Identification of the High and Low Potential Flavins of Liver Microsomal NADPH-Cytochrome P-450 Reductase*

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NADPH-cytochrome P-450 reductase, which catalyzes electron transfer to cytochrome P-450 in liver microsomes in reactions leading to the hydroxylation of a variety of substrates, is known to contain 1 molecule each of FMN and FAD per polypeptide chain. The spectrophotometric changes accompanying stepwise reduction of the oxidized enzyme under anaerobic conditions have recently been described. In addition, the stable semiquinone form produced by air oxidation of the reduced enzyme has been shown to be the 1-electron-reduced species with the flavin of higher potential in the semiquinone state and the other in the oxidized state.

In the present study, FMN was selectively removed from the reductase and the properties of the modified protein were compared with those of the native enzyme. The FMN-depleted enzyme lost the ability to catalyze electron transfer to the phenobarbital-inducible form of liver microsomal cytochrome P-450 in the reconstituted hydroxylation system as well as to cytochrome c and some other artificial acceptors, but retained activity toward ferricyanide and 3-AcPyADP. All catalytic activities were restored when the depleted enzyme was incubated with FMN. From fluorescence measurements a value of 1.3 x 10⁻⁸ m was determined for the FMN dissociation constant under the conditions used. Riboflavin and FAD were also bound by the FMN-depleted enzyme, but less effectively than FMN.

A series of spectrophotometric experiments were carried out to determine whether the properties of the FMN-depleted enzyme correspond to those of the high or low potential flavin of the native enzyme, which have E° values of -0.190 and -0.328 V, respectively. Addition of NADP to the fully reduced, FMN-depleted reductase resulted in significant oxidation of flavin, indicating a midpoint potential for FAD near, rather than above, that of the pyridine nucleotide couple. The semiquinone form of the FMN-depleted reductase, which was produced during air oxidation of NADPH-reduced enzyme or during stepwise photochemical reduction of oxidized enzyme under anaerobic conditions, had spectral characteristics similar to those of the semiquinone of the low potential flavin of the native enzyme and was readily oxidized under aerobic conditions. Addition of oxidized FMN to 1-electron-reduced, FMN-depleted reductase under anaerobic conditions produced an enzyme species with properties similar to those of the 1-electron-reduced form of the native enzyme, thereby indicating that electron transfer from FAD to FMN is thermodynamically favorable. These observations establish that the low and high potential flavins of the reductase are FAD and FMN, respectively.

NADPH-cytochrome P-450 reductase, the membrane-bound flavoprotein which catalyzes electron transfer to P-450Lm, has been purified in functional form following the use of detergent as the solubilizing agent (2–8). Drug metabolism and many other NADPH-dependent oxidative activities of liver microsomes have been reconstituted in a purified enzyme system containing the detergent-solubilized reductase, P-450Lm (now known to be a family of discrete proteins (9)), and phosphatidylcholine (10–12). The reductase is one of several microsomal enzymes previously purified and characterized following release from the membrane by proteolytic treatment (cf. review by Williams (13)). However, it is now understood that such treatment results in cleavage of the polypeptide chain and is accompanied by a loss of ability of the reductase to function in its physiological role (8, 14). Unlike the cytochrome P-450-containing enzyme systems of adrenocortical mitochondria (15–17) and Pseudomonas putida (18–20), the liver microsomal hydroxylation system does not require an iron-sulfur protein as an electron carrier between the flavoprotein and the cytochrome. Characterization of the liver microsomal reductase has shown that it contains 1 molecule each of FMN and FAD per polypeptide chain of molecular weight 76,000 (3, 5, 6, 8, 21) and thus differs from the flavoproteins of the other systems which contain one FAD per polypeptide (22–24).

