Kinetic Mechanism for the Model Reaction of NADPH–Cytochrome P450 Oxidoreductase with Cytochrome c[†]

Daniel S. Sem and Charles B. Kasper*

Appendix: Derivation of the Rate Equations for the Two-Site Ping-Pong Mechanism of NADPH–Cytochrome P450 Oxidoreductase

Daniel S. Sem

McArdle Laboratory for Cancer Research, Medical School, University of Wisconsin, Madison, Wisconsin 53706

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ABSTRACT: The kinetic mechanism of NADPH–cytochrome P450 oxidoreductase (P450R) has been determined for the model reaction with cytochrome c^{3+}. Although initial velocity studies show parallel patterns, consistent with a classical (one-site) ping-pong mechanism that precludes the formation of a ternary NADPH·P450R·cytochrome c^{3+} complex, product and dead-end inhibition results suggest a nonclassical (two-site) ping-pong mechanism [Northrop, D. B. (1969) *J. Biol. Chem. 244,* 5808–5819]. This mechanism is a hybrid of the random sequential (ternary complex) and ping-pong mechanisms, since ternary complexes can form as well as intermediate, modified forms of the enzyme that can be present in the absence of any bound substrate. The complete rate equation is derived for this mechanism, and values for V_{max}, $(V/K)_{NADPH}$, $(V/K)_{cytc}$, and the corresponding Michaelis constants are presented in terms of microscopic rate constants along with the expected product inhibition patterns (Appendix). Inhibition by $NADP^+$ is competitive versus NADPH and uncompetitive versus cytochrome c^{3+}, while inhibition by cytochrome c^{2+} is competitive versus cytochrome c^{3+} and noncompetitive versus NADPH. These inhibition patterns are consistent with the proposed two-site mechanism. This mechanism would give the same initial velocity patterns as the classical one-site ping-pong mechanism, but it allows for the formation of a ternary complex, with NADPH and cytochrome c^{3+} reacting independently at two separate sites on P450R. The $^D(V/K)_{NADPH}$ isotope effect is not affected by cytochrome c^{3+} concentration, consistent with our assumption (in deriving the rate equation) that binding at the two sites is independent. At the high ionic strength used in this study (850 mM), the mechanism is two-site ping-pong, with the electron acceptor site itself reacting with cytochrome c^{3+} in a tetra uni ping-pong manner.

NADPH–cytochrome P450 oxidoreductase (P450R)[1] (NADPH–ferrihemoprotein oxidoreductase, EC 1.6.2.4) is a flavoprotein containing 1 equiv each of FMN and FAD (Iyanagi & Mason, 1973). It is a membrane-bound protein associated with the endoplasmic reticulum (Williams & Kamin, 1962; Phillips & Langdon, 1962) and nuclear envelope (Kasper, 1971) of most eukaryotic cells. The reaction catalyzed by P450R is an electron transfer from NADPH to FAD, then to FMN (Vermilion et al., 1981), and finally to any of a number of cytochromes P450 (Lu et al., 1969). Electrons are also transferred to other microsomal enzymes and to various non-physiologically relevant acceptors like 2,6-dichloroindophenol, menadione, ferricyanide, and cytochrome c^{3+} (Williams & Kamin, 1962).

Previous studies have shown that the stable semiquinone form of P450R, which contains an unpaired electron on the FMN prosthetic group (Vermilion & Coon, 1978a; Iyanagi et al., 1981; Otvos et al., 1986), is reduced by NADPH to give the three-electron-reduced enzyme (Iyanagi & Mason, 1973; Vermilion et al., 1981). This is oxidized in two single-electron transfers to a cytochrome P450/substrate complex or to some other electron acceptor, yielding the stable semiquinone again. Although numerous pre-steady-state kinetic and other studies have been carried out on P450R, yielding a detailed description of the interflavin and substrate-flavin electron transfer steps (Masters et al., 1965a,b; Oprian & Coon, 1982; Iyanagi et al., 1974; Vermilion & Coon, 1978b; Vermilion et al., 1981), there is still no consensus regarding the steady-state kinetic mechanism. Initially, it was proposed that the rat liver P450R went by a sequential mechanism, involving the formation of a ternary complex with NADPH and cytochrome c^{3+} (Phillips & Langdon, 1962). Such a mechanism was also suggested for P450Rs from guinea pig liver (Kobayashi & Rikans, 1984) and artichoke (Benveniste et al., 1986), based on the observation of intersecting initial velocity patterns with substrates NADPH and cytochrome c^{3+}. In contrast, it was suggested that no ternary complex forms with NADPH, cytochrome c^{3+}, and the pig liver enzyme, which is thought to go by a ping-pong mechanism (Masters et al., 1965b). This mechanism has also been proposed for the pig kidney (Fan & Masters, 1974), bovine adrenal cortex (Hiwatashi & Ichikawa, 1979; Kominami et al., 1982, 1984), housefly (Mayer & Durrant, 1979), and army-worm (Crankshaw et al., 1979) enzymes, based on the observation of parallel initial velocity patterns with substrates NADPH and cytochrome c^{3+}. Adding to this confusion over mechanism are numerous reports that $NADP^+$ and 2′-AMP are competitive inhibitors versus NADPH (Neufeld et al., 1955; Phillips & Langdon, 1962; Williams & Kamin, 1962; Ichikawa & Yamano, 1969; Crankshaw et al., 1979; Hiwatashi & Ichikawa, 1979; Mayer

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[1] Abbreviations: P450R, NADPH–cytochrome P450 oxidoreductase; cytc, cytochrome c; 2′-AMP, 2′-adenosine monophosphate; NMR, nuclear magnetic resonance, TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid.

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& Durrant, 1979; Fan & Masters, 1974; Kobayashi & Rikans, 1984), results which are inconsistent with the classical ping-pong mechanism. Indeed, it has been noted that the steady-state kinetic mechanism of P450R is still undefined (Oprian & Coon, 1982).

Our results are consistent with a kinetic mechanism in which NADPH and cytochrome c^{3+} participate in redox reactions at separate sites on P450R. Although other studies[2] are consistent with the presence of separate binding sites for NADPH and cytochrome c^{3+}, the implications of these findings for the interpretation of the steady-state kinetic data have not been explored. We show that the P450R steady-state kinetic mechanism is a two-site ping-pong mechanism in which the "carrier" between the substrate binding sites is the FAD/FMN prosthetic group pair. A number of other enzymes possess multi-site ping-pong mechanisms (Barden et al., 1972; Katiyar et al., 1975; McClure et al., 1971; Northrop, 1969; Tsai et al., 1973), and the theory describing the kinetic behavior of such enzymes is well established (Northrop, 1969; Cleland, 1973, 1977). Although most multi-site ping-pong enzymes possess a mobile carrier (biotin, lipoic acid, or 4′-phosphopantetheine) to transfer a chemical group between substrate binding sites, P450R is unique in that the carrier is stationary since it is electrons that are being transferred between sites, which can be accomplished via tunneling (McClendon, 1988). Previous reports of a ping-pong mechanism for P450R have suggested that no ternary complex forms between P450R, NADPH, and cytochrome c^{3+}. The kinetic mechanism we propose involves the formation of a ternary complex and is consistent with all initial velocity patterns, inhibition studies, and data suggesting the presence of two substrate binding sites.

MATERIALS AND METHODS

NADPH, $NADP^+$, NADH, 2′-AMP, oxidized glutathione, TAPS buffer, horse heart cytochrome *c* (ferric), alcohol dehydrogenase from *Thermoanaerobium brokii*, and glutathione reductase from bovine intestinal mucosa were from Sigma. Ethanol-d_6 (99.5%) and D_2O (99.9%) were from Aldrich. Dipotassium phosphate and KCl were from Mallinckrodt.

Recombinant rat liver P450R was overexpressed in *Escherichia coli* (C-1A) and purified by chromatography on 2′,5′-ADP Sepharose (Yasukochi & Masters, 1976) as described previously (Shen et al., 1989).

Kinetic Assays. Kinetic studies were carried out in 1-cm cuvettes in a 1-mL volume at 25 °C. In all cases, the reaction rate was measured spectrophotometrically by following the reduction of cytochrome c^{3+} ($\epsilon = 21.1$ mM^{-1} cm^{-1}) at 550 nm (Gelder & Slater, 1962). Reaction mixtures contained 100 mM TAPS at pH 8.0, enough KCl to give an ionic strength of 850 mM (calculated), varied concentrations of substrates (NADPH or NADH and cytochrome c^{3+}), and where appropriate, inhibitor. Further details are given in the figure legends. Reactions were initiated by the addition of 0.1–0.5 μg of P450R. Enzyme activity throughout the course of an experiment and between experiments was monitored with a standard activity assay containing 0.1 M potassium phosphate (pH 8.0), 80 μM cytochrome c^{3+}, and 0.5 mM NADPH.

