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# Studies on the Microsomal Mixed-Function Oxidase System: Mechanism of Action of Hepatic NADPH–Cytochrome P-450 Reductase†

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ABSTRACT: The mechanism of hepatic NADPH–cytochrome P-450 reductase has been investigated by using a stopped-flow technique. The reduction of the oxidized native enzyme (FAD–FMN) by NADPH proceeds by both one-electron equivalent and two-electron equivalent mechanisms. The air-stable semiquinone form (FAD–FMNH·) of the native enzyme, which is characterized by an absorption shoulder at 635 nm, is also rapidly reduced to another semiquinone form (FADH·–$FMNH_2$) by NADPH with the disappearance of the shoulder at 635 nm, but the absorbance change at 585 nm is relatively constant. The FAD moiety in the FMN-depleted enzyme is rapidly reduced by NADPH, and reduced FAD is oxidized in successive one-electron steps by $O_2$ or potassium ferricyanide. These results indicate the possibility of intramolecular one-electron transfer between FAD and FMN. The rate of cytochrome P-450 reduction decreases in the presence of FMN-depleted enzyme but is nearly restored to the value of the original enzyme with FMN-reconstituted enzyme. These data suggest that FAD is the low-potential flavin, which serves as an electron acceptor from NADPH. On the other hand, FMN, which is the high-potential flavin, appears to participate as an electron carrier in the process of electron transfer from NADPH to cytochrome P-450 during the mixed-function catalytic cycle.

Hepatic NADPH–cytochrome P-450 reductase (EC 1.6.2.4), the flavoprotein component of a liver microsomal mixed-function oxidase, contains one molecule each of FAD[1] and FMN per molecule of the enzyme, but no other redox-active components (Iyanagi & Mason, 1973). The reductase directly reduces cytochrome P-450 in the presence of phospholipids or detergents without the intermediary of an iron–sulfur protein (Lu & Coon, 1968). The reductase provides the two electrons required for mixed-function oxidation of substrate (Mason, 1958).

We have atempted previously to clarify the redox properties of NADPH–cytochrome P-450 reductase by computer simulating the overall potentiometric and spectrophotometric titration curves (Iyanagi et al., 1974). On the assumption that six components ($F_1$, $F_1H$·, and $F_1H_2$, and $F_2$, $F_2H$·, and $F_2H_2$, where $F_1$ is the high-potential flavin and $F_2$ is the low-potential flavin) derived from the two flavins ($F_1$ and $F_2$) are in equilibrium during the course of the titration of the enzyme with

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[1] Abbreviations used: For purpose of discussion, FAD, FADH·, $FADH_2$, FMN, FMNH·, and $FMNH_2$ are used to symbolize the flavin species; FlH, oxidized flavin; $FlH_2$, neutral flavoprotein semiquinone; and $FlH_3$, neutral fully reduced flavoprotein (Hemmerich et al., 1965); 3-AcPyADP, oxidized 3-acetylpyridine adenine dinucleotide; EPR, electron paramagnetic resonance.

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dithionite, the following set of midpoint potentials which permitted the closest fit of the experimental results was obtained from the computer simulation:

$$F_1 \rightleftharpoons F_1H\cdot \quad E'_{0,1} = -0.110 \text{ V}$$

$$F_1H\cdot \rightleftharpoons F_1H_2 \quad E'_{0,2} = -0.270 \text{ V}$$

$$F_2 \rightleftharpoons F_2H\cdot \quad E'_{0,3} = -0.290 \text{ V}$$

$$F_2H\cdot \rightleftharpoons F_2H_2 \quad E'_{0,4} = -0.365 \text{ V}$$

Recently, Vermilion & Coon (1978b) have provided evidence from a comparison of the redox properties of FMN-depleted enzyme and native enzyme that the low (–0.328 V) and high (–0.190 V) potential flavins are FAD and FMN, respectively. In a series of titration experiments with native enzyme and FMN-depleted enzyme, they also suggest that FAD serves as an acceptor of electrons from NADPH and that there exists a possibility of intramolecular electron transfer between FAD and FMN in the enzyme molecule. However, these suggestions are derived only on the basis of thermodynamic equilibrium results.

In 1965, extensive kinetic studies on the mechanism of steapsin-solubilized pig liver microsomal NADPH–cytochrome *c* (P-450) reductase were published by Henry Kamins laboratory. They have suggested that the two flavins (2 FAD) are not independent but rather interacted with one another, and the simple shuttle mechanism (cycling between two- and four-electron reduced states) for the enzyme has been proposed (Masters et al., 1965). They also reported that the air-stable form, which was proposed to retain two electron-reducing equivalents per two flavin residues, was formed during the catalytic cycle (Masters et al., 1965). However, Iyanagi & Mason (1973) clearly demonstrated that the air-stable form of the enzyme contained only one electron per two flavins, and in addition, the two flavins (FAD and FMN) with different properties (Iyanagi et al., 1974). More recently, Iyanagi et al. (1978) have reported the kinetics of the detergent-solubilized enzyme, which contains equimolar amounts of FAD and FMN, and have showed that the formation of the semiquinone and fully reduced enzyme during the reduction of oxidized enzyme by NADPH occurred independently (Iyanagi et al., 1978), but the experiments were performed under aerobic conditions. However, the detailed mechanism of the electron transfer from a two-electron donor, NADPH, to a one-electron acceptor, cytochrome P-450, has remained obscure.

In the present study, we attempt to elucidate the reaction mechanisms by a stopped-flow technique, focusing on the following points: (1) the reduction mechanism of FMN-depleted enzyme by NADPH and the reoxidation mechanism of the reduced FMN-depleted enzyme by potassium ferricyanide; (2) intramolecular electron-transfer reaction between the two flavins; (3) the reoxidation mechanism of the reduced enzyme by potassium ferricyanide or menadione; (4) the role of each flavin in the process of electron transfer from NADPH to cytochrome P-450. A mechanism for the electron transfer from NADPH to cytochrome P-450 is discussed with special attention focusing on the one- and two-electron-transfer reactions.

## Materials and Methods

*Materials.* The source of materials used for the enzyme purification and assay of the enzyme activities is given elsewhere (Iyanagi et al., 1978). 2′,5′-ADP–Sepharose 4B was purchased from Pharmacia. Potassium bromide (KBr) was obtained from the Kanto Chemical Co. Phosphatidylcholine (type 111-L) was obtained from Sigma. Benzphetamine hydrochloride was a generous gift from Drs. Y. Yasukochi and B. S. S. Masters of the University of Texas Health Science Center at Dallas. All other reagents were of the highest grade available commercially.

*Methods.* Optical spectra were measured with a Hitachi Model 200-10 spectrophotometer, in a sample compartment thermostated at 25 °C. Flavin content was determined by the method of Iyanagi & Mason (1973), using phosphodiesterase. Stopped-flow experiments were performed with a Union Giken RA 401 stopped-flow spectrometer. For anaerobic stopped-flow experiments, 0.1 μM catalase, 10 mM glucose, and 10 units/mL glucose oxidase were added to the reaction mixtures.

Activities of the reductase (native, FMN-depleted, and FMN-reconstituted enzyme) were measured at 25 °C in 0.1 M potassium phosphate buffer, pH 7.7. Rates of reduction of cytochrome *c* and potassium ferricyanide by NADPH were followed by monitoring the absorbance changes at 550 nm, $\epsilon = 19\,500\ M^{-1}\ cm^{-1}$, and at 420 nm, $\epsilon = 1020\ M^{-1}\ cm^{-1}$, respectively. The transhydrogenase activity for 3-AcPyADP was measured as described by Siegel et al. (1974).

