Oxidation–Reduction Properties of Rat Liver Cytochromes P-450 and NADPH–Cytochrome P-450 Reductase Related to Catalysis in Reconstituted Systems†

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ABSTRACT: A series of equilibrium and kinetic measurements involving the oxidation–reduction properties of purified rat liver NADPH–cytochrome P-450 reductase and eight different purified rat liver cytochromes P-450 (P-450s) were carried out. Apparent spin states of P-450 iron were determined in the absence and presence of a number of known substrates by using second-derivative and conventional near-UV absorbance spectroscopy. Many of the substrates examined did not produce significant changes in the apparent iron spin state, even when binding could be demonstrated with equilibrium dialysis. Further, the spin state was not correlated to catalytic activity of the P-450s in reconstituted enzyme systems. The oxidation–reduction potentials were determined for the ferric/ferrous couples of each of the eight P-450s in the presence and absence of known substrates, as well as other proteins suspected of altering the potentials. The midpoint potential ($E_{m,7}$) ranged from −350 to −289 mV for the P-450s under these conditions. In some cases $E_{m,7}$ was raised with the addition of substrates, but the extent of the increase was no greater than +33 mV. The $E_{m,7}$ of one P-450 (P-450$_{\beta NF/ISF-G}$) was not changed significantly when the fraction of high-spin iron varied between 11 and 67%. Steady-state spectral studies provided evidence for the accumulation of an oxygenated ferrous intermediate (or a derived product) of one P-450 (P-450$_{PB-B}$) in the presence of a substrate, cyclohexane. Studies on the donation of electrons from cytochrome b_5 and a series of dyes to this complex suggest that it has an effective $E_{m,7}$ (for reduction) of approximately +50 mV. In studies with one of the P-450s, steady-state spectral studies indicated that the three-electron-reduced form of NADPH–P-450 reductase accumulates, consistent with the view that this form of the reductase is involved in the reduction of P-450 from the ferric to the ferrous state.

Cytochrome P-450[1] is the terminal oxidase of a microsomal mixed-function oxidase system that catalyzes the oxidation of a variety of drugs, steroids, alkanes, and other endogenous and xenobiotic compounds. Much attention has been addressed to the regulation of levels of individual isozymes of this hemoprotein family and to the physical and chemical details of the catalytic mechanism of oxidation. The catalytic mechanism is generally considered to involve substrate binding to the ferric enzyme, reduction of the enzyme to the ferrous form, O_2 binding, introduction of a second electron, release of H_2O, insertion of the second atom of molecular oxygen into the substrate, and release of product from the resulting ferric enzyme [for reviews see Ullrich (1979) and White & Coon (1980)]. While the general features of this scheme are widely accepted, many details of individual steps of the scheme remain to be established.

Recently methods have been developed in this laboratory for the separation and purification of eight isozymes of rat liver microsomal P-450, and the activities of each form toward a variety of substrates were measured in reconstituted enzyme systems containing NADPH–P-450 reductase and di-12:0-GPC (Guengerich et al., 1982). In this report, the relationships between substrate binding, heme iron spin-state transitions, and $E_{m,7}$ values of P-450 ferric/ferrous couples were studied with these P-450s. An $E_{m,7}$ value for the reduction of a ternary oxygenated ferrous P-450 substrate complex was estimated, and the electronic form of NADPH–P-450 reductase involved in ferric P-450 reduction was deduced from steady-state spectral measurements. While studies with soluble reconstituted enzyme systems cannot answer all questions related to the function of these enzymes under the complex conditions involving protein competition and lipid association in the endoplasmic reticulum, the results bear on basic physical properties of the systems in question. The enzymes are catalytically competent in the systems described here, and the hydroxylase activities observed in microsomes are consistent with results obtained in these simplified systems (Guengerich et al., 1982). The results are discussed in terms of thermodynamic parameters of individual steps of the catalytic cycle.

Experimental Procedures

Chemicals. Argon (low-oxygen grade, <0.5 ppm O_2) was purchased from Union Carbide, Linde Division (Somorset, NJ). Copper catalyst (catalyst R3-11, catalog no. 18-3000-00) was obtained from Chemical Dynamics Corp. (South Plainfield, NJ). Benzylviologen, methylene blue, and thionine were obtained from British Drug House (through Gallard-Schlesinger, Carle Place, NY), safranine T was from Fluka Chemical Corp. (Hauppauge, NY), phenosafranine and 3,6-diaminoacridine (proflavin) were from Aldrich Chemical Co. (Milwaukee, WI), and indigodisulfonate was from ICN-K and K (Plainview, NY). (*R*)-Warfarin was a gift of Dr. L. S. Kaminsky, New York Department of Health (Albany, NY). [4-^{14}C]Testosterone was purchased from New England Nuclear Corp. (Boston, MA).

Enzymes. Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) were treated with phenobarbital or β-naphthoflavone as described elsewhere (Guengerich &

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[1] Abbreviations: P-450, cytochrome P-450; EDTA, (ethylenedinitrilo)tetraacetic acid; SKF-525A, (diethylamino)ethyl 2,2-diphenylvalerate; E_h, potential relative to the hydrogen electrode; $E_{m,7}$, midpoint oxidation–reduction potential (relative to the hydrogen electrode) determined at pH 7.0; di-12:0-GPC, L-α-dilauroylglyceryl-3-phosphocholine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Martin, 1980). NADPH–P-450 reductase and the P-450 isozymes P-450$_{UT-A}$, P-450$_{PB-B}$, P-450$_{\beta NF-B}$, P-450$_{PB-C}$, P-450$_{PB-D}$, P-450$_{PB/PCN-E}$, P-450$_{UT-F}$, and P-450$_{\beta NF/ISF-G}$ were purified to electrophoretic homogeneity as described elsewhere (Guengerich et al., 1982). The same reference (Guengerich et al., 1982) describes the electrophoretic, spectral, catalytic, and immunological properties of these P-450s, as well as the immunochemical estimation of levels of each P-450 in microsomes prepared from rats treated in certain ways, the rationale for the nomenclature system, and comparisons with rat P-450 preparations derived by other workers.

Equilibrium Dialysis. P-450$_{PB-B}$ was concentrated on an Amicon PM-30 ultrafiltration device (Amicon Corp., Danvers, MA) and dialyzed for 2 h vs. 0.1 M Tris-HCl buffer (pH 7.6). One-half-milliliter aliquots of the P-450$_{PB-B}$ solution (32 μM P-450) were placed in dialysis bags along with 30 μM di-12:0-GPC and a concentration of (*R*)-warfarin or [4-^{14}C]-testosterone equivalent to that which was subsequently included in the dialysis medium. The dialysis medium consisted of either 20 mL of 0.1 M Tris-HCl buffer (pH 7.6) containing 30 μM di-12:0-GPC and (*R*)-warfarin (5–150 μM), or 10 mL of 0.1 M Tris-HCl buffer (pH 7.6) containing 30 μM di-12:0-GPC and [4-^{14}C]testosterone (2.7 mCi mmol^{-1}) (5–70 μM). Each dialysis bag was rocked in a capped tube (1.8 × 13 cm for (*R*)-warfarin or 1.4 × 11 cm for testosterone) in a horizontal position for 96 h at 23 °C. Equilibrium was reached within 48 h with testosterone. In the case of (*R*)-warfarin, 150-μL aliquots of the dialysis bag and medium were mixed with 2.85 mL of dimethyl sulfoxide, and the (*R*)-warfarin concentrations were determined by fluorescence measurements (excitation 330 mm, emission 395 nm), with correction for protein quenching. Twenty-four data points were collected. In the case of testosterone, 100-μL aliquots of the dialysis bag and medium were dissolved in 10 mL of ACS liquid scintillation cocktail (Amersham-Searle, Arlington Heights, IL), and testosterone concentrations were estimated from radioactivity measurements (the counting efficiency was 93–95% regardless of whether protein was present or not, as judged by external standard ratios). Twenty data points were collected. In both cases, the binding parameters were determined by using linear regression analysis of plots of [bound substrate]/[free substrate] vs. [bound substrate].

