Effect of Ionic Strength on the Kinetic Mechanism and Relative Rate Limitation of Steps in the Model NADPH−Cytochrome P450 Oxidoreductase Reaction with Cytochrome c[†]

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ABSTRACT: Although the kinetic mechanism of the NADPH-cytochrome P450 oxidoreductase (P450R) reaction with cytochrome c^{3+} has been determined at 850 mM ionic strength [Sem, D. S., & Kasper, C. B. (1994) *Biochemistry 33*, 12012−12021], this mechanism is no longer valid at lower ionic strength. At 850 mM ionic strength, the mechanism is two-site ping-pong, and reaction at the electron acceptor site is itself ping-pong. As the ionic strength is decreased below 850 mM, the initial velocity profiles begin to show curvature when cytochrome c^{3+} is the varied substrate. These data are consistent with a mechanism that is still two-site ping-pong, but now with random sequential binding of two molecules of cytochrome c^{3+} at the electron acceptor site. Decreasing ionic strength also causes a change in rate-limiting steps, with $(V/K)_{cytc}$ and $(V/K)_{NADPH}$ increasing while V_{max} decreases (below 500 mM ionic strength). These results are consistent with favorable ionic interactions being important for binding NADPH and cytochrome c^{3+} and with product ($NADP^+$) release becoming the rate-limiting step in V_{max} at low ionic strength. V_{max} decreases significantly at higher ionic strength (>500 mM), while $(V/K)_{NADPH}$ decreases only slightly. The ^DV isotope effect is largest (2.4) at 500 mM ionic strength but decreases at both low and high ionic strength as steps other than hydride transfer become more rate-limiting. $^D(V/K)_{NADPH}$ also decreases at both low and high ionic strength, but to a lesser extent than ^DV. These data are most consistent with a model in which hydride transfer is a significant rate-limiting step in the ionic strength range of 200−750 mM, but since the intrinsic isotope effect is ≥4.3, hydride transfer is not entirely rate limiting. At lower ionic strength, $NADP^+$ release becomes rate-limiting, and at higher ionic strength, some step other than hydride transfer becomes rate-limiting. This step occurs after NADPH binding, but before hydride transfer, so it probably represents a conformational change that must occur prior to hydride transfer. Finally, a novel approach is presented for determining lower limit estimates of the intrinsic isotope effect, the forward commitment to catalysis (C_f), and the V ratio (C_{vf}). This method is applicable to enzymes with ping-pong mechanisms and small reverse commitments to catalysis (C_r).

NADPH−cytochrome P450 oxidoreductase (P450R)[1] (NADPH−ferrihemoprotein oxidoreductase, EC 1.6.2.4) is a member of the dehydrogenase/electron transferase family of flavoproteins (Massey & Hemmerich, 1980) along with ferredoxin−$NADP^+$ reductase. NADH−nitrate reductase, NADH−cytochrome b_5 reductase, NADH−sulfite reductase, and nitric oxide synthase (Porter & Kasper, 1986; Porter, 1991; Karplus et al., 1991; Shen & Kasper, 1993). These enzymes are thought to bind the nicotinamide ring of NADPH and the isoalloxazine ring of FAD in the *exo* orientation (Sem & Kasper, 1992), which distinguishes them from the flavoprotein dehydrogenases related to glutathione reductase (carbon−sulfur transhydrogenases) that bind the two ring systems in the *endo* orientation.

P450R catalyzes the transfer of two electrons from NADPH to the cytochromes P450 (Lu & Coon, 1968) and also to cytochrome b_5 (Enoch & Strittmatter, 1979), fatty acid elongase (Ilan et al., 1981), and heme oxygenase (Schacter et al., 1972). The electron transfer is from NADPH to FAD, then to FMN, and finally to the cytochromes P450 or other electron acceptors (Vermilion et al., 1981). Electrons can be transferred to certain non-physiologically relevant acceptors like cytochrome c^{3+}, dichloroindophenol, ferricyanide, and menadione (Williams & Kamin, 1962). NADPH and the electron acceptor (cytochrome c^{3+}) bind independently at separate sites on P450R (Sem & Kasper, 1994). It is thought that the rate limiting step in the P450R reaction sequence is the hydride transfer from NADPH to FAD (Masters et al., 1965a,b; Oprian & Coon, 1982; Yasukochi et al., 1979), but since this conclusion is based on pre-steady-state studies, it does not provide any information on how rate limiting hydride transfer is relative to product release and a number of other steps in the steady-state reaction sequence.

