Interaction with Arginine 597 of NADPH–Cytochrome P-450 Oxidoreductase Is a Primary Source of the Uniform Binding Energy Used To Discriminate between NADPH and NADH†

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Appendix: Thermodynamic Interpretation of Combined Alternate Substrate/Inhibitor, pH, and Site-Directed Mutagenesis Studies

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ABSTRACT: Site-directed mutagenesis has been used in conjunction with pH and alternate substrate/inhibitor studies to characterize the interactions between NADPH–cytochrome P-450 oxidoreductase (P-450R) and the 2′-phosphate of NADP(H) that provide P-450R with its strong nicotinamide nucleotide specificity. It is known that the 2′-phosphate of NADP(H) is bound to P-450R as the dianion and that interactions between it and residues on P-450R provide 5 kcal/mol of essentially uniform binding energy (preceding paper in this issue). In order to probe these interactions further, Arg597 of P-450R, which is homologous to Arg235 of ferredoxin–$NADP^+$ reductase that forms a salt bridge with the 2′-phosphate of 2′-phospho-AMP in the crystal structure of that complex [Karplus, P. A., Daniels, M. J., & Herriott, J. R. (1991) *Science 251*, 60], was mutated to methionine. The mutant protein, P-450R (R597M), does not appear to have a grossly perturbed tertiary structure on the basis of the observation of similar ^{31}P-NMR chemical shifts for FAD (pyrophosphate) bound to it and wild-type (WT) P-450R, although it is more unstable to urea denaturation. P-450R (R597M) has a K_m for NADPH that is 150 times that of P-450R (WT) and a K_i for $NADP^+$ that is 240 times that of P-450R (WT). In contrast, the R597M mutation has only a modest effect on the K_m for NADH (0.8WT) and the K_i for NAD^+ (2.9WT), indicating that Arg597 must have been interacting specifically with the 2′-phosphate of NADP(H). The R597M mutation has relatively little effect on k_{cat} for NADPH (1.2WT) or NADH (0.6WT), indicating that the mutation is affecting ground and transition states to essentially the same degree, by removing 3 kcal/mol of uniform binding energy. The $NADP^+$ pK_i profile for P-450R (R597M) shows a pK_a of 5.78 for the 2′-phosphate of $NADP^+$, which is bound to P-450R (R597M) as the dianion, but the pK_a of 9.5 for the preferentially protonated enzymic group observed in the P-450R (WT) profile is no longer present. It is argued then that the 2′-phosphate binding pocket of P-450R (WT) has a high positive charge density (>+2) and that Arg597, which is in this binding pocket, has a highly perturbed pK_a of 9.5. Finally, a general theoretical treatment of the thermodynamic consequences of individual and combined perturbations to complementary interacting groups on enzyme and substrate is presented (see Appendix). This allows conclusions to be made regarding the severity of the structural perturbations resulting from a mutation: *local* (affecting interactions with one group on the substrate), *proximal* (affecting interactions with the rest of the substrate), or *global* (affecting interactions in another binding site).

NADPH–cytochrome P-450 oxidoreductase (P-450R)[1] (NADPH–ferrihemoprotein oxidoreductase, EC 1.6.2.4) is a 78 225-Da flavoprotein that is a member of the dehydrogenase/electron transferase family (Massey & Hemmerich, 1980). It contains 1 equiv each of FAD and FMN (Iyanagi & Mason, 1973) and is a peripheral membrane protein, localized on the cytoplasmic side of the endoplasmic reticulum (Williams & Kamin, 1962; Phillips & Langdon, 1962) and nuclear envelope (Kasper, 1971) of a number of eukaryotic cell types. It is an essential component of the MFO system, since it catalyzes the transfer of reducing equivalents from NADPH to the cytochromes P-450.

The source of electrons in the MFO system is NADPH. Consistent with this, P-450R binds NADPH much more tightly than NADH (Prough & Masters, 1976). This specificity is very strong in P-450R, since it has a K_m(NADH)/K_m(NADPH) ratio of 4200 (preceding paper in this issue), which is much larger than that for *Escherichia coli* glutathione reductase (92; Scrutton et al., 1990), for human dihydrofolate reductase (69; Huang et al., 1990); and for FNR (400; Shin & Arnon, 1965). Phillips and Langdon (1962) recognized

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[1] Abbreviations: P-450R, NADPH–cytochrome P-450 oxidoreductase; MFO, mixed function oxidation; FNR, ferredoxin–$NADP^+$ reductase; 2′-phospho-AMP, 2′-phosphoadenosine 5′-monophosphate; WT, wild type; NMR, nuclear magnetic resonance; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; cytc, cytochrome *c*; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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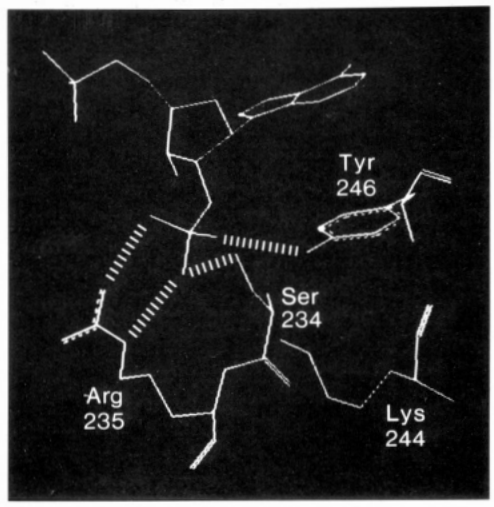
the importance of enzyme binding interactions with the 2′-phosphate, based on the low activity with NADH and the high affinity for the inhibitor 2′-AMP. Although it is not entirely clear why P-450R has evolved to have such a strong preference for NADPH, it could be a result of the relative availability of the NADPH and NADH nicotinamide nucleotides in the cytosol in their correct oxidation states, since it is known that the maximum rate of the MFO system in perfused livers from both fed and fasted mice is limited by the availability of NADPH (Wu et al., 1986). The cytosol is a reducing environment only with regard to NADP(H), not NAD(H) pools (Newsholme & Start, 1973). The evolutionary pressure leading to this nicotinamide nucleotide specificity could also be a result of the metabolic consequences of using one nicotinamide nucleotide pool over the other. Although the effect of cytosolic depletion of NADH is not known (since the MFO system is specific for NADPH), it is known that cytosolic NADPH depletion from MFO activity has the effect of inhibiting fatty acid synthesis and gluconeogenesis (Orrenius et al., 1977; Thurman & Scholz, 1973; Scholz et al., 1973).

This report is a continuation of our ongoing studies of the binding interactions between P-450R and NADPH. Previous studies have shown that P-450R binds NADPH with the nicotinamide ring in the anti conformation, with the enzyme-bound FAD ring located on the exo side of the nicotinamide ring, so that the A-side hydrogen of NADPH can be transferred to the N5 of FAD (Sem & Kasper, 1992). We are now interested in the binding interactions between P-450R and NADPH that allow the enzyme to discriminate so strongly between NADPH and NADH. Specifically, we are interested in the binding interactions with the 2′-phosphate of NADPH. There have been chemical modification studies that have implicated lysyl and arginyl residues in the interaction with this 2′-phosphate (Inano & Tamaoki, 1986; Slepneva & Weiner, 1988; Lumper et al., 1980), specifically, lysine 601 of pig P-450R (Muller et al., 1990), which is homologous to lysine 602 of rat P-450R. Although no crystal structure is available for P-450R to help define these interactions, there is a crystal structure available for FNR (Karplus et al., 1991), which as an $NADP^+$/FAD binding domain that is homologous to residues 267–678 of P-450R (Porter & Kasper, 1985, 1986). The structure of the FNR·2′-phospho-AMP complex shows interactions between the 2′-phosphate and residues Arg235, Ser234, Tyr246, and possibly Lys244 (Figure 1A). These interactions may also occur in the complex with $NADP^+$, which was not crystallized, and may be similar to those that occur in the NADPH·P-450R complex, since these residues are completely conserved among a number of dehydrogenase/electron transferases (Figure 1B).