For the analysis of the redox states of curve 4 of Figure 7, the following extinction coefficients were used: $\Delta\epsilon_{455nm} = 9.7\ mM^{-1}\ cm^{-1}$ ($A_{\text{fully oxidized}} - A_{\text{fully reduced}}$), $\Delta\epsilon_{503nm} = 3.6\ mM^{-1}\ cm^{-1}$ ($A_{\text{air-stable semiquinone}} - A_{\text{fully reduced}}$), and $\Delta\epsilon_{585nm} = 2.3\ mM^{-1}\ cm^{-1}$ ($A_{\text{air-stable semiquinone}} - A_{\text{fully reduced}}$). These values were calculated from Figure 1 of Iyanagi et al. (1978).

The phenobarbital-inducible form of cytochrome P-450 was purified from rabbit liver microsomes as described by Imai & Sato (1974).

*Reconstitution of NADPH–Cytochrome P-450 Reductase Activity in Phospholipid Vesicles.* Detergent-free cytochrome P-450 and reductase (native, FMN-depleted, or FMN-reconstituted enzyme) were incorporated into the membrane of phosphatidylcholine vesicles according to the method of Taniguchi et al. (1979). The rate of reduction of cytochrome P-450 by NADPH in the reconstituted system was measured by following the formation of the CO complex of ferrous cytochrome P-450 at 450 nm, essentially as described by Iyanagi et al. (1978).

*Solubilization and Purification of NADPH–Cytochrome P-450 Reductase.* Detergent-solubilized NADPH–cytochrome P-450 reductase was prepared by the method of Iyanagi et al. (1978) with some modifications (Yasukochi & Masters, 1976). About 15.0 g of microsomal protein was suspended (to 10 mg of protein/mL) in 0.1 M Tris–acetate buffer, pH 7.6, containing 2% Triton N-101, 0.4% sodium deoxycholate, 10% (v/v) glycerol, and 1 mM EDTA. The mixture was stirred for 30 min at 4 °C and centrifuged at 78000*g* for 120 min. The supernatant solution was applied to a DEAE-cellulose (DE-23) column (4.0 × 30 cm) previously equilibrated with 0.1 M Tris–acetate buffer, pH 7.6, containing 20% glycerol, 0.1% Triton N-101, and 1 mM EDTA (buffer A) containing 0.075 M KCl. The DE-23 column was washed with 1 L of buffer A. The reductase was then eluted with buffer A containing 0.5 M KCl. The active fraction was diluted tenfold with buffer A, applied to a second DEAE (DE-52) column (4.0 × 30 cm) previously equilibrated with buffer A, and eluted with a linear gradient, 0–0.5 M KCl in buffer A. The active fractions were dialyzed overnight against 5 L of 25 mM potassium phosphate buffer, pH 7.7, containing 10% glycerol and 0.1% Triton N-101 (25 mM buffer B) and then applied to a hydroxylapatite column (3.0 × 20 cm) equilibrated with 25 mM buffer B; the column was washed with 500 mL of 25 mM

buffer B, and the reductase was eluted with 100 mM buffer B. The active yellow fraction was applied to a 2′,5′-ADP–Sepharose 4B column (1.5 × 10 cm) previously equilibrated with 100 mM buffer B. The column was washed with 500 mL of 25 mM buffer B, and the reductase was eluted with 2 mM $NADP^+$ in 25 mM buffer B. The active fraction was applied to a hydroxylapatite column (1.5 × 15 cm) equilibrated with 25 mM buffer B. The column was washed with 200 mL of 25 mM buffer B, and the reductase then was eluted with 200 mM buffer B. The overall yield of the purified reductase through the preparation was about 30%. Before use, the reductase was dialyzed against 0.1 M potassium phosphate buffer, pH 7.7.

*Preparation of FMN-Depleted Enzyme.* FMN-depleted enzyme was prepared by the method of Vermilion & Coon (1978b) with some modifications. The native enzyme was diluted to a final concentration of about 0.5 μM with 2 M KBr in 0.1 M Tris–acetate buffer, pH 7.6, containing 10% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol, and 0.1% Triton N-101, in a final volume of 100 mL, and was dialyzed against 5 L of the same buffer, changing the outer buffer every day for 10 days. The KBr-dialyzed enzyme was then dialyzed overnight against 5 L of 25 mM potassium phosphate buffer, pH 7.7, containing 10% glycerol and applied to a hydroxylapatite column (1.5 × 15 cm) equilibrated with 25 mM potassium phosphate buffer, pH 7.7, containing 10% glycerol. After the column was washed with 500 mL of the same buffer, the reductase was eluted with 200 mM potassium phosphate buffer containing 10 glycerol. The activity for cytochrome *c*, potassium ferricyanide, and 3-AcPyADP as electron acceptors was 8%, 65%, and 77% that of native enzyme, respectively.

*Preparation of FMN-Reconstituted Enzyme.* The solution (4 mL) of FMN-depleted enzyme (30 μM as FAD) dissolved in 0.1 M potassium phosphate buffer, pH 7.7, containing 10% glycerol and 0.1% Triton N-101, was incubated with a 1.5-fold more excess of FMN for 30 min at 25 °C, and was dialyzed overnight against 1 L of 25 mM potassium phosphate buffer, pH 7.7, containing 10% glycerol, and applied to a hydroxylapatite column (1.5 × 10 cm) equilibrated with 25 mM potassium phosphate buffer, pH 7.7, containing 10% glycerol. The column was washed with 200 mL of the same buffer and eluted with 200 mM potassium phosphate buffer, pH 7.7, containing 10% glycerol.

From fluorescence measurements, a value of $1.5 \times 10^{-8}$ M was determined for the FMN dissociation constant (25 °C). The activity for cytochrome *c* as an electron acceptor was 98% that of native enzyme.

## Results

*Physicochemical Properties of FMN-Depleted, FMN-Reconstituted, and Native Enzyme.* NADPH–cytochrome P-450 reductase contains one molecule each of FAD and FMN per polypeptide chain of molecular weight 78 000 (Iyanagi & Mason, 1973; Vermilion & Coon, 1974; Yasukochi & Masters, 1976; Iyanagi et al., 1978; Black et al., 1979). The $O_2$-stable semiquinone of the reductase, which is characterized by an absorption shoulder at 635 nm, is obtained by reduction with NADPH in the presence of $O_2$ (Iyanagi et al., 1974). On the basis of EPR quantitation, NADPH titrations, and ferricyanide titrations, the $O_2$-stable semiquinone form has been found to be one reducing equivalent more reduced than fully oxidized enzyme (Iyanagi & Mason, 1973; Iyanagi et al., 1978; Vermilion & Coon, 1978a; Yasukochi et al., 1979). The $O_2$-stable semiquinone is not completely reduced by excess NADPH but forms the NADPH-residual semiquinone, which has no shoulder at 635 nm (Iyanagi et al., 1974).

