Reduction Kinetics of Purified Rat Liver Cytochrome P-450. Evidence for a Sequential Reaction Mechanism Dependent on the Hemoprotein Spin State†

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ABSTRACT: The anaerobic reduction kinetics of purified rat liver ferric cytochrome P-450 from phenobarbital-treated rat liver microsomes, reconstituted with saturating NADPH-cytochrome P-450 reductase, have been investigated and were shown not to be monophasic. From experiments correlating changes in the rate of fast-phase reduction with the spin state of the heme iron existing at preequilibrium, data were obtained consistent with a model for spin-state control of cytochrome P-450 reduction wherein the high-spin form of the hemoprotein is more rapidly reduced than the low-spin form. In addition, the temperature dependence of the reduction process in the presence of the substrate benzphetamine was studied. From the results obtained it is suggested that the endothermic nature of the low- to high-spin transition largely accounts for the apparent activation energy observed for the reduction process, with the actual rate constant for reduction of high-spin cytochrome P-450 being relatively temperature insensitive when compared to the rate constant for reduction of the membrane-bound form of the hemoprotein.

One of the early steps in the catalytic sequence of hepatic microsomal cytochrome P-450 is a one-electron reduction of the hemoprotein by NADPH-cytochrome P-450 reductase (Peterson et al., 1977). This process has generally been studied anaerobically under non-steady-state conditions in the presence of saturating concentrations of carbon monoxide. The resultant kinetics do not show simple first-order behavior and have prompted many investigations into the nature of the molecular mechanisms governing this process. The kinetics have previously been resolved into either two (Gigon et al., 1969; Oprian et al., 1979; Peterson et al., 1976, 1978; Taniguchi et al., 1979) or more (Ruf, 1980) phases, which have been described as a composite of two first-order processes or a more complex kinetic behavior consisting of mixed first- and second-order processes (Diehl et al., 1970). Gigon et al. (1969) have explained the above kinetics on the basis of two concurrent first-order processes for the reduction of substrate-free and -bound enzyme, respectively. Peterson et al. (1976), however, proposed the existence of topologically distinct populations of cytochrome P-450 molecules in the microsomal membrane with differing access to NADPH-cytochrome P-450 reductase reducing equivalents, wherein cluster cytochrome P-450 in direct association with NADPH-cytochrome P-450 reductase is reduced at a greater rate than noncluster cytochrome. Other authors, however, using reconstituted systems have suggested that NADPH-cytochrome P-450 reductase and cytochrome P-450 interact through random collisions rather than by forming functional clusters (Taniguchi et al., 1979). In addition to these models, the occurrence of multiphasic kinetics has been attributed to some characteristic of the reductase (Oprian et al., 1979). More recently studies in our laboratories (Cinti et al., 1979; Gibson et al., 1980) and elsewhere (Rein et al., 1977; Ristau et al., 1978) using microsomal and purified systems have shown mammalian cytochrome P-450 heme iron to exist as an equilibrium mixture of low- and high-spin forms, the relative amounts of which may be modulated by both temperature and exogenously added substrates of the heme protein. Alteration in the spin equilibrium results in changes in the apparent midpoint redox potential of the ferric heme iron (Sligar et al., 1979) in direct analogy with the camphor hydroxylase system (Sligar, 1976). These observations in combination with the fact that anaerobic cytochrome P-450 reduction kinetics were stimulated by type I substrates (Gigon et al., 1969) prompted Backes et al. (1980, 1982) to propose an alternative explanation for the observed biphasic reduction kinetics. Working with microsomes, these authors provided considerable kinetic data consistent with a simplified sequential model, which proposed that formation of the high-spin cytochrome P-450 state was a prerequisite for reduction,[1] as follows:

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

where the spin equilibrium constant of the ferric hemoprotein K_{eq} is given by k_1/k_2.

The rate equations describing such a system are

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

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

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

According to this model, the preexisting high-spin cytochrome P-450 content is determined by K_{eq}. As the concentration of reduced hemoprotein is zero prior to NADPH addition, the initial cytochrome P-450 reduction rate simplifies to $k_3[Fe^{3+}_{hs}S]$, as derived from the last equation above. The burst of he-

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[1] Abbreviations: Fe^{3+}_{hs}, high-spin ferric cytochrome P-450; Fe^{3+}_{ls}, low-spin ferric cytochrome P-450; Fe^{2+}, ferrous cytochrome P-450; $Fe^{3+}_{hs}S$, high-spin, substrate-bound ferric cytochrome P-450; $Fe^{3+}_{ls}S$, low-spin, substrate-bound ferric cytochrome P-450; DLPC, dilauroylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid.

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moprotein reduction is followed by a second slower reduction if the rate of formation of the reducible high-spin state is slower than the rate of reduction (k_3). As indicated earlier, there may be forms intermediate between the low-spin and the reducible high-spin states (Backes et al., 1982; Rein et al., 1979).

In the present paper we have studied the anaerobic reduction kinetics of purified rat liver microsomal cytochrome P-450, with a view to delineating the role of the hemoprotein spin state in controlling the reduction process.

Materials and Methods

Purification of Microsomal Enzymes. Male Wistar rats (200 g, University of Surrey Breeders) were pretreated with sodium phenobarbital [0.1% (w/v) in the drinking water for 6 days], and liver microsomal fractions were prepared by differential ultracentrifugation according to Remmer et al. (1966). Microsomal cytochrome P-450 was solubilized and purified according to the method of Guengerich (1978). The final preparation was homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis by the method of Laemmli (1970), exhibiting a specific content of 18 nmol of cytochrome P-450/mg of protein and an A_{417}/A_{280} ratio of 1.5. The hemoprotein used in these studies had an N-terminal amino acid sequence of Met-Glu-Pro-Ser-Ile-Leu. The preparation was catalytically active in the reconstituted system of Haugen et al. (1975), catalyzing the N-demethylation of benzphetamine at rates above 200 nmol of HCHO formed (nmol of cytochrome P-450)$^{-1}$ min^{-1}. Emulgen was removed from the final enzyme preparation by carboxymethyl-Sephadex C-50 chromatography (Imai et al., 1980) and was below the level of detection by using a standard colorimetric assay (Goldstein & Blecher, 1975), in agreement with the high Soret to 280 nm absorbance ratio. The endogenous phospholipid in the final preparation was less than 1 mol/2 mol of cytochrome.