Another reason for studying these interactions is to gain a better understanding of how enzymes bind phosphate groups in general. Such interactions are not well understood, although the phosphate group is ubiquitous in nature (Meiering et al., 1991). Often it serves as a molecular handle by which enzymes hold onto their substrates, providing significant binding energy (Westheimer, 1987; Sem & Cleland, 1991; Knight et al., 1991). The approach we have taken to study these interactions makes use of pH, alternate substrate, and site-directed mutagenesis studies in a synergistic manner to perturb one or more of these interactions and measure the functional consequences of these perturbations. The pH and alternate substrate studies with WT P-450R (preceding paper in this issue) have indicated that the 2′-phosphate of NADPH is bound as a dianion and

A



B

Rat	556	EV--GETLLYYGCRRSDEDYLYREELARFHKD-GALTQLNVAF--SR-EQ	599
S. cerevisiae	568	N•SL•KHI•F••S•NT•--F••QD•WPEYA•KLDGSFEMV••H—••LPN	614
C. tropicalis	558	N•—•K•V•F•••N•EQ•F••KQ•WSEYASVLGENFEMEN••—••QDP	603
P450-BM3	455	SL—••AH••F•••SPH••••Q•••ENAQSE-•II•-HT••—••MPN	498
SiR-FP	483	N———W•FF•NPHFT••F••QV•WQ•YV•E-•V•SRIDL•W—•••D•	522
SpiFNR	194	FN--•LAW•FL•VPT•SS-L••K••FEKM-•E-K•PDNFRLD•AV••-••	237
NOS	1273	NP--CPMV•VF•••Q•KI•HI••••TLQAKNK-•VFRE•YT•Y—•••-•P	1316

Rat	600	A-H——KVYVQHLLKRD-REHLWKLIHEGGAHIYVCGDARNMAKDVQ	641
S. cerevisiae	615	T-K——•••••DK••DY-EDQVFEM•-NN••F•••••••KG•••G•S	655
C. tropicalis	604	T-K——•••••DKILEN-SALVDELL-SS••I•••••••SR••R•••	644
P450-BM3	499	QP——••T••••VMEQ••-GKK•IE•LDQ-•••F•I•••GSQ••PA•E	539
SiR-FP	523	K-E——•I•••DK•REQ-GAE••RW•ND-••••••••••R••A••E	563
SpiFNR	238	T-NEKGE•M•I•TRMAQY-AVE••EMLKKDNTYF•M••-LKG•E•GID	282
NOS	1317	D-RP——•K••DV•QEQLA•SVYRALK•Q•G•••••••V-T••A••L	1359

FIGURE 1: (A) Interactions between spinach ferredoxin–$NADP^+$ reductase and the 2′-phosphate of 2′-phospho-AMP, based on the crystal structure of this complex. This figure was produced with the program HyperChem (from AUTODESK) using the coordinates for 2′-phospho-AMP·FNR (Karplus *et al.*, 1991) obtained from the Protein Data Bank (Bernstein *et al.*, 1977; Abola *et al.*, 1987) at Brookhaven National Laboratory. (B) Amino acid sequence alignment from part of the proposed NADPH binding domains of NADPH–cytochrome P-450 oxidoreductases from rat, yeast (*Saccharomyces cerevisiae* and *Candida tropicalis*), and bacteria (Bacillus *megaterium*), along with sulfite reductase flavoprotein (SiR-FP), spinach ferredoxin–$NADP^+$ reductase (spiFNR), and nitric oxide synthase (NOS). Conserved residues are indicated with dots, and the residues homologous to those shown in Figure 1A, involved in binding interactions with the 2′-phosphate, are marked with asterisks. Reported with permission from Shen and Kasper (1993). Copyright 1993 Springer-Verlag.

contributes 5 kcal/mol uniform binding energy resulting from interactions with enzyme groups, one of which may be the preferentially protonated group with a pK_a of 9.5 observed in the $NADP^+$ pK_i profile. Although no crystal structure is available for WT or mutant P-450R, this approach of making multiple structural perturbations to complementary interacting groups and measuring the functional (with steady-state kinetics) and structural (with ^{31}P-NMR and urea denaturation) consequences of these perturbations approaches the level of rigor suggested by Tsai and Yan (1991) for doing iterative structure–function studies, given the complications of this system: a membrane-bound protein with no crystal structure and a molecular weight too high for high-resolution NMR studies. Thus, our final goal is to present a rigorous approach to structure–function studies that allows interpretation in the absence of crystal structure data. Indeed, the need for reliable approaches to such studies is becoming greater, since the number of protein sequences was 50 times the number of three-dimensional structures as of 1990 (Bowie et al., 1991), and this gap is widening.

MATERIALS AND METHODS

NADPH, NADH, $NADP^+$, NAD^+, FAD, FMN, DTT, EDTA, and horse heart cytochrome *c* (ferric) were from Sigma. Ultrapure urea (≥99.5%) was from Bethesda Research Laboratories. D_2O (100%) and H_3PO_4 were from Aldrich. KCl and potassium phosphate were from Mallinckrodt. All enzymes and reagents used in the site-directed mutagenesis experiments were of the highest purity available from Promega, U.S. Biochemical Corp., and New England Biolabs. The following buffers were used in kinetic studies in the specific pH ranges: MES (5.5–6.5), TAPS (7.0–8.5), and CHES (9.0–10.0). All buffers were from Sigma. Antisera to P-450R and epoxide hydrolase were prepared in male albino rabbits, and the IgG fractions were purified as described previously (Zimmerman & Kasper, 1978).

Site-Directed Mutagenesis. The WT, recombinant rat liver P-450R was overexpressed in *E. coli* (C-1A) using the plasmid pOR263 and purified on a 2′,5′-ADP–Sepharose 4B affinity column (Pharmacia), as described previously (Shen et al., 1989). Site-directed mutagenesis was carried out using the Kunkel method (Kunkel, 1989) with the following oligonucleotide to generate the R597M mutation (CGG→ATG): 5′-CCTTTTCCATGGAGCAG-3′. This oligonucleotide was from GENOSYS and was purified on a Qiagen anion-exchange minicolumn. The mutagenesis was carried out in M13mp19 after a 907-bp *Kpn*I/*Hin*dIII fragment from pOR263 was inserted. The heteroduplex between the mutant oligonucleotide and the recombinant M13mp19 template was extended with T7 DNA polymerase. Mutants were identified by randomly selecting plaques and sequencing the purified template by the method of Sanger et al. (1977). After the 701-bp *Nhe*I/*Hin*dIII fragment containing the mutation was subcloned back into pOR263 [giving pOR263(R597M)], this fragment, and flanking regions in the construct, were sequenced to verify the presence of the desired mutation, along with the absence of other changes. Sequencing was done directly from pOR263(R597M), after it was cut with appropriate endonucleases (*Nco*I/*Kpn*I, *Pst*I/*Nde*I, or *Kpn*I) and one of the strands was digested with T7 gene 6 exonuclease, as described previously (Ruan & Fuller, 1991).

Purification of P-450R (R597M) and Initial Characterization. pOR263(R597M) was expressed in C-1A cells, but P-450R (R597M) could not be purified in the usual manner on the 2′,5′-ADP affinity column, since it did not bind to the resin. Apparently the R597M mutation has disrupted interactions with the 2′,5′-ADP ligand. P-450R (R597M) was purified on a cytochrome *c* affinity column (Sigma) using a modification of the procedure described by Golf et al. (1974). The 40–50 mg of protein obtained from a 500-mL culture was loaded onto a 1- × 9-cm cytochrome *c* affinity column, washed with 20 mL of buffer A (0.1 mM EDTA, 0.05 mM DTT, 10% glycerol, and 50 mM Tris at pH 7.7), then with 10 mL of 80 mM KCl in buffer A, and finally with 10 mL of 30 mM potassium phosphate in buffer A. P-450R (R597M) was eluted with 50 mM oxaloacetate [known to be a competitive inhibitor versus cytochrome *c* (Dombrowski et al., 1980)] in buffer A. P-450R (WT) was also purified on the cytochrome *c* affinity column in the same manner. Both proteins appeared fairly pure by SDS–PAGE, and comparison of the activity for P-450R (WT) purified on the cytochrome *c* column with that for P-450R (WT) purified on the 2′,5′-ADP affinity affinity column indicates a relative purity of 59%, based on specific activities. This serves as a rough estimate of the purity of the P-450R (R597M) purified on the cytochrome *c* affinity column.