Table 1: Dead-End and Product Inhibition of P450R[a]

varied substrates	inhibitor	type of inhibition	inhibition constants (μM)
$cytc^{3+}$	$cytc^{2+}$	competitive	28.4 ± 8.4
	$NADP^+$	uncompetitive	6.53 ± 0.89
	2′-AMP	uncompetitive	39.2 ± 6.0
NADPH	$cytc^{2+}$	noncompetitive	K_{IS} = 80 ± 24 K_{II} = 59 ± 20
	$NADP^+$	competitive	5.24 ± 0.75
	2′-AMP	competitive	19.4 ± 1.9

[a] Reactions were at 25 °C in 0.1 M TAPS, pH 8.0, and an ionic strength of 850 mM. The nonvaried substrate was present at a concentration near its Michaelis constant (30 μM for cytochrome c^{3+} and 3 μM for NADPH).

Preparation of Cytochrome c^{2+}. Cytochrome c^{2+} was prepared by reducing a 1 mM solution of cytochrome c^{3+} (pH 9.0) with 10 mM dithionite. After a 10-min incubation at room temperature, the dithionite was removed by passing the solution over a 1.7 × 25 cm Sephadex G-10 column, collecting the void volume only. The concentrations of cytochrome c^{2+} and residual cytochrome c^{3+} were calculated by measuring the absorbance at 550 nm before and after reduction with dithionite, using previously determined extinction coefficients for reduced (29.5 mM^{-1} cm^{-1}) and oxidized (8.4 mM^{-1} cm^{-1}) cytochrome *c* (Gelder & Slater, 1962). Because of the presence of this residual ferric cytochrome *c*, it was necessary to make appropriate corrections to the concentration of cytochrome c^{3+} (substrate) present in the cytochrome c^{2+} inhibition assays (Table 1).

Preparation of $NADPD_A$. NADPH deuterated in the pro-*R* (A-side) position on the C4 atom of the nicotinamide ring ($NADPD_A$) was synthesized enzymatically with slight modifications of the procedure of Viola et al. (1979) as described previously (Sem & Kasper, 1992). The deuterium was introduced into the A-side of $NADP^+$ by the alcohol dehydrogenase catalyzed oxidation of ethanol-d_6. $NADPD_A$ was purified on an AG MP-1 column (Bio-Rad) and desalted on a Bio-Gel P2 column (Bio-Rad). The fraction of deuteration was determined by incubating a 0.4 mM solution of $NADPD_A$ with oxidized glutathione (0.8 mM) and 2 units/mL of glutathione reductase (a B-side enzyme) in a 10 mM potassium phosphate buffer at pH 8.0 for 15 min. The solution was then filtered through a Centricon 30 membrane (Amicon), lyophilized, and taken up in D_2O. A ^1H NMR spectrum of the oxidized nicotinamide nucleotide was obtained on a Bruker AM500 spectrometer operating at 500.13 MHz. Integration of the aromatic region of this spectrum and comparison of the intensities of the resonances for the C4 (mostly deuterium) and C6 protons of the nicotinamide ring allowed the calculation of the percent deuteration (86%), which is needed for the isotope effect calculation in eq 5.

Concentrations of Stock Solutions. The concentrations of stock solutions of 2′-AMP, $NADP^+$, NADPH, and NADH were determined using known extinction coefficients (P-L Biochemicals, Inc., 1956). The concentrations of stock solutions of cytochrome c^{3+} were determined by reducing with dithionite and measuring the change in absorbance at 550 nm, as described above for determining the concentration of residual cytochrome c^{3+} in stock solutions of cytochrome c^{2+}.

Data Processing. Data were fitted using the FORTRAN programs of Cleland (1979) which perform nonlinear least

[2] Previous studies have demonstrated that the electron transfer is from NADPH → FAD → FMN → cytochrome c^{3+} (Vermilion et al., 1981). Since sequence homology suggests the presence of distinct FAD and FMN binding domains (Porter & Kasper, 1986), and time-resolved fluorescence studies indicate an FAD to FMN distance of 2 nm (Bastiaens et al., 1989), it seems more reasonable that NADPH and cytochrome c^{3+} are binding at separate sites on P450R. Consistent with this conclusion, cytochrome c^{3+} that has been cross-linked with P450R using a water-soluble carbodiimide could be 90% reduced by NADPH (Nisimoto & Otsuka-Murakami, 1988).

squares fits to the specified equations. Most equations were fit in log form since this effectively assumes constant proportional error in the velocity. The data from the initial velocity studies (at 850 mM ionic strength) fit best to the equation for a classical ping-pong mechanism:

$$Y = \frac{V_{max}AB}{(K_AB + K_BA + AB)} \quad (1)$$

where Y is the initial velocity, A and B are the concentrations of NADPH and cytochrome c^{3+}, respectively, and K_A and K_B are their corresponding Michaelis constants. Data from inhibition studies were fitted to equations for competitive (eq 2), uncompetitive (eq 3), and noncompetitive (eq 4) inhibition:

$$\log (Y) = \log \frac{V_{max}A}{K_m(1 + I/K_{is}) + A} \quad (2)$$

$$\log (Y) = \log \frac{V_{max}A}{K_m + A(1 + I/K_{ii})} \quad (3)$$

$$\log (Y) = \log \frac{V_{max}A}{K_m(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (4)$$

where Y is the initial velocity, I is the inhibitor concentration, K_{ii} is the intercept inhibition constant, K_{is} is the slope inhibition constant, A is the varied substrate concentration, and K_m is its apparent Michaelis constant. The nonvaried substrate was present at a level close to its K_m in all cases. The fit giving the smallest σ value, with reasonable standard deviations for all constants, was chosen as the best model to describe the type of inhibition. The primary deuterium isotope effect on $(V/K)_{NADPH}$, $^D(V/K)_{NADPH}$[3] determined using the method of direct comparison (Cleland, 1982) was obtained from a fit to the following equation:

$$\log (Y) = \log \frac{V_{max}A}{K_m(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (5)$$

where Y is the initial velocity, A is the varied substrate (NADPH or $NADPD_A$) concentration, K_m is its corresponding apparent Michaelis constant, F_i is the fraction of deuterium incorporation into the pro-R (A-side) position on the C4 atom of the nicotinamide ring of NADPH, and E_V and $E_{V/K}$ are the isotope effects minus one on V_{max} and $(V/K)_{NADPH}$, respectively. The observed isotope effects will be primary ones since it is known that the A-side (pro-R) hydrogen is transferred (Sem & Kasper, 1992).

RESULTS

Substrate Kinetics. At an ionic strength of 850 mM, a plot of $1/v$ versus 1/[NADPH] at different fixed levels of cytochrome c^{3+} gave a parallel pattern (Figure 1A). Likewise, a plot of $1/v$ versus 1/[cytochrome c^{3+}] at different fixed levels of NADPH also gave a parallel pattern (Figure 1B). These data fit best to the equation for a ping-pong mechanism (eq 1). Although this equation has the form expected for a hexa uni ping-pong mechanism with two of the three substrates being identical, it is also consistent with a two-site ping-pong

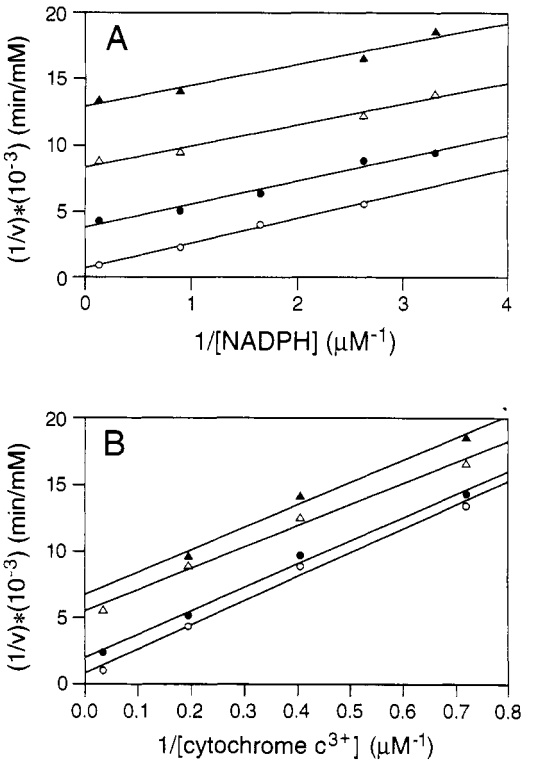

FIGURE 1: Initial velocity pattern for P450R with (A) 1/[NADPH] varied at fixed levels of cytochrome c^{3+} (30, 5.1, 2.5, and 1.4 μM), and (B) 1/[cytochrome c^{3+}] varied at fixed levels of NADPH (7.5, 1.1, 0.38, and 0.30 μM). Open and filled circles and open and filled triangles represent decreasing concentrations of NADPH or cytochrome c^{3+}, as indicated in parentheses. Reactions were carried out in 0.1 M TAPS, pH 8.0, and an ionic strength of 850 mM.