Although both ping-pong (binary complex) and sequential (ternary complex) mechanisms have been proposed for the P450R reaction with cytochrome c^{3+}, the mechanism at high ionic strength is actually a hybrid of these two mechanisms. It is a two-site ping-pong mechanism which allows for formation of a ternary complex (Sem & Kasper, 1994). It was suggested that one reason for the disagreement over the

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[1] Abbreviations: P450R, NADPH−cytochrome P450 oxidoreductase; cytc, cytochrome c^{3+}; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

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kinetic mechanism of P450R is that the various studies were carried out at different ionic strengths (Dignam & Strobel, 1977), since it is known that P450R activity is extremely sensitive to ionic strength (Bilimoria & Kamin, 1973; Phillips & Langdon, 1962; Singh & Piette, 1992; Voznesensky & Schenkman, 1992a,b, 1994). We have extended our previous steady-state kinetic analysis of P450R (Sem & Kasper, 1994) by determining the effect of ionic strength on the kinetic mechanism and on the relative rate limitation of various steps in the reaction sequence. We have found that ionic strength can alter this mechanism and change the relative rate limitation of steps in the steady-state reaction sequence. Although the kinetic mechanism remains two-site ping-pong, decreasing ionic strength does change the reaction from ping-pong to random sequential at the electron acceptor site and also changes the relative rate limitation of the hydride transfer step.

MATERIALS AND METHODS

NADPH, TAPS, MES, CHES, and horse heart cytochrome *c* (ferric) were from Sigma. KCl was from Mallinckrodt and acetic acid was from Fisher. NADPH deuterated in the *pro-R* position on the C4 atom of the nicotinamide ring ($NADPD_A$) was prepared using the procedure of Viola et al. (1979) with slight modifications (Sem & Kasper, 1992). Recombinant rat liver P450R was purified on a 2′,5′-ADP–Sepharose column (Yasukochi & Masters, 1976) as described previously (Shen et al., 1989).

Kinetic Assays. Kinetic assays were performed at 25 °C in a 1-mL cuvette with a path length of 1 cm. Reaction rates were monitored spectrophotometrically at 550 nm using an extinction coefficient of 21.1 mM^{-1} cm^{-1} for cytochrome c^{3+} (Gelder & Slater, 1962). This extinction coefficient is valid over the pH and ionic strength range used in these studies (Sem and Kasper, unpublished results). All assays, with the exception of those used in the pH studies, contained 100 mM TAPS, pH 8.0, and enough KCl to give the desired ionic strength (calculated). The pH dependence of *V/K* values was determined using buffers (acetate, MES, TAPS, and CHES) with pK_a values within 0.5 pH unit of the desired pH. Concentrations of NADPH and cytochrome c^{3+} were varied as described in the figure legends. Reactions were initiated by the addition of P450R (0.1−0.5 μg). Initial rates were linear, although at low ionic strength it was necessary to measure rates after a brief lag period but before product inhibition became significant. Typically, the linear portion of the progress curve was long enough to measure rates reliably. Enzyme activity between experiments and concentrations of stock solutions of cytochrome c^{3+}, NADPH, and $NADPD_A$ were determined as described previously (P-L Biochemicals, 1956; Sem & Kasper, 1994).

Data Processing. Data were fitted to the appropriate equations using the programs of Cleland (1979), which perform a nonlinear least-squares fit. Equations were fit in log form, which effectively assumes constant proportional error in velocity. The data from initial velocity studies at 540 and 300 mM ionic strength fit best to the equation for a two-site ping-pong mechanism, with random sequential binding of two identical substrates (cytochrome c^{3+}) at one of these sites [derived previously by Sem and Kasper (1994)]:

$$v = \frac{V_{max}AB^2}{C_0A + K_BAB + K_AB^2 + AB^2} \quad (1)$$

where v is the initial velocity, C_0 is a constant term, A and B are the concentrations of NADPH and cytochrome c^{3+}, respectively, and K_A and K_B are their corresponding Michaelis constants. The complete data set, which includes a matrix of cytochrome c^{3+} and NADPH concentrations, was fit to eq 1, for experiments carried out at 540 and 300 mM ionic strengths. Sigma values for these fits were 0.061 and 0.12, respectively.

V_{max}, $(V/K)_{NADPH}$, and $(V/K)_{cytc}$ values determined as a function of pH and of ionic strength were obtained by fitting the data to

$$v = \frac{V_{max}C}{C + K_C} \quad (2)$$

where v is the initial velocity, C is the concentration of the varied substrate [NADPH (A in eq 1) or cytochrome c^{3+} (B in eq 1)], and K_C is its Michaelis constant. The nonvaried substrate was maintained at a high concentration (50 μM for NADPH and 100 μM for cytochrome c^{3+}) relative to its K_m (2−16 μM), so that eq 1 simplifies to eq 2. While this simplification is clear when A is the varied substrate (the C_0A and K_BAB terms drop at $B \gg K_B$), it is only valid with B as the varied substrate when B is not too dilute.[2] Empirically, this is achieved when B (cytochrome c^{3+}) is present at >3 μM. Furthermore, since cytochrome c^{3+} shows substrate inhibition (Sem & Kasper, 1994), it was necessary to confine cytochrome c^{3+} concentrations to the range 3−100 μM in order to obtain a reliable fit to eq 2 at all ionic strengths for the determination of V_{max} and (V/K) values.

