Human Cytochrome P450 Oxidoreductase Deficiency Caused by the Y181D Mutation: Molecular Consequences and Rescue of Defect^[S]

Christopher C. Marohnic, Satya P. Panda, Karen McCammon, José Rueff, Bettie Sue Siler Masters, and Michel Kranendonk

Department of Biochemistry, the University of Texas Health Science Center at San Antonio, San Antonio, Texas (C.C.M., S.P.P., K.M., B.S.S.M.); and Department of Genetics/Human Molecular Genetics Research Center, Faculty of Medical Sciences, Universidade Nova de Lisboa, Lisbon, Portugal (J.R., M.K.)

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ABSTRACT:

Patients with congenital adrenal hyperplasia, exhibiting combined CYP17 and CYP21 deficiency, were shown by Arlt et al. (2004) to harbor a 541T→G mutation in exon 5 of *POR* (encoding NADPH-cytochrome P450 reductase, CYPOR), which resulted in a Y181D substitution that obliterated electron transfer capacity. Using bacterial expression models, we examined catalytic and physical properties of the human CYPOR Y181D variant. As purified, Y181D lacked flavin mononucleotide (FMN) and NADPH-cytochrome c reductase (NCR) activity but retained normal flavin adenine dinucleotide binding and NADPH utilization. Titration of the purified protein with FMN restored 64% of wild-type (WT) NCR activity in Y181D with an activation constant of ~2 μ M. As determined by FMN fluorescence quenching, Y181D had $K_d^{FMN} = 7.3 \mu$ M. Biplasmid coexpression of CYPOR and CYP1A2, at the physiological ratio of ~1:10 in the engineered

MK_1A2_POR *Escherichia coli* strain, showed the compromised capacity of Y181D to support CYP1A2-catalyzed metabolism of the procarcinogens 2-aminoanthracene, 2-amino-3-methylimidazo(4,5-*f*)quinoline, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Isolated MK1A2_POR membranes confirmed FMN stimulation of Y181D NCR activity with a 1.6 μ M activation constant. CYP1A2 ethoxyresorufin-O-dealkylase activity of the MK1A2_POR^{Y181D} membranes, undetectable in the absence of added FMN, increased to 37% of MK1A2_POR^{WT} membranes with a 1.2 μ M FMN activation constant. Therefore, we conclude that compromised FMN binding is the specific molecular defect causing *POR* deficiency in patients with Y181D mutation and that this defect, in large part, can be overcome in vitro by FMN addition.

Various developmental abnormalities and medical conditions are attributed to metabolic deficiencies that arise from variation in *POR*, encoding NADPH-cytochrome P450 oxidoreductase [(CYPOR) E.C. 1.6.2.4]. CYPOR activity, mediated by flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) cofactors, is essential to the functions of a number of important microsomal enzymes. Fifty type II cytochrome P450 (P450) monooxygenases require electrons

from CYPOR to catalyze steps in xenobiotic metabolism and developmental biochemical pathways, including sterol metabolism, retinoid homeostasis, and androgen/glucocorticoid synthesis. In addition, heme oxygenases (Schacter et al., 1972), squalene monooxygenase (Ono and Bloch, 1975), and 7-dehydrocholesterol reductase (Nishino and Ishibashi, 2000) require electrons from CYPOR.

POR deficiency (Online Mendelian Inheritance in Man number 201750) encompasses a number of aberrant steroidogenic phenotypes that range widely from the birth defects (midface hypoplasia, skeletal malformations, and ambiguous genitalia) associated with Antley-Bixler syndrome (Antley and Bixler, 1975) to polycystic ovary syndrome (Miller, 2004). In light of the number of *POR* mutations (>50) and polymorphisms (>300) reported within the past few years (Adachi et al., 2004; Arlt et al., 2004; Flück et al., 2004; Fukami et al., 2005; Huang et al., 2005; Homma et al., 2006; Dhir et al., 2007; Hart et al., 2007, 2008; Agrawal et al., 2008; Gomes et al., 2009), it seems likely that further clinical manifestations of *POR* variation will be

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ABBREVIATIONS: CYPOR, NADPH-cytochrome P450 oxidoreductase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; P450, cytochrome P450; NCR, NADPH-cytochrome c reductase; WT, wild-type; PAGE, polyacrylamide gel electrophoresis; BCA, bicinchoninic acid; HPLC, high-performance liquid chromatography; CD, circular dichroism; NFR, NADPH-ferricyanide reductase; EROD, ethoxyresorufin-O-dealkylase; 2AA, 2-aminoanthracene; IQ, 2-amino-3-methylimidazo(4,5-*f*)quinoline; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

revealed in patients as a result of altered responses to xenobiotic exposure, including therapeutic drugs.