Estimation of Spin State of P-450 Iron. A variety of optical and resonance techniques have been used to monitor the iron spin state of P-450 [for reviews see Mannering (1972) and Ullrich (1979)]. In the work presented here, the Soret bands at 392 and 417 nm were used as indicators of the high- and low-spin ferric P-450s, respectively. Instead of the relative changes in the difference spectra which are often used, the second derivatives of the absolute spectra were employed. This method allows direct observation of the peaks at both wavelengths with nearly base-line resolution between them (Figure 1). Moreover, the distance from the peak height to trough follows Beer's law (O'Haver & Green, 1976). The "2 PF" method described in that reference was used here. While each absorbance derivative follows Beer's law, different peaks are not directly comparable to each other. The induced changes in the spectra of P-450$_{PB-B}$ and P-450$_{\beta NF/ISF-G}$ were used to determine that the peak-to-trough relative extinction coefficient in the second derivative spectrum is twice as great for the low-spin band (417 nm) as for the high-spin band (392 nm). Thus, the fraction of high-spin iron in a P-450 sample can be estimated by using the equation

$$\text{fraction high-spin iron} = (2 \times 2\,PF_{392})/[(2 \times 2\,PF_{392}) + 2\,PF_{417}]$$

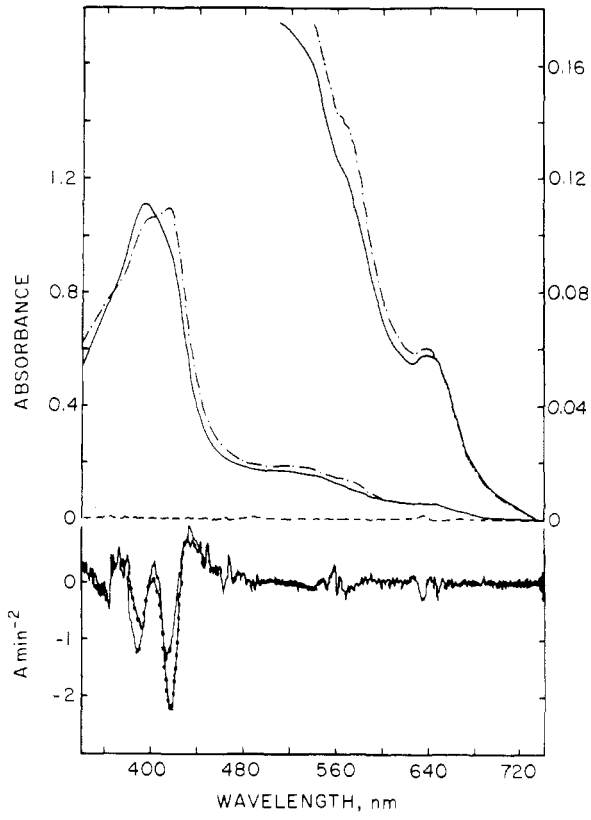

FIGURE 1: Absolute and second-derivative spectra of P-450$_{\beta NF/ISF-G}$ in the presence and absence of testosterone. Spectra were recorded at 25 °C with 4 μM P-450$_{\beta NF/ISF-G}$ in 0.1 M Tris-HCl buffer (pH 7.6) containing 30 μM di-12:0-GPC (Guengerich et al., 1982), both with (●) and without (—) 0.5 mM testosterone, and are uncorrected for dilution effects. Expanded absolute spectra are shown at the right. Second-derivative spectra (lower panels) were obtained for the same samples on a Cary log-derivative accessory with a full-scale setting of 10 *A* min^{-2} and a scanning rate of 2 nm s^{-1}.

A peak centered at 646 nm was present in the visible spectra of P-450 samples which contained a substantial fraction of high-spin P-450 iron, as judged by the Soret spectra. This band has been noted previously and used as a marker of high-spin iron [e.g., see Werringloer & Kawano (1981)]. Spectra of ferric P-450 samples which contained significant absorbance in this region (i.e., P-450$_{\beta NF/ISF-G}$ devoid of added substrates and P-450$_{PB-B}$ samples containing *d*-benzphetamine) were used to determine an extinction coefficient for this band. A_{646} (oxidized vs. reduced difference spectra) was divided by the P-450 concentration: since the second derivative spectra indicated that in each case the P-450 existed as a mixture of spin states (i.e., 30–67% high spin), the apparent ϵ_{646} values were divided by the fraction of P-450 in the high-spin iron configuration to obtain an extinction coefficient that would be expected if the P-450 were completely in the high-spin configuration. The two samples yielded an ϵ_{646} value of 4.9 (±0.1) mM^{-1} cm^{-1}. This value is considerably greater than that calculated by Werringloer & Kawano (1981).

Measurement of $E_{m,7}$ Values. Previous studies have established that dye equilibration techniques can be used to accurately estimate $E_{m,7}$ values of P-450s (Guengerich et al., 1975; Sligar et al., 1979; Waterman & Mason, 1972). Preliminary studies established that the potentials of interest here were in the neighborhood of −300 mV. In most instances, 2.7–3.0 mL of a mixture of the P-450 of interest (3–10 μM), benzylviologen (20 nM), safranine T (0.5 μM), proflavin (1.0 μM), potassium phosphate (pH 7.0, 0.1 M), EDTA (10 mM), *Aspergillus niger* glucose oxidase (10 units mL^{-1}), bovine liver

catalase (450 units mL^{-1}), and glycerol (5–12%, w/w) was placed in a fluorometer cuvette designed for anaerobic work. The cuvette contained 0.3 mmol of mutorotated glucose in a sidearm and a stirring bar in the main compartment. The valve on the cuvette was connected to the manifold of a gas train (Burleigh et al., 1969) which was designed for alternate exposure of the cuvette to vacuum (ca. 20 mmHg) and argon which had been passed through a 5 × 45 cm column of copper catalyst (maintained at 160 °C) and then distilled water. (The lines of the gas train were either glass or copper metal, with four 1-cm connections made with butyl rubber tubing.) Oxygen was removed from the cuvette by using 10 cycles of vacuum and argon (over 1–1.5 h) in the dark; the glucose was added to complete the oxygen scavenging system when the deoxygenation procedure was partially completed. The cuvette valve was closed under positive argon pressure.

UV–visible and fluorescence spectra were recorded on Cary 219 and Varian SF-330 instruments, respectively. Safranine T, when excited at 520 nm, produces a fluorescence emission spectrum with a peak at 570 nm. The reduced dye is not fluorescent. Fluorescence methods were generally used to monitor the potential of the cell since this technique permitted low concentrations of dye to be used which did not interfere with the visible spectra of the P-450s. The absorbance of P-450 at 520 nm did not change considerably during titrations, and changes in fluorescence quenching were not a factor. Control experiments established that the fluorescence was proportional to the concentration of oxidized safranine T in the presence of P-450. The $E_{m,7}$ of safranine T, −289 mV (Clark, 1960), allows accurate potential measurements to be made in the range of −250 to −340 mV. The dyes equilibrated rapidly with the P-450 in these experiments, as verified by repetitive scanning measurements. When low concentrations of indicator dyes were used (that did not significantly interfere with absorbance measurements), spin-state equilibria observed in the presence of substrate such as *d*-benzphetamine did not differ from those observed in the absence of the substoichiometric levels of the dyes. Other studies (Guengerich et al., 1975) suggest that similar $E_{m,7}$ values can be obtained for P-450s in systems using dye equilibration, direct potentiometry in the presence of these dyes, and NADPH/$NADPH^+$-poised systems devoid of dyes. Thus, P-450s do not appear to alter the $E_{m,7}$ values of the dyes [also see Sligar et al. (1979)]. The bulk of the results emphasize differences between $E_{m,7}$ values recorded with the same dye system under various conditions. In some cases (i.e., $P\text{-}450_{PB\text{-}B}$ plus di-12:0-GPC), benzylviologen (ca. 20 μM) was used to verify the estimates obtained with safranine T. Benzylviologen has a useful $E_{m,7}$ (−359 mV; Clark, 1960) but is not fluorescent, and at high concentrations, its optical properties interfere with P-450 in both the Soret and α,β regions.

The anaerobic cuvette, containing the P-450 and dye and maintained at 25 °C, was reduced in a stepwise manner with pulses of light from a 500-W source positioned at a distance of 5 cm. The length of the pulse varied from 3 s, at the start of the reduction, to 5 min, for the last one or two pulses. After each pulse, the cuvette was stirred, and the UV–visible and fluorescence spectra were then recorded. This reduction process usually involved about 10–12 steps over a period of about 2 h. At the end of the series, the cuvette was opened, and solid $Na_2S_2O_4$ was added to establish fully reduced base lines.

At each point, E_h (in mV) of the system was determined by using the formula (Clark, 1960)

$$E_h = E_{m,7} + [RT/(nF)] \ln ([dye^{ox}]/[dye^{red}])$$

where R is the gas constant, T is the absolute temperature, n is the number of electrons transferred, F is the Faraday constant, and the concentrations of oxidized and reduced dye are considered. In the case of safranine T at 25 °C, the Nernst equation becomes

$$E_h = -289 + 29.5 \log [F_i/(F_o - F_i)]$$

where F_i is the fluorescence at a given point i and F_o is the fluorescence of the fully oxidized sample. The reduction of each P-450 was monitored by using absorbance changes, usually in the Soret region. The log ($[P\text{-}450^{ox}]/[P\text{-}450^{red}]$) was plotted vs. E_h. The midpoint potential $E_{m,7}$ and the value of n, equivalent to the slope × 59 mV, were determined by linear regression analysis. Points corresponding to $|\log ([P\text{-}450^{ox}]/[P\text{-}450^{red}])|$ values greater than unity and E_h values outside of the range −250 to −340 mV were not used in these calculations.

