Role of the Conserved Phenylalanine 181 of NADPH−Cytochrome P450 Oxidoreductase in FMN Binding and Catalytic Activity†

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ABSTRACT: NADPH−cytochrome P450 oxidoreductase (P450 reductase, EC 1.6.2.4) is an essential component of the P450 monooxygenase complex and binds FMN, FAD, and NADPH cofactors. Residues Tyr140 and Tyr178 are known to be involved in FMN binding. A third aromatic side chain, Phe181, is also located in the proximity of the FMN ring and is highly conserved in FMN-binding proteins, suggesting an important functional role. This role has been investigated by site-directed mutagenesis. Substitution of Phe181 with leucine or glutamine decreased the cytochrome *c* reductase activity of the enzyme by approximately 50%. Ferricyanide reductase activity was unaffected, indicating that the FAD domain was unperturbed. The mutant FMN domains were expressed in *Escherichia coli*, and the redox potentials and binding energies of their complexes with FMN were determined. The affinity for FMN was decreased approximately 50-fold in the Leu181 and Gln181 mutants. Comparison of the binding energies of the wild-type and mutant enzymes in the three redox states of FMN suggests that Phe181 stabilizes the FMN−apoprotein complex. The amide ^{1}H and ^{15}N resonances of the Phe181Leu FMN domain were assigned; comparison of their chemical shifts with those of the wild-type domain indicated that the effect of the substitution on FMN affinity results from perturbation of two loops which form part of the FMN binding site. The results indicate that Phe181 cooperates with Tyr140 and Tyr178 to play a major role in the binding and stability of FMN.

NADPH−cytochrome P450 reductase is found in the endoplasmic reticulum of most eukaryotic cells and is an essential component of the P450 monooxygenase system (*1*) which is responsible for the metabolism of a wide variety of drugs and environmental chemicals. It is a 78 kDa multidomain molecule comprising a short N-terminal hydrophobic membrane anchor sequence, followed by an FMN-binding domain, similar to the bacterial flavodoxins, and a carboxy-terminal FAD and NADPH-binding domain, similar to ferrodoxin:$NADP^+$ reductase (*2*) but containing a large insert between the conserved FAD binding regions. P450 reductase[1] belongs to a relatively small family of diflavin enzymes which are believed to have evolved as a result of a gene fusion event between ancestral FMN and FAD-containing flavoproteins (*3*, *4*). Recently, two human cytoplasmic diflavin reductases have been discovered, methionine synthase reductase (*5*) and NR1 (*6*), which contain the same domain organization as P450 reductase but lack the membrane anchoring sequence. Other members of the family include the flavoprotein domains of nitric oxide synthase and *Bacillus megaterium* cytochrome P450 BM3, and the reductase subunit of bacterial sulfite reductase (*2*).

P450 reductase mediates the transfer of reducing equivalents from the two-electron donor, NADPH, to the one-electron acceptor, cytochrome P450, via FAD and FMN (*1*). Although its major role appears to be associated with cytochrome P450 and the phase I metabolism of xenobiotic compounds, P450 reductase also has the ability to reduce other exogenous electron acceptors such as cytochrome *c* (*7*), cytochrome b_5 (*8*), and heme oxygenase (*9*). In addition, it has recently been implicated, along with cytochrome b_5, in the bioreductive activation of methionine synthase (*10*), thus pointing to a possible role in the regulation of methionine synthesis. P450 reductase may also play a role in the bioactivation of therapeutic prodrugs through its ability to reduce a range of one-electron acceptors such as the quinone drugs doxorubicin (*11*), mitomycin *c* (*12*), and aromatic *N*-oxides such as the novel benzotriazine, tirapazamine (*13*). Evidence is also emerging that the reductase domain of nitric oxide synthases may similarly reduce such compounds (*14*).

The medium-resolution (2.6 Å) crystal structure of an N-terminally truncated P450 reductase from rat liver has been determined (*15*). This confirms the conclusions from se-

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[1] Abbreviations: P450 reductase, NADPH−cytochrome P450 oxidoreductase; NR1, novel reductase 1; TB, Terrific Broth.

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CPR	*178*	YE.H**F**NAM
NOS1	*889*	YP.H**F**CAF
MTRR	*201*	YT.Y**F**CN
NR1	*202*	YA.K**F**NF
BM3	*574*	WATT**Y**QKV
SR	*157*	YE.F**F**CQS
FDX	*98*	YE.Y**F**CGA

FIGURE 1: Sequence alignment of the region involved in binding the FMN *si*-face (*15*). CPR, human P450 reductase; NOS1, rat neuronal nitric oxide synthase; MTRR, human methionine synthase reductase; NR1, human novel reductase 1; BM3, P450 BM3; SR, *E. coli* sulfite reductase; FDX, *D. vulgaris* flavodoxin. The first amino acid is the residue corresponding to the human P450 reductase Tyr178. Phe181 and the corresponding residues in the other proteins are highlighted in bold.

quence comparisons that the FMN-binding domain is structurally homologous to flavodoxins and the FAD-binding domain to ferredoxin−$NADP^+$ reductase; the insert in the latter domain folds as a third domain in the reductase. The human P450 reductase has been resolved into its constituent domains and the FMN-binding and the FAD/NADPH domains have been individually expressed in *Escherichia coli* (*4*). The structure of the FMN domain has been determined by NMR (*16*) and by X-ray crystallography (*17*). These studies, along with biochemical and site-directed mutagenesis work, have identified the binding sites for the FMN, FAD, and NADPH cofactors and pointed to potential sites of redox partner interaction (*18*, *19*).

The FMN-binding domain is of particular interest as it is the part of the P450 reductase molecule that interacts with cytochrome P450 to transfer electrons during catalysis. As in flavodoxins, in P450 reductase the isoalloxazine ring of FMN is sandwiched between two aromatic side chains, Tyr140 and Tyr178 (*15*, *17*). Tyr178 lies parallel to the *si*-side of the FMN ring, while the second aromatic, Tyr140, located on the *re*-side, lies at an angle of ~40° with respect to the isoalloxazine ring. A similar arrangement is found in the FMN-binding domain of P450 BM3 and in most flavodoxins, apart from *Clostridium* flavodoxin where the *re*-side aromatic side chain residue is replaced with methionine (*20*).