mechanism (Appendix). Only the latter mechanism allows for formation of a ternary complex since it assumes NADPH and cytochrome c^{3+} bind and react independently at separate sites on P450R. Intercept replots were linear for the profiles shown in Figure 1A,B (data not shown). The lack of curvature in the intercept replot for Figure 1A is consistent with a tetra uni ping-pong reaction of cytochrome c^{3+} at site 2 (Appendix). An ionic strength of 850 mM was used in these experiments, since it corresponds to that used in a number of previous kinetic studies with P450R carried out in 0.3 M potassium phosphate buffer. It is also the ionic strength where the kinetic mechanism is simplest, since additional terms must be added to eq 1 to obtain a reasonable fit at lower ionic strength. At low ionic strength, the intercept replots for the profile in Figure 1A show curvature, consistent with random sequential binding of two cytochrome c^{3+} molecules at site 2 (D. S. Sem and C. B. Kasper, manuscript in preparation). This pronounced ionic strength effect on mechanism coupled with the observation of substrate inhibition by cytochrome c^{3+} (vide infra) could be responsible for the conflicting reports on the kinetic mechanism of P450R, since both effects introduce curvature in the initial velocity profiles.

Inhibition Studies. Although both the hexa uni ping-pong and two-site ping-pong mechanisms produce the same initial velocity profiles, described by eq 1, they produce very different product inhibition patterns (Appendix). At 850 mM ionic strength, the product inhibitor cytochrome c^{2+} was competitive against cytochrome c^{3+} and noncompetitive against NADPH (Table 1), while the other product inhibitor $NADP^+$ was uncompetitive against cytochrome c^{3+} and competitive against NADPH (Table 1). The dead-end inhibitor 2′-AMP shows the same inhibition patterns as $NADP^+$ (Table 1). The inhibition patterns are consistent with the two-site ping-pong

[3] The nomenclature of Northrop (1977) and of Cook and Cleland (1981) is used to describe the isotope effect results. $^D(V/K)_{NADPH}$ represents the ratio of the $(V/K)_{NADPH}$ value for hydride transfer with NADPH as substrate to that for deuteride transfer with $NADPD_A$ as substrate (for a "primary" isotope effect).

Table 2: Effect of Cytochrome c^{3+} Concentration on $^{D}(V/K)_{NADPH}$[a]

cytochrome c^{3+} (μM)	$^{D}(V/K)_{NADPH}$
29.6	1.85 ± 0.21
5.14	1.61 ± 0.22
0.46	1.80 ± 0.23

[a] Reactions were at 25 °C in 0.1 M TAPS, pH 8.0, and an ionic strength of 850 mM. NADPH and $NADPD_A$ were varied at the following concentrations: 7.5, 1.1, 0.60, 0.38, and 0.30 μM.

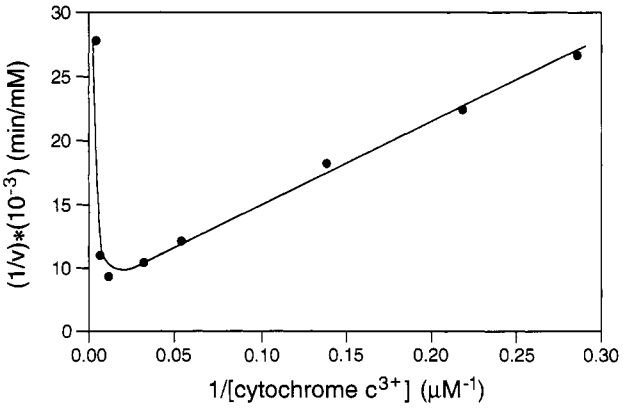

FIGURE 2: Initial velocity as a function of cytochrome c^{3+} concentration, showing the substrate inhibition effect. Reaction conditions were as in Figure 1, except that the ionic strength was 300 mM. Cytochrome c^{3+} was varied from 3.5 to 230 μM in the presence of 50 μM NADPH. Similar patterns were observed at all ionic strengths tested (200–600 mM).

mechanism, with the tetra uni ping-pong reaction of cytochrome c^{3+} at site-2. The slope inhibition constant for 2′-AMP is only 3.7 times that of $NADP^+$, corresponding to a loss of only 0.8 kcal/mol of binding energy upon removal of the nicotinamide mononucleotide half of the $NADP^+$ molecule. As is generally the case with dehydrogenases, the bulk of the ground-state binding energy is provided by the AMP half of $NADP^+$. Inhibition study results are summarized in Table 1.

Isotope Effect Studies. A powerful diagnostic tool for determining kinetic mechanism is the dependence of the $^{D}(V/K)_{app}$ isotope effects on the nonvaried substrate concentration.[3] $^{D}(V/K)_{app}$ differs from $^{D}(V/K)$ in that the nonvaried substrate, cytochrome c^{3+} in these experiments, is not present at saturating levels. At sufficiently high levels of cytochrome c^{3+} ($\gg K_{cytc}$), $^{D}(V/K)_{app} = {}^{D}(V/K)$ (V/K values are for NADPH). How $^{D}(V/K)_{app}$ is expected to vary as a function of the concentration of the nonvaried substrate, for different kinetic mechanisms, has been discussed previously (Cleland, 1986). Briefly, if one substrate can affect the rate of release of another substrate from the Michaelis complex, $^{D}(V/K)_{app}$ will be affected in a way that is diagnostic for a given mechanism. For P450R, $^{D}(V/K)_{NADPH}$ shows no significant variation with cytochrome c^{3+} concentration (Table 2), which indicates that binding of NADPH at site 1 is independent of binding interactions with cytochrome c^{3+} at site 2. This is a central assumption in the derivation of the kinetic mechanism for P450R in the Appendix.

Substrate Inhibition. Cytochrome c^{3+} shows strong substrate inhibition (Figure 2), but only at concentrations >0.1 mM (5–10 times its K_m). It was crucial therefore to maintain cytochrome c^{3+} at sufficiently low concentrations (<0.1 mM) throughout these studies in order to avoid this effect. Although NADPH shows no substrate inhibition, the substrate analog NADH, which has a K_m of 11.6 mM, 4200-fold larger than that of NADPH (Sem & Kasper, 1993a), shows partial

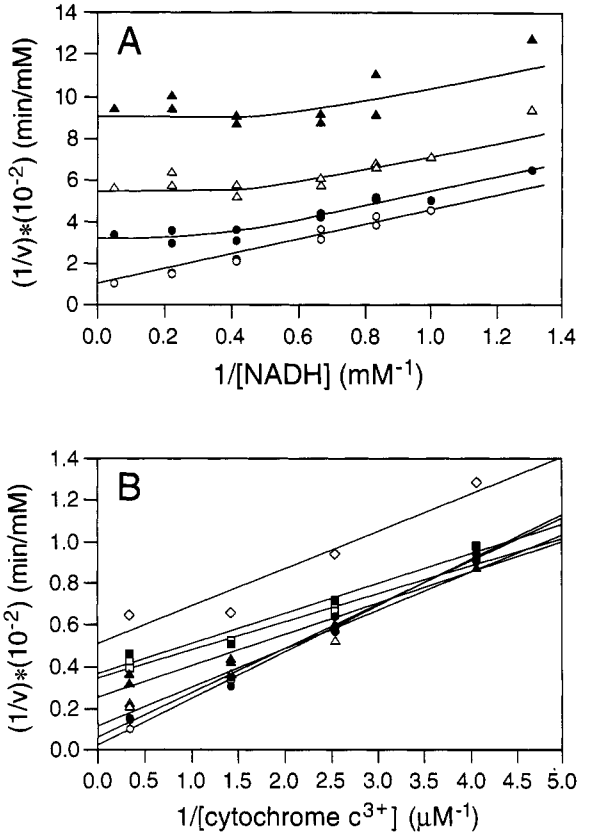

FIGURE 3: Initial velocity pattern for P450R with (A) 1/[NADH] varied at fixed levels of cytochrome c^{3+} (3.0, 0.70, 0.39, and 0.25 μM), and (B) 1/[cytochrome c^{3+}] varied at fixed levels of NADH (20, 4.4, 2.4, 1.5, 1.2, 0.99, and 0.77 mM). Open and filled circles, open and filled triangles, open and filled squares, and open diamonds represent decreasing concentrations of cytochrome c^{3+} or NADH, as indicated in parentheses. Reaction conditions were as in Figure 1.

(competitive) substrate inhibition (Figure 3A,B). The mechanistic significance of this effect is discussed below.