The primary deuterium isotope effects on $(V/K)_{NADPH}$ and V_{max} [$^D(V/K)_{NADPH}$ and ^DV, respectively][3] were determined at various ionic strengths and at various concentrations of cytochrome c^{3+}, using the method of direct comparison (Cleland, 1982). Data were fitted to the following equation:

$$v = \frac{V_{max}A}{K_m(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (3)$$

where v is the initial velocity, A is the varied substrate (NADPH or $NADPD_A$) concentration, K_m is its corresponding Michaelis constant, F_i is the fraction of deuterium incorporation [86%, calculated as described previously (Sem & Kasper, 1994)], and $E_{V/K}$ and E_V are the isotope effects minus one on $(V/K)_{NADPH}$ and V_{max}, respectively. The observed isotope effects are primary since the A-side (*pro-R*) hydrogen is transferred (Sem & Kasper, 1992).

RESULTS

Effect of Ionic Strength on Initial Velocity Profiles. At an ionic strength of 540 mM, although the plot of $1/v$ versus

[2] If $A \gg K_A$, eq 1 can be rearranged and simplified to give $v = V_{max}B/[(C_0/B) + K_B + B]$, which has the form of eq 2 only when $(C_0/B) \ll K_B$, which occurs at relatively high concentrations of B.

[3] The nomenclature used is that of Northrop (1977) and Cook and Cleland (1981). ^DV represents the ratio of the V_{max} value for hydride transfer with NADPH as substrate to that for deuteride transfer with $NADPD_A$ as substrate. $^D(V/K)_{NADPH}$ represents the corresponding ratio for $(V/K)_{NADPH}$ values.

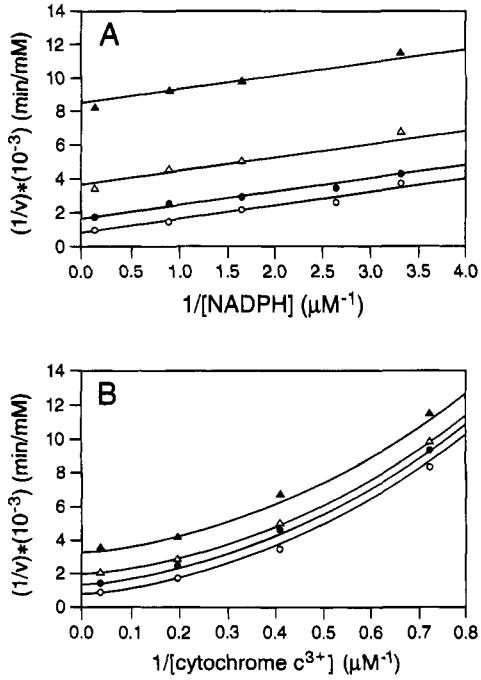


FIGURE 1: Initial velocity patterns for P450R with (A) 1/[NADPH] varied at fixed levels of cytochrome c^{3+} (30, 5.1, 2.5, and 1.4 μM) and (B) 1/[cytochrome c^{3+}] varied at fixed levels of NADPH (7.5, 1.1, 0.60, and 0.30 μM). Reactions were carried out in 0.1 M TAPS, pH 8.0, and an ionic strength of 540 mM. Open and filled circles and open and filled triangles represent decreasing concentrations of cytochrome c^{3+} or NADPH, as indicated above in parentheses. Lines represent a fit of the data to eq 1. Parameters obtained from this are $C_0 = 15.8 \pm 1.5$ μM^2, $V_{max} = 1.36 \pm 0.12$ μM/min, $K_A = 1.04 \pm 0.12$ μM, and $K_B = 3.4 \pm 1.1$ μM.

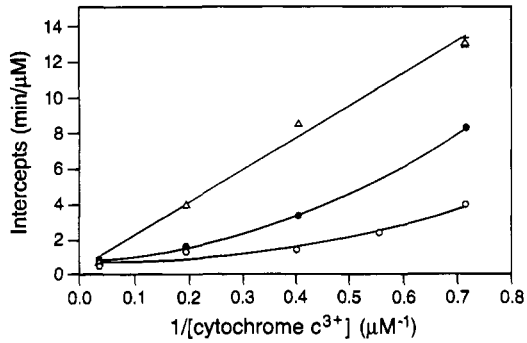


FIGURE 2: Intercept ($1/V_{max}$) replots for initial velocity patterns obtained at ionic strengths of 850 mM (from Sem and Kasper (1994)), 540 mM (Figure 1A), and 300 mM (plot not shown). Open triangles, filled circles, and open circles represent replots of the 850, 540, and 300 mM ionic strength data, respectively.

1/[NADPH] at fixed levels of cytochrome c^{3+} gave parallel lines (Figure 1A), the plot of $1/v$ versus 1/[cytochrome c^{3+}] at fixed levels of NADPH showed increasing curvature at low cytochrome c^{3+} levels (Figure 1B). Similar plots were obtained at 300 mM ionic strength (data not shown). The intercept replots for the data in Figure 1A, and the corresponding data at 300 mM ionic strength, show curvature, though, which is most pronounced at lower ionic strength (Figure 2). Although the high ionic strength (850 mM) data fit best to the equation of a simple ping-pong model (Sem & Kasper, 1994), the curvature observed at low ionic strength in the $1/v$ versus 1/[cytochrome c^{3+}] plots, and in the intercept replots for the $1/v$ versus 1/[NADPH] plots, indicates the presence of an additional, squared term in