In addition to these key residues, a third aromatic side chain, Phe181, lies close to the FMN isoalloxazine ring on the *si*-side at the pyrimidine end, although not in contact with it. This residue is highly conserved in P450 reductase and related enzymes (Figure 1), suggesting a possible role in FMN binding. To investigate the role of this conserved residue, we have replaced Phe181 with Tyr, Leu, and Gln. The biochemical and biophysical characterization of these mutant enzymes indicates that Phe181 plays an important role in stabilizing FMN cofactor binding.

MATERIALS AND METHODS

Materials. Molecular biology enzymes were purchased from Gibco Life Technologies, Inc. [γ-^{35}S]dATP was from Amersham Pharmacia Biotech. All other reagents were purchased from Sigma (Poole, Dorset, U.K.).

Mutagenesis. The cDNA for human NADPH−cytochrome P450 reductase was derived from a human skin fibroblast cDNA library (*4*). The template used for constructing the mutants, pJR4, has been previously described (*21*). It contains the full-length human reductase cDNA fused to an amino-terminal pelB leader sequence in the expression vector pCWori+, a derivative of pHSE (*22*). Mutagenesis was carried out essentially according to the method described by Kunkel (*23*). pJR4, which contains an f1 origin of replication, was transformed into *E. coli* CJ236 (*dut*−, *ung*−). Following infection with M13 KO7 helper phage, single-stranded template DNA was prepared by PEG precipitation of phage particles from culture supernatant and phenol/chloroform extraction according to standard protocols (*24*). Mutagenic oligonucleotides used in this study are listed in Table 1. A selection oligonucleotide, which introduces a silent *Eco*RI restriction site between nucleotides 274 and 277 (encoding Glu37 and Phe38), was also used in conjunction with mutant oligonucleotides to facilitate selection of mutant clones by *Eco*RI digestion. Correct generation of mutants was confirmed by sequencing.

Table 1: Oligonucleotides and Templates Used To Generate P450 Reductase Mutants[a]

	oligonucleotide
wild type	5′-CTTGCCCATGGCATTGAAGTGCTCGTAGGTCTT-GTTC-3′
H180F	5′-CTTGCCCATGGCATTGAAG<u>AA</u>CTCGTAGGTCTT-GTTC-3′
H180L	5′-CTTGCCCATGGCATTGAAG<u>A</u>GCTCGTAGGTCTT-GTTC-3′
H180Q	5′-CTTGCCCATGGCATTGAA<u>C</u>TGCTCGTAGGTCTT-GTTC-3′
F181Y	5′-CTTGCCCATGGCATTG<u>T</u>AGTGCTCGTAGGTCTT-GTTC-3′
F181L	5′-CTTGCCCATGGCATTGA<u>G</u>GTGCTCGTAGGTCTT-GTTC-3′
F181Q	5′-CTTGCCCATGGCATT<u>CTG</u>GTGCTCGTAGGTCTT-GTTC-3′

[a] The mutated sequences are underlined.

Purification of Reductase Mutants. Mutant plasmids were transformed into *E. coli* JM109 cells. Overnight cultures grown in an LB/ampicillin solution (50 μg/mL) at 37 °C were used to inoculate 1 L cultures of Terrific Broth (TB) with 50 mg/mL ampicillin. Cultures were grown at 37 °C to an OD_{600} between 0.6 and 1.0. Following the addition of isopropyl 1-thio-β-D-galactopyranoside (0.5 mM), cultures were grown at 30 °C for 18−24 h. Cells were then harvested by centrifugation and resuspended in 50 mL of 100 mM Tris-HCl, 500 mM sucrose, and 0.5 M EDTA (pH 7.6). Lysozyme was added to 0.25 mg/mL, and cells were incubated on ice with gentle shaking to remove the cell wall. Spheroplasts were pelleted by centrifugation at 2800*g* for 15 min at 4 °C and resuspended in 20 mL of 100 mM potassium phosphate, 6 mM magnesium acetate, 20% glycerol, and 0.1 mM dithiothreitol (pH 7.6). To prepare membrane fractions, spheroplasts were lysed by sonication with several short bursts (10 s) at full power using an MSE sonicator probe. Following a clearing spin at 10000*g* for 15 min at 4 °C, the cell supernatant was ultracentrifuged at 100000*g* using a Sorvall Ultra Pro centrifuge with an A641 rotor. The membrane pellet was resuspended in 30 mL of buffer A [20 mM Tris-HCl (pH 7.7), 10% glycerol, 0.1% Triton X, 1 mM EDTA, 0.1 mM dithiothreitol, and 1 mM PMSF], solubilized by mixing at 4 °C for 30 min, and ultracentrifuged at 100000*g*. Reductase was purified in a two-step process using anion exchange chromatography and affinity purification. The supernatant fraction was passed over a 5 mL Mono Q Hi-Trap column (Pharmacia), pre-equilibrated in buffer A,

and washed with 10 column volumes of buffer A. Column-bound proteins were removed using a step gradient consisting of 40 mL volumes of buffer A containing successively 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl. The reductase-containing fractions, which generally eluted with 0.3−0.4 M NaCl, were pooled and dialyzed overnight at 4 °C against a PBS/10% glycerol mixture. For affinity purification, samples were loaded onto 5 mL of 2′,5′-ADP-Sepharose (Sigma), pre-equilibrated in buffer B [20 mM Tris-HCl (pH 7.7), 150 mM NaCl, 10% glycerol, 1 mM EDTA, and 0.1 mM dithiothreitol]. The column was washed with 50 mL of buffer B and 50 mL of buffer B containing 0.5 M NaCl. Reductase was eluted with buffer B containing 0.5 M NaCl and 10 mM 2′,5′-AMP. Samples were concentrated to ∼2 mL using Centricon concentrators, and FMN was added to a final concentration of 50 μM. Samples were exchanged into a PBS/10% glycerol mixture, and free FMN removed, by size exclusion chromatography using Sephadex PD-10 columns (Pharmacia). Enzymes were stored at −70 °C.

