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Kinetics of Cytochrome P-450 Reduction: Evidence for Faster Reduction of the High-Spin Ferric State†

Wayne L. Backes,‡,§ Paul P. Tamburini,‡ Ingela Jansson,‡ G. Gordon Gibson,‖ Stephen G. Sligar,⊥ and John B. Schenkman*,‡

Department of Pharmacology, University of Connecticut Health Center, Farmington, Connecticut 06032, Department of Biochemistry, Division of Pharmacology and Toxicology, University of Surrey, Guildford, Surrey, GU2 5XH England, and Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

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ABSTRACT: Results are presented that support our hypothesis [Backes, W. L., Sligar, S. G., & Schenkman, J. B. (1980) *Biochem. Biophys. Res. Commun. 97*, 860–867] that the multiphasic reduction kinetics of cytochrome P-450 are, in part, due to the spin equilibrium of the ferric hemoprotein. The disappearance of the high-spin charge-transfer band at 650 nm during reduction of the hemoprotein by NADPH was fast, exhibiting a rate constant greater than that of the fast phase of reduction measured by formation of the carbon monoxide adduct. In contrast, the disappearance of the ferric low-spin form of the cytochrome was at a considerably slower rate. A mathematical expression of the fractional content of high-spin cytochrome P-450 was obtained by comparing the ratio of the initial rate of change in the fraction of total oxidized cytochrome remaining to the initial rate of change in the fraction of high-spin ferric P-450 remaining. Results supporting the model were obtained by using both microsomes and purified cytochrome P-450 RLM5. The calculation from experimental data yielded results that were similar to those obtained by different extrapolation methods used for estimation of the amount of high-spin cytochrome P-450, supporting further the proposed relationship between the spin equilibrium and the reduction kinetics of this hemoprotein.

The metabolism of substrates by hepatic microsomal cytochrome P-450 requires, in addition to substrate and dioxygen binding, two single electron transfers from NADPH–cytochrome P-450 reductase (White & Coon, 1980). The first electron input to cytochrome P-450 from NADPH has been studied by a number of groups and seen to be at least a biphasic process (Gigon et al., 1967; Peterson et al., 1976; Taniguchi et al., 1979; Oprian et al., 1979; Backes et al., 1980, 1982; Ruf, 1980; Blanck et al., 1983) when formation of carbon monoxy–ferrous cytochrome P-450 is monitored. Some of these investigators (Peterson et al., 1976; Taniguchi et al., 1979; Oprian et al., 1979) have attributed the multiphasic reduction kinetics to a functional interaction between the reductase and cytochrome P-450 or to a characteristic of the reductase, while other laboratories (Backes et al., 1980, 1982; Tamburini et al., 1984) have attributed the kinetics to be a consequence of the spin equilibrium between low-spin and high-spin ferric cytochrome P-450.

In previous reports (Backes et al., 1980, 1982) the apparent biphasic kinetics of reduction of hepatic microsomal cytochrome P-450 was shown to be consistent with a sequential model where high-spin cytochrome is initially reduced followed by a slower reduction of low-spin cytochrome subsequent to its conversion to the high-spin state. The kinetics of reduction would therefore be regulated, at least in part, by the preequilibrium between high-spin and low-spin states of the cytochrome. According to the hypothesis, a rapid depletion of the high-spin hemoprotein would be observed on addition of NADPH. This concept requires that a step(s) slower than the direct reduction of high-spin cytochrome exist in order to obtain the observed multiphasic plots. At least one of these steps (though, not necessarily the slow step itself) would be the interconversion between low-spin and high-spin states. Evidence supporting this hypothesis was shown in studies where the extent of reduction in the fast phase was compared to the prereduction levels of high-spin cytochrome P-450 with liver microsomes from untreated rats. A positive correlation was obtained, when the spin state was manipulated either by the addition of substrates or by varying the temperature of the reaction (Backes et al., 1980, 1982). These results demonstrated the relationship between a thermodynamic parameter (the prereduction level of high-spin ferric cytochrome) and the kinetics of reduction.

A general model describing the states of the cytochrome during reduction (Sligar, 1976) has been simplified by the assumption that low-spin cytochrome P-450 is not directly reduced (Sligar et al., 1980; Rein et al., 1979). Results supporting this assumption have been reported by Pierre et al.

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‡ University of Connecticut Health Center.

§ Present address: Department of Pharmacology, Louisiana State University Medical Center, New Orleans, LA 70112.

‖ University of Surrey.

⊥ University of Illinois.