DISCUSSION

Kinetic Mechanism of P450R at 850 mM Ionic Strength. At high ionic strength (850 mM), the initial velocity patterns with varied cytochrome c^{3+} and NADPH are parallel (Figure 1A,B), consistent with a ping-pong mechanism. But, product inhibition results are not consistent with the classical type of ping-pong mechanism (one-site hexa uni in this case) involving modification of the enzyme by the first substrate (NADPH) and followed by reaction with the second and third substrates (cytochrome c^{3+}). This type of mechanism would preclude the formation of a ternary complex and would be predicted to show inhibition by $NADP^+$ that is competitive versus cytochrome c^{3+} and noncompetitive versus NADPH, along with inhibition by cytochrome c^{2+} that is competitive versus NADPH and noncompetitive versus cytochrome c^{3+} (Cleland, 1977). In fact, P450R shows inhibition by $NADP^+$ that is competitive versus NADPH and uncompetitive versus cytochrome c^{3+}, along with inhibition by cytochrome c^{2+} that is competitive versus cytochrome c^{3+} and noncompetitive versus NADPH (Table 1). The dead-end inhibition behavior of 2′-AMP is the same as that for the product inhibitor $NADP^+$. These inhibition results, along with the observation of parallel lines in the initial velocity studies, are consistent with a two-site ping-pong mechanism (Cleland, 1977) in which NADPH and cytochrome c^{3+} bind independently at separate sites on the enzyme with the formation of a ternary NADPH·

P450R·cytochrome c^{3+} complex now possible. The uncompetitive inhibition by $NADP^+$ versus cytochrome c^{3+} indicates that $k_9 \gg k_4$, so electrons are transferred from E_3 to cytochrome c^{3+} more quickly than to $NADP^+$ (Appendix). Also, since inhibition patterns with cytochrome c^{2+} do not show any pronounced curvature, the assumption made in the Appendix that hydride transfer from NADPH (k_3) is much slower than electron transfer to cytochrome c^{3+} (k_9) must be valid. This allowed the dropping of terms C_3, C_4, and C_5 from the equation describing the inhibition by cytochrome c^{2+}, which removes all squared terms in cytochrome c^{3+} (Appendix). It should also be noted that the K_i values reported for 2′-AMP and $NADP^+$ (Table 1) are true dissociation constants,[4] while those for cytochrome c^{2+} are not but are rather a function of many rate constants (Appendix). Further support of the ping-pong mechanism we are proposing comes from studies that have shown that NADPH can reduce P450R, which in turn can reduce NAD^+ (Ichikawa & Yamano, 1969) or 3-acetylpyridine ADP (Vermilion & Coon, 1978a) in the absence of any other electron acceptor (such as cytochrome c^{3+}). These results are consistent with both the one- and two-site ping-pong mechanisms and suggest that in the absence of another electron acceptor the fully reduced P450R (E_4), which is known to form under these conditions (Oprian & Coon, 1982), passes electrons to oxidized pyridine dinucleotide.

The rate equation for a two-site ping-pong mechanism in which reaction at the second (electron acceptor) site is itself tetra uni ping-pong is derived in the Appendix and shown here for the initial velocity condition ($P = Q = 0$):

$$v/E_t = [ABk_3k_9k_{15}/(k_9k_{15} + k_3k_9 + k_3k_{15})]/[AB + AK_{iB}(k_3k_9 + k_3k_{15})/(k_9k_{15} + k_3k_9 + k_3k_{15}) + BK_{iA}(k_9k_{15})/(k_9k_{15} + k_3k_9 + k_3k_{15})] \quad (6)$$

where A and B are the concentrations of NADPH and cytochrome c^{3+}, respectively, and K_{iA} and K_{iB} are their corresponding dissociation constants, E_t is total P450R, and the rate constants are those in eq 7. Comparison of eqs 1 and 6 provides equations for V_{max}, the Michaelis constants for NADPH and cytochrome c^{3+}, and their corresponding V/K values. The equations describing these constants are the same as those derived for a one-site hexa uni mechanism, if it is assumed that binding is in rapid equilibrium and that cytochrome c^{3+} binds with equal affinity to E_3 and E_2. These assumptions were necessary in order to derive the equation for the two-site mechanism in a manageable way, as described in the Appendix. Thus, although the mechanism is two-site ping-pong (based on product inhibition and other results), it is kinetically indistinguishable from the hexa uni ping-pong mechanism in the absence of products. Therefore, values for k_{cat}, k_{cat}/K_m, and K_m were obtained by deriving the rate equation for the hexa uni ping-pong mechanism using the net rate constant method (Cleland, 1975) (not shown) and no longer making the assumptions of rapid equilibrium binding and equal affinity of cytochrome c^{3+} for E_3 and E_2. But, the mechanism is two-site ping-pong with the following reactions occurring at separate sites on the enzyme:[5]

site 1 hydride donor site:

$$E_1 + \text{NADPH} \underset{K_2}{\overset{K_1}{\rightleftharpoons}} E_1\cdot\text{NADPH} \underset{K_4}{\overset{K_3}{\rightleftharpoons}} E_3\cdot\text{NADP}^+ \xrightarrow{K_5} E_3$$

site 2 electron acceptor site:

$$\text{cytc}^{3+} + E_3 \underset{K_8}{\overset{K_7}{\rightleftharpoons}} E_3\cdot\text{cytc}^{3+} \underset{K_{10}}{\overset{K_9}{\rightleftharpoons}} E_2\cdot\text{cytc}^{2+} \xrightarrow{K_{11}} E_2$$

$$\text{cytc}^{3+} + E_2 \underset{K_{14}}{\overset{K_{13}}{\rightleftharpoons}} E_2\cdot\text{cytc}^{3+} \underset{K_{16}}{\overset{K_{15}}{\rightleftharpoons}} E_1\cdot\text{cytc}^{2+} \xrightarrow{K_{17}} E_1 \quad (7)$$

where E_1, E_2, and E_3 are the one ($FMNH^\bullet$/FAD), two ($FMNH_2$/FAD), and three ($FMNH_2$/$FADH^\bullet$) electron-reduced forms of P450R, respectively. The kinetic constants have the form

$$k_{cat} = 1/[1/k_5 + (k_4 + k_5)/k_3k_5 + 1/k_{11} + (k_{10} + k_{11})/k_9k_{11} + 1/k_{17} + (k_{16} + k_{17})/k_{15}k_{17}] \approx 1/[1/k_5 + 1/k_3 + 1/k_{11} + (k_{10} + k_{11})/k_9k_{11} + 1/k_{17} + (k_{16} + k_{17})/k_{15}k_{17}] \quad (8)$$

$$(k_{cat}/K_m)_{NADPH} = \frac{k_1k_3}{k_2(1 + k_4/k_5) + k_3} \approx k_1k_3/(k_2 + k_3) \quad (9)$$

$$(k_{cat}/K_m)_{cytc} = k_7k_{13}/[k_{13}(1 + (k_8/k_9)(1 + k_{10}/k_{11})) + k_7(1 + (k_{14}/k_{15})(1 + k_{16}/k_{17}))] \quad (10)$$

where k_{cat} and k_{cat}/K_m are V_{max} and V/K, respectively, divided by the P450R concentration. It is known that upon reduction of FAD by NADPH, electrons are rapidly equilibrated with FMN, leaving only one of these two electrons on FAD (Oprian & Coon, 1982; Bhattacharyya, et al., 1991). Therefore, $k_4 \ll k_5$, and eqs 8 and 9 can be simplified as shown.

Since Michaelis constants are often used to analyze the effects of mutations on binding interactions between P450R and its substrates, their relationship to true dissociation constants (K_i) is of interest. The Michaelis constant for NADPH is given by

$$K_{NADPH} = K_{iA}[k_{cat}/(k_3k_5/(k_4 + k_5)) + k_{cat}/k_2] \approx K_{iA}[k_{cat}/k_3 + k_{cat}/k_2] \quad (11)$$

where k_{iA} is the dissociation constant (k_2/k_1) for NADPH. If hydride transfer is the rate-limiting step (so $k_{cat} = k_3$ and $k_3 < k_2$), the Michaelis constant becomes equal to K_{iA}. The

[4] In the case of $NADP^+$, since $k_9 \gg k_4$, the K_{ip}'' term is not present (Appendix), and the observed slope inhibition constant, $K_{ip}/(1 + k_4/k_9)$, reduces to K_{ip}, the dissociation constant for $NADP^+$. Furthermore, while the inhibition constant for $NADP^+$ determined with varied NADPH is the true dissociation constant, that obtained with varied cytochrome c^{3+} will be a true dissociation constant if and only if NADPH is present at a concentration equal to its Michaelis constant (Appendix). This is the case in Table 1.