Construction and Purification of FMN Domain Mutants. The FMN-binding domain from each of the reductase mutants was amplified by PCR and cloned into the expression vector pET15b (Novagen, Madison, WI) as described previously (*4*) with minor modifications. Briefly, oligonucleotide primers to the 5′ and 3′ ends of exons 3 and 7 encoding amino acid residues 61−241 were used. The 5′ oligonucleotide contained an *Nde*I restriction site and the 3′ oligonucleotide a *Bam*HI site to enable subcloning into the unique *Nde*I−*Bam*HI sites of the expression vector pET15b. These engineer a His_6 linker and thrombin cleavage site onto the N-terminus of the expressed proteins, enabling purification by nickel−agarose affinity chromatography. Mutant DNAs were transformed into *E. coli* BL21(DE3) plysS for expression. FMN domains were expressed and purified, and the histidine tag was removed by thrombin cleavage as described previously (*4*). Mutant yields were similar to that of the wild type, in the range of 20−30 mg/L of culture.

The uniformly ^{15}N-labeled Phe181Leu FMN-binding domain was expressed and purified as described previously for the wild-type domain (*16*), except that an excess of FMN was added before the cell extract was loaded onto the nickel−agarose column. The yield of purified labeled protein was approximately 10 mg/L of culture.

Activity Assays, Flavin Determination and Spectroscopy. All assays and incubations were carried out in 0.3 M potassium phosphate (pH 7.7) at 25 °C as described previously (*4*). FMN and FAD content was determined by HPLC analysis as previously described (*6*). Absorption spectra were obtained with a Shimadzu UV 2000 spectrophotometer.

For NMR spectroscopy, the wild-type and Phe181Leu FMN-binding domains were concentrated to 0.5−0.8 mM in 40 mM potassium phosphate (pH 6.8) containing 10% ^2H_2O, 0.2 mM DTT, and a molar excess of FMN. NMR experiments were performed at a sample temperature of 298 K, using Bruker DMX500 and DMX600 spectrometers. Assignments of the amide ^{1}H and ^{15}N resonances of the wild-type domain have been reported (*16*); assignment of the mutant was based on ^{1}H−^{15}N NOESY-HSQC and TOCSY-HSQC spectra, obtained using sensitivity-enhanced pulsed field gradient sequences (*25*).

FMN Binding. The apoprotein was prepared by precipitation with trichloroacetic acid essentially as described previously (*16*). The precipitated apoprotein was suspended in 40 mM phosphate (pH 7.4), 0.2 mM DTT, 0.02 mM EDTA, and 20% glycerol, and the solution was centrifuged at 10000*g* for 20 min to remove any undissolved material. A microtiter assay was used to assess FMN binding. In a typical experiment, 100 μL serial dilutions of the apoprotein (0.25−75 μM) in 0.75 μM FMN, 40 mM phosphate (pH 7.4), 0.2 mM DTT, 0.02 mM EDTA, and 20% glycerol were prepared using 96-well microtiter plates (Labsystems). Plates were preincubated at 4 °C for 30 min to allow the system to reach equilibrium. The FMN fluorescence was measured at 25 °C in a Microtiter plate reader (Labsystems); excitation was at 450 nm, and emission was recorded at 535 nm. The FMN that was used was >95% pure according to reverse phase FPLC. The fluorescence of FMN is quenched by protein binding, allowing the equilibrium constant for binding to the protein to be determined from the extent of fluorescence quenching by different concentrations of the apoFMN domain (*26*).

Measurement of Redox Potentials. Redox-dependent spectral changes and midpoint reduction potentials of FMN ($E^{\circ\prime}_1$ = ox/sq; $E^{\circ\prime}_2$ = sq/red) were determined using anaerobic spectroelectrochemistry, as previously described (*27*, *28*). Briefly, enzyme solutions (20−100 μM) were degassed and equilibrated by dialysis into 100 mM Tris-HCl (pH 7.0) under anaerobic conditions (<5 ppm oxygen) within a glovebox (Belle Technology). Spectra were recorded at 25 °C on a Shimadzu 1601 UV−visible spectrophotometer within the glovebox. The reduction potential of the solution was measured by immersion of a Calomel electrode into the enzyme solution (∼8−10 mL). Reduction was achieved by addition of small aliquots of sodium dithionite stock solutions (10 and 1 mM), made up in the same anaerobic buffer. Reoxidation was achieved by similar small additions of 10 and 1 mM stocks of potassium ferricyanide. The reduction potential and absorbance spectrum of the flavoenzyme solution were recorded after each addition of reductant and/or oxidant, after a period of equilibration (∼10 min). Equilibration of the system was expedited by addition of catalytic amounts of mediators to the solution: benzyl viologen and methyl viologen (1 μM), *N*-methylphenazine methosulfate (3 μM), hydroxynaphthoquinone (2 μM), and anthroquinone 2,6-disulfonate (2 μM). Measurements were taken in both reductive and oxidative directions to ensure there was no hysteresis, and at least two separate experiments were performed for each sample.

RESULTS

Enzyme Purification. A series of P450 reductase mutants were generated in which Phe181 and the adjacent His180 residue were substituted with other aromatic, aliphatic, and amide amino acids. The His180 mutants were generated essentially as controls, since the side chain of this residue points out into the solvent, away from the FMN. The enzymes were expressed in *E. coli* and purified using a two-step procedure. Some FMN cofactor is usually lost during the purification process; thus, a molar excess of FMN was added back to purified enzyme preparations following the final 2′,5′-ADP affinity step, and unbound FMN was removed by size exclusion chromatography. Typical yields

