Enzyme–Substrate Binding Interactions of NADPH–Cytochrome P-450 Oxidoreductase Characterized with pH and Alternate Substrate/Inhibitor Studies†

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ABSTRACT: The pH dependence of the kinetic parameters for the reaction catalyzed by NADPH–cytochrome P-450 oxidoreductase (P-450R) has been determined, using various substrates and inhibitors. All V_{max} and (V/K) profiles show pK_as of 6.2–7.3, for an acidic group that is preferentially unprotonated for catalysis, and of 8.1–9.6, for a basic group that is preferentially protonated for catalysis. The presence of the wrong ionization state for both of these groups is tolerated more at lower ionic strength (300 mM) than at higher ionic strength (850 mM). Ionization of the basic group has a more pronounced effect on binding of substrate (cytochrome *c* or dichloroindophenol) than on catalysis, since ionization has only a 2-fold effect on V_{max} with cytochrome *c*, and only a 5-fold effect on V_{max} with dichloroindophenol, while (V/K) for both substrates continues to drop at high pH with no sign of reaching a plateau. Therefore, this basic group affects predominantly substrate binding and, to a lesser extent, catalysis. It is most likely located on the surface of the protein at the cytochrome *c*/dichloroindophenol binding site, near the FMN prosthetic group. The $NADP^+$ pK_i profile shows a pK_a of 5.95 for the 2′-phosphate of $NADP^+$, which is bound to P-450R as the dianion, and a pK_a of 9.53 for an enzyme group that must be protonated in order to bind $NADP^+$. Removal of the 2′-phosphate of NADPH leads to a loss of 5.0 kcal/mol of ground-state and 6.0 kcal/mol of transition-state binding energy, while removal of the 2′-phosphate of $NADP^+$ leads to a loss of 4.7 kcal/mol of ground-state binding energy. Thus, the 2′-phosphate is providing roughly 5 kcal/mol of uniform binding energy, resulting from all of the enzyme interactions with this group. The $(V/K)_{cytc}$ pH profile at 300 mM ionic strength fits best to a model assuming ≤2 lysines with pK_as of 10.6 that are involved in binding interactions between P-450R and cytochrome *c*. There is also a group with a pK_a of 7.27 that must be unprotonated for cytochrome *c* to bind to P-450R. These binding interactions between cytochrome *c* and P-450R are not significant at higher ionic strength (850 mM).

NADPH–cytochrome P-450 oxidoreductase (P-450R)[1] (NADPH–ferrihemoprotein oxidoreductase, EC 1.6.2.4) is a membrane-bound flavoprotein associated with the endoplasmic reticulum (Williams & Kamin, 1962; Phillips & Langdon, 1962) and nuclear envelope (Kasper, 1971) of a variety of cell types. It contains one mole each of FAD and FMN (Iyanagi & Mason, 1973), bound noncovalently, and catalyzes the transfer of two reducing equivalents from NADPH to FAD, then to FMN (Vermilion et al., 1981), and finally to any of a number of cytochromes P-450 (Lu et al., 1969) as well as other microsomal enzyme systems, including heme oxygenase (Schacter et al., 1972) and the fatty acid desaturation/cytochrome b_5 (Enoch & Strittmatter, 1979) and fatty acid elongation (Ilan et al., 1981) systems. It is the A-side hydrogen of NADPH that is transferred to FAD, and the nicotinamide ring is in the anti conformation (Sem & Kasper, 1992). Electrons can also be transferred to various alternate electron acceptors such as cytochrome *c*, menadione, or DCIP (Williams & Kamin, 1962). Since P-450R catalyzes the transfer of electrons between nicotinamide nucleotides (obligate two-electron or hydride donors/acceptors) and obligate one-electron donors/acceptors, it is a member of the dehydrogenase/electron transferase family (Massey & Hemmerich, 1980), along with ferredoxin–$NADP^+$ reductase, NADH–nitrate reductase, NADH–cytochrome b_5 reductase, and NADPH–sulfite reductase (Karplus et al., 1991; Porter & Kasper, 1986).

The kinetic mechanism of P-450R has been reported as ping-pong (Masters et al., 1965; Kominami et al., 1982), with hydride transfer from NADPH to the N5 of FAD probably representing the rate-limiting step (Yasukochi et al., 1979; Vermilion et al., 1981), consistent with our observations (unpublished results). Although no crystal structure is available for P-450R, the geometric relationship between the nicotinamide and FAD rings has been determined using NMR (Sem & Kasper, 1992), and they are in the exo orientation. Crystal structures are available for spinach ferredoxin–$NADP^+$ reductase (Karplus et al., 1991), which is homologous to the FAD and NADPH binding domains of P-450R, and for bacterial flavodoxin (Watenpaugh et al., 1973), which is homologous to the FMN binding domain of P-450R (Porter & Kasper, 1986). The purpose of this study is to characterize the enzyme–substrate interactions of P-450R, involving ionizable groups that play a role in binding and/or catalysis. One can characterize these interactions using steady-state kinetic pH profile studies. An analysis of the pH variation of the kinetic parameters V_{max} and V_{max}/K_m[2] for substrates and alternate substrates, and of K_i for inhibitors, allows one to identify the number of functionally important ionizable

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[1] Abbreviations: P-450R, NADPH–cytochrome P-450 oxidoreductase; NMR, nuclear magnetic resonance; DCIP, 2,6-dichloroindophenol; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, *N*-[tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; cytc, cytochrome *c*.

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groups, their apparent pK_a values, their preferred ionization states, and their roles in binding and/or catalysis (Cleland, 1982a).

Alternate substrate and site-directed mutagenesis (topic of following paper in this issue) studies are quite informative when used in conjunction with pH studies, since they allow one to probe the effects of removing the functional groups on the substrate and enzyme, respectively, that may be responsible for pK_as in certain pH profiles. An analysis of the V_{max}, (V/K), and K_i profiles for P-450R, using various substrates and inhibitors, has allowed us to propose a model describing the roles of ionizable groups in binding and catalysis. This model is further tested using alternate substrates/inhibitors (DCIP, NADH, $NADP^+$, and NAD^+) and, in the following paper, altered enzyme (R597M) (Sem & Kasper, 1993).

MATERIALS AND METHODS

NADPH, $NADP^+$, NAD^+, NADH, and horse heart cytochrome *c* (ferric) were from Sigma. KCl was from Mallinckrodt. D_2O (100%), H_3PO_4, and DCIP were from Aldrich. The following buffers were used in kinetic studies and titrations in the specified pH ranges: acetate (4.5–5.0), MES (5.5–6.5), TAPS (7.0–8.5), and CHES (9.0–10.0). Acetic acid was from Fisher, while the remaining buffers were from Sigma.

Recombinant P-450R was purified from *Escherichia coli* after overexpression of the rat liver gene contained in the plasmid pOR263 as described previously (Shen et al., 1989). Protein concentration was determined using the assay of Lowry et al. (1951).

^{31}P-NMR spectra for the NADPH and $NADP^+$ titration curves were obtained with a Bruker AM500 spectrometer operating at 202.46 MHz, using a $\pi/4$ observation pulse. Chemical shifts were referenced to 85% H_3PO_4. Experiments were performed at 25 °C using a 5-mm NMR tube, with D_2O present in a coaxial tube to provide the lock. The titration samples contained 10 mM NADPH or $NADP^+$, 100 mM buffer, and enough KCl to give an ionic strength of 850 mM.