Reporting on three patients with congenital adrenal hyperplasia, Arlt et al. (2004) found altered urinary sterol profiles indicative of combined CYP17 and CYP21 deficiencies but no mutations in either *CYP* gene, leading them to pursue *POR* genetics. Two of the patients had a 531T→G mutation in *POR* exon 5, encoding a CYPOR Y181D substitution. In the same study, a bacterially expressed Y181D protein, lacking 46 N-terminal residues, was devoid of measurable NADPH-cytochrome *c* reductase (NCR) activity. The capacity of heterologously expressed CYPOR Y181D to support mitomycin C-induced apoptosis in a cell culture model was further shown to be completely diminished compared with wild-type (WT) CYPOR (Wang et al., 2007).

Bacterial membranes, containing human CYPOR Y181D (truncated by 27 N-terminal residues), were devoid of detectable NCR activity and were unable to support 17 α -hydroxylase and 17,20 lyase activities of CYP17A1 (Huang et al., 2005). Likewise, CYP1A2 and CYP2C19 activities were not supported by Y181D (Agrawal et al., 2008).

Shen et al. (1989) previously characterized the orthologous Y178D variant of rat liver CYPOR using a bacterially expressed construct. FMN incorporation and NCR activity were both abolished by the mutation. A maximum of ~17% of WT NCR activity could be restored on addition of FMN, with half-maximal activation achieved at [FMN] = 3.4 μ M.

The structure of the full cytosolic catalytic domain of rat CYPOR (Wang et al., 1997), as well as that of the FMN binding domain of human CYPOR (Zhao et al., 1999), shows that in the WT enzyme, Y181 forms stacking interactions with the *si*-face of FMN (Fig. 1). Substitution of the tyrosyl phenol with the carboxylic acid side chain of aspartate would remove a large hydrophobic surface interaction and introduce a strong negative charge to the FMN binding site with deleterious implications for FMN incorporation, oxidation/reduction cycling, and docking interactions with acidic surface residues of microsomal electron acceptors.

FIG. 1. Structural alignment of the FMN binding domain of rat CYPOR [Protein Data Bank = 1amo (Wang et al., 1997), rendered in gray] with that of human CYPOR [Protein Data Bank = 1blc (Zhao et al., 1999), rendered in blue] with root mean square deviation = 0.27 Å. Human residue Y143, in green (orthologous rat Y140 in cyan), is positioned behind the *re*-face of FMN. Human Y181, in red (orthologous rat Y178 in magenta), forms a stacking interaction with the *si*-face of FMN. Figure was rendered by using PyMOL (De Lano, 2002).

Recently solved crystal structures of a modified rat CYPOR variant (Hamdane et al., 2009) and of a yeast-human CYPOR chimera (Aigrain et al., 2009) have provided evidence for a mechanistic domain rearrangement, described as the “open-closed” transition, whereby the NADPH/FAD binding domain, held in close proximity to the membrane-anchored FMN binding domain in the closed state, moves up to ~90 Å away in the open state, subsequently exposing FMN to interactions with a multitude of microsomal electron-accepting protein partners. Although specific amino acids within the hinge region of the connecting domain have been implicated by deletion [Δ T²³⁶-E²³⁹] (Hamdane et al., 2009), the mechanisms triggering or actuating conformational dynamics are currently unresolved. The hypothesis of the conformational change driven by redox states of FAD and FMN, as well as by NADPH occupancy, necessitates a role for FMN with which Y181D substitution would likely interfere.

Here, we have characterized the deleterious nature of the Y181D mutation using purified human CYPOR combined with a bacterial model of the membrane-localized CYPOR-CYP1A2 functional interaction. The extent to which FMN binding is compromised by the mutation and rescue of function is described as a possible indication for therapeutic modalities.

Materials and Methods

Site-Directed Mutagenesis. The construction of bacterial expression plasmids encoding OmpA3-fused, full-length human CYPOR (holo) and N-terminally truncated human CYPOR (Δ 66), as well as the methods used to mutagenize them, have been described in detail previously (Marohnic et al., 2006). The oligonucleotides used to insert the 531T→G substitution, encoding Y181D, were as follows: (sense) 5'-ggg aac aag acc gat gag cac ttc aat gcc-3' and (antisense) 5'-ggc att gaa gtg ctc atc ggt ctt gtt ccc-3'.

