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# Localization of the Free Radical on the Flavin Mononucleotide of the Air-Stable Semiquinone State of NADPH–Cytochrome P-450 Reductase Using $^{31}P$ NMR Spectroscopy†

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*Received February 25, 1986; Revised Manuscript Received June 24, 1986*

ABSTRACT: Microsomal NADPH–cytochrome P-450 reductase is the only mammalian flavoprotein known to contain both FAD and FMN as prosthetic groups. The discovery of the air-stable semiquinone [Masters, B. S. S., Kamin, H., Gibson, Q. H., & Williams, C. H., Jr. (1965) *J. Biol. Chem. 240*, 921–931] and its identification as a one-electron-reduced state [Iyanagi, T., & Mason, H. S. (1973) *Biochemistry 12*, 2297–2308] have engendered a number of studies to elucidate its unique catalytic mechanism. In this paper, $^{31}P$ NMR spectroscopy is utilized to probe the localization of the free radical in this air-stable semiquinone form and to ascertain the environments of the FAD and FMN prosthetic groups as affected by the paramagnetic ion Mn(II). Consistent with conclusions drawn from studies utilizing FMN-free reductase [Vermilion, J. L., & Coon, M. J. (1978) *J. Biol. Chem. 253*, 8812–8819], the free radical was shown to reside on the FMN moiety by the broadening of its characteristic resonance in the $^{31}P$ NMR spectrum. In addition, the effect of the paramagnetic ion Mn(II) was determined on the four resonances attributable to FAD and FMN and the additional ones contributed by $NADP^+$ resulting from the oxidation of the physiological reductant NADPH. The addition of Mn(II) had little effect on the line widths of the FMN and FAD signals but resulted in an increase in their intensities due to a decrease in $T_1$ relaxation times. On the other hand, the pyrophosphate resonances of bound $NADP^+$ were only minimally affected by the paramagnetic ion, indicating that the pyrophosphate moiety of $NADP^+$ is more sequestered from the solvent than the pyrophosphate of FAD. These studies demonstrate the utility of $^{31}P$ NMR as a direct probe of the environments of the phosphorus-containing cofactors of NADPH–cytochrome P-450 reductase under various conditions, including changes in redox state.

NADPH–cytochrome P-450 reductase (EC 1.6.2.4) was first purified and shown to contain 2 mol of flavin/mol of enzyme protein by Masters et al. (1965a). These workers also demonstrated the existence of an air-stable semiquinone form of the flavoprotein. The identification of the prosthetic groups of the reductase as FAD *and* FMN and the determination of the air-stable semiquinone as a one-electron-reduced state were made by Iyanagi and Mason (1973). Subsequently, a number of studies have appeared in the literature addressing the nature of its mechanism of catalysis (Iyanagi et al., 1974, 1981; Vermilion & Coon, 1978a,b; Yasukochi et al., 1979; Vermilion et al., 1981; Oprian & Coon, 1982). Various biophysical methods have been applied to the study of NADPH–cytochrome P-450 reductase in order to understand the spatial and electronic relationships governing the interactions of the two flavin prosthetic groups, including EPR spectroscopy (Iyanagi & Mason, 1973), fluorescence quantum yield and emission anisotropy measurements (Blumberg et al., 1982), X-ray diffraction of enzyme crystals (Sugiyama et al., 1983), and resonance Raman spectroscopy (Sugiyama et al., 1985). These experimental approaches have not yet yielded definitive results.

In an attempt to ascertain the location of the free radical of the air-stable semiquinone state of NADPH–cytochrome P-450 reductase and to probe the environments of the prosthetic flavins, FAD and FMN, $^{31}P$ NMR spectroscopy was applied to oxidized and one-electron-reduced samples of the flavoprotein. The effects of the addition of the paramagnetic ion Mn(II) on the $^{31}P$ resonances attributable to FAD, FMN, the reductant (NADPH), and residual 2′-AMP (used in affinity chromatography) were also examined. The results show that the free radical of the semiquinone state resides on the FMN prosthetic group. Line-broadening effects produced by Mn(II) were restricted to the residual 2′-AMP used in eluting the reductase from the 2′,5′-ADP–Sepharose 4B affinity

† Supported by a grant from the National Institutes of Health (HL 30050) to B.S.S.M.
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0006-2960/86/0425-7220$01.50/0 

column and differential perturbations, i.e., increased intensities, resulting from shortened spin–lattice relaxation times ($T_1$), were obtained only with the $^{31}$P resonances assigned to FAD and FMN but not to $NADP^+$. These results suggest differences in the environments of the two flavins, which permit the localization of the free radical on a single prosthetic group (FMN), and show differences in exposure of the flavin phosphates, as opposed to pyridine nucleotide reductant phosphates, to the paramagnetic ion, Mn(II), probe.

## Materials and Methods

*Preparation of Microsomes from Porcine Liver.* Fresh pig livers were obtained from a local abattoir (Patrick Cudahy, Inc., Cudahy, WI), and microsomes were prepared by using the $CaCl_2$ precipitation procedure of Cinti et al. (1972), as modified by Yasukochi and Masters (1976).

*Purification of Reductase.* The procedure used for purification of NADPH–cytochrome P-450 reductase (also referred to as reductase in this paper) was that of Yasukochi and Masters (1976) with some modifications. Reductase from porcine liver microsomes was solubilized with pancreatic steapsin following the procedure of Williams and Kamin (1962) with modifications made by Masters et al. (1965a). Ten to fifteen grams of microsomal protein was used for each solubilization.

The subsequent purification proceeded as follows: The resultant solution was centrifuged at 106000*g* for 90 min. The supernatant was applied directly to a 1500-mL bed volume column of DE-52 (Whatman), equilibrated with 25 mM Tris-HCl buffer, pH 7.7, containing 1 mM EDTA, 0.05 mM dithiothreitol, and 0.02% $NaN_3$, in a sintered glass funnel. After sample application, the DE-52 was washed with 2 L of the DE-52 equilibration buffer followed by 2 L of a similar buffer containing 0.12 M KCl. The reductase was then eluted with 0.3 M KCl in the equilibration buffer.

The eluate was applied to a 20-mL bed volume column of 2′,5′-ADP–Sepharose 4B (Pharmacia Fine Chemicals). The column was washed with 1 L of a 150 mM Tris-HCl buffer, pH 7.65, containing 1 mM EDTA and eluted with 150 mL of a 100 mM Tris-HCl buffer, pH 7.65, containing 4 mM 2′-AMP (P-L Biochemicals) and 1 mM EDTA.