The spectrophotometric titration of DCIP at 600 nm was carried out at 25 °C. The samples contained 50 μM DCIP, 100 mM buffer, and enough KCl to give an ionic strength of 300 mM.

Kinetic Assays. Kinetic studies were carried out in 1-cm cuvettes in a 1-mL volume by measuring absorbance changes at 550 nm in the cytochrome *c* assays ($\Delta\epsilon = 21.1\ mM^{-1}\ cm^{-1}$) (Gelder & Slater, 1962) and at 600 nm in the DCIP assays. The extinction coefficient for DCIP was calculated at each pH (discussed later). Initial velocities were determined at 25.0 °C, and reactions were initiated by the addition of enzyme. Background rates in the absence of enzyme were subtracted from initial velocities when significant. Reaction mixtures contained the appropriate buffer at 100 mM, enough KCl to give an ionic strength of 300 or 850 mM, varied levels of cytochrome *c* or DCIP (from 0.2 to 5 times their respective K_m values), varied levels of NADPH or NADH (from 0.2 to 5 times their respective K_m values), and P-450R. In calculating the ionic strength of a given reaction mixture, to determine how much KCl had to be added to achieve the specified ionic strength, the following pK_a values were used: MES (6.02), TAPS (8.19), and CHES (9.23) (Ellis & Morrison, 1982); acetic acid (4.76) (Perrin & Dempsey, 1974); DCIP (5.86), NADPH (5.91), and $NADP^+$ (5.81) (this paper). pH profiles were extended as far as possible at the pH extremes, until enzyme stability or substrate inhibition became a problem. P-450R was found to be stable at the pH extremes for the 0.5–1 min required to obtain a steady-state velocity. Thus, any decrease in V_{max} and (V/K) at these pH values is not due to protein unfolding or flavin loss. Activity of the P-450R stock solution, throughout and between experiments, was determined with a standard activity assay containing 0.1 M potassium phosphate (pH 8.0), 80 μM cytochrome c^{3+}, and 0.5 mM NADPH, to monitor and correct for minor changes in activity resulting from denaturation and flavin loss.

Data Processing. Data were fitted using modified versions of the FORTRAN programs of Cleland (1979), which employ the nonlinear least-squares method. All equations were used in log form (except eq 2) in the fitting process because this effectively assumes constant proportional error in the measured parameter (Y). All plots of kinetic data contain error bars for each data point, corresponding to one standard deviation (if not visible, the error is less than the radius of the point). V_{max}, (V/K), and K_m values (at 300 mM) were obtained from a fit to eq 1, with the nonvaried substrate (NADPH) present at saturating levels ($>10K_m$):

$$Y = \frac{V_{max}A}{K_m + A} \tag{1}$$

where Y is the initial velocity and A is the substrate (DCIP or cytochrome *c*) concentration. Reliable (V/K) and K_m values for NADPH could not be determined in this manner, since cytochrome *c* shows substrate inhibition at concentrations above its K_m. (V/K) values for cytochromic *c*, NADPH, and NADH, at 850 mM ionic strength, could be obtained from a fit to eq 1 with nonsaturating levels of the nonvaried substrate, since at this ionic strength a ping-pong mechanism applies (unpublished results), so the concentration of one substrate will not affect (V/K) for the other. V_{max}, (V/K), and K_m values were obtained from a fit to eq 2 for a ping-pong mechanism (at 850 mM ionic strength):

$$Y = \frac{V_{max}AB}{(K_AB + K_BA + AB)} \tag{2}$$

where Y is the initial velocity, A and B are the two substrate concentrations, and K_A and K_B are their corresponding Michaelis constants. Although it is often assumed that P-450R has a simple ping-pong mechanism, this is only true at high ionic strength (850 mM). At lower ionic strength the mechanism is more complicated, and eq 2 is no longer valid (unpublished results). It is therefore not possible to obtain reliable estimates of all the kinetic contants at lower ionic strength, although if NADPH is maintained at saturating levels, the kinetic equation simplifies to eq 1, allowing the calculation of V_{max}, $V/K)_{cytc}$, and $(V/K)_{DCIP}$ but not $(V/K)_{NADPH}$. The fit to eq 2 was required to obtain V_{max}, K_m(NADPH), and K_m(NADH) values at 850 mM ionic strength since saturation with cytochrome *c* is not possible due to substrate inhibition. K_i values for the competitive inhibitors $NADP^+$ and NAD^+ were obtained from a fit to

$$Y = \frac{V_{max}A}{K_m(1 + I/K_i) + A} \tag{3}$$

where K_i is the competitive inhibition constant, I is the concentration of the inhibitor ($NADP^+$ or NAD^+), and A is the concentration of the varied substrate (NADPH). Titration curves for NADPH, $NADP^+$, and DCIP (Figure 1) were fitted

[2] V_{max}/K_m is written henceforth as $(V/K)_{substrate}$, where the subscript identifies the substrate with this pseudo-first-order rate constant.

to

$$Y = \frac{[Y_L(H/K) + Y_H]}{(H/K + 1)} \quad (4)$$

where Y is the ^{31}P-NMR chemical shift (NADPH and NADP$^+$ profiles) or the extinction coefficient (DCIP profile), H is the proton concentration, K is the acid dissociation constant, and Y_L and Y_H are the low- and high-pH plateau values, respectively, for Y. pH profiles consistent with a model where one acidic group must be unprotonated and one basic group must be protonated (Figures 2–4), were fitted to

$$Y = \frac{Y_0}{(1 + H/K_1 + K_2/H)} \quad (5)$$

where K_1 and K_2 are the acid dissociation constants for the acidic and basic groups, respectively, Y is V_{max}, (V/K), or $1/K_i$, and Y_0 is the value for Y when both groups are in their preferred ionization state. The V_{max} pH profile with NADPH and cytochrome c as substrates at 300 mM ionic strength (Figure 5A) was fitted to

$$Y = \frac{Y_H}{(H/K_2 + 1)} + \frac{Y_M}{(H/K_1 + K_2/H + 1)} + \frac{Y_L}{(K_1/H + 1)} \quad (6)$$

where Y is V_{max}, K_1 and $_{K2}$ are the acid dissociation constants for the acidic and basic groups, respectively, and Y_L, Y_M, and Y_H are the low-, intermediate-, and high-pH plateau values, respectively, for Y. The $(V/K)_{cytc}$ pH profile, with NADPH as the other substrate at 300 mM ionic strength (Figure 5B), fitted to

$$Y = \frac{Y_H}{(H/K_1 + 1)^2(K_2/H + 1)(K_3/H + 1)} + \frac{Y_L}{(K_1/H + 1)^2} \quad (7)$$

where Y is $(V/K)_{cytc}$, K_1 is the acid dissociation constant for each of two acidic groups, K_2 and K_3 are the acid dissociation constants for two basic groups, and Y_L and Y_H are the low- and high-pH plateau values, respectively, for Y. The V_{max} pH profile with NADPH and DCIP as substrates at 300 mM ionic strength (Figure 6A) was fitted to

$$Y = \frac{Y_H}{(H/K_2 + 1)} + \frac{Y_L}{(H/K_1 + 1)(K_2/H + 1)} \quad (8)$$

where Y is V_{max}, K_1 and K_2 are the acid dissociation constants for the acidic and basic groups, respectively, and Y_L and Y_H are the low- and high-pH plateau values, respectively, for Y. The $(V/K)_{DCIP}$ pH profile, with NADPH as the other substrate at 300 mM ionic strength (Figure 6B), was fitted to

$$Y = \frac{Y_0}{[1 + H/K_2 + K_3/H + H^2/(K_1K_2)]} \quad (9)$$

where Y is $(V/K)_{DCIP}$, K_1 and K_2 are the acid dissociation constants for two acidic groups, K_3 is the acid dissociation constant for a basic group, and Y_0 is the plateau value for Y when all groups are in their preferred ionization states.