Aside from the ability to catalyze electron transfer to P-450Lm, many characteristics of the detergent-solubilized enzyme, in particular its oxidation-reduction properties (8), are not substantially different from those of the trypsin-solubilized enzyme as described in detail by Iyaniyi et al. (25). The spectrophotometric results of titration with dithionite or NADPH under anaerobic conditions are qualitatively and quantitatively similar for the two proteins, and no evidence for the presence of oxidation-reduction-active groups other than flavin was obtained. The spectral changes which accompany the overall conversion of the oxidized enzyme to the reduced form during reductive titration are complex. The enzyme stabilizes the semiquinone form of each flavin as the neutral, blue radical, and the characteristic long wavelength absorbance and EPR signal are observed during stepwise reduction (8, 25). The spectral changes observed during re-

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† The abbreviations used are: P-450Lm, liver microsomal cytochrome P-450; DCIP, dichloroindophenol. The individual forms of rabbit liver microsomal cytochromes P-450 are numbered according to their relative electrophoretic mobilities when submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis; P-450Lm is the form induced by phenobarbital.
ductive titration are best explained assuming that the midpoint potentials for the two half-reactions, $\text{F} = \text{FH}_2$ and $\text{F} = \text{FH}_2$, are quite different for each flavin. To interpret the spectral changes which accompany the overall reduction of oxidized enzyme to the reduced form with dithionite, Iyanagi et al. (25) combined spectrophotometric and potentiometric data to obtain computer-generated titration curves for several possible oxidation-reduction mechanisms. A very close fit to the experimentally determined spectrophotometric titration curve was obtained when midpoint potentials were assigned as follows:

1. $\text{F} = \text{FH}_2$, $E' = -0.110 \text{ V}$
2. $\text{F} = \text{FH}_2$, $E' = -0.270 \text{ V}$
3. $\text{F} = \text{FH}_2$, $E' = -0.290 \text{ V}$
4. $\text{F} = \text{FH}_2$, $E' = -0.365 \text{ V}$

The two flavins are represented as $\text{F}_1$ and $\text{F}_2$ by the order in which they are titrated. The calculated midpoint potential of the high potential flavin is $-0.190 \text{ V}$, and that of the low potential flavin is $-0.328 \text{ V}$. The semiquinone forms of the two flavins have slightly different spectral properties and differ dramatically in their reactivity toward oxygen. A partially reduced form of the enzyme containing the high potential flavin in the semiquinone state is stable under aerobic conditions for several days. This species, which is readily produced by air oxidation of either NADPH- or dithionite-reduced enzyme, is generally referred to as the air-stable semiquinone form and was characterized as the 1-electron reduced species, $\text{F} = \text{F} + \text{H}_2$, in previous studies (8, 21, 25).

In earlier experiments with the trypsin-solubilized reductase, Baggot and Langdon (26) described the inactivation of the enzyme by photoreoxidation at neutral pH in the presence of ammonium sulfate. The activity of the resulting preparation could be restored by the addition of flavin, and reactivation by FMN was more rapid than with FAD (26). Our later studies with the detergent-solubilized enzyme (27) showed that this procedure, as well as a method involving treatment of the enzyme with high concentrations of KBr (28), results in the selective removal of FMN. In the experiments described in the present paper, the spectral and oxidation-reduction properties of the FMN-depleted enzyme were compared with those of the native enzyme in order to determine whether FAD is the high or low potential flavin.

**EXPERIMENTAL PROCEDURES**

Protein concentrations were estimated by the method of Lowry et al. (29) with bovine serum albumin as the standard. Tris buffer was the chloride salt and phosphate buffer the potassium salt. Unless stated otherwise, all buffer solutions contained 10% glycerol and 0.1 mM EDTA. A Radiometer GK 2302C electrode was employed for pH measurements, and spectra were recorded in an Aminco DW-2 or a Cary 219 recording spectrophotometer with the temperature maintained at 10-12°C by use of a Lauda K2-R circulating water bath.

**Purification of Microsomal Enzyme**—The detergent-solubilized reductase was purified from liver microsomes of phenobarbital-induced rats by a procedure described in detail earlier (8) which incorporates a recently introduced affinity column chromatography step (6) but with 2',5'-AMP-agarose in place of the corresponding Sepharose derivative. The procedure was modified slightly to include the addition of phenylmethanesulfonyl fluoride at a final concentration of 0.1 mM to all buffers used in the final column chromatographic step. The resulting preparations catalyzed the reduction of 62 to 68 pmol of cytochrome c/min/mg of protein when assayed at 20°C in 0.3 M phosphate buffer, pH 7.7.