2′-AMP not bound to the eluted reductase was removed by repeated concentration on an Amicon ultrafilter using a PM-10 membrane and dilution with fresh 100 mM Tris-HCl buffer, pH 7.65, containing 1 mM EDTA. Bound 2′-AMP was removed from the enzyme by passing it over a column of charcoal and Celite in a 1:2 ratio. Column size was determined by flavin content (based on $E^{mM}_{454nm} = 10.8$), with a ratio of 1 mg of charcoal–Celite/nmol of flavin in the preparation. This procedure is critical in the effective removal of 2′-AMP or $NADP^+$ from the reductase, but it can also result in the removal of FMN.

Enzyme stripped of FMN during chromatography on charcoal–Celite was reconstituted by incubation for 30 min at 4 °C with a 2.5-fold molar excess of FMN (Sigma Chemicals). Free FMN was removed by washing the preparation on an Amicon ultrafilter using a PM-10 membrane, followed by two cycles of dialysis against 2 L of 100 mM Tris-HCl buffer, pH 7.65, containing 1 mM EDTA. For Mn(II) perturbation experiments, EDTA was omitted from the final wash and dialysis buffers. Samples were concentrated to 255–360 μM flavin by using an Amicon Centricon 10 microconcentrator prior to NMR spectroscopy.

Enzyme preparations were shown to contain three bands by SDS–PAGE using the procedure of Laemmli (1970), modified for use with slab gels. The major band migrated with an apparent molecular weight of approximately 68 000, while the two minor bands exhibited apparent molecular weights of 54 000 and 22 000. The lower molecular weight bands appear to be proteolytic fragments of the intact reductase and the data of Fan and Masters (1974) suggest that, at least, the 54-kilodalton fragment possesses enzymatic activity. A recent report of Haniu et al. (1984) on porcine liver reductase confirmed the presence of these proteolytic fragments. Specific activities were in the range of 19–28 μmol of cytochrome *c* reduced $min^{-1}$ (mg of protein)$^{-1}$, and turnover numbers were in the range of 720–900 mol of cytochrome *c* reduced $min^{-1}$ (mol of flavin)$^{-1}$. Flavin analysis by the method of Faeder and Siegel (1973), using purified FAD and FMN as standards, showed the enzyme preparations to contain 1 mol each of FAD and FMN/mol of enzyme. The flavin content of the reductase was consistently equimolar in FAD and FMN in all preparations tested.

NADPH–cytochrome *c* reductase activity was assayed at 25 °C in 50 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA, according to the procedure of Masters et al. (1967). Since the reductase is highly sensitive to ionic strength (Phillips & Langdon, 1962), comparison of activities with other preparations must be made with caution. All spectral and kinetic measurements were performed using a Hewlett-Packard Model 8450A diode-array spectrophotometer with a Model 89100a temperature-control accessory.

*Generation of the Neutral Blue Semiquinone Form of NADPH–Cytochrome P-450 Reductase.* Generation of the neutral blue semiquinone form of NADPH–cytochrome P-450 reductase was accomplished by three different methods. Anaerobic chemical reductions were carried out with NADPH or NADH (P-L Biochemicals) by adding a solution of reductant (0.6 mol of reductant/mol of flavoprotein or 1.2 electron equiv of reductant/4 electron equiv of enzyme-bound flavin) to the sample. All solutions were made anaerobic by bubbling with purified argon for 15 min prior to their use. Photochemical reductions were also carried out under anaerobic conditions and followed the procedure of Massey et al. (1978) in buffer containing 1 mM EDTA and 20 μM 5-deazaisoalloxazine (a generous gift of Dr. Frank Frerman), using a Sylvania 625-W Sun-Gun held at a distance of 10 cm from the sample tube. Since photoreduction usually overreduced the flavoprotein, photoreduced samples were exposed to air and allowed to reoxidize to the air-stable, neutral blue semiquinone form of the reductase. Samples were then made anaerobic once again by bubbling for 15 min with Ar prior to NMR spectroscopy. On the basis of comparisons of initial and final flavoprotein spectra (using $E^{mM}_{454nm} = 10.8$ for oxidized NADPH–cytochrome P-450 reductase and $E^{mM}_{454nm} = 7.0$ for the one-electron-reduced flavoprotein), it was determined that chemically reduced samples prior to NMR measurements were greater than 85% semiquinone and photoreduced samples were nearly 100% semiquinone. Reoxidation of the air-stable semiquinone form of the enzyme was accomplished by adding a 2-fold molar excess of $K_3Fe(CN)_6$ in 10 μL of buffer to the sample.

*NMR Methods.* $^{31}$P NMR spectra were measured at 101.2 MHz with a Bruker WM-250 spectrometer. All spectra were recorded at 10 ± 2 °C with broad-band proton decoupling using a spectral width of 5000 Hz, 8K data points, 45° pulse width, and 0.82-s pulse repetition rate. A total of 90 000–170 000 transients were typically acquired for each spectrum, requiring approximately 20–39-h accumulation time. Chemical shifts are reported relative to external 85% phosphoric acid. NMR samples of ca. 1.8 mL were contained in 10-mm NMR

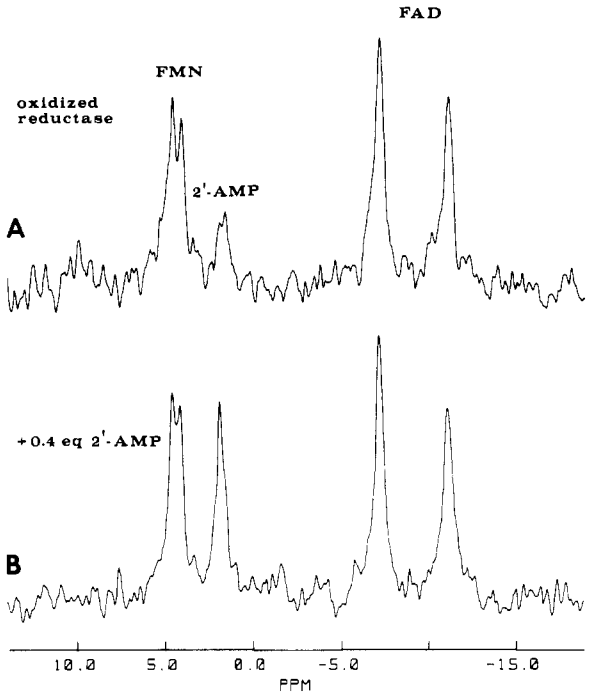


FIGURE 1: $^{31}P$ NMR spectra of oxidized NADPH–cytochrome P-450 reductase at pH 7.65: (A) native enzyme (0.18 mM), 90 000 transients, line broadening 10 Hz; (B) same enzyme to which 0.4 equiv of 2′-AMP had been added, with spectral parameters identical with those used for spectrum A. The assignments in spectrum A are based on considerations given in the text.

tubes in buffer containing 10% $D_2O$ to provide the field-frequency lock. Spectra of the semiquinone form of the reductase were obtained by using air-tight screw-capped NMR tubes (Wilmad 513-7-TR) with an atmosphere of purified argon over the samples. Spin–lattice ($T_1$) relaxation time measurements were performed by using the "intensity ratio" method of progressive saturation (Freeman et al., 1972). The accuracy of the reported values is estimated to be ±20%. Nuclear Overhauser enhancements (NOEs) were measured as the ratio of the intensities of the resonances in spectra acquired with and without proton decoupling.

