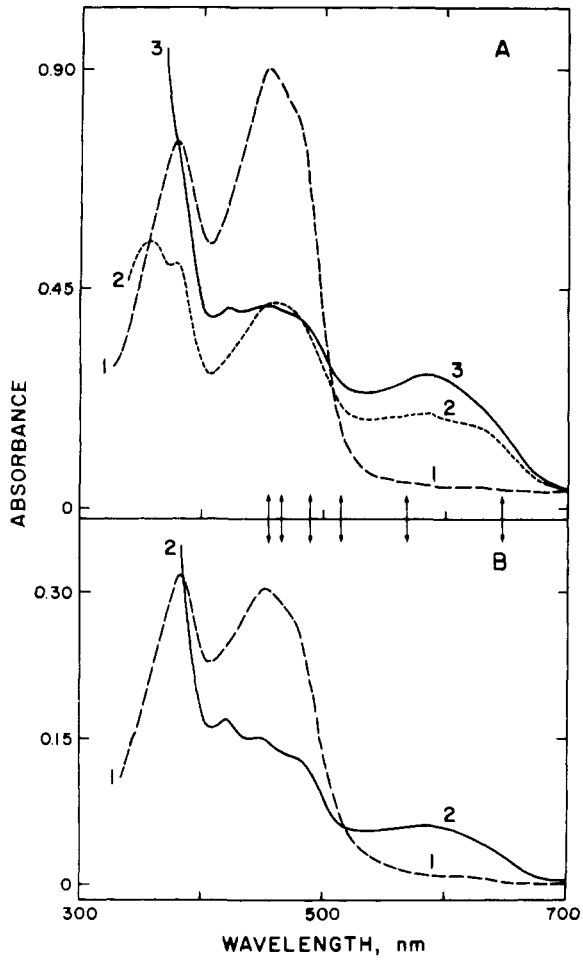


FIGURE 1: Electronic absorption spectra of NADPH-cytochrome P-450 reductase. (A) Native enzyme, 0.40 mM. (1) (--) fully oxidized; (2) (···) reduced aerobically with 1.0 mM NADPH at 25 °C for 120 min; (3) (—) as in (2) but reduced anaerobically. (B) FMN-depleted protein, 0.26 mM. (1) (--) fully oxidized; (2) (—) reduced anaerobically with 1.0 mM NADPH at 25 °C. The double-headed arrows indicate the positions of the laser lines used for resonance Raman excitation of the oxidized and partially reduced species.

Resonance Raman Spectra of Oxidized NADPH-Cytochrome P-450 Reductase. The resonance Raman spectra of the native and FMN-depleted forms of the reductase are shown in Figure 2. These data were obtained by spontaneous Raman scattering from frozen solutions of the enzyme samples (~77 K) without addition of any exogenous fluorescence quenchers. The weak Raman spectrum was extracted from the total signal after computer subtraction of the fluorescence background (Loehr et al., 1979). At least 14 bands are clearly observed in the spectra of the oxidized flavin species in the 800–1800-cm^{-1} interval; these are identified in Table I with the common notations for weak (w), medium (m), and strong (s) relative intensities. Although the spectra shown in Figure 2 were obtained with 457.9-nm excitation, other laser lines at 454.5, 465.8, and 488.0 nm, which all give resonance enhancement of oxidized flavin vibrational modes, were also used to record resonance Raman spectra from both frozen and liquid samples. We noted that the Raman spectra of the native vs. the FMN-depleted forms of the oxidized P-450 reductase were indistinguishable. In Table I, we list averaged frequencies obtained from the Raman spectra of four separate enzyme preparations that include four spectra of the holoenzyme and two of the FMN-depleted protein; in addition, we obtained three spectra of the aerobically reduced species, [FAD; FMNH·], by excitation within the ~450-nm band of the