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(1982) with cytochrome P-450$_{cam}$. The model has been further simplified by the assumption that the only effect of substrate is on the equilibrium between high-spin and low-spin ferric cytochrome:

$$Fe^{3+}_{ls} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Fe^{3+}_{hs} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} Fe^{2+}$$

where Fe^{3+} is used to designate the ferric hemoprotein and Fe^{2+} the ferrous states and the subscripts hs and ls represent high-spin and low-spin states, respectively. Data supportive of this assumption has been reported (Tamburini et al., 1984). In order to obtain biphasic kinetics with this model, the rate constants controlling the conversion from low-spin to the directly reducible high-spin state (k_1 and k_2) must be smaller than the rate constant of reduction of that high-spin species (k_3). The rate of formation of the reduced carbon monoxide complex is expressed by the rate equation

$$d[Fe^{2+}]/dt = k_3[Fe^{3+}_{hs}] - k_4[Fe^{2+}] \quad (1)$$

where $k_4[Fe^{2+}]$ has been assumed to be negligible. As also pointed out earlier, the changes in the high- and low-spin ferric states with time can be expressed as

$$d[Fe^{3+}_{ls}]/dt = k_2[Fe^{3+}_{hs}] - k_1[Fe^{3+}_{ls}] \quad (2)$$

$$d[Fe^{3+}_{hs}]/dt = k_1[Fe^{3+}_{ls}] + k_4[Fe^{2+}] - (k_2 + k_3)[Fe^{3+}_{hs}] \quad (3)$$

Cytochromes P-450 undergo substrate-induced type I spectral changes (Schenkman et al., 1967) characterized by a shift in Soret absorbance from 417 to 390 nm. These spectral changes have been correlated with a low- to high-spin change of the ferric hemoprotein (Whysner et al., 1970; Mitani & Horie, 1969; Kominami et al., 1979; Tsai et al., 1970). Concomitant with the increase in 390-nm absorbance is the appearance of a ferric absorption band at 650 nm. This latter absorption band has been attributed to a high-spin ferric charge-transfer transition (Brill & Williams, 1961; Brill, 1977; Werringloer et al., 1979).

The purpose of this study was to further test the sequential model described above. Evidence capable of discriminating between this model and one in which spin state is not involved in the reduction kinetics was obtained by comparing the kinetics of disappearance of high-spin ferric cytochrome with the kinetics of formation of the carbon monoxide bound ferrous cytochrome. Both microsomal cytochrome P-450 and purified cytochrome P-450 RLM5 reconstituted with NADPH–cytochrome P-450 reductase and dilauroylphosphatidylcholine (DLPC)[1] were utilized.

Materials and Methods

Male Sprague-Dawley CD rats (Charles River Breeding Laboratories) were maintained on laboratory chow (Purina) and water ad libitum. The rats (225–275 g) were decapitated, the livers were removed and perfused with cold 0.9% NaCl to remove blood, and microsomes were prepared by using the calcium aggregation method (Cinti et al., 1972) as described previously (Backes et al., 1982).

Cytochrome P-450 RLM5 was purified by using a modification of a previously described procedure (Cheng & Schenkman, 1982, Schenkman et al., 1982). After the 45 mM fraction from the carboxymethyl-Sepharose CL-6B column was pooled, the sample was dialyzed against 5 mM sodium phosphate–25% glycerol, pH 7.25 (buffer A). An hydroxylapatite column (1.5 × 7 cm) was equilibrated with buffer A, and up to 150 nmol of cytochrome P-450 was applied to the column. The column was washed with 2 column volumes of buffer A, and the cytochrome was eluted by using a linear sodium phosphate gradient. The initial buffer was 10 mM sodium phosphate, 25% glycerol, 0.5% Emulgen 911, 0.2% sodium cholate, 0.1 mM DTT, and 0.1 mM EDTA, pH 7.45, and the final buffer was 80 mM sodium phosphate, 25% glycerol, 0.5% Emulgen 911, 0.2% sodium cholate, 0.1 mM DTT, and 0.1 mM EDTA, pH 7.25. The flow rate of the column did not exceed 3 mL/h. Fractions containing cytochrome P-450 RLM5 were pooled (Cheng & Schenkman, 1982). The purified cytochrome was concentrated, and Emulgen 911 was removed by applying the protein to a 1.5 × 3 cm hydroxylapatite column after dialysis against buffer A. The column was washed with buffer A until the Emulgen 911 absorbance at 280 nm dropped to zero. The protein was then eluted with 300 mM potassium phosphate–25% glycerol, pH 7.25, and immediately dialyzed against 50 mM sodium phosphate–25% glycerol pH 7.25. The final preparation contained 15–17 nmol of cytochrome P-450 (mg of protein)$^{-1}$.

NADPH–cytochrome P-450 reductase was purified according to a modification of the method of Yasukochi & Masters (1976). The eluate from the 2′,5′-ADP–agarose affinity column contained both the native reductase and a lower molecular weight contaminant (M_r 68 000). The contaminant was removed by chromatography on a Sephadex G-100 column (1.5 × 60 cm).

Cytochrome P-450 RLM5 and the reductase were reconstituted with DLPC prepared by a modification of Enoch et al. (1977). DLPC (32 mM) was suspended in 50 mM sodium phosphate buffer, pH 7.25, containing 25% glycerol, 0.1 M sodium chloride, and 5 mM EDTA and sonicated by using a Branson bath sonicator (Shelton, CT) until clarification. This procedure forms vesicles of 250-Å diameter. The DLPC (4 μmol) was then incubated for 15 min at room temperature with 10 nmol of reductase and 10 nmol of cytochrome P-450 RLM5. The preparation was then mixed with the other assay components.