## RESULTS

*$^{31}P$ NMR Spectrum of Oxidized Reductase.* NADPH–cytochrome P-450 reductase contains equimolar amounts of noncovalently, but tightly, bound FMN and FAD, which are expected to contribute one and two resonances, respectively, to the $^{31}P$ NMR spectrum of the enzyme. The observed spectrum of steapsin-solubilized reductase at pH 7.65, shown in Figure 1A, is more complex than anticipated, containing five resolved resonances instead of three. No changes in the chemical shifts of any of these resonances were observed when the buffer was adjusted to pH 6.8,[1] suggesting that they all arise from enzyme-bound species that remain in the same ionization state over this pH range.

The two most upfield resonances, at −7.33 and −11.25 ppm, are readily assigned to the two phosphorus nuclei of the pyrophosphate moiety of FAD. The resonances of free FAD at the same pH appear at −9.87 and −10.55 ppm (Table I). The greatly increased separation between the two resonances when FAD is bound to the reductase may be attributed to differences in the protein environments of the two phosphoryl groups and/or to the imposition by the enzyme of an altered conformation about the phosphodiester linkage. The latter possibility would be expected to give rise to a change in the $^{31}P$–$^{31}P$ spin–spin coupling constant between the phosphoryl groups, which for free FAD is 21 Hz. Unfortunately, owing to the much greater line widths of the resonances from enzyme-bound FAD (approximately 40 Hz at half-height), it is not possible to measure the $^{31}P$–$^{31}P$ coupling constant, since neither signal exhibits any resolved splitting. Nevertheless, it can be estimated, by comparing the line shapes of the observed resonances with those generated by spectral simulations using a range of coupling constants and line widths, that the coupling constant for bound FAD must be significantly smaller (≲10 Hz) than for free FAD. Spin–lattice relaxation times ($T_1$) were also measured and found to be quite different for the two FAD resonances. The most upfield signal at −11.25 ppm has the longest $T_1$ (6.5 s), while that of the other FAD signal is 3.6 s. A practical consequence of this large difference in $T_1$, the origin of which cannot be determined without more detailed relaxation studies, is that the most upfield member of the pair of FAD signals will always have a lower intensity, owing to its differential attenuation under the partially relaxed conditions used to acquire all of the spectra presented in this paper. The NOE values of the upfield and downfield signals were also measured and found to be 1.27 and 1.20, respectively.

The small resonance at 1.7 ppm in Figure 1A has a $T_1$ of 2.9 s and an integrated area indicating that its origin is not FMN, but rather a species whose concentration is approximately one-third that of FMN and FAD (and the enzyme). Furthermore, in spectra of different enzyme preparations the intensity of this resonance has been found to be somewhat variable, suggesting that it might arise from residual 2′-AMP that remains bound to the protein following its use during the purification procedure to elute the reductase from the 2′,5′-ADP–Sepharose 4B affinity column (see Materials and Methods). 2′-AMP is a potent competitive inhibitor of the enzyme [$K_i$ = 3 × $10^{-5}$ M (Phillips & Langdon, 1962)], presumably because of its strong interaction with the NADPH binding site, and it is therefore not surprising that its complete removal by charcoal–Celite treatment is difficult to achieve. To confirm the assignment of this signal, 0.4 equiv of 2′-AMP was added to the enzyme used to obtain the spectrum in Figure 1A. As shown in Figure 1B, the only resonance affected was that at 1.7 ppm, which increased in intensity by about 2.5-fold.

Table I: $^{31}P$ Chemical Shifts of Nucleotide and Flavin Cofactors and Their Complexes with NADPH–Cytochrome P-450 Reductase[a]

| | monoester phosphate | pyrophosphate |
|---|---|---|
| FMN | 5.14 | |
| FAD | | −9.87, −10.55 |
| $NADP^+$ | 4.08 | −10.61, −10.95 |
| NADH | | −10.55[b] |
| 2′-AMP | 3.98 | |
| reductase[c] | [4.40, 3.98][d] (FMN) 1.70 (2′-AMP) | −7.33, −11.25 (FAD) |
| reductase–$NADP^+$[e] | [4.33, 3.97][d] (FMN) 1.57 (NADP, 2′-AMP)[f] | −7.25, −11.05 (FAD) −9.40, −12.60 ($NADP^+$) |

[a] Chemical shifts are in parts per million relative to external $H_3PO_4$ (85%). Downfield shifts are positive. Samples of the free nucleotide and flavin cofactors were 5 mM in buffer, consisting of 100 mM Tris-HCl and 1 mM EDTA, pH 7.65. [b] The resonances of the two pyrophosphate nuclei are too similar to be resolved. [c] The reductase as isolated contains equimolar amounts of FMN and FAD, plus variable substoichiometric amounts of 2′-AMP (see Figure 1 and text for assignments). [d] Two partially resolved resonances from FMN are usually observed, possibly arising from the presence of a proteolytic fragment in the reductase preparations (see Results). [e] The complex resulting from addition of 0.7 equiv of $NADP^+$ to the reductase (from Figure 2A). [f] The 2′-phosphate resonance of $NADP^+$ superimposes upon that of the residual 2′-AMP bound to the reductase.

[1] Lower pH values could not be used due to the loss of stability and flavin content of NADPH–cytochrome P-450 reductase.

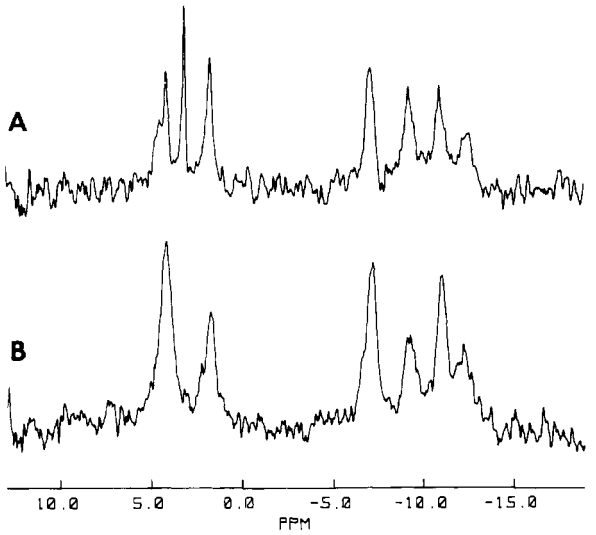


FIGURE 2: $^{31}$P NMR spectra of the complex between oxidized NADPH–cytochrome P-450 reductase and $NADP^+$ at pH 7.65 in the absence (A) and presence (B) of added Mn(II) ion: (A) native enzyme (0.14 mM) plus 0.7 equiv of $NADP^+$, 116 000 transients, line broadening 15 Hz; (B) same conditions as for spectrum A, in the presence of 0.20 mM $MnSO_4$. The narrow resonance at 3.00 ppm in spectrum A is from a small amount of contaminating inorganic phosphate.