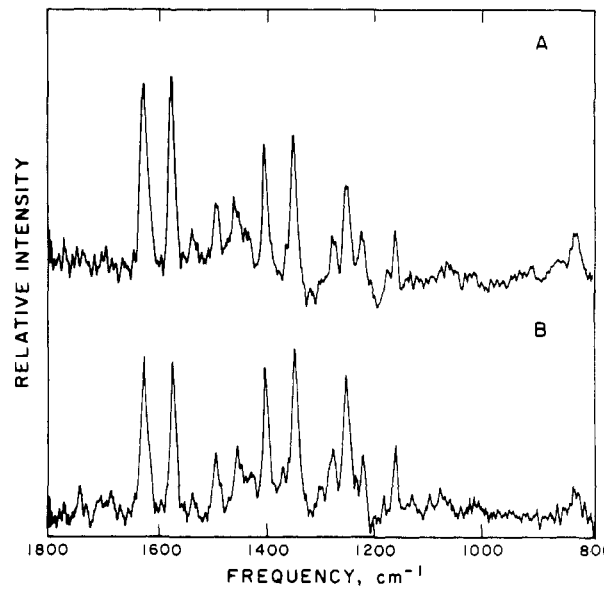


FIGURE 2: Resonance Raman spectra of oxidized NADPH-cytochrome P-450 reductase in the 800–1800-cm^{-1} region obtained with 457.9-nm excitation from frozen solution samples at ~77 K. (A) Native enzyme, 0.23 mM, corresponding to [FAD; FMN]; spectral conditions were a laser power at the sample Dewar of 50 mW, scan rate 2.5 cm^{-1}/s, slits 8 cm^{-1}, and sum of 16 scans; data are shown with a 13-point smooth. (B) FMN-depleted enzyme, 0.25 mM, [FAD; -]; spectral conditions as for (A) except for a scan rate of 2.0 cm^{-1}/s and an accumulation of 18 scans.

oxidized FAD. Thus, the averaged values are based upon *nine* observations for all but the bands marked very weak (vw), for which only limited confirmation was attainable (three to four independent observations). The standard deviations of the resonance Raman spectral bands are listed in Table I and provide a good measure of the precision of the present data.

Resonance Raman Spectra of NADPH-Cytochrome P-450 Reductase Semiquinones. Representative Raman spectra of intermediates formed by NADPH reduction of the reductase obtained with 568.2-nm excitation are illustrated in Figure 3. The overall quality of these spectra is considerably lower than that of the oxidized species, which presumably relates to the relatively low absorbance of the semiquinones in the 550–650-nm region and the rich fluorescence of these protein samples. All of these spectra were recorded from liquid samples maintained at ~2 °C; the strong fluorescence background was computer subtracted to enhance the relative intensity of the weak Raman signals. The spectra are considerably simpler since only some five or six bands are reliably observed. The common feature at ~1460 cm^{-1} is also observed in the Raman spectrum of the glycerol-containing buffer itself and is therefore discounted as arising from the flavins.

Aerobically reduced native reductase forms the O_2-stable FMNH· semiquinone with no concomitant change in the FAD moiety, i.e., [FAD; FMNH·]. Excitation of these species within the 450-nm band generates a resonance Raman spectrum typical of oxidized flavins (see above). However, excitation within the semiquinone absorption gives rise to an entirely new vibrational spectrum that is dominated by a peak at ~1612 cm^{-1} (Figure 3A). Results based on six independent experiments, five with 568.2-nm and one with 647.1-nm excitation, from three different protein preparations yielded an averaged frequency set with standard deviations of the six observations as given in Table I. The highest quality spectra were obtained with 568.2-nm excitation, and similar spectra, but of lower quality, were seen with 647.1-nm irradiation. Use of 514.5-nm light, however, yielded neither a spectrum of

Table I: Resonance Raman Spectral Data for Oxidized and Semiquinonoid Forms of NADPH-Cytochrome P-450 Reductase[a]

[FAD; FMN][b] and [FAD; −],[b] oxidized	[FADH·; $FMNH_2$],[c] O_2 unstable	[FADH·; −],[c] O_2 unstable	[FAD; FMNH·],[c] O_2 stable
1764 (4.8), wv			
1700 (6.6), vw			
1680 (5.5), vw			
1629 (1.6), s			
	1611 (0.0), s	1611 (1.0), s	1611 (2.8), s
1576 (1.5), s			
1537 (3.4), w	1539 (1.0), m	1543 (3.0), m	1532 (1.6), m
1496 (2.6), m			
1460 (4.3), m			
1434 (4.9), wv, sh			
1403 (1.4), s			
	1377 (3.5), m	1378 (1.0), m	1388 (1.0), m
1348 (1.0), s			
1302 (2.6), vw	1305, w		1304 (1.5), w
1278 (1.1), m			
	1265, w	1262, w	1268 (4.6), w
1252 (1.5), s			
1225 (2.4), m	1225 (1.8), s	1227, m	1227 (1.2), m
1176 (2.2), w, sh			
1159 (1.1), m			
829 (2.0), m			