The concentration of total protein after purification of P-450R (R597M) on the cytochrome *c* affinity column was determined using the assay of Lowry et al. (1951). The concentration of P-450R (R597M) was determined by generating a standard curve from the band intensities obtained from the densitometric analysis of a Western blot containing 5 lanes of increasing concentrations (30 → 65 ng) of P-450R (WT) that had been purified to homogeneity on a 2′,5′-ADP affinity column. This blot also contained 6 lanes of the P450R (R597M) purified on the cytochrome *c* affinity column, whose concentration was determined using this standard curve. Comparison of the concentration of P-450R (R597M) obtained from the densitometric analysis of this Western blot and the total protein concentration obtained from the Lowry assay gives a purity of 44%. All activity measurements were therefore corrected for this level of purity, as were calculations of flavin content.

In order to determine if there were any competing cytochrome *c* reductase activities in this protein preparation [other than from P-450R (R597M)], the protein was immunoprecipitated, and residual activity in the supernatant was measured. P-450R (WT or R597M), at 25 μg/mL, was incubated in 1 mL of a 10% glycerol/0.1 M potassium phosphate solution at pH 8.0, with ca. 10^{-6} titer of purified rabbit anti-P-450R or, as a control, rabbit anti-epoxide hydrolase. The solution was incubated 3 h at 5 °C, at which point 20 μL of a protein A suspension [a 10% solution of essentially nonviable *Staphylococcus aureus* cells (Sigma)] was added, and the immunoprecipitate was removed by centrifugation. The activity in the supernatant was then assayed with the standard activity assay (described later).

The flavin content of P-450R (R597M) was determined using the spectrofluorometric assay of Faeder and Siegel (1973). The FAD and FMN used to generate the standard curve for this assay were purified using HPLC, on a Spherogel TSK DEAE-SPW column, and eluting with a linear gradient of 120–800 mM NaCl in 50 mM Tris at pH 8.0, as described by Porter et al. (1987). Flavin was removed from P-450R (R597M) for the assay by heating at 100 °C for 5 min in the dark and then centrifuging to remove protein.

Urea Stability Study. The effect of incubation with urea on protein stability was determined for both the WT and mutant enzymes after purification on the cytochrome *c* affinity column. P-450R (WT or R597M) was incubated for 2 h at 25 °C in a 0.1 M TAPS buffer at pH 8.0, containing 0–3.5 M urea (prepared fresh daily), and enough KCl to give an ionic strength of 300 mM. Protein activity, expressed as a percentage of that in the absence of urea, was determined using the standard activity assay (described below). This assay is unaffected by the presence of up to 0.1 M urea, which is more than the amount of urea that was introduced in the assay of the most concentrated (3.5 M) reaction. This standard activity assay, used also to measure P-450R (WT) activity after purification on the two affinity columns, and to measure residual P-450R (WT and R597M) activity in the immunoprecipitation study, contained 0.1 M potassium phosphate at pH 8.0, 80 μM cytochrome *c* (ferric), and 0.5 mM NADPH, in addition to P-450R, and was carried out at 25 °C.

^{31}P-NMR Characterization of P-450R (WT and R597M). P-450R (WT and R597M) was purified on a cytochrome *c* affinity column, concentrated using a collodian apparatus, and dialyzed against a 0.1 M Tris buffer, pH 7.7. Experiments were performed at 15 °C using a 10-mm tube containing 1.7 mL of sample. The samples contained 0.20 μM WT or 0.14 μM R597M P-450R, 10% D_2O, and 0.1 M Tris at pH 7.7.

Table I: Kinetic and Inhibition Constants for Nicotinamide Nucleotides with WT and R597M Mutant P-450R[a]

substrate[b] 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)
		WT		
NADPH(cytc)	5.09 ± 0.30	1,860 ± 100	2.74 ± 0.25	
cytc(NADPH)	5.09 ± 0.30	312 ± 12	16.4 ± 1.6	
NADH(cytc)	0.928 ± 0.016	0.0800 ± 0.0024	11600 ± 600	
$NADP^+$				5.1 ± 1.1
NAD^+				14,200 ± 4,700
		R597M		
NADPH(cytc)	6.34 ± 0.33	15.8 ± 0.6	401 ± 31	
cytc(NADPH)	6.34 ± 0.33	283 ± 12	22.5 ± 2.1	
NADH(cytc)	0.522 ± 0.011	0.0536 ± 0.0020	9740 ± 540	
$NADP^+$				1220 ± 210
NAD^+				40900 ± 9,200
		R597M/WT Ratios		
NADPH(cytc)	1.2	8.5×10^{-3}	150	
cytc(NADPH)	1.2	0.91	1.4	
NADH(cytc)	0.56	0.67	0.84	
$NADP^+$				240
NAD^+				2.9

[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. [b] The other substrate is indicated in parentheses.

Spectra were obtained with a Bruker AM500 spectrometer operating at 202.46 MHz, using a $\pi/4$ observation pulse, and broad-band proton decoupling. Chemical shifts were referenced to 85% H_3PO_4.

Kinetic Assays. Kinetic studies were carried out at 25 °C in 1-cm curves in a 1-mL volume, by measuring absorbance changes at 550 nm, from the reduction of cytochrome *c* ($\Delta\epsilon$ = 21.1 mM^{-1} cm^{-1}) (Gelder & Slater, 1962). Reaction mixtures contained 100 mM of the appropriate buffer, enough KCl to give an ionic strength of 850 mM, varied levels of cytochrome *c* (from 0.2 to 5 times its K_m), varied levels of NADPH or NADH (from 0.2 to 5 times their respective K_ms), and P-450R. In the inhibition studies, the competitive inhibitor, $NADP^+$ or NAD^+, was present at 0, 0.75, and 2 times its K_i value, NADPH was varied from 0.2 to 5 times its K_m, and cytochrome *c* was present at 30 μM. To determine how much KCl had to be added to achieve an ionic strength of 850 mM, the ionic strength of a given reaction mixture was calculated using the following pK_a values: MES (6.02), TAPS (8.19), and CHES (9.23) (Ellis & Morrison, 1982); NADPH (5.91) and $NADP^+$ (5.81) (preceding paper in this issue). The pK_i and $(V/K)_{NADPH}$ pH profiles could not be extended above pH 10.0 or below pH 5.6 since the enzyme is too unstable, and at lower pH such high substrate/product concentrations are needed in the assays, due to the decreased binding affinity, that nonspecific binding effects are observed. Activity of P-450R stock solutions was assayed and corrected for denaturation and flavin loss as described in the preceding paper (Sem & Kasper, 1993).

Data Processing. Data were fitted using modified versions of the FORTRAN programs of Cleland (1979). The fit to equations in log form assumes constant proportional error in the measured parameter (Y). Error bars corresponding to 1 standard deviation are shown in the pK_i $(V/K)_{NADPH}$ pH profiles (if not visible, the error is less than the radius of the point). The data from the initial velocity studies of R597M (Figure 4A) and WT (Figure 4B) P-450R fit best to the equation for a 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 two substrate (NADPH and cytochrome *c*) concentrations, and K_A and K_B are their corresponding Michaelis constants. The fit to eq 1 was used to calculate the V_{max}, (V/K), and K_m values for P-450R (WT and R597M) shown in Table I. The K_i values in Figure 5A ($NADP^+$) and Table I (NAD^+ and $NADP^+$) were obtained from a fit to eq 2 for competitive inhibition:

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

where K_i is the competitive inhibition constant, I is the inhibitor ($NADP^+$ or NAD^+) concentration, and A is the concentration of the varied substrate (NADPH). The pK_i profile for $NADP^+$ acting as a competitive inhibitor of P-450R (R597M) (Figure 5A) was fitted to eq 3 for a model assuming one acidic group that must be unprotonated for binding:

$$\log (Y) = \log \frac{Y_0}{(1 + H/K_1)} \quad (3)$$

where K_1 is the acid dissociation constant of this acidic group, Y is $1/K_i$, and Y_0 is the value of Y when this acidic group is fully unprotonated. $(V/K)_{NADPH}$ values in the pH profile shown in Figure 5B were obtained from a fit to eq 4, with the nonvaried substrate (cytochrome *c*) present at the subsaturating level of 30 μM:

$$\log (Y) = \log \frac{V_{max}A}{K_m + A} \quad (4)$$

where Y is the initial velocity and A is the substrate (NADPH) concentration. Although cytochrome *c* could not be maintained at saturating levels due to substrate inhibition, the level of cytochrome *c* does not affect $(V/K)_{NADPH}$, since the mechanism is ping-pong at 850 mM ionic strength (unpublished results). The $(V/K)_{NADPH}$ pH profile (Figure 5B) was fitted to eq 5 for a model assuming one acidic group that is preferentially unprotonated and one basic group that is preferentially protonated:

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

where K_1 and K_2 are the acid dissociation constants for the acidic and basic groups, respectively, Y is $(V/K)_{NADPH}$, and Y_0 is the value of Y when both groups are in their preferred ionization states.

