Role of Ser457 of NADPH−Cytochrome P450 Oxidoreductase in Catalysis and Control of FAD Oxidation−Reduction Potential†

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Received March 11, 1996; Revised Manuscript Received May 15, 1996⊗

ABSTRACT: Site-directed mutagenesis of Ser457 of NADPH−cytochrome P450 oxidoreductase demonstrates that this residue plays a major role in both hydride transfer from NADPH to FAD and modulation of FAD redox potential. Substitution of Ser457 with alanine or cysteine decreases the rates of reduction of the substrates cytochrome *c* and potassium ferricyanide approximately 100-fold, while substitution with threonine produces a 20-fold decrease in activity. No changes are observed in K_m^{NADPH}, $K_i^{NADP^+}$, or flavin content, indicating that these substitutions have no effect on cofactor binding but affect catalysis only. $K_m^{cyt\ c}$ values are decreased in parallel with the observed decreases in the rates of the reductive half-reaction. Stopped-flow studies with the S457A mutant show a 100-fold decrease in the rate of flavin reduction. The primary deuterium isotope effect on k_{cat} for cytochrome *c* reduction increases from 2.7 for the wild-type enzyme to 9.0 for the S457A mutant, consistent with a change in the rate-determining step from $NADP^+$ release in the wild-type enzyme to hydride transfer in the S457A mutant. The primary deuterium isotope effect on k_1 for flavin reduction at high ionic strength (I = 535 mM) increases from 12.2 for the wild-type enzyme to >20 for the S457A mutant, consistent again with an increase in the relative rate limitation of hydride transfer. Furthermore, anaerobic titration of S457A indicates that the redox potential of the FAD semiquinone has been decreased. Data presented in this study support the hypothesis that Ser457 is involved in hydrogen bonding interactions which stabilize both the transition state for hydride transfer and the reduced FAD.

The microsomal and nuclear envelope flavoprotein NADPH−cytochrome P450 oxidoreductase (P450R)[1] mediates the transfer of electrons from NADPH to cytochrome P450 and other microsomal proteins such as cytochrome b_5, heme oxygenase, and fatty acid elongase, as well as to non-physiological electron acceptors such as cytochrome *c*, ferricyanide, dichloroindophenol, and menadione [for recent reviews, see Porter (1991) and Shen & Kasper (1993)]. This protein contains unique binding domains for FMN and FAD (Iyanagi & Mason, 1973; Porter & Kasper, 1986; Shen & Kasper, 1993), and electron transfer proceeds from NADPH to FAD to FMN to cytochromes *c* or P450 (Vermilion & Coon, 1981; Kurzban & Strobel, 1986).

Sequence comparisons have shown that P450R is a member of a family of FMN- and FAD-containing enzymes which also includes nitric oxide synthase and the bacterial sulfite reductase α-subunit (Porter, 1991; Shen & Kasper, 1993). Comparison of the sequence of the amino-terminal FMN-binding domain of P450R with that of the bacterial flavodoxins identified potential FMN-binding residues (Porter & Kasper, 1986), which were confirmed by site-directed mutagenesis (Shen *et al*., 1989). The FAD/NADPH binding domain is related to another class of flavoproteins, the transhydrogenases (Porter & Kasper, 1986; Karplus *et al*., 1991; Porter, 1991; Shen & Kasper, 1993), which includes ferredoxin−$NADP^+$ reductase (FNR), NADH−nitrate reductase, and NADH−cytochrome b_5 reductase, all of which abstract the *pro-R* (A-side) hydrogen of NADPH (Drysdale *et al.*, 1961; Krakow *et al.*, 1965; Guerro & Vennesland, 1975; Karplus *et al.*, 1991; Sem & Kasper, 1992). The conformation of the bound nicotinamide has been shown to be *anti* in P450R (Sem & Kasper, 1992) but has not been established for other members of this family. Sequence comparisons of this family of proteins have shown conservation of many of the FAD- and NADPH-binding amino acids identified in the FNR crystal structure (Karplus *et al.*, 1991; Shen & Kasper, 1993; Bruns & Karplus, 1995). Site-directed mutagenesis in conjunction with alternate substrate and pH studies have demonstrated that one of these residues, Arg597 of rat liver P450R (Arg235 of FNR), interacts with the 2′-phosphate of NADPH and is responsible for the strong nucleotide specificity of P450R (Sem & Kasper, 1993b). In contrast, substitution of a cysteine, previously identified in the human enzyme as essential for catalysis (Haniu *et al.*, 1986, 1989), but not well-conserved (Shen & Kasper, 1993), was found to have only small effects on NADPH binding and catalysis (Shen *et al.*, 1991).

The crystal structure of FNR places Ser96 in close proximity to the isoalloxazine ring of FAD, suggesting a role for this residue in hydride transfer and/or stabilization of the reduced flavin (Karplus *et al.*, 1991; Bruns & Karplus, 1995).

† This research was supported by Grants CA22484 and CA0920 from the National Institutes of Health. This study made use of the National Magnetic Resonance Facility at Madison which is supported in part by NIH Grant RR02301 from the Biomedical Research Technology Program, Division of Research Resources. Equipment in the facility was purchased with funds from this program, the University of Wisconsin, the NSF Biological Instrumentation Program (Grant DMB-8415048), the NIH Shared Instrumentation Program (Grant RR02781), and the U.S. Department of Agriculture.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations: P450R, NADPH−cytochrome P450 oxidoreductase; FNR, ferredoxin−$NADP^+$ reductase; PCR, polymerase chain reaction; AcPyrADP, 3-acetylpyridine adenine dinucleotide phosphate; e^-, electron equivalents.

S0006-2960(96)00587-9 CCC: $12.00

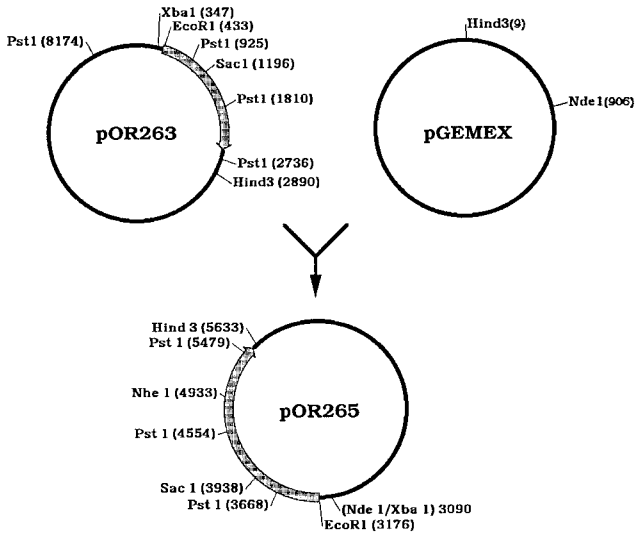

FIGURE 1: Construction of NADPH−cytochrome P450 oxidoreductase expression vector, pOR265. Shaded arrows represent the P450R coding sequence.

