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CYTOCHROME P-450 REDUCTION EXHIBITS BURST KINETICS

by

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SUMMARY

Cytochrome P-450 reduction kinetics can be described by sequential reactions involving a rapid reduction of cytochrome P-450 in the high spin state, followed by a slower reduction controlled by formation of high spin P-450 from the low spin configuration. The burst kinetics observed would be the result of the equilibrium between low and high spin states prior to addition of reducing equivalents. The initial reduction velocity (burst) can therefore be described as $v_i = k_3 m_{hs}^0$ and the slower velocity observed at longer times is controlled by the net rate of formation of the high spin conformation.

INTRODUCTION

Electron transfer between NADPH-cytochrome P-450 reductase and the much greater microsomal content of cytochrome P-450 does not follow simple first or second order kinetics. When plotted in semilogarithmic form, apparent biphasic kinetics result. This was shown not to be due to a mixture of cytochrome species nor to the presence of residual oxygen (1).

Early studies on the kinetics suggested the "fast phase" was a more rapid reduction of the cytochrome P-450-substrate complex than of uncomplexed cytochrome P-450 (1). Other studies suggested the apparent biphasicity is the result of concurrent first order processes, the reduction of cytochrome P-450 molecules clustered around a single reductase and a slower reduction of cytochrome P-450 molecules not directly associated with a cluster (2,3).

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Since hepatic cytochrome P-450 appears to exist in a temperature dependent spin equilibrium (4-7) with substrates differentially perturbing this equilibrium (8), a model embracing the newer findings was needed. The model we developed is one of sequential reactions, the terminal step being reduction of the cytochrome. The model predicts "burst" kinetics similar to that obtained with microsomes.

METHODS

Liver microsomes from male CD rats (Charles River Breeding Laboratories) were prepared as described earlier (5). Kinetics and spectra were recorded on an Aminco DW2 spectrophotometer. Temperature was controlled by a Lauda K-2R water bath circulator and monitored in the cuvet with a YSI probe (Yellow Springs).

Cytochrome P-450 reduction was measured by observation of the formation of the carbon monoxide complex of cytochrome P-450 after the addition of NADPH to a final concentration of 0.3mM. Complex formation was monitored at 450-490nm on an Aminco DW2 spectrophotometer using the dual wavelength mode. Anaerobiosis was insured by the use of an oxygen scavenging system (glucose oxidase-catalase). The assays were performed in 0.1M sodium phosphate buffer (pH 7.5).

RESULTS AND DISCUSSION

Reduction of cytochrome P-450 under carbon monoxide in an oxygen-free system yields an apparent biphasicity when disappearance of ferric cytochrome is plotted (100%-reduced CO complex) in semi-logarithmic form (figure 1A). This effect was assumed earlier to be due to concurrent first order reactions (1-3). Equations describing concurrent reactions are:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \qquad \text{and} \qquad B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} D \qquad \text{eq. 1}$$

A and B can be non-equilibrating substrate bound and substrate free forms of the cytochrome, respectively, or cluster cytochrome and non-cluster cytochrome under slow exchange limitation. C and D are indistinguishable reduced cytochromes P-450. Differential equations describing these reactions are:

$$\frac{dA}{dt} = -k_1[A] + k_2[C] \quad \text{and} \quad \frac{dB}{dt} = -k_3[B] + k_4[D] \qquad \text{eq. 2}$$

Since the reduced form of each is trapped with carbon monoxide, k_2 C and k_4 D will express minimal rates. The reaction followed will thus be the sum of the appearance of C and D at any time. This can be designated as E. Thus:

$$\frac{dE}{dt} = k_1[A] + k_3[B] \qquad \text{eq. 3}$$

where E = C + D

Even if interactions between C and D exist via a back reaction, since the back reaction approaches zero in the presence of saturating levels of carbon monoxide, little effect would be observed.

A computer model utilizing equation 3 indicates that concurrent first order processes can yield semilogarithmic plots which are similar to the observed reaction kinetics of cytochrome P-450 reduction. However, if the kinetics are based upon A and B being substrate bound and substrate free cytochrome or cluster and non-cluster cytochrome, the addition of saturating levels of substrate should convert all of form A to form B. If this occurs, then cytochrome P-450 reduction would appear monophasic. If A and B represent non-interconvertible forms of the cytochrome, only one of which can be "activated" by substrate, then biphasic plots could be obtained. However, even with "highly purified" cytochrome P-450 LM_4 and LM_2 biphasic plots are seen in the reconstituted system (9,10). Further, increasing the level of NADPH-cytochrome P-450 reductase to 1:1 stoichiometry with cytochrome P-450 does not eliminate the "biphasic" nature of the semilogarithmic plot (10). Hence, the concurrent reaction model also does not adequately describe cytochrome P-450 reduction kinetics.