[5] In the pre-steady-state, E_0 is reduced to E_2 and E_4 by NADPH and then oxidized by a one-electron transfer (Iyanagi & Mason, 1973; Iyanagi et al., 1978, 1981; Vermilion & Coon, 1978b; Yasakochi et al., 1979; Vermilion et al., 1981; Oprian & Coon, 1982) to cytochrome c^{3+} to give E_1 and E_3, respectively. In the steady-state reactions we are monitoring, P450R cycles between E_1 and E_3 states (Iyanagi & Mason, 1973; Vermilion et al., 1981). Although an E_2 to E_4 cycle is possible (Masters et al., 1965b; Iyanagi et al., 1974, 1981), it is not likely to be significant since E_4 accumulates only at high NADPH concentrations in the pre-steady-state and in the absence of an electron acceptor (Iyanagi et al., 1974, 1981; Oprian & Coon, 1981). But, a small fraction of E_2 to E_4 cycling cannot be ruled out based on our results, as it would have little effect on the kinetic profiles, assuming the redox state of the enzyme does not have a significant effect on affinity for substrates and products.

observation of a large $^{D}(V/K)_{NADPH}$ isotope effect (Table 1 and D. S. Sem and C. B. Kasper, manuscript in preparation) suggests that hydride transfer is largely (but not entirely) rate limiting, so K_{NADPH} is probably a close approximation to the true K_i. The Michaelis constant for cytochrome c^{3+} is given by

$$K_{cytc} = K_{iB1}[k_{cat}/(k_9k_{11}/(k_{10} + k_{11})) + k_{cat}/k_8] + K_{iB2}[k_{cat}/(k_{15}k_{17}/(k_{16} + k_{17})) + k_{cat}/k_{14}] \quad (12)$$

where K_{iB1} ($=k_8/k_7$) and K_{iB2} ($=k_{14}/k_{13}$) are the dissociation constants for cytochrome c^{3+} from the E_3 and E_2 enzyme forms, respectively.

What makes the two-site ping-pong mechanism unique from the one-site mechanism is the expected product and dead-end inhibition patterns (see Appendix and discussion above), along with the fact that a ternary complex can form since the two substrates are binding independently at separate sites. Further support that binding at the two sites is independent is provided by the $^{D}(V/K)_{NADPH}$ isotope effect results. If NADPH binding and reaction at site 1 were enhanced by the presence of cytochrome c^{3+} at site 2, the $^{D}(V/K)_{NADPH}$ isotope effect would be expected to decrease as the concentration of cytochrome c^{3+} increased, a result of the forward commitment to catalysis[6] increasing, which is clearly not the case (Table 2).

Cytochrome c^{3+} shows substrate inhibition at concentrations 5–10 times its K_m. This could be a result of nonspecific protein–protein interactions between P450R and cytochrome c^{3+} that lead to the formation of nonproductive complexes (Figure 2). NADH shows partial competitive substrate inhibition versus cytochrome c^{3+} (Figure 3A,B). This inhibition pattern is somewhat unexpected and suggests that either NADH is inhibiting by binding nonspecifically at the cytochrome c^{3+} site or possibly by forcing P450R into a redox state or conformation that binds/reacts more slowly with cytochrome c^{3+}. If the latter were true, then the two binding sites would not in fact be totally independent of each other, as assumed. Indeed, previous studies have suggested that binding interactions with the 2′-phosphate of NADPH affect, through a conformational change, binding interactions at the cytochrome c^{3+} binding site (Sem & Kasper, 1993a,b). But, our kinetic model has been consistent with no interaction between sites 1 and 2 thus far, and isotope effect results argue further against interactions between sites. This apparent contradiction is resolved if P450R has a conformation that is retained throughout the steady-state reaction sequence, but is different with NADPH and NADH as substrates. Thus, binding at sites 1 and 2 would be independent as assumed, but only once the steady-state is reached, and a different steady-state conformation of P450R would be present depending upon whether interactions with the 2′-phosphate of NADPH are possible. An NADPH-induced hysteresis effect like this has been observed in dihydrofolate reductase (Penner & Frieden, 1985). This would explain why significantly different pK_a's were observed in $(V/K)_{NADPH}$ and $(V/K)_{NADH}$ pH profiles for P450R for what appeared to be the same enzymic group—an effect which could not be explained based on different degrees of substrate stickiness (Sem & Kasper, 1993a).

In summary, we have shown that the kinetic mechanism of the model P450R reaction with cytochrome c^{3+} is two-site ping-pong, with cytochrome c^{3+} itself binding in a tetra uni ping-pong manner at site 2. We have derived the steady-state kinetic equation that corresponds to this model, the equations describing the kinetic constants V_{max}, $(V/K)_{cytc}$, and $(V/K)_{NADPH}$, and the corresponding Michaelis constants. Such a model is consistent with the observed inhibition patterns and predicts the formation of ternary (NADPH or $NADP^+$)·(P450R)·(cytochrome c^{3+} or cytochrome c^{2+}) complexes. This mechanism only applies at high ionic strength (850 mM) and serves as a mechanistic foundation for the interpretation of results at lower ionic strength (D. S. Sem and C. B. Kasper, manuscript in preparation).

[6] The forward commitment to catalysis (Cleland, 1982) is the ratio of the rate constant for hydride transfer to the net rate constant for NADPH release for the reaction sequence in eq 7.

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REFERENCES

Barden, R. E., Fung, C. H., Utter, M. F., & Scrutton, M. C. (1972) *J. Biol. Chem. 247*, 1323–1333.
Bastiaens, P. I. H., Bonants, P. J. M., Muller, F., & Visser, A. J. W. G. (1989) *Biochemistry 28*, 8416–8425.
Benveniste, I., Gabriac, B., & Durst, F. (1986) *Biochem. J. 235*, 365–373.
Bhattacharyya, A., Lipka, J. J., Waskell, L., & Tollin, G. (1991) *Biochemistry 30*, 759–765.
Cleland, W. W. (1973) *J. Biol. Chem. 248*, 8353–8355.
Cleland, W. W. (1975) *Biochemistry 14*, 3220–3224.
Cleland, W. W. (1977) *Adv. Enzymol. 45*, 273–387.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.
Cleland, W. W. (1982) *CRC Crit. Rev. Biochem. 13*, 385–428.
Cleland, W. W. (1986) in *Investigations of Rates and Mechanisms of Reactions* (Bernasconi, C. F., Ed.) Vol. 6, pp 791–870, Wiley, New York.
Cook, P. F., & Cleland, W. W. (1981) *Biochemistry 20*, 1790–1796.
Crankshaw, D. L., Hetnarski, K., & Wilkinson, C. F. (1979) *Biochem. J. 181*, 593–605.
Fan, L. L., & Masters, B. S. S. (1974) *Arch. Biochem. Biophys. 165*, 665–671.
Gelder, B. F. V., & Slater, E. C. (1962) *Biochim. Biophys. Acta 58*, 593–595.
Hiwatashi, A., & Ichikawa, Y. (1979) *Biochim. Biophys. Acta 580*, 44–63.
Ichikawa, Y., & Yamano, T. (1969) *J. Biochem. 66*, 351–360.
Iyanagi, T., & Mason, H. S. (1973) *Biochemistry 12*, 2297–2308.
Iyanagi, T., Makino, N., & Mason, H. S. (1974) *Biochemistry 13*, 1701–1710.
Iyanagi, T., Anan, F. K., Imai, Y., & Mason, H. S. (1978) *Biochemistry 17*, 2224–2230.
Iyanagi, T., Makino, R., & Anan, F. K. (1981) *Biochemistry 20*, 1722–1730.
Kasper, C. B. (1971) *J. Biol. Chem. 246*, 577–581.
Katiyar, S. S., Cleland, W. W., & Porter, J. W. (1975) *J. Biol. Chem. 250*, 2709–2717.
Kobayashi, S., & Rikans, L. E. (1984) *Comp. Biochem. Physiol. 77B*, 313–318.
Kominami, S., Ogishima, T., & Takemori, S. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Eds.) pp 715–718, Elsevier North Holland, New York.
Kominami, S., Hara, H., Ogishima, T., & Takemori, S. (1984) *J. Biol. Chem. 259*, 2991–2999.
Lu, A. Y. H., Junk, K. W., & Coon, M. J. (1969) *J. Biol. Chem. 244*, 3714–3721.
Masters, B. S. S., Bilimoria, M. H., Kamin, H., & Gibson, Q. H. (1965a) *J. Biol. Chem. 240*, 4081–4088.
Masters, B. S. S., Kamin, H., Gibson, Q. H., & Williams, C. H., Jr. (1965b) *J. Biol. Chem. 240*, 921–931.