Table I: FAD–FMN Contents of Native and FMN-Depleted and -Reconstituted NADPH–Cytochrome P-450 Reductase

| enzyme | FAD (%) | FMN (%) | FMN/FAD ratio | fluorescence of enzyme bound flavins (%) |
|---|---|---|---|---|
| native enzyme | 52 | 48 | 0.92 | 2.7 |
| FMN-depleted enzyme | 95 | 5 | 0.05 | 0.5 |
| FMN-reconstituted enzyme | 55 | 45 | 0.82 | 1.0 |

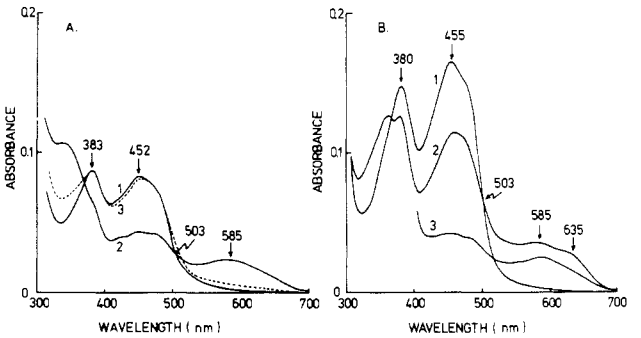



FIGURE 1: Absorption spectra of FMN-depleted and reconstituted reductase. (A) Oxidation–reduction of FMN-depleted enzyme. (Curve 1) Oxidized FMN-depleted enzyme (7.4 μM flavin; molar ratio of FMN to FAD, 0.05) in 0.1 M phosphate buffer, pH 7.7, containing 20% glycerol and 0.1% Triton N-101. (Curve 2) Air-unstable semiquinone recorded at 50 s, after the addition of 40 μM NADPH in the presence of air. (Curve 3) 20 min after addition of 40 μM NADPH. (B) Spectra of FMN-reconstituted enzyme. (Curve 1) oxidized FMN-reconstituted enzyme (15.0 μM flavin; molar ratio of FMN to FAD, 0.82) in 0.1 M phosphate buffer, pH 7.7, containing 20% glycerol and 0.1% Triton N-101. (Curve 2) Air-stable semiquinone prepared by adding 40 μM NADPH in the presence of $O_2$ to FMN-reconstituted enzyme and allowing the solution to stand for 20 min. (Curve 3) After additional NADPH (400 μM final concentration) under anaerobic conditions (NADPH-residual semiquinone).

For determination of whether or not the NADPH-residual semiquinone form of the enzyme is attributable to the same flavin (FAD or FMN) as the $O_2$-stable semiquinone, the spectral and oxidation–reduction properties of the FMN-depleted, FMN-reconstituted, and native enzyme were compared. Flavin contents of the native, FMN-depleted, or FMN-reconstituted enzyme are summarized in Table I. FMN-depleted enzyme contained 95% of the FAD present in the native enzyme, but the content of FMN decreased to only 8% of the original amount of FMN (data not shown). Thus, FMN in the enzyme was shown to be selectively removed from the reductase, in agreement with results of Vermilion & Coon (1978b). The FMN-depleted enzyme still retains significant catalytic activity for ferricyanide and 3-AcPyADP as electron acceptors, but the activity for cytochrome *c* was 8% that of native enzyme (see Materials and Methods). The FMN-depleted enzyme binds FMN strongly ($K_d = 1.5 \times 10^{-8}$ M) (see Materials and Methods). The spectra of the reconstituted enzyme are shown in Figure 1B. The FAD moiety in FMN-depleted enzyme is readily reduced by NADPH; the reduced form reoxidizes in air to a flavin semiquinone species (Figure 1A, curve 2) very similar to the NADPH residual semiquinone formed from native or reconstituted reductase in that no shoulder at 635 nm is present in its absorption spectrum (Figure 1B, curve 3). Similar results have been reported by Vermilion & Coon (1978b), and they indicate that the semiquinone species derived from FAD is air unstable and spectrally similar to the NADPH residual semiquinone. The

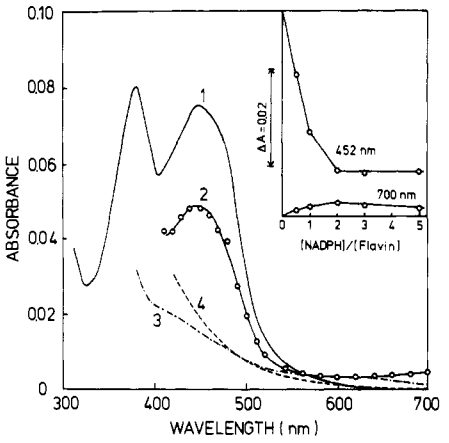



FIGURE 2: Absorption spectra of FMN-depleted enzyme reduced by NADPH. (Curve 1) Oxidized FMN-depleted enzyme (7 $\mu$M flavin; molar ratio of FMN to FAD, 0.05) in 0.1 M phosphate buffer, pH 7.7. (Curve 2) Spectrum obtained at 200 ms, constructed from the stopped-flow experiments at various wavelengths. FMN-depleted enzyme was mixed with NADPH anaerobically. Final concentrations were 7 $\mu$M enzyme and 14 $\mu$M NADPH in 0.1 M phosphate buffer, pH 7.7. (Curve 3) Spectrum reduced by an NADPH-generating system consisting of glucose-6-phosphate dehydrogenase, 0.5 unit/mL, glucose 6-phosphate (10 mM), and $NADP^+$ ($10^{-6}$ M). (Curve 4) Spectrum of $Na_2S_2O_4$ reduced enzyme. (Inset) Oxidation–reduction levels at 200 ms were plotted against [NADPH]/[flavin].

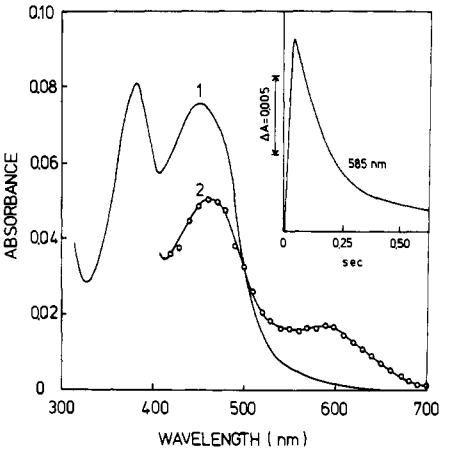



FIGURE 3: Absorption spectrum of the semiquinone formed during potassium ferricyanide oxidation of the reduced FMN-depleted enzyme. (Curve 1) Oxidized FMN-depleted enzyme (7 $\mu$M flavin; molar ratio of FMN to FAD, 0.05) in 0.1 M potassium phosphate buffer, pH 7.7. (Curve 2) Spectrum obtained at 200 ms, which is constructed from the stopped-flow experiments at various wavelengths. FMN-depleted enzyme was mixed with NADPH plus potassium ferricyanide. Final concentrations were 7 $\mu$M enzyme, 7 $\mu$M NADPH, and 7 $\mu$M potassium ferricyanide, and 0.1 M phosphate buffer, pH 7.7. (Inset) Formation and decay of the semiquinone at 585 nm. Final concentrations were 7 $\mu$M enzyme, 7 $\mu$M NADPH, 56 $\mu$M potassium ferricyanide, and 0.1 M potassium phosphate buffer, pH 7.7.

formation of the $O_2$-stable semiquinone is recovered in FMN-reconstituted reductase (Figure 1B, curve 2).