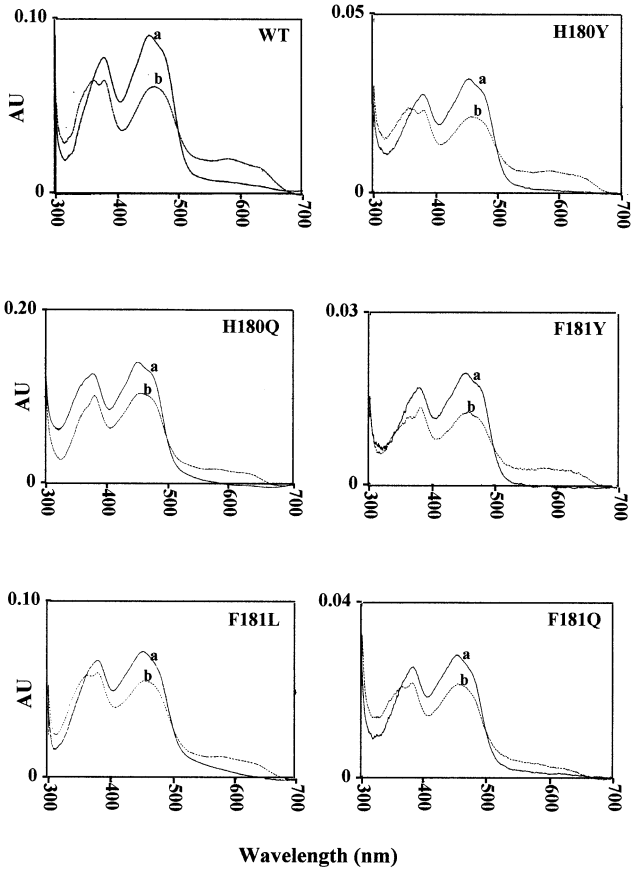

FIGURE 2: UV−visible absorbance spectra of the recombinant wild-type P450 reductase (WT) and His180Tyr (H180Y), His180Gln (H180Q), Phe181Tyr (F181Y), Phe181Leu (F181L), and Phe181Gln (F181Q) mutants. In each panel, the final spectrum of the oxidized (a) and reduced semiquinone (b) is shown. The reduced semiquinone was produced following the addition of a 5-fold molar excess of NADPH.

of full-length P450 reductase ranged between 2 and 4 mg of pure enzyme per liter of culture. The yields of mutant enzymes were similar, apart from His180Leu for which we were unable to produce a soluble recombinant enzyme, suggesting that this substitution significantly destabilizes the enzyme. Flavin analysis of purified enzymes showed that wild-type and mutant enzymes contained a normal 1:1 FMN:FAD ratio. The visible absorption spectrum of each mutant was determined following addition of NADPH (Figure 2). The overall spectral characteristics of the mutant enzymes were similar to those of the wild type. The oxidized enzymes had absorption maxima at 275, 380, and 455 nm. The addition of NADPH caused flavin reduction, with a decrease in absorption in the 450 nm region and the appearance of a broad band at 584 nm, indicative of neutral blue semiquinone on one or both flavins (see also ref *29*).

Reduction of Cytochrome c and Ferricyanide by Mutant Enzymes. The effects of the mutations on enzyme activity were measured by using as electron acceptors cytochrome *c*, which measures the extent of electron flow from the FMN domain, and potassium ferricyanide, which measures the extent of direct transfer of electrons from FAD (*30*, *31*). The measured specific activities are summarized in Table 2. Substitution of the histidine residue or replacing Phe181 with an aromatic tyrosine residue did not significantly affect the rates of either cytochrome *c* or ferricyanide reduction.

Table 2: Cytochrome *c* and Ferricyanide Reductase Activities of P450 Reductase Mutants[a]

	cytochrome *c*		ferricyanide	
	without FMN	with FMN[b]	without FMN	with FMN
wild type	15 ± 1	17 ± 0.5	26 ± 2	22 ± 3
H180F	22 ± 1	21 ± 2	35 ± 11	33 ± 5
H180Q	17 ± 1	17 ± 1	30 ± 8	34 ± 8
F181Y	14 ± 1	17 ± 1	31 ± 6	29 ± 9
F181L	8 ± 1	8 ± 0.5	28 ± 6	28 ± 9
F181Q	8 ± 0.5	9 ± 0.5	26 ± 4	26 ± 5

[a] Specific activity is expressed as the number micromoles of substrate reduced per minute per milligram of enzyme. Values represent the means and standard deviations of three assays. Measurements were performed at 25 °C. [b] Assays carried out in the presence of 50 μM FMN.

Table 3: Kinetic Analysis of Cytochrome *c* Reduction[a]

	k_{cat} (min^{-1})	K_M (μM)		k_{cat} (min^{-1})	K_M (μM)
wild type	857 ± 105	16 ± 6	F181Y	857 ± 67	20 ± 5
H180F	714 ± 173	17 ± 13	F181L	357 ± 41	19 ± 7
H180Q	786 ± 94	22 ± 8	F181Q	500 ± 38	16 ± 4

[a] Reactions were carried out in 0.3 M potassium phosphate, 50 mM NADPH, 50 μM free FMN, and varying amounts of cytochrome *c* (0.01−200 mM). Reactions were initiated by the addition of NADPH following preincubation for 2 min at 25 °C. Values are expressed as the means and standard deviations of three experiments.

However, the cytochrome *c* reductase activities of the Phe181Leu and Phe181Gln mutants were decreased by ~50% compared to that of the wild type, while the ferricyanide reduction rates were unaffected. Activities were not significantly altered by the addition of an excess of the FMN cofactor to the assay solutions. The steady-state kinetic constants of the mutant enzymes for cytochrome *c* reduction, summarized in Table 3, show that the decreased activity of the Phe181Leu and Phe181Gln mutants arises from a decrease in k_{cat}, the K_M value being essentially unaffected. The K_M values obtained for the wild-type and mutant enzymes are in the range of 5−22 μM reported for mammalian P450 reductases (*7*, *18*, *32*, *33*).

Redox Potential and FMN Binding. FMN-binding domain constructs were used to analyze the effects of mutations of Phe181 on the binding of FMN and the redox potential of the bound FMN. We have previously shown that the FMN and FAD domains can be dissected and expressed separately to produce correctly folded, functional proteins (*4*, *16*, *17*, *29*, *34*). Mutant constructs were prepared containing residues 61−241 (exons 3−7) corresponding to the FMN domain region used for NMR (*16*) and crystallography (*17*). High levels of expression of the soluble protein were achieved in *E. coli*, and the domain was purified to homogeneity.