Protein Expression and Purification. Histidine-tagged, soluble Δ 66 CYPOR proteins were bacterially expressed and purified as described previously (Marohnic et al., 2006). Membrane-bound CYPOR proteins were also expressed, detergent-extracted, and 2'5'ADP-Sepharose 4B (GE Healthcare, Uppsala, Sweden) affinity-purified as described previously (Marohnic et al., 2006; Huber et al., 2009). The purity of each preparation was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Fractions exhibiting single-band purity were pooled and concentrated (Centriprep 30; Millipore Corporation, Billerica, MA) before quantification by oxidized flavin absorbance (total flavin $\epsilon_{454\text{ nm}} = 21.4\text{ mM}^{-1}\text{cm}^{-1}$) and by the micro-bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) according to standard protocol. Aliquots were stored under liquid N₂.

Flavin Content Analysis. Flavins were extracted into water from 50 μ M protein samples by boiling for 5 min. Boiled samples were immediately chilled on ice before centrifugation at 14,000g for 10 min to precipitate aggregated protein. Samples were then diluted 5-fold in mobile phase before high-performance liquid chromatography (HPLC) analysis using a Hewlett-Packard (Palo Alto, CA) Series 1100 HPLC equipped with a G1316A diode-array detector. A Thermo Fisher Scientific (Waltham, MA) Hypersil Gold column (150 $\times$ 2.1 mm, 5 μ m) was fitted with a guard column packed with Nova-Pak C18 Guard-Pak inserts (Waters, Milford, MA). The column was equilibrated with the mobile phase buffer (filtered and degassed) consisting of 94% 10 mM (NH₄)₂HPO₄, pH 5.5, and 6% acetonitrile. The flow rate was 0.75 ml/min, and flavins were monitored at 473 nm with $\epsilon^{\text{FAD}} = 10.1\text{ mM}^{-1}\text{cm}^{-1}$ and $\epsilon^{\text{FMN}} = 8.0\text{ mM}^{-1}\text{cm}^{-1}$. Extinction coefficients were determined by measurement of standards contained in the mobile phase buffer. Integration and analysis were performed using Agilent Technologies (Santa Clara, CA) ChemStation software (Rev. B.03.02).

Spectroscopic Analysis. UV/visible absorbance spectra ($\lambda = 250$ –900 nm) were recorded using an Agilent Technologies 8453 diode-array spectrophotometer. Circular dichroism (CD) spectra were recorded in the far UV ($\lambda = 180$ –300 nm) and near UV/visible ($\lambda = 300$ –600 nm) regions using a Jasco (Easton, MD) J815 spectropolarimeter. Far UV measurements were made of 0.7 μ M protein samples contained in 50 mM KPi, pH 7.0, in a quartz cylindrical cuvette with a path length of 0.1 cm. Near UV/visible measurements were made of 30 μ M protein samples contained in 50 mM MOPS, pH 7.0, in a quartz cylindrical cuvette with a path length of 1.0 cm.

Fluorometric Analysis of FMN Binding. FMN fluorescence quenching on equilibrium binding to Y181D $\Delta 66$ protein was measured as described previously (Vermilion and Coon, 1978) using a Horiba Jobin Yvon (Edison, NJ) FluoroMax-3 with $\lambda_{\text{ex}} = 450$ nm and $\lambda_{\text{em}} = 530$ nm, with a 100-ms integration time in antibleaching mode. At constant [enzyme], measurements were carried out while titrating a 2-ml sample in 50 mM KPi, pH 7.4, and 0.5 mM EDTA with FMN from 0 to 50 μM . After measurement of OD_{ex} and OD_{em} at each point in the titration, inner filter effects were corrected as described by Lakowicz (2006). A further correction was applied for dilution factor before plotting F_{corr} versus [FMN]. The difference in F_{corr} in the presence and absence of enzyme, ΔF , was plotted versus [FMN]. The resulting data were fitted to a single binding-site hyperbola using SigmaPlot (Systat Software, Inc., San Jose, CA) to estimate K_d .