The phenobarbital-inducible form of cytochrome P-450, P-450.M, was purified from rabbit liver microsomes as described elsewhere (30, 31). The final preparations were homogeneous as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Preparation and Assay of FMN-depleted Reductase—The following procedure was carried out at 4°C. The native enzyme was diluted to a final concentration of approximately 0.5 μM (30 to 35 μg of protein/ml) with 2 mM KBr in 0.05 M potassium pyrophosphate buffer, pH 8.5, containing glycerol but no EDTA, in a final volume of 150 ml. Care was taken to exclude light from all enzyme solutions containing KBr. The solution was concentrated to 50 ml or less in a 200-ml Amicon ultrafiltration apparatus equipped with an XM-50 membrane, and the volume was readjusted to 150 ml by the addition of the KBr-containing buffer. This process was repeated several times, and the solution was then concentrated to 15 ml and dialyzed overnight against 1 liter of 0.05 M phosphate buffer, pH 7.7, with one change of the buffer solution. Recovery of FMN-dependent reductase activity following this treatment was 65 to 70%, determined from the rate of cytochrome c reduction observed when an appropriate aliquot of enzyme was diluted in the usual assay mixture supplemented with FMN (0.4 μM) and incubated for 4 min at 30°C prior to initiation of the reaction with NADPH. Several KBr-treated reductase preparations were pooled (10 to 15 mg of protein, total), and deoxycholate and Renex 690 were added at a final concentration of 0.1% each. The mixture was applied to a column (0.7 x 5 cm) of 2',5'-ADP-agarose previously equilibrated with 0.1 M phosphate buffer, pH 7.5, containing 1.1% Renex. The column was washed with 20 ml of equilibrating buffer, and the reductase was eluted with the same buffer mixture containing 2 mM 2'-AMP. The eluted fractions were pooled and dialyzed against 0.05 M Tris buffer, pH 7.6. Excess KBr was removed by adsorption of the enzyme onto calcium phosphate gel (150 mg) as described previously (8), except that 0.25 M phosphate buffer was used to elute the reductase. The final preparation (containing 1 to 2 mg of protein/ml) was dialyzed against 0.1 M phosphate buffer, pH 7.7, and stored at -20°C. Recovery of FMN-dependent cytochrome c reductase activity from the combined affinity chromatography and calcium phosphate steps was about 70%.

**Photochemical Reduction Procedure**—Photoreduction of enzyme flavin was carried out under anaerobic conditions by the method of Massey and Hemmerich (32); the gas phase was nitrogen, treated to remove traces of residual oxygen as described elsewhere (33). After anaerobiosis was achieved, a solution of 3,10-dimethyl-5-deazaisoafoxazine (henceforth referred to as deazaflavin) was added from a side arm of the sealed vessel at a final molar concentration equal to 5 to 10% that of the enzyme-bound flavin. The experimentally determined extinction coefficients of 21.2 and 10.1 x 10^4 M^-1 cm^-1 were used to calculate the concentration of native and FMN-depleted reductase, respectively, at the wavelength of maximal absorbance in the 450 nm region. An extinction coefficient at 398 nm of 12.0 x 10^4 M^-1 cm^-1 was used to determine the concentration of deazaflavin solutions (34). The light source was a Smith-Victor “sun gun” operated at 70 W by use of a variable transformer and positioned approximately 7 cm from the sample. During illumination, the sample was immersed in a circulating ice-water bath.

**Materials**—The source of materials used for enzyme purification and assay of enzymatic activities are given elsewhere (8). FMN and FAD were purified as previously described (8). Potassium bromide was purified as previously described (9). Potential sources of contamination were removed: High-purity KBr was obtained from J. T. Baker and phenylmethanesulfonyl fluoride from Eastman. Deazaflavin was a generous gift from Dr. V. Massey of this department.