*Mechanism of NADPH Reduction of FMN-Depleted Reductase.* The spectrum of the oxidized form of FMN-depleted enzyme has absorption maxima at 452 and 383 nm (Figure 1A, curve 1). When the oxidized FMN-depleted enzyme (FAD) is mixed with NADPH under anaerobic conditions, a large decrease at 452 nm is observed, and a new, flat absorption band in the long-wavelength region from 500 to 700 nm appears (Figure 2, curve 2), but does not shown an absorption maximum at 585 nm, which is characteristic of a neutral semiquinone form. A similar spectrum with a flat absorption band around 700 nm has been observed in NADH–cytochrome $b_5$ reductase (Iyanagi, 1977) and NADPH–adrenodoxin reductase (Lambeth & Kamin, 1976). The absorbance change at 700 nm observed during the reduction with NADPH parallels that at 452 nm (data not shown). These results indicate that this band is due to the formation of a charge-transfer complex between $NADP^+$ and reduced flavin. However, the absorbance at 452 nm decreases to approximately 60% of its initial value even when two NADPH/total flavin or greater is employed (Figure 2, inset). The rate of flavin reduction by NADPH is decreased by the addition of $NADP^+$, and the final level of reduction is also decreased (data not shown). However, the fully reduced enzyme is observed only in the presence of an NADPH-generating system under anaerobic conditions (Figure 2, curve 3).

*Semiquinone Form of FMN-Depleted Reductase.* As shown in Figure 1A, a spectrum resembling that of the neutral form of flavin semiquinone is observed during air reoxidation of NADPH-reduced, FMN-depleted enzyme. For determination of whether or not this spectrum did indeed reflect a one-electron-containing species, oxidized FMN-depleted enzyme was mixed with equimolar NADPH plus ferricyanide in the stopped-flow apparatus, as described by Lambeth & Kamin (1977). As shown in Figure 3 (curve 2), the spectrum obtained at 200 ms after mixing is very similar to that of $O_2$-sensitive species obtained in Figure 1A (curve 2). The kinetics of the formation and decay of the semiquinone species in the presence of excess ferricyanide are shown in the inset of Figure 3. The

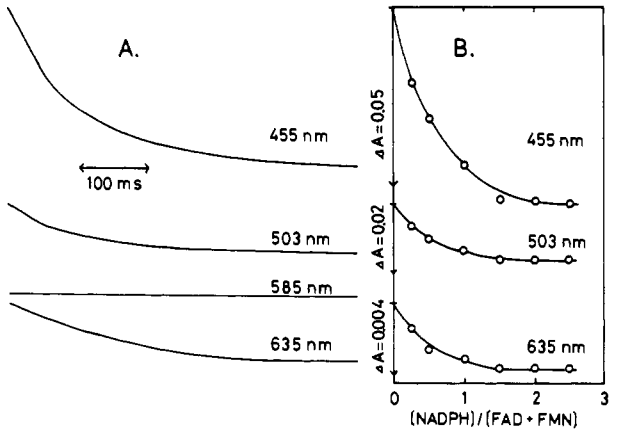



FIGURE 4: Reduction of air-stable semiquinone by several concentrations of NADPH. (A) Air-stable semiquinone was mixed with NADPH anaerobically, and absorbance changes were recorded at four different wavelengths. Final concentrations were 14 $\mu$M air-stable semiquinone, 14 $\mu$M NADPH, and 0.1 M potassium phosphate buffer, pH 7.7. (B) The oxidation–reduction levels at 500 ms were plotted against [NADPH]/[FAD + FMN]. Air-stable semiquinone was prepared by adding NADPH (2 NADPH/total flavin) in the presence of air (Iyanagi et al., 1978). Therefore, the reaction system contains $NADP^+$ (2 $NADPH^+$/total flavin).

absorbance increase at 585 nm due to the formation of the semiquinone occurs without a lag phase.

*Mechanism of NADPH Reduction of the $O_2$-Stable Semiquinone.* As described already, the semiquinone derived from FMN-depleted reductase is unstable to $O_2$ and has an absorption maximum at 585 nm, without showing an absorption shoulder at 635 nm (Figure 1A, curve 2). On the other hand, the spectrum of the air-stable semiquinone form is characterized by an absorption shoulder at 635 nm (Figure 1B, curve 2). Hence, the air-stable semiquinone form of the native enzyme, which is a one-electron-reduced species, is designated as FAD–$FMNH\cdot$. When the $O_2$-stable semiquinone of the native enzyme is mixed with NADPH under anaerobic conditions, the rapid decrease in absorbance at 455 and 503 nm [the isosbestic point between the oxidized and the one-electron-reduced, $O_2$-stable semiquinone or $O_2$-unstable

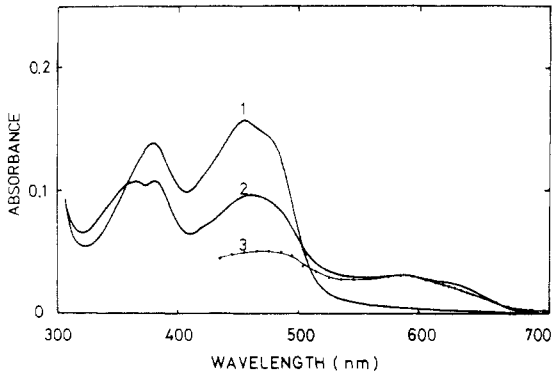



FIGURE 5: Spectra obtained at 500 ms under the same conditions described in Figure 4A. (Curve 1) Oxidized enzyme, 14 $\mu$M. (Curve 2) Air-stable semiquinone, 14 $\mu$M. (Curve 3) Spectrum obtained at 500 ms after enzyme was mixed with NADPH.

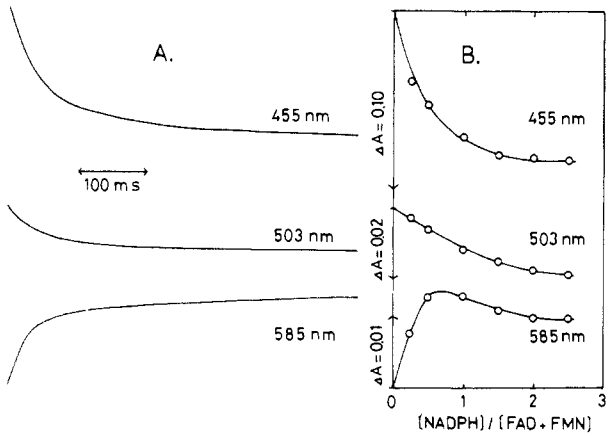



FIGURE 6: Reduction of the oxidized enzyme by several concentrations of NADPH. (A) Oxidized enzyme was mixed with NADPH anaerobically, and absorbance changes were recorded at three different wavelengths. Final concentrations were 14 $\mu$M oxidized enzyme, 14 $\mu$M NADPH, and 0.1 M potassium phosphate buffer, pH 7.7. (B) Oxidation–reduction levels at 500 ms were plotted against [NADPH]/[FAD + FMN].

semiquinone (Figure 1B and 2B)] is observed, but the absorbance at 585 nm is relatively constant (Figure 4A). The change at 635 nm parallels those at 455 nm and at 503 nm (Figure 4B). This indicates that the semiquinone species ($O_2$ stable, a shoulder at 635 nm, FAD–FMNH·) is converted to the semiquinone of the other flavin (FADH·–$FMNH_2$, $O_2$ sensitive, absorption at 585 nm) because the shoulder at 635 nm disappears while the semiquinone absorption at 585 nm remains. In fact, such a spectrum is observed after mixing the $O_2$-stable semiquinone with NADPH under anaerobic conditions (Figure 5, curve 3). In this figure, the spectrum obtained at 500 ms after $O_2$-stable semiquinone is mixed with NADPH corresponds to the three-electron-reduced state of the reductase.