The midpoint redox potentials of the wild type and the F181 mutants of the FMN domain were determined using anaerobic spectroelectrochemistry, as previously described (*27*, *28*, *35*), and are shown in Table 4. The largest change occurred when phenylalanine was replaced with glutamine, resulting in more negative redox potentials than in the wild-type domain, particularly for the oxidized/semiquinone step (E_1, changed from −45 to −85 mV) but also to a lesser extent for the semiquinone/reduced step (E_2, changed from −282 to −297 mV). The flavin reduction potentials of the other mutant proteins were the same, within error, as those of the wild-type reductase.

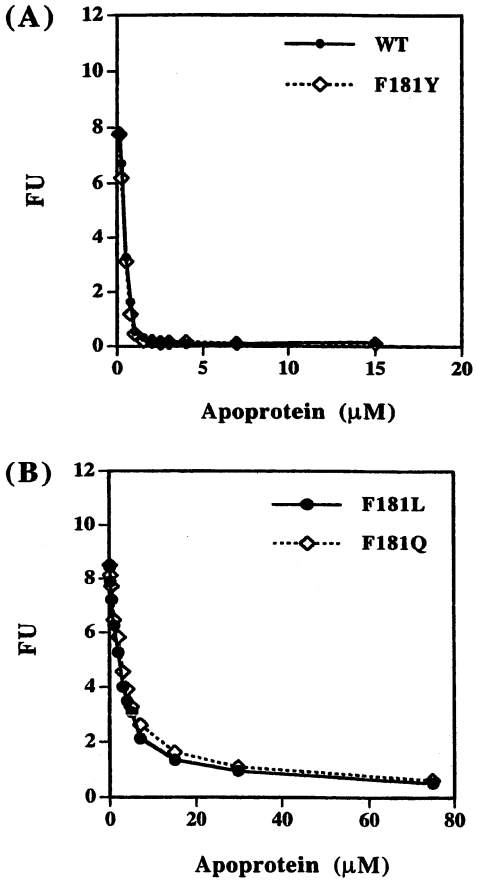

FIGURE 3: Determination of the dissociation constants for FMN and the apoFMN domains. An estimate of the K_d was obtained from the experimental points close to the equivalence point that lie off the linear parts of the titration curve. Reactions were carried out in 50 mM potassium phosphate at pH 7.0 and 25 °C.

Table 4: Midpoint Redox Potentials of Wild-Type and Mutant FMN Domains

	E_1 (ox/sq) (mV vs SHE)	E_2 (sq/red) (mV vs SHE)	E_{12} (ox/red) (mV vs SHE)
wild type	−45.2 ± 4.0	−281.6 ± 4.6	−163.4 ± 4.3
F181Y	−45.3 ± 4.1	−285.2 ± 4.0	−165.3 ± 4.0
F181L	−42.3 ± 5.2	−289.1 ± 5.6	−165.7 ± 5.4
F181Q	−84.6 ± 4.7	−297.4 ± 6.9	−191.0 ± 5.8

To assess the effects on cofactor binding, FMN was removed by unfolding and refolding the protein to produce the apo form. We have previously shown by NMR spectroscopy (*16*) that the apoprotein obtained in this way has a folded structure substantially similar to that of the holoprotein, as established in more detail for flavodoxins (*36*). The apoFMN domain was added to oxidized FMN to obtain an estimate of the K_d for the complex as shown in Figure 3. The value obtained for the wild-type FMN domain was 4×10^{-8} M, comparable to the published value of 2×10^{-8} M for the whole reductase (*26*). Replacement of Phe181 with tyrosine had little effect on FMN binding, but replacement with a nonaromatic residue led to an approximately 50-fold decrease in affinity, to $170-210 \times 10^{-8}$ M (Table 5).

The affinities of binding of the protein for the three redox forms of FMN are thermodynamically linked to the redox potentials (*37*). Thus, using the measured redox potentials of the wild-type and mutant domains and the measured dissociation constants for the complexes with oxidized FMN, the binding energies for all three redox forms for binding to wild-type and mutant domains can be derived. The free energy changes on binding (ΔG_{ox}, ΔG_{sq}, and ΔG_{red}) are shown in Table 5. For the wild type and all the mutant proteins, the most stable complex is that with reduced FMN, while the semiquinone formed the least stable complexes. The complexes of the Phe181Leu and Phe181Gln mutants with oxidized and semiquinone FMN were similarly destabilized by 2.2−2.5 kcal/mol relative to the wild type (Table 5). However, the reduced FMN complex of the Phe181Gln mutant was more destabilized than that of the Phe181Leu mutant (3.5 vs 2.5 kcal/mol, respectively, relative to that of the wild type), reflecting the more negative redox potential of the oxidized/semiquinone (E_1) step. Thus, replacement of Phe181 with a nonaromatic residue reduces the affinity of the protein for FMN.

Table 5: Dissociation Constants of Oxidized FMN−ApoFMN Domain Complexes and Free Energies of the Corresponding Oxidized, Semireduced, and Reduced Complexes[a]

	K_d (μM)	ΔG_{ox} (kcal/mol)	ΔG_{sq} (kcal/mol)	ΔG_{red} (kcal/mol)
wild type	0.042 ± 0.007	−10.06 ± 1.7	−9.06 ± 1.5	−11.96 ± 1.9
F181Y	0.039 ± 0.012	−10.11 ± 3.0	−9.02 ± 2.7	−11.92 ± 3.6
F181L	1.715 ± 0.307	−7.87 ± 1.4	−6.69 ± 1.2	−9.66 ± 1.7
F181Q	2.089 ± 0.287	−7.75 ± 1.1	−6.39 ± 0.9	−8.38 ± 1.2

[a] Values were determined from the fluorometric titrations of FMN with wild-type or mutant apoFMN domains shown in Figure 3. They are the means and standard deviations from at least three points that lie off the linear points of the titration curve. Free energy values were calculated from the equations $\Delta G_{ox} = RT \ln K_d$, $\Delta G_{sq} = \Delta G_{ox} - F(E_2 - E_{2free})$, and $\Delta G_{red} = \Delta G_{ox} - F(E_2 + E_1 - E_{2free} - E_{1free})$ (*42*), where R, T, and F are the gas constant, temperature in kelvin, and Faraday constant, respectively.