Results

Optical Spectral Changes of P-450 in the Presence of Substrates. The effects of known substrates on the spin states of P-450 were examined by using the second-derivative technique described under Experimental Procedures, and the results are presented in Table I. Only some of the known substrates tested yielded substantial changes in the apparent spin state. The list of substrates which did produce changes includes *d*-benzphetamine ($P\text{-}450_{PB\text{-}B}$, $P\text{-}450_{PB\text{-}D}$), cyclohexane ($P\text{-}450_{PB\text{-}B}$), and testosterone ($P\text{-}450_{UT\text{-}F}$). $P\text{-}450_{\beta NF/ISF\text{-}G}$ exists primarily in the high-spin form as isolated. While other workers have apparently isolated this isozyme in the presence of a bound ligand (Ryan et al., 1980; Fisher et al., 1981), the spectra of our ferrous preparations suggest that such material is not present here and is not responsible for the high-spin character (Guengerich et al., 1982). Moreover, the same enzyme can be isolated in the high-spin state from rats treated with other chemicals such as β-naphthoflavone (Guengerich et al., 1982). Some of the substrates produced small shifts in the spin state of $P\text{-}450_{\beta NF/ISF\text{-}G}$ to the low-spin form.

In general, the more hydrophobic compounds (e.g., cyclohexane, testosterone, and benzphetamine) seemed to be more likely to produce spin-state conversions. However, the observed rates of catalytic activity showed no apparent relation to the extent of spin conversion. For instance, $P\text{-}450_{PB\text{-}B}$ metabolized aminopyrine and *d*-benzphetamine at similar rapid rates (Guengerich et al., 1982), although aminopyrine did not perturb the spin state and *d*-benzphetamine did. *d*-Benzphetamine shifted the iron to the high-spin form to similar extents in $P\text{-}450_{PB\text{-}B}$ and $P\text{-}450_{PB\text{-}D}$, yet the difference in catalytic activity between the two forms was greater than an order of magnitude (Guengerich et al., 1982). Three P-450s ($P\text{-}450_{PB\text{-}B}$, $P\text{-}450_{PB\text{-}C}$, and $P\text{-}450_{UT\text{-}F}$) all metabolized testosterone at similar rates, and some (but not all) of the products were the same in the three cases (Guengerich et al., 1982).

In order to determine if extensive binding of these substrates occurred under the conditions in which the spectra were obtained, equilibrium dialysis studies were carried out with testosterone and (*R*)-warfarin, using $P\text{-}450_{PB\text{-}B}$. Both substrates were bound in a saturable manner. Roughly one molecule of (*R*)-warfarin (1.3 at infinite substrate concentration) was bound to each monomeric unit of $P\text{-}450_{PB\text{-}B}$, and the calculated K_D was 20 μM. The binding curve obtained with testosterone contained a less saturable, lower affinity component at the higher concentrations used. Without correction for this component, the K_D was 36 μM, and 2.0 molecules of testosterone were bound to each monomeric $P\text{-}450_{PB\text{-}B}$ unit at infinite substrate concentration.

Table I: Comparison of High-Spin Iron Content and Reduction of High-Spin Iron in Reconstituted Cytochrome P-450 Systems with Rates of Catalytic Turnover

P-450 isozyme	additions[a] (concn)	% of Fe in high-spin form (Fe^{3+} state)[b]	% of high-spin Fe^{3+} reduced[c]	catalytic turnover no. (min^{-1})[d]
$P\text{-}450_{UT\text{-}A}$	none	<5	[e]	(32)[f]
$P\text{-}450_{UT\text{-}A}$	(*R*)-warfarin (0.3 mM)	<5		1.3
$P\text{-}450_{UT\text{-}A}$	ethylmorphine (7 mM)	<5		44
$P\text{-}450_{UT\text{-}A}$	testosterone (0.5 mM)	<5		14
$P\text{-}450_{PB\text{-}B}$	none	<5		(56)[f]
$P\text{-}450_{PB\text{-}B}$	SKF-525A (5 μM or 0.1 mM)	<5		
$P\text{-}450_{PB\text{-}B}$	aminopyrine (5 mM)	<5[g]		85
$P\text{-}450_{PB\text{-}B}$	ethylmorphine (7 mM)	<5		24
$P\text{-}450_{PB\text{-}B}$	(*R*)-warfarin (0.3 mM)	<5		0.23
$P\text{-}450_{PB\text{-}B}$	testosterone (0.5 mM)	<5		12
$P\text{-}450_{PB\text{-}B}$	cyclohexane (20 mM)	50		30[h]
$P\text{-}450_{PB\text{-}B}$	*d*-benzphetamine (0.5 mM)	31[i]	81	72
$P\text{-}450_{\beta NF\text{-}B}$	none	<5		(38)[f]
$P\text{-}450_{\beta NF\text{-}B}$	acetanilide (5 mM)	<5		46
$P\text{-}450_{\beta NF\text{-}B}$	(*R*)-warfarin (0.3 mM)	<5		3.6
$P\text{-}450_{\beta NF\text{-}B}$	*d*-benzphetamine (0.5 mM)	<5		13
$P\text{-}450_{PB\text{-}C}$	none	<5		(22)[f]
$P\text{-}450_{PB\text{-}C}$	ethylmorphine (7 mM)	<5		54
$P\text{-}450_{PB\text{-}C}$	(*R*)-warfarin (0.3 mM)	<5		1.1
$P\text{-}450_{PB\text{-}D}$	none	<5		(9)[f]
$P\text{-}450_{PB\text{-}D}$	*d*-benzphetamine (0.5 mM)	23	<20	3
$P\text{-}450_{PB\text{-}D}$	aminopyrine (7 mM)	<5		7
$P\text{-}450_{PB\text{-}D}$	(*R*)-warfarin (0.3 mM)	<5		0.12
$P\text{-}450_{PB/PCN\text{-}E}$	none	<5		(18)
$P\text{-}450_{PB/PCN\text{-}E}$	(*R*)-warfarin (0.3 mM)	<5		0.14
$P\text{-}450_{UT\text{-}F}$	none	<5		(47)
$P\text{-}450_{UT\text{-}F}$	(*R*)-warfarin (0.3 mM)	<5		<0.05
$P\text{-}450_{UT\text{-}F}$	testosterone (0.5 mM)	51	39	17
$P\text{-}450_{\beta NF/ISF\text{-}G}$	none	61	33	(61)
$P\text{-}450_{\beta NF/ISF\text{-}G}$	aminopyrine (7 mM)	48	79	29
$P\text{-}450_{\beta NF/ISF\text{-}G}$	(*R*)-warfarin (0.3 mM)	45	56	0.03
$P\text{-}450_{\beta NF/ISF\text{-}G}$	testosterone (0.5 mM)	33		0.5
$P\text{-}450_{\beta NF/ISF\text{-}G}$	*d*-benzphetamine (0.5 mM)	60		11

[a] Additions were made in water, as neat compounds (cyclohexane) or as 0.5 M ethanol solutions (testosterone). [b] See Figure 1 for general conditions and methodology. Unless noted otherwise, NADPH–P-450 reductase was not present. [c] Reconstituted enzyme systems, containing 4 μM P-450 and 4 μM NADPH–P-450 reductase (see Figure 3 for other additions), were prepared and divided into two 150-μL cuvettes, at 37 °C. After a base line was obtained, NADPH (0.5 mM, in the presence of a regenerating system) was added to the sample cuvette, and spectra were recorded. The traces obtained within the first 1–2 min were used in these estimations. The ϵ_{646} for oxidized vs. reduced P-450 was 4.9 mM^{-1} cm^{-1} (see Experimental Procedures). [d] From Guengerich et al. (1982), unless otherwise indicated. The units (min^{-1}) indicate nmol of product formed min^{-1} (nmol of P-450)$^{-1}$. [e] A blank indicates that the percent of high-spin P-450 iron reduced was not determined. [f] Numbers in parentheses indicate rates of NADPH oxidation in the absence of substrate (Guengerich et al., 1982). The units (min^{-1}) indicate nmol of NADPH oxidized min^{-1} (nmol of P-450)$^{-1}$. [g] A concentration of NADPH–P-450 reductase equivalent to that of the P-450 was added in a separate experiment, and identical results were obtained. [h] Macdonald et al. (1982). [i] Similar results were obtained in the presence and absence of 30 μM di-12:0-GPC.

Oxidation–Reduction Potentials of P-450 Ferric/Ferrous Couples. Preliminary studies indicated that di-12:0-GPC had a negligible effect on the observed $E_{m,7}$ of $P\text{-}450_{PB\text{-}B}$ (Table II). Since other studies on the catalytic activities were carried out in the presence of this phospholipid (Guengerich et al., 1982), $E_{m,7}$ values of all eight P-450 isozymes were determined under such conditions. In these studies, a difference in $E_{m,7}$ of >|10 mV| is considered significant. The $E_{m,7}$ values ranged from −350 ($P\text{-}450_{PB/PCN\text{-}E}$) to −299 mV ($P\text{-}450_{\beta NF\text{-}B}$) (Table II). In several cases, substrates which are metabolized by the individual P-450s were added to the systems, and $E_{m,7}$ values were determined. The substrate cyclohexane raised the $E_{m,7}$ of $P\text{-}450_{PB\text{-}B}$ from −311 to −286 mV, in the presence of di-12:0-GPC. *d*-Benzphetamine and (*R*)-warfarin did not affect the $E_{m,7}$ of $P\text{-}450_{PB\text{-}B}$. The $E_{m,7}$ of $P\text{-}450_{UT\text{-}A}$ was raised 14 mV by the presence of ethylmorphine, the $E_{m,7}$ of $P\text{-}450_{UT\text{-}F}$ was raised 33 mV by the presence of testosterone, and the $E_{m,7}$ of $P\text{-}450_{PB\text{-}D}$ was raised 24 mV by the presence of *d*-benzphetamine. The $E_{m,7}$ values for $P\text{-}450_{PB\text{-}C}$ and $P\text{-}450_{PB/PCN\text{-}E}$ were unchanged in the presence of ethylmorphine and (*R*)-warfarin, respectively.