RESULTS

pH Profile Theory. A determination of the pH dependence of V_{max} (V/K), and K_1 is useful in the characterization of the roles of ionizable groups on the enzyme and substrate. The K_i profile provides true thermodynamic pK_a values for groups that are involved in binding, while the V_{max} profile yields apparent pK_as for groups involved in catalytic–isomerization–product release steps (but not binding), and the (V/K) profile gives pK_as for groups involved in binding and/or catalysis–isomerization–product release. While pK_as in V_{max} profiles can be perturbed in either direction, the pK_as in (V/K) profiles can only be perturbed outward. That is, pK_as for groups that ionize on the acidic limb of the profile (these groups, that are preferentially unprotonated, are henceforth referred to as acidic groups) can be perturbed to the left on the profile, to lower pH, while pK_as for groups that ionize on the basic limb of the profile (these groups, that are preferentially protonated, will be referred to as basic groups) can be perturbed to the right on the profile, to higher pH. This perturbation is most pronounced for sticky substrates but is usually <1 pH unit. A sticky substrate is one that, upon formation of the Michaelis complex, undergoes reaction to form products much faster than it is released from this complex (Cleland, 1982b). Therefore, slow alternate substrates (which are nonsticky) give unperturbed pK_as. For a thorough discussion of the theory behind pH profile studies, consult Cleland (1982a).

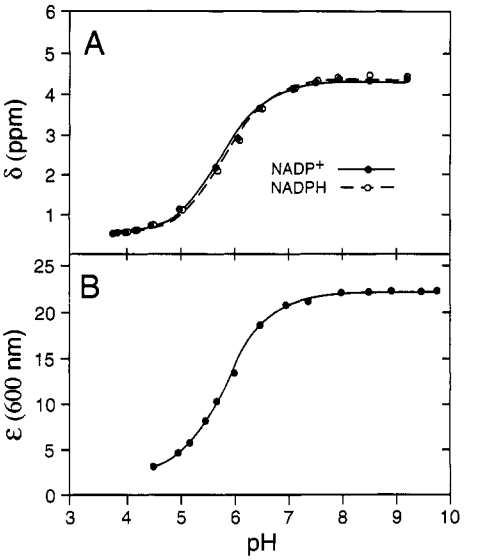

FIGURE 1: (A) pH variation of the ^{31}P-NMR chemical shift of the 2′-phosphate of NADPH(○) and NADP$^+$(·) at an ionic strength of 850 mM. Both data sets were fitted to eq 4 with pK_a values of 5.91 ± 0.04 for NADPH and 5.81 ± 0.04 for NADP$^+$. (B) pH variation of the extinction coefficient for DCIP (at 600 nm) at an ionic strength of 300 mM. The data were fitted to eq 4 with a pK_a value of 5.86 ● 0.01.

Substrate Characterization. In order to facilitate interpretation of pH profile results, pK_a values were determined for all substrates and inhibitors (except cytochrome c) under the same conditions used in the pH profile studies. pK_a values of 6.1 and 6.4 have been reported for NADPH and NADP$^+$, respectively (Feeney et al., 1975), in 100% D_2O containing 500 mM KCl at 11 °C. Values of 6.52 ± 0.03 and 6.40 ± 0.03 have also been reported for NADPH and NADP$^+$, respectively (Mas & Colman, 1984), in 10% D_2O containing 10% glycerol at 25 °C. Since the presence of D_2O will affect the pK_a of the 2′-phosphate, as will ionic strength, it was necessary to determine the pK_as for NADPH and NADP$^+$ under the conditions of our experiments. At 25 °C and an ionic strength of 850 mM (in the absence of D_2O), the pK_a of the 2′-phosphate of NADP$^+$ is 5.81 ± 0.04, while that for NADPH is 5.91 ± 0.04 (Figure 1A). The pK_a for the hydroxyl group of DCIP at 25 °C and an ionic strength of 300 mM is 5.86 ± 0.01 (Figure 1B), and the effect of this ionization on the extinction coefficient for DCIP is described by eq 4. The extinction coefficients used in obtaining the DCIP pH profiles were calculated at each pH with this equation,[3] which assumes

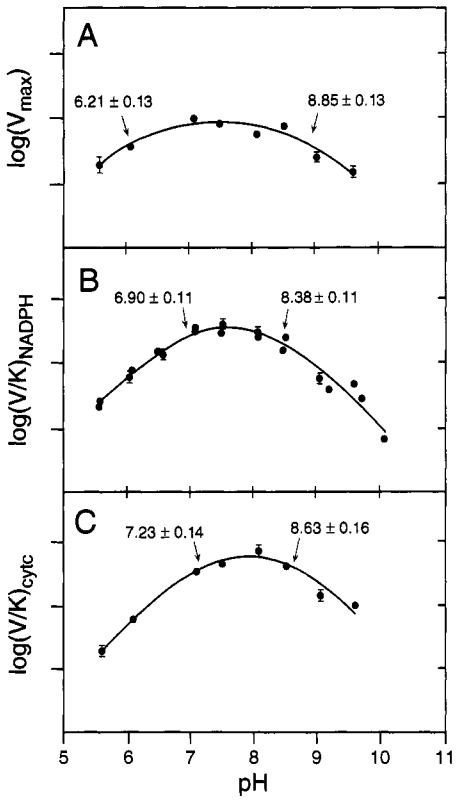

FIGURE 2: Effect of pH on (A) log (V_{max}), (B) log $(V/K)_{NADPH}$, and (C) log $(V/K)_{cytc}$ at an ionic strength of 850 mM. All three data sets were fitted to eq 5, which assumes one pK_a for an acidic group that is preferentially unprotonated and one pK_a for a basic group that is preferentially protonated. The acidic and basic groups have pK_a values of 6.21 ± 0.13 and 8.85 ● 0.13, respectively, in the log (V_{max}) profile, a 6.90 ± 0.11 and 8.38 ± 0.11, respectively, in the log $(V/K)_{NADPH}$ profile, and of 7.23 ± 0.14 and 8.63 ± 0.16, respectively, in the log $(V/K)_{cytc}$ profile. The units of V_{max} and (V/K) are arbitrary, with each demarcation on the y-axis representing one log unit [a 10-fold change in V_{max} or (V/K)].

an extinction coefficient of 21.0 mM^{-1} cm^{-1} at pH 7.0 (Steyn-Parve & Beinert, 1958).