[a] Data presented in the table are averaged results from multiple experiments (three to nine determinations) with the standard deviations given in parentheses. Abbreviations: m, medium; s, strong; sh, shoulder; v, very; w, weak. [b] Excitation wavelengths were 454.5–488.0 nm. [c] Excitation wavelengths were 568.2 and 647.1 nm.

oxidized nor semiquinone species. Thus, in addition to the strong 1611-cm^{-1} band, other features are present at 1532, 1388, 1304, 1268, and 1227 cm^{-1} that are attributable to the FMNH· semiquinone species.

Anaerobic reduction of the FMN-depleted protein yields the FADH· semiquinone, whose spectrum is shown in Figure 3B. Again, the dominant Raman band is at 1612 cm^{-1}. Interestingly, a sample illuminated with 488.0-nm light gave a superposition of frequencies due to both the semiquinone species and the oxidized FAD, indicating that the sample (prepared in a 1:1 stoichiometry of reductant to protein) was incompletely reduced. The average values of the [FADH·; −] frequencies reported in Table I are based on three experiments from two independent protein preparations. Increasing the quantity of reductant, however, has no effect on the resonance Raman spectrum of the semiquinonoid species.

Spectrum C of Figure 3 was obtained from native enzyme reduced anaerobically with a 3-fold excess of NADPH. The Raman spectrum of [FADH·; $FMNH_2$] is of acceptable quality and is essentially identical with that of the analogous FMN-depleted sample (Figure 3B). The averaged results in Table I are based on three data sets from two preparations obtained with 568.2- (two spectra) and 647.1-nm excitations. The complete absence of any oxidized species contributing to the [FADH·; $FMNH_2$] spectrum (Figure 3C) was verified by the observation that a sample with only a 2-fold excess of reductant gave only a very weak resonance Raman spectrum when excited at 465.8 nm. Since $FMNH_2$ does not yield resonance-enhanced Raman modes, all of the features observed with long-wavelength excitation must arise from the semiquinonoid species.

Two significant differences stand out in a comparison of the spectra of the O_2-stable vs. O_2-unstable semiquinonoid species (Table I). The [FAD; FMNH·] species has peaks at ~1532 and 1388 cm^{-1}, whereas in both of the FADH·-containing samples the analogous peaks are shifted by approximately 10 cm^{-1} in *opposite* directions, to ~1540 and 1378 cm^{-1}. The reliability of the frequency values, as revealed by the tabulated standard deviations of the means, puts these differences beyond experimental errors. Moreover, no temperature-dependent shifts in band positions or relative intensities could be discerned as has been reported for the resonance Raman spectra of adrenodoxin reductase (Kitagawa et al., 1982). Thus, the different frequencies of these related chromophores (FADH· and FMNH·) must reflect alterations in their chemical environments that are either not present or not Raman detectable in the oxidized species (FAD and FMN).

Discussion

Oxidized NADPH-Cytochrome P-450 Reductase. The resonance Raman spectra of native and FMN-depleted enzymes, obtained by excitation within their 450-nm absorption bands, are virtually indistinguishable (Figure 2). A plausible explanation for this observation is that the chemical environments of the two flavins (FAD and FMN) are similar enough not to suffer detectable disturbances of their electronic structures. This conclusion is supported by X-ray crystallographic results obtained from FAD- and FMN-containing proteins in that the patterns of hydrogen bonding at the isoalloxazine chromophores are very similar (Schirmer & Schulz, 1983; Smith et al., 1983; Adman, 1979) although other substantial differences may exist (Schulz et al., 1982). On the other hand, the FMN moiety is relatively easily removed (reversibly) from the reductase whereas the FAD is not (Vermilion & Coon, 1978b; Nisimoto & Shibata, 1982), but the significance of this in structural terms is not readily determinable. Husain & Massey (1978) point out that "flavoproteins exhibit a great degree of variation in their ease of resolution into flavin and apoenzyme, ...the factors that govern the value of K_d vary much from one protein to another". One possibility is that NADPH-free FAD-binding domain is blocked by a tyrosyl group and is not accessible to solvent, as in glutathione reductase (Nisimoto et al., 1984; Nisimoto & Shibata, 1982; Schirmer & Schulz, 1983), whereas this is not the case for the FMN-binding domain if the isoalloxazine moiety lies in a hydrophobic pocket as in flavodoxins (Smith et al., 1983; Adman, 1979).