*Mechanism of NADPH Reduction of the Oxidized Enzyme.* When the oxidized enzyme (FAD–FMN) is mixed with NADPH under anaerobic conditions, a decrease in absorption at 455 nm and an increase change at 585 nm are observed, as shown in Figure 6A. The maximum concentration of the semiquinone (increase at 585 nm at 500 ms) is observed when 0.5 mol of NADPH is added per mol of the enzyme (2 mol of flavin) (Figure 6B). However, when 2 NADPH/total flavin or greater is employed, the increase in absorbance at 585 nm is not as great (Figure 6B). The formation of semiquinone (increase at 585 nm) occurs without any lag phase (Figure 6A). The spectra obtained at 500 ms after the oxidized enzyme is mixed with NADPH (0.5 NADPH or 1 NADPH/

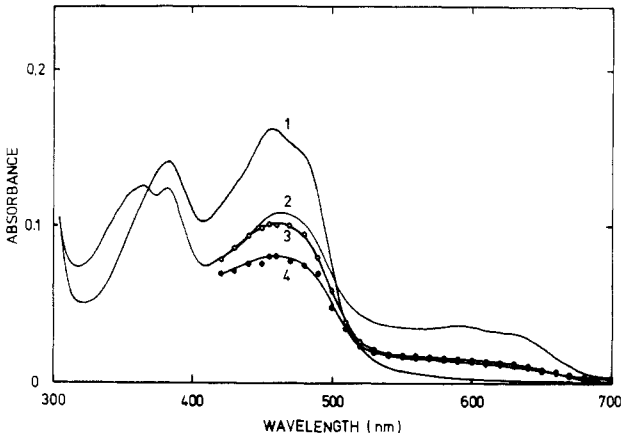



FIGURE 7: Spectra obtained at 500 ms under the same conditions described in Figure 6. (Curve 1) Oxidized enzyme, 14 $\mu$M. (Curve 2) Air-stable semiquinone, 14 $\mu$M. (Curves 3 and 4) Spectra obtained at 500 ms after enzyme was mixed with the ratios of 0.5 NADPH/total flavin and 1 NADPH/total flavin, respectively.

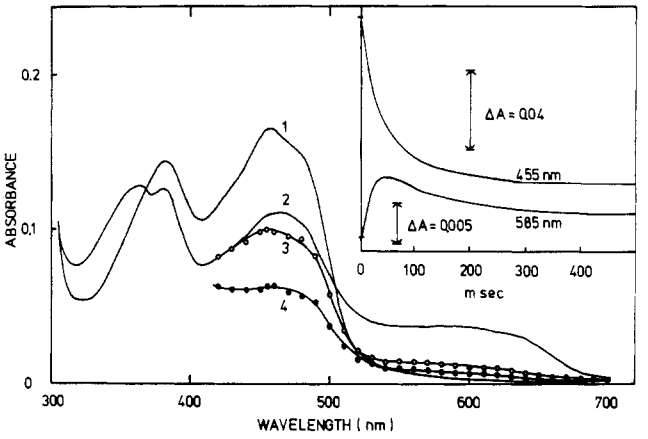



FIGURE 8: Spectra obtained at 50 and 500 ms under the same conditions described in Figure 6. (Curve 1) Oxidized enzyme, 14 $\mu$M. (Curve 2) Air-stable semiquinone, 14 $\mu$M. (Curves 3 and 4) Spectra obtained at 50 and 500 ms after the enzyme was mixed with the ratio of 10 NADPH/total flavin, respectively. (Inset) Time course of the reduction of the enzyme by NADPH (140 $\mu$M).

total flavin) are shown in Figure 7 (curves 3 and 4). From the absorbance changes at 455 (the sum of the formation of semiquinone and reduced forms), 503 (the formation of fully reduced flavin species), and 585 nm (the formation of both fully reduced and semiquinone flavin species), the amount of semiquinone and reduced forms in Figure 7 (curve 4) was calculated to be 17% and 37% of the total flavin, respectively. Therefore, about 46% of the total electrons of NADPH added (at the ratio of one NADPH/total flavin) is transferred to the oxidized enzyme within 500 ms. When 10 NADPH/total flavin is employed, the absorbance change at 585 nm increases and then decays, as shown in the inset of Figure 8. The spectra obtained at 50 (peak at 585 nm) and 500 ms are shown in Figure 8 (curves 3 and 4, respectively).

*The Mechanism of the Oxidation by Ferricyanide and Menadione.* The oxidized enzyme (FAD–FMN) was mixed with NADPH plus ferricyanide solution in the stopped-flow apparatus. The results are shown in Figure 9A. The decrease in the absorbance at 455 nm parallels that at 585 nm, but the absorbance change at 503 nm, which would decrease on the formation of a fully reduced flavin species, is relatively constant. These results indicate that the two-electron-reduced forms of the enzyme do not accumulate measurably during the oxidation by ferricyanide. The air-stable semiquinone form was observed at 500 ms of the reaction (data not shown).

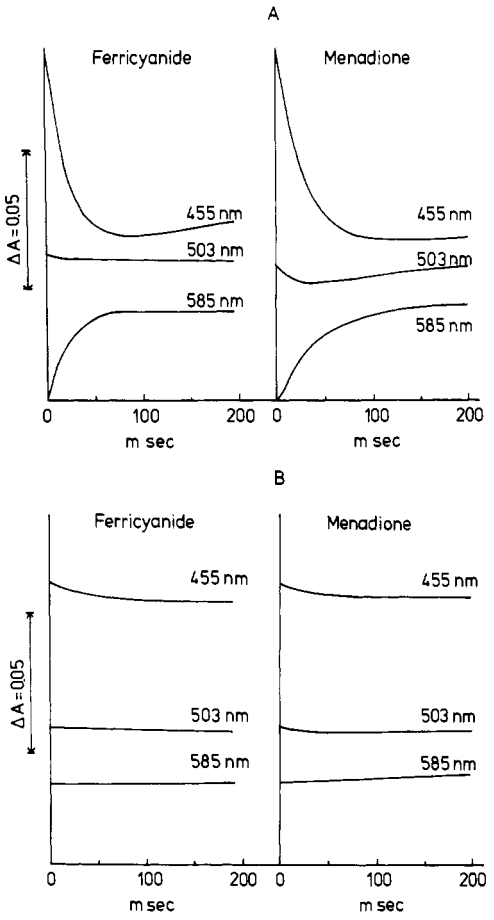



FIGURE 9: Change of absorption with time at three wavelengths during reaction of potassium ferricyanide or menadione and NADPH–cytochrome P-450 reductase with NADPH followed in a stopped-flow apparatus. Oxidized (A) or air-stable (B) semiquinone were mixed with NADPH plus potassium ferricyanide or menadione anaerobically, and absorbance changes were recorded at three different wavelengths. Final concentrations were 14 $\mu$M enzyme [(A) oxidized; (B) air-stable semiquinone], 14 $\mu$M NADPH, 28 $\mu$M potassium ferricyanide or 14 $\mu$M menadione, and 0.1 M potassium phosphate buffer, pH 7.7.