This result not only confirms the assignment of this signal to enzyme-bound 2′-AMP but, due to the absence of any resonance intensity arising from free 2′-AMP at 3.98 ppm (Table I), also permits an estimate to be made of the binding affinity of the enzyme for 2′-AMP ($K_B \gtrsim 10^6$ $M^{-1}$).[2]

The remaining two signals at 3.98 and 4.40 ppm both have $T_1$ values of 3.0 s and NOE values of 1.20 and have a combined integrated area equal to that of each of the FAD resonances (after correction for differential attenuation due to $T_1$ differences by using the equations in the paper of Gupta et al. (1977)). This would be expected for a stoichiometry of 1 mol of FMN and 1 mol of FAD/mol of reductase. For this reason they are assigned to the 5′-phosphate group of enzyme-bound FMN. The detection of two FMN signals rather than one was not expected and implies that the enzyme solution consists of two substates, which have slightly different FMN binding environments. The amounts of the two forms, which are assessed by the relative heights of the 3.98 and 4.40 ppm signals, have been found to be quite variable. However, the *total* integrated area of the two resonances remains constant relative to the FAD signals. There is no concurrent change noted in the catalytic properties of the reductase during these measurements, which argues against denaturation of the enzyme as the origin of the two FMN resonances.

*$^{31}$P NMR Spectrum of the $NADP^+$–Reductase Complex.* The first step in the catalytic cycle of reduction of cytochrome P-450 by the reductase is a two-electron transfer from NADPH to the enzyme (Kamin et al., 1966; Yasukochi et al., 1979). Since no information is available concerning the structure of the binary complex of the enzyme with NADPH or its oxidized product, $NADP^+$, it was of interest to measure the $^{31}$P NMR parameters of the three resonances expected from enzyme-bound $NADP^+$ as well as to determine what, if any, perturbations of the flavin resonances are induced by $NADP^+$ binding. In Figure 2A is shown a spectrum of reductase to which 0.7 equiv of $NADP^+$ has been added. In Table I are listed the chemical shifts of the resonances and their assignments as discussed below. A spectrum of the reductase taken prior to $NADP^+$ binding (not shown) indicates that the sample was contaminated with a small amount of free inorganic phosphate which gives rise to the sharp signal at 3.00 ppm in Figure 2A, as well as approximately 0.3 equiv of residual 2′-AMP, as judged by the intensity of its resonance at 1.7 ppm. Because of the expectation that 2′-AMP and $NADP^+$ compete for the same binding site on the enzyme and the affinity is greater for $NADP^+$ by approximately 10-fold (Phillips & Langdon, 1962; Prough & Masters, 1976), the amount of $NADP^+$ added (0.7 equiv) was kept substoichiometric to prevent displacement of the bound 2′-AMP. In other experiments (not shown), when a full 1.0 equiv of $NADP^+$ is bound, a signal from free 2′-AMP is observed at 3.98 ppm, which superimposes on that of the upfield member of the FMN pair of resonances, thereby complicating analysis of the spectrum.

Two resonances, at −9.4 and −12.6 ppm, in the upfield region of the spectrum in Figure 2A are not present in the spectrum of the reductase alone and are therefore assigned to the pyrophosphate phosphoryl groups of bound $NADP^+$. As was the case for the FAD pyrophosphate resonances, binding of $NADP^+$ induces a downfield shift of one resonance from −10.61 to −9.40 ppm and an upfield shift of the other from −10.95 to −12.60 ppm relative to those observed for free $NADP^+$ (Table I). Both $NADP^+$ pyrophosphate signals are more broadened than the corresponding FAD signals, particularly the upfield member of the pair. This broadening may in part be attributable to a larger, though still unresolved, $^{31}$P–$^{31}$P spin-coupling interaction. The 2′-phosphate resonance of bound $NADP^+$ appears at 1.57 ppm, where it is superimposed upon that of bound 2′-AMP. The near identity of the 2′-phosphate signals from these two strong competitive inhibitors of the enzyme provides persuasive evidence that these 2′-nucleotides interact with the same binding site. It is difficult, however, to draw any meaningful conclusions regarding the nature of this interaction solely from the observed chemical shift. It is noted that the bound $NADP^+$ chemical shift of about 1.6 ppm is closer to that of the monoanion form of the 2′-phosphate of $NADP^+$ (0.4 ppm) than to the shift of the dianion (4.2 ppm) (Mas & Colman, 1984). However, the known sensitivity of phosphate ester chemical shifts to O–P–O bond angle distortions or torsional angle differences (Gorenstein, 1981) makes any such correlation with ionization state highly speculative. Finally, it is observed that only very minor chemical shift changes in the flavin phosphorus resonances are induced by $NADP^+$ binding. The only significant change is a small, but reproducible, 0.2 ppm downfield shift of the most upfield FAD resonance (Table I), which suggests that the $NADP^+$ binding site may be closer to that of FAD than FMN (see Discussion).

*Effect of Manganese Addition.* The amount of paramagnetic broadening of a resonance induced by added Mn(II) ion (0.2 mM) is frequently used as a qualitative indicator of the degree to which a particular nucleus in a macromolecule is exposed to solvent (James, 1975). For example, the absence of any broadening of the FMN phosphate resonance of *Azotobacter* flavodoxin (Edmondson & James, 1982) or the FAD pyrophosphate resonances of glucose oxidase (James et al., 1981) in the presence of Mn(II) has been taken as evidence that the phosphate-containing portion of these flavin coenzymes in their respective flavoproteins is "buried" in the protein and is inaccessible to bulk solvent. The effect of Mn(II) on the spectrum of the reductase is shown in Figure

[2] The estimate of $K_B$ is based on the law of mass action for a single binding equilibrium and the assumption that at least 90% of the added 2′-AMP is bound to the enzyme.