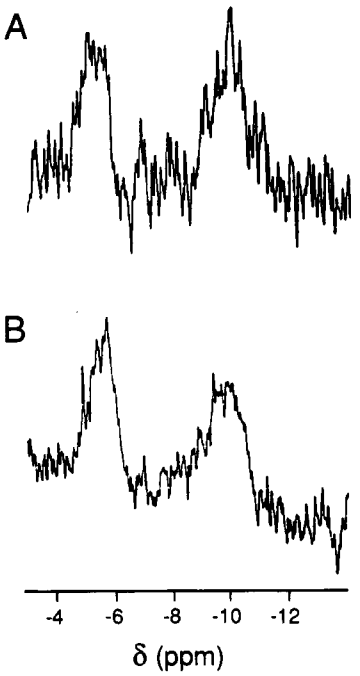


FIGURE 2: ^{31}P-NMR spectra of P-450R, at pH 7.7 and 15 °C, showing the FAD pyrophosphate resonances for (A) R597M and (B) WT proteins, both purified on a cytochrome *c* affinity column. The spectrum in (A) is the average of 35 855 transients, while that in (B) is the average of 40 098 transients. A line broadening of 10 Hz was used for the exponential multiplication in both spectra.

RESULTS

Purification of P-450R (R597M) and Determination of Purity. The 40–50 mg of total protein obtained from a 500-mL culture of *E. coli* [C-1A, containing the expression plasmid pOR263(R597M)] had to be purified on a cytochrome *c* affinity column, since it had no affinity for the 2′,5′-ADP–Sepharose resin. Although the protein was 44% pure on the basis of a comparison of Lowry assays and immunoassays, immunoprecipitation experiments showed that the observed cytochrome *c* reductase activity was due only to P-450R. Cytochrome *c* reductase activity was completely (>98%) eliminated upon immunoprecipitation with rabbit anti-P-450R but was not affected (±3%) by the control treatment with rabbit anti-epoxide hydrolase. Thus, there are no competing cytochrome *c* reductase activities in this protein preparation. The activity that is present is due only to P-450R.

Structural Characterization of P-450R (R597M). The structure of the P450R gene [in pOR263(R597M)] was changed only at the desired point of mutation: CGG → ATG. The tertiary structural consequences of this mutation were assessed qualitatively with ^{31}P-NMR. The FMN resonance was obscured by the large phospholipid resonance (not shown), which has a chemical shift similar to that for FMN (Narayanasami et al., 1992). The pyrophosphate of FAD bound to R597M (Figure 2A) or WT (Figure 2B) P-450R has resonances at −5.5 and −10.0 ppm, while that for free FAD has resonances at −9.9 and −10.6 ppm (Otvos et al., 1986).

The flavin content of P-450R (R597M) was measured with the spectrofluorometric assay of Faeder and Siegel (1973). The FMN content is 62% and the FAD content is 87%, while the FAD and FMN content of P-450R (WT) is usually >80–90%. But, a 1-h incubation of 2 μM P-450R (R597M) with 4 μM FMN in buffer A, followed by overnight dialysis against buffer A, increases the FMN/FAD ratio from 0.71 to 0.83, closer to the value of ca. 0.90–1.0 observed for P-450R (WT).

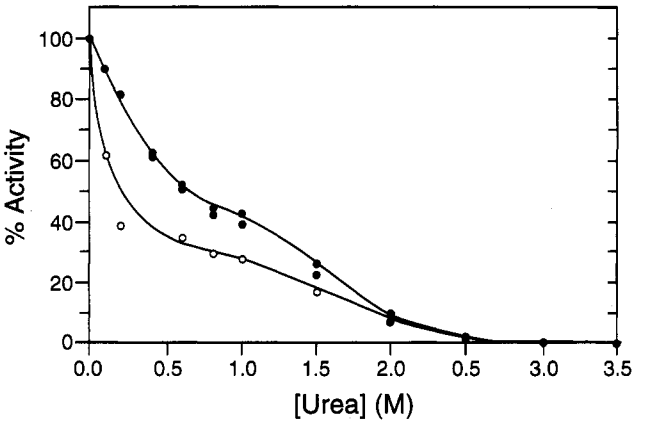


FIGURE 3: Urea denaturation curves for WT (●) and R597M (○) P-450R at pH 8.0, 25 °C, and an ionic strength of 300 mM. Half-maximal activity was observed at 0.22 M urea for R597M and 0.64 M urea for WT P-450R. Activity is the initial velocity for cytochrome c^{3+} reduction, obtained using the standard activity assay, and is expressed as a percentage of that in the absence of urea. Since electron transfer to cytochrome c^{3+} is measured, any decrease in activity will reflect unfolding and/or flavin loss (which is itself probably a result in partial unfolding). The curves shown are hand-drawn.

The relative stabilities of WT and R597M P-450R were compared by monitoring their degree of unfolding, quantitated using the standard activity assay, as a function of increasing urea concentration. Since activity is used as a measure of protein unfolding, any unfolding that has an adverse effect on substrate binding or on the electron transfer reaction will affect the urea stability profile (Figure 3). The profiles for both WT and R597M P-450R appear biphasic, indicating a minimum of two steps in the unfolding process, before activity is completely lost. The initial decrease in activity is probably a result of FMN loss, since it is bound less tightly than FAD and is known to dissociate at these lower urea concentrations (Masters et al., 1992; Narayanasami et al., 1992). P-450R (R597M) is more unstable to urea denaturation, with half-maximal activity at 0.22 M urea, in comparison with P-450R (WT), which has a half-maximal activity at 0.64 M urea. This tendency to lose FMN more readily in the R597M mutant is consistent with the low FMN content observed for this mutant protein after purification and may represent a lower stability of the folded state of P-450R relative to the unfolded state, as a result of the R597M mutation.

Functional Characterization of P-450R (R597M). (*a*) *Steady-State Kinetic Mechanism and Constants.* The double-reciprocal plots of initial velocity versus NADPH concentration, at four fixed levels of cytochrome *c* concentration, show parallel line patterns for R597M (Figure 4A) and WT (Figure 4B) P-450R. These patterns are consistent with a ping-pong mechanism for both WT and R597M P-450R (although the evidence for a ping-pong mechanism is more extensive for P-450R(WT) (unpublished results)]. The mutation has not altered the kinetic mechanism, thus allowing us to obtain the kinetic constants k_{cat}, k_{cat}/K_m, and K_m for both enzymes using the same equations (eqs 1 and 4). The kinetic constants for WT and R597M P-450R are summarized in Table I, along with the *x*-fold change in these constants as a result of the R597M mutation. With NADPH and cytochrome *c* as substrates, k_{cat} is not affected by the mutation, while $(k_{cat}/K_m)_{NADPH}$ is decreased 120-fold and K_m(NADPH) is increased 150-fold. In contrast, $(k_{cat}/K_m)_{cytc}$ and K_m(cytc) are only affected slightly by the mutation. So the mutation affects NADPH but not cytochrome *c* binding. Furthermore, when NADH is the substrate instead of NADPH, only minor changes are seen in $(k_{cat}/K_m)_{NADH}$ and K_m(NADH) as a result