The free FAD and FMN chromophores have very similar resonance Raman spectra. Schmidt et al. (1983) show no differences greater than 2 cm^{-1}, although Benecky et al. (1979) report some 3–6-cm^{-1} differences, especially in the hydrogen bond sensitive 1230–1260-cm^{-1} region; such H bonding could

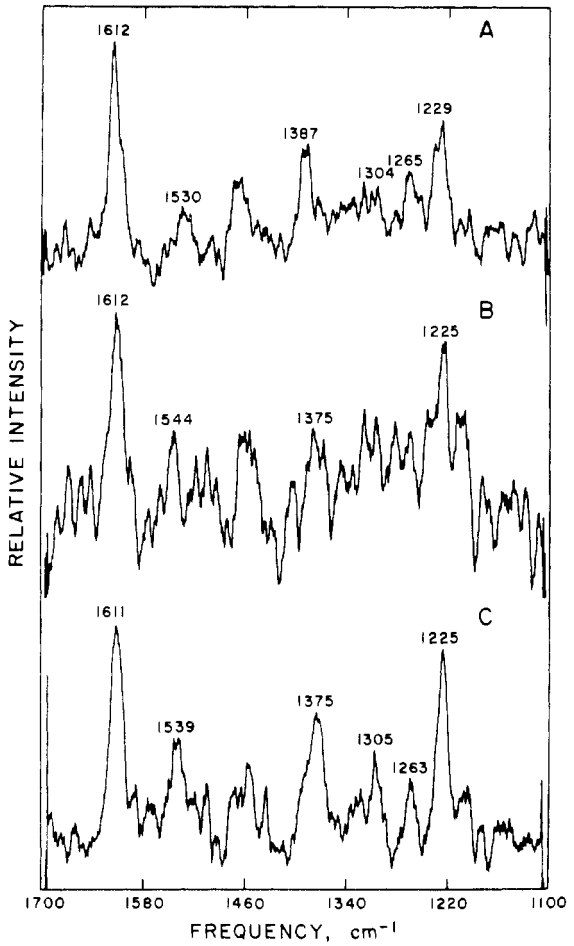
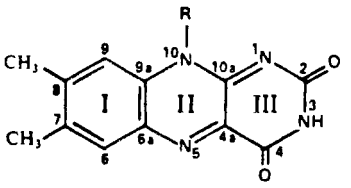


FIGURE 3: Resonance Raman spectra of the semiquinonoid species of NADPH-cytochrome P-450 reductase in the 1100–1700-cm^{-1} region obtained with 568.2-nm excitation from aqueous solution samples at ~2 °C. (A) Native enzyme, 0.47 mM, reduced aerobically with 1.0 mM NADPH, [FAD; FMNH·]; spectral conditions were a laser power at the sample of 50 mW, scan rate 2.5 cm^{-1}/s, slits 10 cm^{-1}, and sum of five scans; data are shown with a 17-point smooth. (B) FMN-depleted protein, 0.87 mM, reduced anaerobically with 0.87 mM NADPH, [FADH·; –]; spectral conditions as for (A) except for a scan rate of 2 cm^{-1}/s, accumulation of 19 scans, and a presentation with a 25-point smooth. (C) Native enzyme, 0.57 mM, reduced anaerobically with 1.7 mM NADPH, [FADH·; $FMNH_2$]; spectral conditions as for (B) except for a laser power of 65 mW and a total of 15 scans.