Hepatic cytochrome P-450 exists as a mixture of high and low spin configurations in both substrate free and substrate bound states (4-8). This requires that any model developed to explain reduction kinetics of cytochrome P-450 incorporate all possible equilibrium states of the cytochrome.

Previous studies by our groups have demonstrated that the spin equilibrium of the hemoprotein regulates the redox equilibrium (11). Since substrates which

alter the spin equilibrium (8), influence the rate of reduction of the cytochrome (1) , the same simplified model used to examine the redox equilibrium (11) can be used to describe the reduction kinetics of cytochrome P-450.

For simplicity we have adopted the earlier symbols of Sligar (12) where m represents the monooxygenase, superscript o and r indicate oxidized and reduced, respectively, and subscript ls and hs indicate low spin and high spin ferric form of the cytochrome. The reaction described by this model is:

$$m^o_{ls} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} m^o_{hs} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} m^r_{hs} \qquad \text{eq. 5}$$

where the presence or absence of substrate is only assigned the function of influencing the spin equilibrium. This is reasonable in view of the linear relationship obtained between spin equilibrium and midpoint potential regardless of the absence or presence of different substrates (11).

A computer model of cytochrome P-450 reduction depicted in equation 5 was developed using the differential equations below:

$$\frac{dm^o_{ls}}{dt} = k_2 m^o_{hs} - k_1 m^o_{ls} \qquad \text{eq. 6}$$

$$\frac{dm^o_{hs}}{dt} = k_4 m^r_{hs} + k_1 m^o_{ls} - (k_2+k_3) m^o_{hs} \qquad \text{eq. 7}$$

$$\frac{dm^r_{hs}}{dt} = k_3 m^o_{hs} - k_4 m^r_{hs} \qquad \text{eq. 8}$$

Implicit in equation 5 is the assumption that transition from the ferric low spin state to the ferrous high spin state proceeds through the ferric high spin state, a process governed by the spin equilibrium constant (see review ref. 13), and that at time t = 0 before addition of NADPH an equilibrium exists between low and high spin forms. Such an equilibrium has already been demonstrated for both microsomal and purified cytochrome P-450 (5-7). This

provides the initial P-450 available for reduction with the initial velocity governed by the amount of m^o_{hs} and the forward rate constant, k_3 (eq. 8) since prior to addition of NADPH there is no m^r_{hs}. Further, in the presence of carbon monoxide which aids visualization of m^r_{hs}, as well as traps the hemoprotein in a CO complex, m^r_{hs} is essentially absent, thereby maintaining the velocity as $v = k_3 m^o_{hs}$. If $k_1 = k_2 = 0$ or if m^o_{ls} equals zero the reaction will yield a linear semilogarithmic plot. If m^o_{ls} does not equal zero and $k_1 = k_2 = 0$, the plot will approach an asymptote at a level determined by the amount of m^o_{ls}. However, since low spin and high spin are present in equilibrium, k_1 and k_2 do not equal zero. From the fact that the spin equilibrium changes with temperature (5-7) these constants have finite values. Prior to addition of NADPH, at t=0 an equilibration has been set up where $m^o_{ls} k_1 = m^o_{hs} k_2$. After addition of NADPH, m^o_{hs} is consumed. Due to the decreased concentration of m^o_{hs} the low spin-high spin equilibrium is perturbed resulting in the net formation of high spin cytochrome from the low spin form. This will influence the reduction kinetics of cytochrome P-450, to an extent dependent upon the values of k_1 and k_2 relative to k_3. When the excess of m^o_{hs} is consumed the rate of reduction cannot exceed the rate of m^o_{hs} formation, $k_1 m^o_{ls} - k_2 m^o_{hs}$.

Computer models indicate that when k_3 is greater than k_1 and k_2 burst kinetics are observed (Fig. 1b). If k_1 is much larger than k_3 a break in the semilogarithmic plot is not seen. The similarity of figure 1 to figure 1a indicates that in microsomes and most reconstituted monooxygenase systems, the rate constants k_1 and k_2 are smaller than k_3, the forward rate constant for the reductase.