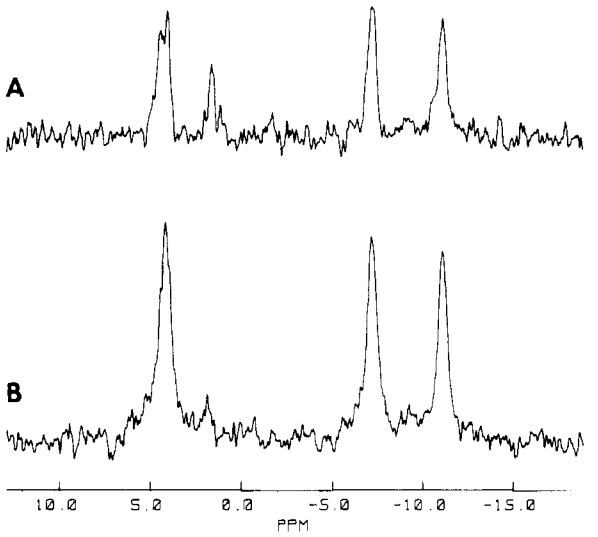


FIGURE 3: $^{31}P$ NMR spectra of oxidized NADPH–cytochrome P-450 reductase at pH 7.65 in the absence (A) and presence (B) of added Mn(II) ion: (A) native enzyme (0.15 mM), 135 000 transients, line broadening 15 Hz; (B) same conditions as for spectrum A, in the presence of 0.20 mM $MnSO_4$.

3. Whereas there is virtually no change observed in the line widths of the FMN and FAD resonances, the 2′-AMP signal at 1.7 ppm is broadened almost beyond detection. This result indicates that the 2′-phosphate group of bound AMP is in much closer proximity to the Mn(II) ions in solution than the phosphorus nuclei of the FMN and FAD, owing either to its binding near the surface of the enzyme or in a cleft to which solvent has access.

The absence of an appreciable effect of Mn(II) on the line widths of the already broad FMN and FAD resonances does not necessarily imply that these coenzymes are so deeply buried in the interior of the protein ($\gtrsim$10 Å from the surface) that there is no paramagnetic contribution to their relaxation. Instead, the paramagnetic contribution could be of a magnitude that is quite small relative to the natural transverse relaxation rate ($1/T_2$), thereby causing only minimal broadening. At the same time, it could be relatively large compared to the much smaller spin–lattice relaxation rate ($1/T_1$). It appears that just such a situation is encountered with NADPH–cytochrome P-450 reductase as indicated in Figure 3 by the approximate doubling in the intensity of the resonances from FMN and FAD in the presence of Mn(II) when spectra of the enzyme with and without Mn(II) were accumulated under identical conditions. The intensity increases are a direct consequence of more efficient spin–lattice relaxation, which causes each resonance to be less attenuated by the effects of incomplete relaxation between pulses. From the observed changes in intensity, it can be calculated (Gupta, 1977) that the $T_1$ values of the FMN resonance and the downfield FAD resonance must have decreased to approximately 0.5 s (from about 3 s) and that of the upfield FAD signal to approximately 3 s (from 6.5 s). Paramagnetic contributions of these magnitudes to $T_2$ relaxation would, on the other hand, be hardly noticeable since they would induce line width increases of less than 2 Hz. From these observations it may be concluded that both FMN and FAD are bound to the reductase so as to shield the phosphate-containing portions of the coenzymes from interaction with bulk solvent, but are not buried deeply enough in the protein interior to preclude some influence of the paramagnetic ions in solution.

The effect of Mn(II) on the $^{31}P$ NMR properties of the $NADP^+$–reductase complex is shown in Figure 2B. Comparison with the spectrum of the complex in the absence of Mn(II) (Figure 2A) indicates that the only signal whose line width is dramatically affected is that of the contaminating free inorganic phosphate at 3.0 ppm, which, as expected, is broadened beyond detection. The resonances from the FMN and FAD in the presence of $NADP^+$ respond to the presence of Mn(II) (Figure 2B) the same as in the absence of $NADP^+$, (Figure 3), showing primarily the effects of shortened $T_1$s (greater intensity). The pyrophosphate resonances of the bound $NADP^+$ at −9.4 and −12.6 ppm are only minimally broadened by the Mn(II) and, unlike the FAD resonances, have intensities that are virtually the same as in the absence of Mn(II). Thus, it can be inferred that the binding site of the pyrophosphate portion of $NADP^+$ extends farther into the protein interior than that of FAD. Interpreting the effect of Mn(II) on the 2′-phosphate of bound $NADP^+$ is somewhat more complicated because, as discussed in the preceding section, the 1.6 ppm resonance in Figure 2A is a superposition of 2′-phosphate signals from the approximately 70% of the enzyme that contains $NADP^+$ (only 0.7 equiv of $NADP^+$ was added) and the remaining 30% that contains 2′-AMP bound to the $NADP^+$ binding site. Since it was shown in Figure 3 that Mn(II) causes the disappearance of the 2′-AMP resonance, it can be inferred that the 1.6 ppm signal in Figure 2B arises solely from $NADP^+$. Since its intensity is significantly greater than that which is assumed to contribute (70%) to the 1.6 ppm resonance in Figure 2A, it is concluded that Mn(II) causes some reduction in its $T_1$ relaxation time, though not as much as is experienced by FMN and FAD. What is most noteworthy about the 1.6 ppm resonance in Figure 2B is not its intensity, however, but the fact that its line width is narrow enough to permit its detection at all. This contrasts with the severe broadening of the 2′-phosphate signal of 2′-AMP, which, according to the arguments presented earlier, is believed to interact with the enzyme in a manner virtually identical with that of the 2′-phosphate of $NADP^+$. What would therefore appear to be the most likely explanation for this difference is binding of the nicotinamide and/or pyrophosphate portions of the coenzyme in such a manner as to shield the 2′-phosphate of $NADP^+$ from access to solvent.

*Effect of Reduction to the Air-Stable Semiquinone Form.* The one-electron-reduced form of the reductase contains an air-stable flavin semiquinone whose unpaired electron offers a natural paramagnetic probe of its environment. Since the enzyme contains two flavins, FMN and FAD, it has been of considerable interest to establish which of them is the site of localization of the free radical. On the basis of several absorption spectroscopic and kinetic studies employing FMN-depeleted enzyme (Vermilion & Coon, 1981; Iyanagi et al., 1981), there is evidence suggesting that the unpaired electron may reside on the FMN in this redox state since stabilization of the semiquinone state is dependent upon the presence of FMN or an appropriate analogue in its binding site. Direct confirmation of this conclusion was sought by determining to what extent, if any, the $^{31}P$ resonances of the two flavin coenzymes would be paramagnetically broadened upon generation of the semiquinone. Calculations using the Solomon–Bloembergen equation (Solomon, 1955; Bloembergen, 1957) indicate that significant line broadening ($\gtrsim$20 Hz) can be expected for a phosphorus nucleus located within 10 Å of the flavin free radical. However, because of uncertainty in the value used ($10^{-8}$ s) in the calculations for the electron spin relaxation time (Palmer et al., 1971) and the fact that the unpaired electron spin density is distributed in an unknown manner over the flavin ring system, it is not possible to deduce