arise from intramolecular H-bond formation between the flavin and the adenine that would not be possible in FMN. A rather broad range of resonance Raman frequencies has been reported from different laboratories for the spectra of free flavins (Table II). In part, this arises from uncertainties in peak positions in CARS spectra (Dutta et al., 1977; Irwin et al., 1980; Visser et al., 1983). Nevertheless, these data are useful for comparative studies of the effects of protein interactions with these cofactors. In the case of riboflavin binding protein, it has been documented that binding of riboflavin causes only a small perturbation of the resonance Raman spectrum of the bound chromophore (Kitagawa et al., 1979; Nishina et al., 1980; Bowman & Spiro, 1981; Copeland et al., 1984). However, the frequencies observed for a variety of flavodoxins as a whole, while themselves encompassing a 4–25-cm^{-1} range for the peak positions in different proteins, exhibit values that clearly tend to be lower than those for the unbound chromophores. On an absolute scale, 10 out of 12 of the 13 marker bands have frequencies lower by 1–15 cm^{-1} than in the spectra of the free flavin molecules (Table II). These lowered frequencies have generally been interpreted as arising from strong hydrogen-bonding interactions with the proteins (Dutta & Spiro, 1980; Irwin et al., 1980; Schmidt et al., 1983; Visser et al., 1983). The resonance Raman spectral data for the P-450 reductase indicate that the frequency pattern follows that of the flavodoxins in being decidedly at or below the lowest values shown by the free flavins. At least seven of the 13 resonance Raman bands of the reductase have frequencies that are shifted ~5 cm^{-1} to lower energies relative to the FAD and riboflavin values given by Schmidt et al. (1983). Five of these, bands II, III, VI, X, and XII, have vibrational contributions from rings II and III (Nishina et al., 1978; Kitagawa et al., 1979; Schopfer & Morris, 1980; Bowman & Spiro, 1981; Schmidt et al., 1983) that would be involved in hydrogen-bonding interactions of the heteroatoms of these rings. The ring and atom numbering scheme of the isoalloxazine group is



This interpretation of the Raman results is supported by the X-ray crystallographic finding that the flavins in flavoproteins undergo extensive bonding and nonbonding interactions with the protein (Adman, 1979; Schirmer & Schulz, 1983; Smith et al., 1983).

When the differences between selected resonance Raman frequencies of free FMN and those of the P-450 reductase are compared with flavodoxin data according to the scheme of Visser et al. (1983), the reductase most closely resembles the flavodoxin from *Desulfovibrio desulfuricans*; however, a comparison of relative intensities also provides a good fit to the spectrum of the flavodoxin from *Megasphaera elsdenii*. The resonance Raman spectrum of P-450 reductase, thus, appears to resemble selected features of both A- and B-type flavodoxins. The presence of an intense feature at ~1280 cm^{-1} has been attributed to C4=O H-bonding interactions (Visser et al., 1983). The reductase clearly has a band of moderate intensity at 1278 cm^{-1} in support of such a condition. Crystallographic data available for the structure of *D. vulgaris* flavodoxin (Watenpaugh et al., 1973) show that hydrogen bonding of the flavin occurs through the N3–H proton and both C2=O and C4=O carbonyls. NMR data for *M. els-*

Table II: Comparative Resonance Raman Spectral Data for Oxidized Flavins and Flavoproteins

band[a]	free FAD, FMN, riboflavin[b]	riboflavin on RBP[c]	flavodoxins[d]	P-450 reductase[e]
I	1628–1635	1631	1624–1631	1629
II	1581–1585	1583–1584	1576–1580	1576
III	1547–1548	1546–1548	1531–1556	1537
IV	1500–1508	1500–1504	1497–1505	1496
V	1461–1467	1463–1465	1446–1460	1460
VI	1407–1416	1407–1410	1404–1409	1403
VII	1353–1359	1355–1357	1352–1358	1348
VIII	1302–1303	1302–1303		1302
IX	1277–1282	1282	1275–1295	1278
X	1250–1261	1250–1252	1252–1257	1252
XI	1228–1233	1229	1225–1231	1225
XII	1178–1192	1179–1180	1176–1181	1176
XIII	1158–1170	1161	1159–1166	1159

[a] Band numbering from Bowman & Spiro (1981). [b] Data from Benecky et al. (1979), Dutta et al. (1978), Irwin et al. (1980), Kitagawa et al. (1979), Schmidt et al. (1982, 1983), and Schopfer & Morris (1980). [c] RBP = riboflavin-binding protein; data from Bowman & Spiro (1981), Copeland et al. (1984), Dutta et al. (1978), Kitagawa et al. (1979), and Nishina et al. (1980). [d] Data compiled by Visser et al. (1983). [e] This work.