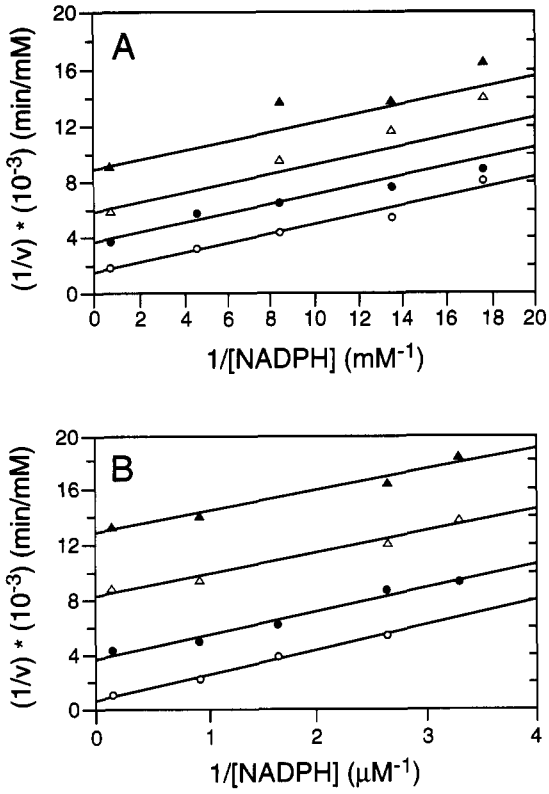


FIGURE 4: Double-reciprocal plots of initial velocities as a function of NADPH concentration, with each line representing a different cytochrome *c* concentration. The experiment was carried out at pH 8.0, 25 °C, and an ionic strength of 850 mM, and NADPH and cytochrome *c* were both varied from 0.2 to 5 times their respective K_m values. The plots for (A) R597M and (B) WT P-450R represent the fit of the data to eq 1. Open and filled circles and open and filled triangles represent decreasing concentrations of cytochrome c^{3+}, respectively (29.6, 5.14, 2.46, and 1.39 μM).

of the R597M mutation. Hence, the mutation has a much larger effect with NADPH as substrate than with NADH, which lacks the 2′-phosphate of NADPH. Results consistent with these were obtained with the product inhibitors $NADP^+$ and NAD^+. The competitive inhibition constant for $NADP^+$ is increased 240-fold due to the mutation, while that for NAD^+ is increased only 2.9-fold due to the mutation (Table I). The larger effect from the R597M mutation is therefore seen with $NADP^+$, which differs from NAD^+ only in that it has a 2′-phosphate.

(b) Chemical Mechanism Probed with pH Studies. To characterize the binding interactions between $NADP^+$ and P-450R (R597M), the effect of pH on K_i, the inhibition constant for $NADP^+$, was determined (Figure 5A). This pK_i profile will give true thermodynamic pK_a values, and only for groups that are involved in binding interactions between $NADP^+$ and P-450R. pK_i decreases at low pH as a group with a pK_a of 5.78 ± 0.06 is protonated, but shows no decrease at high pH like that observed in the P-450R (WT) profile (preceding paper in this issue). The group with a pK_a of 5.78 is probably the 2′-phosphate of $NADP^+$, which is known to ionize with a pK_a of 5.81 (preceding paper in this issue), while the group with a pK_a of 9.5, observed in the P-450R (WT) profile, is not present in this profile.

To further characterize the enzyme–substrate binding interactions between NADPH and P-450R (R597M), the effect of pH on $(V/K)_{NADPH}$ was determined (Figure 5B). This $(V/K)_{NADPH}$ profile will provide information on groups that are involved in binding and/or catalysis. Furthermore, since NADPH binds to P-450R (R597M) with such low

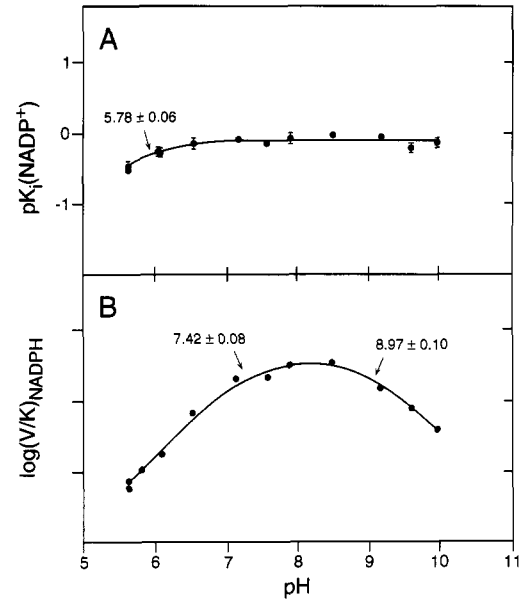


FIGURE 5: (A) Effect of pH on K_i, the inhibition constant for $NADP^+$, which is a competitive inhibitor versus NADPH in the P-450R-(R597M) reaction. The ionic strength was 850 mM and cytochrome *c* was present at 30 μM. The data were fitted to eq 3. pK_i decreases at low pH as a group with a pK_a of 5.78 ± 0.06 is protonated. K_i is in units of millimolar. (B) Effect of pH on log $(V/K)_{NADPH}$ for P-450R(R597M), with cytochrome *c* acting as the other substrate (present at 30 μM), at an ionic strength of 850 mM. Data were fitted to eq 5. $(V/K)_{NADPH}$ decreases at low pH as an acidic group with a pK_a of 7.42 ± 0.08 is protonated and also decreases at high pH as a basic group with a pK_a of 8.97 ● 0.10 is deprotonated. $(V/K)_{NADPH}$ has arbitrary units, with each demarcation on the *y*-axis representing one log unit [a 10-fold change in $(V/K)_{NADPH}$].

affinity (K_m is increased 150-fold relative to WT), the pK_as in this profile will be true thermodynamic pK_as, unperturbed by stickiness effects. Log $(V/K)_{NADPH}$ decreases below a pK_a of 7.42 ± 0.08 with a slope of 1 due to protonation of a group that must be unprotonated for activity and above a pK_a of 8.97 ± 0.10 with a slope of −1 due to deprotonation of a group that must be protonated for activity. Although the pK_a of 5.78 observed in the pK_i profile would be expected to show up in this $(V/K)_{NADPH}$ profile, there is not enough low-pH data to define this pK_a with confidence (a fit assuming a second acidic pK_a did give a value of 5.2 ± 0.3, though, which is within 2 standard deviations of 5.78).

DISCUSSION

Selection and Construction of the R597M Mutation of P-450R. P-450R has very strong specificity for its nicotinamide nucleotide substrate, binding NADPH 4200 times more tightly than NADH. The binding interactions between P-450R and NADPH that are responsible for this specificity must be with the 2′-phosphate, which is absent on NADH. Previous studies have indicated that the 2′-phosphate is bound as the dianion and that removal of it leads to a loss of 5.0 kcal/mol of uniform binding energy, resulting from the disruption of all interactions between it and the enzyme (preceding paper in this issue). Since the purpose of this study is to begin defining these enzyme groups and their contributions to substrate specificity using site-directed mutagenesis, information correlating protein primary structure and binding of the 2′-phosphate was needed. The crystal structure of the FNR·2′-phospho-AMP complex (Figure 1A) shows the interactions between FNR and the 2′-phosphate of 2′-phospho-AMP, which may also occur in the FNR·$NADP^+$ complex, which was not crystallized (Karplus et al., 1991).

The region of the FNR primary structure involved in this interaction shows strong homology with P-450Rs from a number of sources, along with other dehydrogenase/electron transferases (Figure 1B). Since the four residues of FNR involved in interactions with the 2′-phosphate (Arg235, Ser234, Tyr246, and Lys244) are completely conserved, we hypothesized that the residues of P-450R which are homologous to these four might be involved in binding interactions with the 2′-phosphate of NADPH in P-450R. Chemical modification studies have also implicated basic residues (Lys and Arg) in this interaction in P-450R (Inano & Tamaoki, 1986; Slepneva & Weiner, 1988; Lumper et al., 1980), one of which [Lys601 of porcine P-450R (Muller et al., 1990)] is homologous to Lys602 of rat P-450R. Since arginine is positively charged and therefore capable of stronger interactions with the negatively charged 2′-phosphate than Ser234 and Tyr246, and since the role of Lys244 of FNR is questionable (being more distant from the 2′-phosphate in the FNR structure), this study has focused on the role of Arg597 of P-450R, which is homologous to Arg235 of FNR. Future studies will address the role of the remaining three residues.