The $E_{m,7}$ values of $P\text{-}450_{PB\text{-}B}$ were examined under a variety of conditions suspected of affecting the system. The nonionic detergent Lubrol PX (used in P-450 isolations) raised the $E_{m,7}$ slightly. The enzymes NADPH–P-450 reductase and cytochrome b_5 lowered the $E_{m,7}$ value only very slightly. In these experiments, cytochrome b_5 was completely reduced before $P\text{-}450_{PB\text{-}B}$ was. In the experiment with the NADPH–P-450 reductase, the changes in A_{455} (which occurred before P-450 reduction) were used to determine an apparent $E_{m,7}$ of −260 mV with an *n* value of 2.34. These changes correspond to the addition of three electrons to the flavoprotein, on the basis of the spectra reported elsewhere (Iyanagi & Mason, 1973; Vermilion & Coon, 1978a). Iyanagi et al. (1974) have de-

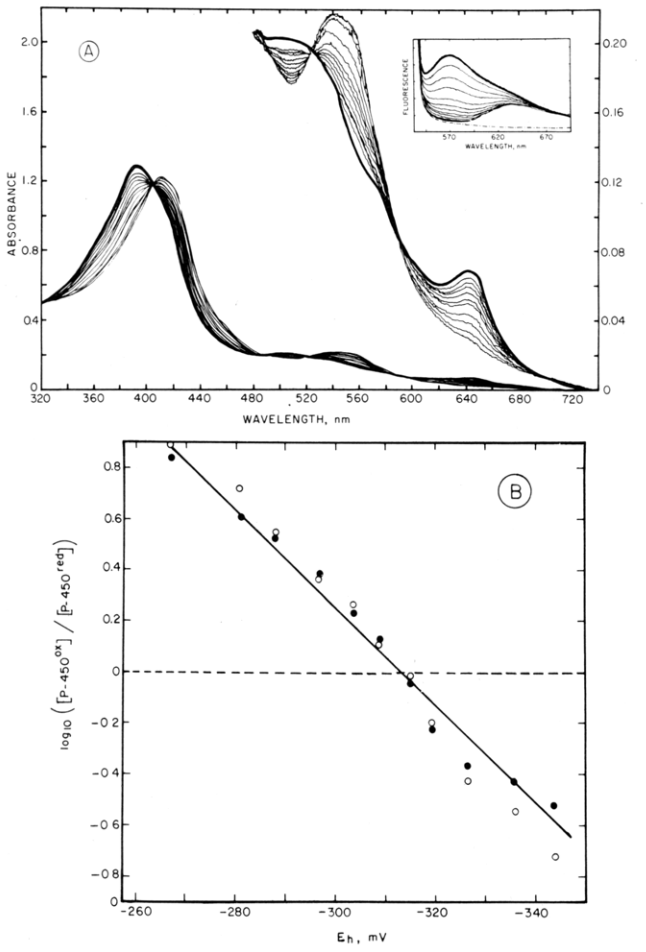

FIGURE 2: Estimation of $E_{m,7}$ of P-450$_{\beta NF/ISF\text{-}G}$. (A) Stepwise photochemical reduction of a mixture of safranine T and P-450$_{\beta NF/ISF\text{-}G}$. The cuvette contained P-450$_{\beta NF/ISF\text{-}G}$ (8.65 μM), potassium phosphate (0.1 M, pH 7.0), di-12:0-GPC (75 μM), EDTA (10 mM), proflavin sulfate (1.0 μM), safranine T (0.5 μM), glucose oxidase (9 IU mL^{-1}), catalase (430 IU mL^{-1}), glucose (0.1 M), and glycerol (5%, v/v) under argon gas. Initial absorbance and fluorescence (inset) measurements are shown with a heavy solid line. Subsequent spectra following stepwise illumination are shown with lighter lines. The fluorescence spectrum following reduction of the sample with solid $Na_2S_2O_4$ is also shown in the inset (●). (B) The data from part A are plotted in the form of the Nernst equation by using concentrations of oxidized and reduced P-450 calculated from measurements made at 390 (○) and 420 nm (●).

termined individual $E_{m,7}$ values of −171, −260, and −281 for these three steps in the purified flavoprotein which had been treated to remove a small hydrophobic peptide. The data collected here probably represent the aggregate of the three individual steps. During the reduction of the P-450, the blue absorbance of the flavin semiquinone remained rather constant and decreased with further reduction. The A_{595} changes were used to calculate an $E_{m,7}$ of −337 mV (n = 0.99) for this step, in comparison to the −371 mV reported by Iyanagi et al. (1974). These results are consonant with the parameters obtained by Iyanagi et al. (1974) under more precise conditions, in the absence of P-450.

Mild heat treatment of P-450$_{PB\text{-}B}$ completely converted the enzyme to cytochrome P-420, as judged by the absorption spectrum of the ferrous–carbonyl complex. This preparation yielded an $E_{m,7}$ of −164 mV, considerably more positive than that of the native P-450$_{PB\text{-}B}$.

P-450$_{\beta NF/ISF\text{-}G}$ was an enzyme of special interest in these studies, as this P-450 is typically isolated predominantly in the high-spin ferric state. This particular preparation contained 67% high-spin iron and 33% low-spin iron as judged by second-derivative spectroscopy (see above). The $E_{m,7}$ of this preparation was −304 mV. The $E_{m,7}$ was essentially identical in the presence of di-12:0-GPC. The spectra obtained in this titration are presented in Figure 2. Nearly all of the spectral traces passed through the clear isosbestic points at 587, 533, 498, and 404 nm. Calculation of $E_{m,7}$ using the spectral changes at four different wavelengths gave values that differed by no more than 7 mV. An $E_{m,7}$ value of −310 mV was found when the same system was gradually oxidized with oxygen and the intermediate traces were used for calculations. The addition of 0.22 M butanol to P-450$_{\beta NF/ISF\text{-}G}$ resulted in conversion to a form in which the iron was 89% low spin, consonant with the spectral changes described in a rabbit liver P-450 by White & Coon (1982). The $E_{m,7}$ of the butanol-treated enzyme was −297 mV, not significantly different than in the case of the predominantly high-spin form.

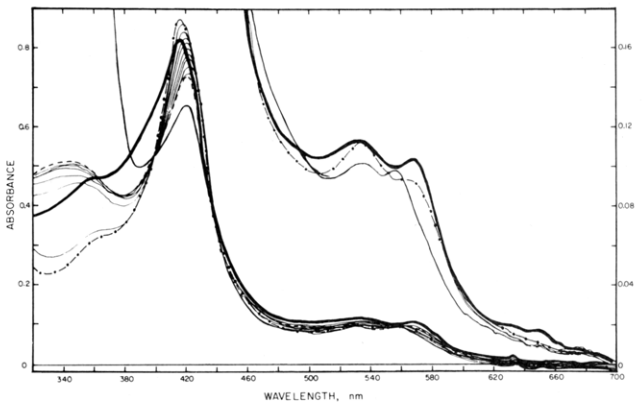

FIGURE 3: Steady-state spectra of P-450$_{PB\text{-}B}$ during catalytic turnover in the presence of cyclohexane. A solution of P-450$_{PB\text{-}B}$ (9.1 μM), NADPH–P-450 reductase (5.7 μM), di-12:0-GPC (0.28 mM), Tris-HCl (pH 7.6, 50 mM), glucose 6-phosphate (50 mM), glucose-6-phosphate dehydrogenase (1.5 IU mL^{-1}), catalase (5 μg mL^{-1}), cyclohexane (10 mM), EDTA (0.2 mM), and glycerol (7.5%, v/v) was placed in the sample cuvette, and a solution containing all components except P-450$_{PB\text{-}B}$ and NADPH–P-450 reductase was placed in the reference cuvette (total volume 200 μL). The spectrum was recorded at 25 °C (very heavy solid line). Two microliters of 10 mM NADPH was added to each cuvette, and the spectrum was recorded immediately (●). Subsequent spectra, recorded every 130 s, are also shown (—). The last of the series of consecutively recorded spectra is shown as a dashed line (---), and the fully reduced (solid $Na_2S_2O_4$) spectrum is also presented (heavy solid line).