Kinetic Analysis of Purified CYPOR Variants. 1) NADPH-ferricyanide reductase (NFR) measurements were made in triplicate in 96-well format using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA). Ferricyanide reduction was measured as the rate of decrease in absorbance at 420 nm using the extinction coefficient, $\epsilon_{420\text{ nm}} = 1.02\text{ mM}^{-1}\text{cm}^{-1}$ with [ferricyanide] = 1 mM. The reaction was contained in 50 mM KPi buffer, pH 7.0, [CYPOR] = 10 nM. [NADPH] was varied over a range from 0.1 to 200 μM . An NADPH-regenerating system was used, which consisted of 10 mM isocitrate, 0.5 units of isocitrate dehydrogenase, and 5 mM MgCl_2 . Reactions were monitored for 5 min, and rates were extrapolated from the linear range of the kinetic traces. Plots of rate versus NADPH concentration were fitted to the Michaelis-Menten equation, using SigmaPlot (Systat Software, Inc.), to determine k_{cat} and K_m values. 2) NCR activity was measured as described previously (Marohnic et al., 2006).

Construction of MK_1A2_POR Cell Models. Coexpression of CYPOR variants with CYP1A2 in the BMX100-derived MK_1A2_POR strain of *Escherichia coli* was achieved using a biphasic system described previously (Kranendonk et al., 1999). Plasmid pLCM_POR contained human WT *POR* cDNA, based on National Center for Biotechnology Information sequence NM_000941, encoding the CYPOR consensus sequence NP_000932, lacking the first three amino acids. This plasmid was modified to contain the mutagenized Y181D allele or no cDNA [plasmid pLCM (Kranendonk et al., 1998)]. The three plasmids were transfected into strain MK_1A2, already containing pCWhCYP1A2, the expression plasmid for human CYP1A2 (Fisher et al., 1992; Kranendonk et al., 1998), yielding the humanized bacterial cell models MK_1A2_POR^{WT}, MK_1A2_POR^{Y181D}, and MK_1A2_POR^{Null}, respectively. Culture and induction conditions, as well as membrane preparation procedures, were the same as reported previously (Kranendonk et al., 2008). CYP1A2 and CYPOR expression were quantified by reduced CO-difference spectroscopy and immunoblotting, respectively, also as previously reported (Kranendonk et al., 2008), substituting a monoclonal primary antibody to human CYPOR (Santa Cruz product sc-55477; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

MK_1A2_POR Enzyme Activities. NCR activity of the membranes was determined as described before (Kranendonk et al., 2008). Where indicated, FMN was added to a final concentration of 10 μM , before starting the reaction with NADPH. Activities are reported as the mean $\pm$ S.D. of triplicate determinations.

CYP1A2-mediated ethoxyresorufin-*O*-dealkylase (EROD) activity of membrane preparations was determined using the method described by Burke et al. (1994) in microplate format. Two types of EROD assays were performed, varying the ethoxyresorufin concentration ($\leq 5\text{ }\mu\text{M}$) either in the absence or presence of 10 μM FMN or varying the FMN concentrations with fixed ethoxyresorufin concentration (2.5 μM). The solvent dimethyl sulfoxide concentration was maintained at 0.2% (v/v) throughout. k_{cat} and K_m were extrapolated from a plot of the mean $\pm$ S.D., determined in triplicate, of reaction rate versus ethoxyresorufin concentration according to the Michaelis-Menten equation.

MK_1A2_POR Mutagenicity Assays. Whole cell mutagenicity assays were performed as described previously (Kranendonk et al., 2008). CYP1A2-mediated bioactivation is expressed in terms of mutagenic activity [L-arginine prototrophic (revertant) colonies per nanomole of compound or in revertant colonies per micromole of compound]. Mutagenicity values were determined from the linear portion of the dose-response curve, with each dose tested at least in triplicate.

Statistical Analysis. Statistical analysis was performed by applying the Student's *t* test, using GraphPad Software Inc. (San Diego, CA) software.

Results

Characterization of Purified CYPOR Variants. WT human CYPOR, as well as the mutated Y181D variant, was bacterially expressed and purified in both soluble ($\Delta 66$) and membrane anchor-containing (holo) isoforms. Each of the purified enzymes exhibited the predicted molecular mass on SDS-PAGE of 72 kDa for $\Delta 66$ and 77 kDa for holo (Supplemental Fig. 1).

Both WT isoforms, when purified, were green because of the protein-bound content of oxidized FAD (yellow) and air-stable FMN semiquinone (blue). In contrast, both Y181D isoforms were bright yellow on purification, suggesting either altered flavin content or flavin oxidation/reduction potentials. Representative $\Delta 66$ UV/visible spectra are shown in Supplemental Fig. 2. The visible fingerprint of air-stable neutral blue FMN semiquinone, a broad peak at approximately 600 nm, was apparent for WT but not for Y181D.