Structural Consequences of the R597M Mutation. The structural perturbation introduced in the R597M mutant P-450R is effectively only the removal of the guanido group of Arg597, since the $-CH_2CH_2SCH_3$ of methionine should be fairly isosteric with the $-CH_2CH_2CH_2NH-$ of arginine. The ^{31}P-NMR spectra of FAD bound to R597M and WT P-450R (Figure 2, panels A and B, respectively) have been obtained as a probe of gross structural changes in the FAD binding pocket, which is probably in close proximity to the NADPH binding pocket [since electrons are transferred from NADPH to FAD (Vermilion et al., 1981)]. These spectra show pyrophosphate resonances at −10.0 and −5.5 ppm for both WT and R597M P-450R.[2] Since ^{31}P-NMR chemical shifts are very sensitive to environment, they are a good qualitative measure of gross conformational/environmental changes in the vicinity of the phosphorus nuclei. The fact that free FAD has pyrophosphate resonances at −10.6 and −9.9 ppm (Otvos et al., 1986) implies a large change in environment for one of the pyrophosphate phosphorus nuclei upon binding of FAD to P-450R and demonstrates the sensitivity of ^{31}P-NMR chemical shifts to such changes. But, since the chemical shifts for the pyrophosphate of FAD are the same in the WT and R597M P-450R complexes, the environment around them must be similar in both proteins, implying that no gross conformational change has occurred. Although this is only cursory evidence that the structure of P-450R has not been greatly disrupted by the R597M mutation, functional evidence will be presented later that supports this conclusion.

The urea denaturation curves for WT and R597M P-450R (Figure 3) have a biphasic shape, due probably to the loss of FMN followed by FAD (Masters et al., 1992; Narayanasami et al., 1992), as the protein unfolds. The curve for P-450R (R597M) is shifted to lower urea concentration relative to P-450R (WT) such that the concentration of urea giving half-maximal activity is shifted from 0.64 to 0.22 M, indicating that removal of Arg597 has decreased the stability of the folded state relative to the unfolded state. It appears that FMN is lost more readily in P-450R (R597M), consistent with the low FMN content (62%) of this protein after purification. Although this mutation has apparently decreased the stability of the folded state, due to introduction of unfavorable interactions or removal of favorable interactions, this does not necessarily mean that structure has been disrupted. Indeed, functional evidence will be presented that is consistent with little disruption of the tertiary structure due to the R597M mutation, as suggested by the ^{31}P-NMR results.

Functional Consequences of the R597M Mutation: Kinetic Characterization. (a) Kinetic Mechanism and Constants. Initial velocity studies indicate that the R597M mutation has not altered the kinetic mechanism of P-450R (Figure 4), which still appears to be ping-pong for P-450R (R597M). This allows for the determination of the kinetic constants for both proteins using the same equations (eqs 1 and 4). The kinetic results for WT and R597M P-450R, summarized in Table I, show a 150-fold increase in K_m for NADPH due to the mutation, with no significant effect on k_{cat}. Since K_m is a fair approximation of a binding constant [based on the observation of large ^DV and $^D(V/K)_{NADPH}$ isotope effects (unpublished results)], one can conclude that the R597M mutation affects only binding (uniform binding) and not catalysis (catalysis of elementary steps). To see if Arg597 interacts specifically with the 2′-phosphate of NADPH, and not with other parts of this molecule, NADH was used as an alternate substrate. With NADH as substrate, little effect is seen on K_m (0.8WT) and k_{cat} (0.6WT). Thus, Arg597 interacts specifically with the 2′-phosphate of NADPH. Similar results were obtained with product inhibitors $NADP^+$ and NAD^+. Specifically, the R597M mutation decreases binding affinity for $NADP^+$ 240-fold but decreases affinity for NAD^+ a relatively modest 2.9-fold.

(b) Functional Evidence Consistent with the Lack of a Globally Disruptive Structural Change. Since structural information on P-450R (WT or R597M) is scant, it is necessary to exercise caution in interpreting the functional consequences of a structural perturbation, because the nature of the perturbation is not clearly defined. It is therefore important to make use of functional information that helps to define the extent of a structural perturbation. The large decrease in binding affinity for NADP(H), coupled with the small effect seen on NAD(H) affinity, is strong evidence that removal of Arg597 disrupts interactions *locally* with the 2′-phosphate but has little effect on *proximal* binding interactions with the remainder of the NADP(H) molecule. Furthermore, since the K_m for cytochrome *c*, which binds at a separate site on P-450R (Nisimoto & Otsuka-Murakami, 1988; Porter & Kasper, 1986), is affected very little by the R597M mutation (1.4WT), the mutation must not create a *global* disruption of structure. Thus, kinetic results with additional substrates and alternate substrates/inhibitors can provide information on how disruptive a mutation is: *locally*, *proximally*, or *globally*.

(c) pH Studies. Just as removing functional groups on substrate or enzyme can disrupt binding interactions, so can changes in pH if protonation/deprotonation of the group(s) involved in the interaction decreases the strength of the interaction. The pK_as for these ionizable groups can be obtained from an analysis of the pH variation of the kinetic constants [V_{max}, (V/K), and $1/K_i$], but they will no longer be observed in the pH profile if the ionizable group has actually

[2] Although the ^{31}P-NMR chemical shifts for WT and R597M P-450R (−5.5 and −10.0 ppm) differ from those previously reported [−7.3 and −11.3 ppm (Otvos et al., 1986)], the difference could reflect the purification used. Resonances at −5.5 and −10.0 ppm have been reported for the cloned rat liver reductase, in addition to the usual two FAD resonances (Narayanasami et al., 1992). So, the purification on the cytochrome *c* affinity column and concentration with the collodian apparatus may somehow be selecting for the "conformation" of P-450R yielding the −5.5 and −10.0 ppm resonances. In any case, the important point is that the ^{31}P-NMR resonances for the FAD bound to WT and R597M P-450R have essentially the same chemical shifts.

been removed from the enzyme or substrate. The pH variation of $1/K_i$ for $NADP^+$ (Figure 5A) shows a decrease in affinity as the pH drops below a pK_a of 5.78 (for the 2′-phosphate of $NADP^+$). The group with a pK_a of 9.5 observed in the profile for P-450R (WT) (preceding paper in this issue) is no longer present in the P-450R (R597M) pK_i profile, consistent with this ionization having been for Arg597. Although the apparent loss of this group with a pK_a of 9.5 could be the result of a mere perturbation of another group's pK_a upon removal of Arg597, it would have to be an extremely local effect in light of the kinetic results with NAD(H) and cytochrome *c* that argue against *proximal* and *global* perturbations, respectively, and the ^{31}P-NMR results that argue against a *global* perturbation. That is, if Arg597 is not the group with a pK_a of 9.5, it must be sufficiently close in space to the group with a pK_a of 9.5 that it can perturb this group's pK_a, and also bind the 2′-phosphate of NADP(H), yet not affect binding interactions with the rest of the NADP(H) molecule, with cytochrome *c*, or with the FAD pyrophosphate. It is more reasonable that the group with a pK_a of 9.5 is Arg597. This pK_a is perturbed 2–3 pH units lower than that expected for arginine in an aqueous environment (Creighton, 1984). Such large perturbations for basic amino acid pK_as are not without precedent: lysyl ϵ-amino groups in acetoacetate decarboxylase and *O*-acetylserine sulfhydrolase have pK_as of 5.9 (Schmidt & Westheimer, 1971) and 8.1 (Cook et al., 1992) respectively. However, little is known regarding such perturbations for arginyl guanido groups. It is somewhat surprising that the 2′-phosphate of $NADP^+$ still appears to be binding preferentially as the dianion, as suggested by the presence of the pK_a of 5.78 in the $NADP^+$ pK_i profile. The 2′-phosphate binding site of P-450R must still be capable of stabilizing the 2′-phosphate with a −2 charge on it to a greater extent than that with a −1 charge, even in the absence of the positive charge on Arg597, which clearly interacted with the 2′-phosphate. This suggests there is a high positive charge density, possibly greater than +2, in the 2′-phosphate binding site of P-450R (WT).