Cytochrome P-450 reduction was measured both in microsomes and in a reconstituted system at 35 °C. For the microsomal system, samples were prepared for use in the stopped-flow spectrophotometer (Aminco-Morrow) in two flasks, one containing microsomes, glucose, ±benzphetamine, and phosphate buffer and the other flask containing NADPH, glucose, ±benzphetamine, and phosphate buffer. Each sample was gassed by bubbling with carbon monoxide for 1 min, the glucose oxidase and catalase were added, and gassing was continued for an additional 30 s. The preparations were then loaded into the stopped-flow spectrophotometer of 1-cm light path. The presence of glucose, glucose oxidase, and catalase comprised a deoxygenating system capable of decreasing the oxygen content below detectable levels (<0.8 μM) within seconds. The final concentrations of the assay components after mixing were 3.6 μM cytochrome P-450 (4 mg of microsomal protein/mL), 7 mM glucose, 0.7 unit/mL glucose oxidase, 0.4 unit/mL catalase, 0.3 mM NADPH, and, where indicated, 1.0 mM benzphetamine in 100 mM sodium phosphate buffer, pH 7.5.

When purified cytochrome P-450 RLM5 was used, a mixture of reductase, RLM5, and DLPC was present instead of microsomes. The final concentrations of components were 1 mM DLPC, 4.0 μM reductase, 2.7 μM RLM5, 7 mM glucose, 0.7 unit/mL glucose oxidase, 0.4 unit/mL catalase, 0.5 mM

[1] Abbreviations: cytochrome P-450 RLM5, one of the forms of cytochrome P-450 isolated from untreated rat liver, with a molecular weight of 52 000; reductase, NADPH–cytochrome P-450 reductase isolated from PB-treated male rats; DLPC, L-α-dilauroylphosphatidylcholine; PB, phenobarbital; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

NADPH, and, where indicated, 1 mM benzphetamine, in 50 mM sodium phosphate buffer, pH 7.25, containing 25% glycerol.

Data were collected on an Aminco DW-2 split beam recording spectrophotometer in the dual wavelength mode linked to either a Tektronix storage oscilloscope or a Northstar Horizon II microcomputer.

The absolute spectra of both high-spin and low-spin ferric cytochrome P-450 RLM5 were measured in the presence of an equimolar concentration of reductase at 35 °C, under similar conditions as used for the reduction assays, in order to determine the relative proportion of high-spin cytochrome present in the assay medium. The absorption of the reductase was subtracted from the total absorbance by having reductase present in the reference cuvette. Final conditions were the following: (sample cuvette) 1.5 μM RLM5, 1.5 μM reductase, and 0.6 mM DLPC in 50 mM sodium phosphate, pH 7.25, containing 25% glycerol; (reference cuvette) 1.5 μM reductase and 0.6 mM DLPC in the same buffer. When added, 1 mM benzphetamine was present in both cuvettes. Estimates of the fraction of high-spin cytochrome in the absence and presence of benzphetamine were made by using a difference extinction coefficient of 126 mM^{-1} cm^{-1} for the 390–422 nm wavelength couple (Cinti et al., 1979) and by temperature variation (Tamburini et al., 1984).

When the estimates of the fraction of high-spin cytochrome in the absence and presence of benzphetamine were used, the theoretical absorbance of preparations of RLM5 that were completely low spin and completely high spin could be calculated at any wavelength by using simultaneous equations of the form

$$(\epsilon_{ls})[ls] + (\epsilon_{hs})[hs] = Abs_{total}$$

where [ls] and [hs] are the concentrations of low-spin and high-spin cytochrome, respectively. The values ϵ_{ls}, ϵ_{hs}, and Abs_{total} represent the extinction coefficient of low-spin cytochrome, the extinction coefficient of high-spin cytochrome, and the total measured absorbance of the sample at a given wavelength.

Cytochrome P-450 content was determined from the 450–490-nm absorption of the ferrous–carbon monoxide complex by using a difference extinction coefficient of 91 mM^{-1} cm^{-1} (Omura & Sato, 1964).

Results

Reduction of Microsomal Cytochrome P-450. The kinetics of reduction of substrate-free microsomal cytochrome P-450 followed at three different wavelengths is shown in Figure 1. Reduction (curve a) monitored at 450–490 nm measured the absorption maximum of the carbon monoxide adduct of ferrous cytochrome P-450. Disappearance of high-spin cytochrome P-450 (curve b) was measured at 650–700 nm. Disappearance of low-spin cytochrome (curve c) was monitored at 420–490 nm. Unfortunately, this latter wavelength pair is not free of interfering absorbances of other microsomal pigments, as well as the ferrous and ferric high spin cytochrome; thus, this tracing can only be used as a qualitative indication of changes in low-spin cytochrome. When followed at 450 nm (curve a), cytochrome P-450 was reduced in a multiphasic process; an initial fast phase (about 5 s) was followed by a slower phase(s), and 80% reduction was obtained within 30 s. High-spin cytochrome P-450 (curve b) declined rapidly on initiation of the reduction reaction, attaining a pseudo steady state within 5 s (a similar time frame to the first phase of reduction measured at 450 nm). Observation of the disappearance of absorption at 420 nm (curve c) produced findings different from those