pH Profile Studies at 850 mM Ionic Strength. The pH variation of V_{max} for P-450R at an ionic strength of 850 mM with NADPH and cytochrome c as substrates is shown in Figure 2A. Log (V_{max}) decreases below a pK_a of 6.21 with a slope of 1 due to the protonation of a group that must be unprotonated for activity and above a pK_a of 8.85 with a slope of −1 due to deprotonation of a group that must be protonated for activity. Similar pK_as are observed in the $(V/K)_{NADPH}$ profile (6.90 and 8.38) (Figure 2B) and in the $(V/K)_{cytc}$ profile (7.23 and 8.63) (Figure 2C), probably reflecting ionization of the same two groups. To determine if either of these pK_as are for the 2′-phosphate of NADPH, or for some enzyme group interacting with the 2′-phosphate, the (V/K) profile for NADH (a slow alternate substrate) was obtained (Figure 3). $(V/K)_{NADH}$ decreases below a pK_a of 7.12 and above a pK_a of 9.61. Neither of these pK_as, which are probably for the same two groups that ionize in the V_{max}, $(V/K)_{NADPH}$, and $(V/K)_{cytc}$ profiles (Figure 2), can be for the 2′-phosphate, which is absent on NADH, or for enzyme groups that interacted with the 2′-phosphate. To further probe enzyme–NADP(H) binding interactions, the effect of pH on the inhibition constant for the competitive inhibitor $NADP^+$ was

[3] Substituting the calculated values for Y_L, Y_H, and K in eq 4, the extinction coefficient at 600 nm for DCIP is given by ϵ (mM^{-1} cm^{-1}) = [2.41 $(H/10^{-5.86})$ + 22.3]/[$(H/10^{-5.86})$ + 1].

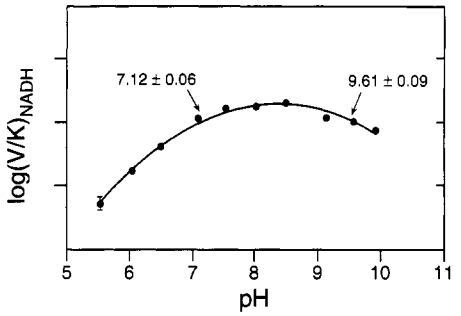

FIGURE 3: Effect of pH on log $(V/K)_{NADH}$, with cyt c acting as the other substrate, at an ionic strength of 850 mM. Data were fitted to eq 5. $(V/K)_{NADH}$ decreases at low pH as an acidic group with a pK_a of 7.12 ± 0.06 is protonated, and at high pH as a basic group with a pK_a of 9.61 ± 0.09 is deprotonated. $(V/K)_{NADH}$ has arbitrary units, with each demarcation on the y-axis representing one log unit [a 10-fold change in $[V/K)_{NADH}$].

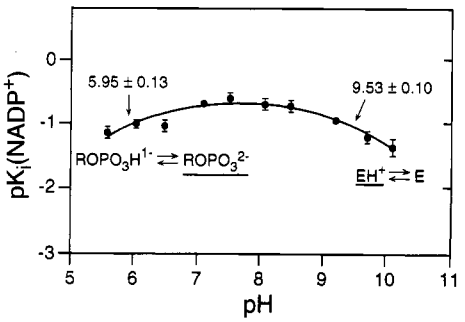

FIGURE 4: Effect of pH on K_i, the inhibition constant for $NADP^+$, which is a competitive inhibitor versus NADPH in the P-450R reaction, at an ionic strength of 850 mM with cytochrome c present at 30 μM. The data were fitted to eq 5. pK_i decreases at low pH as an acidic group with a pK_a of 5.95 ● 0.13 is protonated and at high pH as a basic group with a pK_a of 9.53 ± 0.10 is deprotonated. EH^+ is a protonated enzymic group and $ROPO_3^{2-}$ is $NADP^+$ with the 2′-phosphate present as the dianion. K_i is in units of micromolar.

determined. The pK_i profile (Figure 4), like the (V/K) profile for the slow alternate substrate NADH (Figure 3), gives true thermodynamic pK_a values, but it provides information on binding only. pK_i decreases at low pH as a group with a pK_a of 5.95 is protonated and at high pH as a group with a pK_a of 9.53 is deprotonated. The group with a pK_a of 5.95 is probably the 2′-phosphate of $NADP^+$, which is known to ionize with a pK_a of 5.81 (Figure 1A), while the group with a pK_a of 9.53 must be an enzymic group that has to be protonated for binding.

pH Profile Studies at 300 mM Ionic Strength. Although many previous kinetic studies with P-450R have been carried out at high ionic strength (850 mM, using a 0.3 M phosphate buffer at pH 7.7–8.0), we decided to determine pH profiles at lower ionic strength since it is possible that a change in ionic strength may alter the profiles by affecting the strength of certain ionic interactions (Wijnands et al., 1984). This is a real possibility since P-450R is known to be very sensitive to ionic strength (Phillips & Langdon, 1962; Bilimoria & Kamin, 1973). An ionic strength of 300 mM was chosen, since this is the ionic strength used in a number of other kinetic studies (which used a 0.1 M potassium phosphate buffer at pH 7.7–8.0). It was not possible to obtain reliable pH profiles at lower ionic strengths, since the kinetic mechanism becomes more complicated with initial velocity–double reciprocal plots showing curvature (unpublished results). NADPH was maintained at a saturating level of 0.5 mM, and V_{max} and $(V/K)_{cytc}$ values were obtained by fitting the data to eq 1 [saturating levels of cytochrome c could not be achieved, since

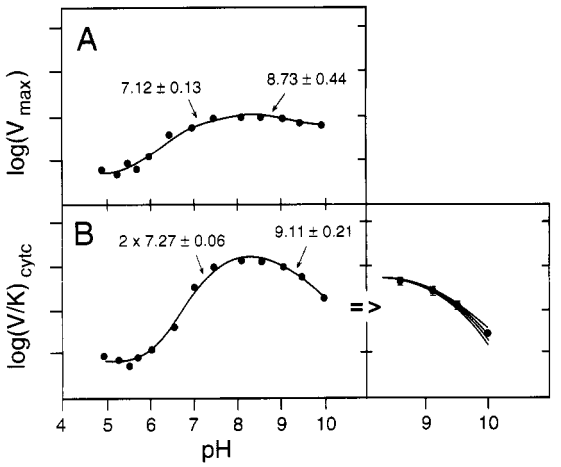

FIGURE 5: Effect of pH on (A) V_{max} and (B) $(V/K)_{cytc}$ at an ionic strength of 300 mM, with NADPH present as the other substrate. The V_{max} data were fitted to eq 6. V_{max} decreases 33-fold and plateaus at low pH, as an acidic group with a pK_a of 7.12 ± 0.13 is protonated, and decreases 2-fold and then plateaus at high pH, as a basic group with a pK_a of 8.73 ± 0.44 is deprotonated. The $(V/K)_{cytc}$ data were fitted to eq 7. $(V/K)_{cytc}$ decreases 350-fold [log $(V/K)_{cytc}$ decreases with a slope of 2] and then reaches a plateau at low pH as two acidic groups with an average pK_a of 7.27 ± 0.06 are protonated. $(V/K)_{cytc}$ decreases at high pH as a basic group with a pK_a of 9.11 ± 0.21 is deprotonated, and decreases further as ≤2 groups with pK_a values of ca. 10.6 are deprotonated. The data fit best to eq 7 which assumes one pK_a of 10.6 ± 0.9. Theoretical curves are shown for models assuming O groups (by subtracting log $[1/(K_3/H + 1)]$ from the fit to eq 7), 1 group (the fit to eq 7), 2 groups (by adding log $[1/(K_3/H + 1)]$ to the fit to eq 7), and 3 groups (by adding 2·log $[1/(K_3/H + 1)]$ to the fit to eq 7) with average pK_a values of 10.6. The curves for 0–3 of these basic groups are shown staggered from top to bottom in the expanded portion of the $(V/K)_{cytc}$ profile. The units of V_{max} and (V/K) are arbitrary, with each demarcation on the y-axis representing one log unit [a 10-fold change in V_{max} or (V/K)].