**RESULTS**

**Preparation and Properties of FMN-depleted NADPH-Cytochrome P-450 Reductase**—Preliminary experiments reported elsewhere (27) indicated that the selective removal of FMN from the reductase could be accomplished by photoradiation in the presence of 40% ammonium sulfate or by dialysis against 2 M KBr. To avoid lengthy exposure of the protein to light, the latter procedure was used exclusively in the present studies. Dialysis of flavoproteins against high concentrations of KBr is a mild procedure frequently used to obtain apoenzyme preparations suitable for reconstitution studies (28, 35, 36), but the pH at which dissociation of the flavin occurs most readily varies with the particular enzyme. Although significant dissociation of FMN from NADPH-cytochrome P-450 reductase was observed in the presence of high concentrations of salt in the pH range from 6.5 to 9.0, loss of FAD was minimal at relatively high pH, and selective removal of FMN was found to be optimal in the pH range from 8.0 to 9.0. Since FMN removal by KBr dialysis was no more than 70% complete when concentrated protein solutions were used, the large scale procedure finally adopted was...
diaphragm of dilute enzyme solutions with 2 m KBr-containing buffer in an ultrafiltration apparatus. This procedure consistently resulted in removal of 85 to 95% of the FMN from the enzyme. The analysis of a typical preparation is given in Table I.

Preparations of reductase purified by the two-step procedure employing affinity chromatography (8) apparently contain a contaminating protease which may result in significant conversion of the native enzyme to the 69,000 molecular weight form during KBr treatment. This conversion, which was not observed in the absence of KBr, was inhibited by the addition of the protease inhibitor, phenylmethanesulfonyl fluoride. Proteolysis during KBr treatment was completely prevented when the later stages of purification of the native enzyme were carried out with buffers supplemented with phenylmethanesulfonyl fluoride, as described under "Experimental Procedures." Preparations of FMN-depleted reductase were routinely examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate to verify that proteolysis had not occurred.

After removal of KBr by dialysis, the FMN-depleted reductase was submitted to affinity chromatography on 2',5'-ADP-agarose as a precautionary measure to ensure removal of any denatured protein. As shown in Fig. 1, the native and FMN-depleted enzymes behaved similarly when eluted from the column with 2'-AMP in an increasing linear concentration gradient. In both cases, the peak fractions were eluted at about 2 mM adenylate. When NADP was used in place of adenylate, both the native and the FMN-depleted reductase were eluted more readily; the peak fractions were obtained in the range of 45 to 50 mM NADP. These observations provided the first indication that the ability of the enzyme to interact with pyridine nucleotide is not significantly altered by FMN removal.

Relationship between Flavin Content and Catalytic Activity of FMN-depleted Reductase—The catalytic activities of the native and the FMN-depleted reductase are summarized in Table II. The preparation of FMN-depleted reductase used in these experiments contained 95% of the FAD present in the native enzyme but only 12% of the original amount of FMN (see Table I). In each case, activities were also determined after preincubation of the enzyme with FMN. Since the native enzyme showed approximately a 5% increase in activity when preincubated with FMN, and the ratio of FMN to FAD was slightly less than the theoretical value of 1.0, the activities determined in the presence of added FMN were assigned a value of 100% for comparison with those of the FMN-depleted reductase. With P-450LM2, cytochrome c, menadione, and DCIP, the activity of the FMN-depleted reductase was very low; values of 11, 12, 17, and 11%, respectively, of that obtained for the native enzyme supplemented with FMN were observed. These relative activities correspond closely to the amount of FMN remaining in the preparation and indicate that, with the possible exception of menadione, the FMN-depleted reductase has negligible activity with these acceptors. When the FMN-depleted reductase was assayed after preincubation with FMN, the ability to reduce these acceptors was nearly completely restored. In contrast to the results obtained with these four acceptors, the FMN-depleted preparation catalyzed the reduction of ferricyanide and 3-Ac-
Proceed via an FMN-independent pathway, although the pres-
fold and that of the transhydrogenase-type reaction slightly
significant rates. It appears that the latter two reactions may
indicated by the ability of the FMN-depleted enzyme to
electrons from NADPH without the participation of FMN, as
catalyze the reduction of ferricyanide and 3-AcPyADP at
PyADP was determined for both the native and FMN-de-
pleted enzymes in the presence of a saturating amount of
NADPH, and the values were found not to differ significantly.