NADPH-cytochrome P-450 reductase (EC 1.6.2.4) was purified to electrophoretic homogeneity from the same animals by the method of Yasukochi & Masters (1976) with the following modifications for the effective removal of the nonionic detergent emulgen. Following the high ionic strength wash, 2′,5′-ADP–agarose-bound NADPH-cytochrome P-450 reductase was washed with 10 mM potassium phosphate buffer, pH 7.7, containing 25% glycerol, 0.02 mM EDTA, and 0.2 mM dithiothreitol, until the A_{280} of the wash decreased to zero and elution of the reductase was achieved with the same buffer containing 0.7 mM 2′-AMP. Fractions containing NADPH-cytochrome P-450 reductase were pooled and dialyzed twice for successive 8-h periods against 2 L of 50 mM potassium phosphate, pH 7.25, containing glycerol (20% v/v) to remove bound 2′-AMP. This procedure resulted in essentially detergent-free, highly purified NADPH-cytochrome P-450 reductase with a specific activity of 19 units/mg of protein (11.4 nmol/mg) when assayed in the presence of 0.1 M potassium phosphate buffer, pH 7.25 at 25 °C. One unit of reductase activity is defined as 1 μmol of cytochrome *c* reduced min^{-1}.

Spectral Determinations. Spectra were recorded on an Aminco DW-2 recording spectrophotometer operating in either the dual wavelength or split beam mode. Temperature was maintained by using a Lauda K-2R circulating water bath in thermal contact with the cuvette or stopped-flow cell and monitored by using a Y.S.I. (Yellow Springs Instrument Co.) probe.

Cytochrome P-450 reduction kinetics were followed by using dual wavelength difference spectrophotometry (450 minus 490 nm) using both plunger-type anaerobic cells (American Instrument Co.) and stopped-flow spectrophotometry. In the former method, the cuvette contents (3.0 mL) comprised 100 mM potassium phosphate buffer, pH 7.25, containing glycerol (16% v/v), glucose (6.7 mM), glucose oxidase (13 units mL^{-1}), catalase (590 units mL^{-1}), probe-sonicated DLPC (50 μg/mL), cytochrome P-450 (0.45 μM), and NADPH-cytochrome P-450 reductase. In preliminary experiments to determine the optimal concentrations of DLPC and NADPH-cytochrome P-450 reductase, 50–100 μg mL^{-1} DLPC was found to be optimal, and saturation of cytochrome P-450 reduction kinetics with respect to both initial rates and extents was achieved by using 2 units mL^{-1} (1.2 μM) NADPH-cytochrome P-450 reductase. All data to be presented were obtained in the presence of saturating NADPH-cytochrome P-450 reductase. The cells were gassed with carbon monoxide [deoxygenated according to Meites & Meites (1948)] for 1.5 min prior to the addition of glucose oxidase and catalase and subsequently for 2.5 min. After temperature equilibration for 3 min (25 °C), the reaction was initiated by the addition of NADPH to a final concentration of 0.3 mM via the well of the anaerobic plunger.

Stopped-flow experiments were performed with an Aminco-Morrow stopped-flow apparatus, and data were recorded on a storage oscilloscope. Reactions were initiated by rapidly mixing an equal volume of an anaerobic solution of cytochrome P-450 and NADPH-cytochrome P-450 reductase from one driving syringe with an anaerobic solution of NADPH from the other syringe. All other components were present in equal concentrations in either syringe-producing final concentrations after mixing of the following: glucose, 6.7 mM, catalase, 590 units mL^{-1}, glucose oxidase, 13 units mL^{-1}, and DLPC, 100 μg mL^{-1}; benzphetamine, 1.28 mM, cytochrome P-450, 0.45 μM, NADPH-cytochrome P-450 reductase, 2 units mL^{-1} (1.2 μM), and NADPH, 0.3 mM in 90 mM potassium phosphate buffer, pH 7.25, containing glycerol (18% v/v).

Spectral changes were monitored through 1-cm light path with a spectral bandwidth of 3.0 nm by using either method. The time course of reduction of cytochrome P-450 was conveniently monitored by the increase in absorbance at 450 nm of the ferrous carbon monoxide complex. Under these experimental conditions the rate of binding of carbon monoxide to the ferrous hemoprotein was not rate limiting (Diehl et al., 1970; Omura et al., 1965; Gray, 1982).

The effectiveness of the glucose oxidase/catalase deoxygenation system was confirmed by directly measuring the oxygen tension of the system under the same experimental conditions as used in the kinetic studies. This was achieved by using a Clark-type oxygen electrode of home design. Starting with the aerobic reconstituted system, the deoxygenating system removed oxygen to below the limit of detection (0.8 μM) within 30 s.

Spin-State Analysis and Spectral Titrations with Benzphetamine. Both temperature- and substrate-induced spin-state changes were determined by using cytochrome P-450 in the complete reconstituted system as described above but in the absence of NADPH.