A serine or threonine at this position, which corresponds to Ser457 of P450R, is conserved in all members of this family. The current study explores the role of Ser457 in catalysis and suggests an essential role for Ser457 in both hydride transfer from NADPH to FAD and regulation of FAD redox potentials.

MATERIALS AND METHODS

To facilitate DNA sequencing of the mutant constructs, the expression plasmid pOR265 was constructed by ligating the 2.5 kbp *Hin*d3−*Xba*1 fragment of pOR263 (Shen *et al.*, 1989), carrying the *omp*A signal peptide and complete P450R coding sequence, into the plasmid pGEMEX (Promega), cut with *Nde*1 and *Hin*d3 (Figure 1). The host TG1 was used for expression of P450R. Although the level of expression was lower, P450R produced by this vector was identical in all respects to that produced by pOR263. Higher levels of expression could be obtained in the host JM109(DE3), which contains the T7 polymerase; however, formation of inclusion bodies precluded its use.

Site-directed mutagenesis was performed using PCR. The following 5′-oligonucleotides were synthesized on an Applied Biosystems synthesizer:

S457A	5'-CACGCCTGCAGGCCCGATACTACGCCATTG-3'
S457T	5'-CACGCCTGCAGGCCCGATACTACACCATTG-3'
S457C	5'-CACGCCTGCAGGCCCGATACTACTGCATTG-3'
S460A	5'-CATTGCCGCATCCTCCA-3'
S460A2	5'-TGGAGGATGCGGCAATG-3'
FAD2	5'-GGCAAGCTTAATGCGGTAGT-3'
1550	5'-CCTGGATGTCATCATGTCTC-3'

Each S457 oligonucleotide contained the desired mutation and a *Pst*1 site at the 5′-end to facilitate cloning of the PCR fragment. PCR reactions contained 10 mM Tris, pH 8.3, 50 mM KCl, 6.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each dNTP, 10 μg/mL pOR265 DNA, 1 μM S457 mutagenic oligonucleotide, 1 μM FAD2 oligonucleotide (hybridizing to bases 5622−5641 of pOR265), and 0.05 unit/μL AmpliTaq polymerase (Perkin-Elmer). Reaction conditions were as follows: 94 °C, 1 min; 43 °C, 1 min; 72 °C, 2 min; 25 cycles, followed by extension for 5 min at 72 °C. Ampliwax (Perkin-Elmer) was used according to the manufacturer's instructions. After removal of free oligonucleotides by centrifugation through a 100 000-MW cutoff filter (Millipore), PCR-amplified DNA fragments were digested with *Pst*1 and *Nhe*1 and purified by agarose gel electrophoresis. The *Pst*1-*Nhe*1 fragment containing the mutation and the 616 bp *Sac*1-*Pst*1 fragment spanning bases 3938−4554 of pOR265 were ligated into pOR265 cut with *Sac*1 and *Nhe*1 (Figure 1). The S460A mutant was prepared by the method of Higuchi *et al.* (1988), using the S460A/FAD2 and S460A2/1550 primer pairs and pOR265 plasmid template in the first two PCR reactions, with reaction conditions as described above. The products of the first two PCR reactions were combined and used as templates for the final PCR reaction, using the 1550/FAD2 primer pair and an annealing temperature of 55 °C. The final PCR reaction product was digested with *Pst*1 and *Nhe*1 and processed as described for the Ser457 mutants. All mutant plasmids were characterized by restriction mapping and sequencing of the PCR-amplified regions and cloning sites.

Expression and purification of recombinant P450R were carried out as described previously (Shen *et al.*, 1989), except that cultures were grown and induced at 28 °C instead of 37 °C. Growth temperature was found to have no effect on the activities of the wild-type or Ser457 proteins; however, proper folding of some P450R mutants has been found to be sensitive to growth temperature (Shen & Kasper, unpublished). Therefore, as a precaution, all cultures were grown at 28 °C.

Protein was assayed by the BCA method (Smith *et al.*, 1985), and FMN and FAD were determined by the method of Faeder & Siegel (1973). Cytochrome *c*, ferricyanide, and AcPyrADP activities were assayed as described previously (Shen *et al.*, 1991), except that reactions were initiated by addition of P450R.

For studies of the primary deuterium isotope effect, A-side NADPD was synthesized as described previously (Sem & Kasper, 1992). Protein was removed by filtration through a Centriprep-10 membrane (Amicon) and NADPD purified by high-performance liquid chromatography on a Spherogel TSK column equilibrated in 25 mM potassium phosphate, pH 8.0. Fractions with a 340/260 absorbance ratio >0.42 were pooled and used in steady-state and stopped-flow studies. The fraction of deuteration was determined as described previously (Sem & Kasper, 1994). Primary deuterium isotope effects were determined by the method of direct comparison (Cleland, 1982). Data were fitted to the following equations, which assume, respectively, equal isotope effects on V_{max} and K_m^{NADPH}, different isotope effects on V_{max} and V_{max}/K_m^{NADPH}, and isotope effects on V_{max} only (Sweet & Blanchard, 1991).

$$v = \frac{V_{max}A}{[(K_m + A)(1 + F_iE)]} \quad (1)$$

$$v = \frac{V_{max}A}{[K_m(1.0 + F_iE_{V/K}) + A(1 + F_iE_v)]} \quad (2)$$

$$v = \frac{V_{max}A}{[K_m + A(1.0 + F_iE_v)]} \quad (3)$$

where v is the initial velocity, A is the concentration of NADPH, F_i is the fraction of deuterium label, E is the isotope effect minus 1 on V_{max} and V_{max}/K_m, $E_{V/K}$ is the isotope effect minus 1 on V_{max}/K_m, and E_v is the isotope effect minus 1 on V_{max}.

Visible absorption spectra and anaerobic reduction rates for the S457A protein were measured at 28 °C on a Beckman 7500 diode-array spectrophotometer. Anaerobic reactions contained 50 mM Tris, pH 7.7, 10 mM glucose, 5 units/mL glucose oxidase, 1 μM methyl viologen, and P450R at the concentrations indicated. Cuvettes were sealed and oxygen was removed by subjecting the samples to several cycles of evacuation followed by flushing with N_2 purified by passage through a scrubbing tower containing 0.5% sodium dithionite, 0.05% 2-anthroquinonesulfonate, and 0.4% NaOH. NADPH or dithionite was dissolved in 50 mM Tris, pH 7.7, made anaerobic as described above, and added to protein samples with a gas-tight syringe.

