Kinetics of Hepatic Cytochrome P-450 Reduction: Correlation with Spin State of the Ferric Heme†

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ABSTRACT: The reduction kinetics of cytochrome P-450 are known to be biphasic, with a rapid initial phase and a slower subsequent phase, both of which appear linear in semilogarithmic plots. The present report demonstrates that these biphasic reduction kinetics can be described in terms of a preequilibrium between high- and low-spin ferric states. Computer simulations are used which express the rate and extent of the fast phase or burst as being due to the initial proportion of high-spin cytochrome P-450. According to this simplified sequential model, the slow phase of reduction is controlled by the rate of formation of high-spin cytochrome P-450. The substrate-induced alterations in the reduction kinetics are likewise consistent with the model, which indicates that type I compounds exert their effect by virtue of a decrease in the rate constant controlling the shift from high-spin to low-spin ferric cytochrome. The model is further supported by the influence of temperature on the spin equilibrium and reduction kinetics. Potential influences of other intermediate steps in the reduction and assumptions in the hypothesis are described.

One of the initial steps in the reaction sequence of hepatic cytochrome P-450 is one-electron reduction of the hemoprotein (Peterson et al., 1977). This reductive process has generally been studied anaerobically in the presence of carbon monoxide, and the reduction kinetics have been described as two simultaneous first-order processes (Gigon et al., 1969). To explain their results, these investigators proposed that the two phases of reduction are due to substrate-bound (fast phase) and substrate-free (slow phase) cytochrome P-450. Other investigators have suggested that the reduction kinetics can be described as simultaneous first- and second-order processes (Diehl et al., 1970) and four simultaneous first-order processes (Ruf, 1980). Peterson et al. (1976) proposed a model for the biphasic reduction kinetics wherein 8–10 cytochrome P-450 molecules clustered around a reductase molecule produced the fast phase of reduction. Cytochrome P-450 molecules that were not part of the cluster would then produce the slow phase of reduction by subsequent association with reductase after lateral diffusion through the microsomal membrane. Other investigators (Taniguchi et al., 1979; Gander & Mannering, 1980), however, suggested that reductase and cytochrome P-450 do not form functional clusters but interact through random collision of the two protein components.

Recent reports from our laboratories (Sligar, 1976; Pierson & Cinti, 1977; Cinti et al., 1979; Gibson et al., 1980) and other laboratories (Rein et al., 1977; Ristau et al., 1978; Lange et al., 1977) have shown cytochrome P-450 species from different sources to be capable of undergoing temperature-dependent spin transitions, as well as substrate-dependent spin transitions (Schenkman et al., 1967; Sligar, 1976; Waterman et al., 1973; Jansson et al., 1980). Further studies on the spin equilibrium have shown it to markedly influence the redox potential of the cytochrome (Sligar, 1976; Sligar et al., 1979).

This report extends these previous studies with respect to the influence of spin state on the reduction kinetics of hepatic cytochrome P-450. The results indicate that in the presence of carbon monoxide the anaerobic reduction of cytochrome P-450 can be described by two sequential first-order processes which result in "burst" kinetics. The results are consistent with the hypothesis that the spin state of cytochrome P-450 controls its reduction.

Materials and Methods

Male Sprague-Dawley CD rats (Charles River Breeding Laboratories) were maintained on laboratory chow (Purina) and water ad libitum. Rats (200–250 g) were decapitated, and the livers were removed and perfused with ice-cold 0.9% NaCl to remove blood. Microsomal fractions were prepared by differential centrifugation using a rapid calcium aggregation technique (Cinti et al., 1972) and then were washed with 0.15 M KCl. The washed microsomes were suspended in 90 mM sodium phosphate buffer, pH 7.5. Spectra were recorded in an Aminco DW-2 split beam/recording spectrophotometer in either the dual-wavelength or dual-beam mode. Temperature was maintained by a Lauda K-2R circulating water bath connected to the cuvette chamber and was monitored by using a YSI temperature probe directly in the cuvette. The cytochrome P-450 in the microsomes used in this study exhibited 100% temperature sensitivity, as determined by the method of Cinti et al. (1979).

Cytochrome P-450 reduction kinetics were measured by dual-wavelength difference spectroscopy (450 nm minus 490 nm) in the spectrophotometer using Aminco anaerobic cells (American Instrument Co.). The cells were gassed with deoxygenated carbon monoxide for 2 min and brought into temperature equilibration for 5 min. The final concentrations of the assay components were 6.7 mM glucose (Mallinckrodt), 13 units/mL glucose oxidase (Sigma Type II), 590 units/mL catalase (Sigma C-30), 90 mM sodium phosphate (Baker) buffer, pH 7.5, and 1 mg of microsomal protein/mL. Anaerobiosis was maintained under carbon monoxide by using an oxygen scavenger consisting of glucose, glucose oxidase, and catalase as described earlier (Jansson & Schenkman, 1978). The final volume of the assay was 3.0 mL, and the reaction was initiated by the addition of 30 μL of 30 mM NADPH (Sigma C-30; final concentration 0.3 mM) in the plunger of the anaerobic cell. Binding of carbon monoxide to the ferrous hemoprotein was not rate limiting under con-