Mayer, R. T., & Durrant, J. L. (1979) *J. Biol. Chem. 254*, 756–761.
McClendon, G. (1988) *Acc. Chem. Res. 21*, 160–167.
McClure, W. R., Lardy, H. A., Wagner, M., & Cleland, W. W. (1971) *J. Biol. Chem. 246*, 3579–3583.
Neufeld, E. F., Kaplan, N. O., & Colowick, S. P. (1955) *Biochem. Biophys. Acta 17*, 525–535.
Nisimoto, Y., & Otsuka-Murakami, H. (1988) *Biochemistry 27*, 5869–5876.
Northrop, D. B. (1969) *J. Biol. Chem. 244*, 5808–5819.
Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 122–152, University Park Press, Baltimore, MD.
Oprian, D. D., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 8935–8944.
Otvos, J. D., Krum, D. P., & Masters, B. S. S. (1986) *Biochemistry 25*, 7220–7228.
Penner, M. H., & Frieden, C. (1985) *J. Biol. Chem. 260*, 5366–5369.
Phillips, A. H., & Langdon, R. G. (1962) *J. Biol. Chem. 237*, 2652–2660.
P-L Biochemicals, Inc. (1956) Circular OR-10, pp 1–21.
Porter, T. D., & Kasper, C. B. (1986) *Biochemistry 25*, 1682–1687.
Sem, D. S., & Kasper, C. B. (1992) *Biochemistry 31*, 3391–3398.
Sem, D. S., & Kasper, C. B. (1993a) *Biochemistry 32*, 11539–11547.
Sem, D. S., & Kasper, C. B. (1993b) *Biochemistry 32*, 11548–11558.
Shen, A. L., Porter, T. D., Wilson, T. E., & Kasper, C. B. (1989) *J. Biol. Chem. 264*, 7584–7589.
Tsai, C. S., Burgett, M. W., & Reed, L. J. (1973) *J. Biol. Chem. 248*, 8348–8352.
Vermilion, J. L., & Coon, M. J. (1978a) *J. Biol. Chem. 253*, 2694–2704.
Vermilion, J. L., & Coon, M. J. (1978b) *J. Biol. Chem. 253*, 8812–8819.
Vermilion, J. L., Ballou, D. P., Massey, V., & Coon, M. J. (1981) *J. Biol. Chem. 256*, 266–277.
Viola, R. E., Cook, P. F., & Cleland, W. W. (1979) *Anal. Biochem. 96*, 334–340.
Williams, C. H., Jr., & Kamin, H. (1962) *J. Biol. Chem. 237*, 587–595.
Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337–5344.
Yasukochi, Y., Peterson, J. A., & Masters, B. S. S. (1979) *J. Biol. Chem. 254*, 7097–7104.

APPENDIX: DERIVATION OF RATE EQUATIONS FOR TWO-SITE PING-PONG MECHANISM OF NADPH–CYTOCHROME P450 OXIDOREDUCTASE

General Framework. Two methods exist for the derivation of rate equations for multi-site ping-pong mechanisms. According to the method outlined by Northrop (1969), the rate equation for a two-site ping-pong mechanism can be derived if one assumes that the rate of reaction at each site is proportional to the fraction of the enzyme in the correct form to react with substrate and the fractional occupation of the site by substrate. This assumption is not made in Cleland's method (Cleland, 1973), but in the case of P450R, this is a reasonable assumption and simplifies the algebra considerably, so we have used Northrop's method. This method also assumes that (a) there are separate sites on the enzyme where reactions can occur, (b) binding of substrate or product at one site does not affect binding at the other site, (c) binding occurs with equal affinity to all forms (redox states) of the enzyme, and (d) binding of substrates and products is in rapid equilibrium. The last two assumptions are necessary in order to make use of Cha's simplification (Cha, 1968) of the King and Altman method (King & Altman, 1956). These assumptions reduce the number of terms in the final rate equation by over 2 orders of magnitude.[7]

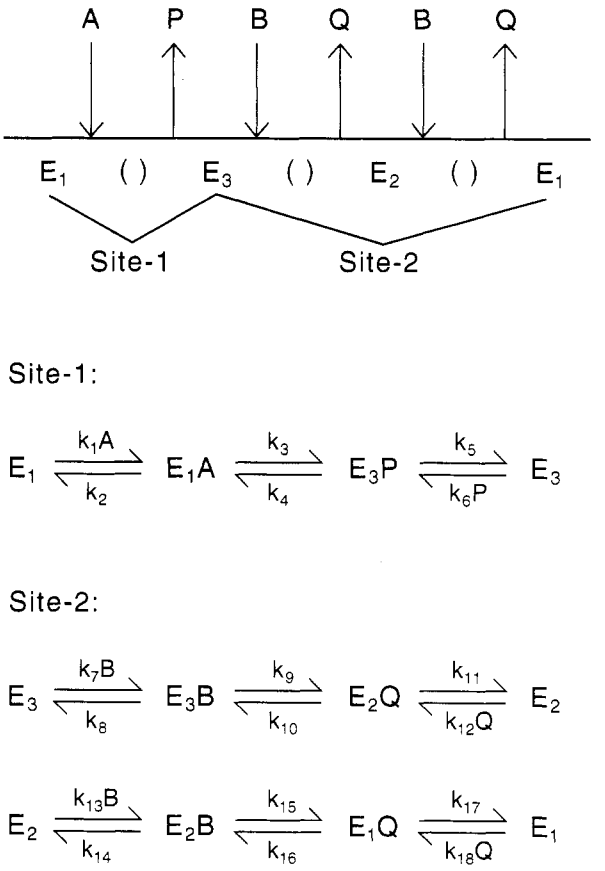


FIGURE 4: Kinetic scheme for a two-site ping-pong mechanism with uni uni reaction at site 1 and tetra uni ping-pong reaction at site 2. *A*, *B*, *P*, and *Q* are concentrations of NADPH, cytochrome c^{3+}, $NADP^+$ and cytochrome c^{2+}, respectively. E_1, E_2, and E_3 are the one ($FMNH^{\bullet}/FAD$), two ($FMNH_2/FAD$), and three ($FMNH_2/FADH^{\bullet}$) electron-reduced forms of P450R, respectively. Although substrates (*A* and *B*) and products (*P* and *Q*) can bind to E_1, E_2, and E_3 (as in Figure 2), only the complexes shown here are catalytically competent.

Derivation for Tetra Uni Ping-Pong Reaction at Site 2. The kinetic scheme for a two-site ping-pong mechanism with uni uni reaction at site 1 and tetra uni ping-pong reaction at site 2 is shown in Figure 4. Since sites 1 and 2 can be occupied independently of each other, a number of different complexes can form with P450R. The complexes that can form in this mechanism are shown in Figure 5. In this scheme, binding is independent at the two sites (assumptions a and b) so, for example, *E*, *EA*, and *EP* all bind *B* with equal affinity. Furthermore, binding occurs with equal affinity to E_1, E_2, and E_3 (assumption c). Since the binding scheme shown in Figure 5 is in rapid equilibrium (assumption d), the fractional occupation (f_x) of site 1 or site 2 can be calculated from the dissociation constants (Cha, 1968). It can be shown that

$$f_A = \frac{EA + EAB + EAQ}{E_t} = \frac{A/K_{iA}}{1 + A/K_{iA} + P/K_{iP}} \quad (A1)$$

[7] This is based on the fact that the complete rate equation for transcarboxylase, which has a simpler mechanism with uni/uni reactions at both sites, is predicted to have more than 1000 terms (Northrop, 1969).

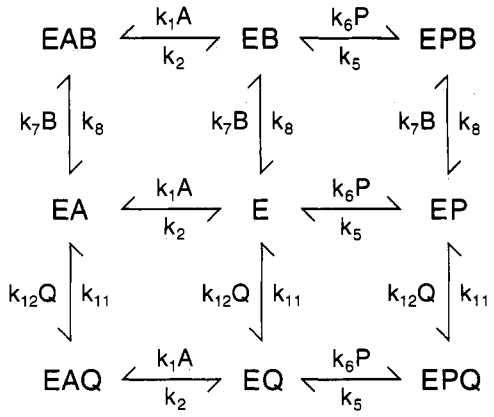


FIGURE 5: Scheme showing the various complexes that can form in the mechanism described in Figure 4. E is E_1, E_2, or E_3, and A, B, P, and Q are as in Figure 4. These binding steps are assumed to be in rapid equilibrium.