Aerobic stopped-flow studies were carried out on a Cary spectrophotometer equipped with an Olis stopped-flow apparatus or an Olis RSM 1000 rapid scanning spectrophotometer. Anaerobic reduction rates were measured on a Beckman 7500 diode-array spectrophotometer. NADPH was present at a 10-fold molar excess relative to flavin. Data were fitted to the following equations:

$$A_t = A_0 + [A_1e^{(-k_1t)}] \quad (4)$$

$$A_t = A_0 + [A_1e^{(-k_1t)}] + [A_2e^{(-k_2t)}] \quad (5)$$

RESULTS

Spectral Properties. Substitution of either Ser457 or the adjacent Ser460 had no significant effects on the visible absorption spectra of either the oxidized or air-stable semiquinone forms of P450R. The oxidized forms of the wild-type (Figure 2A) as well as the mutant (Figure 2B−E) proteins had spectral properties characteristic of the rat liver enzyme, with peaks at 384 and 452 nm and a shoulder at approximately 485 nm (Iyanagi & Mason, 1973). Small variations in the 485 nm shoulder were not reproducible between different protein preparations. All four mutant proteins could be reduced by NADPH. Addition of NADPH to the wild-type or any of the mutant proteins under aerobic conditions produced spectra typical of the air-stable semiquinone (FAD/FMNH•), having a decreased absorbance at 452 nm and a long-wavelength band with a broad maximum at approximately 585 nm and a shoulder at 630 nm, indicative of electron transfer from NADPH to FAD to FMN.

Enzymatic Activity. Substitution of alanine, threonine, or cysteine for Ser457 markedly decreased the rates of reduction of cytochrome *c* and ferricyanide, as well as transhydrogenase activity with AcPyrADP. Specific activities in the presence of saturating substrate concentrations are shown in Table 1. Cytochrome *c* and ferricyanide reductase activities of the S457A and S457C mutants were decreased to approximately 1% of wild-type, while the S457T mutant retained approximately 6% activity. Transhydrogenase activity for all of the Ser457 mutants was decreased to about 6−11% of wild-type. Replacement of a nearby seryl residue (460) with alanine had no effect on enzymatic activities (Table 1), suggesting that decreased catalytic activities were not due to conformational changes in this region.

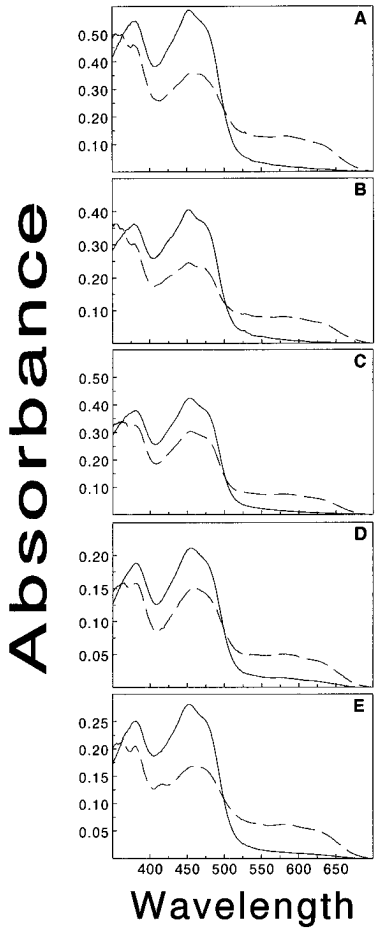

FIGURE 2: Visible absorption spectra of the oxidized (—) and air-stable semiquinone (- - -) forms of wild-type and mutant P450R proteins. Semiquinone spectra were obtained by adding NADPH under aerobic conditions and recording spectra after equilibrium had been reached (>15 min). A, wild-type; B, S457A; C, S457T; D, S457C; E, S460A.

Table 1: Catalytic Activities of Ser457 and S460 Mutants

	specific activity [μmol/(min·mg)]		
protein	cytochrome *c*[a]	ferricyanide[b]	AcPyrADP[c]
P450R	51.5 ± 9.0 (13)	102 ± 7.9 (4)	1.95 ± 0.2 (3)
S457A	0.62 ± 0.05 (7)	0.74 ± 0.13 (4)	0.13 ± 0.02 (3)
S457C	0.39 ± 0.03 (3)	0.6 ± 0.3 (3)	0.22 (2)
S457T	3.02 ± 0.22 (4)	5.5 ± 0.9 (3)	0.11 ± 0.01 (3)
S460A	43.1 ± 2.6 (3)	112 ± 25 (3)	3.1 ± 0.1 (3)

[a] Reactions contained 0.27 M potassium phosphate, pH 7.7, 65 μM cytochrome *c*, and 50 μM NADPH. Values are mean ± SD (*n*). [b] Reactions contained 0.27 M potassium phosphate, pH 7.7, 500 μM potassium ferricyanide, and 100 μM NADPH. Values are mean ± SD (*n*). [c] Reactions contained 0.27 M potassium phosphate, pH 7.7, 100 μM NADPH, and 100 μM AcPyr ADP. Values are mean ± SD (*n*).

Steady-State Kinetics. Substitutions at Ser457 impaired catalysis only, with no effect on pyridine nucleotide binding. All three substitutions at Ser457 decreased k_{cat} for cytochrome *c* reduction, with no significant changes in K_m^{NADPH} or $K_i^{NADP^+}$ (Table 2). S457A and S457C k_{cat} values were decreased to about 1% of wild-type, while S457T k_{cat} was reduced to 5%. For the wild-type as well as all three mutant enzymes, $NADP^+$ was a competitive inhibitor versus NADPH and $K_i^{NADP^+}$ values were unchanged from wild-type.

Table 2: Kinetic Properties of Ser457 and Ser460 Mutants[a]

protein	K_m^{NADPH} (μM)	$K_m^{cyt\ c}$ (μM)	k_{cat} (min^{-1})	K_i^{NADP+} (μM)	E^b I = (475 mM)	E^b I = (70 mM)
P450R	6.2 ± 0.7 (4)	16.3 ± 1.7 (4)	5421 ± 744 (4)	18.8 ± 1.9 (3)	2.7	1.3
S457A	6.3 ± 0.5 (5)	<1 (3)	60 ± 6 (5)	18.2 ± 2.1 (4)	9.0	6.8
S457C	7.2 ± 0.5 (4)	<1 (3)	43 ± 1 (3)	19.2 (2)	ND[c]	
S457T	7.4 ± 1.0 (4)	2.2 ± 0.3 (3)	268 ± 16 (4)	17.9 (1)	ND	
S460A	5.3 ± 0.9 (4)	15.0 ± 1.0 (4)	4496 ± 903 (4)	18.6 ± 4.5 (3)	ND	

[a] Reactions contained 0.27 M potassium phosphate, pH 7.7, 65 μM cytochrome *c* (for K_m^{NADPH}, K_i^{NADP+}) and 50 μM NADPH (for $K_m^{cyt\ c}$), with varying amounts of NADPH or cytochrome *c*. Reactions were preincubated at 28 °C for 2 min and initiated by addition of protein. Values are mean ± SD (*n*). [b] Primary deuterium isotope effect on V_{max} and V_{max}/K_m^{NADPH} at high (475 mM) and low (70 mM) ionic strength. [c] ND, not determined.