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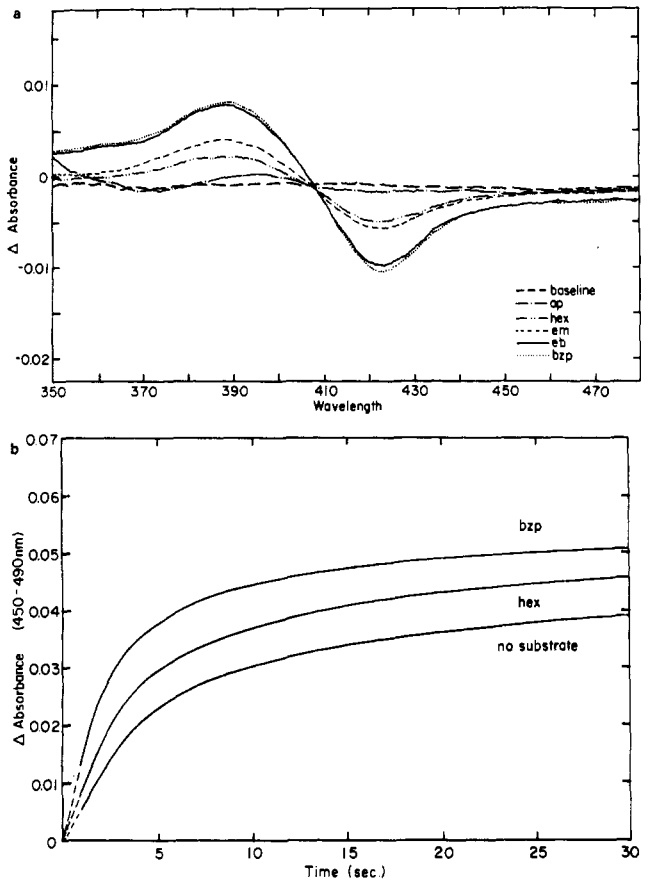

FIGURE 1: (a) Substrate-induced spectral changes by a variety of type I substrates; 0.33 mM hexobarbital, 1.67 mM aminopyrine, 0.83 mM ethylmorphine, 1.5 mM ethylbenzene, and 0.33 mM benzphetamine. The cytochrome P-450 concentration was 0.71 nmol/mg of protein, and the protein concentration was 1 mg of protein/mL. The temperature of the assay was 25 °C. (b) The time course for reduction of cytochrome P-450 without substrate addition and after the addition of 0.33 mM hexobarbital and 0.33 mM benzphetamine. The protein concentration was 1 mg of protein/mL. Assay conditions were as in (a), containing the deoxygenation medium described under Materials and Methods.

ditions used in this study (Omura et al., 1965).

Mathematical modeling of the reduction of cytochrome P-450 was carried out by using a North Star Horizon 2 microcomputer for numerical integrations. Values for the rate constants and starting concentrations of the cytochrome in high- and low-spin states according to the following model were input:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} C$$

where A,[1] B, and C represent all low spin oxidized, all high spin oxidized, and reduced cytochrome P-450, respectively. An integration constant of 0.01 s (50 times smaller than the fastest rate constant for k_3) was used for the numerical integration. The computer program will be supplied on request.

Results

The addition of various substrates to cytochrome P-450 has been shown to produce a reversible shift in the wavelengths

[1] Abbreviations: A, low spin oxidized cytochrome P-450; B, high spin oxidized cytochrome P-450; C, reduced cytochrome P-450; K_{eq}, the spin equilibrium constant ([HS]/[LS]); cytochrome P-450$_{cam}$, cytochrome P-450 isolated from *Pseudomonas putida*; cytochrome P-450$_{LM4}$, cytochrome P-450 isolated from β-naphthaflavone-treated rabbits; FpT, NADPH–cytochrome P-450 reductase; k_{burst}, the observed rate constant for the initial phase of reduction; k_s, the observed rate constant for the slow phase of reduction.

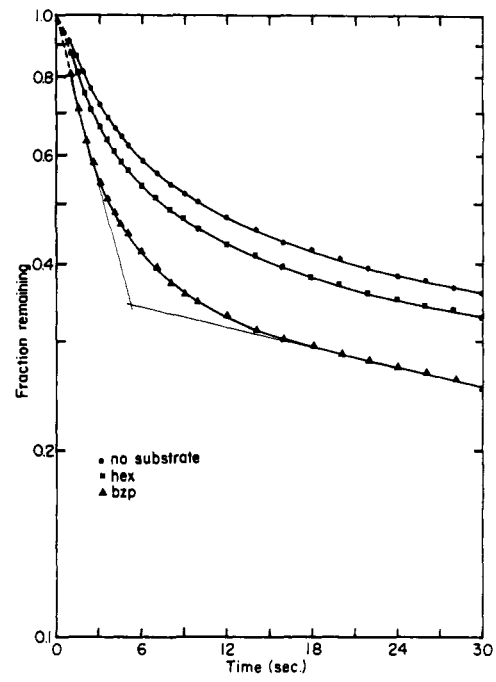

FIGURE 2: First-order plot of the reduction of cytochrome P-450. Conditions are as in Figure 1.