When menadione was used as electron acceptor instead of ferricyanide, very similar results were obtained, except that the rate of the formation of semiquinone at 585 nm was about half and the small decrease in the absorbance at 503 nm was observed (Figure 9A). On the other hand, when the air-stable semiquinone form was mixed with NADPH plus ferricyanide or menadione solution, the absorbance at 455, 503, and 585 nm almost did not change, as shown in Figure 9B. At 500 ms of the reaction, the air-stable semiquinone form was observed as a main spectral species (data not shown). These results indicate that the air-stable semiquinone form is a predominant intermediate formed during the enzyme catalysis.

*The Role of FMN in Electron Transfer from NADPH to Cytochrome P-450.* The ratio of FMN to FAD in FMN-depleted enzyme was 0.05 (Table I), and its NADPH-cytochrome *c* reductase activity was 8% of that for the native enzyme (see Materials and Methods) whereas the activities for ferricyanide and 3-AcPyADP as electron acceptors were 65% and 77% that of the native enzyme (see Materials and Methods). In the reconstituted enzyme, these activities were restored nearly to those of the native enzyme. Furthermore, the FAD moiety in the FMN-depleted enzyme is rapidly reduced by NADPH (Figure 2, curve 2). These results suggest that FMN is necessary for the transfer of electrons to various electron acceptors, in agreement with results by Vermilion & Coon (1978b). The reductase (native, FMN-depleted, or

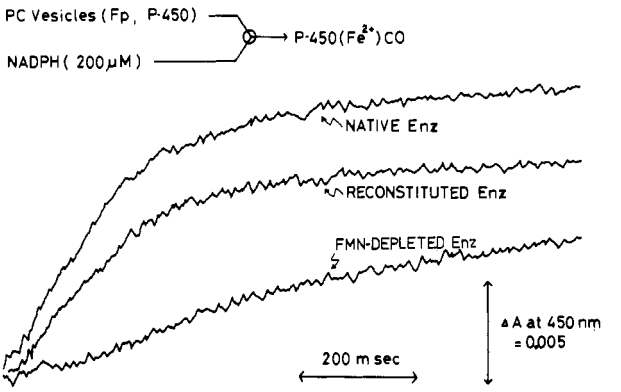



FIGURE 10: Time course of the reduction of cytochrome P-450 by native, FMN-depleted, and FMN-reconstituted NADPH–cytochrome P-450 reductase. The rate of cytochrome P-450 reduction was recorded by following the formation of the CO complex of ferrous cytochrome P-450 in the presence of 1 mM benzphetamine. Final concentrations were 0.2 $\mu$M cytochrome P-450, 0.1 $\mu$M (as per mole of enzyme) reductase, 100 $\mu$M NADPH, and 0.1 M potassium phosphate buffer, pH 7.25.

FMN-reconstituted enzyme) and cytochrome P-450 were incorporated into the phospholipid vesicles. The system contained the reductase, cytochrome P-450, and phosphatidylcholine at a molar ratio of 0.5:1:400. As shown in Figure 10, the rate of cytochrome P-450 reduction was markedly decreased in the presence of the FMN-depleted enzyme but was restored to a value approximately 70% that of the original enzyme with FMN-reconstituted enzyme.

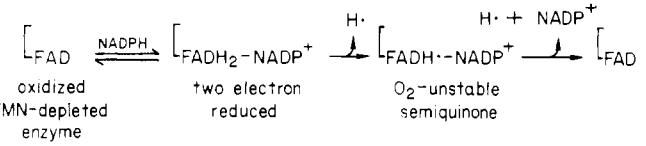
## Discussion

The electron transport system of the hepatic microsomal cytochrome P-450 mixed-function oxidase differs from *Pseudomonas putida* and adrenal mitochondria containing the reductase, non-heme iron protein, and cytochrome P-450; namely, iron–sulfur protein with a $g = 1.94$ signal is not detected in hepatic microsomes (Miyake et al., 1967; Peisach & Blumberg, 1970). More recently, a number of workers have demonstrated that the detergent-solubilized hepatic microsomal NADPH–cytochrome P-450 reductase directly reduces cytochrome P-450 in the presence of detergents or phospholipids (Lu & Coon, 1968; Yasukochi & Masters, 1976; Vermilion & Coon, 1978a; Iyanagi et al., 1978; Taniguchi et al., 1979). The presence of two flavins with different oxidation–reduction properties suggests that they may have individual roles in catalysis; e.g., one flavin accepts two reducing equivalents from NADPH and the other acts as a one-electron carrier in the electron transfer from NADPH to cytochrome P-450 (Iyanagi et al., 1974; Vermilion & Coon, 1978b). Furthermore, NADPH–cytochrome *c* (P-450) reductase in the endoplasmic reticulum of eukaryotic cells has been shown to contain one molecule each of FAD and FMN per molecule of the enzyme (Iyanagi, 1974; Yasukochi & Masters, 1976; Dignam & Strobel, 1977; Aoyama et al., 1978; Mayer & Durrant, 1979; Madyastha & Coscia, 1979; Hiwatashi & Ichikawa, 1979). If these enzymes are directly involved in the microsomal reduction of cytochrome P-450 during the mixed-function oxidase cycle, it is possible that the electron transfer sequence is

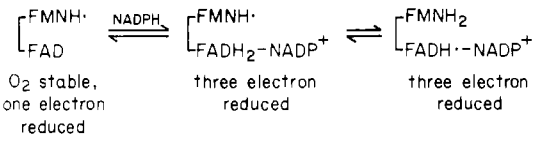
NADPH → fp (FAD → FMN) → cytochrome P-450

The two electrons from NADPH to cytochrome P-450 have been suggested to be transferred to cytochrome P-450 by two sequential steps (Estabrook et al., 1968). Therefore, it seems to be important to study the exact electron distribution among the various reduced forms of the two flavin components of the reductase.

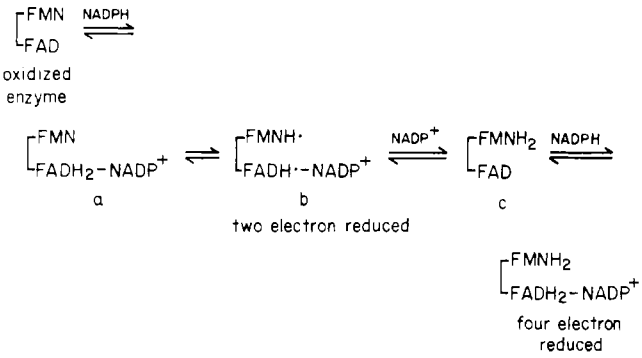
Scheme I




Scheme II




Scheme III




Vermilion & Coon (1978b) have prepared FMN-depleted enzyme, measured its kinetics and oxidation–reduction properties, and provided evidence that the low- and high-potential flavins are FAD and FMN, respectively. We have confirmed and extended these observations in the present study. Since the semiquinone derived from FMN-depleted enzyme is unstable to $O_2$ and without an absorption shoulder at 635 nm (Figure 1A, curve 2), the low-potential flavin is probably FAD because the redox couple, $F_2H\cdot/F_2$ ($E'_{0,3}$ = −0.290 V), is capable of one-electron reduction of $O_2$ [$E'_0$ = −0.27 to −0.33 V (Sawada et al., 1975)] whereas the high-potential redox couple, $F_1H\cdot/F_1$($E'_{0,1}$ = −0.110 V), is not. Furthermore, the oxidized FMN-depleted enzyme (FAD) is rapidly reduced by NADPH, but the maximum decrease at 452 nm is about 40% of the initial value (Figure 2, curve 2). This result suggests that the midpoint potential of enzyme-bound FAD is near that or more negative than that of the NADPH/$NADP^+$ couple. The reoxidation of the $NADP^+$-bound reduced FAD proceeds via a neutral (blue) semiquinone species (Figure 3), indicating that the reduced FAD is oxidized in sucessive one-electron steps. These results may be summarized by Scheme I. The step of dissociation of $NADP^+$ from the reduced enzyme is unclear at present. As reported previously in NADPH–adrenodoxin reductase and NADH–cytochrome $b_5$ reductase, the formation of the complex between the reduced flavin and $NAD(P)^+$ caused a shift in the redox potentials of the enzyme-bound FAD (Lambeth & Kamin, 1976; Iyanagi, 1977). In the present work, however, these problems remain unresolved.