$K_m^{cyt\ c}$ values were reduced to less than 5% of wild-type, consistent with the two-site ping-pong mechanism proposed for this enzyme and a decrease in the rate of the reductive half-reaction (Matthews, 1991; Sem & Kasper, 1994). No changes in any of the steady-state parameters were observed with the S460A mutant.

To assess the relative rate limitation of hydride transfer in the S457A mutant, the deuterium isotope effect on cytochrome *c* reduction with [(4*S*)-H,(4*R*)-D]NADPD was determined. For both the wild-type and S457A enzymes, the best fit was obtained by assuming equal isotope effects on V_{max} and V_{max}/K_m^{NADPH} (eq 1). Wild-type primary deuterium isotope effects at high (475 mM) and low (70 mM) ionic strength (Table 2) were similar to those reported previously (Sem & Kasper, 1994, 1995). The S457A mutation increased the deuterium isotope effects 3- and 5-fold, respectively, at high and low ionic strength. Similar changes in the magnitude of the isotope effect were observed with ferricyanide as the electron acceptor (data not shown), also consistent with an increase in the relative rate limitation of hydride transfer.

Kinetics of Flavin Reduction. Stopped-flow studies were performed to confirm that the decreases in k_{cat} were due to decreases in the rate of flavin reduction. Figure 3 shows absorbance changes for the wild-type and mutant proteins at 452 and 585 nm upon reduction by NADPH at low ionic strength (I = 35 mM). Rate constants and isotope effects calculated from these absorbance changes at low and high (I = 535 mM) ionic strength (Table 3) are consistent with hydride transfer to FAD being rate-limiting for k_{cat}. At low ionic strength, reduction of the wild-type enzyme under aerobic conditions was biphasic, with an initial fast phase, k_1 = 55.0 s^{-1}, accounting for about 80% of the absorbance change, followed by a slower phase, k_2 = 4.05 s^{-1} , which accounted for about 20% of the absorbance change (Figure 3A and Table 3). Although these rates were determined under aerobic conditions, reoxidation of the enzyme has been shown to be very slow (1−2 min^{-1}) (Iyanagi *et al.*, 1978), and the rate constants are comparable to those obtained with rat liver P450R in 100 mM potassium phosphate, pH 7.0, under anaerobic conditions (Oprian & Coon, 1982). At high ionic strength, k_1 was nearly doubled, but k_2 was decreased 62% (Table 3) and the slow phase accounted for nearly half of the absorbance change (not shown). At both high and low ionic strength, aerobic reduction of the S457A protein was monophasic (Figure 3B) and 100 times slower, with k_1 values of 0.58 s^{-1} and 0.68 s^{-1} at low and high ionic strength, respectively (Table 3). A similar, monophasic, reduction rate (k_1 = 0.33 s^{-1}) was observed under anaerobic conditions (Figure 3C and Table 3). The rate of reduction of the S457T protein was approximately 2% of the wild-type rate (Table 3).

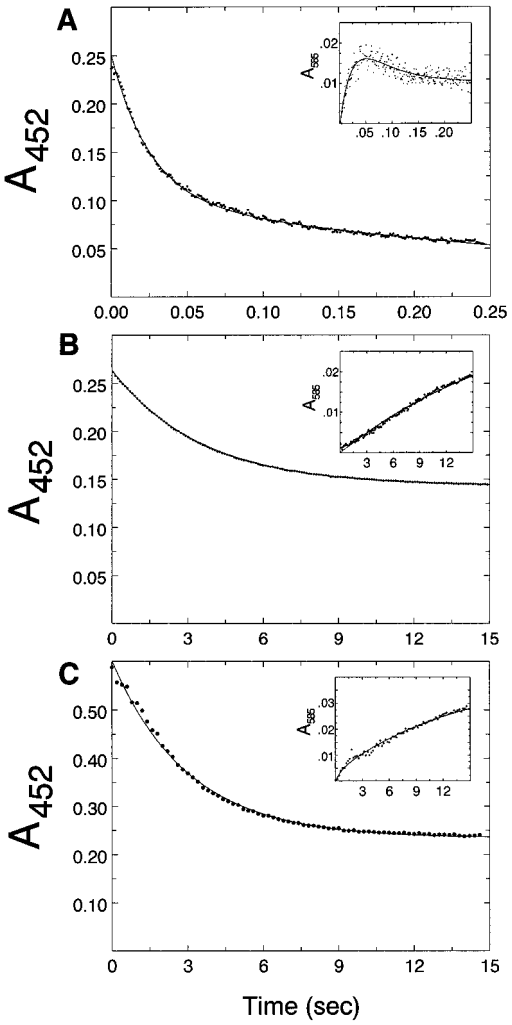

FIGURE 3: Kinetics of reduction of wild-type and mutant P450R by NADPH at low ionic strength. Absorbance changes at 452 and 585 nm (inset) are shown. (A) Reduction of wild-type P450R under aerobic conditions. The solid lines are fits of the data to eq 5. (B) Reduction of S457A protein under aerobic conditions. The solid lines are fits of the data to eq 4. (C) Reduction of S457A under anaerobic conditions. The solid lines are fits of the data points to eqs 4 (452 nm) and 5 (585 nM).

In addition to decreases in the rate of flavin reduction, a decrease in the extent of reduction of the S457A mutant was also observed. The wild-type enzyme was nearly completely reduced by NADPH, with the final absorbance at 452 nm being <25% of the initial absorbance (Figure 3A). However, the final A_{452} of the S457A mutant was 41% (anaerobic) to 55% (aerobic) of the initial A_{452}, indicating only partial reduction of the flavin (Figure 3, panels C and B).