of absorption of the hemoprotein (Schenkman et al., 1967) which has been correlated with a shift in the spin equilibrium by using mammalian (Mitani & Horie, 1969) and bacterial cytochrome P-450 (Peterson, 1971; Waterman et al., 1973; Tsai et al., 1970). The addition of hexobarbital, benzphetamine, aminopyrine, ethylmorphine, or ethylbenzene (Figure 1a) to microsomes produces type I spectral changes indicative of shifts in the spin equilibria from the predominantly low spin to favor the high-spin state. The differences in the magnitude of the spectral changes with the various substrates reflect the differing abilities of different substrates to perturb the equilibrium between high- and low-spin forms of the cytochrome. In general, substrates for the P-450 hemoprotein can influence both the extent and the direction of the spin shift (Jansson et al., 1980).

Addition of NADPH to anaerobic microsomes under a carbon monoxide atmosphere resulted in reduction kinetics which cannot be described as a simple first-order process (Figures 1b and 2). At 25 °C, reduction proceeds in a burst which is essentially complete within 15 s, followed by a much slower second phase. When describing the biphasic reduction kinetics of cytochrome P-450, three parameters were measured: The rate of the fast burst phase was estimated as the initial rate of reduction measured from a first-order plot (Figure 2). The rate of the slow phase was taken as the rate determined at longer times. Finally, the *extent* of the slow and fast phases was estimated from the intersection of extrapolated burst and slow phases for reasons to be described in detail later.

The effect of perturbation of the spin equilibrium on the reduction of cytochrome P-450 with the different substrates used in Figure 1 is shown in Figure 2. Each of the substrates increased the initial rate of the burst and its extent, but none changed the slope of the slow-phase reduction. When the initial rates of the fast phase were divided by the extents of the respective bursts, the same rate constant was obtained (0.25 s^{-1}), independent of the substrate present. The spectral changes obtained from the hemoprotein titration with various type I substrates (Figure 1a) were plotted vs. the extent of the burst (obtained from Figure 2). The results, shown in Figure 3, demonstrate a correlation between the extent of the burst and

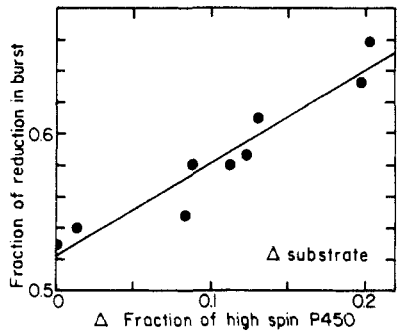

FIGURE 3: Correlation between substrate-induced spectral change and the fraction of cytochrome P-450 reduction in the burst. From left to right the points represent the following: (1) no substrate, (2) 1.7 mM aminopyrine, (3) 0.33 mM hexobarbital, (4) 3.3 μM benzphetamine, (5) 0.83 mM ethylmorphine, (6) 8.3 μM benzphetamine, (7) 17 μM benzphetamine, (8) 1.5 mM ethylbenzene, and (9) 0.33 mM benzphetamine. Conditions are as in Figure 1. For the determination of the Δ fraction of high-spin P-450, an extinction coefficient of 126 mM^{-1} cm^{-1} was used for the 387–421-nm wavelength pair, as determined by the convergence fit of Cinti et al. (1979).

the magnitude of the spin shift seen with microsomal cytochrome P-450 on substrate binding. These data suggest that the substrate-mediated increase in the rate of the burst is simply a reflection of a shift in the spin equilibrium of the ferric hemoprotein.

Another method of perturbing the spin equilibrium of hepatic cytochrome P-450 is through temperature variation. As shown by Cinti et al. (1979) with hepatic microsomal cytochrome P-450, an increase in temperature causes an increase in the amount of high spin cytochrome. In Figure 4 is shown a plot of the amount of high-spin cytochrome P-450 [taken from van't Hoff plots, as in Cinti et al. (1979)] at different temperatures vs. the extent of the observed burst in the reduction kinetics. A linear relationship was obtained with a slope of approximately unity. These results provide further support of the suggestion of spin-state control of cytochrome P-450 reduction (Backes et al., 1980).