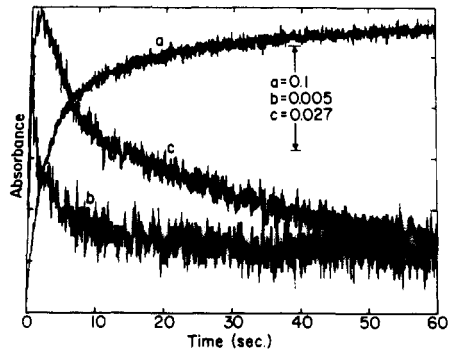


FIGURE 1: Kinetics of reduction of cytochrome P-450. Three tracings are shown. These are (a) the appearance of the CO adduct, measured at 450–490 nm, (b) the disappearance of high-spin cytochrome, measured at 650–700 nm, and (c) the disappearance of absorbance at 420–490 nm. The assay medium was as described under Materials and Methods. Microsomal protein concentration was 4 mg/mL. The medium also contained 1.0 mM benzphetamine. Microsomes contained 0.9 nmol of P-450/mg of protein. Time zero initiates triggering of the trace.

obtained when high-spin cytochrome absorption was measured. Initially, the absorbance at 420 nm did not decline immediately but began to decline after about 2 s. The decrease in 420-nm absorbance was slower than the absorbance change at 650 nm. As expected, when benzphetamine was present, it increased the proportion of high-spin cytochrome P-450. When reduction was measured at these wavelengths in the presence of benzphetamine, the rate and magnitude of the disappearance of 650-nm absorbance was increased (not shown) as well as the fraction of the reduction at 450 nm occurring in the fast phase, as shown earlier (Backes et al., 1982).

The kinetics of formation of reduced cytochrome and disappearance of the high-spin form were then examined in more detail in order to obtain further evidence that would distinguish between the sequential model presented here and one where spin state has no influence on the rate of reduction. In this analysis the time course of reduction (or disappearance of high-spin cytochrome) was measured as a fraction of the total absorbance change (ΔA_T) produced by dithionite addition. The fractional initial rates measured at both 450 (f_{450nm}) and 650 nm (f_{650nm}) are shown in Table I. In each case the fractional initial rate measured at 450 nm was smaller than that observed at 650 nm. This difference is indicative of a faster rate of reduction of high-spin than of low-spin cytochrome P-450. The underlying rationale for this conclusion can be illustrated as follows. If spin state was not involved in the reduction process, both low-spin and high-spin cytochrome would have the same apparent rate constant for reduction. The fractional initial rate of both high-spin and low-spin disappearance would be identical; therefore, the fractional initial rate of formation of the reduced form would also be identical. If, however, high-spin cytochrome was reduced faster than the low-spin form, then at any given time a larger fraction of high spin would have been transformed when compared to the fraction of total cytochrome P-450 which was reduced.

The initial rate of high-spin disappearance ($\Delta A_{650nm}/\Delta t$) can be defined in terms of a rate constant and the concentration of high-spin ferric cytochrome ($k_3[Fe^{3+}_{hs}]$). When expressed as a fraction of the dithionite reducible absorbance ($\Delta A_{T\,650nm}$), the fractional initial rate (f_{650nm}) can be described as follows:

$$f_{650nm} = \frac{\Delta A_{650nm}/\Delta t}{\Delta A_{T\,650nm}} = \frac{k_3[Fe^{3+}_{hs}]}{[Fe^{3+}_{hs}]} \quad (4)$$

Table I: Fractional Initial Rates of Change of Ferrous and High-Spin Ferric Cytochrome P-450 during Reduction[a]

preparation	fraction high spin[b]	f_{450nm} (s^{-1})	f_{650nm} (s^{-1})	f_{450nm}/f_{650nm}[c]
microsomes				
-substrate	0.48	0.17	0.37 ± 0.02 (3)	0.46
+benzphetamine (1.3 mM)	0.68	0.35 ± 0.029 (2)	0.50 ± 0.07 (3)	0.70
RLM5 (+1 mM benzphetamine)				
reductase:RLM5 (1.5:1)		1.02 ± 0.03 (3)	1.49 ± 0.09 (3)	0.68
reductase:RLM5 (1:1)	0.69	0.80 ± 0.03 (3)	1.18 ± 0.06 (4)	0.68
reductase:RLM5 (1:)[d]	0.69	0.78	1.26	0.62

[a] Values ± SEM are indicated, and the number of determinations is shown in parentheses after the SEM. [b] The fraction of high-spin cytochrome P-450 was estimated by the method of Cinti et al. (1979) from the absorption spectrum of the oxidized cytochrome. [c] The value for f_{450nm}/f_{650nm} represents an estimate of the amount of high-spin cytochrome P-450 present prior to reduction as estimated from kinetic data. [d] The value for f_{650nm} was measured at 433 nm.