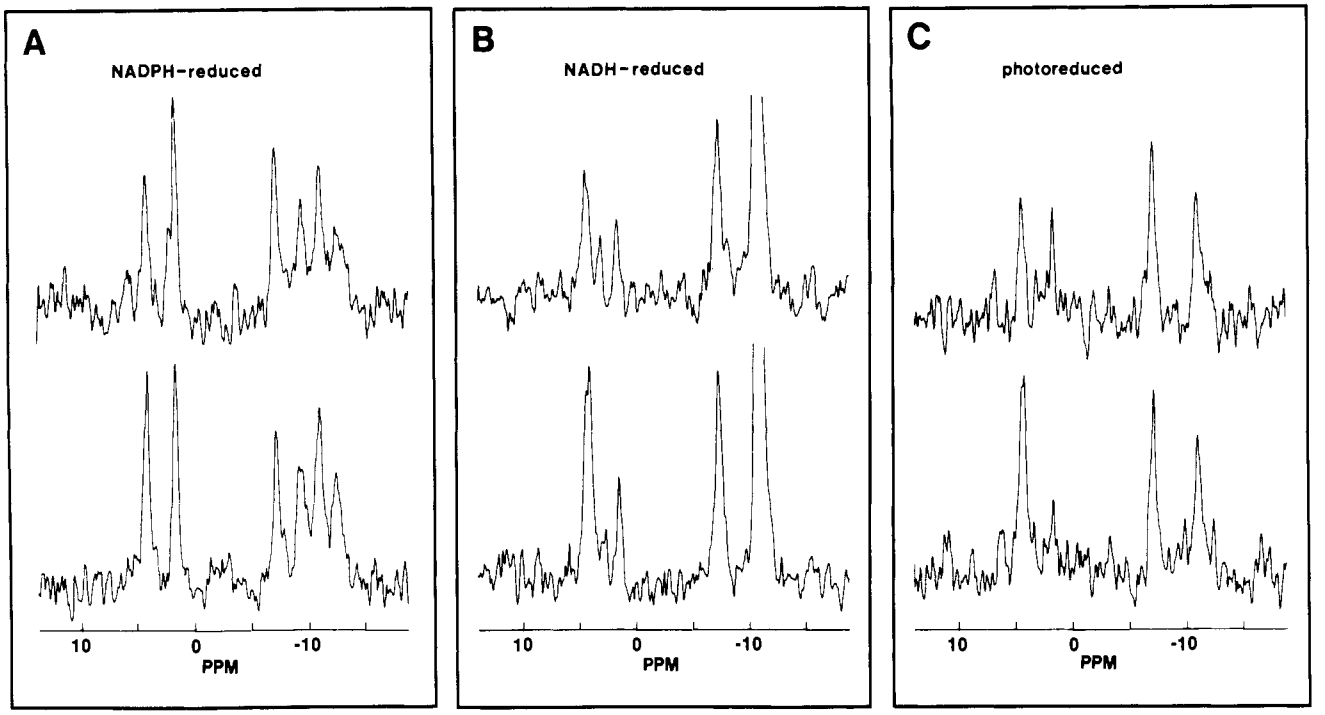


FIGURE 4: $^{31}$P NMR spectra of the oxidized (bottom) and semiquinone (top) forms of NADPH–cytochrome P-450 reductase at pH 7.65. The semiquinone was generated by three different methods: anaerobic reduction with NADPH (A), anaerobic reduction with NADH (B), and photoreduction (C). (A, top) Native reductase (0.13 mM) reduced with 0.9 equiv of NADPH, 170 000 transients, line broadening 20 Hz. (A, bottom) Identical sample of native reductase (0.13 mM) taken from the same enzyme preparation to which was added 0.9 equiv of $NADP^+$ with spectral parameters identical with spectrum A (top). (B, top) Native reductase (0.13 mM) reduced with 1.2 equiv of NADH, 135 000 transients, line broadening 20 Hz. The large off-scale resonance at 10.8 ppm, which obscures the most upfield FAD resonance is from $NAD^+$ (the much weaker binding of $NAD^+$ compared to $NADP^+$ causes its free and bound resonance to be exchange-averaged). (B, bottom) Same as spectrum B (top) after enzyme reoxidation with excess (2 equiv) $K_3Fe(CN)_6$. (C, top) Native reductase (0.13 mM) reduced photochemically (see Materials and Methods), 170 000 transients, line broadening 20 Hz. (C, bottom) Same as spectrum C (top) after enzyme reoxidation with excess (2 equiv) $K_3Fe(CN)_6$. The semiquinone contents of the reduced enzyme samples from spectra A–C determined spectrophotometrically after the NMR spectra were run were 87%, 83%, and 62%, respectively.

reliable distances between the phosphorus and the flavin from the magnitude of the observed broadening. Nevertheless, the calculations assure that at least some measurable broadening will be detected in the $^{31}$P resonance(s) from the flavin coenzyme on which the radical is localized, thereby enabling its unambiguous identification. Several $^{31}$P NMR studies of other flavoenzymes containing either FMN (Favaudon et al., 1980; Edmondson & James, 1982; Moonen & Müller, 1982) or FAD (James et al., 1981) have confirmed this expectation by demonstrating paramagnetic line broadening in the semiquinone states ranging from approximately 40 Hz to several hunderd Hz.

In Figure 4 are shown spectra of the air-stable semiquinone form of the reductase generated by three different methods: anaerobic chemical reduction using the native reductant, NADPH, anaerobic chemical reduction using the less efficient reductant, NADH (Prough & Masters, 1976), or photochemical reduction (Massey et al, 1978). To facilitate comparison, a spectrum of the corresponding oxidized enzyme acquired under identical conditions appears beneath each spectrum of the semiquinone. Immediately evident upon examination of all three pairs of spectra is the fact that neither the chemical shifts, line widths, nor intensities of the two FAD resonances are influenced by semiquinone formation, which is in accord with existing evidence that it is FMN, not FAD, that stabilizes the free radical. Similarly, the 2′-phosphate and pyrophosphate resonances of bound $NADP^+$ are also unchanged in the semiquinone form (Figure 4A). The absence of any perturbation of the FAD and $NADP^+$ signals indicates that their phosphate-containing moieties are at least 12 Å distant from the FMN semiquinone, and there is no apparent difference in their environments in the two redox states.