The effect of temperature on the rate of the fast, k_{burst}, and slow, k_s, phase is shown in Figure 5. Arrhenius plots of the inital extrapolated rate constants of reduction in both the presence and absence of the type I substrate benzphetamine are shown in Figure 5a. In both cases the temperature-dependence plots were concave downward, indicating that perhaps the observed reduction rate constant, k_{burst}, actually represents a complex function. From the results presented in Figure 4, which demonstrate that both the spin state of cytochrome P-450 and the extent of the burst are increased with increasing temperature, and because increasing temperature also increases the activity of the reductase toward a number of electron acceptors (Schenkman, 1972), the results in Figure 5a may be an expression of these effects, i.e., a composite of temperature effects on both spin state and activity of the reductase. So that this hypothesis could be tested, the data in Figure 5a were corrected for the influence of temperature on the spin equilibrium by dividing k_{burst} by the extent of the burst. The resulting values, when plotted in the Arrhenius manner (Figure 5b), yielded a linear relationship. In addition, the differences in the rate constants due to the presence of benzphetamine (open circles) were largely eliminated (Figure 5b). Similar results were obtained with aminopyrine and hexobarbital (not shown). The slopes of the lines were 14.5 kcal/mol both in the presence and in the absence of benzphetamine and represent the activation energy for NADPH–cytochrome P-450 reductase reducing high-spin cytochrome P-450. In Figure 5c is shown the temperature dependence of

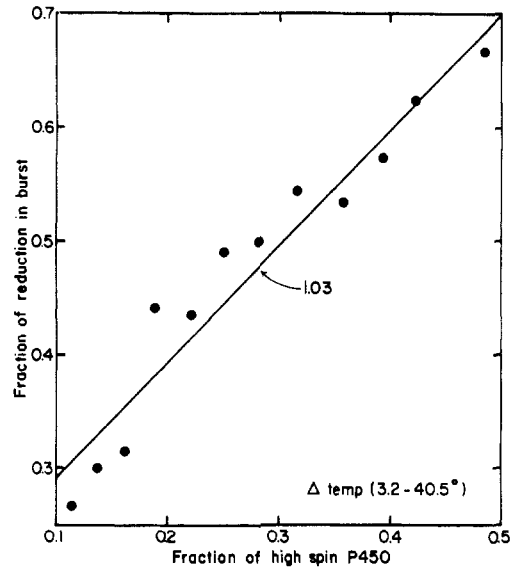

FIGURE 4: Correlation between fraction of high spin cytochrome P-450 and fraction of cytochrome P-450 reduction in the burst. The amount of high spin was altered by variation of temperature. From left to right the temperatures were 3.2, 6.8, 10.3, 13.7, 17.4, 20.6, 24, 27.4, 31.2, 34.4, 37.3, and 40.5 °C. The assays used 1 mg of microsomal protein/mL containing 0.99 nmol of P-450/mg of protein. This preparation exhibited 100% temperature sensitivity and an extinction coefficient of 126 mM^{-1} cm^{-1} as determined by the method of Cinti et al. (1979).

the rate constant of the slow phase of cytochrome P-450 reduction. This Arrhenius plot is linear, uninfluenced by the presence of type I substrates, and has an energy of activation of 16.8 kcal/mol.

Up to this point, we have shown that the spin state of cytochrome P-450 can be correlated with the extent and velocity of reduction of the hemoprotein. A simplified mathematical model utilizing these observations and the current understanding of the spin state of cytochrome P-450 has been developed (Backes et al., 1980). According to this model, cytochrome P-450 reduction is the result of two sequential first-order reactions

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} C$$

where A, B, and C represent all low-spin ferric, high-spin ferric, and ferrous states of cytochrome P-450, respectively.

The differential equations describing the above scheme are

$$d[A]/dt = k_2[B] - k_1[A] \quad (1)$$

$$d[B]/dt = k_4[C] + k_1[A] - (k_2 + k_3)[B] \quad (2)$$

$$d[C]/dt = k_3[B] - k_4[C] \quad (3)$$

The equilibrium between A and B represents the spin equilibrium between low- and high-spin ferric states which exist before the addition of reducing equivalents in the form of NADPH. On addition of NADPH the reduction reaction is initiated, and the concentration of the hemoprotein in the high-spin state is rapidly diminished. Since the concentration of reduced cytochrome at time zero is negligible, eq 3 simplifies to

$$d[C]/dt = k_3[B] \quad (4)$$

which describes the initial burst of reduction. The initial velocity of reduction thus depends upon the amount of high-spin cytochrome P-450 present, due to preequilibrium, and