Table 3: Reduction of P450R and S457A Proteins by NADPH[a]

	low ionic strength[b]			high ionic strength[c]		
	k_1 (s^{-1})	k_2 (s^{-1})	k_1^H/k_1^D	k_1 (s^{-1})	k_2(s^{-1})	k_1^H/k_1^D
P450R (aerobic)	55.0 ± 3.3 (9)	4.05 ± 0.48 (9)	3.4	97.2 ± 7.5 (3)	1.52 ± 0.3 (3)	12.2
S457A (aerobic)	0.58 ± 0.06 (8)		6.2	0.68 ± 0.11 (4)		>20
S457A (anaerobic)	0.33 (2)		ND[d]	ND		ND
S457T (aerobic)	1.43 ± 0.02 (4)	0.05 ± 0.003 (4)	ND	ND	ND	ND

[a] Rate constants were obtained by monitoring absorbance changes at 452 nm and fitting the data to eqs 4 or 5. Values are mean ± SD (*n*). [b] Reactions were carried out in 50 mM Tris, pH 7.7, at 28 °C. [c] Reactions were carried out in 50 mM Tris, pH 7.7, 500 mM KCl, at 28 °C. [d] ND, not determined.

Formation of flavin semiquinone was monitored through absorbance changes at 585 nm (Iyanagi & Mason, 1973). Addition of NADPH under low ionic strength conditions to wild-type P450R produced an initial increase in absorbance at 585 nm, with a rate constant similar to that observed for flavin reduction ($k = 51.4\ s^{-1}$), followed by a slight decrease (Figure 3A, inset). This is in accord with results reported for anaerobic NADPH reduction of rat liver P450R (Oprian & Coon, 1982) and consistent with rapid interflavin electron transfer following reduction of FAD by NADPH. Reduction of the S457A protein under anaerobic conditions, however, produced an initial absorbance increase at 585 nm ($k \sim 1.3\ s^{-1}$), followed by a further slow absorbance increase ($k \sim 0.03\ s^{-1}$) (Figure 3C, inset). Reduction of the S457A protein under aerobic conditions produced an initial lag followed by a slow absorbance change (Figure 3B, inset).

The deuterium isotope effect on the wild-type k_1 for flavin reduction was 3.4 at low ionic strength and increased to 12.2 at high ionic strength (Table 3). Sugiyama *et al.* (1975) have reported a value of 5.1 for the rabbit liver enzyme; however, the ionic strength used is not known. The isotope effect on reduction of the S457A mutant was 6.2 at low ionic strength, which is comparable to that obtained for k_{cat} for cytochrome *c* reduction at low ionic strength (Table 2). At high ionic strength, the isotope effect was increased to greater than 20.

Reduction of P450R by NADPH. The decrease in the extent of NADPH reduction of the S457A mutant (Figure 3B and C) suggests a change in the flavin redox potential. To investigate the oxidation−reduction properties of FMN and FAD in the S457A mutant, separate titrations with NADPH and dithionite were carried out. Reduction of P450R with dithionite or NADPH produces visible absorption spectra which are characteristic for each redox state of the enzyme (Iyanagi *et al.*, 1974; Vermilion & Coon, 1978a). Titrations of the wild-type and S457A proteins with NADPH are shown in Figure 4. Titration of the wild-type enzyme with NADPH proceeded in a manner identical to that reported for the rat liver enzyme (Iyanagi *et al.*, 1974; Vermilion & Coon, 1978a). Addition of up to 0.5 mol of NADPH/mol of enzyme to the fully oxidized wild-type protein produced the spectrum of the air-stable semiquinone (FAD/FMNH•), characterized by an absorbance decrease at 452 nm, isosbestic points at 363 and 502 nm, and appearance of a long-wavelength absorbance with a maximum at 585 nm and a shoulder at 630 nm (Figure 4A,E−G). Further addition of NADPH (Figure 4B,E−G) led to production of the 3-electron-reduced form ($FMNH_2$/FADH•), with absorbance decreases at 452 and 502 nm, corresponding to $FMNH_2$ formation, and a shift in the long-wavelength absorbance to 592 nm with loss of the 630 nm shoulder, corresponding to formation of the FAD semiquinone (FADH•).

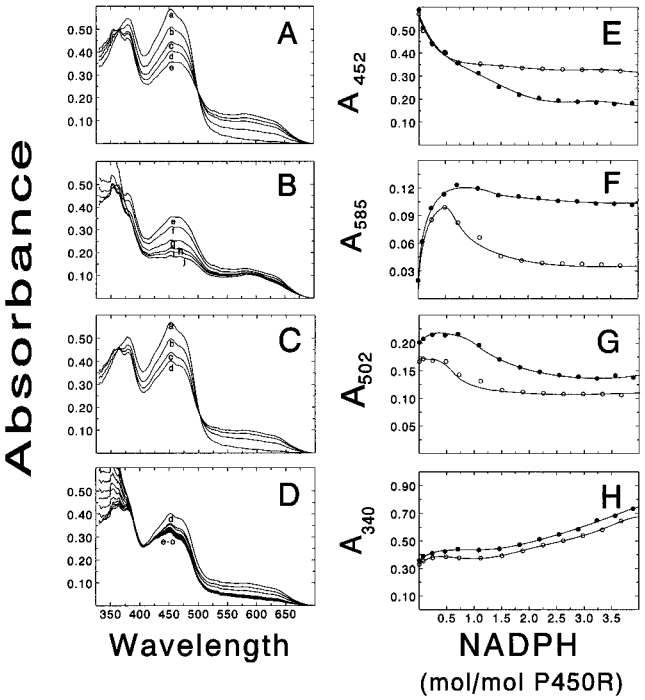

FIGURE 4: NADPH titration of wild-type and S457A P450R. Panels A and B represent a continuous titration of wild-type protein with NADPH under anaerobic conditions. Spectrum a is that of the oxidized enzyme, while b−j were recorded after stepwise addition of NADPH. Panels C and D represent a continuous titration of the S457A protein with NADPH under anaerobic conditions. Spectrum a is that of the oxidized enzyme, while b−o were recorded after stepwise addition of NADPH. Some intermediate spectra have been omitted for clarity. Panels E−H display the associated absorbance changes at the indicated wavelengths for the wild-type (●) and S457A (○) proteins as a function of mol of NADPH/mol of protein.

Since the midpoint potential of the FADH•/$FADH_2$ couple is below that of the NADPH/$NADP^+$ couple, NADPH is unable to fully reduce P450R (Iyanagi *et al.*, 1974; Vermilion & Coon, 1978a). Further addition of NADPH to the 3-electron-reduced enzyme produced minimal absorbance changes in the flavin spectra (Figure 4,E−G) and increases at 340 nm indicative of NADPH accumulation (Figure 4H).