NMR Spectroscopy. Since the side chain of Phe181 does not make contact with the bound FMN in the structure of the intact reductase (*15*) or that of the isolated FMN-binding domain (*17*), we used NMR spectroscopy to obtain an indication of the extent of any structural changes which might result from the Phe181Leu substitution and perhaps account for the observed functional effects. After the protein sample had been exchanged into NMR buffer and concentrated, the $^1H-^{15}N$ HSQC spectrum of the Phe181Leu mutant contained a number of broad signals in addition to the majority of sharp cross-peaks. Addition of 2 molar equiv of FMN led to a marked sharpening of the broad signals, to give a well-resolved spectrum with uniform line widths (Figure 4). This is consistent with a partial loss of FMN from the mutant during sample preparation.

Comparison of the HSQC spectra of the wild-type and Phe181Leu domains (Figure 4) shows that the majority of the amide resonances of the protein are unaffected by the substitution, but a limited number show significant changes of up to 0.5 ppm in the ^1H dimension and up to 2.5 ppm in the ^{15}N dimension. This indicates that the overall fold of the protein is preserved in the mutant, but suggests that there are some more or less local structural changes. The resonances of the mutant domain were assigned by using NOESY-HSQC and TOCSY-HSQC experiments, allowing a detailed comparison of the chemical shifts of the wild-type and mutant proteins and the identification of the regions of the domain affected by the substitution.

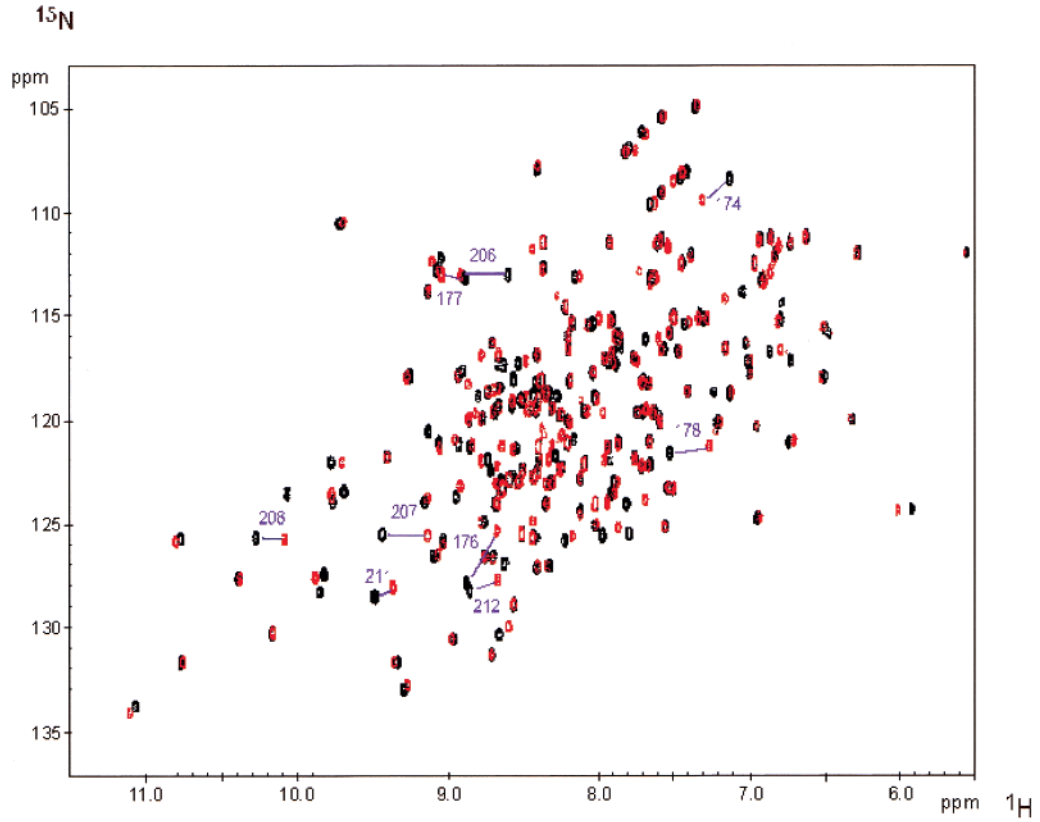

FIGURE 4: $^{1}H-^{15}N$ HSQC spectra of wild-type (black) and Phe181Leu (red) FMN domains of human P450 reductase. Differences in the chemical shifts of a number of individual residues are indicated.