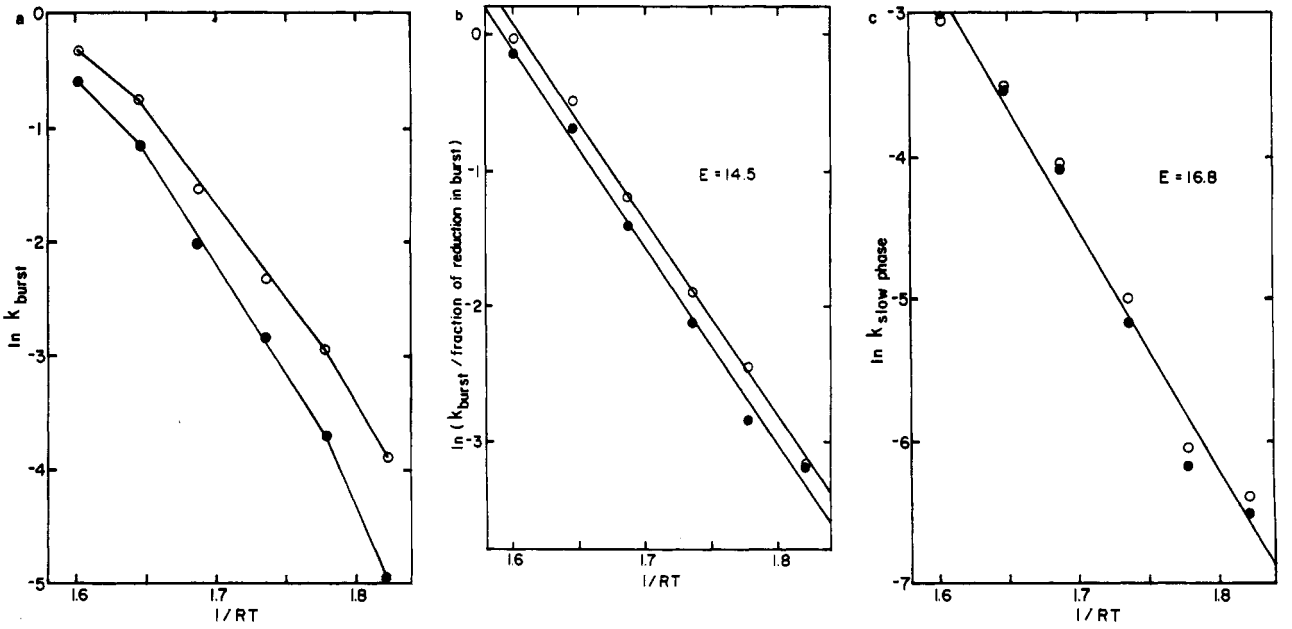

FIGURE 5: (a) Arrhenius plot for the burst rate of cytochrome P-450 reduction. The assays used 1 mg of microsomal protein/mL in Aminco anaerobic cells containing 0.97 nmol of P-450/mg of microsomal protein. Closed circles and open circles represent absence and presence of 0.37 mM benzphetamine, respectively. (b) Arrhenius plot for the burst rate correction for temperature-dependent changes in the spin state of cytochrome P-450. (c) Arrhenius plot for the slow phase of reduction of cytochrome P-450.

upon the rate constant k_3. The initial burst perturbs the equilibrium, and this causes low-spin cytochrome to shift to the high-spin state, which can subsequently become reduced. This sequence could explain the two phases of reduction under conditions where k_3 is larger than k_1 and k_2.

Implicit in the above model are certain assumptions. (1) Only cytochrome P-450 in the high-spin state can be directly reduced; low-spin cytochrome P-450 is reducible only on conversion to the high-spin configuration. This assumption is reasonable, since the redox potential of the high-spin cytochrome extrapolates to −175 mV while that of the low-spin state approaches −400 mV (Sligar et al., 1979). Further, ferrocytochrome P-450$_{cam}$ has been shown to be virtually completely high spin by Mossbauer spectroscopy (Sharrock et al., 1973) and magnetic susceptibility (Champion et al., 1975). From the similarity of magnetic circular dichroism spectra (Dawson et al., 1978) of hepatic ferrocytochrome P-450 to those of bacterial ferrocytochrome P-450, it was concluded the former must also exist in a high-spin ($s = 2$) configuration. From these considerations it is clear that in order to reach the high-spin ferrous state, low-spin ferric P-450 must pass through an intermediate state (Rein et al., 1979). (2) A preequilibrium between low- and high-spin ferric P-450 exists, described by the equilibrium constant $K_{eq} = k_1/k_2$. (3) Substrate affects the reduction kinetics solely by influencing the spin equilibrium. Although the rate and extent of reduction of the cytochrome are increased or decreased by substrates (Gigon et al., 1969; Schenkman, 1972), these compounds exert their effects before addition of reducing equivalents. Spin equilibrium shifts were shown to influence the redox potential of the cytochrome, and the latter was linearly related to the amounts of high- and low-spin configurations regardless of whether substrate was present or absent (Sligar et al., 1979). (4) The reduction of cytochrome P-450 is a pseudo-first-order reaction with respect to reductase concentration; i.e., the reduced reductase concentration is essentially unchanged throughout the reaction.

The feasibility of this model was tested by variation of the parameters in eq 1–3, using the program described under Materials and Methods, and by synthesizing theoretical curves for comparison with kinetic data. The effects of the rate constants k_1 and k_2 on the reduction kinetics are shown in Figure 6a. For this series of plots, both k_1 and k_2 were varied in a manner such that their ratio (K_{eq}) was not changed; K_{eq} was set equal to 1, and k_4 was set at 0 for these simulations. The resultant plots were biphasic with the burst rate not changing as k_1 and k_2 were varied. The slow-phase rate constant was, however, influenced markedly by variation of these constants, increasing as they increase and decreasing as they decrease. Extrapolating the slow phases to zero time caused them to intersect at a common point (solid circle) which was empirically observed to be equal in value (ordinate) to the amount of low-spin cytochrome. When k_1 was equal to k_3, the biphasicity of the plots disappeared. In this study the intercept of the burst and slow-phase extrapolations has been used as an estimate of the fraction of low-spin cytochrome P-450. The use of this estimate was based upon empirical observation of kinetic data as well as the results in Figure 6a. The latter results indicate the intercept of the slow phases is equal to the fraction of low-spin cytochrome P-450 at initiation of the reaction. However, since the slow phase is not readily varied alone, experimentally, the intercept of burst and slow-phase extrapolations was chosen as the next best measurement, giving a more accurate estimation of the fraction of low-spin cytochrome than extrapolation of the slow-phase slope to the ordinate.