Spectral changes associated with reduction of the S457A enzyme to the 1-electron-reduced (FAD/FMNH•) stage paralleled those seen for the wild-type enzyme (Figure 4C,E−G). As with the wild-type enzyme, approximately 0.5 mol of NADPH/mol of protein was sufficient to produce the air-stable semiquinone. However, further addition of NADPH up to 4 mol/mol of protein produced only a small additional decrease in A_{452} (Figure 4D,E). The increase in absorbance at 340 nm was the same as that seen with wild-type protein, indicating that NADPH was being oxidized to the same extent (Figure 4H). A_{585} decreased (Figure 4F) with

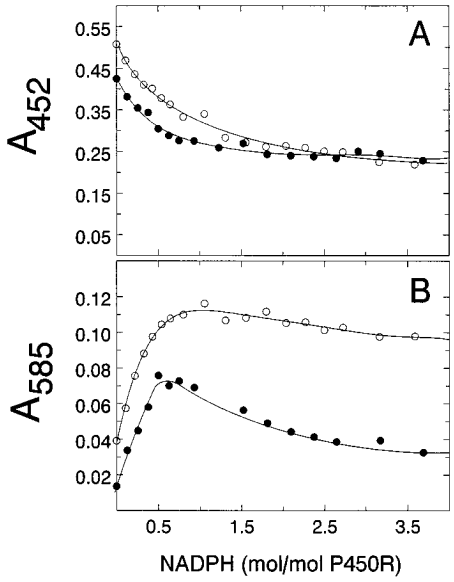

FIGURE 5: NADPH titration of S457C and S457T proteins under anaerobic conditions. Absorbance changes at 452 (A) and 585 (B) nm are plotted as a function of mol of NADPH/mol of P450R for the S457C (●) and S457T (○) proteins.

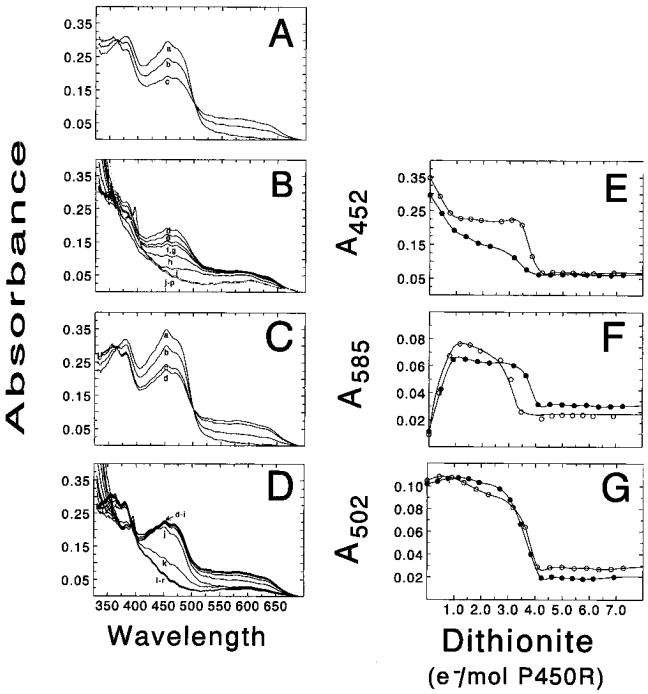

FIGURE 6: Dithionite titration of wild-type and S457A P450R. Panels A and B represent a continuous titration of wild-type protein with sodium dithionite under anaerobic conditions. Spectrum a is that of the oxidized enzyme, while spectra b−p were recorded after stepwise addition of dithionite. Panels C and D represent a continuous titration of S457A protein with sodium dithionite under anaerobic conditions. Spectrum a is that of the oxidized enzyme. Spectra b−r were recorded after stepwise addition of dithionite. Some intermediate spectra have been removed for clarity. Panels E−G display the associated absorbance changes at the indicated wavelengths for the wild-type (●) and S457A (○) proteins as a function of electron equivalents (e^-)/mol of P450R.

no shift in maximum absorbance or loss of the 630 nm shoulder, consistent with reduction of $FMNH^\bullet$ to $FMNH_2$ and no $FADH^\bullet$ formation (Figure 4D). A_{502} decreased concomitantly with A_{585}, also consistent with reduction of $FMNH^\bullet$ to $FMNH_2$ (Figure 4F,G).

Addition of 0.5 mol of NADPH/mol of protein to the S457C and S457T proteins also produced the air-stable semiquinone. Further addition of NADPH to the 1-electron-reduced S457T enzyme produced absorbance changes similar to those observed for the wild-type enzyme, namely, decreases in A_{452} (Figure 5A) but not A_{585} (Figure 5B), and alterations in the long-wavelength absorbance characteristic of $FADH^\bullet$ formation. However, the magnitude of the A_{452} decrease was slightly less than that observed for the wild-type protein (Figure 5A). Addition of NADPH to the 1-electron-reduced S457C enzyme produced changes similar to those seen for the S457A mutant. Absorbance at 452 nm decreased only slightly (Figure 5A), while A_{585} did decrease (Figure 5B), again indicating reduction of $FMNH^\bullet$ with no $FADH^\bullet$ formation.

Reduction of P450R by Dithionite. As with the NADPH titration, spectral changes associated with dithionite reduction of the wild-type protein paralleled those reported for the liver enzyme (Figure 6A,B,E−G) (Iyanagi *et al.*, 1974; Vermilion & Coon, 1978a). Addition of up to 1 electron equivalent (e^-) of dithionite/mol of enzyme to the fully-oxidized wild-type protein produced the spectrum of the air-stable semiquinone ($FAD/FMNH^\bullet$) (Figure 6A,E−G). Further addition of dithionite produced the 2-electron-reduced ($FAD/FMNH_2$; $FADH^\bullet/FMNH^\bullet$) and 3-electron-reduced forms of the enzyme ($FADH^\bullet/FMNH_2$; $FADH_2/FMNH^\bullet$) (Figure 6B), characterized by continuing decreases in A_{452} (Figure 6B,E), and a shift in the long-wavelength spectrum to that of the FAD semiquinone with no change in A_{585} (Figure 6B,F). Full reduction was observed after addition of $4e^-$/mol of protein, with absorbance decreases at all three wavelengths (Figure 6B,E−G).

Addition of $1e^-$/mol of S457A produced the same air-stable semiquinone ($FAD/FMNH^\bullet$) spectrum as the wild-type enzyme (Figure 6C,E−G). However, further addition of dithionite up to $3e^-$/mol of protein did not produce any further decreases in A_{452} (Figure 6D,E). In contrast to the wild-type protein, a continual decrease in A_{585} was observed after addition of $1e^-$/molecule of protein, with no shift to 592 nm or loss of the 630 nm shoulder (Figure 6D,F). A_{502} also began to decrease after addition of $1e^-$/mol of protein, consistent with reduction of $FMNH^\bullet$ to $FMNH_2$ (Figure 6D,G). As with the wild-type enzyme, fully-reduced S457A protein was produced with $4e^-$/mol of protein (Figure 6D,E−G).