The $(V/K)_{NADPH}$ profile (Figure 5B) shows an acidic group with a pK_a of 7.42 that is preferentially unprotonated and a basic group with a pK_a of 8.97 that is preferentially protonated. Since NADPH is not sticky with mutant P-450R (since binding affinity for NADPH is so low), these pK_as are true thermodynamic pK_as (Cleland, 1982). They are most likely for the same two catalytic groups with pK_as in the V_{max} and (V/K) profiles for P-450R (WT) (preceding paper in this issue). The pK_as for these acidic and basic groups are perturbed upward 0.5–0.6 pH unit relative to those of 6.9 and 8.4 observed in the $(V/K)_{NADPH}$ profile for P-450R (WT) (preceding paper in this issue). Although the perturbation of the acidic pK_a could be the result of a decrease in substrate stickiness (Cleland, 1982), the "outward perturbation" of the basic pK_a must be due to an actual change in the environment around this group, possibly resulting from a partial disruption of the interaction with the 2′-phosphate, suggested to play a role in selecting the conformation of P-450R bound by substrate, thereby affecting the environment around this group which is near the FMN binding site (preceding paper in this issue).

Thermodynamic Model Describing Interactions between Arg597 and the 2′-Phosphate. There are three major classes of enzyme–substrate binding interactions (Albery & Knowles, 1976): uniform binding (where all intermediates and transition states are bound with equal affinity), differential binding (where some intermediates are bound more tightly than others), and catalysis of elementary steps (where a transition state is bound preferentially). One would expect disruption

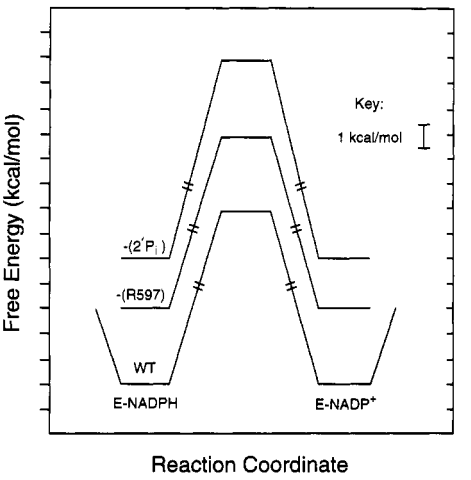


FIGURE 6: Free energy profile showing the changes in free energy (at 25 °C) for the P-450R·NADPH and P-450R·$NADP^+$ ground-state intermediates, and the transition state intermediate for hydride transfer, as a result of removing the substrate/product 2′-phosphate (−2′P_i), or the enzyme guanido group of Arg597 (−Arg597). Only internal (enzyme-bound) states are shown, thus avoiding the need for specifying standard-state substrate or product concentrations, and the height of the hydride transfer transition-state barrier is not known. Changes in ground- and transition-state binding energies are based on data from Table I of the Appendix. E is P-450R.

of interactions involved in uniform binding to affect predominantly K_m and k_{cat}/K_m, disruption of interactions involved in catalysis of elementary steps to affect predominantly k_{cat} and k_{cat}/K_m, and disruption of interactions involved in differential binding to give mixed affects (Fersht, 1985; Albery & Knowles, 1976). Our studies (preceding paper in this issue) have shown that P-450R uses 5 kcal/mol predominantly uniform binding energy to bind the 2′-phosphate of NADP(H), with an additional 1 kcal/mol of binding energy used to stabilize the transition state (catalysis of elementary steps). The thermodynamic consequences of removing the 2′-phosphate, and therefore all interactions with it, are shown in the free energy profile in Figure 6. Removal of Arg597 from the enzyme leads to a less of 3 kcal/mol uniform binding energy. That is, removal of this one interaction destabilizes the enzyme–substrate, product, and transition-state complexes to the same degree. Hence, large effects are seen on K_m(NADPH) and K_i($NADP^+$), but k_{cat} is unaffected since ground and transition states are destabilized to the same degree. The thermodynamic consequences of removing Arg597 are also summarized in Figure 6. The binding energy of 3 kcal/mol for the salt bridge made by Arg597 of P-450R is similar to the values reported for other enzymes: 2.9 kcal/mol for an amino group in chymotrypsin (Fersht, 1972), 4.3 kcal/mol for an amino group in tyrosyl-tRNA synthetase (Santi & Pena, 1973), and 2.2 kcal/mol for an amino group in thrombin (Stone et al., 1991). Arg597 represents only one of what may be many interactions with the 2′-phosphate, and yet removal of it leads to a loss of 60% of the total binding energy of the 2′-phosphate group. This estimate is probably an upper limit since removal of Arg597 may actually be disrupting other *local* interactions with the 2′-phosphate group. But, since the effect is so large, and since this mutation has been shown to be nondisruptive in a *global* or *proximal* sense, we conclude that Arg597 plays a major role in binding the 2′-phosphate of NADP(H). An important role for lysyl and arginyl residues in binding the 2′-phosphate of NADP(H) has also been established for dihydrofolate reductase (Huang et al., 1990) and glutathione reductase (Scrutton et al., 1990), respectively, using site-directed mutagenesis. The strong specificity that P-450R has for NADPH over NADH is due in large part to a strong

uniform binding interaction with Arg597 and a binding pocket with a high positive charge density. Since charge–charge interactions in a nonpolar environment are some of the strongest noncovalent binding interactions, it makes sense that P-450R should have a binding site that selectivity binds the 2′-phosphate with a −2 charge on it, rather than with a −1 charge. This affinity for the charge phosphate could be made even stronger by having a binding site with a positive charge density greater than +2, which appears to be the case in P-450R.

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APPENDIX: THERMODYNAMIC INTERPRETATION OF COMBINED ALTERNATE SUBSTRATE/INHIBITOR, pH, AND SITE-DIRECTED MUTAGENESIS STUDIES

The approach we have taken combines perturbations to the substrate and to the enzyme using alternate substrate/inhibitor, pH, and site-directed mutagenesis studies. Since this approach is very informative when studying complementary interacting groups on an enzyme and substrate, a discussion of its general usefulness, along with a thermodynamic description of the expected changes in binding energies, is presented. If an enzyme group R_i is thought to be involved in a binding interaction with a group P on substrate S, then removal of R_i from the enzyme should affect the binding of S–P. If it affects the binding of a substrate that binds at another site, then the mutation is *globally disruptive*. If it affects the binding of substrate S after group P has been removed from it, then the mutation has been *proximally disruptive*. If the mutation affects only bridging of S–P, but not S, then it is *locally disruptive*. Furthermore, if either group P or R_i is an ionizable group, then it should have a pK_a that will show up in pK_i [or V_{max} or (V/K)] profiles, provided they ionize in the experimentally accessible pH range. When the ionizable group is removed from the enzyme or substrate, its pK_a will no longer be present in the profile, allowing assignment of the pK_a to a specific group and confirming its functional role in binding (or catalysis).

A discussion of the thermodynamic equations that describe the interactions between one group (P) on substrate S and n groups (R_1, R_2, ..., R_n) on enzyme E, is given below. The binding energy for group P ($\Delta\Delta G_P^*$) is given by

$$\Delta\Delta G_P^* = \sum_{i=1}^{n} (\Delta\Delta G_{R_i}^*) \quad (1)$$

where $\Delta\Delta G_{R_i}^*$ is the binding energy of group R_i on the enzyme.[1] The asterisk indicates a true binding energy, which is used to stabilize a ground- or transition-state intermediate. $\Delta\Delta G$ terms lacking an asterisk represent calculated binding energies, based on changes in K_m, K_i, or k_{cat}/K_m. The net observable effect of removing group R_j (for $i = j$) on binding affinity for substrate can be expressed as the sum of the *local disruption energy* ($\Delta\Delta G_{R_j}^* + IE_j$) and the *proximal disruption energy* (PD_j), from the mutation, given by:

$$\Delta\Delta G_{R_j} = \Delta\Delta G_{R_j}^* + IE_j + PD_j \quad (2)$$

which is a combination of the binding energy for group R_j ($\Delta\Delta G_{R_j}^*$), and the thermodynamic effect of *locally* disrupting the $n - 1$ neighboring interactions with group P (the interaction energy, IE_j) in addition to *proximally* disrupting interactions with the rest of S (the *proximal disruption energy*, PD_j).[2] The presence of this interaction energy term is often blamed for the lack of additivity when mutations are combined:

$$\Delta\Delta G_P \neq \sum_{i=1}^{n} (\Delta\Delta G_{R_i}) \quad (3)$$

[1] This is the incremental binding energy for the group R_i that has been removed from the enzyme (Fersht et al., 1980). It is not a true binding energy, though. Rather, it is an apparent binding energy for group R_i that has been corrected for the effects of disruption of other *local* and *proximal* interactions between enzyme and substrate [contained in the reorganization energy term discussed by Wells et al. (1991)]. It differs from the true binding energy for group R_i because of changes in the solvation state of the active site upon removal of R_i (Wells et al., 1991).