When k_4 was given a finite value in the computer simulation instead of being set at 0, a deviation from linearity was encountered in the slow phase (Figure 6b) due to the approach of the system to equilibrium. At any k_4 value (even very small ones), the greater the amount of reduced cytochrome at a given time the earlier deviation from linearity occurs and the more difficult to extrapolate back to a common intersect point on the burst extrapolation. In all instances (Figure 6), as both k_1 and k_2 increased the slow phase rate increased.

The influence of the individual constants k_1 and k_2 on the observed reduction velocity was then examined by simulation. As seen in Figure 7a, increasing the value of k_1 at constant values of k_2 increased the rates of both the burst and slow phase and the extent of reduction in the burst. Since the ratio

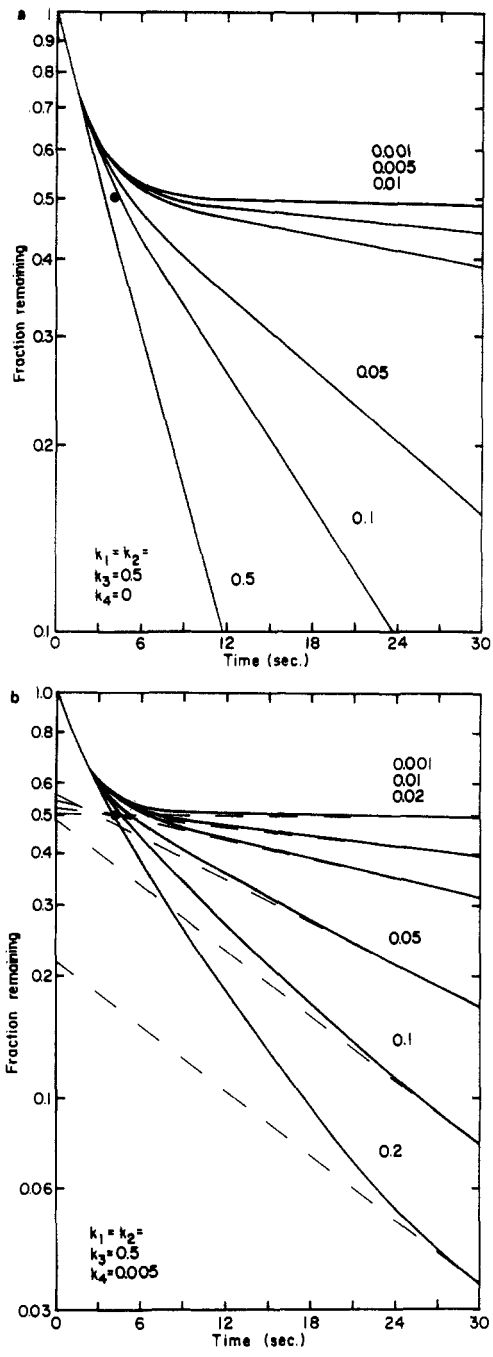

FIGURE 6: (a) Computer simulation of the effect of k_1 and k_2 on the rate of reduction. These rate constants were varied in a manner such that K_{eq} was unchanged ($K_{eq} = 1$). The rate constants were $k_3 = 0.5$ and $k_4 = 0$; k_1 and k_2 were varied from 0.001 to 0.5 as shown on curves. The closed circle represents the intersection of the slow-phase extrapolations and is equal to the fraction of low-spin cytochrome. (b) Effect of k_1 and k_2 in the presence of a small value for k_4. The rate constants k_3 and k_4 were 0.5 and 0.005, respectively; k_1 and k_2 were varied from 0.001 to 0.2 as shown on the curves. All other conditions are described in (a).