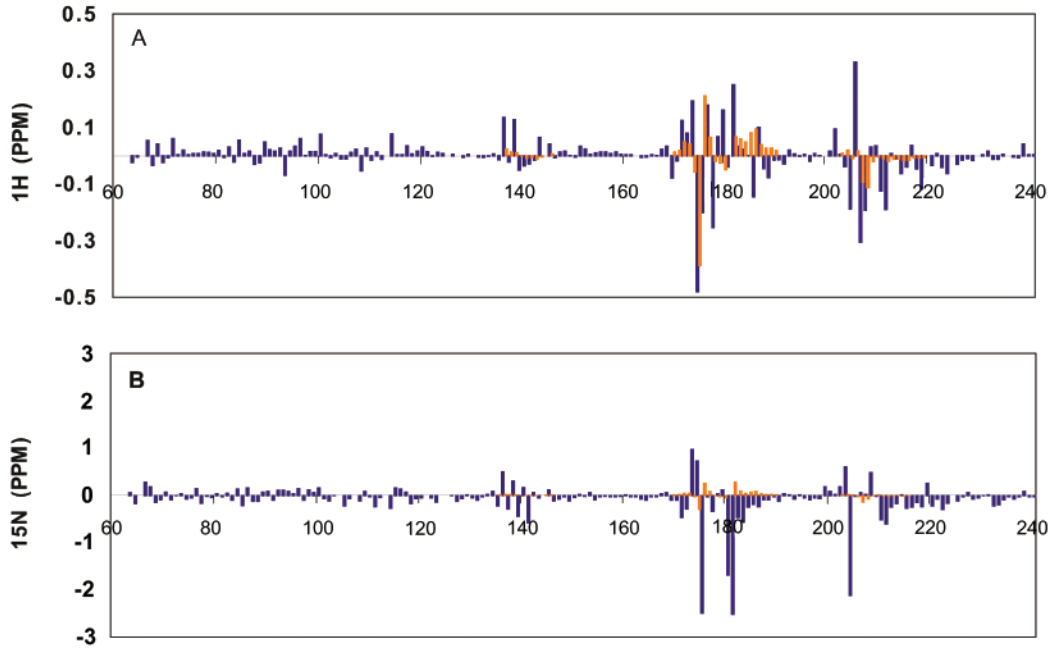

FIGURE 5: Differences in the ^{1}H and ^{15}N chemical shifts (blue) between wild-type and Phe181Leu FMN domains as a function of residue number. The orange bars show the calculated changes in chemical shift (arising from ring current effects) expected if the phenyl ring of Phe181 were removed with no other change in structure.

The chemical shift changes are summarized in Figure 5; the most affected residues can be seen to fall into three regions of the protein sequence: residues 170−184 (around the site of mutation), residues 202−224, and residues 137−144 (smaller effects). Since we have replaced an aromatic residue with an aliphatic one, chemical shift changes would be expected even in the absence of any structural change. We, therefore, calculated the effects expected on the amide chemical shifts if the phenyl ring of Phe181 was removed without any other changes in the structure of the protein (Figure 5). These effects were large only for residues very close to the substitution (residues 175 and 176), but significant effects (>0.05 ppm for ^{1}H) were predicted for a number of residues (174−186, 207, and 208). It is clear that

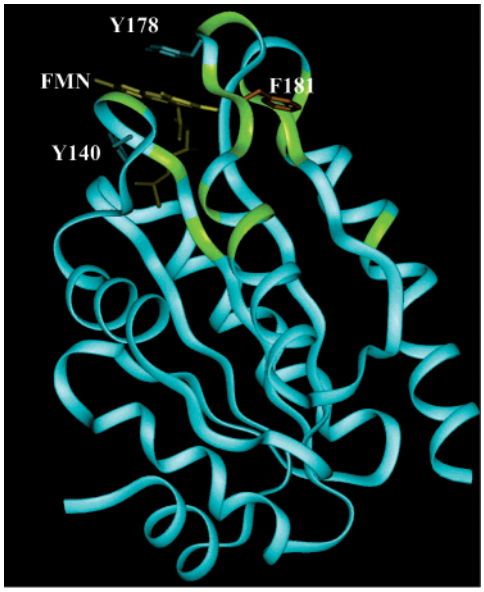

FIGURE 6: Structure of the FMN domain of human P450 reductase, showing in green the residues showing significant changes in amide chemical shift on substitution of Phe181 with leucine.

the simple absence of the aromatic ring and its magnetic anisotropy cannot account for the observed effects; indeed, in many instances, the calculated shifts were in the opposite direction with respect to the observed shifts. We conclude that some degree of structural change must be involved. The residues likely to be involved in this change, as indicated by the changes in the amide chemical shift, are indicated on a diagram of the FMN domain in Figure 6. It can be seen that they comprise parts of β-strand 4 and α-helix 6 (*17*) and the intervening loop (residues 175−182) which includes both Phe181 and the key FMN-binding residue Tyr178, part of β-strand 5 and the following loop, and residues 137−144 near the *re*-face of the isoalloxazine ring and Tyr140.

DISCUSSION

The essential features of the FMN binding site are highly conserved in human P450 reductase and related enzymes (*17*), and the predominant role of aromatic interactions, particularly Tyr178 at the *si*-face and Tyr140 at the *re*-face of the flavin, has been well documented (*15*, *18*). However, there is also a third highly conserved aromatic residue, Phe181, raising the question of whether this side chain could also play a significant role in FMN binding. To investigate this further, mutants were constructed and the effects on catalytic activity, FMN binding, and redox properties were analyzed. Following the substitution of Phe181 with non-aromatic residues, the enzyme activity with cytochrome *c* as an electron acceptor was decreased by ∼50%. Although this is not a particularly large difference, ferricyanide reduction was unaffected, thus pointing toward a specific detrimental effect of the mutation on electron transfer via the FMN domain.

The major effect associated with the substitution of the Phe181 residue is on the FMN binding characteristics of the enzyme. Both glutamine and leucine mutants show a 50-fold increase in K_d for FMN (Table 5), corresponding to a ΔG of 2.3 kcal/mol. Reduction potential measurements indicate that there is only minimal perturbation of the redox properties of the FMN in all of the human CPR FMN domain F181 mutants (Table 4). The potentiometry experiments were performed at flavoprotein concentrations of ∼25 μM, well in excess of the K_d values for FMN binding to wild-type and mutant proteins (K_d values in range of 0.04−2.1 μM). This means that even in the case of mutant F181Q (which shows the weakest binding of FMN) more than 90% of the FMN will be bound to the enzyme under the conditions used for potentiometric titrations. From this, we can conclude that the major role of F181 in the CPR structure is to stabilize binding of the FMN cofactor. It is notable that there is a small change in the two-electron reduction potential of the flavin in mutant F181Q ($E_{12} = -191.0 \pm 5.8$ vs -161.5 ± 7.3 mV for the wild type), explained largely by the effect on the oxidized/semiquinone couple ($E_1 = 84.6 \pm 4.7$ vs -43.0 ± 7.0 mV for the wild type). Since the vast majority of the FMN remains bound to the F181Q mutant, this small effect on the stability of the semiquinone form may be explained by interactions between the side chain amide of the glutamine in F181Q and the flavin ring system.