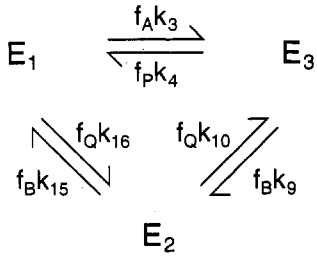


FIGURE 6: Scheme showing the interconversion of enzyme forms for the mechanism in Figure 4. The f_A and f_P terms represent the fraction of P450R with site 1 occupied by A and P, respectively, while f_B and f_Q are the corresponding terms for occupation of site 2 by B and Q, respectively.

$$f_P = \frac{EP + EPB + EPQ}{E_t} = \frac{P/K_{iP}}{1 + A/K_{iA} + P/K_{iP}} \quad \text{(A2)}$$

$$f_B = \frac{EB + EAB + EPB}{E_t} = \frac{B/K_{iB}}{1 + B/K_{iB} + Q/K_{iQ}} \quad \text{(A3)}$$

$$f_Q = \frac{EQ + EAQ + EPQ}{E_t} = \frac{Q/K_{iQ}}{1 + B/K_{iB} + Q/K_{iQ}} \quad \text{(A4)}$$

where $E_t = E + EA + EB + EP + EQ + EAB + EBP + EAQ + EPQ$, and the K_i terms are the dissociation constants for A, B, P, and Q. Making use of these terms, one can then use the method of King and Altman (1956) to determine the fraction of enzyme in the correct form to react (E_1/E_t, E_2/T_t, or E_3/E_t) based on the scheme in Figure 6 describing the interconversion of enzyme forms. This yields the following rate equation:

$$v/E_t = [(k_3k_9k_{15})(AB^2 - PQ^2/K_{eq})]/[AB^2(k_9k_{15} + k_3k_9 + k_3k_{15}) + ABK_{iB}(k_3k_9 + k_3k_{15}) + B^2K_{iA}k_9k_{15} + ABQ(K_{iB}/K_{iQ})(k_3k_9 + k_9k_{16} + k_3k_{10} + k_3k_{15}) + B^2P(K_{iA}/K_{iP})(k_9k_{15} + k_4k_{15}) + BP(K_{iA}K_{iB}/K_{iP})k_4k_{15} + BQ(K_{iA}K_{iB}/K_{iQ})k_9k_{16} + AQ(K_{iB}^2/K_{iQ})k_3k_{10} + AQ^2(K_{iB}^2/K_iQ^2)(k_3k_{10} + k_{10}k_{16}) + PQ(K_{iA}K_{iB}^2/(K_{iP}K_{iQ}))(k_4k_{10} + k_4k_{16}) + BPQ(K_{iA}K_{iB}/(K_{iP}K_{iQ}))(k_4k_{10} + k_4k_{16} + k_9k_{16} + k_4k_{15}) + PQ^2(K_{iA}K_{iB}^2/(K_{iP}K_{iQ}^2))(k_4k_{10} + k_4k_{16} + k_{10}k_{16}) + Q^2(K_{iA}K_{iB}^2/K_{iQ}^2)k_{10}k_{16}] \quad \text{(A5)}$$

where K_{eq} is the equilibrium constant for the reaction catalyzed by P450R. In the absence of products ($P = Q = 0$), this simplifies to

$$v/E_t = [ABk_3k_9k_{15}/(k_9k_{15} + k_3k_9 + k_3k_{15})]/[AB + AK_{iB}(k_3k_9 + k_3k_{15})/(k_9k_{15} + k_3k_9 + k_3k_{15}) + BK_{iA}(k_9k_{15})/(k_9k_{15} + k_3k_9 + k_3k_{15})] \quad \text{(A6)}$$

which is of the same form as the equation for a classical ping-pong mechanism:

$$\frac{V}{E_t} = \frac{ABV_{max}}{AB + AK_B + BK_A} \quad \text{(A7)}$$

where K_A and K_B are Michaelis constants. The V_{max}, K_A, and K_B values derived using the net rate constant method (Cleland, 1975) in the paper reduce to those shown here if it is assumed that there is rapid equilibrium binding of all substrates and products and that cytochrome c^{3+} binds with equal affinity to E_3 and E_2.

Product Inhibition Patterns. In the presence of P ($Q = 0$), eq A5 reduces to

$$v = \frac{ABV_{max}}{AB + AK_B + BK_A(1 + P/K_{iP}') + K_AK_{iB}(P/K_{ip}'')} \quad \text{(A8)}$$

where $K_{ip}' = K_{ip}/(1 + k_4/k_9)$ and $K_{ip}'' = K_{ip}(k_9/k_4)$. In reciprocal form, the equation describing the product inhibition by P with varied A is

$$1/v = (1/V_{max})(1 + K_B/B) + (K_A/V_{max})(1/A)(1 + P/K_{iP}' + (K_{iB}/B)(P/K_{ip}'')) \quad \text{(A9)}$$

which predicts competitive inhibition. The corresponding equation describing the product inhibition by P with varied B is

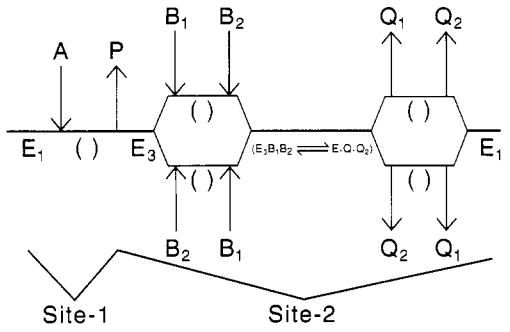
$$1/v = (1/V_{max})(1 + (K_A/A)(1 + P/K_{iP}')) + (1/B)(K_B/V_{max})[1 + (K_{A/A})(K_{iB}/K_B)(P/K_{ip}'')] \quad \text{(A10)}$$

which predicts noncompetitive inhibition. If $k_9 \gg k_4$ (E_3 passes electrons to cytochrome c^{3+} faster than to $NADP^+$), then $K_{ip}'' \gg K_{ip}'$, and uncompetitive inhibition would be observed. Conversely, if $k_4 \gg k_9$, competitive inhibition would be observed.

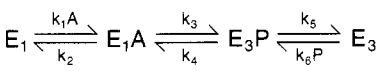
In the presence of Q ($P = 0$), eq A5 reduces to

$$v = AB^2V_{max}/[AB^2 + AB(K_B + (Q/K_{iQ})C_1 + B^2K_A + B(Q/K_{iQ})C_2 + A(Q/K_{iQ})C_3 + A(Q^2/K_{iQ})C_4 + (Q^2/K_{iQ}^2)C_5] \quad \text{(A11)}$$

where $C_1 = K_{iB}(k_3k_9 + k_9k_{16} + k_3k_{10} + k_3k_{15})/\text{DEN}$; $C_2 = K_{iA}K_{iB}(k_9k_{16})/\text{DEN}$; $C_3 = K_{iB}^2(k_3k_{10})/\text{DEN}$; $C_4 = K_{iB}^2(k_3k_{10} + k_{10}k_{16})/\text{DEN}$; $C_5 = K_{iA}K_{iB}^2(k_{10}k_{16})/\text{DEN}$; and $\text{DEN} = (k_9k_{15} + k_3k_9 + k_3k_{15})$. This will give curved initial velocity patterns, unless C_1 and $C_2 \gg C_3$, C_4, and C_5. Actually, since k_{10} and k_{16} are expected to be quite small (they represent the rates of reduction of E_2 and E_1, respectively, by cytochrome c^{2+}), inspection of the equations for C_1–C_5 reveals that C_1 and C_2 will be the predominant terms if $k_3 \ll k_9$ (hydride transfer is slower than electron transfer). If this is the case, as expected for P450R, then the equation describing product inhibition by Q with varied B is



Site-1:



Site-2:

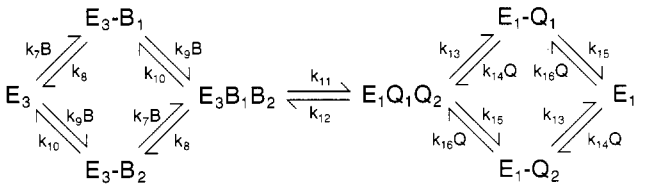


FIGURE 7: Kinetic scheme for a two-site ping-pong mechanism with uni uni reaction at site 1 and bi bi random sequential reaction at site 2. Nomenclature is as in Figure 4, with subscripts on B and Q indicating the subsite (1 or 2) occupied in site 2.

$$1/v = (1/V_{max})(1 + K_A/A) + (1/B)(K_B/V_{max} + (C_1/V_{max})(Q/K_{iQ}) + (QC_2)/(AK_{iQ}V_{max})) \quad (A12)$$

which predicts competitive inhibition. The corresponding rate equation describing product inhibition by Q with varied A is

$$1/v = (1/V_{max})(1 + K_B/B + (C_1/B)(Q/K_{iQ})) + (1/A)(K_A/V_{max} + (QC_2)/(BK_{iQ}V_{max})) \quad (A13)$$

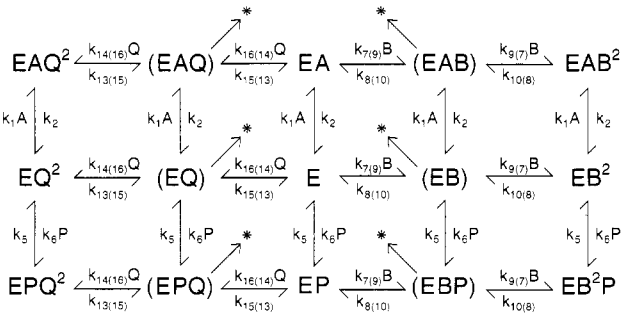
which predicts noncompetitive inhibition.