cytochrome c shows substrate inhibition; furthermore, the mechanism no longer fits the simple ping-pong model described by eq 2, so a $(V/K)_{NADPH}$ profile could not be obtained]. With cytochrome c acting as the electron-accepting substrate, V_{max} decreases 33-fold as a group with a pK_a of 7.12 is protonated and decreases only 2-fold as a group with a pK_a of 8.73 is deprotonated (Figure 5A). Although (V/K) pH profiles can have a "hump" like that seen in Figure 5A, due only to the ionization of an acidic group and the presence of certain ratios of rate constants, this is not possible with V_{max} profiles (Cleland, 1982b). Hence, there must also be a basic group ionizing in this V_{max} profile as well as in the DCIP V_{max} profile discussed later. These are probably the same two groups that showed up in the V_{max}, $(V/K)_{cytc}$, and $(V/K)_{NADPH}$ pH profiles at 850 mM ionic strength (Figure 2), but the ionization of these groups has a less pronounced effect at lower ionic strength. $(V/K)_{cytc}$ decreases 350-fold at low pH as two groups with an average pK_a of 7.27 are protonated (Figure 5B). One of the two ionizations at 7.27 is probably for the same group seen in the V_{max} profile, while the other may be for a group involved in a binding interaction between cytochrome c and P-450R. $(V/K)_{cytc}$ decreases also at high pH as a group with a pK_a of 9.11 is deprotonated, probably the same group observed in the V_{max} profile with a pK_a of 8.73. Since V_{max} is hardly affected by this ionization, but $(V/K)_{cytc}$ is affected to a larger extent, this group may be involved in a binding interaction with cytochromic c, in addition to having an effect on catalysis. The $(V/K)_{cytc}$ profile fit best to an equation assuming two basic groups in addition to the two acid groups discussed previously. This fit gave a pK_a of 10.6 for the additional basic group. It is known that there are a number of lysines on cytochrome c involved in binding interactions with other proteins (Koppenol & Margoliash, 1982), and these would be expected to show up in the $(V/K)_{cytc}$ profile. Thus, although the pK_a of lysine is outside the accessible range of our pH profile, theoretical curves have been calculated for models assuming 0, 1, 2, or 3 groups with pK_a values of 10.6, to get an estimate of the number of lysines that may be involved in binding interactions with cytochrome c. The curve fit best to the model assuming ≤2 lysines, as can be seen by comparing the experimental values for log $(V/K)_{cytc}$ at pH 10 and 9.5 with the theoretical values from models assuming 0, 1, 2, or 3 lysines (Table I).

Table I: Experimental and Modeled Values for Log $(V/K)_{cytc}$[a]

log $(V/K)_{cytc}$	experimental	0 Lys	1 Lys	2 Lys	3 Lys
pH 10.0	0.33 ± 0.02	0.44	0.34	0.24	0.14
pH 9.5	0.83 ± 0.05	0.83	0.80	0.77	0.73

[a] Modeled values of log $(V/K)_{cyt}$ were obtained as described in the legend for Figure 5B, for the two high-pH data points in that profile.

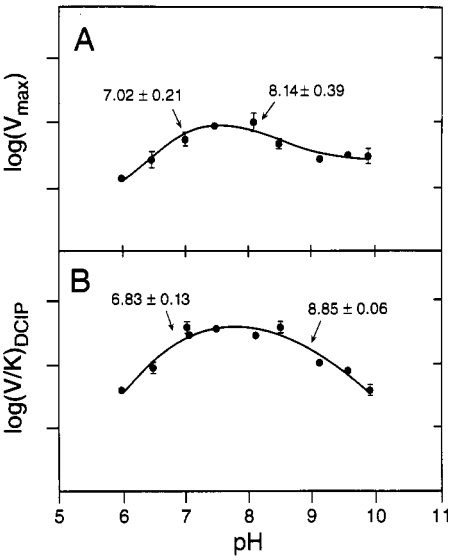

FIGURE 6: Effect of pH on (A) V_{max} and (B) $(V/K)_{DCIP}$ at an ionic strength of 300 mM, with NADPH acting as the other substrate. The V_{max} data were fitted to eq 8. V_{max} decreases at low pH, as an acidic group with a pK_a of 7.02 ± 0.21 is protonated, and decreases 5-fold and reaches a plateau at high pH, as a basic group with a pK_a of 8.14 ± 0.39 is deprotonated. The $(V/K)_{DCIP}$ data were fitted to eq 9. $(V/K)_{DCIP}$ decreases at low pH as an acidic group with a pK_a of 6.83 ± 0.13 is protonated and decreases further as another acidic group with a pK_a of 5.92 ± 0.37 is protonated. $(V/K)_{DCIP}$ decreases at high pH as a basic group with a pK_a of 8.85 ± 0.06 is deprotonated. The units of V_{max} and (V/K) are arbitrary, with each demarcation on the y-axis representing one log unit [a 10-fold change in V_{max} or (V/K)].

To determine which, if any, of these ionizable groups are present on the substrate, cytochrome c, V_{max} and (V/K) profiles were determined with DCIP as the electron-accepting substrate. The V_{max} profile decreases at low pH as a group with a pK_a of 7.02 is protonated and decreases 5-fold at high pH as a group with a pK_a of 8.14 is deprotonated (Figure 6A). These are probably the same two groups that showed up in the V_{max} profile with cytochrome c acting as the electron acceptor. The $(V/K)_{DCIP}$ profile decreases at low pH as a group with a pK_a of 6.83 is protonated, but the second pK_a around 7 that showed up in the $(V/K)_{cytc}$ profile (Figure 5B) is no longer present (Figure 6B). At lower pH, $(V/K)_{DCIP}$ decreases further as a group with a pK_a of 5.9 [probably DCIP itself, which has a pK_a of 5.86 (Figure 1B)] is protonated. At high pH, $(V/K)_{DCIP}$ decreases as a basic group with a pK_a of 8.85 is protonated, as with $(V/K)_{cytc}$.

Table II: Kinetic and Inhibition Constants for Nicotinamide Nucleotides[a]

substrate or inhibitor	$k_{cat} \times 10^{-3}$ [μmol min^{-1} (μmol of P-450R)$^{-1}$]	k_{cat}/K_m (min·μM)$^{-1}$	K_m (μM)	K_i (μM)
NADPH	5.09 ± 0.30	1860 ± 100	2.74 ± 0.25	
NADH	0.928 ± 0.016	0.0800 ± 0.0024	11600 ± 600	
$NADP^+$				5.1 ± 1.1
NAD^+				14200 ● 4700

[a] Studies were carried out at 25 °C in a 100 mM TAPS buffer (pH 8.0) with KCl added to give an ionic strength of 850 mM. The electron accepting substrate was cytochrome *c*, which was present at a fixed concentration of 30 μM in the K_i determinations and varied from 0.2 to 5 times its K_m in the initial velocity studies with NADPH and NADH.