The concentration of each protein preparation was determined both as a function of oxidized flavin absorbance and by the micro-BCA assay (Pierce). Both methods yielded similar results for WT $\Delta 66$ (2.0 ± 0.2 mol flavin/mol protein) and holo (2.1 ± 0.2 mol flavin/mol protein) samples, indicating complete cofactor incorporation. Concentrations based on flavin absorbance were at least 2-fold lower than the corresponding values obtained by micro-BCA for Y181D samples with molar flavin/protein ratios of 1.0 ± 0.2 for Y181D $\Delta 66$ and 1.1 ± 0.2 for Y181D holo. These results suggested that Y181D proteins, as purified, were deficient in flavin.

Therefore, HPLC-based measurements of flavin content were performed (Fig. 2). FAD and FMN standards had retention times of ~ 4.7 and ~ 7.2 min, respectively. WT $\Delta 66$ exhibited the full complement of both FAD and FMN at a 1:1 ratio. Depending on the batch of protein tested, volume of buffers used in purification, and extent of postpurification dialysis, Y181D $\Delta 66$ had FMN content ranging from $<1\%$ up to nearly 10% of protein concentration but contained nearly the full complement of FAD ($\sim 95\%$ of protein concentration). In previous studies of FAD-deficient $\Delta 66$ CYPOR mutants (Y459H and V492E), overnight incubation with excess FAD resulted in increased FAD/protein ratios (Marohnic et al., 2006). In contrast, overnight incubation of Y181D $\Delta 66$ with 100-fold molar excess of FMN, followed by

Fig. 2. Flavin analysis: typical HPLC elution profiles of FAD and FMN standards are shown above those of flavins extracted from CYPOR $\Delta 66$ preparations of WT and Y181D variants. Integration of elution peaks was used to calculate FAD/FMN ratios.

separation of protein-bound and free flavins by gel filtration, yielded no measureable increase in protein-bound FMN content (data not shown), showing the strong contribution of FMN concentration to the equilibrium between FMN binding and dissociation from the Y181D-substituted protein. Considering the relatively small number of molecular contacts between CYPOR and FMN (compared with the CYPOR-FAD interaction), the loss of key hydrophobic/aromatic surface interactions between the phenol side-chain atoms of Y181 and the FMN isoalloxazine ring, as well as the introduction of a negatively charged carboxylic acid by substitution with aspartate, diminished the stability of the CYPOR-FMN interaction.

CD spectra were collected for each of the $\Delta 66$ samples in the both the far-UV (Fig. 3A) and near-UV/visible (Fig. 3B) regions. Comparison of WT and Y181D far-UV CD spectra suggested that Y181D substitution had no major effect on protein secondary structure. However, the absence of protein-bound FMN in the Y181D variant became apparent on comparison of near-UV/visible CD spectra of the $\Delta 66$ samples. WT CYPOR had negative CD bands centered at approximately 360 and 450 nm, indicative of flavin structural conformation and microenvironment polarity, with the strongly negative character of protein-bound FMN canceling out the positive character contributed by protein-bound FAD in the band at approximately 360 nm. The Y181D spectrum had a strong positive band centered at 375 nm, verifying the presence of bound FAD, but the negative band at approximately 450 nm (attributable to bound FMN) was weakened. Addition of 60 μM ($2 \times [\text{protein}]$) FMN to the WT sample had a small additive effect on the spectrum, contributing positive character at approximately 325 nm. In contrast, addition of 60 μM FMN to the Y181D sample had a more dramatic effect, causing the spectrum to

more closely resemble that of WT, with dual negative bands at 375 and 460 nm, presumably a result of FMN binding to the apoprotein. This finding contrasted with the previous observation that overnight treatment of Y181D with saturating FMN, followed by separation of bound/free flavins by size exclusion, yielded no observable increase in protein-bound FMN content. Taken together, these results emphasize the contribution of [FMN] to the equilibrium involving FMN association/dissociation within the Y181D protein.

Binding to CYPOR, as well as many other proteins, has been shown to quench the fluorescence of FMN as a result of stacking interactions with aromatic residues. Titrations of buffer and Y181D $\Delta 66$ were performed with increasing concentrations of FMN. After corrections for dilution and inner filter effects, FMN fluorescence (F_{corr}) and [FMN] displayed linear proportionality in buffer alone (Fig. 4A). When the titration was repeated in the presence of 10 μM Y181D $\Delta 66$ (Fig. 4A), the correlation between F_{corr} and [FMN] was a smooth dose-dependent curve. The difference in $F_{\text{corr}} \pm \text{protein}$ (FMN fluorescence quenching or ΔF) was plotted versus [FMN] (Fig. 4B), yielding a saturation curve with apparent $K_d = 7.3 \pm 1.1 \mu\text{M}$. This value represented a ~ 560 -fold increase compared with 13 nM, the value determined by Vermilion and Coon (1978) using the same method, and WT rat liver CYPOR, depleted of FMN by denaturation and refolding.