Steady-State Spectral Investigation of P-450 during Catalytic Turnover. Absolute and various difference spectra have been reported for ferrous P-450/oxygen complexes with purified rabbit liver P-450s (Guengerich et al., 1976; Bonfils et al., 1981), rat liver microsomes (Estabrook et al., 1971), and a bacterial P-450 (Ishimura et al., 1971; Tyson et al., 1972). The rat liver P-450$_{PB\text{-}B}$ formed what appeared to be a ferrous/oxygen complex (or product thereof) during steady-state turnover in the presence of NADPH, NADPH–P-450 reductase, cyclohexane, and O_2 (Figure 3). As the reaction proceeded, the level of O_2 in the cuvette was depleted, and the spectra shifted toward that of the ferrous form, which was verified later by the addition of $Na_2S_2O_4$.

The absolute spectrum of the oxygenated ferrous complex resembled that of the rabbit P-450$_{LM\text{-}2}$ complex. The absorbance of the complex appeared to be greater than that of the low-spin ferric P-450, in apparent contrast to other cases (Ishimura et al., 1971; Tyson et al., 1972; Bonfils et al., 1981). This observation can be explained by the partial conversion of high-spin P-450 (λ_{max} 392 nm) to the ferrous oxygenated complex (λ_{max} 418 nm) in the presence of ferric low-spin P-450 (λ_{max} 417 nm). Unsuccessful attempts were also made to

Table II: Oxidation-Reduction Potentials of Ferric/Ferrous Couples of Cytochromes P-450[a]

P-450 isozyme	additions (concn)	$E_{m,7}$ (mV)
P-450$_{PB-B}$	none	−318, −323, −323, −313, −316[b]
	di-12:0-GPC (75 μM)	−311
	di-12:0-GPC (75 μM), *d*-benzphetamine (1 mM)	−320
	di-12:0-GPC (75 μM), *d*-benzphetamine (6 mM)	−306
	di-12:0-GPC (75 μM), (*R*)-warfarin (0.3 mM)	−301
	di-12:0-GPC (75 μM), cyclohexane (20 mM)	−300,[c] −286[d]
	Lubrol PX, 1% (w/v)	−305
	cytochrome b_5 (equimolar)	−324
	NADPH-P-450 reductase (equimolar)	−322
	conversion to cytochrome P-420[e]	−164
P-450$_{UT-A}$	di-12:0-GPC (75 μM)	−321
	di-12:0-GPC (75 μM), ethylmorphine (30 mM)	−307
P-450$_{\beta NF-B}$	di-12:0-GPC (75 μM)	−299
P-450$_{PB-C}$	di-12:0-GPC (75 μM)	−336
	di-12:0-GPC (75 μM), ethylmorphine (30 mM)	−346
P-450$_{PB-D}$	di-12:0-GPC (75 μM)	−313
	di-12:0-GPC (75 μM), *d*-benzphetamine (5 mM)	−289
P-450$_{PB/PCN-E}$	di-12:0-GPC (75 μM)	−350
	di-12:0-GPC (75 μM), (*R*)-warfarin (0.3 mM)	−346
P-450$_{UT-F}$	di-12:0-GPC (75 μM)	−327
	di-12:0-GPC (75 μM), testosterone (0.1 mM)	−294
P-450$_{\beta NF/ISF-G}$	none	−304
	none (reoxidation)	−310
	di-12:0-GPC (75 μM)	−306,[f] −308,[g] −312,[h] −313[i]
	1-butanol (0.22 M)	−297
phenobarbital-treated rat liver microsomes	none	−362[j]

[a] $E_{m,7}$ values were estimated by using the general methods described under Experimental Procedures. The value of n ranged from 0.85 to 1.22, and the correlation coefficient r^2 for the plot of log ([P-450^{ox}]/[P-450^{red}]) vs. E_h ranged from 0.93 to 0.999 unless otherwise indicated. Data presented for each condition are from a single experiment unless indicated otherwise. [b] The five values were determined in separate experiments. The mean of the values is −319 mV, and the SD is 4 mV. [c] Data calculated from ΔA_{414} measurements. [d] Data calculated from ΔA_{393} measurements. [e] Prepared by heating at 56 °C for 5 min. In this determination, indigodisulfonate ($E_{m,7}$ = −116 mV; Clark, 1960) and phenosafranine ($E_{m,7}$ = −252 mV; Clark 1960) were also used as indicators. [f] Data calculated from ΔA_{646} measurements. [g] Data calculated from ΔA_{554} measurements. [h] Data calculated from ΔA_{392} measurements. [i] Data calculated from ΔA_{420} measurements. [j] n = 0.78, and r^2 = 0.88. Safranine T reduction was monitored spectrophotometrically.

observe ferrous/oxygen complexes in the cases of other P-450s and substrates. P-450$_{PB-B}$ metabolized *d*-benzphetamine to a product which bound ferrous P-450 and formed a spectral complex at 455 nm (Werringloer & Estabrook, 1973); formation of this interfering species was already evident in the first spectral traces recorded. P-450$_{\beta NF/ISF-G}$ did not form a discernible spectral complex. P-450$_{\beta NF-B}$, in the presence of acetanilide, appeared to remain largely in the ferric state during turnover. Advantage was taken of this observation to examine the spectrum of NADPH–P-450 reductase during catalytic turnover (see below).

The extinction coefficient for the band at 646 nm (4.9 mM^{-1} cm^{-1}) was used in conjunction with the steady-state spectral data to estimate the fraction of the high-spin ferric P-450 that was reduced to the ferrous state. Sample and reference cuvettes both contained P-450, NADPH–P-450 reductase, di-12:0-GPC, phosphate buffer, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase. NADPH was added to the sample cuvette in each case, and difference spectra were recorded. When significant portions of high-spin iron were present in the ferric state, a sizable fraction of the high-spin ferric iron was reduced, but the data suggest that reduction was not always complete (Table I).

The changes in the Soret region of the spectra were also measured under these conditions (cf. Figure 3). Under the conditions used (see Table I), only minor changes were observed with P-450$_{\beta NF-B}$, P-450$_{PB-D}$, and P-450$_{PB/PCN-E}$. Substantial changes were observed with the other P-450s, but quantitative estimates are not presented due to uncertainty in assignment of spectra of all potential catalytic intermediates.

Oxidation–Reduction Potential of Oxygenated Ferrous P-450. In order to estimate an $E_{m,7}$ value for reduction of the ferrous/oxygen complex of P-450, the abilities of proteins and dyes of known $E_{m,7}$ to donate electrons to this complex were examined. Cytochrome b_5 (accepted $E_{m,7}$ = +20 mV) (Velick & Strittmatter, 1956; Weber et al., 1971) and each of the dyes methylene blue ($E_{m,7}$ = +9 mV), thionine ($E_{m,7}$ = +59 mV), and dichlorophenolindophenol ($E_{m,7}$ = +217 mV) (Clark, 1960) were used in individual experiments. All of these reduced compounds are readily autoxidizable, however, with the exception of dichlorophenolindophenol. Therefore, each of the compounds was reduced photochemically under anaerobic conditions and oxygenated at 4 °C. The kinetics of oxidation were determined. P-450$_{PB-B}$, in the presence of cyclohexane, was photochemically reduced along with each of the potential electron donors, and the rate of reoxidation of each of the dyes or cytochrome b_5 was followed kinetically at 4 °C after oxygenation. Thus, ferrous P-450$_{PB-B}$ bound O_2, and an increased rate of reoxidation of cytochrome b_5 or a dye under such conditions is believed to represent transfer of an electron to oxygenated ferrous P-450$_{PB-B}$. Catalase and superoxide dismutase were included in these experiments to rule out oxidation of cytochrome b_5 and the dyes by H_2O_2 or superoxide anion.