Table III: Thermodynamic Effects of Removing the 2′-Phosphate of NADPH[a]

modification	$\Delta\Delta G_b$ (kcal/mol)	$\Delta\Delta G_b^*$ (kcal/mol)
NADPH → NADH	5.0	6.0
$NADP^+ \rightarrow NAD^+$	4.7	

[a] Free energy changes (at 25 °C) were calculated as described by Fersht (1985): $\Delta\Delta G_b = -RT \ln (K_{NADPH}/K_{NADH})$ or $\Delta\Delta G_b = -RT \ln (K_{NADP^+}/K_{NAD^+})$ and $\Delta\Delta G_b^* = RT \ln [(k_{cat}/K_m)_{NADPH}/(k_{cat}/K_m)_{NADH}]$, where R is the gas constant and T is the temperature in kelvins.

Alternate Substrate and Inhibitor Studies at 850 mM Ionic Strength. The values for k_{cat}, k_{cat}/K_m, and K_m were compared with NADPH and NADH as substrates, to probe the role of the 2′-phosphate of NADPH (Table II). k_{cat} is decreased a modest 5.5-fold, while K_m increases 4200-fold due to removal of the 2′-phosphate group in going from NADPH to NADH.[4] If K_m is taken as an approximation of a dissociation constant [this is a fair assumption since there are large deuterium isotope effects on V_{max} and $(V/K)_{NADPH}$, consistent with hydride transfer being a major rate limiting step (unpublished results)], this would correspond to a loss of 5.0 kcal/mol of ground-state binding energy (Table III). The effect on k_{cat}/K_m [since hydride transfer is a major rate limiting step in k_{cat}/K_m (unpublished results)] would correspond to a loss of 6.0 kcal/mol of the hydride transfer transition-state binding energy upon removal of the 2′-phosphate group. The values of K_i for the competitive (versus NADPH) inhibitors $NADP^+$ and NAD^+ were compared, to probe the role of the 2′-phosphate of $NADP^+$ (Table II). Removal of the 2′-phosphate group from the product $NADP^+$, giving NAD^+, causes a 2800-fold increase in K_i, which corresponds to a 4.7 kcal/mol loss of ground-state binding energy.

DISCUSSION

pH Profiles at 850 mM Ionic Strength. The V_{max} profile with NADPH and cytochrome *c* as substrates (at 850 mM ionic strength) shows that a group with a pK_a of 6.21 must be unprotonated, while a group with a pK_a of 8.85 must be protonated for maximum activity (Figure 2A). Similar pK_as are observed in the $(V/K)_{NADPH}$ (Figure 2B) and $(V/K)_{cytc}$ (Figure 2C) profiles, probably for the same two groups, which appear to be playing a role in catalysis, but not substrate binding. The ca. 0.8 pH unit perturbation of the acidic pK_a in the V_m profile could reflect a slow product release step, or possibly just solvent exclusion from the active site (Cleland, 1982a). No additional pK_as are observed in these two (V/K) profiles, indicating a lack of ionizable groups (that ionize in this pH range) that play a role in substrate binding. The (V/K) profile for NADH shows the same two pK_as that were observed in the $(V/K)_{NADPH}$ profile, but the basic pK_a of 8.38 has been perturbed to 9.61, while the acidic pK_a of 7.12 is essentially the same as that of 6.90 observed in the $(V/_K)_{NADPH}$ profile (Figure 3). The perturbation of the basic pK_a cannot be due to substrate stickiness (NADH should be less sticky than NADPH), as the shift is in the wrong direction for this sort of effect. The ionization state of this catalytic group affects the fraction of the NAD(P)H·P-450R complex that goes on to products (Cleland, 1982a), and its pK_a reflects its microenvironment in the substrate-free enzyme. Therefore, there must be multiple conformations of P-450R in the absence of substrate, and the conformation bound by NADPH differs from that bound by NADPH in that this basic group is exposed to a different environment. This selection of different enzyme conformations by different substrates is reminiscent of the situation observed in phosphoglucomutase (Ray et al., 1993) and suggests a role for the 2′-phosphate in controlling the conformation of P-450R. This basic group probably is not close to the 2′-phosphate though, since our results indicate that it is near the FMN binding site (discussed later), and the FAD ring, which is part of the NADPH binding site [since the nicotinamide ring must reside near the isoalloxazine ring of FAD for hydride transfer to occur (Vermilion et al., 1981; Sem & Kasper, 1992)] is thought to be 2 nm from FMN based on fluorescence studies (Bastiaens et al., 1989). It has been suggested that binding of NADPH induces a significant conformational change in P-450R (Vermilion et al., 1981), or at least selectively binds and populates a unique conformation of P-450R, which could be driven in part by interactions with the 2′-phosphate group, as is the case with dihydrofolate reductase (Bystroff & Kraut, 1991).

The pK_i profile for $NADP^+$ shows that a group with a pK_a of 5.95 is preferentially unprotonated while a group with a pK_a of 9.53 is preferentially protonated for $NADP^+$ to bind to P-450R (Figure 4). Since the 2′-phosphate of $NADP^+$ has a pK_a of 5.81 (Figure 1A), and a change in substrate ionization state would be expected to affect binding affinity, the pK_a observed at 5.95 is probably for this 2′-phosphate. This would mean that the 2′-phosphate is present as the dianion when bound to P-450R. The ^{31}P-NMR chemical shift of the 2′-phosphate of $NADP^+$ bound to P-450R was found by Otvos et al. (1986) to be closest to that for the 2′-phosphate of free $NADP^+$ in the monoanionic form. Clearly, as suggested by these authors, correlations between ^{31}P-NMR chemical shift and ionization state of free phosphate esters are not strong enough to be useful in predicting the ionization state of a protein-bound phosphate ester, since many factors (O–P–O torsion angle, local environment, etc.) can affect chemical

[4] Prough and Masters (1976) have reported a 23 000-fold difference in K_m values for NADPH and NADH, with no difference in V_{max} for the two substrates. The discrepancies between these results and ours are most likely a result of the different assay conditions used. Prough and Masters used a 50 mM potassium phosphate buffer at pH 7.7, which would give an ionic strength of 138 mM. In addition to the ionic strength difference, the presence of phosphate could affect NADH binding by binding in the 2′-phosphate binding pocket of P-450R, as observed in the crystal structure of the NADH·glutathione reductase complex (Pai et al., 1988).

shifts. Indeed, reliance on the ^{31}P-NMR results would have led to the wrong assignment of the ionization state for the 2′-phosphate of NADP$^+$ bound to P-450R. The pK_a at 9.53 must be for a protonated enzyme group, since NADP$^+$ has no ionizations in this range. One possible role for this enzyme group is to interact with this 2′-phosphate (topic of following paper in this issue). The two pK_as observed in this pK_i profile do not appear in the $(V/K)_{NADPH}$ pH profile (Figure 2B), so they must have been perturbed outside of the pH range of the $(V/K)_{NADPH}$ profile due to stickiness effects. Although NADH is a slow substrate, and should therefore show pK_as that are not perturbed by stickiness effects, it lacks the 2′-phosphate and therefore must lack the acidic pK_a observed in the NADP$^+$ pK_i profile. The pK_a at 9.61 in the $(V/K)_{NADPH}$ profile (Figure 3) is probably not for the same group that is present in the pK_i profile (9.53), because the catalytic group with a pK_a of 8.38 should also ionize in the $(V/K)_{NADPH}$ profile. It is more likely then that the pK_a for this catalytic group has shifted from 8.38 in the $(V/K)_{NADPH}$ profile to 9.61 in the $(V/K)_{NADPH}$ profile, as discussed earlier, and that the enzyme group with a pK_a of 9.53 that shows up in the NADP$^+$ pK_i profile does not appear in the $(V/K)_{NADH}$ profile because it was involved in an interaction with the 2′-phosphate of NADPH, and therefore does not affect NADH binding. Results of the following paper (Sem & Kasper, 1993) indicate that this is in fact the case.