Temperature-dependent spin-state changes were recorded in the dual wavelength mode (390 minus 420 nm) while the cuvette temperature was increased over a 1-h period from 14 to 40 °C. The cuvette temperature was simultaneously monitored by using a temperature probe (Yellow Springs Instruments) inserted directly into the cuvette. The data obtained was analyzed by a regression fit routine wherein the correlation coefficient served as a convergence parameter for the least-squares fit of

$$\ln K_{eq} = \ln \frac{A_L - A_T}{A_T - A_H} = \frac{\Delta S}{R} - \frac{\Delta H}{RT}$$

where A_H and A_L are the maximal and minimal absorbance differences (390 − 420 nm) occurring with 100% high-spin and 100% low-spin cytochrome P-450 respectively, characterized by a difference extinction coefficient of 126 mM^{-1} cm^{-1} (Cinti et al., 1979; Gibson et al., 1980) and A_T is the absorbance difference (390 − 420 nm) of the cytochrome P-450 Soret region at temperature T. The absorbance difference, A_T, corresponds to an equilibrium mixture of low- and high-spin cytochrome P-450, and the spin equilibrium constant (K_{eq}) is defined as the ratio of $Fe^{3+}_{hs}/Fe^{3+}_{ls}$. ΔS and ΔH are the entropy and enthalpy changes, respectively, characterizing the spin equilibrium process. R is the gas constant (1.99 cal deg^{-1} mol^{-1}).

Spectral titrations of cytochrome P-450 with benzphetamine were determined in the complete reconstituted system in the absence of NADPH, and the spectral binding parameters (K_s and ΔA_{max}) were determined by double-reciprocal plots (Schenkman et al., 1967). The increase in the high-spin component of cytochrome P-450 in the presence of a given concentration of benzphetamine was determined from the magnitude of the corresponding type I spectral change (Schenkman et al., 1967) by using the difference extinction coefficient (390 − 420 nm) of 126 mM^{-1} cm^{-1}.

Other Assays. Cytochrome P-450 was determined by the method of Omura & Sato (1964) using an extinction coefficient of 91 mM^{-1} cm^{-1} for the absorbance difference 450 − 490 nm of the dithionite-reduced carbon monoxide complex. The concentration of NADPH-cytochrome P-450 reductase was determined by using an extinction coefficient of 21.4 mM^{-1} cm^{-1} at 456 nm of the oxidized flavoprotein (French & Coon, 1979). Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard, and the presence of interfering reagents was allowed for by suitable additions to standard curves.

The kinetics of reduction of cytochrome *c* by NADPH-cytochrome P-450 reductase were studied in a 3.0 mL volume comprising 2.2 μM cytochrome *c* and 0.17 unit mL^{-1} NADPH-cytochrome P-450 reductase in 100 mM potassium phosphate buffer, pH 7.25. The reaction was initiated with NADPH (0.3 mM final) and the production of reduced cytochrome *c* measured as the increase in absorbance at 550 nm measured in the split beam mode by using an extinction coefficient of 20.4 mM^{-1} cm^{-1}.

Results

Effectiveness of the Deoxygenating System. The importance of maintaining complete anaerobiasis when studying cytochrome P-450 reduction kinetics particularly concerning the slow-phase components has been emphasised (Gigon et al., 1969). In the present study an enzymatic oxygen scavenging system comprised of glucose, glucose oxidase, and catalase was employed, the effectiveness of which was confirmed by directly measuring the oxygen tension of the system under the same experimental conditions as used in the kinetic studies. The results of such analyses indicated that under these conditions all dithionite-reducible oxygen was effectively removed by the scavenging system.

Spectral Interaction of Benzphetamine with Cytochrome P-450. The hypsochromic shift of the Soret band of mammalian cytochrome P-450 (from 418 to 390 nm) observed after the addition of various type I substrates has previously been correlated with a change in the hemoprotein spin equilibrium (Gibson et al., 1980; Kumaki et al., 1978; Mitanie & Horie, 1969; Rein et al., 1977; Tsai et al., 1970), resulting in a partial conversion to the high-spin form. In order to correlate changes in the reduction kinetics of cytochrome P-450 with changes in the spin state of the heme iron, it was first necessary to select a suitable substrate capable of interacting with the cytochrome to produce large type I spectral changes. Benzphetamine appeared ideal for this purpose, as addition of this substrate to cytochrome P-450 could be controlled so as to yield graded increments in the magnitude of the spectral change and thus the spin state of the heme iron at 25 °C (Figure 1). Also of importance was the fact that the relatively high affinity of benzphetamine for the enzyme preparation (K_s = 77 μM) (Figure 1) meant that it was possible to achieve effective saturation of the hemoprotein with substrate, resulting in greater than 30% high-spin conversion relative to the substrate-free enzyme.

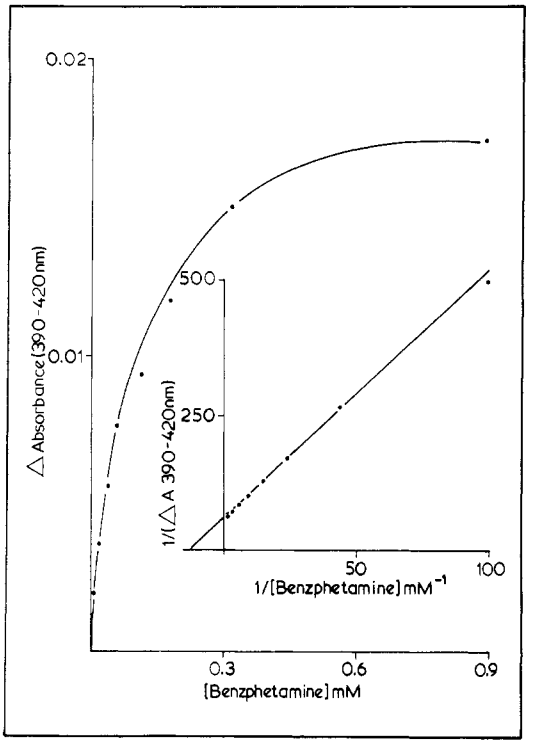


FIGURE 1: Spectral titration of purified cytochrome P-450 with benzphetamine. Cytochrome P-450 (0.45 μM) was titrated (in the presence of the complete reconstituted enzyme system) with concentrations of benzphetamine designed to give graded incremental increases in the fraction of high-spin cytochrome P-450, under conditions similar to those employed in the kinetic studies. (Inset) Double-reciprocal plot of the spectral titration data yielding a gross spectral dissociation constant of 77 μM.