$NADP^+$ Difference Spectrum. The difference spectrum produced upon binding of $NADP^+$ to P450R provides a probe for interactions between FAD and the oxidized pyridine nucleotide. The difference spectrum obtained upon addition of $NADP^+$ to wild-type P450R is characterized by a trough at 435 nm and a peak at 500 nm (Figure 7A) and is similar to that of the rat liver enzyme (Vermilion & Coon, 1978b) and FNR (Aliverti *et al.*, 1995). The S457A mutation produced marked changes in the difference spectrum (Figure 7B), with a shift in the position of the trough and the peak to 395 and 482 nm, respectively, and a decrease in the magnitude of the absorbance change, consistent with alterations in the relative conformation and/or orientation of the nicotinamide and flavin rings. Similar alterations have been observed with the Ser96 mutants of FNR (Aliverti *et al.*, 1995).

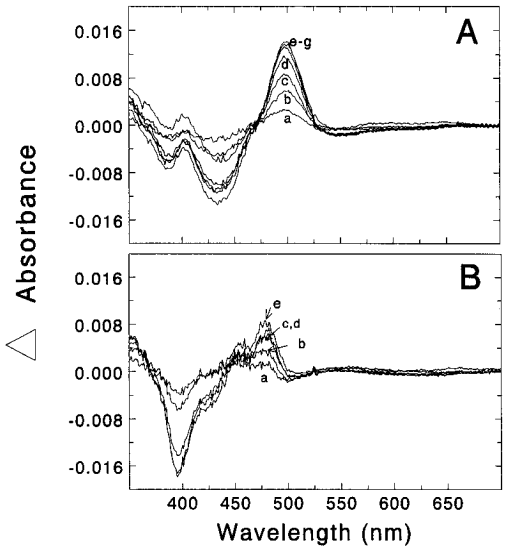

FIGURE 7: $NADP^+$ difference spectra of wild-type and S457A P450R. Difference spectra were recorded after addition of increasing amounts of $NADP^+$ to the wild-type (A) and S457A (B) proteins. Protein concentrations were 20.5 μM (wild-type) and 29.0 μM (S457A). Concentrations of $NADP^+$ (μM) for the wild-type protein were as follows: a, 5; b, 10; c, 15; d, 20; e, 25; f, 30; and g, 35. Concentrations of $NADP^+$ (μM) for the S457A protein were as follows: a, 5; b, 10; c, 20; d, 35; and e, 71.

DISCUSSION

Removal of the hydroxyl-containing side chain of Ser457 of P450R produces dramatic decreases in the rate of flavin reduction as well as in catalytic activity with the substrates cytochrome *c*, which accepts electrons from FMN, and ferricyanide and AcPyrADP, which accept electrons from FAD. Substitutions at Ser457 affect catalysis only, with no effects on flavin content, K_m^{NADPH}, or $K_i^{NADP^+}$. The decrease in $K_m^{cyt\ c}$ concomitant with a decrease in the velocity of the reductive half-reaction is consistent with the two-site ping-pong mechanism proposed for this enzyme (Matthews, 1991; Sem & Kasper, 1994). The specific effect of the Ser457 mutations on catalysis, together with the absence of any effect of the nearby Ser460 substitution, suggests that the effects of the Ser457 mutations are not due to gross conformational changes in this region. This is further supported by the observation that the crystal structures of mutants of the homologous residue of FNR, Ser96, show only minimal structural changes (Aliverti *et al.*, 1995).

P450R-mediated electron transfer from NADPH to 1-electron acceptors begins with 2-electron reduction of FAD via hydride transfer from NADPH, followed by rapid 1-electron transfers to FMN and electron transfer from $FMNH_2$ to 1-electron acceptor (Vermilion *et al.*, 1981; Bhattacharya *et al.*, 1991). The transhydrogenase reaction with AcPyrADP, however, involves 2-electron transfers from reduced FAD to AcPyrADP, and ferricyanide is reduced via 1-electron transfer from reduced FAD. Reduction of FAD by NADPH has been shown by stopped-flow studies to proceed through rapid formation of an enzyme−NADPH complex, followed by rate-limiting hydride transfer, rapid electron transfer to FMN, and $NADP^+$ dissociation (Oprian & Coon, 1982).

Although the S457A mutant is able to effect electron transfer from NADPH to FMN, overall rates of reduction of the substrates cytochrome *c* and ferricyanide are decreased approximately 100-fold and are associated with a similar decrease in the rate of flavin reduction. (Reduction of AcPyrADP is reduced only 10-fold; however, k_{cat} for this reaction is 33-fold lower than that of cytochrome *c* and steps other than hydride transfer may be rate-limiting.) Since electron transfer proceeds from NADPH to FAD to FMN with no evidence of direct electron transfer between NADPH and FMN, the decreased k_{cat} may be due to decreases in either the rate of interflavin electron transfer or the rate of reduction of FAD, which in turn depends on the rate and/or extent of formation of the enzyme−NADPH complex and the rate of hydride transfer. The decrease in $K_m^{cyt\ c}$ is consistent with a decrease in the rate of reduction of P450R at either the FMN or FAD sites. The kinetics of the absorbance changes at 585 nm are consistent with rapid interflavin electron transfer in the S457A protein, although it is possible that the observed spectral changes in the long-wavelength region are due to charge-transfer complex formation rather than semiquinone formation.

The decrease in ferricyanide reductase activity and the large increase in the deuterium kinetic isotope effect are evidence for a decrease in the rate of hydride transfer to FAD. Hydride transfer is partially rate-limiting for wild-type P450R (Sem & Kasper, 1994); any further decrease in the rate of hydride transfer in the absence of other changes would be expected to increase this rate limitation and so increase the deuterium isotope effect. Comparison of the relative magnitudes of the deuterium isotope effect on k_{cat} for cytochrome *c* reduction shows that hydride transfer is indeed more rate-limiting in the S457A mutant. In accord with previous results on wild-type isotope effects from this laboratory (Sem & Kasper, 1994, 1995), identical isotope effects were obtained for V_{max} and V_{max}/K_m^{NADPH}. The magnitude of the isotope effect was ionic strength-dependent, decreasing from 2.7 at 475 mM ionic strength to 1.3 at 50 mM ionic strength. Since the isotope effect for the first-order rate constant for flavin reduction was found to be 5.1 for the rabbit liver enzyme (Sugiyama *et al.*, 1984), it has been concluded that hydride transfer is partially rate-limiting at high, but not low, ionic strength (Sem & Kasper, 1995). The ionic strength-sensitive rate-limiting step has been postulated to be $NADP^+$ release (Phillips & Langdon, 1962). For the S457A mutant, the isotope effect is again identical for V_{max} and V_{max}/K_m^{NADPH} and increases to 3.3-fold over wild-type at high ionic strength and 5.2-fold at low ionic strength, consistent with an increase in the relative rate limitation of hydride transfer.