The initial rate of formation of ferrous cytochrome ($\Delta A_{450nm}/\Delta t$) can also be expressed as a fraction of the dithionite-reducible absorbance ($\Delta A_{T\,450nm}$). Since initially only high-spin ferric hemoprotein would be reduced, the fractional initial rate for ferrous cytochrome P-450 formation can be written as follows:

$$f_{450nm} = \frac{\Delta A_{450nm}/\Delta t}{\Delta A_{T\,450nm}} = \frac{k_3[Fe^{3+}_{hs}]}{[Fe^{3+}_{total}]} = \frac{k_3[Fe^{3+}_{hs}]}{[Fe^{3+}_{hs}] + [Fe^{3+}_{ls}]} \quad (5)$$

When the ratio of the fractional initial rates is taken at 450 and 650 nm, a value can be obtained that represents the preincubation fraction of high-spin cytochrome P-450. This method obviates the need for extinction coefficient determinations:

$$\frac{f_{450nm}}{f_{650nm}} = \frac{k_3[Fe^{3+}_{hs}]/([Fe^{3+}_{hs}] + [Fe^{3+}_{ls}])}{k_3[Fe^{3+}_{hs}]/[Fe^{3+}_{hs}]} = \frac{[Fe^{3+}_{hs}]}{[Fe^{3+}_{hs}] + [Fe^{3+}_{ls}]} = \frac{Fe^{3+}_{hs}}{E_t} \quad (6)$$

where E_t = total ferric hemoprotein. Data obtained with microsomes, calculated by using eq 6, are shown in Table I. The f_{450nm}/f_{650nm} ratios (0.46 and 0.70) determined in the absence and presence of benzphetamine are in excellent agreement with the fraction of high spin (0.48 and 0.68) estimated from the Soret absorbance (Backes et al., 1982).

Reduction Kinetics of RLM5. The kinetics of RLM5 reduction in a reconstituted system at equimolar hemoprotein and reductase concentrations are shown in Figure 2. The reaction measuring the reduced carbon monoxide complex (at 450 nm) proceeded initially in a burst followed by a slower phase(s) of reduction. In the presence of benzphetamine (open circles) the reduction of cytochrome P-450 proceeded generally in the same manner as shown in the absence of substrate (closed circles). However, the initial burst of the reaction was faster, and the fraction of reduction in the first phase was significantly greater in the presence of benzphetamine. In contrast to earlier observations with microsomes (Backes et al., 1980, 1982) the slope of the slow phase was also increased by benzphetamine. Estimates of the amount of high-spin cytochrome from the intercept of the fast- and slow-phase extrapolations of first-order plots (41% and 76% in the absence and presence of benzphetamine) agreed closely with estimates obtained from absolute spectra (28% and 69% in the absence and presence of benzphetamine).

Experiments similar to those done with microsomes were performed by using the reconstituted system in the presence of benzphetamine (Table I). The f_{450nm} was smaller than the f_{650nm} in accord with the microsomal data; however, both the fractional initial rates obtained from the reconstituted system were larger than the corresponding microsomal values. This might be expected due to the higher degree of saturation of the RLM5 with reductase in the reconstituted system. The ratio of f_{450nm}/f_{650nm} (Table I) produced a value (0.68) that reflected the prereduction fraction of high-spin cytochrome P-450. This value is in excellent agreement with the fraction of high-spin RLM5 estimated from the Soret absorbance. Further, changing the molar proportion of reductase to RLM5 from 1.5:1 to 1:1 did not appreciably influence the calculated proportion of high-spin form.

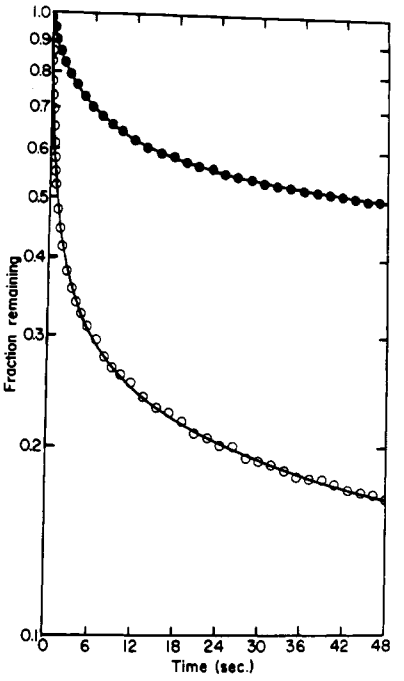


FIGURE 2: Reduction of RLM5 by NADPH. First-order plot of cytochrome P-450 RLM5 reduction in the absence (●) and presence (○) of 1 mM benzphetamine. Reduction was measured at 450–490 nm by using the formation of the carbon monoxide–ferrous complex. Cytochrome P-450 RLM5 (2.5 μM) was reconstituted with reductase (2.5 μM) in 1 mM DLPC.

Spin-State Estimation and Construction of Theoretical Low-Spin and High-Spin Spectra for RLM5. Absolute spectra of RLM5 were obtained under conditions similar to those used for the reduction assays both in the absence and presence of benzphetamine (Figure 3, top). In view of the potential effect of the reductase on the spin equilibrium of cytochrome P-450 (French et al., 1980), the absolute spectra were recorded in the presence of equimolar reductase and RLM5. Spectral contributions of the reductase were eliminated by adding an equal amount of reductase to both cuvettes as described under Materials and Methods. By use of these spectra (Figure 3, top) the fraction of high-spin cytochrome was calculated (Cinti et al., 1979; Tamburini et al., 1984). Values of 28% and 69% were obtained in the absence and presence of benzphetamine.