The reduction of cytochrome P-450 thus involves "burst" kinetics, where the initial level of the substrate, m^o_{hs}, drops rapidly from controlling the velocity of reduction to a level where the formation of m^o_{hs} becomes the limiting rate (eq. 7). Burst kinetics would also explain the apparent early deviation from linearity seen in product formation from substrates which are rapidly metabolized by the monooxygenase.

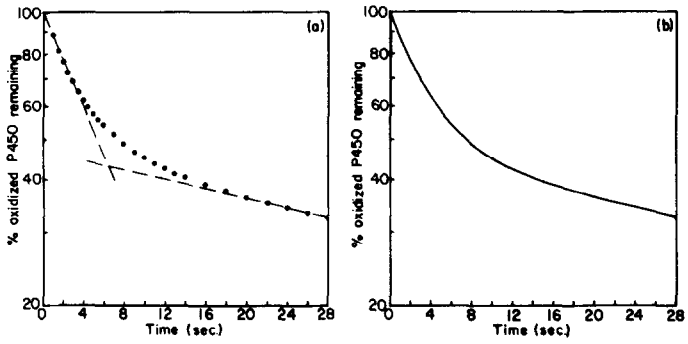

Figure 1. Reduction kinetics of cytochrome P-450. a) Microsomes (1mg/ml) in 0.1M potassium phosphate buffer, pH 7.5 containing an oxygen scavenger system under carbon monoxide. The reaction was initiated at 25.1° with 0.3mM NADPH. b) Computer simulation of sequential reaction mechanism described in equation 5, where m_{ls} = 41.5%, m_{hs} = 58.5%, k_1 = 0.0136s^{-1}, k_2 = 0.00965s^{-1}, k_3 = 0.24700s^{-1} and k_4 = 0.00247s^{-1}.

An indication of the validity of this kinetic model resides in the apparent non-linearity of Arrhenius plots of initial rate constant for reduction (k_i) (fig. 2a). The plot has a break at about 15°C which indicates that electron transfer to cytochrome P-450 is a complex function; the functions involved are the temperature dependence of the spin equilibrium and the temperature sensitivity of the reductase.

Although extrapolation of the "slow phase" back towards t=0 to gain an estimation of the amount of m^0_{hs} is technically incorrect for this model, it

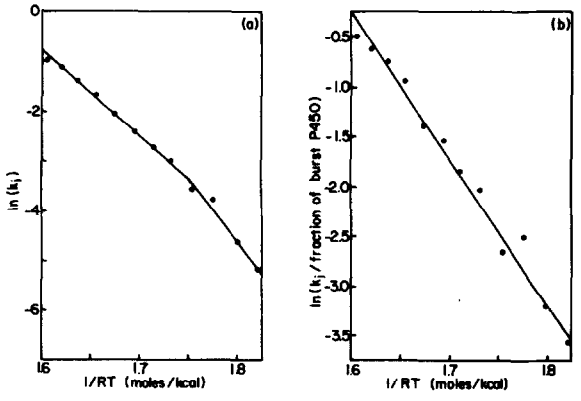

Figure 2. Arrhenius plots of initial observed rate constants for reduction of cytochrome P-450. a) uncorrected; b) corrected for initial proportion of high spin cytochrome P-450, i.e., $k_i/m^0_{hs}=k_3$. Conditions were as in figure 1a, at different temperatures (see text).

does provide a reasonable estimate of the proportion of low spin cytochrome P-450 because k_1 and k_2 are much slower than k_3 (a detailed analysis of the influence of rate constants on the burst kinetics will be published elsewhere by Backes, Sligar, Cinti and Schenkman). When the rate constants (k_i) used in figure 2a were corrected for the differing amounts of m^0_{hs} at the different temperatures and were replotted, the resultant Arrhenius plot yielded a straight line, the slope of which (15 kcal/mole) is the activation energy of NADPH-cytochrome P-450 reductase (fig. 2b).

CONCLUSIONS

The kinetics of cytochrome P-450 reduction can best be explained in terms of burst kinetics where the initial reaction is governed by the amount of high spin cytochrome. Subsequently, the rate becomes controlled by the rate of formation of the high spin cytochrome. These observations indicate that the rate constants for the spin equilibrium are slower than that for reduction of the cytochrome.

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