Reduction Kinetics of Cytochrome P-450. In reports by Oprian et al. (1979) and Taniguchi et al. (1979) with purified rabbit cytochrome P-450 isozymes, the addition of NADPH to an anaerobic reconstituted system, containing the cytochrome, NADPH-cytochrome P-450 reductase, and phospholipid under a carbon monoxide atmosphere, resulted in reduction kinetics which could not be described by a single first-order process. Cytochrome P-450 isolated from phenobarbital-pretreated rats in the present study also behaved in this manner in the reconstituted system (Figure 2) and qualitatively resembled the kinetics observed when microsomes were used (Backes et al., 1982), in that reduction proceeds in a rapid burst, tailing off to a much slower second phase. As demonstrated in Figure 2, increasing concentrations of benzphetamine markedly influenced the reduction kinetics. At low levels of the drug, when only a small shift toward the high-spin state was noted (Figure 1), the stimulation of the rate of the

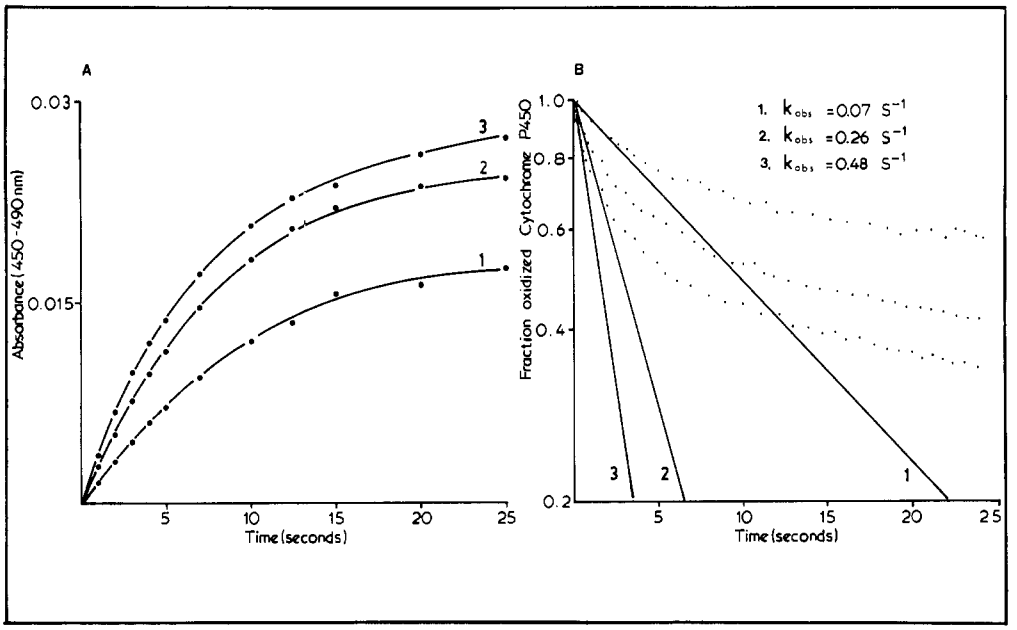


FIGURE 2: Influence of benzphetamine on the reduction kinetics of purified cytochrome P-450. The time course of cytochrome P-450 reduction in a reconstituted enzyme system at 25 °C under anaerobic conditions was determined as described under Materials and Methods. The cytochrome P-450 concentration was 0.45 μM. (A) Time course of cytochrome P-450 reduction as monitored by the absorbance increment between 450 and 490 nm; (1) substrate free; (2) plus 60 μM benzphetamine; (3) plus 900 μM benzphetamine. (B) Semilogarthmic plot of the data shown in (A) above, where (1), (2), and (3) refer to the same concentrations of benzphetamine as in (A) above.

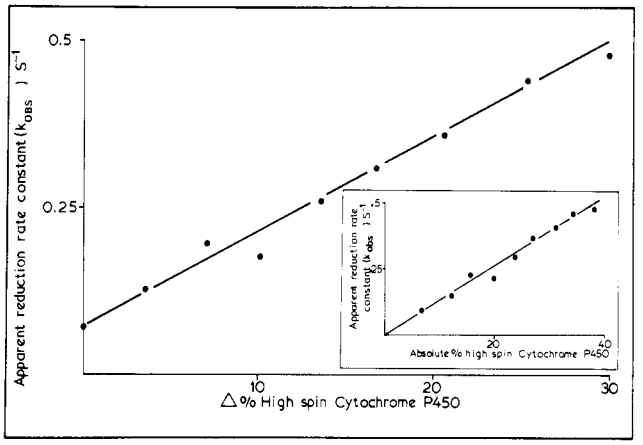


FIGURE 3: Correlation of the apparent fast-phase rate constant for cytochrome P-450 reduction (k_{obsd}) with the hemoprotein high-spin content. The cytochrome P-450 high-spin content was varied by adding increasing concentrations of the substrate benzphetamine (as in Figure 1). The rate constants for fast-phase reduction of cytochrome P-450 were determined at 25 °C under anaerobic conditions as described under Materials and Methods. The inset shows the dependence of the fast-phase rate constant on the absolute cytochrome P-450 high-spin content, determined as described in the text.

fast phase of reduction was considerably lower than when higher levels of benzphetamine were present (Figure 2). As with microsomes (Backes et al., 1982), substrate addition did not cause a change in the slope of the slow phase of reduction. Although such kinetics were suggested as being a property of the reductase (Oprian et al., 1979), no such biphasicity was observed either with cytochrome *c* (data not shown) or with cytochrome b_5 as substrate for the reductase (I. Jansson, personal communication).