Table III: Comparative Resonance Raman Data for Flavin Semiquinones

					P-450 reductase[e]		proposed assignments[f]	
N^5-Me-FMNH·[a]	N^5-Me-FADH·[a]	*C.* MP fd·[b]	Rf· RBP[c]	FADH· AdR[d]	FADH·	FMNH·	band	mode
1611	1611	1611	1617	1611	1611	1611	II	ring II (?)
1590	1590							
1516	1516	1535[g]	1533		1540	1532	III	C4a–N5, C10a–N1
(1476)[h]								
1443	1432							
			1407					
		1391[i]	1393[j]					
1376	1372	1378[i]			1378	1388	VI	C2–N1
(1351)[h]								
1334	1330		1350	1344				
		1314		1310	1305	1304	X	
1282	1278	1269	1273	1271	1264	1268	XI	rings II and III
1224	1224	1232	1230		1226	1227	XII	rings II and III
1179	1174					1200	XIII	rings II and III
						1118		
			878					
				785				
			747	743				
			550	546				

[a] N^5-Methyl derivatives of FMN and FAD semiquinones (Benecky et al., 1983). [b] *Clostridium* MP flavodoxin semiquinone (Dutta & Spiro, 1980). [c] Irradiated riboflavin on riboflavin binding protein (Nishina et al., 1980). [d] Adrenodoxin reductase semiquinone (Kitagawa et al., 1982). [e] This work. [f] See discussion in text. [g] The original report shows a band at ~1535 cm^{-1} that is not discussed by the authors. [h] These peaks were observed only by excitation into the higher energy π–π^* band. [i] These two bands collapse into a single band at 1386 cm^{-1} in D_2O solution. [j] Shifts to 1387 cm^{-1} in D_2O solution.

denii flavodoxin (Van Schagen & Müller, 1981) also support hydrogen bonding through the C4=O. The presence of resonance Raman bands shifted to lower frequencies and features of notable intensity at ~1280 and 1250 cm^{-1} suggest that the flavin(s) of the NADPH-cytochrome P-450 reductase is (are) also hydrogen bonded through both carbonyls and the heteroatom of ring III.

From a study of the solvent dependence of the high-frequency resonance Raman bands of flavins, Schmidt et al. (1983) have established four resonance Raman criteria that indicate the presence of H bonding. Thus, when the solvent conditions permit hydrogen bonding, then (1) band IX is retained. (2) Band II shifts by several wavenumbers to lower energy. (3) The intensities of bands III and IV are weak and medium, respectively; in solvents of low dielectric strength and poor hydrogen-bonding ability, the intensity pattern for these two features is reversed. (4) The frequency of band X is observed at a relatively high value. With the possible exception of the last item, which appeared to be of limited utility even in the model systems studied, all of these criteria for a strongly hydrogen-bonding system are met by the resonance Raman spectrum of the NADPH-cytochrome P-450 reductase (Figure 2; Table II). For the reductase, in particular, (1) band IX at 1278 cm^{-1} is clearly present with an intensity that is relatively even greater than that in the aqueous FAD, FMN, and riboflavin spectra shown by Schmidt et al. (1983). (2) Band II at 1576 cm^{-1} is among the lowest values reported for any flavin system (Visser et al., 1983) and, in fact, lies some 5–9 cm^{-1} below that in aqueous flavins. (3) The intensities of bands III and IV in the reductase at 1537 cm^{-1} (weak) and 1496 cm^{-1} (medium) are as expected. Thus, the present data clearly speak to the fact that the flavins of the reductase are hydrogen bonded to appropriate donor/acceptor groups of the protein as also demonstrated by X-ray crystallography of other proteins (Schirmer & Schulz, 1983; Smith et al., 1983; Adman, 1979). The aforementioned observation that the resonance Raman spectra of the holoenzyme and the FMN-depleted form are identical confirms that both isoalloxazines undergo strong interactions with the protein.

In addition to the 13 "standard" bands (Bowman & Spiro, 1981) observed in the resonance Raman spectra of the oxidized forms of the reductase, we have noted a few weaker features above band I (Table I). Although their origin remains unsettled since we have not rigorously established that these features are resonance-enhanced modes of the chromophore, they are too high in energy to be typical "protein" bands (Parker, 1983). However, it is of interest that in Bowman and Spiro's normal coordinate analysis several bands were calculated to lie >1630 cm^{-1}, i.e., ν_{10} to ν_{13} at 1783, 1731, 1713, and 1645 cm^{-1}, respectively. The 1731-cm^{-1} band was assigned as band I (observed at ~1630 cm^{-1}). If these previously unreported, weak features are indeed correct and can be corroborated in future studies, they may prove to be useful in further calculations involving the complex vibrational behavior of the isoalloxazine ring system.