It is concluded from these results that the reactions with P-
450Lm2, cytochrome c, menadione, and DCIP proceed via an
FMN dependent pathway, but that the enzyme can accept
electrons from NADPH without the participation of FMN, as
indicated by the ability of the FMN-depleted enzyme to
catalyze the reduction of ferricyanide and 3-AcPyADP at
significant rates. It appears that the latter two reactions may
proceed via an FMN independent pathway, although the pre-
ence of FMN enhances the rate of ferricyanide reduction 1.3-
fold and that of the transhydrogenase-type reaction slightly
over 2-fold.

Flavin Binding to FMN-depleted Reductase—The fluores-
cence of the native enzyme is less than 1% of that of free
FMN, and the FMN-depleted reductase is virtually nonflu-
orescent. Accordingly, the equilibrium constant for the reac-
tion, reductase ≡ FMN-depleted reductase + FMN, can be
determined by the extent of fluorescence quenching in a
titration of the depleted enzyme with the free flavin. The
results of such an experiment are shown in Fig. 2. With
correction of the total flavin concentration of the FMN-de-
pleted preparation for the presence of a small amount of
native enzyme, 0.91 mol of FMN was bound per mol of FMN-
depleted reductase. A dissociation constant of $1.3 \times 10^{-8}$ M
was obtained from this experiment, performed at 12°C in 0.1
m phosphate buffer, pH 7.7.

The ability of FAD and riboflavin to bind to the FMN-
depleted reductase was determined indirectly in experiments
in which the enzyme preparation was incubated with varying
concentrations of either flavin in the cytochrome c reductase
assay mixture prior to initiation of the reaction with NADPH.
The rate of cytochrome c reduction, corrected for the activity
observed in the absence of flavin, was used as a measure of
the amount of reactivated enzyme produced, and the data
were analyzed by double reciprocal plots of velocity versus
flavin concentration. Both FAD and riboflavin were effective
in restoring the ability of the FMN-depleted reductase to
reduce cytochrome c; the maximal velocities determined with
these flavins were 100 and 71%, respectively, of that obtained
with FMN. However, the concentration of FAD required for
half-maximal activity was 100 times greater, and that for
riboflavin nearly 16 times greater, than for FMN. Although
the FMN-depleted reductase does not exhibit absolute specific-
ity for FMN, the results indicate that FMN is bound the
most tightly of the three flavin derivatives.

Comparison of Spectral and Oxidation-Reduction Proper-
tries of High and Low Potential Flavins of Native Enzyme—
As outlined above, the midpoint potentials of the two flavins
are widely separated, and that of the low potential flavin (F2)
is very near that of the NADPH/NADP couple. The semi-
quinone form of F2 is stabilized by the enzyme, and the
midpoint potential for the conversion of the 3-electron-re-
duced enzyme (F2H2—F2H) to the fully reduced form
(F2H2—F2) is about 45 mV below that of the NADPH/
NADP couple. Consequently, the 4-electron-reduced form
cannot be produced by the addition of a stoichiometric quan-
ty of NADPH. The fully reduced enzyme is readily produced
with dithionite, but due to the difficulties in handling and
quantitating anaerobic solutions of this reductant, a more
convenient method for complete reduction of the low potential
flavin was sought. Under carefully controlled conditions,
flavin-catalyzed reduction by EDTA and light has been used
for the stepwise reduction of many flavoproteins (38, 39). The
use of deazaflavins as catalysts in the EDTA-light reaction
was recently introduced and shown to be an even more effi-
cient method for reducing oxidation-reduction proteins of very
low potential (32, 40). As indicated in the earlier studies (32),
the 4-electron-reduced form of the reductase may be obtained
by deazaflavin-catalyzed photoreduction in the presence of
EDTA. The results of such an experiment are shown in Fig.
3A. Several spectra were recorded at intermediate stages
during the reduction process, and the sequence of spectral
changes corresponds to that seen during reductive titration
with dithionite. Following irradiation of the anaerobic solution
of oxidized enzyme for 20 s, a substantial amount of the
semiquinone form of the high potential flavin was produced
The spectra recorded after 40 and 60 s of irradiation corre-
spond to those seen after the addition of approximately 2 and
3 reducing equivalents, respectively, during titration with
dithionite; after a total of 140 s of irradiation the fully reduced
enzyme was obtained. No further spectral changes were ob-
served upon continued irradiation. When deazaflavin was
omitted, even with EDTA concentrations as high as 20 mm,
the rate of reduction was greatly diminished, and after 24 h of
continuous irradiation, the extent of reduction was no greater
than that observed after only 40 s in the presence of deaza-
flavin. As is the case with both NADPH- and dithionite-
reduced enzyme, exposure of the photochemically reduced
enzyme to oxygen yields the air-stable semiquinone form.