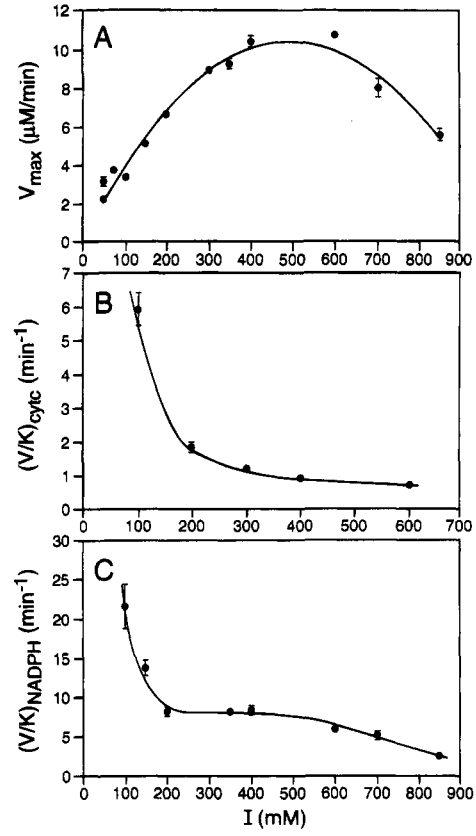


FIGURE 3: Effect of ionic strength on (A) V_{max}, (B) $(V/K)_{cytc}$, and (C) $(V/K)_{NADPH}$. Reactions were carried out in 0.1 M TAPS, pH 8.0, with ionic strength varied by the addition of appropriate levels of KCl. For $(V/K)_{cytc}$ determinations, NADPH was present at 50 μM and cytochrome c^{3+} was varied at 90, 31, 19, 7.2, 4.5, and 3.4 μM. For $(V/K)_{NADPH}$ determinations, cytochrome c^{3+} was present at 100 μM and NADPH was varied at 4.8, 1.1, 0.54, 0.37, and 0.29 μM. V_{max} values were obtained from both of these data sets.

cytochrome c^{3+}. The data at lower ionic strength were therefore fitted to eq 1.

Effect of Ionic Strength on Kinetic Constants. V_{max} is a maximum (±20% variation) at an ionic strength of 250−700 mM but decreases at higher and lower ionic strengths (Figure 3A). There is a 5-fold decrease in V_{max} in going from 500 to 50 mM ionic strength. $(V/K)_{cytc}$ and $(V/K)_{NADPH}$ both increase as ionic strength decreases (Figure 3 panels B and C, respectively) but are relatively insensitive to ionic strength effects at higher ionic strength (>300 mM). $(V/K)_{NADPH}$ shows a modest decrease at ionic strengths above 400 mM. $(V/K)_{cytc}$ is only slightly more sensitive to ionic strength since it increases 6-fold in going from an ionic strength of 500 to 100 mM, while $(V/K)_{NADPH}$ increases 3-fold for the same change in ionic strength.

Relative V/K Values. $(V/K)_{NADPH}$ is generally larger than $(V/K)_{cytc}$ for P450R. Although the $(V/K)_{NADPH}/(V/K)_{cytc}$ ratio does not vary in a systematic way with pH or ionic strength, it is always in the range 4−14 for ionic strengths of 50−850 mM (pH 8.0) and pH 5−10 (ionic strength 850 mM) (data not shown).

Effect of Ionic Strength on Isotope Effects. ^{D}V has a maximum value of 2.0−2.5 at an ionic strength of 250−700 mM (Figure 4A). It decreases at higher ionic strength, and at lower ionic strengths it extrapolates to a value of 1.0 when the ionic strength is zero. The isotope effect at the physiologically relevant ionic strength of 150 mM is ca 1.7.

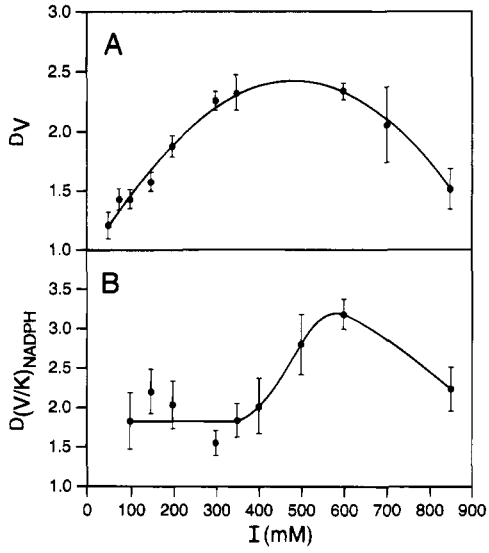


FIGURE 4: Effect of ionic strength on (A) ^{D}V and (B) $^{D}(V/K)_{NADPH}$ primary isotope effects. Reaction conditions were as in Figure 3. In the determination of each isotope effect, cytochrome c^{3+} was present at 100 μM and both NADPH and NADPD$_A$ were varied at 4.8, 1.1, 0.54, 0.37, and 0.29 μM.