The only resonance that is significantly affected by reduction of the enzyme is that of the FMN phosphate at about 4 ppm. However, it is not the line width of the signal that appears to be altered but rather the intensity. In the semiquinone spectra in Figure 4A–C, the FMN resonance is reduced in intensity by approximately 40% compared to that of the oxidized enzyme. A possible origin of at least part of this intensity reduction is a diminished NOE that might result from the contribution to $T_1$ relaxation of the FMN phosphate of the semiquinone free radical. However, since the NOE of the FMN signal in the oxidized reductase is only 1.20, its complete abolition could account for a reduction in signal intensity of only 17%. Another explanation for this result is that the observed resonance is actually a superposition of two FMN signals, one of which is paramagnetically broadened beyond the limits of detection and the other which is narrow enough to be observed. Possible reasons why only a fraction of the reductase gives rise to a broadened FMN resonance are discussed later (Discussion).

## Discussion

Since the early studies of Masters et al. (1965a) and Kamin et al. (1966) on the first preparations of liver microsomal NADPH–cytochrome P-450 reductase (then called NADPH–cytochrome *c* reductase) demonstrated *two* flavin prosthetic groups, its mechanism of catalysis has been of great interest. When it became apparent in the mid-1960s that its physiological function involved the reduction of cytochrome(s) P-450 in endoplasmic reticulum electron-transport systems, this interest intensified. NADPH–cytochrome P-450 reductase was

the first flavoprotein in which the catalytic cycle was demonstrated to involve a semiquinone form as the most oxidized partner in the catalytic oxidation–reduction cycle (Masters et al., 1965a,b). The nature of this semiquinone form was not clarified until the studies of Iyanagi and Mason (1973) and Iyanagi et al. (1974) identified the prosthetic groups as FAD and FMN and showed that the air-stable semiquinone form contained only one electron per mole of enzyme (or one electron per two flavins). This work then established NADPH–cytochrome P-450 reductase as the only mammalian flavoprotein to contain both FAD and FMN as prosthetic groups and set into motion a number of subsequent studies to elucidate its mechanism.

The demonstration that NADPH–cytochrome P-450 reductase directly reduces cytochrome P-450 in microsomal electron-transport systems (Lu & Coon, 1968; Yasukochi & Masters, 1976; Vermilion & Coon, 1978a; Iyanagi et al., 1978) documented a fundamental difference between microsomal and mitochondrial or bacterial cytochrome P-450 mediated systems. The experiments of Lu and Coon (1968), in which the first reconstitution of a cytochrome P-450 mediated system was effected, showed unequivocally that the liver microsomal cytochrome P-450 catalyzed oxygenation of fatty acids required only the reductase flavoprotein and cytochrome P-450 in the presence of phospholipids. There was no requirement for an iron–sulfur protein to mediate electron transport between the flavoprotein and heme protein (cytochrome P-450). It is now well established that the endoplasmic reticulum of *all* hepatic and extrahepatic tissues contains cytochrome P-450 systems in which electron transfer is mediated *directly* via NADPH–cytochrome P-450 reductase. It has also been suggested that this flavoprotein represents a more recently evolved electron-transport process, portions of which share flavin-binding domains homologous with more primitive forms (Porter & Kasper, 1985).

Since the studies of Iyanagi and Mason (1973) and Iyanagi et al. (1974) have shown that the air-stable semiquinone form of NADPH–cytochrome P-450 reductase is a one-electron-reduced state, it is of interest to determine the location of the unpaired electron on either the FAD or the FMN. Iyanagi et al. (1974) established the redox potentials of the various components in equilibrium ($F_1$, $F_1H^\bullet$, $F_1H_2$, $F_2$, $F_2H^\bullet$, and $F_2H_2$), but did not identify the low- and high-potential flavins. This is of particular importance since the FAD prosthetic group has been identified by Vermilion and Coon (1978b) as the entrance flavin, i.e., the port of entry of electrons from NADPH, the physiological reductant, in experiments utilizing preparations of FMN-depleted reductase. Vermilion and Coon (1978b) then determined that the low-potential flavin was FAD and the high-potential flavin was FMN on thermodynamic grounds from spectral titration studies. Interestingly, Iyanagi et al. (1974) also pointed out the similarity between the redox potential of adrenodoxin [between –0.305 and –0.270 V (Cooper et al., 1970; Huang & Kimura, 1973)] and the potentials ($F_1H_2/F_1H^\bullet$ = –0.270 V and $F_2H^\bullet/F_2$ = –0.290 V) of the two intermediate redox states of NADPH–cytochrome P-450 reductase.

The redox potentials of NADPH–cytochrome P-450 reductase must be poised to donate electrons, one at a time, to two different redox states of cytochrome P-450: the oxidized, substrate-bound form and the reduced, substrate-bound, oxygenated form. It is, therefore, important to characterize the structure–function relationships of the FAD and FMN prosthetic groups as they relate to pyridine nucleotide reductant, to each other within the reductase molecule, to the protein environment, and ultimately to the electron acceptor(s).

$^{31}P$ NMR affords an approach in which stable states of the reductase can be studied using naturally abundant $^{31}P$ as a probe. The formation and stability of the air-stable semiquinone form of NADPH–cytochrome P-450 reductase was first reported by Masters et al. (1965a) and its quantitative generation was shown by Yasukochi et al. (1979) in electron paramagnetic resonance (EPR) experiments in which the NADPH-reduced semiquinone was compared to a photochemically reduced standard of flavodoxin. Since recent studies of Moonen and Müller (1982) demonstrated a line-broadening effect on the $^{31}P$ NMR spectrum of FMN bound to *Megabacterium elsdenii* flavodoxin as the enzyme was reduced to its semiquinone state, similar experiments were designed to attempt to localize the free radical on one of the two flavins of NADPH–cytochrome P-450 reductase. At the same time, the environments of the FAD and FMN phosphates were examined, utilizing the paramagnetic broadening effects of Mn(II) to determine their exposure to solvent.

The $^{31}P$ NMR spectra of NADPH–cytochrome P-450 reductase, obtained with *130–180* $\mu$M enzyme protein (*255–360* $\mu$M in total flavin), revealed five resolved resonances (Figure 1A). The small resonance at 1.7 ppm has been attributed to residual 2′-AMP utilized in eluting the reductase from the 2′,5′-ADP–Sepharose 4B affinity column (Figure 1B). The remaining four resonances are attributable to the prosthetic flavins (upfield at –7.33 and –11.25 ppm to FAD and downfield at 3.98 and 4.40 ppm to FMN). Addition of $NADP^+$ to the enzyme produces additional resonances at –9.4 and –12.6 ppm attributable to the bound form of $NADP^+$ and superimposes a third resonance at 1.57 ppm upon that of bound 2′-AMP (Figure 2A,B).