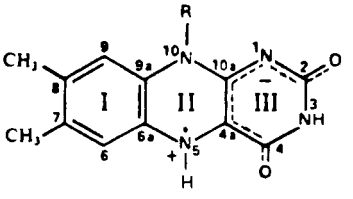
Semiquinonoid Forms of NADPH-Cytochrome P-450 Reductase. Upon aerobic or anaerobic reduction of the reductase with NADPH, semiquinonoid and fully reduced forms of the two flavins, FAD and FMN, are produced in amounts predictable from the potentials of the four one-electron redox couples (Iyanagi et al., 1974; Oprian & Coon, 1982). The relative concentrations of the various species can be determined spectrophotometrically, or estimated stoichiometrically. In the absence of O_2, both semiquinones, FADH· and FMNH·, are formed. The low-potential FADH· is unstable to O_2 and rapidly disappears, but the high-potential FMNH· is O_2 stable. In addition, the FMN-depleted form of the reductase is readily prepared, and the three redox states of the remaining FAD can be formed in the absence of the FMN species. This combination of experimental approaches has made it possible to identify the resonance Raman bands characteristic of redox states of each flavin (Table I) and to compare them with the spectra of semiquinonoid states of other flavins and flavoproteins (Table III).

The most striking feature of the presently available resonance Raman data of the semiquinonoid species is the dramatic simplification of their spectra. Even though we were not able to obtain very satisfactory resonance Raman spectra of the reductase, a comparison of our results with other published semiquinone data indicates that the loss of detail is a general phenomenon (Table III). In the 1100–1700-cm^{-1} interval, oxidized flavins have upward of 13 bands that are repeatedly

confirmed in many laboratories; but for the half-dozen examples of semiquinone resonance Raman spectra, it appears that the number of bands is reduced approximately 2-fold. No systematic studies of semiquinone species have been attempted to aid in the assignment of their resonance Raman spectra: not only are the semiquinone forms less stable, but their resonance Raman spectral intensities are also reduced.

The preparation of the N^5-methylated derivatives of FAD and FMN has brought to light model compounds with their accordant ease of study and has been very fruitful for comparison with oxidized species and preliminary assignments of the semiquinonoid forms (Benecky et al., 1983). The resonance Raman spectra of these semiquinone model compounds exhibit a greater number of bands than is observed for the proteins (Table III). This is due, in part, to the higher quality of the model compound spectra but is also an indication of some differences between the chromophores in the models and the proteins. Although bands at ~1611 and 1375 cm^{-1} are common features to both, the model compounds have additional strong bands at 1590 and 1330 cm^{-1}, which are actually more reminiscent of the doublets observed in the spectra of the oxidized flavins (e.g., Figure 2) than the protein semiquinones (Figure 3). The characteristic semiquinone peak at 1611 cm^{-1} has been assigned to a stretching mode of ring II, since it shows no ring I substituent dependence (Benecky et al., 1983). This peak could be analogous to band II of the oxidized flavins; the shift from ~1580 to 1611 cm^{-1} is similar to the shift from 1584 to 1618 cm^{-1} that occurs upon protonation of pyrazine (Foglizzo & Novak, 1970). However, 1611-cm^{-1} bands also appear in the two N^5-methyl derivatives (Benecky et al., 1983), and the 1617-cm^{-1} band of riboflavin semiquinone shows very little sensitivity to C and N isotope substitutions in rings II and III (Nishina et al., 1983). Thus, the specific assignment of the 1611-cm^{-1} feature that is apparently common to the flavin semiquinones remains uncertain.