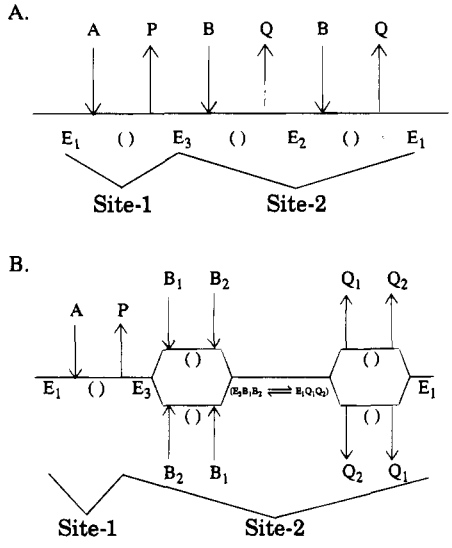


FIGURE 5: Kinetic schemes for the two-site ping-pong mechanism of P450R. The reaction at site 2 is (A) tetra-uni ping-pong at high ionic strength and (B) random sequential at low ionic strength. A, B, P, and Q are NADPH, cytochrome c^{3+}, NADP$^+$, and cytochrome c^{2+}, respectively. E_1, E_2, and E_3 are the one- (FMNH$^\bullet$/FAD), two- (FMNH$_2$/FAD), and three-(FMNH$_2$/FADH$^\bullet$) electron-reduced forms of P450R, respectively.

The $^{D}(V/K)_{NADPH}$ isotope effect is >2.5 at 450−700 mM ionic strength and appears to decrease somewhat at higher ionic strength but decreases only to a plateau value of around 1.8 at lower ionic strength (Figure 4B).

DISCUSSION

Effect of Ionic Strength on Kinetic Mechanism. Previous studies (Sem & Kasper, 1994) have demonstrated that the kinetic mechanism of P450R at 850 mM ionic strength is two-site ping-pong, with NADPH and cytochrome c^{3+} binding independently at separate sites. There is also tetra-uni ping-pong reaction of two cytochrome c^{3+} molecules at the electron acceptor site (Figure 5A). The rate equation corresponding to this model [derived by Sem and Kasper (1994)] is

$$1/v = (K_{iB}C_B/V_{max})(1/B) + (K_{iA}C_A/V_{max})(1/A) + 1/V_{max} \quad (4)$$

where v is the initial velocity, A and B are the concentrations of NADPH and cytochrome c^{3+}, respectively, and K_{iA} and K_{iB} are their corresponding dissociation constants. C_A and C_B are kinetic terms reflecting the relative rate limitation of hydride and electron transfer. As the ionic strength decreases to 540 and 300 mM, the kinetic mechanism becomes more complicated, with initial velocity patterns showing curvature when cytochrome c^{3+} is the varied substrate (540 mM, Figure 1B; 300 mM, data not shown). Furthermore, the intercept replots (Figure 2) for the initial velocity patterns with NADPH acting as the varied substrate (540 mM, Figure 1A; 300 mM, data not shown) show curvature. This curvature is consistent with a model in which two molecules of cytochrome c^{3+} bind in a random sequential manner at the electron acceptor site (Figure 5B). Although this is the first report of a 2:1 stoichiometry for cytochrome c^{3+} binding to P450R, recent excited-state quenching studies of cytochrome c oxidase have demonstrated a 2:1 stoichiometry for cytochrome c^{3+} binding in this system (Zhou & Hoffman, 1994). The rate equation corresponding to this model [derived by Sem and Kasper (1994)] is

$$1/v = (K_{iB}''C_B/V_{max})(1/B^2) + (K_{iB}'C_B/V_{max})(1/B) + (K_{iA}C_A/V_{max})(1/A) + 1/V_{max} \quad (5)$$

where all constants are as defined in eq 4 and K_{iB}' is the sum of the dissociation constants ($K_{iB1} + K_{iB2}$) for cytochrome c^{3+} binding to the two subsites of site 2 (the electron acceptor site), while K_{iB}'' is the product of the two cytochrome c^{3+} dissociation constants ($K_{iB1}K_{iB2}$). Thus, a decrease in ionic strength, which increases the affinity for cytochrome c^{3+} (vide infra), results in the sequential binding of two molecules of cytochrome c^{3+}, while at higher ionic strength (850 mM) the two cytochrome c^{3+} molecules bind one at a time at this site, in a ping-pong fashion (Sem & Kasper, 1994). More formally, the mechanism changes from two-site ping-pong with a tetra-uni ping-pong reaction at the second site, at high ionic strength, to two-site ping-pong with a bi-bi random sequential reaction at the second site, at lower ionic strength. The overall kinetic scheme describing the reaction at site-2 at all ionic strengths is summarized in Figure 6. At high ionic strength, kinetic path-1 predominates and initial velocity profiles are described by eq 4, while at lower ionic strength kinetic path-2 predominates[4] and initial velocity profiles are described by eq 5.

Effect of Ionic Strength on Rate-Limiting Steps. The two kinetic constants V_{max} and V/K pertain to different physical phenomena (pseudo-zero-order and first-order reactions, respectively) and can therefore vary independently. These constants were derived previously for the models in Figure 5A,B using the method of Northrop (1969) and assuming rapid equilibrium binding of all substrates and products. But

[4] Kinetic path 2 is a simplified version of that shown in Figure 5B for the random sequential binding of two molecules of cytochrome c^{3+}. This is a reasonable simplification since the two substrates binding in random sequential order are identical (both cytochrome c^{3+}), so there is little distinction between random and ordered sequential mechanisms.