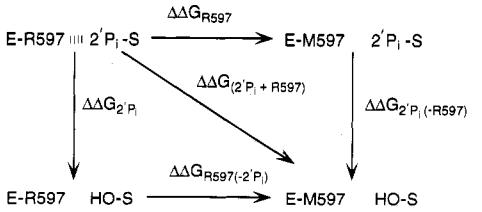

FIGURE 1: Thermodynamic box describing the effects of removing the 2′-phosphate of NADP(H) and Arg597 of P-450R, separately and in combination. The change in binding energy resulting from the perturbation to enzyme or substrate structure is given by $\Delta\Delta G_{R597}$, $\Delta\Delta G_{2'P_i}$, or $\Delta\Delta G_{(2'P_i+R597)}$ for removal of R597, the 2′-phosphate, or both, respectively, $\Delta\Delta G_{2'P_i(-R597)}$ for removal of the 2′-phosphate in the absence of R597, and $\Delta\Delta G_{R597(-2'P_i)}$ for removal of R597 in the absence of the 2′-phosphate. $2'P_i$-S is NADP(H) and E is P-450R.

Table I: Thermodynamic Effects of Removing Arginine 597 of P-450R[a]

modification	$\Delta\Delta G_b$ (kcal/mol)	$\Delta\Delta G_b^{\neq}$ (kcal/mol)
	WT, $\Delta\Delta G_{2'P_i}$	
NADPH → NADH	5.0	6.0
$NADP^+ \rightarrow NAD^+$	4.7	
	R597M, $\Delta\Delta G_{2'P_i(-R597)}$	
NADPH → NADH	1.9	3.4
$NADP^+ \rightarrow NAD^+$	2.1	
	WT → R597M, $\Delta\Delta G_{R597}$	
NADPH	3.0	2.8
$NADP^+$	3.2	
	WT → R597M, $\Delta\Delta G_{R597(-2'P_i)}$	
NADH	−0.10	0.24
NAD^+	0.63	

[a] Free energy changes (at 25 °C) were calculated as described by Fersht (1985): $\Delta\Delta G_{2'P_i(\pm R597)} = -RT \ln (K_{NADP(H)}/K_{NAD(H)})$ or $\Delta\Delta G_{R597(\pm 2'P_i)} = -RT \ln (K_{WT}/K_{R597M})$, and $\Delta\Delta G_{2'P_i(\pm R597)}^{\neq} = RT \ln [(k_{cat}/K_m)_{NADPH}/(k_{cat}/K_m)_{NADH}]$ or $\Delta\Delta G_{R597(\pm 2'P_i)}^{\neq} = RT \ln [(k_{cat}/K_m)_{WT}/(k_{cat}/K_m)_{R597M}]$, where R is the gas constant and T is the temperature in kelvins.

which may be reasonable since one might expect PD to be small for most conservative mutations. The presence or absence of additivity when mutations are combined has been discussed and studied at length (Blacklow et al., 1991; Wells, 1990; Carter et al., 1984; Kuliopulos et al., 1990; Horovitz & Rigbi, 1985). This discussion pertains to the consequences of removing a group R_j on the enzyme separately and in combination with removal of a group P on the substrate (Figure 1), when R_j and P are complementary interacting groups. In the case of P-450R, R_j is Arg597 and $\Delta\Delta G_{R_j}$ = 3.0 kcal/mol for NADPH and 3.2 kcal/mol for $NADP^+$ (Table I), while P is the 2′-phosphate group of NADP(H) and $\Delta\Delta G_P$ = 5.0 kcal/mol for NADPH and 4.7 kcal/mol for $NADP^+$ (Table I). Henceforth, R_j will be referred to as Arg597 (or R597) and P as $2'P_i$.

The observed binding energy of Arg597 when the $2'P_i$ group is not present on NADP(H) is given by $\Delta\Delta G_{R597(-2'P_i)}$ (Figure 1), which is −0.10 kcal/mol for NADH and 0.63 kcal/mol for NAD^+ (Table I). This is much smaller than the 3.0–3.2 kcal/mol of binding energy observed for Arg597 when the $2'P_i$

[2] It has been assumed that the interaction energy and *proximal disruption energy* are both zero for removal of the $2'P_i$ group, so that $\Delta\Delta G_P = \Delta\Delta G_P^*$. It seems reasonable that IE_p should be zero since there are not likely to be other substrate interactions with R597. PD_p will also be close to zero, unless the NAD(H) molecule adopts a different orientation in the active site than NADP(H), thereby disrupting more remote interactions with residues in the active site.

group was present and is evidence that Arg597 interacts specifically with the 2′P_i group of NADP(H). In fact, any change in binding affinity when the 2′P_i group is absent must be due to disrupted interactions with the rest of the NADP(H) molecule, or *proximal* disruptions. Hence, the observed binding energy of Arg597, when the 2′P_i is absent, is given by

$$\Delta\Delta G_{R597(-2'P_i)} = PD_{R597} \quad (4)$$

where PD_{R597} is the *proximal disruption energy*, which should be close to zero if Arg597 is interacting with only the 2′P_i.

Finally, the observed binding energy of the 2′P_i group when Arg597 is not present on P-450R is $\Delta\Delta G_{2'P_i(-R597)}$ (Figure 1), which is 1.9 kcal/mol for NADPH and 2.1 kcal/mol for $NADP^+$ (Table I). This is considerably smaller than the 4.7–5.0 kcal/mol binding energy observed for the 2′P_i when Arg597 was present. This argues that although Arg597 was contributing much to the binding energy of the 2′P_i, there are still remaining interactions with the 2′P_i, the $n-1$ groups discussed earlier, possibly Ser596, Tyr604, and Lys602 on the basis of homology with FNR (for the sake of this discussion, it will be assumed that these are the only other groups interacting with the 2′P_i), providing 2 kcal/mol binding energy in the absence of Arg597. This is probably an underestimate of the contribution of these groups since it is decreased in magnitude by the interaction energy of Arg597 (see below). The observed binding energy of the 2′P_i group, in the absence of Arg597, is given by

$$\Delta\Delta G_{2'P_i(-R597)} = \Delta\Delta G_{2'P_i} - (\Delta\Delta G_{R597}{}^* + IE_{R597}) \quad (5)$$

which is obtained from eqs 2 and 4 and the thermodynamic box in Figure 1. If, for the sake of this discussion, the remaining $n-1$ groups are assumed to be Ser596, Tyr604, and Lys602, then this equation is equivalent to

$$\begin{aligned}\Delta\Delta G_{2'P_i(-R597)} &= [\sum_{i=1}^{n-1} (\Delta\Delta G_{R_i}{}^*)] - IE_{R597}\{R_i \neq R597\} \quad (6)\\ &= \Delta\Delta G_{S596}{}^* + \Delta\Delta G_{Y604}{}^* + \Delta\Delta G_{K602}{}^* - IE_{R597}\end{aligned}$$

which is obtained by substituting eq 1 into eq 5. So, $\Delta\Delta G_{2'P_i(-R597)}$ gives the sum of all the other binding energy contributions from the $n-1$ ($= 3$) remaining enzymic groups that interact with the 2′P_i, less the interaction energy of Arg597.

Mention should also be made that the combined removal of Arg597 and the 2′P_i is expected to be completely nonadditive, since the groups are involved in a binding interaction with each other. Specifically

$$\Delta\Delta G_{(2'P_i+R597)} \neq \Delta\Delta G_{R597} + \Delta\Delta G_{2'P_i} \quad (7)$$

but rather

$$\Delta\Delta G_{(2'P_i+R597)} = \Delta\Delta G_{2'P_i} + PD_{R597} \quad (8)$$

Thus, the effect of the combined change should differ from the effect of removing just the 2′P_i only to the extent that removing Arg597 disrupts interactions with the rest of the NADPH molecule.

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