The intense band I of the oxidized flavins, which is sensitive to changes in ring I substituents, does not appear to be present in any of the spectra of semiquinonoid species. The disappearance of this band may be related to a loss of resonance coupling of the vibrational modes associated with ring I. Such a loss of resonance can arise from changes in the skeletal structure of the isoalloxazine group, from electronic rearrangements, or a combination of both effects. Several authors (Müller et al., 1970; Ahmad et al., 1981) have suggested that the predominant electronic structure of neutral semiquinone can best be represented by a structure in which the π-electron density is largely localized on rings I and III, with an altered conjugation across the three rings:



X-ray crystallographic results on the semiquinonoid forms of flavodoxins show the ring system to be essentially planar as in the oxidized form (Watenpaugh et al., 1976; Smith et al., 1977). Since several vibrational bands in the oxidized flavin spectra have a major contribution from ring I modes, a loss of resonance with this benzenoid structural unit would serve to reduce the number of possible resonance Raman bands in the semiquinone species. According to Bowman and Spiro's (1981) normal-mode analysis, bands I, IV, V, VIII, and IX have predominantly ring I motions, and it is just these bands that appear to be missing from the spectra of the semiquinones (Table III). The remaining eight bands belonging to the set of 13 characteristic flavin bands involve contributions from rings II and III (i.e., bands II, III, VI, VII, and X–XIII). These bands are most frequently discussed in the context of hydrogen-bonding interactions of flavins (see above).

The present proposal not only provides a basis for the reduced number of resonance Raman bands in the spectra of the semiquinones but also allows us to explain the differential behavior of the FMNH· and FADH· semiquinones of cytochrome P-450 reductase in terms of differences in hydrogen bonding. The FADH· species has bands III and VI at 1540 and 1378 cm^{-1}, respectively, whereas in the FMNH· species they are located at 1532 and 1388 cm^{-1}. Band III involves stretching of the C4a–N5 (28%) and C10a–N1 (14%) bonds, where the percentages refer to the relative contributions of the calculated modes determined for the oxidized species by Bowman & Spiro (1981). Band VI contains a stretch of the C2–N1 bond in the assigned normal mode. Since hydrogen-bonding interactions generally serve to decrease the vibrational frequencies of the affected bonds, we are in a position to propose that in the semiquinonoid forms of the reductase the FMNH· species is "more strongly" interacting with the protein through its N5–H proton, whereas the FADH· species invokes its N1 atom as a hydrogen-bond acceptor to the greater extent. The similarity in frequencies of the remaining resonance Raman bands at 1305, 1265, and 1225 cm^{-1}, which are most likely associated with bands X–XIII that have predominantly ring III contributions, indicates that the interactions through the carbonyl groups of the two flavins are similar.

Support for our Raman spectral hypothesis of structural alterations upon semiquinone formation in the reductase comes from X-ray crystallographic results. For the FMN-containing flavodoxin from *Clostridium* MP, Smith et al. (1977) have shown that a 1-equiv reduction is accompanied by formation of a new hydrogen bond between N5–H and the peptide carbonyl group of Gly-57 that rotates into position when the semiquinone is formed. This similarity is strengthened by the observation of N5–H hydrogen bonding in *Desulfovibrio vulgaris* flavodoxin semiquinone by Watenpaugh et al. (1976). No crystallographic results are yet available for FAD vs. FADH· binding domains.

Finally, we note with interest that the resonance Raman data for *Clostridium* MP flavodoxin and riboflavin binding protein bear further parallels to the P-450 reductase. Band III of the FMNH· semiquinone appears at ~1535 cm^{-1} in the spectrum of the flavodoxin and at 1533 cm^{-1} in riboflavin binding protein (Table III). These frequencies are consistent with a hydrogen-bonded N5–H, as proposed for the P-450 reductase, and support the hypothesis of a common FMN binding domain.

Added in Proof

The nucleotide sequence of rat liver NADPH-cytochrome P-450 reductase has recently been reported (Porter & Kasper, 1985). These authors compared the polypeptide sequence derived from the cDNA with the amino acid sequences of six other flavoproteins and found evidence for the presence of two discrete flavin binding domains in the reductase. A long segment exhibits sequence homology to the FMN-binding domain of bacterial flavodoxins, whereas a shorter segment is homologous with the FAD-binding regions of fumarate reductase from *E. coli* and D-amino acid oxidase. These results support our spectroscopic analysis for the presence of two distinct FAD and FMN sites in their one-electron reduced states of the enzyme.

ACKNOWLEDGMENTS

We thank Barbara L. Fischer for painstaking enzyme preparations and Andrew K. Shiemke and Lawrence J. Willis for assistance with the Raman experiments.

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