In addition to differences in oxidation-reduction potential, the semiquinone forms of the two flavins may be distinguished by their spectra and reactivity toward oxygen. The experiment shown in Fig. 3B illustrates these differences. Spectra A and B are those of the oxidized and photoreduced enzyme produced in the experiment shown in 3A. Oxidized pyridine nucleotide (1.05 mol/mol of FAD) was then added anaerobically, resulting in oxidation of the low potential flavin to yield, at equilibrium, a mixture which contained predominantly the 3-electron-reduced species, F₁H₂--F₂H. (Spectrum C). The reaction vessel was subsequently opened to air, and within several minutes the spectral changes were complete, and Spectrum D was recorded. This species is the air-stable semiquinone form which has been characterized in previous studies as F₁H₂-F₂H. (8, 21, 25). The semiquinone form of the low potential flavin, which was observed under anaerobic conditions following oxidation of the reduced enzyme with NADP, shows maximal absorbance in the long wavelength region at 592 nm and does not exhibit the pronounced shoulder at 630 nm which is characteristic of the semiquinone form of the high potential flavin. As this experiment demonstrates, the semiquinone form of F₂ is not appreciably air-stable, and exposure of partially or fully reduced enzyme to oxygen yields the air-stable species, F₁H₂-F₂H.

Air Oxidation of NADPH-reduced, FMN-depleted Reductase—As indicated by its ability to catalyze ferricyanide reduction at a rate nearly that observed for the native enzyme, the FMN-depleted reductase is rapidly reduced by NADPH. In an experiment not shown, complete reduction of enzyme flavin was observed in the presence of an NADPH-generating system under anaerobic conditions. The spectrum of the oxidized, FMN-depleted reductase, which has absorbance maxima at 455 and 384 nm, and the spectral changes following the addition of an excess of NADPH under aerobic conditions are presented in Fig. 4. Oxidation of reduced flavin was rapid under these conditions, and within 2 min after the addition of NADPH a substantial amount of semiquinone was produced. This species exhibited maximal absorbance in the long wavelength region at 592 nm, and no shoulder was evident at 630 nm. The spectra recorded during the next 20 min indicate that the semiquinone was further oxidized at an appreciable rate, resulting in recovery of over 90% of the absorbance of the initial oxidized enzyme at 455 nm. The residual absorbance at 585 nm and slightly lower final absorbance at 455 nm seen at 20 min in Fig. 4 may be attributed to the presence of about 10% of the total protein in this preparation as native enzyme. This experiment shows that the semiquinone produced by air oxidation of the reduced, FMN-depleted reductase is similar to the low potential flavin of the native enzyme both in spectral characteristics and in its reactivity toward oxygen.