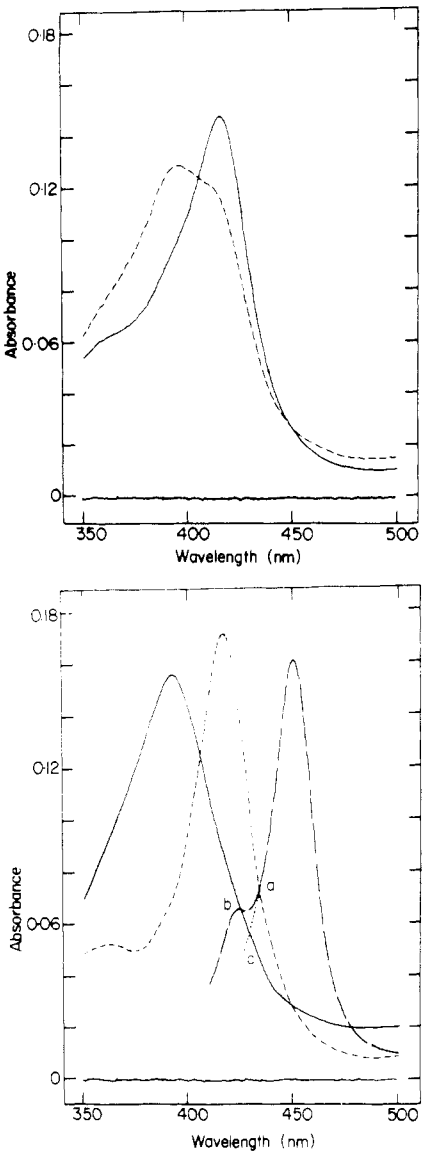


FIGURE 3: Spectrum of cytochrome P-450 RLM5 in a reconstituted system with reductase and dilauroylphosphatidylcholine. NADPH–cytochrome P-450 reductase and DLPC were mixed and divided into two test tubes. To one of these tubes was added cytochrome P-450 RLM5. After a 15-min incubation at room temperature, both samples were diluted to 1 mL and placed in a Cary 219 spectrophotometer (DLPC + reductase + RLM5 in the sample and DLPC + reductase in the reference), and (top panel) the spectrum was recorded at 35 °C (solid line). Benzphetamine was added to a final concentration of 1 mM to each cuvette, and the spectrum was recorded (dashed line). The final concentrations of components were 1.5 μM RLM5, 1.5 μM reductase, and 0.6 mM DLPC. (Bottom panel) Both cuvettes in the top panel were bubbled with carbon monoxide for 1 min and reduced with sodium dithionite (– –). The remaining curves in the bottom panel represent the theoretical spectra, pure low-spin (- - -) and pure high-spin (—) cytochrome P450 RLM5. a = isosbestic point of the low-spin ferric form and ferrous–CO complex; b = apparent isosbestic point of high-spin ferric cytochrome P-450 and the ferrous–CO complex; c = extrapolated isosbestic point between high-spin ferric form and ferrous–CO complex of cytochrome P-450.

The above data permitted construction of the theoretical low-spin and high-spin spectra for RLM5 by using simultaneous equations as described under Materials and Methods. These spectra are illustrated in the bottom panel of Figure 3. The actual spectrum of the carbon monoxide adduct of ferrous RLM5 is superimposed on these theoretical spectra. A small peak was present at 423 nm. This peak was present whether NADPH or dithionite was used as the reductant. The observed spectrum of RLM5 after carbon monoxide and dithionite was

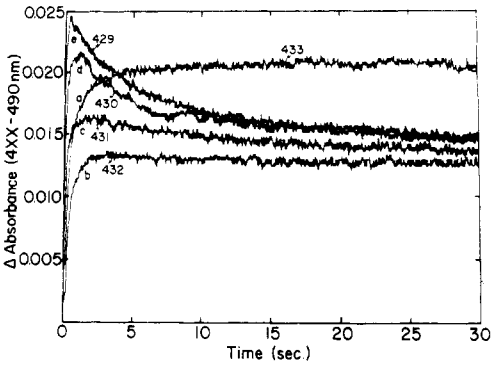


FIGURE 4: Reduction of RLM5 at various wavelengths. Time course of cytochrome P-450 RLM5 reduction measured at a variety of wavelengths by using 490 nm as the base wavelength. Conditions are the same as in Figure 3 in the presence of 1 mM benzphetamine.

stable for about 5 min after which some conversion to cytochrome P-420 occurred.

The calculated spectra in Figure 3 (bottom) show two wavelengths of particular interest with respect to cytochrome P-450 reduction. The first is at about 433 nm (point a), where ferrous carbon monoxide bound RLM5 is isosbestic with the low-spin cytochrome. The other wavelength represents the isosbestic point between high-spin RLM5 and the ferrous carbon monoxide form (point b). The presence of the 423-nm spectral component in the ferrous CO adduct prevented the precise localization of the wavelength where ferric high-spin and CO–ferrous forms are isosbestic. However, since the 423-nm peak did not appear to change during the reduction process, it was possible to ignore it in order to estimate the isosbestic wavelength. Toward this end, the lower wavelength side of the 450-nm peak was extrapolated to a point where it intersected the high spin spectrum (Figure 3, bottom, dotted line). The calculated point of intersection at about 430 nm (point c) placed the actual isosbestic point at approximately that wavelength. The wavelength range from 429 to 433 nm was utilized to provide additional evidence for the sequential reduction model.