Correlation of the Rate of Reduction with the Spin State of Ferric Cytochrome P-450 Heme Iron. Figure 3 shows data strongly suggesting a role for the spin state in modulation of the fast phase of cytochrome P-450 reduction. A plot of the apparent reduction rate constant (k_{obsd}), extrapolated to time zero, vs. the change in the percent high-spin cytochrome produced by the addition of benzphetamine yielded a linear plot. The plot did not go through the origin, indicating the existence of some high-spin cytochrome before the addition of exogenous substrate. Extrapolation to the abscissa suggests the amount to be 6%. The temperature dependence of the spin equilibrium of the cytochrome was therefore determined in order to allow calculation of the amount of high-spin cytochrome P-450 in Figure 3. As seen in Table I, at 25 °C this amount was found to be 7%, in excellent agreement with the extrapolation in Figure 3. Correcting for this preexisting amount of high-spin component (Figure 3, inset) allowed a plot of the apparent initial burst rate constant vs. absolute percent high-spin cytochrome. The resultant plot was linear and passed through the origin.

Table I: Parameters Describing the Substrate-Free Spin Equilibrium of Cytochrome P-450 in the Reconstituted System[a]

temp (°C)	K_{eq}	% high spin cytochrome P-450
15.0	0.042	4.1
20.0	0.058	5.5
25.0	0.079	7.3
30.0	0.106	9.6
35.0	0.141	12.4
40.0	0.186	15.7

[a] The spin equilibrium constant for the reaction $Fe^{3+}_{ls} \rightleftharpoons Fe^{3+}_{hs}$ is given by $K_{eq} = Fe^{3+}_{hs}/Fe^{3+}_{ls}$ characterized by the thermodynamic parameters of ΔH = 10.69 kcal mol^{-1} and ΔS = 30.8 cal deg^{-1} mol^{-1}.

Effect of Temperature upon Cytochrome P-450 Reduction Kinetics. Figure 4A shows the temperature dependence of the apparent fast-phase rate constant (k_{obsd}) in the presence of a saturating concentration of benzphetamine. The temperature dependence of this reaction was not a linear function and exhibited an apparent discontinuity at approximately 20 °C, suggesting that there was more than one component contributing to the overall activation energy profile (Backes et al., 1982). The above experimental observation can be rationalized in terms of the temperature dependence of both the activity of NADPH-cytochrome P-450 reductase itself and the fraction of high-spin cytochrome P-450, the latter being primarily dictated by the enthalpy of the low-spin/high-spin transition

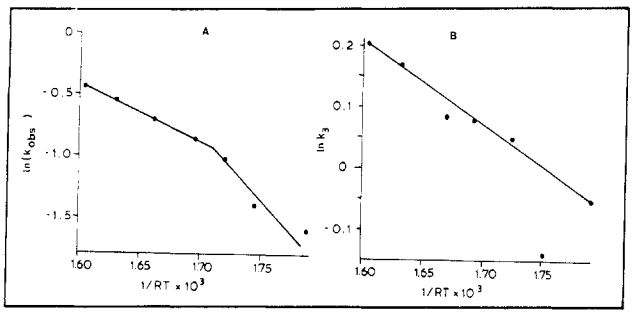


FIGURE 4: Influence of temperature on the fast-phase reduction kinetics of cytochrome P-450. The fast-phase reduction kinetics of purified cytochrome P-450 were obtained by stopped-flow spectrophotometry in the presence of saturating benzphetamine and reductase, as described under Materials and Methods. (A) Temperature dependence of the apparent, experimentally determined fast-phase rate constant (k_{obsd}). (B) Temperature dependence of the cytochrome P-450 fast-phase reduction rate constant corrected for the high-spin content of the hemoprotein (k_3) as described in the text and in Table II.

Table II: Effect of Temperature on Cytochrome P-450 Reduction Kinetics

temp (°C)	k_{obsd} (s^{-1})[a]	fraction high-spin cytochrome P-450[b]	k_3[c]
9.3	0.203	0.22	0.92
15.5	0.244	0.28	0.87
20.0	0.348	0.33	1.06
25.0	0.423	0.39	1.08
34.0	0.490	0.45	1.09
35.0	0.606	0.51	1.19
40.0	0.698	0.57	1.22

[a] k_{obsd} is defined as the apparent rate constant for fast-phase reduction and was determined as the initial gradient of linear portion of the fast phase seen in semilogarthmic analysis, in the presence of saturating levels of benzphetamine. [b] The fraction of high-spin cytochrome P-450 in the reaction $Fe^{3+}_{ls}S \rightleftharpoons Fe^{3+}_{hs}S$ is defined as the ratio of $Fe^{3+}_{hs}S/(Fe^{3+}_{hs}S + Fe^{3+}_{ls}S)$. [c] k_3, which is the true rate constant for reduction of high-spin cytochrome P-450, is defined as the ratio of k_{obsd}/fraction high-spin cytochrome P-450.

of the heme iron. The temperature dependence of the spin state was determined for the cytochrome in the presence of substrate (Table II) as in the absence of substrate (Table I). Accordingly, when the data shown in Table II was used to correct the apparent reduction rate constants for the amount of high-spin hemoprotein present, the temperature dependence of the rate constant (k_3) for fast-phase reduction was obtained, as shown in Figure 4B. It was observed that the apparent temperature dependence of cytochrome P-450 reduction was largely removed, which yielded an activation energy of only 1.45 kcal mol^{-1} as compared to values of about 5.3 kcal mol^{-1} above and 8.3 kcal mol^{-1} below the breakpoint for the two apparent linear components in the uncorrected plot (Figure 4A). In addition, the deviation from linearity was also effectively removed, which implied that the correction for concentration of high-spin cytochrome P-450 was conceptually correct for the deconvolution of this complex process. Similar observations were made with liver microsomes (Backes et al., 1980) where Arrhenius plots of k_{obsd} were nonlinear until corrected for the concentration of high-spin cytochrome in the microsomes. In contrast to the reconstituted system above, the corrected activation energy observed with microsomes was 15 kcal mol^{-1}.