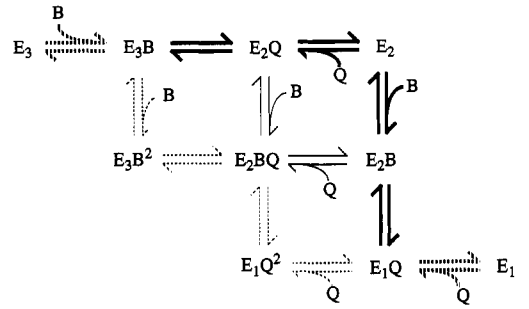
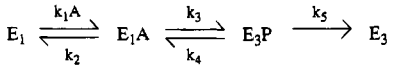


FIGURE 6: Overall kinetic scheme for reaction at site 2 at all ionic strengths. At high ionic strength, kinetic path 1 is taken (bold arrow), corresponding to the ping-pong reaction in Figure 5A. At low ionic strength, kinetic path 2 is taken (dashed arrows), corresponding to the (random[4]) sequential reaction in Figure 5B. At intermediate ionic strengths both paths, including steps shown with narrow solid lines, would be operative. Bold and dashed arrows represent regions of overlap between kinetic paths 1 and 2. B and Q are as defined in Figure 5.

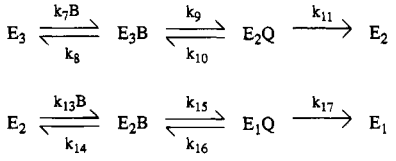
A.

Site-1:



B.

Site-2:



C.

Site-2:

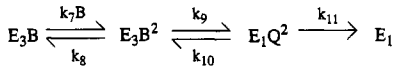


FIGURE 7: Kinetic schemes for reaction at sites 1 and 2 under limiting conditions of high and low ionic strength. Reaction at site 1 (A) is the same at all ionic strengths, while reaction at site 2 changes from ping-pong at high ionic strength (B) to sequential at low ionic strength (C). The sequential reaction at low ionic strength starts from the E_3B complex under conditions where $B \gg K_{iB1}$ (see eq 5). The two electron transfer steps present in kinetic path 2 in Figure 6 are shown as one step here (k_9), in order to simplify the mechanism and rate equations, as are the two product (Q) release steps (represented here by k_{11}). A, B, P, and Q are as defined in Figure 5.

it was noted (Sem & Kasper, 1994) that the final rate equation derived in this manner is the same as that derived for a one-site mechanism using the net rate constant method (Cleland, 1975a), with the addition of terms for substrate binding and product release steps (a result of removing the rapid equilibrium binding assumption). Figure 7 summarizes the kinetic scheme for reaction at site 1 (Figure 7A) and site 2 (Figure 7B) at high ionic strength and at site 1 (Figure 7A) and site 2 (Figure 7C) at low ionic strength. At low ionic strength, initial velocity profiles are curved but become linear at relatively high cytochrome c^{3+} concentrations. This occurs when [cytochrome c^{3+}] > K_{iB1} in eq 5, which results in saturation of the first subsite in site 2. The kinetic scheme at site 2 then simplifies from kinetic path 2 in Figure 6 to that shown in Figure 7C at low ionic strength. Since V_{max} and $(V/K)_{cytc}$ values were measured under these conditions, kinetic constants were derived for the simplified scheme in Figure 7A,C using the net rate constant method, and they have the following form:

$$k_{cat} = \frac{k_3}{1 + C_{vf} + C_r} \approx \frac{k_3}{1 + C_{vf}} \quad (6)$$

$$(k_{cat}/K_m)_{NADPH} = \frac{k_3}{K_{iA}(1 + C_r + C_f)} \approx \frac{k_3}{K_{iA}(1 + C_f)} \quad (7)$$

$$(k_{cat}/K_m)_{cytc} = \frac{k_9}{K_{iB}(1 + C_r' + C_f')} \approx \frac{k_9}{K_{iB}(1 + C_f')} \quad (8)$$