The time course of reduction monitored at several different wavelengths is shown in Figure 4. At 433 nm, a rapid initial increase in absorbance was observed within 4–5 s which was followed by a much slower second phase of absorbance increase. At 432 nm, an initial rapid increase in the absorbance was observed for about 2 s followed by a very slow decrease after about 5 s (Figure 4). As the monitored wavelength was further decreased, this pattern persisted with an absorbance increase followed by a decrease. However, at the lower wavelengths, both the magnitude and duration of the initial absorbance increases were diminished, and the subsequent decrease in absorbance became larger.

These absorbance changes can be explained by the simplified sequential model. At any given wavelength each of the three pertinent species (low-spin ferric, high-spin ferric, and ferrous) have their own characteristic extinction coefficients (see bottom panel of Figure 3). Prior to reduction the total absorbance is dependent on the amount of low-spin and high-spin forms and their extinction coefficients at each wavelength. An increase in absorbance would be observed on reduction if the reduced form had a larger extinction coefficient than its precursor; if the reduced form had a smaller extinction coefficient than its precursor, then a decrease in absorbance would be observed. No absorbance change would be observed if the reduced form had the same extinction coefficient as its precursor. If high-spin cytochrome P-450 was reduced substantially faster than low-spin cytochrome P-450, then the

absorbance changes with time monitored at the Fe^{3+}_{ls}/Fe^{2+} isosbestic point (point a in Figure 3, bottom panel) should exhibit only a rapid increase in absorbance, corresponding to the reduction of Fe^{3+}_{hs}. Further, the absorbance changes measured at the Fe^{3+}_{hs}/Fe^{2+} isosbestic point (point c, Figure 3, bottom) should exhibit a lag followed by a slower decrease in absorbance corresponding to the slow interconversion of Fe^{3+}_{ls} to Fe^{3+}_{hs}. Examination of the kinetics at wavelengths between these isosbestic points would yield a combination of rapid increases and slow decreases in absorbance. The results in Figure 4 are in accord with these expectations and support a model evoking a faster rate of reduction of the high-spin cytochrome than of the low-spin cytochrome. For example, the nature of the absorbance changes observed at 432 and 433 nm (Figure 4) suggests that the Fe^{3+}_{ls}/Fe^{2+} isosbestic point lies between these two wavelengths, in close agreement with the value estimated from the theoretical spectra of Figure 3 (bottom). The kinetics observed in the estimated region of the Fe^{3+}_{hs}/Fe^{2+} isosbestic point were not entirely as expected. At 430 nm, the estimated wavelength of the isosbestic point (Figure 3, bottom), a small rapid increase in absorbance was observed, preceding the expected lag and slow decrease in absorbance. The initial rapid increase in absorbance may be accounted for by the fact that Fe^{3+}_{hs}/Fe^{2+} points are not isosbestic at 490 nm, due to greater absorbance of the Fe^{3+}_{hs} at this wavelength. Since the absorbance changes measured in Figure 4 were all relative to 490 nm, a small rapid decrease in absorbance at 490 nm upon high-spin RLM5 reduction manifests itself as a small increase in $\Delta A_{430-490nm}$. A similar effect is observed with microsomes (Figure 1, curve c). The rapid phase of high-spin cytochrome P-450 reduction, measured at the Fe^{3+}_{ls}/Fe^{2+} isosbestic point, is not much affected by absorbance changes at 490 nm due to Fe^{3+}_{ls} removal because Fe^{3+}_{ls} and Fe^{2+} absorbances are similar at 490 nm (Figure 3, bottom). Thus, the changes in absorbance at this latter wavelength are due mainly to reduction of high-spin P-450.

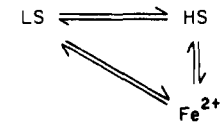
Since 433 nm is very close to the Fe^{3+}_{ls}/Fe^{2+} isosbestic point, the rate of absorbance change at this wavelength may be used in conjunction with eq 4 to obtain an additional estimate of the fractional initial rate of high-spin cytochrome P-450 reduction. The f_{433nm} was 1.26 s^{-1}, and a value of 0.78 s^{-1} was obtained at 450 nm. The ratio of these initial rate values produced an estimate of high-spin cytochrome P-450 of 62%, which is consistent with those values presented in Table I.