Discussion

The reduction of cytochrome P-450 by NADPH-cytochrome P-450 reductase first requires complex formation between the hemoprotein and reductase followed by electron transfer (Oprian et al., 1979). In the present study, the reduction kinetics of homogeneous cytochrome P-450 were measured at a reductase to cytochrome P-450 value of 2.7/1 and were found not to be monophasic. Higher ratios of the reductase to hemoprotein produced no further changes in the kinetics. Similar observations have been made elsewhere with a rabbit liver isozyme (Oprian et al., 1979). It is clear therefore that any model which attempts to explain these kinetics in terms of differences in the accessibility of cytochrome P-450 subpopulations [e.g., cluster vs. noncluster cytochrome P-450 (Peterson et al., 1976)] to a limiting supply of reductase molecules may be discounted.

It has been suggested that the lack of monophasicity in the reduction kinetics of cytochrome P-450 is not due to the cytochrome but to a property of the reductase (Oprian et al., 1979). However, as discussed by Vermilion et al. (1981), this hypothesis was weakened as no difference was seen when reduction was initiated by the air-stable semiquinone, as compared with fully oxidized reductase (Oprian et al., 1979), indicating that internal electron rearrangement within the reductase flavin groups was probably not the rate-limiting step. Titration to saturation with either the reductase or NADPH also failed to convert the reaction to monophasic kinetics (Oprian et al., 1979), and this could be confirmed in our laboratories. Furthermore, reduction of cytochrome *c* or cytochrome b_5 (I. Jansson, personal communication) by the reductase only yielded monophasic, first-order plots. For these reasons, we suggest that the observed reduction kinetics are probably not a characteristic feature of the reductase.

According to our simplified model, the multiphasic nature of cytochrome P-450 reduction is explained by assuming that the rate of formation of high-spin cytochrome P-450 subsequent to its removal by reduction is slower than the rate of high-spin cytochrome P-450 reduction governed by k_3 (see below). The spin interconversion process was represented by a single-step process wherein the slow phase was controlled by the magnitude of the rate constant for the formation of high-spin cytochrome P-450. In reality, however, temperature-jump experiments suggest that the spin interconversion process of microsomal cytochrome P-450 is at least biphasic, exhibiting relaxation times which are too fast to account for the slow phase of microsomal cytochrome P-450 reduction (Yang & Tsong, 1980).

In order to explain this inconsistency, we have postulated that formation of a reducible high-spin ferric hemoprotein may be comprised of two sequential reactions (Backes et al., 1982):

$$Fe^{3+}_{ls} \overset{\text{fast}}{\rightleftharpoons} Fe^{3+}_{hs} \overset{\text{slow}}{\rightleftharpoons} Fe^{3+}_{hs*} \overset{e^-}{\rightleftharpoons} Fe^{2+}_{hs}$$

where Fe^{3+}_{hs} and Fe^{3+}_{hs*} are spectrally indistinguishable. Rein et al. (1979) and Sligar have also postulated such an intermediate to explain the anomalies in the published spin-state–redox correlations in cytochrome P-450 (Sligar et al., 1979). In the model of Rein et al. (1979), the slow process is suggested to represent a change from a hexacoordinate to a pentacoordinate heme iron. Similarly, the observation that the rate of formation of the substrate-induced, type I spectral change of purified rabbit liver cytochrome P-450 represents an extremely rapid reaction (Smettan et al., 1978) would also seem inconsistent with the interpretation of the kinetic data reported herein.

However, it does not necessarily follow that a substrate-induced spin-state change is kinetically the same as a spin-state change produced by either temperature perturbation or ferric hemoprotein removal. Thus, the spin interconversion of pu-

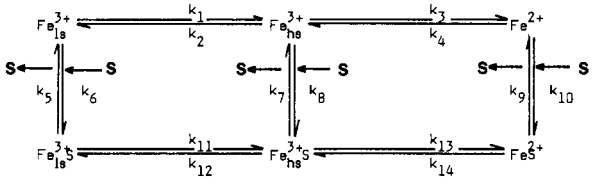
rified, phenobarbital-induced, rat liver microsomal cytochrome P-450 studied in the presence of benzphetamine using temperature-jump techniques has been shown to be slower than the substrate-induced spin-state change (Tsong & Yang, 1978). The biphasic nature of this process observed by these latter authors is consistent with the involvement of at least two sequential equilibria as postulated by Backes et al. (1982). Preliminary temperature-jump studies on the isozyme isolated in our laboratory and used in the present studies indicates similar complexities in the spin interconversion reaction. Thus, in the presence of benzphetamine, at least four relaxation rates were observed occurring in the dead time of the instrument, in 50 ms, in 250 ms, and in seconds. The interpretation of such data in terms of the sequential reactions model for anaerobic hemoprotein reduction will depend upon whether the kinetically distinct phases of the spin interconversion process correspond to different conformational states of cytochrome P-450 which are spectrally indistinguishable, the way in which such states are interrelated (e.g., whether the component interconversions occur sequentially or in parallel), and which of these states (if not all of them) are directly reducible by NADPH-cytochrome P-450 reductase.

Data in support of the simplified model has been obtained in our laboratory (Backes et al., 1982), where a linear correlation was observed between the rate of fast-phase reduction and the spin shift produced by preequilibration of microsomal cytochrome P-450 with a series of structurally dissimilar type I compounds. These results have since been confirmed elsewhere from a study of the interaction of rabbit liver microsomal cytochrome P-450 with a series of benzphetamine analogues (Blanck et al., 1983). However, analysis of the above microsomal data is complicated by the fact that hepatic microsomes exhibit a reductase to cytochrome P-450 ratio of approximately 1/10 and contain more than one isozyme of cytochrome P-450. In the present study, a linear correlation was demonstrated for the first time between the spin state and the rate of first-electron reduction of a homogeneous cytochrome P-450 isozyme, functionally reconstituted with a saturating concentration of reductase. This result more clearly demonstrates the role of the hemoprotein spin state in determining the rate of electron transfer to cytochrome P-450.