where k_{cat} is V_{max} divided by the P450R concentration, and

$$C_f = k_3/k_2 \quad (9)$$

$$C_r = k_4/k_5 \approx 0 \quad (10)$$

$$C_f' = k_9/k_8 \quad (11)$$

$$C_r' = k_{10}/k_{11} \approx 0 \quad (12)$$

$$C_{vf} = k_3/k_{11} + k_3/[k_9k_{11}/(k_{10} + k_{11})] + k_3/k_5 \quad (13)$$

where the rate constants are those given in Figure 7A,C. C_f-(C_f') and C_r(C_r') are the forward and reverse commitments, respectively. They represent the ratio of the rate constant for hydride transfer to that for NADPH (C_f) or $NADP^+$ (C_r) release from P450R and the ratio of the rate constant for electron transfer to that for cytochrome c^{3+} (C_f') or cytochrome c^{2+} (C_r') release from P450R (Cleland, 1982). Since stopped-flow studies show no accumulation of $FADH_2$ after reduction of P450R with NADPH, the thermodynamically favorable electron transfer to $FMNH^\bullet$ is very fast and irreversible (Iyanagi et al., 1974; Oprian & Coon, 1982), so the C_r terms in eqs 6 and 7 drop out. The C_r' term also drops out of eq 8 since electron transfer to cytochrome c^{3+} is thermodynamically very favorable based on reduction potentials (Iyanagi et al., 1974; Walsh, 1979), indicating that this electron transfer step is also irreversible. C_{vf} is the V ratio and represents the ratio of the hydride transfer rate constant to all other "net rate constants" in the forward reaction sequence, excluding substrate binding steps (Cleland, 1982). These kinetic constants have a similar form at high ionic strength (Sem & Kasper, 1994), with the only difference being due to the occurrence of two nearly identical reaction cycles at site 2 (Figure 7B). Therefore, eqs 6−8 are sufficient for a qualitative discussion of rate limiting steps at all ionic strengths, under conditions where $B > K_{iB1}$ (eq 5).

$(V/K)_{NADPH}$ and $(V/K)_{cytc}$ both increase significantly at low ionic strength, while V_{max} actually decreases at low ionic strength (Figure 3A-C). Such an effect on V_{max} must be due to increasing rate limitation of the product release step (rate constants k_5 and/or k_{11}, which are not present in the V/K equations). The fact that both $(V/K)_{DAPH}$ and $(V/K)_{cytc}$ increase at low ionic strength must mean that binding of NADPH and cytochrome c^{3+} to P450R, to form productive complexes, becomes tighter (K_{iA} and K_{iB} decrease). Therefore, there must be favorable ionic interactions between P450R and its substrates, NADPH and cytochrome c^{3+}.

Although it has been suggested that such interactions do not exist between P450R and cytochrome P450 (Voznesensky & Schenkman, 1992a,b, 1994), it does appear that they are important in forming the complex between cytochrome c^{3+} and P450R. Indeed, previous studies (Sem & Kasper, 1993a) have demonstrated that ≤2 lysine residues provide ionic stabilization of the cytochrome c^{3+}/P450R complex at 300 mM ionic strength. Furthermore, site-directed mutagenesis, alternate substrate, and pH studies have shown that ionic interactions play a key role in binding NADPH, particularly through interactions with the 2′-phosphate (Sem & Kasper, 1993a,b).

Isotope effect studies with $NADPD_A$ have been carried out to determine how rate-limiting hydride transfer is relative to other steps in the reaction sequence. The decrease in the rate of hydride transfer, caused by substitution of a deuterium for a hydrogen, is the intrinsic isotope effect (Cleland, 1982). For rabbit P450R, $NADPD_A$ transfers a deuteride ion 5.1-fold more slowly than NADPH transfers a hydride ion to FAD (Sugiyama & Mason, 1984), so the intrinsic isotope effect (k_{3H}/k_{3D} for the reaction shown in Figure 7A) is 5.1. The maximum possible isotope effect on V_{max} and $(V/K)_{NADPH}$ is the intrinsic isotope effect, which would only be observed if hydride transfer were totally rate limiting. Usually it is decreased by the presence of commitment terms (C_f and C_r) and the V ratio, C_{vf}. In the case of P450R, the equations describing the isotope effects on V_{max} and V/K (derived from eqs 6 and 7) are given by

$$^{D}(V/K)_{NADPH} = \frac{^{D}k_3 + C_f + C_r{}^{D}K_{eq}}{1.0 + C_f + C_r} \approx \frac{^{D}k_3 + C_f}{1.0 + C_f} \quad (14)$$

$$^{D}V = \frac{^{D}k_3 + C_{vf} + C_r{}^{D}K_{eq}}{1.0 + C_{vf} + C_r} \approx \frac{^{D}k_3 + C_{vf}}{1.0 + C_{vf}} \quad (15)$$

where

$$^{D}K_{eq} = k_{3H}k_{4D}/k_{3D}k_{4H} \quad (16)$$

and $^{D}K_{eq}$ is the equilibrium isotope effect on hydride transfer. It is clear from Figure 4A that ^{D}V decreases at both high and low ionic strength, with a maximum value at 250−700 mM. In fact, it extrapolates to zero isotope effect ($^{D}V = 1$) at low ionic strength, consistent with step(s) other than hydride transfer being entirely rate-limiting at low ionic strength. Since $^{D}(V/K)_{NADPH}$ does not extrapolate to zero isotope effect at low ionic strength (Figure 4B), the effect on ^{D}V must be due to differences between the C_{vf} and C_f terms (consistent with our assumption that the C_r term is absent). That is, product release becomes rate-limiting for V_{max} at low ionic strength. This is consistent with a previous report of rate-limiting release of $NADP^+$, based on stopped-flow studies (Oprian & Coon, 1982),[5] and the general observation that product release often limits the rate of enzymatic reactions, particularly dehydrogenases (Cleland, 1975b). The fact that $^{D}(V/K)_{NADPH}$ decreases at low ionic strength must be attributed to an increase in C_f, due to the higher relative affinity for NADPH at lower ionic strength (k_2 decreases). The decrease in $^{D}(V/K)_{NADPH}$, ^{D}V, and V_{max}, and to a lesser extent in $(V/K)_{NADPH}$, at high ionic strength is more difficult to explain. One could argue that the more pronounced decrease in V_{max} and ^{D}V at high ionic strength is a result of rate-limiting product release, resulting from an increased strength of hydrophobic interactions. This is reasonable since both hydrophobic and ionic interactions are likely to play a role in binding interactions with NADPH and cytochrome c^{3+}. It is also possible that at high ionic strength NADPH forms a lower proportion of the catalytically active Michaelis complex. This would then explain the decrease that is also observed in $(V/K)_{NADPH}$ and $^{D}(V/K)_{NADPH}$ at high ionic strength and is consistent with the formation of an E·NADPH complex that undergoes a slower conformational change at higher ionic strength to an E*·NADPH complex, which must form for hydride transfer to occur.