Oxidation of Reduced, FMN-depleted Reductase by NADP—To determine whether the midpoint potential of the FMN-depleted reductase is near, or whether it is above that of the NADPH/NADP couple, an experiment analogous to that described earlier with the native enzyme (see Fig. 3) was performed. An anaerobic solution of FMN-depleted enzyme was irradiated in the presence of EDTA and deazaflavin, and spectra were recorded at several intermediate stages in reduction. Equilibration of the oxidized, reduced, and semiquinone species was much slower than with the native enzyme, and several hours were required before a stable spectrum was obtained after each exposure to light. As shown in Fig. 5A, the series of spectra recorded when equilibration was complete...
are consistent with the conversion of a single oxidized flavin species to the semiquinone, followed by subsequent reduction to the fully reduced form, and indicate considerable stabilization of the semiquinone by the FMN-depleted enzyme. The spectrum of the semiquinone produced during photoreduction was identical with that of the species transiently observed during air oxidation of the NADPH-reduced, FMN-depleted reductase (Fig. 4). If FAD is the high potential flavin, the addition of NADP to the fully reduced enzyme should not result in oxidation of the flavin, but if it is the flavin with the midpoint potential of \(-0.328\) V, significant oxidation of flavin should be observed. As shown in Fig. 5B, Spectrum C, the addition of NADP (1.07 mol/mol of FAD) immediately produced a mixture containing both oxidized and reduced flavin. When it was assumed that only these two species contribute to the absorbance at 455 nm, calculations based on the observed absorbance at this wavelength indicated the presence of 45 and 55% of the total flavin as the oxidized and reduced forms, respectively. These values were used to calculate the absorbance contributed by the enzyme at 340 nm in order to estimate the amount of NADPH produced. A value of 7.56 m\(\text{M}\) was obtained, which represents 47% of the total concentration of pyridine nucleotide added. These calculations are consistent with the conclusion that Spectrum C is that of a mixture containing approximately equal amounts of the oxidized and reduced forms of flavin and pyridine nucleotide. The results may be explained by a rapid reaction of reduced enzyme with the added pyridine nucleotide to immediately generate an equilibrium mixture of the oxidized and reduced forms of each, and, since about equal concentrations of each species were produced, indicate that the midpoint potential of the FMN-depleted reductase is very near that of the NADPH/NADP couple. At the present time the nature of the low, flat absorbance band also observed at very long wavelengths is not known. Spectrum C is not stable with time, and the slow changes, which resulted in a decrease in absorbance at 455 nm and an increase at 592 nm, may be ascribed to the intermolecular disproportionation of the oxidized and reduced forms of the enzyme to yield semiquinone. At 11 h, a substantial quantity of semiquinone had been produced (Spectrum D), and the broad absorbance band at longer wavelengths had disappeared. The observed \(A_{592}\) was 71% of that expected if all of the flavin were present as the semiquinone.

As was also evident from the stepwise photochemical reduction (Fig. 5A), these results demonstrate that there is considerable thermodynamic stabilization of the semiquinone form of the FMN-depleted reductase; thus, the midpoint potentials for the two half-reactions, \(F = FH^+\) and \(FH^- = FH_2^-\), are very likely above and below, respectively, that of the NADPH/NADP couple. These oxidation-reduction properties correspond to those of the low potential flavin of the native enzyme. When the reaction mixture was exposed to air, nearly complete oxidation of enzyme flavin was observed within several minutes (Spectrum E).

**Electron Transfer from FAD to FMN**—If, as indicated by the above experiments, the FMN-depleted enzyme contains the low potential flavin, it should be possible to demonstrate, subsequent to flavin binding, the transfer of electrons from FAD to added FMN. In the experiment shown in Fig. 6, oxidized, FMN-depleted reductase (Spectrum A) was irradiated in the presence of EDTA and deazaflavin under anaerobic conditions to a stage representing about 50% reduction. When equilibration following the last exposure to light was complete, Spectrum B was recorded. The predominant species in this mixture was the semiquinone; at this stage approximately one reducing equivalent per molecule of flavin had been introduced into the system. Oxidized FMN (1.04 mol/mol of FMN-depleted reductase) was then added anaerobically. The first spectrum recorded, which is not shown, indicated very little change in the total absorbance at 585 nm, but a shoulder at 630 nm was easily discerned, and the long wavelength-absorbing species more closely resembled the air-stable semiquinone of the native enzyme than that of the FMN-depleted reductase. A substantial increase at 456 nm was also observed, but additional slow changes took place over a period of hours which resulted in a further increase in absorbance at 585 nm and a slight decrease at 456 nm. Spectrum C, which was recorded after 6 h, resembles very closely that of the 1-electron-reduced or air-stable semiquinone form of the native enzyme. It is likely that the initial solution of 50% reduced, FMN-depleted reductase was an equilibrium mixture containing small amounts of oxidized and reduced flavin as well as the semiquinone, and that the rapid spectral changes observed following FMN addition reflected facile 1-electron transfer from those molecules existing in the semiquinone state to oxidized enzyme-bound FMN. The slower
spectral changes are consistent with a slow intermolecular disproportionation between $F_i$,$H_2$ and $F_i$,$F_2$ (produced by the addition of FMN to those molecules of depleted enzyme initially present in the fully reduced form) and $F_i$,$F_2$ (produced by the addition of FMN to those molecules of depleted enzyme initially present in the fully oxidized form). Since the enzyme stabilizes the semiquinone form of the high potential flavin to an even greater extent than it does the low, the equilibrium favors formation of $F_i$,$H_2$. The anaerobic solution was then exposed to air to ascertain whether the semiquinone produced (Spectrum C) was indeed that of the high potential flavin and therefore resistant to air oxidation. No spectral changes were observed in the long wavelength region upon aeration, as shown by Spectrum D, and only a slight increase in absorbance was observed at 456 nm. These results, which confirm that the species produced following the addition of oxidized FMN to the partially reduced, FMN-depleted reductase represents the 1-electron-reduced or air-stable semiquinone form of the native enzyme, are those expected if the depleted enzyme preparation contains the low potential flavin. Oxidation of the air-stable species by the addition of ferricyanide (10 nmol in 2 μl) gave Spectrum E.