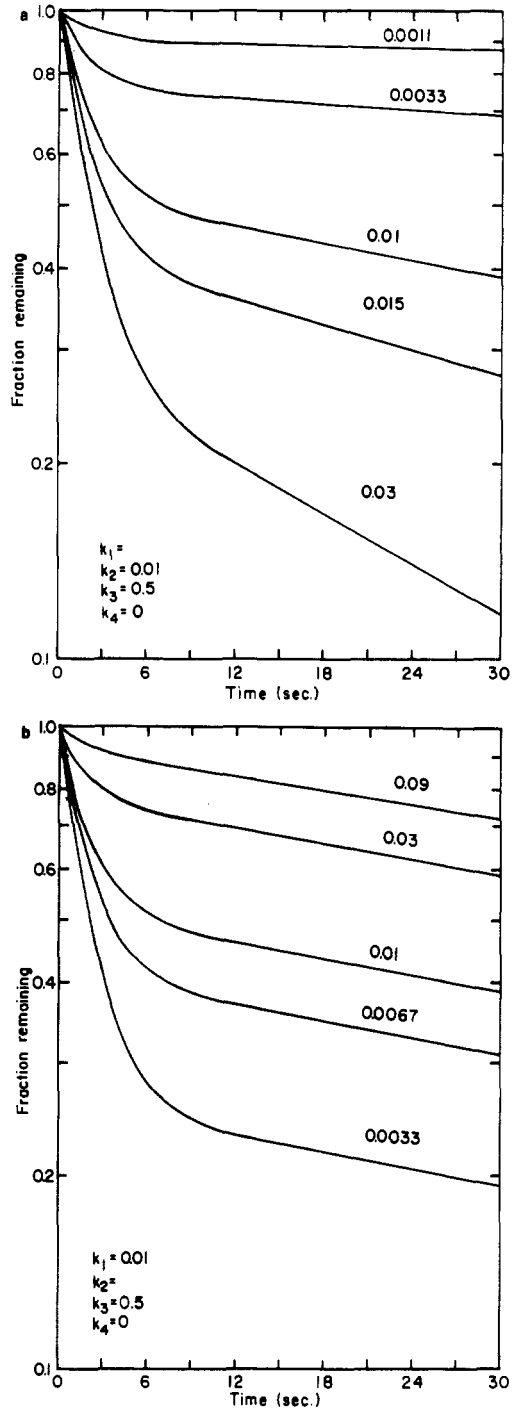

FIGURE 7: (a) Simulation of reduction of cytochrome P-450 by using the sequential model described in eq 1–3. Variation of K_{eq} by modification of k_1. The rate constant k_1 was varied from 0.0011 to 0.03 as shown on the curves; k_2, k_3, and k_4 were 0.01, 0.5, and 0, respectively. The values of K_{eq} were 0.111, 0.333, 1, 1.5, and 3. (b) Computer simulation of cytochrome P-450 reduction. Variation of K_{eq} by modification of k_2. The rate constant k_2 was varied from 0.09 to 0.0033 as shown on the curves; k_1, k_3, and k_4 were 0.01, 0.5, and 0, respectively. The values of K_{eq} were the same as those in Figure 7a.

of the constants k_1/k_2 is the spin equilibrium constant, as expected the increase in high-spin state increased the burst rate and extent.

When k_1 was held constant and the value of k_2 was decreased, the spin equilibrium constant was affected just as in the prior instance. However, as seen in Figure 7b, only the burst rates were increased, with the corresponding slow-phase slopes being parallel. Note that these resultant plots mimic the kinetic data with type I substrates in Figure 2. These results suggest that type I substrates owe their shift of the spin equilibrium and enhancement of the burst to an ability to decrease the value of k_2. Further, from the lack of influence of k_2 on the slow phase, it would appear that k_1 strongly influences the slow phase of reduction. In agreement with the kinetic data, dividing the apparent rate constants of the burst by the extents of the burst in Figure 7b gave the same burst rate constant for all plots. Similar effects were obtained with Figure 2.

Strictly speaking, the use of the above model is an oversimplification, since in these studies the reductase concentration

is not saturating. Variation of reductase concentration could alter the kinetics of the model from a first-order to a second-order process. For example, a decrease in the reductase (FpT) concentration would affect k_3[B] because k_3 could be expressed as k_3'[FpT]. However, since reduction of the reductase is much faster than reduction of cytochrome P-450 [as determined by a 30-fold greater rate of transfer of electrons from NADPH to ferricyanide (Jansson & Schenkman, 1977)], in essence there is no change in the concentration of reduced reductase when NADPH is present in excess.

Discussion

In prior studies we have examined the equilibrium between high-spin and low-spin states of hepatic ferricytochrome P-450. The equilibrium was shown to be temperature dependent (Pierson & Cinti, 1977; Cinti et al., 1979) and to be influenced by the presence of substrates of the monooxygenase (Jansson et al., 1980; Schenkman et al., 1981). The spin equilibrium was shown further to influence the reduction–oxidation equilibrium of the cytochrome (Sligar et al., 1979). The data reported in this paper extend the prior studies to propose an explanation of the biphasic reduction kinetics exhibited by the monooxygenase.

A sequential model was proposed for cytochrome P-450 reduction in which the constraints of the system which lead to biphasic or burst kinetics are caused by values for the rate constants governing the spin equilibrium which are much lower than those for reduction (Backes et al., 1980). Only the high-spin form of the cytochrome is reduced, and this causes a rapid depletion of the high-spin form of the ferric hemoprotein, whereupon reduction becomes limited by the formation of more high-spin hemoprotein.