In contrast to the above observations, Kominani & Takemori (1982) failed to observe a correlation between the hemoprotein spin state and reduction rate of purified cytochrome $P\text{-}450_{21}$ from bovine adrenal cortex when measured in the presence and absence of progesterone and 17α-hydroxyprogesterone. While interesting, these experiments cannot be compared with the present study as they were performed in a reconstituted system devoid of phospholipid and containing 0.5% emulgen. Under such conditions, it is likely that the interactions between the functional protein components may be altered, consequently altering the mechanism and rate of electron transfer. In addition, nonionic detergents have been shown to affect the spin state of cytochrome P-450 (Denk, 1979). It is also apparent that these authors did not measure the spin state and reduction rate of cytochrome P-450 under strictly comparable conditions.

Further data consistent with our hypothesis of spin-state control of cytochrome P-450 reduction was provided from a study of the temperature dependence of the fast phase of reduction. Thus, a break in the Arrhenius plot of the initial reduction rate constant (k_{obsd}) for cytochrome P-450 at different temperatures was observed, which suggested that more than one process was influencing the apparent fast-phase rate constant. Since the spin equilibrium of cytochrome P-450 is temperature dependent, correction was made for the different amounts of ferric high-spin cytochrome P-450 at the different temperatures. This resulted in a linear Arrhenius plot providing additional support for the concept (Backes et al., 1980, 1982) that the initial rate of the fast phase is governed by the amount of high-spin cytochrome; in earlier studies with liver microsomes (Backes et al., 1980, 1982) similar rate constants both in the presence and in the absence of substrate at any temperature were obtained. This indicates that the primary effect of substrate on the reduction kinetics is on the spin-state equilibrium. In a similar manner, Arrhenius plots of the initial rate constant for cytochrome P-450 reduction in microsomes at different temperatures were nonlinear (Backes et al., 1982). Correcting the apparent rate constant for the amount of high-spin cytochrome also yielded a straight line (Backes et al., 1980) with an activation energy of 15 kcal mol^{-1} for the reductase with cytochrome P-450 as its substrate. With the purified, reconstituted system, however, the activation energy for the reduction of cytochrome P-450 by NADPH-cytochrome P-450 reductase was only 1.45 kcal mol^{-1}, a value substantially lower than with microsomes.

Equations have been developed (see introduction) to express our hypothesis in mathematical terms. These equations would apply both in the presence and in the absence of saturating substrate. At subsaturating levels of substrate, the model must be expanded to include all possible states as follows:



where the preequilibrium conditions prior to the addition of reducing equivalents are given by

$$k_2[Fe^{3+}_{hs}] = k_1[Fe^{3+}_{ls}] \quad (1)$$

$$k_5[Fe^{3+}_{ls}] = k_6[Fe^{3+}_{ls}][S] \quad (2)$$

$$k_{12}[Fe^{3+}_{hs}S] = k_{11}[Fe^{3+}_{ls}S] \quad (3)$$

$$k_7[Fe^{3+}_{hs}S] = k_8[Fe^{3+}_{hs}][S] \quad (4)$$

and

$$[E]_t = [Fe^{3+}_{ls}] + [Fe^{3+}_{hs}] + [Fe^{3+}_{ls}S] + [Fe^{3+}_{hs}S] \quad (5)$$

$[E]_t$ is the total enzyme and k_5 to k_{14} are the appropriate rate constants to account for the presence of substrate.

Under the assumption that only high-spin species of the cytochrome are directly reduced, then the initial rate of reduction (V_{obsd}) would be

$$V_{obsd} = k_3[Fe^{3+}_{hs}] + k_{13}[Fe^{3+}_{hs}S] \quad (6)$$

The assumption is made that the ferrous substrate-bound and substrate-free forms are spectrally indistinguishable. In a similar manner, the ferric low-spin, substrate-bound and substrate-free states are spectrally indistinguishable, as are the corresponding high-spin species. The rate constants for reduction (k_3 and k_{13}) are larger than those associated with the shift in the spin equilibrium (k_1 and k_{11}), and prior to the addition of reducing equivalents, the four ferric species are in a preequilibrium.

An equation describing the relationship of the initial rate of reduction (V_{obsd}) to the substrate concentration (S) results

by substituting eq 1–5 into eq 6:

$$k_{obsd} = \frac{V_{obsd}}{[E]_t} = \frac{k_3k_1/k_2 + k_{13}k_{11}k_6[S]/(k_{12}k_5)}{1 + k_1/k_2 + k_6[S]/k_5 + k_{11}k_6[S]/(k_{12}k_5)} \quad (7)$$

Under the limits described by this equation, in the absence of substrate ([S] = 0), the above equation simplifies to

$$k_{obsd} = \frac{V_{obsd}}{[E]_t} = k_3\left(\frac{Fe^{3+}_{hs}}{Fe^{3+}_{ls} + Fe^{3+}_{hs}}\right) = k_3f_{hs} \quad (8)$$

where the term f_{hs} represents the fraction of high-spin cytochrome P-450 in the absence of substrate.

In the presence of saturating amounts of substrate ([S] $\rightarrow \infty$), then the resultant expression (from eq 7) simplifies to

$$k_{obsd} = \frac{V_{obsd}}{[E]_t} = \frac{k_{13}[Fe^{3+}_{hs}S]}{[Fe^{3+}_{ls}S] + [Fe^{3+}_{hs}S]} = k_{13}f_{hss} \quad (9)$$

where f_{hss} equals the fraction of high-spin cytochrome P-450 in the presence of saturating substrate.