The results are shown in Figure 4. Oxidation of cytochrome b_5 and methylene blue was markedly stimulated by the presence of oxygenated ferrous P-450. The oxidation of thionine was enhanced only slightly. When the experiment was carried out in the presence of reduced dichlorophenolindophenol, less than 5% of the dye was oxidized. On the basis of these results, the effective $E_{m,7}$ for the couple involving transfer of an additional electron to a ferrous P-450/O_2 complex is estimated to be about +50 mV. This estimate assumes that the electron transfers are kinetically efficient in all cases and that abortive decomposition of the oxygenated ferrous

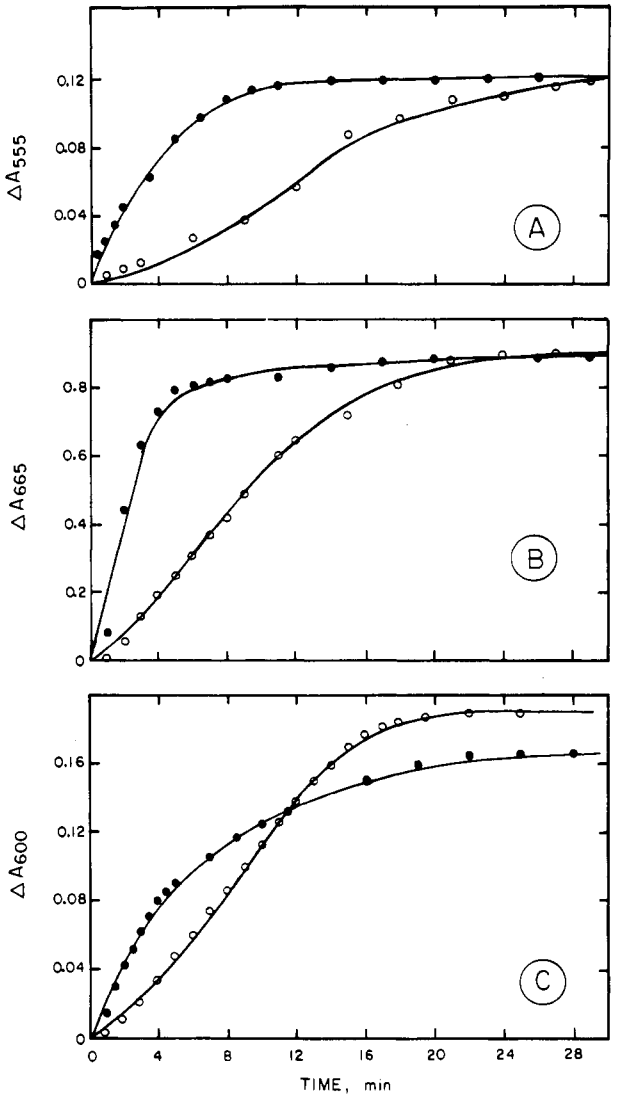

FIGURE 4: Effect of P-450 on kinetics of oxidation of reduced cytochrome b_5, methylene blue, and thionine. In each case, a solution of 15 μM cytochrome b_5 (A), methylene blue (B), or thionine (C) was photochemically reduced in the absence (O) or presence (●) of an equimolar concentration of P-450$_{PB-B}$ in a 3.0 mL volume of 0.1 M potassium phosphate buffer (pH 7.0) containing di-12:0-GPC (50 μM), proflavin (1.0 μM), EDTA (10 mM), glycerol [15% (v/v)], benzyl viologen (0.1 μM), catalase (1300 IU mL^{-1}), and superoxide dismutase (52 IU mL^{-1}). In parts B and C, other additions included 0.2 μM riboflavin, 0.1 μM safranine T, 0.2 μM thionine (part B only), and 0.2 μM methylene blue (part C only) to enhance electron transfer. After photoreduction (monitored spectrophotometrically), the cuvette was cooled to 4 °C, and 2 mL of air was bubbled through the opened cuvette while the cuvette was stirred in the spectrophotometer. Repetitive spectra were recorded within 15 s after oxygenation, and stirring was continued.

P-450 complex is not so extensive as to compete under these conditions. The similarity of the electron-transfer properties of methylene blue and thionine (Clark, 1960) is sufficient enough to argue that strictly kinetic differences in the behavior of these dyes are small.

Oxidation–Reduction Properties of NADPH–P-450 Reductase. The question of which electronic forms of NADPH–P-450 reductase participate in the P-450 catalytic cycle has been raised before and partially addressed by others (Iyanagi et al., 1978; Vermilion et al., 1981). Experiments are difficult to perform because the absorption spectrum of the P-450 heme tends to obscure that of the flavin. When the spectral state of P-450$_{\beta NF-B}$ was examined during aerobic turnover in the presence of acetanilide, the changes in the

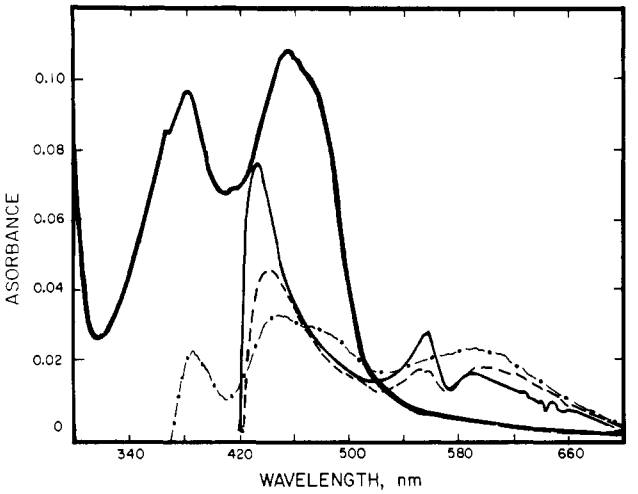

FIGURE 5: Spectra of NADPH–P-450 reductase obtained under steady-state conditions during catalytic turnover. A solution containing P-450$_{\beta NF-B}$ (11.9 μM), di-12:0-GPC (38 μM), Tris-HCl buffer (pH 7.6, 67 mM), glucose-6-phosphate dehydrogenase (1 IU mL^{-1}), catalase (3 μg mL^{-1}), and acetanilide (6.7 mM) was divided into two microcuvettes (150 μL total volume each), and a base line was recorded at 25 °C. Forty-eight microliters of 10 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA and 20% (v/v) glycerol was added to the reference cuvette, and the same amount of buffer containing 23.6 μM NADPH–P-450 reductase was added to the sample cuvette (heavy solid line). Two microliters of 10 mM NADPH was added to both cuvettes, and spectra were recorded every 120 s. The first (●), second (- - -), and fifth (—) recorded spectra are shown.

spectrum of the heme were found to be quite small (see above). For this reason, the flavin spectra in this system were considered more closely.

Figure 5 shows the spectra obtained during aerobic turnover in this system. After the first one or two traces, the system became partially anaerobic, and ferrous P-450 began to accumulate. The A_{595}:A_{455} ratio in the first trace, during which the system is in an aerobic steady state, was 0.57. Comparison of this value to the data of Vermilion & Coon (1978b) shows correspondence to 74% reduction, i.e., input of three electrons. As the sample became more anaerobic, the absorbance in the 440-nm region increased, but the absorbance at 460 nm, which is more reflective of the flavins as opposed to heme, was constant.

Other studies also indicated that NADPH–P-450 reductase, when mixed with NADPH and O_2 in the absence of P-450, was reduced to a three-electron-containing species (A_{595}:A_{455} = 0.59). This state has also been shown by others to predominate when NADPH is added to NADPH–P-450 reductase under anaerobic conditions (Iyanagi & Mason, 1973; Vermilion & Coon, 1978a). If an NADPH-generating system was used, this state of the flavoprotein was maintained (in the presence of O_2). When the system was allowed to reoxidize, the one-electron-reduced state was reached (A_{595}:A_{455} = 0.33; cf. Iyanagi & Mason, 1973; Vermilion & Coon, 1978a). Since the same electronic state predominated in reactions including and excluding P-450, the rates of NADPH–P-450 reductase turnover were determined in the absence and presence of P-450s to verify that coupling actually occurred. At 25 °C, the reductase oxidized NADPH at a rate of 1.8 min^{-1} in the absence of P-450. When a 1.2-fold excess of P-450$_{\beta NF-B}$ was added, the rate increased to 6.8 min^{-1} and then to 12 min^{-1} when 2 mM acetanilide was also added. If one assumes that the P-450s are not stimulating the direct reaction of the flavoprotein with O_2, then the results indicate that the reductase is tightly coupled to the P-450 under the conditions in which the steady-state spectra were obtained. Thus, the three-

electron-reduced form of NADPH–P-450 reductase appears to be the predominant electronic species which accumulates during the steady-state reduction of P-450 or O_2.

Discussion

The $E_{m,7}$ values for the ferric/ferrous couples of eight purified rat liver P-450s were determined under a variety of conditions (Table II). Most of the P-450s, in the presence of phospholipid and the absence of substrate, had $E_{m,7}$ values in the range −300 to −320 mV. P-$450_{PB\text{-}C}$ and P-$450_{PB/PCN\text{-}E}$ had $E_{m,7}$ values in the range of −340 to −350 mV. Cytochrome P-420 formation, involving perturbation of the sulfhydryl ligand of the P-450 heme (Ullrich, 1979), raised the apparent $E_{m,7}$ significantly. The apparent iron spin state was not correlated with substrate binding, and the $E_{m,7}$ for the oxidation–reduction couple was not correlated with either substate binding or the P-450 spin state. The maximum change in $E_{m,7}$ observed in the presence of a substrate was +33 mV (P-$450_{UT\text{-}F}$ with testosterone). Since the higher $E_{m,7}$ was observed under conditions in which 50% of the iron was high spin, an increase of no more than +66 mV in $E_{m,7}$ might be expected to accompany a complete low- to high-spin iron transition, if this result is representative. In the case of P-$450_{\beta NF/ISF\text{-}G}$, varying the high-spin iron fraction from 11 to 67% did not produce a significant change in $E_{m,7}$ (Table II and Figure 2). The significance of differences in $E_{m,7}$ values of P-450s depends upon the effective $E_{m,7}$ of the system which drives the mixed-function oxidases. The data presented here need to be considered with caveats about comparison to systems involving microsomal membranes, in which changes in spin state are often more pronounced (Peterson, 1980). Nevertheless, the systems considered here have been found to metabolize substrates efficiently (Table I), and dramatic changes in spin state were found in some cases, without correlation to changes in $E_{m,7}$ for ferric/ferrous P-450 couples.