Dead-End Inhibition by an NADPH Analog. If an NADPH analog (A′) can bind without reacting in site 1, the following additional complexes can form: $E{\cdot}A'$, $E{\cdot}A'{\cdot}B$, and $E{\cdot}A'{\cdot}Q$. Adding these complexes to the equilibrium shown in Figure 5A and repeating the previous derivation leads to the following equation:

$$v = \frac{ABV_{max}}{AB + AK_B + BK_A(1 + A'/K_{iA}')} \quad (A14)$$

where K_{iA}' is the dissociation constant for A'. This predicts competitive inhibition in the A' versus A profile and uncompetitive inhibition in the A' versus B profile.

Derivation for Bi Bi Random Sequential Reaction at Site 2. The kinetic scheme for a two-site ping-pong mechanism with bi bi random sequential binding at site 2 is shown in Figure 7. Since two molecules of B or Q (or one of each) can occupy the two subsites of site 2, a number of additional complexes can form with P450R that could not form in the previous mechanism, as shown in Figure 8. As before, the fractional occupation of sites 1 and 2 can be calculated from dissociation constants:



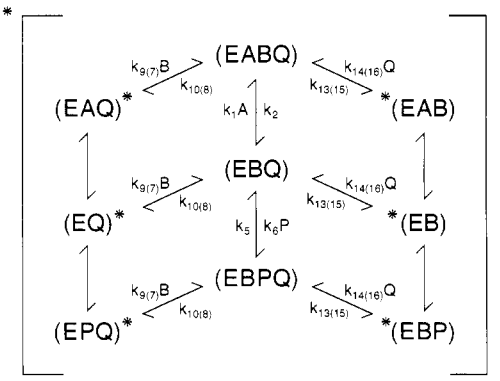


FIGURE 8: Scheme showing the various complexes that can form in the mechanism described in Figure 7. Nomenclature is as in Figure 5. These binding steps are assumed to be in rapid equilibrium. All complexes in parentheses represent two species, since site 2 has two subsites which can be occupied by B and/or Q [for example, (EB) is really EB_1 or EB_2]. Kinetic constants leading to or from these complexes are written for the sequential occupation of subsite 1 followed by subsite 2 (e.g., $E \rightarrow EB_1 \rightarrow EB_1B_2$) with subscripts for the reverse binding order (e.g., $E \rightarrow EB_2 \rightarrow EB_1B_2$) given in parentheses. Finally, the arrows pointing to the asterisks indicate that this scheme is continued into a third dimension, shown below in brackets, with formation of the $(EABQ)$, (EBQ), and $(EBPQ)$ complexes.

$$f_A = (EA + EAB_1 + EAB_2 + EAB^2 + EAQ_1 + EAQ_2 + EAQ^2 + EAB_2Q_1 + EAB_1Q_2)/E_t$$

$$= \frac{A/K_{iA}}{1 + A/K_{iA} + P/K_{iP}} \quad (A15)$$

$$f_P = \frac{P/K_{iP}}{1 + A/K_{iA} + P/K_{iP}} \quad (A16)$$

$$f_B = \frac{EB^2 + EAB^2 + EPB^2}{E_t}$$

$$= [B^2/(K_{iB1}K_{iB2})]/[1 + B^2/(K_{iB1}/K_{iB2}) + B/K_{iB}' + Q^2/(K_{iQ1}K_{iQ2}) + Q/K_{iQ}' + BQ/K_{iBQ}'] \quad (A17)$$

$$f_Q = [Q^2/(K_{iQ1}K_{iQ2})]/[1 + B^2/(K_{iB1}K_{iB2}) + B/K_{iB}' + Q^2/(K_{iQ1}K_{iQ2}) + Q/K_{iQ}' + BQ/K_{iBQ}'] \quad (A18)$$

where $E_t = E + EA + EAB_1 + EAB_2 + EAB^2 + EAQ_1 + EAQ_2 + EAQ^2 + EAB_1Q_2 + EAB_2Q_1 + EB_1 + EB_2 + EB^2 + EP + EQ_1 + EQ_2 + EQ^2 + EPB_1 + EPB_2 + EB_1Q_2 + EB_2Q_1 + EPB_1Q_2 + EPB_2Q_1 + EPB^2 + EPQ_1 + EPQ_2 + EPQ^2$; $K_{iB}' = (K_{iB1}K_{iB2})/(K_{iB1} + K_{iB2})$; and $K_{iBQ}' = (K_{iB1Q2}K_{iB2Q1})/(K_{iB1}Q_2 + K_{iB2Q1})$. These terms can be used with the scheme in Figure 9 to derive the rate equation as described previously:

$$v = [k_3k_{11}(AB^2 - PQ^2/K_{eq})]/[AB^2(k_3 + k_{11}) + A_{KiB1}K_{iB2}k_3 + B^2{}_{KiA}k_{11} + AB(K_{iB1}K_{iB2}/K_{iB}')\,k_3 + AQ^2(K_{iB1}K_{iB2}/(K_{iQ1}K_{iQ2}))(k_3 + k_{12}) + AQ(K_{iB1}K_{iB2}/K_{iQ}')k_3 + B^2P(K_{iA}/K_{iP})(k_4 + k_{11}) + PB(K_{iA}K_{iB1}K_{iB2}/(K_{iP}K_{iB}'))k_4 + ABQ(K_{iB1}K_{iB2}/K_{iBQ}')k_3 + BPQ(K_{iA}K_{iB1}K_{iB2}/(K_{iP}K_{iBQ}'))k_4 + P(K_{iA}K_{iB1}K_{iB2}/K_{ip})k_4 + PQ(K_{iA}K_{iB1}K_{iB2}/(K_{iP}K_{iQ}'))k_4 + Q^2(K_{iA}K_{iB}K_{iB2}/(K_iQ_1K_{iQ2}))k_{12} + PQ^2(K_{iA}K_{iB1}K_{iB2}/(K_{iP}K_{iQ1}K_{iQ2}))(k_4 + k_{12})]$$ (A19)

In the absence of products ($P = Q = 0$), this simplifies to

$$v = [AB^2k_3k_{11}/(k_3 + k_{11})]/[AB^2 + AK_{iB1}K_{iB2}k_3/(k_3 + k_{11}) + B^2K_{iA}k_{11}/(k_3 + k_{11}) + ABK_{iB1}K_{iB2}k_3/(K_{iB}'(k_3 + k_{11}))]$$ (A20)

which can be written as

$$\frac{v}{E_t} = \frac{AB^2V_{max}}{AB^2 + AK_B + B^2K_A + ABK_B/K_{iB}'}$$ (A21)

In reciprocal form this is

$$1/v = 1/V_{max} + (1/B)(K_B/V_{max})(1/B + 1/K_{iB}') + (1/A)(K_A/V_{max})$$ (A22)

which would give curved initial velocity patterns with varied

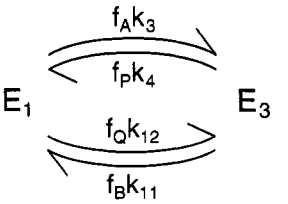


FIGURE 9: Scheme showing the interconversion of enzyme forms for the reaction described in Figure 7. The fractional occupation terms (f_X) are as defined in Figure 6, except that f_B and f_Q now represent the binding of two molecules of B and Q, respectively.

B. Although the $1/v$ versus $1/A$ profile would give straight lines, the intercept replots would be curved. The curvature in both plots would be most pronounced at low concentrations of B, when $B \leq K_{iB}'$. Inspection of eq A21 also reveals that the loss of the AK_B term (this term reflects formation of the EB^2 complex) would produce an equation similar in form to eq A7, for the classical ping-pong mechanism. Thus, conditions that favor formation of the EB^2 complex would produce the curved initial velocity patterns discussed here.

REFERENCES

Cha, S. (1968) *J. Biol. Chem. 243*, 820–825.

Cleland, W. W. (1973) *J. Biol. Chem. 248*, 8353–8355.

Cleland, W. W. (1975) *Biochemistry 14*, 3220–3224.

King, E. L., & Altman, C. (1956) *J. Phys. Chem. 60*, 1375–1378.

Northrop, D. B. (1969) *J. Biol. Chem. 244*, 5808–5819.