Table 1: Effect of Cytochrome c^{3+} Concentration on $^{D}V_{app}$[a]

cytochrome c^{3+} (μM)	$^{D}V_{app}$
29.6	2.20 ± 0.14
5.14	1.54 ± 0.10
2.46	1.43 ± 0.08
1.39	1.08 ± 0.04

[a] Assays were carried out at 25 °C in 100 mM TAPS, pH 8.0, and an ionic strength of 850 mM. NADPH was varied at 49, 1.1, 0.69, 0.38, and 0.30 μM.

Novel Method for Estimating $^{D}k_3$, C_f, and C_{vf}. Experiments in which cytochrome c^{3+} concentration was varied around its K_m (from $0.08K_m$ to $1.8K_m$) have shown that ^{D}V is quite sensitive to the concentration of cytochrome c^{3+} (Table 1). Derivation of the equation that describes ^{D}V (or rather $^{D}V_{app}$) under these conditions revealed that eq 15 describes $^{D}V_{app}$ only if C_{vf} is modified such that

$$C_{vf}' = C_{vf} + \frac{k_3}{k_{cat}}\frac{K_{cytc}}{[\text{cytochrome } c^{3+}]} \quad (17)$$

to account for the fact that cytochrome c^{3+} is no longer saturating. Making use of eqs 6, 15, and 17, it can be shown[6] that

$$1/(^{D}V_{app} - 1) = (1 + r)m \quad (18)$$

where r is K_{cytc}/[cytochrome c^{3+}] and

$$m = (1 + C_{vf})/(^{D}k_3 - 1) \quad (19)$$

A plot of $1/(^{D}V_{app} - 1)$ versus $(1 + r)$, using the data in Table 1, yields a value of 0.30 for m.[7] Since $C_{vf} \geq 0$, it must be true (from eq 19) that

[5] Rate-limiting $NADP^+$ release was suggested on the basis of these studies, but this conclusion was thought to be inconsistent with the steady-state data that suggested a ternary complex could not form. The two-site ping-pong mechanism we have demonstrated (Sem & Kasper, 1994) allows for the formation of a ternary complex, thus reconciling the steady-state and stopped-flow results.

[6] From eq 6, the ratio k_3/k_{cat} is $(1 + C_{vf})$. Making this substitution into eq 17 and then substituting C_{vf}' in eq 15 with this equation for C_{vf} yields $^{D}V_{app} = [^{D}k_3 + C_{vf} + (1 + C_{vf})r]/[1 + C_{vf} + (1 + C_{vf})r]$. Subtracting 1.0 from both sides of this equation, rearranging terms, and inverting yields eq 18.

[7] Since the y-intercept for eq 18 is the origin, this point was included in the fit. The last point ($^{D}V_{app} = 1.08$) could not be included in the fit. Since it is so close to 1.0, even a small error in $^{D}V_{app}$ would lead to a large error in the ratio $1/(^{D}V_{app} - 1)$.

Table 2: Lower Limits for C_{vf} and C_f[a]

ionic strength (mM)	C_{vf}	C_f
170	3.7	3.1
550	1.4	0.5
850	5.6	1.5

[a] These are lower limits based on the fact that $^Dk_3 \geq 4.3$.

$$^Dk_3 \geq 1 + (1/m) \quad (20)$$

Thus, $^Dk_3 \geq 4.3$ for P450R. Substitution of this lower limit for $^Dk_3$ into eqs 14 and 15 yields lower limits for the C_f and C_{vf} terms, which have been determined at several ionic strengths (Table 2). Since $C_{vf} \geq 1.4$ at the ionic strength where hydride transfer is most rate-limiting (550 mM), at least one other step in the steady-state reaction sequence must be as slow as the hydride transfer step. Hydride transfer is even less rate-limiting at lower ionic strength where $NADP^+$ (and NADPH) release becomes very slow, as reflected in the increased value(s) for C_{vf} (and C_f).

The variation of $^DV_{app}$ with substrate concentration is a novel diagnostic tool for the assessment of the relative rate limitation of an isotope-sensitive step that should prove to be generally useful for enzymes with ping-pong mechanisms. In cases where the reverse commitment is small, it allows for the estimation of lower limits for $^Dk_3$, C_f, and C_{vf}.

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