According to eq 7, a linear relationship with the fraction of high-spin cytochrome is not obtained if k_3 does not equal k_{13}. However, under the condition where $k_3 = k_{13}$, eq 7 simplifies to

$$k_{obsd} = \frac{V_{obsd}}{[E]_t} = k_3\left(\frac{Fe^{3+}_{hs} + Fe^{3+}_{hs}S}{Fe^{3+}_{ls} + Fe^{3+}_{hs} + Fe^{3+}_{ls}S + Fe^{3+}_{hs}S}\right) = k_3f_{hs}^*$$

where f_{hs}^* is the fraction of the cytochrome present in the high-spin state *at any substrate concentration.*

In this model, if the rate constants k_3 and k_{13} are equal (CO binding of the reduced species essentially eliminates the effect of k_4 and k_{14}), the only effect of substrate on the reduction kinetics will be seen as an increase in the initial rate in proportion to the increase in the total amount of high-spin cytochrome. The data in Figure 3 suggest that this is the case, since at zero substrate levels some high-spin cytochrome still exists and there is a corresponding burst. Determination of the amount of high-spin cytochrome P-450 in the absence of substrate from temperature studies (Table I) indicates that this relates to the initial rate of reduction, just as the substrate-induced high-spin species does, also fitting the linear plot (Figure 3, inset). From the absolute dependence of k_{obsd} on the fraction of high-spin cytochrome P-450 (Figure 3, inset), it would appear that the direct reduction of low-spin cytochrome does not significantly contribute to the initial rate of reduction of the hemoprotein.

The data presented in this paper clearly show that the influence of substrate on the reduction kinetics of ferric cytochrome P-450 is secondary to its effects on the spin equilibrium of the hemoprotein.

Registry No. Fe, 7439-89-6; cytochrome P-450, 9035-51-2; NADPH-cytochrome P-450 reductase, 9039-06-9; monooxygenase, 9038-14-6.

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Gonadotropin Modulation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity in Desensitized Luteinized Rat Ovary†

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ABSTRACT: These studies were done to examine the effect of gonadotropin on rat luteal 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity (the rate-limiting step in cholesterol biosynthesis) in ovaries of pregnant mare's serum gonadotropin (PMSG)–human chorionic gonadotropin (hCG) primed rats. Administration of hCG stimulated HMG CoA reductase activity in a time- and dose-dependent manner: significant increases were noted within 4 h, with maximum effects (30–40-fold increases) seen 24 h after hCG (25 IU) administration. This effect was specific in that only LH, of several hormones tested, was as effective as hCG in stimulating HMG CoA reductase activity, and no change in the activity of either liver microsomal HMG CoA reductase or luteal microsomal NADPH-cytochrome *c* reductase was seen after hCG. The gonadotropin-induced increase in HMG CoA reductase activity seemed to be due to a net increase in enzyme activity, not to a change in the phosphorylated/dephosphorylated state of the enzyme. Pretreatment of animals with aminoglutethimide, an inhibitor of the conversion of cholesterol to steroid (pregnenolone), prevented the hCG-induced rise in HMG CoA reductase activity, whereas treatment with 4-aminopyrazolo[3,4-*d*]pyrimidine (4-APP), which depletes cellular cholesterol content, led to striking increases in enzyme activity. However, the combined effects of 4-APP and hCG were additive, suggesting that the stimulating effect of hCG on HMG CoA reductase activity is not entirely due to a depletion of cellular sterol content of luteinized ovaries. Similarly, cholesteryl ester and cholesterol syntheses as measured by [^{14}C]acetate conversion were also increased by hCG and 4-APP treatment. Furthermore, hCG compared to 4-APP was the preferred stimulator of cholesteryl ester, and combined treatments resulted in synergistic action. In summary, hCG stimulates HMG CoA reductase activity in luteinized ovaries. This effect appears to be tissue, hormone, and enzyme specific and not entirely as a function of a reduction in plasma or tissue cholesterol concentrations. Thus, gonadotropin regulation of ovarian function does not seem to be limited to steroidogenesis but also involves profound effects on cellular cholesterol metabolism.

Several recent reports have raised the possibility that disturbances in intracellular sterol metabolism may be involved in the process by which rat luteal cells become "desensitized" to gonadotropin stimulation. Thus, there is mounting evidence that rat luteal cells preferentially utilize high density lipoprotein (HDL) derived cholesterol for steroidogenesis (Azhar & Menon, 1981; Azhar et al., 1981; McNamara et al., 1981; Schuler et al., 1981a,b; Brout et al., 1982; Gwynne & Strauss, 1982), and it appears that a substantial fall in intracellular cholesteryl ester content occurs early in the development of human chorionic gonadotropin (hCG) induced desensitization (Azhar et al., 1983). Furthermore, it has been shown that the decline in steroidogenesis seen in luteinized ovaries can be overcome, at least early in the process, by the addition of HDL. The present study was undertaken in an effort to define further the changes in sterol metabolism that take place in hCG-desensitized luteal cells and has focused on defining the effects of hCG on HMG CoA reductase activity, the rate-limiting enzyme in cholesterol biosynthesis. The results to be presented include that HMG CoA reductase activity is increased approximately 30–40-fold in hCG-desensitized luteal cells. This effect is tissue, enzyme, and hormone specific and does not appear to be a simple function of the intracellular depletion of cholesteryl ester that is associated with hCG-induced desensitization.

Experimental Procedures

Materials

Purified human chorionic gonadotropin (CR-121; biopotency 13 450 IU/mg) was kindly provided by Dr. R. E. Canfield, College of Physicians and Surgeons of Columbia University, New York, NY, through the Center for Population Research of the National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD. Ovine luteinizing hormone (o-LH NIAMDD-OLH-22; biopotency 9 units/mg) and rat prolactin (NIAMDD-rat prolactin-B-2; biopotency 20 IU/mg) were gifts from Pituitary Hormone Distribution Program, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD. The following chemicals were supplied by Sigma Chemical Co., St. Louis, MO: DL-3-hydroxy-3-methylglutaryl-CoA; DL-mevalonic acid lactone; cytochrome *c*; glucose-6-phosphate dehydrogenase (240 units/mg of protein; product no. G-4134); cholesterol; cholesteryl oleate;

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