The air-stable semiquinone form is confirmed to be a one-electron reduced species. There are two possible one-electron reduced states, FADH·–FMN and FAD–FMNH·. Since the semiquinone derived from FMN-depleted enzyme (FAD) is unstable to $O_2$ (Figure 1A, curve 2), the air-stable semiquinone form is designated as FAD–FMNH·. When this form is mixed with NADPH, the decrease in the absorbance at 455 nm is accompanied by a negligible change of absorbance at 585 nm (Figure 4A), indicating the reduction of the oxidized flavin molecule contained in the $O_2$-stable semiquinone form. Presumably, oxidized FAD accepts two electrons from NADPH after which one electron is transferred intramolecularly to FMNH·. Since the decrease in absorbance at 635 nm occurs without any lag phase, the reduction of oxidized FAD by NADPH should be the rate-limiting step. As shown in Figure 4B, the concentration of each redox state in Scheme II is governed by the NADPH concentrations. At higher molar ratios of NADPH to reductase, the three-electron-reduced species, with the flavin of lower potential in the semiquinone state (FADH·–$FMNH_2$), is increased.

NAD(P)H is a two-electron donor (the direct transfer of a hydride equivalent from NAD(P)H to flavin is generally accepted) for the flavin dehydrogenase (Hemmerich, 1977). Therefore, when the oxidized enzyme (FAD–FMN) is mixed with NADPH, the two- and four-electron-reduced forms should be observed without forming any semiquinone species. However, in this case, the formation of semiquinone as judged by the increase at 585 nm occurs, in fact without any lag phase (Figure 6A). The extent of semiquinone formation depends on the molar ratios of NADPH to reductase (Figure 6B). There is no significant intermolecular electron transfer between the enzyme molecules within 500 ms. This is supported by the fact that the rates of reduction of the enzyme by NADPH do not depend on the concentration of the enzyme (data not shown). Therefore, the spectrum obtained at this time (Figure 7, curve 3) is the mixture of two- and four-electron-reduced forms of the enzyme. There are several possible two-electron-reduced states: $FADH_2$–FMN, FAD–$FMNH_2$, and FADH·–FMNH·. If we assume that there is only 1 binding site for NADPH per 2 flavins, the results suggest that FAD accepts two electrons from NADPH first, and then electrons are transferred to FMN by the intramolecular route; during the proceses of complete reduction, FAD in species c accepts a second pair of electrons from NADPH.

According to Scheme III, we can expect that, at low molecular ratios of NADPH to the reductase, the two-electron-reduced form would predominate over the four-electron-reduced form, and, at high concentration of NADPH, the four-electron-reduced form increases with decrease in the semiquinone species. Such results are demonstrated in Figures 6B, 7, and 8.

Masters et al. (1965) reported the oxidation–reduction states of steapsin-solubilized NADPH–cytochrome $c$ (P-450) reductase during its reduction by NADPH and reoxidation by potassium ferricyanide, menadione, and 2,6-dichlorophenolindophenol. They demonstrated the involvement of a partially reduced form of the enzyme during catalysis and also suggested that this reduced form may be an intermediate of the catalytic cycle involved in one-electron reduction of several electron acceptors (Masters et al., 1965). This partially reduced oxidation–reduction state is identified as the one-electron-reduced semiqunone form of the enzyme. In the present studies, we also found that the air-stable semiquinone form is the predominant intermediate observed during oxidation of the reduced enzyme by potassium ferricyanide or menadione (Figure 9). However, during a one-electron oxidation reactions of the three-electron-reduced species ($FADH_2$–FMNH· or FADH·–$FMNH_2$) by potassium ferricyanide or menadione, biradical species (FADH·–FMNH·) of the enzyme could be produced. Such an intermediate was not observed, as shown in Figure 9. Since the air-stable semiquinone is inactive toward such electrons acceptors (Masters et al., 1965), the reduction of the oxidized enzyme by NADPH should be the rate-limiting step. Namely, the reactions of electron acceptors with the reduced flavin species and intramolecular electron transfer

between FAD and FMN are fast.

The present studies have also confirmed that the FMN-depleted enzyme retains ferricyanide reductase and transhydrogenase activities, but it is incapable of transferring electrons to cytochrome P-450 (Vermilion & Coon, 1978b). As shown in Figure 10, the activity of the FMN-depleted enzyme, which is incorporated into the membrane of phosphatidylcholine vesicles by the cholate dialysis method (Taniguchi et al., 1979), was very slow. But, the ability to reduce the cytochrome P-450 was nearly restored with FMN-reconstituted enzyme. Therefore, the results indicate that the enzyme-bound FMN interacts directly with cytochrome P-450. In the reconstituted system, the rate-limiting reaction step is between the reductase and cytochrome P-450 (Taniguchi et al., 1979). The reductase is therefore probably kept in the reduced forms during catalysis. Since the $O_2$-stable semiquinone is inactive toward several electron acceptors, such as cytochrome *c*, ferricyanide, and menadione, and, in addition, is rapidly reduced to a three-electron-reduced form by NADPH, the FAD–FMN pairs may cycle between the one-electron (FAD–FMNH·) and three-electron (FADH·–$FMNH_2$) reduced states to donate two electrons to cytochrome P-450. The redox potential of the couples, $FMNH_2$/FMNH· ($E'_{0,2}$) and FADH·/FAD ($E'_{0,3}$), of the reductase may play an important role in intramolecular electron transfer and in the donation of electrons to cytochrome P-450. The values of redox potential, $E'_{0,2}$ (−0.270 V) and $E'_{0,3}$ (−0.290 V), are very close to one another and therefore permit one-electron transfer between flavins at a constant oxidation–reduction potential. $E'_{0,2}$ (−0.270 V) of the three-electron-reduced form may be capable of supplying the first electron to the substrate-bound, ferric cytochrome P-450 [$E'_0$ = −0.237 V, in the presence of benzphetamine as substrate (Sligar et al., 1979)] and/or the second electron to the substrate-bound, ferrous oxycytochrome P-450, which may have a more positive redox potential than that of substrate-bound ferric cytochrome P-450 (Gunsalus et al., 1973).