pH Profiles at 300 mM Ionic Strength. Since ionic strength may affect which ionizations are observed in pH profiles (Wijnands et al., 1984), and since P-450R is known to be very sensitive to changes in ionic strength (Phillips & Langdon, 1962), it was necessary to obtain pH profiles at the lower ionic strength of 300 mM. Kinetic studies of P-450R are more difficult at lower ionic strength, since the kinetic mechanism becomes more complicated (unpublished results), so $(V/K)_{NADPH}$ profiles could not be determined.

The V_{max} profile with cytochrome *c* and NADPH acting as substrates (Figure 5A) shows an acidic group with a pK_a of 7.12 that is preferentially unprotonated and a basic group with a pK_a of 8.73 that is preferentially protonated. These are probably the same two catalytic groups that showed up in the V_{max} profile at 850 mM ionic strength, but ionization to the wrong ionization states at this lower ionic strength has a smaller effect on V_{max}, since protonation of the acidic group causes only a 33-fold decrease in V_{max} and deprotonation of the basic group causes a modest 2-fold decrease in V_{max}. Apparently, the incorrect ionization states for these two groups are tolerated better at lower ionic strength. These two ionizations also show up in the $(V/K)_{cytc}$ profile (Figure 5B), with pK_as of 7.27 and 9.11, respectively, in addition to another acidic group with a pK_a of 7.27. Since the pK_a for this second acidic group did not appear in the V_{max} profile, it must be involved in binding interactions between cytochrome *c* and P-450R. Furthermore, since the pK_a for this acidic group was not observed in profiles at higher ionic strength, it is likely to be an ionic interaction that decreases in strength as ionic strength increases. Protonation of both acidic groups causes a combined 350-fold decrease in $(V/K)_{cytc}$. Deprotonation of the basic group with a pK_a of 9.11 has a much more pronounced effect on $(V/K)_{cytc}$ than on V_{max}, indicating that its effect is mostly on binding interactions between cytochrome *c* and P-450R (Figure 5B), although ionization of this group in the Michaelis complex also has a modest effect on catalysis (Figure 5A). This basic group has a dual role in binding and catalysis, although the effect on catalysis is less significant at the lower ionic strength of 300 mM.

The $(V/K)_{cytc}$ pH profile fit best to a model assuming two basic groups (Figure 5B) in addition to the two acidic groups just discussed. The fit gave a pK_a of 10.6 for the additional basic group. Although this ionization is unfortunately outside of the pH range of this profile, making any fit speculative, a group ionizing in this range will still have a significant effect on $(V/K)_{cytc}$ at pH 10. There are surface lysines surrounding the heme crevice of cytochrome *c* (which would be expected to have pK_as in this range) that are thought to play a role in binding interactions with carboxyl groups on the surfaces of cytochromes *c* oxidase, reductase, and peroxidase (Koppenol & Margoliash, 1982). The residues of cytochrome *c* most frequently involved in binding interactions with other electron transfer proteins are lysines 13, 27, 72, 86, and 87, and they surround the exposed portion of the heme, in the interaction domain of cytochrome *c* (Margoliash & Bosshard, 1983). Similar binding interactions have been proposed between other electron transfer protein pairs: adrenodoxin/adrenodoxin reductase, adrenodoxin/cytochrome P-450$_{scc}$ (Lambeth et al., 1984; Coghlan & Vickery, 1991), cytochrome P-450/P-450R (Nadler & Strobel, 1988), and P-450R/cytochromes *c*, b_5, and P-450 (Tamburini & Schenkman, 1986). Although ionic interactions play an important role in a number of electron transfer protein–protein interactions, there are cases where hydrophobic and van der Waals interactions play a significant, if not dominant, role (Stayton & Sligar, 1990). Since the question of how many lysines are involved in the cytochrome *c*/P-450R binding interactions is of such interest (as a model of P-450R/substrate binding interactions and of nonspecific protein–protein binding interactions in general), we have modeled the $(V/K)_{cytc}$ profile assuming 0–3 lysines with pK_a values of 10.6, in order to get an estimate of how many lysines are involved in binding (Table I and Figure 5B). The data are most consistent with an average of ≤2 lysines (probably 1) involved in binding cytochrome *c* to P-450R, at an ionic strength of 300 mM. This is a reasonable number of interactions, although slightly less than the number of ionic interactions throught to be present in the following electron transfer protein–protein complexes: between cytochrome *c* and cytochrome *c* oxidase (3 interactions) (Millet et al., 1983), cytochrome b_5 (5–7 interactions) (Stonehuerner et al., 1979; Ng et al., 1977), cytochrome *c* peroxidase (5 interactions) (Poulos & Kraut, 1980), or flavodoxin (4 interactions) (Simondsen & Tollin, 1982); P-450R and cytochrome P-4501A2 (7–10 interactions) (Shimiu et al., 1991); cytochrome *c* and adrenodoxin reductase (4 interactions) (Geren & Millett, 1981); cytochrome b_5 and cytochrome b_5 reductase (5 interactions) (Dailey & Strittmatter, 1979); and cytochrome c_2 and the photosynthetic reaction center (4 interactions) (Knaff et al., 1991). But, since cytochrome *c* is not a natural substrate for P-450R, a high degree of complementarity between the charged surfaces of P-450R and cytochrome *c* would not be expected. Although previous studies have suggested that charge pairing interactions between lysines and carboxyl groups are important for the binding of charged electron acceptors to P-450R (Shimizu et al., 1991; Tamburini & Schenkman, 1986), the recent results on Voznesensky and Schenkman (1992a,b) suggest electrostatic interactions may actually be unfavorable. These studies focused mainly on interactions with cytochrome P-450, although it was suggested that the ionic interactions between cytochrome c^{3+} and P-450R are also unfavorable, based on the observation of a decreased rate of electron transfer (V_{max}) to cytochrome c^{3+} at high ionic strength and an increased rate at high glycerol. Our results also show that V_{max} increases at high ionic strength,

but since $(V/K)_{cytc}$ decreases at high ionic strength (unpublished results), we have concluded that favorable ionic interactions are important for binding cytochrome c^{3+} to P-450R. This interpretation may not apply to interactions with cytochrome P-450, since Voznesensky and Schenkman (1992a,b) have shown that its K_m decreases at high ionic strength (and increases at high glycerol), but it does explain our results and those of Voznesenski and Schenkman (1992a,b) with cytochrome c^{3+}. Cross-linking studies suggest that lysine 13 of cytochrome *c* is involved in binding interactions between cytochrome *c* and P-450R (Nisimoto, 1986), and the cross-linked complex is itself catalytically competent (Nisimoto & Otsuka-Murakami, 1988). Site-directed mutagenesis of residues in two acidic clusters on the surface of P-450R shows modest effects on K_m for cytochrome *c* (Shen & Kasper, 1990), implying that these ionic interactions play some role in binding, with no one interaction being particularly important. It is possible that cytochrome *c* binds to P-450R in a number of different orientations, such that no one interaction is vital for binding. Lysine 13 could interact with a number of different residues on P-450R, yielding a variety of catalytically competent complexes. This type of multiplicity of complex conformation has been suggested for cytochrome *c* binding interactions with other proteins (Poulos et al., 1987; Northrup et al., 1988; Peerey et al., 1991; Zhou & Kostic, 1991), and is a result of a number of fairly nonspecific ionic and hydrophobic interactions which favor different binding orientations, many of which are adequate for electron transfer. The P-450R/cytochrome *c* complex appears to have an average of ≤2 such ionic interactions, one of which may involve lysine 13 of cytochrome *c*.