Similarly, the increased isotope effect on the rate of flavin reduction is also consistent with a slower rate of hydride transfer. Moreover, although hydride transfer is predominantly rate-limiting for FAD reduction, the effects of ionic strength on both the rate and the deuterium isotope effect for flavin reduction suggest the presence of another partially rate-limiting step which is independent of Ser457. An increase in ionic strength from 35 to 500 mM increases the rate of wild-type fast-phase flavin reduction (k_1) 1.8-fold, with a nearly 4-fold increase in the isotope effect, suggesting the presence of another partially rate-limiting step, either enzyme−NADPH complex formation or interflavin electron transfer, which is faster (less rate-limiting) at high ionic strength. Reduction of related flavoproteins, such as FNR and phthalate dioxygenase reductase, has been shown to proceed through formation of one or more charge-transfer complexes in which the flavin and pyridine nucleotide are oriented for efficient hydride transfer (Batie & Kamin, 1986;

Gassner *et al.*, 1994). Stopped-flow studies have suggested that reduction of P450R by NADPH does proceed through rapid formation of an enzyme−NADPH complex (Oprian & Coon, 1982; Kamin *et al.*, 1966), although a charge-transfer complex has not been observed spectroscopically (Oprian & Coon, 1982).

NADPH and dithionite titration data show that, in addition to affecting the rate of FAD reduction, the Ser457A mutation also decreases the redox potential of the FAD/FADH$^{\bullet}$ redox couple. Reaction of the FAD/FMNH$^{\bullet}$ semiquinone form of the wild-type enzyme with NADPH produces either $FADH_2$/FMNH$^{\bullet}$ or $FMNH_2$/FADH$^{\bullet}$, with the latter favored thermodynamically (Oprian & Coon, 1982). However, NADPH reduction of the S457A FAD/FMNH$^{\bullet}$ semiquinone results in NADPH oxidation and reduction of FMNH$^{\bullet}$ with no evidence of FADH$^{\bullet}$ production. Although FAD must be reduced by NADPH, as evidenced by oxidation of NADPH, the equilibrium between the FAD/FADH$^{\bullet}$ and FMNH$^{\bullet}$/$FMNH_2$ couples is shifted toward reduction of FMNH$^{\bullet}$; i.e., the redox potential of the FAD/FADH$^{\bullet}$ couple in the S457A mutant has been lowered. Ser457 in the wild-type enzyme then must act to stabilize the semiquinone of FAD (FADH$^{\bullet}$), through a direct hydrogen-bonding interaction or indirectly by donating a hydrogen bond to Asp575, which has been shown to be within hydrogen-bonding distance in the related protein FNR (Karplus *et al*., 1991; Bruns & Karplus, 1995). The inability of the S457A mutant to stabilize the FADH$^{\bullet}$ semiquinone raises a question as to how electron transfer to FMN proceeds in this mutant. However, since k_{cat} for cytochrome *c* reduction is decreased to the same extent as both flavin reduction and ferricyanide reduction and a rapid absorbance increase at 585 nm is observed upon addition of NADPH, interflavin electron transfer does not appear to be rate-limiting.

A serine or threonine is found at the position corresponding to Ser457 of P450R (Ser96 of FNR) in members of the FNR family (Karplus *et al*., 1991; Shen & Kasper, 1993), and substitution of the homologous residues in FNR and NADH−cytochrome b_5 reductase has been shown to produce large decreases in enzymatic activity (Aliverti *et al.*, 1995; Shirabe *et al*., 1994). Although the FAD-binding sites of these related enzymes are highly conserved, the effects of mutations at Ser457 highlight subtle variations in the active sites of these proteins (Aliverti *et al.*, 1995; Shirabe *et al.*, 1994). The effects of the S457A mutation on catalysis and rate of flavin reduction, which are intermediate between those of the S96G and S96V mutants of FNR, are consistent both with the degree of exclusion of water from the active site and interference with nicotinamide binding. In contrast, corresponding mutants of NADH−cytochrome b_5 reductase showed either unchanged or increased rates of flavin reduction. K_m^{NADPH} is unaffected by any of the Ser457 substitutions, but is increased slightly (2- to 3-fold) by substitutions in FNR and NADH−cytochrome b_5 reductase. Finally, the degree of destabilization of the FAD semiquinone varies with the protein and amino acid substitution, and the S96G mutant of FNR is unique in that the redox potential of FAD appears to be increased.

The results presented here demonstrate that Ser457 of P450R is an important catalytic residue as a consequence of its role in facilitating hydride transfer from NADPH to FAD. Ser457 may facilitate hydride transfer by stabilizing the transition state or by controlling the orientation and/or proximity of the isoalloxazine and nicotinamide rings. Studies of NAD^+-linked dehydrogenases as well as dihydrofolate reductase have implicated a transition state for hydride transfer in which a developing carbonium ion at C-4 of nicotinamide is stabilized by hydrogen-bonding interactions with the protein (Rotberg & Cleland, 1991; Brown & Kraut, 1992; Sakowicz *et al.*, 1993). The magnitudes of the isotope effects seen with the S457A protein are larger than those expected from the relative energies of C−H versus C−D bonds and approach the range seen with hydrogen tunneling. Tunneling is favored by close packing of the reactants and exclusion of solvent, both of which may be facilitated by Ser457 (Klinman, 1989). Finally, since hydride transfer is believed to require a bent geometry and an optimum distance of 2.6 Å (Wu & Houk, 1987), Ser457 may facilitate the correct geometry for hydride transfer. Observation of an altered $NADP^+$ difference spectrum is consistent with an altered conformation of the bound nicotinamide. Similar arguments hold for the role of Ser457 in control of FAD redox potential.

The observed alterations in redox potential can be linked to the decreased rate of FAD reduction through Marcus theory in which the activation energy of a reaction is expressed in terms of two or more components: an intrinsic thermodynamic barrier energy, which is dependent upon the redox potentials of the reactants in the electron-transfer complex (FAD and NADPH), and work terms involved in bringing the reactants and products together in the proper geometry, as has been proposed for *p*-hydroxybenzoate hydroxylase (Palfrey *et al.*, 1994). Results presented here indicate that the observed rate decreases seen with the Ser457 mutations are likely to be a result of alterations in both the FAD redox potential and the orientation and proximity of the flavin and nicotinamide groups.

ACKNOWLEDGMENT

We are grateful to Timothy Culligan for technical assistance, to Drs. Dexter Northrup and Brian Fox for use of their stopped-flow spectrophotometers, to Dr. Frank Simpson for assistance with the stopped-flow experiments, to Dr. Daniel Sem for many helpful discussions, and to Kristen Adler for preparation of the manuscript.

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BI960587N