Discussion

In this report, a new approach was taken to examine our hypothesis that lack of monophasic reduction kinetics of cytochrome P-450 is the result of faster reduction of the ferric high-spin state. In this study the kinetics of reduction of cytochrome P-450 was examined by using both microsomal preparations and a reconstituted system containing cytochrome P-450 RLM5. The experiments were designed to discriminate between a model where high-spin cytochrome is reduced more rapidly than the low-spin state and one where both forms are reduced at the same rate. The kinetics of the reaction were measured at different wavelengths, such that the formation of the carbon monoxide–ferrous complex as well as the disappearance of high-spin ferric cytochrome P-450 would be monitored. A slower absorbance change was observed at 420 nm, a region wherein low-spin cytochrome P-450 absorbs. The data showed a rapid disappearance of high-spin cytochrome that corresponded with the fast phase of formation of the ferrous complex (Figure 1). When plotted as a fraction of the dithionite-reduced absorbance values, the initial rate of formation of the reduced species (f_{450nm}) was smaller than the initial rate of disappearance of the high-spin species (f_{650nm}), an observation that supports spin-state involvement in the reduction process. As indicated by eq 6, the ratios of the fractional initial rates shown in Table I yield values representing the fraction of high-spin cytochrome P-450 present prior to reduction. These values were determined under various conditions and compared with an independent estimate of the fraction of high-spin cytochrome. Excellent agreement was obtained. Finally, cytochrome P-450 reduction was monitored at wavelengths where ferrous cytochrome and both ferric forms absorbed (Figure 3, bottom). The time course of absorbance changes expected by the sequential model were compared with the experimental data. The time course of absorbance changes agreed with what would be expected by the sequential model. For example, at 433 nm (where Fe^{3+}_{ls} and Fe^{2+} approach an isosbestic point) a rapid absorbance change was observed which was similar to that seen at 650 nm. As we move toward 430 nm (a region approaching the isosbestic point between Fe^{3+}_{hs} and Fe^{2+}), the initial rapid absorbance increase becomes smaller in extent, and the subsequent slower decrease in absorbance becomes pronounced; i.e., the absorbance changes with time begin to resemble more closely those expected at the Fe^{3+}_{hs}/Fe^{2+} isosbestic point. A small initial increase in absorbance observed even at 430 nm may be accounted for, at least in part, by the greater absorbance of Fe^{3+}_{hs} at 490 nm than Fe^{3+}_{ls} or Fe^{2+}.

The simplified model, applied to microsomes, assumes a single homogeneous form of cytochrome P-450. However, liver microsomes of the untreated rat contain a number of cytochrome P-450 enzymes (Agosin et al., 1979; Cheng & Schenkman, 1982; Schenkman et al., 1982). The observation that the reduction of microsomal cytochrome P-450 was qualitatively similar to the reduction of RLM5, when measured at 650 and 450 nm, implies that the different enzymes in hepatic microsomes exhibit similar reducibilities; i.e., each enzyme is reduced much faster in the high-spin state than in the low-spin state. Further, the present studies clearly obviate any hypothesis that does not take into account differences in the rates of reduction of high- and low-spin states of cytochrome P-450. For example, if spin state was uninvolved in the reduction process (Oprian et al., 1979), both low-spin and high-spin cytochromes would have the same rate constant for reduction. Thus, for a cytochrome preparation that exists 60% in the high-spin state prior to reduction, exactly 60% of the remaining oxidized cytochrome would exist in the high-spin state throughout the time course of the reaction. Since this is contrary to our observations (Table I), the results in this report rule out such a model.

Though the results show that high-spin cytochrome P-450 is reduced faster than the low-spin form, the precise mechanism remains to be elucidated. The direct reduction of low-spin cytochrome could occur, but at a significantly slower rate. If this occurs, the following model for reduction is seen:



Studies by Pierre et al. (1982), however, indicate that low-spin cytochrome $P\text{-}450_{cam}$ is not photoreducible. Similar conclusions were suggested from studies with hepatic cytochrome P-450 purified from phenobarbital-pretreated rats, where NADPH was used as a reducing agent (Tamburini et al., 1984).

In our simplified model, only two states are shown for the ferric cytochrome (low spin and high spin). These observed

equilibria are undoubtably composed of a number of microscopic equilibrium states including hexacoordinate low spin, hexacoordinate high spin, and pentacoordinate high spin as well as the respective substrate-bound forms of these species. In any perturbation, the microscopic rate constants that control the overall rate of conversion between low-spin and high-spin states would not significantly alter the predictions of the simplified model, as long as the rate of appearance of the *directly reducible* high-spin species from the low-spin state is severalfold slower than its rate of reduction.

Despite the data supporting the sequential model, some recent reports have appeared, which suggest that the rate constant for the substrate-evoked spectral change may be many times faster than the rate constant for reduction (Tsong & Yang, 1978; Ziegler et al., 1982). However, the reduction kinetics of cytochrome P-450 is obviously a complex matter. Studies such as above are not directly comparable with reduction studies as shown here since they were not performed in the presence of saturating levels of the reductase. It is implicit in our model that the rate of the fast phase of reduction reflects electron transfer within the reductase-bound high-spin P-450 complex. Various studies have shown these two proteins to form a tight bimolecular complex (Oprian et al., 1979; French et al., 1980), an essential prerequisite for efficient electron transfer. Further, in our studies a concentration of the reductase producing saturation of the kinetics was employed, which implied that all of the cytochrome P-450 was in the reductase-bound state. The presence of a large ligated protein like the reductase may, depending upon the mechanism of complex formation, impair the rate of conformational change in the cytochrome P-450 molecule. Although the studies reported here, and elsewhere (Oprian et al., 1979), using saturating levels of reductase, do not yield monophasic reduction kinetics, it is still possible that interaction between the two proteins plays a part in the observed kinetics. Thus, complex formation between cytochrome P-450 and its reductase involves both hydrophobic and electrostatic components. An additional complication may involve heterogeneity of complex formation, i.e., hydrophobic binding or electrostatic interaction or a mixture of these components. Although precise details of the reaction mechanism have yet to be elucidated, the results presented in this paper nevertheless unambiguously indicate the differential reduction rates for high-spin and low-spin forms of ferric cytochrome P-450.

Registry No. NADPH, 53-57-6; cytochrome P-450, 9035-51-2; monooxygenase, 9038-14-6.

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