Since it is not clear which of the above-mentioned pK_as are for groups that are present on P-450R or on cytochrome *c* (except for the pK_a of 5.95 that has been assigned to the 2′-phosphate of NADP$^+$), pH profiles have been obtained with DCIP acting as the electron-accepting substrate, in place of cytochrome *c*. While cytochrome *c* has many ionizations in the experimental pH range, complicating interpretation of pH profiles, DCIP has only one ionization for its hydroxyl group, which has a pK_a of 5.86 (Figure 1B). The V_{max} profile with NADPH and DCIP acting as substrates shows an acidic group with a pK_a of 7.02 that is preferentially unprotonated and a basic group with a pK_a of 8.14 that is preferentially protonated (Figure 6A). These are the same two catalytic groups that showed up in the V_{max} profile with cytochrome *c* acting as the electron-accepting substrate (Figure 5A), so they must be present on P-450R rather than cytochrome *c*. Deprotonation of the basic group causes a 5-fold decrease in V_{max}. These two ionizations also show up in the $(V/K)_{DCIP}$ profile (Figure 6B), with pK_as of 6.83 and 8.85, respectively, in addition to the pK_a of 5.9 for DCIP itself, which is preferentially unprotonated (possessing a −1 charge). Since the additional group with a pK_a of 7.27 observed in the $(V/K)_{cytc}$ profile (Figure 5B) does not appear in the $(V/K)_{DCIP}$ profile, it must be involved in binding interactions only with cytochrome *c*, not DCIP. Such an interaction was also proposed by Phillips and Langdon (1962) on the basis of their measurements of the pH dependence of P-450R activity, although their study did not identify the origin of the effect (V_{max} or V/K). As was observed in the profiles with cytochrome *c* acting as the electron-accepting substrate, deprotonation of the basic group has a larger effect on $(V/K)_{DCIP}$ than on V_{max}, indicating that its ionization affects mostly binding interactions between DCIP and P-450R, while ionization of this group in the Michaelis complex has a smaller effect on catalysis. This basic group has a dual role in binding and catalysis, affecting the binding of both DCIP and cytochrome *c*. Since electrons are thought to be transferred from FMN to the electron-accepting substrate (Vermilion et al., 1981), this basic group must be on the surface of the protein near the FMN binding site [and the cytochrome P-450/cytochrome *c*/DCIP binding site(s)], possibly having its effect on V_{max} by either interacting directly with FMN or by affecting the conformation/orientation of the electron transfer complex between P-450R and DCIP or cytochrome *c*.

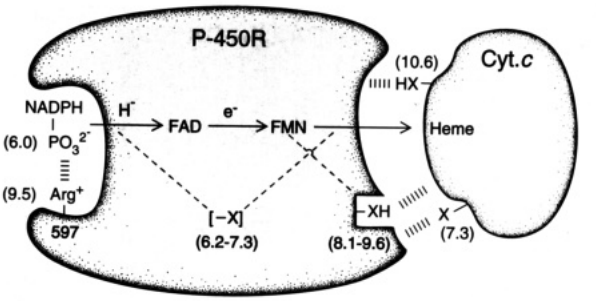

FIGURE 7: Model showing proposed roles for various ionizable groups in binding and catalysts. The preferred ionization states of the groups are indicated as X for the conjugate base or XH for the conjugate acid. The estimated value of the pK_a for each functional group is given in parentheses.

Proposed Model/Summary. The proposed roles for the various ionizable groups that affect binding and/or catalysis with P-450R are summarized in the model in Figure 7. pK_a values are shown in parentheses and are rounded off to two significant figures. The 2′-phosphate of NADP$^+$ (and probably NADPH) is bound preferentially as the dianion and has a pK_a of 6.0. The results of the following paper (Sem & Kasper, 1993) will show that the enzyme group with a pK_a of 9.5 that plays a role in binding NADPH is actually Arg597 and is involved in an ionic interaction with the 2′-phosphate of NADPH. Removal of the 2′-phosphate from NADPH leads to a loss of 5.0 kcal/mol of ground-state and 6.0 kcal/mol of transition-state binding energy (Table III). Likewise, removal of the 2′-phosphate from NADP$^+$ leads to a loss of 4.7 kcal/mol of ground-state binding energy (Table III). Thus, all interactions between P-450R and the 2′-phosphate of NADPH are contributing no more than 5 kcal/mol of essentially uniform binding energy,[5] with possibly an additional 1 kcal/mol of binding energy only being realized in the transition state. There are also two catalytic groups residing on P-450R, one with an apparent pK_a of 6.2–7.3 that must be unprotonated and one with an apparent pK_a of 8.1–9.6 that must be protonated. The basic group affects the predominantly binding of DCIP and cytochrome *c* at lower ionic strength, so it must be on the protein surface, near the FMN and the cytochrome *c*/DCIP binding site. These groups may play a role in catalysis by interacting with the flavins, possibly to manipulate their redox potentials, as was shown to occur in flavodoxin (Ludwig et al., 1990). There may also be a need for an acid/base catalyst near the N5 of the FAD prosthetic group, since a proton is generated in the course of the electron transfer reaction (the A-side hydrogen of NADPH is transferred to the N5 of FAD and must ultimately be released into solution). It is also possible that one or both of these pK_as

[5] There are three classes of enzyme–substrate binding interactions, as described by Albery and Knowles (1976): uniform binding where all ground and transition states are bound with equal affinity, differential binding where some ground states are bound more tightly than others, and catalysis of elementary steps where certain transition states are bound most tightly.

could be for the flavins themselves. FMN, FMNH•, and $FMNH_2$ in their unbound states have pK_as of 10.0–10.4 (at N3), 8.6 (at N5), and 6.5 (at N1), respectively (Draper & Ingraham, 1968; Theorell & Nygaard, 1954), although these pK_as can be perturbed more than 4 pH units when the flavins are protein-bound (Ludwig et al., 1990). Finally, there are ≤2 lysines (one of which may be lysine 13) on cytochrome *c* that are important for binding to P-450R, and there is one group with a pK_a of 7.3 that is preferentially unprotonated and also plays a role in binding cytochrome *c* to P-450R, but only at lower ionic strength. At higher ionic strength where the importance of ionic interactions is diminished, hydrophobic interactions may predominate (Zhou & Kostic, 1991).

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