The $E_{m,7}$ data are in accord with values reported by others for liver microsomal P-450s in microsomes and purified preparations (Guengerich et al., 1975; Sligar et al., 1979; Waterman & Mason, 1972). In a bacterial P-450, binding of the substrate camphor is reported to be accompanied by a nearly complete low- to high-spin iron transition and an increase in $E_{m,7}$ of −303 to −173 mV (Sligar et al., 1979). The correlation of substrate binding, low- to high-spin state transition, and large positive change in $E_{m,7}$ for the ferric/ferrous couple may be unique to this bacterial P-450. Binding of the substrate *d*-benzphetamine to purified rabbit liver P-$450_{LM\text{-}2}$ did not significantly affect the $E_{m,7}$ (Guengerich et al., 1975) under conditions in which the substrate was shown to be bound stoichiometrically (Coon et al., 1976). If the binding of CO to ferrous P-450 is independent of *d*-benzphetamine binding, the results of Werringloer & Kawano (1980) indicate that binding of *d*-benzphetamine raised the $E_{m,7}$ of P-450 in rat liver microsomes by +30 mV. Sies & Kandel (1970) showed that the apparent $E_{m,7}$ of P-450 in perfused rat liver, as estimated from NADPH/$NADP^+$ equilibria, was actually lowered by hexobarbital or aminopyrine. Light & Orme-Johnson (1981) found that a bovine adrenal mitochondrial P-450 existed as a mixture of low- and high-spin forms under conditions where binding of substrates was stoichiometric and that $E_{m,7}$ was a function of which substrate was bound, but not the spin state. On the other hand, Sligar et al. (1979) used techniques similar to those described here and reported that the $E_{m,7}$ of a partially purified rat liver P-450 (which cannot be equated with any of the forms considered here) was changed from −300 mV in the absence of substrate (10% high-spin P-450 iron) to −237 mV in the presence of benzphetamine (38% high-spin P-450 iron) or −225 mV in the presence of hexobarbital (35% high-spin P-450 iron). (The ability of the P-450 preparation to metabolize these substrates was not considered.) However, these results were calculated with the assumption that safranine T is a one-electron instead of a two-electron donor (Clark, 1960). The corrected values for the unbound and benzphetamine- and hexobarbital-bound P-450s are −294, −257, and −263 mV, respectively. Thus, the maximum increase in $E_{m,7}$ observed in the presence of substrate in that study was about +37 mV.

An $E_{m,7}$ of about +50 mV was estimated for the reduction of an oxygenated ferrous P-$450_{PB\text{-}B}$/cyclohexane complex by using the relative abilities of materials of known $E_{m,7}$ to transfer electrons to this complex (Figure 4). The kinetics observed for the reoxidation of P-$450_{PB\text{-}B}$ and cytochrome b_5 at 4 °C are consonant with the results of Bonfils et al. (1981) (which were obtained with rabbit liver enzymes in micellar systems), or at least with the large slow phases. The decomposition of the oxygenated ferrous P-450 complex probably prevented complete electron transfer, although the complex appeared to be involved in oxidation of at least half of the reduced cytochrome b_5 or methylene blue (Figure 4). The similarity of methylene blue and thionine (Clark, 1960) suggests that the effects on the oxidation of these two dyes can be attributed to differences in thermodynamic properties, as opposed to kinetic differences. Werringloer & Kawano (1980) reported an effective $E_{m,7}$ of +25 mV for a P-450/*d*-benzphetamine/CO complex in rat liver microsomes and reasoned that this was a useful model for the oxygenated ferrous complex. This view assumes that the P-450s under consideration have similar affinities for CO and O_2, but the estimate is consistent with that reported here. The results presented here suggest that transfer of electrons from cytochrome b_5 to the oxygenated ferrous P-450 complex can occur, on thermodynamic grounds, and may help in the understanding of the role of cytochrome b_5 in P-450-mediated reactions (Bonfils et al., 1981; Imai, 1981; Noshiro et al., 1980, 1981; Sugiyama et al., 1980; Werringloer & Kawano, 1980). The "oxygenated ferrous P-450 complex" referred to here may not necessarily be the Fe^{2+}–O_2 species but a resonance form or derivative (Ullrich, 1979). However, the complex is probably not the final formal perferryl oxenoid species ($Fe^V = O$) presumed to be involved in electron (hydrogen atom) abstraction (Groves et al., 1981), as the potential for oxidation is probably still too low. For instance, the potentials for one-electron oxidations of sulfides and amines are approximately +1 V (Watanabe et al., 1980; Shono et al., 1982).

Direct observation of spectral states of NADPH–P-450 reductase during reduction of P-450 is difficult because of the high extinction coefficient of heme relative to that of flavin. A system was used here where the heme spectra changed very little during steady-state catalytic turnover; i.e., P-$450_{\beta NF\text{-}B}$ appeared to accumulate primarily in the ferric form during catalysis of acetanilide hydroxylation, as well as with (*R*)-warfarin or in the absence of substrates (data not presented). Under these conditions, the three-electron-reduced form of NADPH–P-450 reductase also appeared to accumulate, as judged by the ratio of semiquinone to near-UV absorbance (Figure 5) (Iyanagi et al., 1974; Vermilion & Coon, 1978a). Since both ferric P-$450_{\beta NF\text{-}B}$ and the three-electron-reduced form of NADPH–P-450 reductase accumulated under these conditions, the initial electron transfer appears to be rate limiting and involves reduction of ferric iron by three-electron-reduced NADPH–P-450 reductase. Iyanagi et al. (1974) reported that NADPH–P-450 reductase transfers electrons

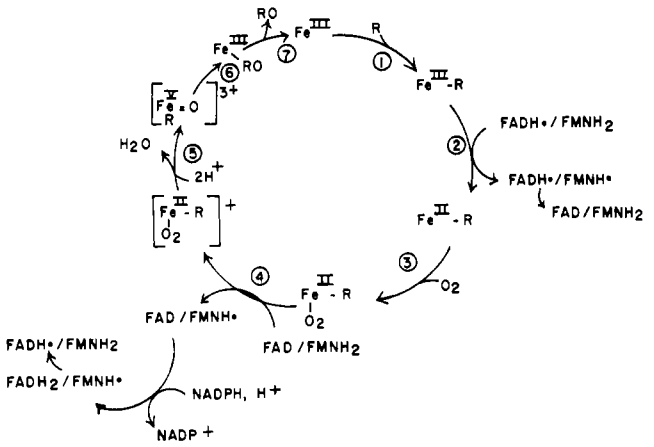

FIGURE 6: Working scheme for the catalytic mechanism of P-450. See the text for discussion.

in four one-electron steps, which have $E_{m,7}$ values of −110, −270, −290, and −365 mV. Coon and his associates have provided evidence to support the hypothesis that electrons are transferred via the pathway NADPH → FAD → FMN → P-450, with FAD serving as the low-potential flavin and FMN serving as the high-potential flavin (Vermilion et al., 1981). Further, they have recently ascribed the individual $E_{m,7}$ values of −110 mV to the FMN/FMNH· couple, −270 mV to the FMNH·/FMNH$_2$ couple, −290 mV to the FAD/FADH· couple, and −365 mV to the FADH·/FADH$_2$ couple (Oprian & Coon, 1982). Thus, a reasonable hypothesis would involve oxidation of the FMNH$_2$/FADH· to the FMNH·/FADH· form of the reductase by ferric P-450, with equilibration to FMNH$_2$/FAD. The FMNH$_2$/FAD form could donate another electron to the oxygenated ferrous P-450 complex to form the FMNH·/FAD species and then be reduced to the FADH$_2$/FMNH· species by NADPH in a two-electron process. The FADH$_2$/FMNH· could undergo internal electron transfer to regenerate FADH·/FMNH$_2$ and reinitiate the cycle (Figure 6).

A working scheme for the catalytic action of P-450 is presented in Figure 6. Step 1, binding of the substrate, is exergonic, with a release of 4–9 kcal mol^{-1} (based on K_D values of 10^{-3}–10^{-6} M), but the binding energy is not stored in the form of a spin-state transition. The overall free energy involved in going from low-spin ferric iron to the diamagnetic ferrous/oxygen complex should be independent of the route taken; i.e., the existence of spin transitions along the pathway should not affect this net energy. The reduction of ferric iron (step 2) requires 6–8 kcal mol^{-1} ($E_{m,7}$ of −350 to −280 mV), provided through the driving force of the NADPH/NADP$^+$ couple. If a K_D of 10^{-6} M is assumed for binding of O_2 to ferrous P-450 (step 3) (Ishimura et al., 1971; Ullrich, 1979), such binding results in a gain of 8–9 kcal mol^{-1}. The addition of a second electron to this system (step 4) appears to be thermodynamically favorable ($E_{m,7}$ = +50 mV, −1 kcal mol^{-1}). Little is known about step 5, in which the dioxygen bridge is broken and protonation of the divalent oxygen atom occurs, although the process raises the energy of the system to the point preceding step 6, in which electron (or hydrogen atom) abstraction and oxygen rebound occur. The final step (7) requires energy for product release, probably slightly less than the energy gained by substrate binding (4–8 kcal mol^{-1}).