"Our proposed model", mechanism 4 (Iyanagi et al., 1974), and present results may be summarized by eq 1–5, where

$$\text{FAD–FMN} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{NADP}^+\text{–FADH}_2\text{–FMN} \quad (1)$$

$$\text{NADP}^+\text{–FADH}_2\text{–FMN} \rightleftharpoons \text{NADP}^+\text{–FADH·–FMNH·} \rightleftharpoons \text{FAD–FMNH}_2 + \text{NADP}^+ \quad (2)$$

$$\text{FAD–FMNH}_2 + \text{RH–Fe}^{3+}\text{ (P-450)} \rightleftharpoons \text{FAD–FMNH·} + \text{RH–Fe}^{2+}\text{ (P-450)} + \text{H}^+ \quad (3)$$

$$\text{FAD–FMNH·} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{NADP}^+\text{–FADH}_2\text{–FMNH·} \quad (4)$$

$$\text{NADP}^+\text{–FADH}_2\text{–FMNH·} = \text{NADP}^+\text{–FADH·–FMNH}_2 + \text{RH–Fe}^{2+}\text{O}_2\text{ (P-450)} \rightleftharpoons \text{NADP}^+\text{–FADH·–FMNH·} = \text{FAD–FMNH}_2 + \text{NADP}^+ + \text{Fe}^{3+}\text{ (P-450)} + \text{ROH} + \text{OH}^- \quad (5)$$

FAD–FMNH· is the $O_2$-stable semiquinone and RH–$Fe^{3+}$ (P-450) is the substrate-bound oxidized form of cytochrome P-450.

The three-electron-reduced form, which is produced by the reduction of the air-stable semiquinone by NADPH, can supply the first as well as second electrons to cytochrome P-450 (eq 6 and 7). Furthermore, we must take into account the

$$\text{NADP}^+\text{–FADH}_2\text{–FMNH·} = \text{NADP}^+\text{–FADH·–FMNH}_2 + \text{RH–Fe}^{3+}\text{ (P-450)} \rightleftharpoons \text{NADP}^+\text{–FADH·–FMNH·} = \text{FAD–FMNH}_2 + \text{NADP}^+ + \text{RH–Fe}^{2+}\text{ (P-450)} + \text{H}^+ \quad (6)$$

$$\text{FAD–FMNH}_2 + \text{RH–Fe}^{2+}\text{O}_2\text{ (P-450)} \rightleftharpoons \text{FAD–FMNH·} + \text{Fe}^{3+}\text{ (P-450)} + \text{ROH} + \text{OH}^- \quad (7)$$

four-electron-reduced form (Masters et al., 1965), which can donate two electrons to cytochrome P-450 (eq 8–10). However, we cannot exclude the possibility that partially reduced FMN, FMNH·, as well as fully reduced FMN, $FMNH_2$, participates in electron transfer to the substrate-bound ferrous oxycytochrome P-450 (Iyanagi et al., 1974). The proposed mechanism described above needs more detailed information, especially concerning the kinetics between the reductase and cytochrome P-450.

$$\text{FAD–FMNH}_2 + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{NADP}^+\text{–FADH}_2\text{–FMNH}_2 \quad (8)$$

$$\text{NADP}^+\text{–FADH}_2\text{–FMNH}_2 + \text{RH–Fe}^{3+}\text{ (P-450)} \rightleftharpoons \text{NADP}^+\text{–FADH}_2\text{–FMNH·} + \text{RH–Fe}^{2+}\text{ (P-450)} + \text{H}^+ \quad (9)$$

$$\text{NADP}^+\text{–FADH}_2\text{–FMNH·} = \text{NADP}^+\text{–FADH·–FMNH}_2 + \text{RH–Fe}^{2+}\text{O}_2\text{ (P-450)} \rightleftharpoons \text{NADP}^+\text{–FADH·–FMNH·} = \text{FAD–FMNH}_2 + \text{NADP}^+ + \text{Fe}^{3+}\text{ (P-450)} + \text{ROH} + \text{OH}^- \quad (10)$$

In summary, hepatic microsomal NADPH–cytochrome P-450 reductase contains two flavins, FAD and FMN, in equimolar quanties, which have different redox properties. Namely, FAD is able to accept two reducing equivalents from NADPH whereas FMN appears to serve as a one-electron carrier in the process of electron transfer from NADPH to cytochrome P-450 during the mixed-function oxidase catalytic cycle.

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# Computation of Enzyme–Substrate Specificity†

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ABSTRACT: The present study reports the development of a new procedure for the theoretical computation of enzyme–substrate specificities. The immediate goal has been to identify experimental data with which computations may be effectively compared, examine the underlying theoretical principles, and demonstrate feasibility. The experimental systems treated are hydrolyses catalyzed by chymotrypsin of Ac-Trp-$NH_2$, of Ac-Phe-$NH_2$, and of the Hein–Niemann "locked" substrate derived from phenylalanine; this may be designated as Lock-HN-$OCH_3$. For Trp and Phe, the L enantiomers are substrates while the D enantiomers are inhibitors, thus indicating differences of 7 kcal/mol or more in $\Delta\Delta G^{\ddagger}$ (D–L). For the "locked" substrate, the D enantiomer is the better substrate and $\Delta\Delta G^{\ddagger}$ (D–L) is −4 to −6 kcal/mol. We have used molecular mechanics to compute steric energies of models for the transition states for these hydrolyses and have been able to reproduce the experimental $\Delta\Delta G^{\ddagger}$ values surprisingly well even with a relatively primitive model. The differences in computed steric energies are not due to any one major term but are rather the consequences of summations of a large number of small terms. The new method shows promise of developing into a useful probe for the quantitative study of biochemical systems.

Many aspects of enzyme chemistry have been worked out in impressive detail. Yet one essential aspect has remained elusive: how to predict quantitatively the energies of interaction of substrates and enzymes so that analogous catalytic systems may be designed and synthesized at will. Such quantitative computational capability is likewise essential to the understanding of many other phenomena of biochemistry at the molecular level.

The size of enzyme molecules poses severe problems of logistics, and the need to obtain interaction energies to about 1 kcal/mol in order to predict rates even to a factor of 8 seems out of reach. However, we have in the past several years been employing the technique of molecular mechanics in a special way to estimate steric effects on reaction rates and have been able to estimate relative free energies of activation to about 0.5 kcal/mol in ester hydrolysis and in $S_N2$ cyclization reactions (DeTar & Tenpas, 1976; DeTar et al., 1978; DeTar & Luthra, 1980). It is promising, therefore, to apply this methodology to enzyme–substrate systems to see what sorts of results may be obtained.

Applications of molecular mechanics to problems of protein structure have a long history. Examples include prediction of conformations (Ramachandran et al., 1963; Scheraga, 1971; Momany et al., 1975; Momany, 1976; White & Morrow, 1977), estimation of energies of crystals of peptides and related molecules (Momany et al., 1974a,b; Hagler & Lifson, 1974; Hagler et al., 1974, 1976, 1979a–c; Hagler & Bernstein, 1978; Lifson et al., 1979; Dauber & Hagler, 1980), treatment of the complex problems of protein folding (Levitt, 1974; Honig et al., 1976; Levitt & Warshell, 1975; Finkelstein & Ptitsyn, 1977; Cohen & Sternberg, 1980; Lesk & Chothia, 1980; Hagler et al., 1979a–c), studies of ring conformations of proline (Ramachandran et al., 1970; Venkatachanlam et al., 1974, 1975; DeTar & Luthra, 1977, 1979), studies of binding of peptides to proteins (Platzer et al., 1972a,b; Bosshard, 1974; Pincus et al., 1976), and examination of protein dynamics as in the behavior of an internal aryl ring in interacting with a peptide system (McCammon & Karplus, 1979).

To calculate theoretically the rate of a reaction it is sufficient to calculate $\Delta G^{\ddagger}$, the free energy of activation. This can be represented as the difference in the free energy of formation of the transition state and of the reactant state. In particular it is not necessary to trace such details as reaction trajectories, nor is it necessary to take specific account of minor energy minima, identifiable experimentally perhaps as conformational

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