The model is supported by a biphasic Arrhenius plot of the apparent fast-phase rate constants obtained at different temperatures and the ability to obtain a straight line by correcting the apparent initial rate constants for the amount of high-spin cytochrome. Obviously, the rates plotted in semilogarithmic manner will be k_3[B], and if [B] as well as k_3 changes with temperature, one must correct for changing [B] in order to obtain the true temperature dependence of k_3. A further indication of the validity of this procedure was the same burst rate constant obtained by dividing the slope of the burst by its extent for reductions in which different type I substrates were present (Figure 2). The linearity obtained in plots of increasing high-spin cytochrome P-450 (determined from substrate-induced spectral changes) and the increasing magnitude of the burst further support the sequential model (Figures 3 and 4).

Substrates shift the equilibrium between the high- and low-spin state of the ferric cytochrome and also alter the rate and extent of the burst portion of the reduction kinetics. From computer models, in which variation of the individual rate constants was carried out, it would appear that substrates alter the value of k_2 and not k_1, increasing the burst merely by increasing the amount of high-spin cytochrome. Substrates do not influence the slope of the slow phase at all in semilog plots. Computer models indicate that the burst rate and extent are influenced by the ratio of k_1/k_2, i.e., the spin equilibrium constant. With cytochrome P-450$_{LM4}$, Oprian et al. (1979) showed the extent of reduction in the burst was approximately 75%. According to our spin-state model for reduction, this result would suggest that in their reconstituted system cytochrome P-450$_{LM4}$ was approximately 75% high spin. Consistent with this suggestion is the large shoulder at 420 nm in the absolute spectrum of this cytochrome [Vatsis et al. (1979), Figure 1; Haugen & Coon (1976), Figures 6, 7, and 9]. In a similar manner, studies with cytochrome P-450$_{LM2}$ show an extent of reduction in the burst (Iyanagi et al., 1978) of about 40%, which would be consistent with a lower fraction of high-spin cytochrome.

The proposed sequential model

$$A \rightleftharpoons B \rightleftharpoons C$$

is a first approximation for describing P-450 reduction and may be expanded to include a number of additional equilibrium states, for example, substrate-bound and substrate-free high- and low-spin forms and reductase-free and reductase-bound states. As indicated above, substrate influences the reduction kinetics solely by affecting the spin equilibrium (between A and B). Reductase is suggested to bind with the same affinity to both low- and high-spin states of the enzymes, since it does not affect the spin equilibrium in our reconstituted system (data not shown). According to this model, however, only high-spin reductase-bound cytochrome P-450 can be reduced; reductase-free cytochrome cannot be reduced unless it combines with the reductase. This constraint modifies the above model. The agreement between computer models and observed kinetic data, as well as the similar enthalpies obtained from ratios of high- to low-spin cytochrome P-450 and amounts of burst to slow-phase cytochrome P-450 (Backes et al., 1980), strongly supports our hypothesis that the kinetics of reduction of the hemoprotein are controlled by the spin equilibrium constant.

In the simplified model spectrally indistinguishable species are lumped together as a single entity; i.e., "A" represents all low-spin forms of the hemoprotein characterized by a Soret maximum at 418 nm and "B" represents those species in a high-spin state absorbing at about 390 nm. Temperature-jump studies by Tsong & Yang (1978) can be construed as being in support of this model. In the absence of substrates, the cytochrome P-450 showed no rapid spin shifts. We have also seen such lack of change in temperature-jump experiments both in microsomes and with highly purified cytochrome P-450 in the absence of substrates (W. L. Backes, W. Windsor, T. M. Schuster, and J. B. Schenkman, unpublished data). However, on a slower time scale (equilibrium studies), a significant spin shift was observed with temperature with these preparations. When considered with the data in Figure 4, these observations support the involvement of the spin-state equilibrium in the observed burst kinetics. In the presence of benzphetamine, Tsong & Yang (1978) saw multiple relaxations, indicating that more than a single process occurs in the temperature-induced spin shift of the substrate-bound cytochrome. This observation raises the possibility of multiple low-spin and high-spin states, which would support possible expansions of the proposed model. In the studies of Tsong & Yang (1978), the temperature jump was resolved into two relaxations, a fast relaxation with a first-order rate constant of 32 s^{-1} and a slower relaxation with a rate constant of 3.7 s^{-1}. Stopped-flow studies by Blanck et al. (1977), where the substrate-induced spin-state change was determined, produced a rate constant of 26 s^{-1} at similar benzphetamine concentrations. Therefore, the fast phase of the temperature jump and the reported substrate-induced rate constants appear to be similar, although probably too fast to cause the biphasic reduction kinetics. The slower relaxation observed by temperature jump may, however, be involved in some manner in the slow phase of cytochrome P-450 reduction.

Possible expansions of this model are currently under investigation, one of which evokes a very rapid substrate-induced spin shift to a high-spin state followed by a slower configurational change to another high-spin form of the cytochrome.

$$A \overset{\text{fast}}{\rightleftharpoons} B \overset{\text{slow}}{\rightleftharpoons} B^* \overset{\text{fast}}{\rightleftharpoons} C$$

This possibility could explain the linear relationship with a slope of 0.6 observed between the spin shift and the change in the fraction of cytochrome reduced in the burst (Figure 3).

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