Having established the diminished capacity of purified Y181D $\Delta 66$

FIG. 3. CD spectroscopy: each spectrum is the average of three scans (see keys in insets). A, far-UV CD: spectra of $\Delta 66$ WT and Y181D at 0.7 μM protein. B, near-UV/visible CD: spectra of $\Delta 66$ WT and Y181D, as purified, at 30 μM total flavin concentration, and after addition of 60 μM FMN. The spectrum of free FMN (60 μM) is also shown.

FIG. 4. FMN fluorescence quenching. A, FMN fluorescence was measured as a function of [FMN] in the absence (buffer only) or presence of Y181D $\Delta 66$ protein. Measurements were made after 5-min equilibration after each FMN addition. Each point (F_{corr}) is the average $\pm$ S.D. of 10×100 -ms signal integrations, corrected for dilution and inner filter effects. The [FMN] range from 0 to 12 μM is expanded in the inset. The solid line is the best-fit of the buffer titration, whereas the dashed line is drawn to illustrate that the Y181D titration deviates significantly from linearity at low [FMN] but approaches linearity at high [FMN]. B, fluorescence quenching (ΔF) was calculated by subtraction of the free FMN F_{corr} (buffer only) from the F_{corr} measured in the presence of 10 μM Y181D $\Delta 66$ and was plotted versus [FMN] before single parameter hyperbolic curve-fitting ($R^2 = 0.981$) to determine K_d .

protein to maintain bound FMN, the catalytic effects of FMN deficiency were assayed. NFR activity was measured for WT and Y181D, both in the presence and absence of exogenous FMN (added to the reaction at 1000-fold molar excess). Michaelis-Menten parameters for both holo and $\Delta 66$ isoforms are reported in Table 1. Y181D $\Delta 66$ retained 80% of WT $\Delta 66$ NFR turnover (1980 min^{-1}) in the absence of added FMN, confirming previous observations in CYPOR that ferricyanide reductase activity is localized within the FAD domain (Vermilion and Coon, 1978). The Y181D $\Delta 66$ variant also exhibited a similar K_m^{NADPH} to WT $\Delta 66$, with respective values of 3.4 and 4.6 μM . Although both k_{cat} and K_m^{NADPH} of WT $\Delta 66$ were affected by FMN, the catalytic efficiency (k_{cat}/K_m) was not. In the Y181D $\Delta 66$ reaction, FMN addition decreased k_{cat} (34%) with no significant effect on K_m^{NADPH} . Among the holo isoforms, addition of FMN consistently decreased NFR activity without affecting NADPH utilization. With the exception of the WT $\Delta 66$ studies, these results suggested that FMN had an inhibitory effect that was not competitive with NADPH. Therefore, FMN was hypothesized to be acting as an electron-accepting substrate for both WT and Y181D $\Delta 66$ variants. In a preliminary study, reduction of a 20 μM solution of FMN was monitored at 446 nm under conditions of saturating NADPH (200 μM) in a cuvette open to the atmosphere. No activity was observed for either WT or Y181D regardless of enzyme concentration (up to 1 μM). Although it appeared from these studies that FMN was not acting as a substrate, NADPH consumption (at 340 nm) revealed that electron transfer was occurring, albeit slowly. WT was turning over NADPH at a rate of $4.6 \pm 0.4 \text{ min}^{-1}$ and Y181D at $5.9 \pm 0.6 \text{ min}^{-1}$. When the same reactions were measured in the absence of FMN, those rates decreased 6-fold ($0.8 \pm 0.2 \text{ min}^{-1}$) for WT and 8-fold ($0.7 \pm 0.3 \text{ min}^{-1}$) for Y181D. Based on these results, it was hypothesized that FMN was acting to promote NADPH oxidase activity in both WT and Y181D. When the same experiments were repeated under semiaerobic conditions in a sealed cuvette in buffer that had been partially scrubbed of oxygen, FMN reduction was observed after a short lag period, during which NADPH was consumed at the usual rate. On complete reduction of FMN, NADPH consumption also stopped. Leaving FMN out of the reaction mixture had the same effect under semiaerobic conditions as it did under aerobic conditions, the only difference being the excursion of NADPH consumption. FAD had an identical effect on both WT and Y181D. Cumulatively, these studies showed that free flavins promoted low levels of NADPH oxidase activity by purified CYPOR.