Although the approaches used in this work have been addressed almost exclusively toward thermodynamic parameters, some discussion of kinetics is appropriate. On the basis of the literature available, steps 1, 3, and 7 (Figure 6) are probably all relatively rapid (White & Coon, 1980 and references therein). Step 2 is rapid enough not to be rate limiting under some conditions (Iyanagi et al., 1978), but in other situations this step could be limiting (see above). Step 4 could be limiting in some cases, as suggested by the enhancement of some hydroxylation rates observed with the addition of cytochrome b_5 (Imai, 1981; Sugiyama et al., 1980). Very little is known about step 5. Step 6 can also be rate limiting in some cases; e.g., kinetic deuterium isotope effects can be observed in some situations (Miwa et al., 1980), and Watanabe et al. (1980) have shown that the V_{max} for sulfide oxidation can be correlated with the ease of removal of electrons. Thus, steps 2, 4, and 6 can all probably be rate limiting under different circumstances.

What, then, determines the rates, regioselectivity, and substrate specificity of P-450-catalyzed reactions? The results presented here suggest that neither spin state nor the kinetics of ferric P-450 reduction are sufficient to explain the observed differences observed under various conditions. Three factors are hypothesized to interact to control rates as well as regioselective patterns, within each isozymic form of P-450 under consideration. First, different substrates probably exert some control of rates of electron transfer (steps 2 and 4), although the basis is believed to be primarily kinetic and not thermodynamic (see above). Second, physical factors related to enzyme/substrate topography control the juxtaposition of substrates within the active site, at least in the case of large substrates, to influence both the regioselectivity of hydroxylation and the rates of hydroxylation (within step 6) by positioning of atoms in relationship to the activated iron/oxygen complex. Finally, the potentials of oxidizable atoms for electron transfer and oxygen rebound (in step 6) play a role in the ease of these steps. Further investigations with P-450s and appropriate chemical models will be required to elucidate the details of these processes.

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Registry No. P-450, 9035-51-2; di-12:0-GPC, 18194-25-7; (*R*)-warfarin, 5543-58-8; ethylmorphine, 76-58-4; testosterone, 58-22-0; SKF-525A, 62-68-0; aminopyrine, 58-15-1; cyclohexane, 110-82-7; *d*-benzphetamine, 156-08-1; acetanilide, 103-84-4; NADPH–cytochrome P-450 reductase, 9039-06-9; monooxygenase, 9038-14-6.

References

Bonfils, C., Balny, C., & Maurel, P. (1981) *J. Biol. Chem. 256*, 9457–9465.

Burleigh, B. D., Jr., Foust, G. P., & Williams, C. H., Jr. (1969) *Anal. Biochem. 27*, 536–544.

Clark, W. M. (1960) *Oxidation-Reduction Potentials of Organic Systems*, Williams and Wilkins, Baltimore, MD.

Coon, M. J., Haugen, D. A., Guengerich, F. P., Vermilion, J. L., & Dean, W. L. (1976) in *The Structural Basis of Membrane Function* (Hatefi, Y., & Djavadi-Ohaniance, L., Eds.) pp 409–427, Academic Press, New York.

Estabrook, R. W., Hildebrandt, A. G., Baron, J., Netter, K. J., & Leibman, K. (1971) *Biochem. Biophys. Res. Commun. 42*, 132–139.

Fisher, G. J., Fukushima, H., & Gaylor, J. L. (1981) *J. Biol. Chem. 256*, 4388–4394.

Groves, J. T., Haushalter, R. C., Nakamura, H., Nemo, T. E., & Evans, B. J. (1981) *J. Am. Chem. Soc. 103*, 2884–2886.

Guengerich, F. P., & Martin, M. V. (1980) *Arch. Biochem. Biophys. 205*, 365–379.

Guengerich, F. P., Ballou, D. P., & Coon, M. J. (1975) *J. Biol. Chem. 250*, 7405–7414.

Guengerich, F. P., Ballou, D. P., & Coon, M. J. (1976) *Biochem. Biophys. Res. Commun. 70*, 951–956.
Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., & Kaminsky, L. S. (1982) *Biochemistry 21*, 6019–6030.
Imai, Y. (1981) *J. Biochem. (Tokyo) 89*, 351–362.
Ishimura, Y., Ullrich, V., & Peterson, J. A. (1971) *Biochem. Biophys. Res. Commun. 42*, 140–146.
Iyanagi, T., & Mason, H. S. (1973) *Biochemistry 12*, 2297–2308.
Iyanagi, T., Makino, N., & Mason, H. S. (1974) *Biochemistry 13*, 1701–1710.
Iyanagi, T., Anan, F. K., Imai, Y., & Mason, H. S. (1978) *Biochemistry 17*, 2224–2230.
Light, D. R., & Orme-Johnson, N. R. (1981) *J. Biol. Chem. 256*, 343–350.
Macdonald, T. L., Burka, L. T., Wright, S. T., & Guengerich, F. P. (1982) *Biochem. Biophys. Res. Commun. 104*, 620–625.
Mannering, G. J. (1972) in *Fundamentals of Drug Metabolism and Disposition* (LaDu, B. N., Mandel, H. G., & Way, E. L., Eds.) pp 206–252, Williams and Wilkins, Baltimore, MD.
Miwa, G. T., Garland, W. A., Hodshon, B. J., Lu, A. Y. H., & Northrop, D. B. (1980) *J. Biol. Chem. 255*, 6049–6054.
Noshiro, M., Harada, N., & Omura, T. (1980) *J. Biochem. (Tokyo) 88*, 1521–1535.
Noshiro, M., Ullrich, V., & Omura, T. (1981) *Eur. J. Biochem. 116*, 521–526.
O'Haver, T. C., & Green, G. L. (1976) *Anal. Chem. 48*, 312–318.
Oprian, D. D., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 8935–8944.
Peterson, J. A. (1980) in *Microsomes, Drug Oxidations, and Chemical Carcinogenesis* (Coon, M. J., Conney, A. H., Estabrook, R. W., Gelboin, H. V., Gillette, J. R., & O'Brien, P. J., Eds.) pp 455–467, Academic Press, New York.
Ryan, D. E., Thomas, P. E., & Levin, W. (1980) *J. Biol. Chem. 255*, 7941–7955.
Shono, T., Toda, T., & Oshino, N. (1982) *J. Am. Chem. Soc. 104*, 2639–2641.
Sies, H., & Kandel, M. (1970) *FEBS Lett. 9*, 205–208.
Sligar, S. G., Cinti, D. L., Gibson, G. G., & Schenkman, J. B. (1979) *Biochem. Biophys. Res. Commun. 90*, 925–932.
Sugiyama, T., Miki, N., & Yamano, T. (1980) *J. Biochem. (Tokyo) 87*, 1457–1467.
Tyson, C. A., Lipscomb, J. D., & Gunsalus, I. C. (1972) *J. Biol. Chem. 247*, 5777–5784.
Ullrich, V. (1979) *Top. Curr. Chem. 83*, 67–104.
Velick, S. F., & Strittmatter, P. (1956) *J. Biol. Chem. 221*, 265–275.
Vermilion, J. L., & Coon, M. J. (1978a) *J. Biol. Chem. 253*, 2694–2704.
Vermilion, J. L., & Coon, M. J. (1978b) *J. Biol. Chem. 253*, 8812–8819.
Vermilion, J. L., Ballou, D. P., Massey, V., & Coon, M. J. (1981) *J. Biol. Chem. 256*, 266–277.
Watanabe, Y., Iyanagi, T., & Oae, S. (1980) *Tetrahedron Lett. 21*, 3685–3688.
Waterman, M. R., & Mason, H. S. (1972) *Arch. Biochem. Biophys. 150*, 57–63.
Weber, H., Weis, W., & Staudinger, H. (1971) *Z. Physiol. Chem. 352*, 109–110.
Werringloer, J., & Estabrook, R. W. (1973) *Life Sci. 13*, 1319–1330.
Werringloer, J., & Kawano, S. (1980) in *Microsomes, Drug Oxidations, and Chemical Carcinogenesis* (Coon, M. J., Conney, A. H., Estabrook, R. W., Gelboin, H. V., Gillette, J. R., & O'Brien, P. J., Eds.) pp 469–478, Academic Press, New York.
Werringloer, J., & Kawano, S. (1981) in *Biochemistry, Biophysics, and Regulation of Cytochrome P-450* (Gustafsson, J.-A., Carlstedt-Duke, J., Mode, A., & Rafter, J., Eds.) pp 359–362, Elsevier/North-Holland, New York.
White, R. E., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315–356.
White, R. E., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 3073–3083.