Upon $NADP^+$ binding the only flavin resonance that is altered is that at –11.25 ppm, which is consistently shifted downfield by 0.2 ppm. This shift may be indicative of the $NADP^+$ and FAD binding sites being in close proximity. This would be consistent with the findings of Vermilion and Coon (1978b) and Iyanagi et al. (1981) that enzyme-bound FAD serves as the low-potential electron acceptor from NADPH. The observation (Figure 2A,B) that the FAD and FMN resonances, and not the $NADP^+$ resonances, are appreciably perturbed by the addition of Mn(II) suggests that the prosthetic groups, while shielded from bulk solvent, are still influenced by the paramagnetic ion; i.e., the spin–lattice relaxation rates are decreased. In addition, the contribution of bound $NADP^+$ to the 1.6 ppm resonance indicates that strong binding interactions are contributed by the nicotinamide and/or pyrophosphate portions of the pyridine nucleotide, since the signal at 1.7 ppm due to 2′-AMP is broadened almost beyond detection by Mn(II) addition (Figure 3B).

Having assigned the various resonances from bound FAD, FMN, $NADP^+$, and 2′-AMP, it was possible to examine the effects of reduction of NADPH–cytochrome P-450 reductase to the air-stable semiquinone state. All three of the experiments shown in Figure 4 demonstrate that the FMN phosphate resonances at about 4 ppm are altered in intensity by approximately 40% in the semiquinone state of NADPH–cytochrome P-450 reductase compared to those for the oxidized enzyme. At least a partial explanation of this result is provided by the difficulty encountered in preparing a reductase sample for NMR that remained 100% in the semiquinone form for the entire period of time (~35 h) needed to acquire the spectra. (On the basis of spectrophotometric analyses of the samples at the conclusion of the NMR acquisitions, the semiquinone compositions of the enzyme solutions prepared

by reduction with NADPH, by reduction with NADH, and by photochemical means were 87%, 83%, and 62%, respectively). NMR spectra of these samples are therefore expected to contain a contribution from the oxidized form which, if electron transfer (intermolecular) between the oxidized and reduced forms is slow on the NMR time scale, will result in superimposable spectra of those two redox states (see Moonen & Müller, 1982). It is estimated that if the FMN phosphate resonance of the semiquinone form is paramagnetically broadened by more that about 100 Hz, it would be impossible to detect in the presence of the narrower signal from the residual oxidized protein at the same chemical shift. In such a case, the spectral consequence of semiquinone production would be a reduction in the intensity of the FMN signal, as seen in Figure 4.

This explanation satisfactorily accounts for the observation of a narrow FMN resonance in spectra of the semiquinone, but seems insufficient to explain the intensity of the signal, which is significantly greater than that expected to arise from the approximately 20–40% fraction of the reductase that remains in the oxidized form. Although we do not understand at present the reason for this discrepancy, its origin may be related to the fact that the FMN phosphate in the oxidized enzyme, as discussed earlier, gives rise to two nearly superimposable resonances at about 4.4 and 4.0 ppm, rather than one. This feature is not as apparent in the spectra of the oxidized reductase in Figure 4 as it is in those of Figure 1, owing to the application of a 20-Hz digital line-broadening function of the former to improve sensitivity. Nevertheless, two FMN signals are indeed present in all three spectra of oxidized enzyme in Figure 4, with the upfield member of the pair exhibiting the greater intensity. In the semiquinone spectra, however, there appears to be only one FMN resonance whose chemical shift of about 4.3 ppm corresponds most closely with that of the downfield member of the pair. This observation suggests that it may only be the most upfield FMN resonance that becomes paramagnetically broadened in the semiquinone state.

The detection of two FMN signals could arise from the existence of two substates of the reductase in which the FMN exists in two slightly different binding environments. Whether these are conformational states in dynamic equilibrium with one another or represent different forms of the enzyme is not known. The former situation is favored by the observation that a single preparation of enzyme gives rise to different ratios of the 4.4 and 4.0 ppm resonances, although this metamorphosis is not accompanied by changes in catalytic properties. Another possibility is suggested by the observation that the protease-solubilized pig liver reductase invariably reveals three bands on SDS gel electrophoresis, with apparent molecular weights of 68 000, 54 000, and 22 000. It could be postulated that the lower molecular weight species, considered to be proteolytic fragments of the solubilized enzyme, may possess slightly altered FMN binding sites which give rise to the 3.98 ppm 5′-phosphate signal. Confirmation of this possibility and evaluation of its significance must await the outcome of studies that are in progress to establish the origin of the dual FMN signals.

These results indicate quite clearly that the free radical of the air-stable semiquinone state of NADPH–cytochrome P-450 reductase resides on the FMN. This result is especially interesting in light of the kinetic experiments (Masters et al., 1965a; Yasukochi et al., 1979; Oprian & Coon, 1982), which indicate more rapid equilibration of reducing equivalents between the two flavins than the introduction of reducing equivalents from NADPH, indicating spatial contiguity between the FAD and FMN. If NADPH interacts with FAD as the entrance flavin, the two-electron reduction (by hydride ion) must involve this prosthetic group first and subsequent rapid equilibration of the reducing equivalents must follow. Yasukochi et al. (1979) suggested, from aerobic stopped-flow spectrophotometric experiments, that only one of the two flavins is reduced rapidly by NADPH, followed by rapid intramolecular electron transfer from one flavin to the other and subsequently by a much slower reaction with $O_2$ to form the one-electron-reduced air-stable semiquinone. The data of Vermilion and Coon (1978a,b) established that FAD interacts with the electron donor(s) and FMN interacts with the electron acceptors and, recently, Porter and Kasper (1985) have presented evidence from cDNA-derived sequencing data on rat liver reductase that two discrete flavin-binding domains exist. These data and those reported in the present manuscript support the hypothesis that the FAD and FMN prosthetic groups of NADPH–cytochrome P-450 reductase interact rapidly during electron transfer, but a thermodynamically stable semiquinone involving only FMN is formed after reduction in air. These events require spatial contiguity of the two flavins on the one hand, but spatial separation preventing delocalization of the semiquinone free radical on the other hand.

## ACKNOWLEDGMENTS

We are indebted to Steven Ugent for his technical assistance during a Summer Program in Undergraduate Research fellowship in Dr. Masters' laboratory. We thank Patrick Cudahy, Inc., for their generosity in supplying porcine livers gratis throughout these studies. We appreciate the advice of Dr. Albert Girotti in setting up and performing the photoreduction experiments in his laboratory.

**Registry No.** FMN, 146-17-8; FAD, 146-14-5; $NADP^+$, 53-59-8; NADH, 58-68-4; 2′-AMP, 130-49-4; FAD radical, 73651-30-6; NADPH–cytochrome P-450 reductase, 9039-06-9; Mn, 7439-96-5.

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