**DISCUSSION**

It was previously suggested by Iyanagi et al. (25) that the two flavins of the reductase may have distinct roles in catalysis, since they exhibit quite different oxidation-reduction properties. Studies by Siegel and co-workers (41, 42) on the flavoprotein component of sulfite reductase, a bacterial enzyme which also contains FMN and FAD and functions in an electron transfer role, give precedent to the idea that one member of a two-flavin pair may serve as acceptor of electrons from reduced pyridine nucleotide, and the other as donor of electrons to the oxidized substrate. Experiments with the native and FMN-depleted forms of the bacterial enzyme provided evidence for a mechanism of electron transfer which involves the initial reaction of reduced pyridine nucleotide with FAD and subsequent transfer of electrons to FMN and ultimately to an oxidized acceptor as two successive equi- tential 1-electron transfers from the fully reduced form of FMN (41). Since our studies with the FMN-depleted reductase have shown that enzyme containing only FAD is readily reduced by NADPH and that electron transfer from FAD to FMN is thermodynamically favorable, such a mechanism might also be envisioned for the FMN-dependent reactions catalyzed by NADPH-cytochrome P-450 reductase. In view of our present knowledge regarding the identity of the two flavins, assignment of a role for FAD as the entry site for reducing equivalents from NADPH is also compatible with the results of earlier stopped flow studies by Masters et al. (43) which indicated that the air-stable semiquinone form of the enzyme ($F_i$,$H_2$) is rapidly reduced by NADPH. The FMN requirement for the reactions with P-450$M_3$, cytochrome c, DCIP, and menadione could be explained by assuming that partially or fully reduced FMN interacts directly with these acceptors. Since other studies have shown that the air-stable semiquinone form reduces cytochrome c only very slowly (43), a mechanism involving direct electron transfer from FAD instead of $F_i$ to cytochrome c would be favored with this acceptor. However, several differences between the properties of both the native and FMN-depleted forms of NADPH-cytochrome P-450 reductase and the bacterial flavoprotein should be noted. The spectral changes observed during reductive titration of the bacterial enzyme (44) are quite different from those observed with NADPH-cytochrome P-450 reductase (8, 25), indicating that the oxidation-reduction properties of the two flavin components differ in the respective proteins. In addition, the FMN-depleted bacterial enzyme is not a competent ferriyanide reductase and retains only the transhydrogenase-type activity of the native enzyme, whereas the FMN-depleted cytochrome P-450 reductase catalyzes both reactions. Thus, in the case of cytochrome P-450 reductase the possibility that FAD, as well as FMN, may participate directly in electron transfer to 1-electron acceptors cannot be excluded. It was also found that the ferriyanide reductase and transhydrogenase-type activities of the FMN-depleted enzyme were enhanced by the addition of FMN. Since the number of binding sites on the enzyme for pyridine nucleotide is not yet known, this observation cautions that further studies are required to eliminate the possibility that FMN may also react independently with NADPH.

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