Having shown that NFR function was retained by the Y181D

variant, despite the absence of FMN, the thermal stability of the WT and Y181D $\Delta 66$ isoforms was evaluated by measurement of residual NFR activity after incubation of aliquots for 5 min at temperatures ranging from 10 to 100°C (Supplemental Fig. 3). The profiles of the two proteins were nearly indistinguishable with loss of 50% of NFR activity occurring between 48 and 52°C .

NCR activity, known for CYPOR to require protein-bound FMN, was also determined for both WT and Y181D variants. Michaelis-Menten parameters are reported in Table 1. WT $\Delta 66$ exhibited a $k_{\text{cat}}/K_m^{\text{NADPH}} = 220 \text{ min}^{-1} \mu\text{M}^{-1}$, whereas WT holo was nearly three times more efficient with a $k_{\text{cat}}/K_m^{\text{NADPH}} = 650 \text{ min}^{-1} \mu\text{M}^{-1}$. Reaction rates were beneath the limit of detection when either $\Delta 66$ or holo Y181D was assayed, in agreement with previously published findings using bacterially expressed, *N*-truncated proteins (Arlt et al., 2004; Huang et al., 2005; Wang et al., 2007). On the other hand, purified Y181D was shown by HPLC to retain a small proportion of bound FMN, which would hypothetically confer at least some small amount of NCR activity. However, the assay required large dilution of the purified enzymes before activity measurements, and fluorometric studies suggested that dilution would favor dissociation of FMN from the Y181D $\Delta 66$ protein. Addition of a 1000-fold molar excess of FMN to the reactions restored NCR activity to both Y181D isoforms. With a $k_{\text{cat}} = 420 \text{ min}^{-1}$ and $K_m^{\text{NADPH}} = 1.2 \mu\text{M}$, the FMN-stimulated Y181D $\Delta 66$ gained 53% of WT turnover and 159% of WT catalytic efficiency. Y181D holo was likewise activated by the addition of FMN with a catalytic efficiency of $290 \text{ min}^{-1} \mu\text{M}^{-1}$, 45% that of WT holo. Addition of FMN to WT NCR assays had an inhibitory effect ($\sim 20\%$).

To verify FMN K_d determination from fluorescent methods, saturation of NCR activity by FMN titration was measured for WT and Y181D $\Delta 66$ variants. The resulting titration curves are presented in Fig. 5. The WT enzyme was not significantly affected by FMN titration; therefore, no K_d could be determined. Using a single binding site model, the data for Y181D gave an apparent $K_d^{\text{FMN}} = 2.1 \pm 0.6 \mu\text{M}$. This value was 3.5-fold lower than was determined from fluorometric studies but still represented an approximate 160-fold increase over the 13 nM reported for WT rat liver CYPOR (Vermilion and Coon, 1978).

Expression of Human CYP1A2 with Variant POR Alleles in MK_1A2_POR Cell Models. The soluble catalytic domains of CYPOR and microsomal P450s, produced using truncated con-

FIG. 5. FMN dependence of NCR activity of purified $\Delta 66$ CYPOR variants: reaction rates of $\Delta 66$ WT (—) and Y181D (---) were determined in triplicate. Error bars represent + S.D. Estimates of K_d^{FMN} were made by fitting the data to a single binding site hyperbolic function using SigmaPlot (Systat Software, Inc.). Fitting parameters are reported in the inset; “no fit” means hyperbolic fitting did not converge such that K_d^{FMN} was not determined.

TABLE 1

Michaelis-Menten kinetic parameters for purified CYPOR variants

Twenty micromolar added to +FMN reactions.

Enzyme	NFR		NCR	
	k_{cat}	K_m^{NADPH}	k_{cat}	K_m^{NADPH}
	min^{-1}	μM	min^{-1}	μM
WT $\Delta 66$				
–FMN	1980 ± 370	4.6 ± 0.4	660 ± 10	3.0 ± 0.1
+FMN	2660 ± 280	6.2 ± 1.1	660 ± 90	2.9 ± 0.1
WT holo				
–FMN	1890 ± 330	5.5 ± 0.4	780 ± 20	1.2 ± 0.1
+FMN	1560 ± 160	5.0 ± 0.7	630 ± 10	1.1 ± 0.1
Y181D $\Delta 66$				
–FMN	1580 ± 530	3.4 ± 0.5	ND*	ND*
+FMN	1040 ± 180	3.1 ± 0.9	420 ± 10	1.2 ± 0.1
Y181D holo				
–FMN	2440 ± 470	4.6 ± 0.4	ND*	ND*
+FMN	1430 ± 220	4.4 ± 0.7	260 ± 10	0.9 ± 0.1

* ND, activity not detected.