The results are completely consistent with FMN stabilization as the primary role of residue F181 in human cytochrome P450 reductase. Conservative replacement of the phenylalanine with tyrosine leads to only a minor increase in the dissociation constant for FMN (to 0.05 μM), but removal of the aromatic ring system (F181L) or introduction of an amine group (F181Q) results in approximately 50-fold increases in K_d (Table 4). Potentiometric data indicate that, under conditions in which we expect >95% of the FMN to be enzyme-bound, the reduction potentials for the flavins are not altered significantly in mutants F181Y and F181L, and changed only significantly for the oxidized/semiquinone couple in mutant F181Q. The fact that the reduction potential of the FMN in F181Q becomes slightly more negative ($E_{12} = -191.0 \pm 5.8$ vs -161.5 ± 7.3 mV for the wild type) indicates that there is a thermodynamically stronger driving force for electron transfer from the flavin to the heme of the P450s with which the CPR interacts. Therefore, we would expect this mutant to be adversely affected in catalytic properties only through its poorer ability to bind FMN, and not because of effects on the redox properties of the flavin system.

The FMN cofactor binds in a shallow groove at the C-terminal end of the β-strands in the FMN-binding domain (*15*−*17*). The isoalloxazine ring interacts with residues 140−148 and 175−183 and is sandwiched between the aromatic residues of Tyr140 and Tyr178 at the C-terminal ends of strands β3 and β4, respectively (*16*). The "outer" Tyr178 residue lies parallel to the isoalloxazine ring, while the "inner" Tyr140 lies at an angle of ∼40°. By contrast, the aromatic ring side chain of Phe181 does not interact directly with FMN but faces away from the FMN isoalloxazine ring (Figure 6). This side chain is packed in an environment defined by the side chains of Asn182, Gly174, and Asp207, and the main chain of residues 174−176 (Figure 7). The FMN isoalloxazine ring is held in place through hydrogen bond interactions between its N1, O2, N3, and O4 atoms and the backbone atoms of residues Asn175, His180, Asn182, and Gly162, three of which are in the same β4−α6 loop as Phe181 and the outer Tyr178 which is known to be important for FMN binding, and it is possible that packing

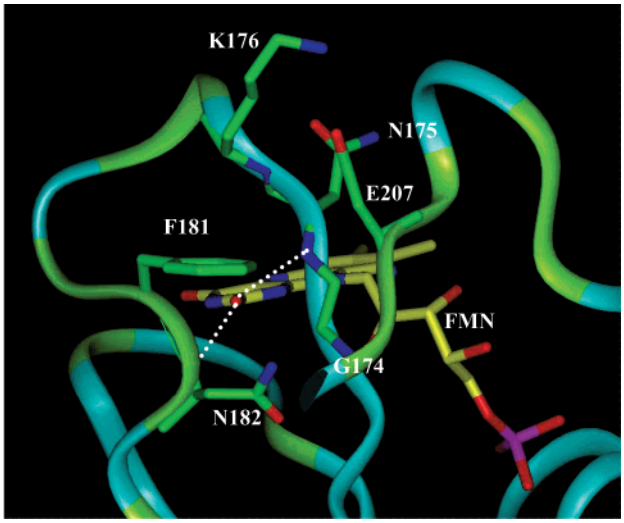

FIGURE 7: Environment of the Phe181 binding site in human P450. The dashed line shows the hydrogen bonds between the O2 atom of FMN and Asn122 and Asn175. The FMN molecule and residue side chains discussed in the text are denoted. The residues showing significant changes in amide chemical shift on substitution of Phe181 with leucine are shown in green. The ribbon figure was generated from PDB entry 1b1c using InsightII.

of the Phe181 side chain is important for these interactions between the FMN and the protein.

Some support for this comes from the changes in amide chemical shift seen in the NMR spectra. The majority of residues affected by the Phe181Leu substitution are in the β4−α6 and adjacent β5−α7 loops, consistent with a local change in structure arising from the change in packing on the phenylalanine to leucine substitution. This structural change might be expected to affect residues 175, 178, and 182 which interact directly with the FMN, and indeed the backbone amides of Asn175 and Asn182, which form hydrogen bonds with FMN O2, show changes in ^{1}H and ^{15}N chemical shifts in the mutant as compared to the wild-type FMN domain.

Changes in these interactions might weaken the overall interaction between FMN and the enzyme, as observed experimentally, and are also likely to lead to a modest change in the exact position of the isoalloxazine ring in the binding site which might contribute to the decrease in catalytic activity and to the changes in the chemical shifts of residues 137−144 near the *re*-face of the isoalloxazine ring. In this context, it is interesting that replacement of Tyr140 with aspartate increases the K_d for FMN binding and decreases enzyme activity ~5-fold (*18*), effects qualitatively similar to those of the Phe181 substitutions reported here. It is possible that subtle changes in the positioning of FMN could have a detrimental effect on the efficiency of electron transfer either from FAD to FMN or from FMN to the redox partner. This might explain how a decreased affinity for FMN can result in a decrease in K_{cat} for cytochrome *c* in the Phe181 mutants. In this regard, it is important to note that interactions with cytochrome P450, the physiological partner of P450 reductase, are currently being investigated, and we have found that nonconservative substitutions of Phe181 also result in decreased P450 reductase activities (data not shown).

The same arrangement of aromatic residues (two "sandwiching" the isoalloxazine ring and a third corresponding to Phe181) is found in the crystal structures of rat P450 reductase (*15*), bacterial P450 BM3 (*38*), and bacterial flavodoxins, including those from *Anaboena* 7120 (*39*), *Ancystis nidulans* (*40*), and *Desulfovibrio vulgaris* (*41*). This suggests a similar role for the aromatic residue in position 181 in these molecules as well. A notable exception is the *Clostridium beijerinckii* flavodoxin (*20*) which contains a nonaromatic glycine residue in the position homologous to Phe181. However, inspection of the crystal structure reveals the existence of a possible alternative aromatic residue, Tyr88, which occupies a position in three-dimensional space similar to that of Phe181.

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