TABLE 2
MK_1A2_POR membrane contents

MK_1A2_POR	P450 Content	CYPOR Content ^a	P450/CYPOR	Cytochrome <i>c</i> Reduction ^a	
				–FMN	+FMN ^d
	pmol/mg	pmol/mg	molar ratio	nmol reduced/(min/mg)	
Model					
Null	64 ± 12	— ^b	— ^b	0.9 ± 0.8	4.1 ± 0.8
WT	113 ± 3	10.6 ± 0.4	~11	33.9 ± 1.1	38.3 ± 3.1
Y181D	129 ± 18	~10.6 ^c	~12	2.1 ± 0.7	28.2 ± 0.4

^a $n \geq 3$.

^b Not detectable.

^c Values estimated by comparison of Western blot band intensity to CYPOR_WT.

^d Ten micromolars FMN added to reaction.

structs, are generally compromised in the ability to interact productively (Black et al., 1979). For productive alignment of the two proteins, insertion of both proteins in an appropriate lipid micro-environment is considered essential (Hlavica et al., 2003). Therefore, to test the effect of the Y181D POR mutation on P450 activity, we made use of the bacterial cell model MK1A2_POR, containing CYP1A2 and CYPOR correctly inserted into the membrane. Initially, MK_1A2_POR cell models were characterized with respect to CYPOR and CYP1A2 expression. Membrane fractions were derived from MK_1A2_POR^{WT}, MK_1A2_POR^{Y181D}, and MK_1A2_POR^{null} strains, and expression levels were evaluated (Table 2). CYP1A2 expression, measured by reduced CO difference spectra, ranged from 64 to 129 pmol/mg protein, values that represent contents normally obtained for CYP1A2 in this cell model (Kranendonk et al., 2008). CYPOR expression levels were verified by SDS-PAGE analysis (Supplemental Fig. 4A), with a 77 kDa CYPOR band present in WT and Y181D strains but absent from the null strain. CYPOR expression was confirmed by immunoblotting with a monoclonal antibody directed against the N-terminal region of human CYPOR (Supplemental Fig. 4B). CYPOR protein levels were equal in the WT and Y181D strains and undetectable in the null strain. This indicated that Y181D substitution was not detrimental to CYPOR expression or stability in the MK_1A2 cell model.

In the absence of added FMN, membranes prepared from the Y181D strain showed only slightly higher NCR activity than those prepared from the null strain (Table 2). If background activity (CYPOR-nonspecific, as indicated in the null strain) was subtracted from both WT and Y181D activities, the Y181D strain retained only ~3% WT NCR activity. This low level of NCR activity suggested that Y181D, when membrane-bound and not subjected to extensive washing during preparation, may have retained somewhat more FMN than purified Y181D proteins, although direct determination of CYPOR-specific flavin contents of the membranes was not made. On addition of FMN, the NCR activity of Y181D-containing membranes increased more than 15-fold, confirming that the observed FMN deficiency and activation of Y181D CYPOR were not unique to the purified enzymes.

TABLE 3
MK_1A2_POR EROD kinetic parameters

	WT	Y181D	Null
–FMN			
k_{cat} (min ^{–1})	0.66 ± 0.02	nd	nd
$K_m^{ethoxyresorufin}$ (μM)	1.32 ± 0.09	nd	nd
+FMN (10 μM)			
k_{cat} (min ^{–1})	0.58 ± 0.03	0.24 ± 0.01	nd
$K_m^{ethoxyresorufin}$ (μM)	1.23 ± 0.14	1.08 ± 0.05	nd

nd, EROD activity < limit of detection.

The apparent FMN affinity of the Y181D variant, as derived from a single-binding site saturation plot of NCR activity versus [FMN] (Fig. 6A), was 1.6 μM, in good agreement with the value derived for the purified Δ66 preparation (2.1 μM).

Effect of the Y181D POR Mutation on CYP1A2 Activity in Membranes. The effect of the Y181D substitution on CYPOR interaction with one of its natural redox partners, CYP1A2, was tested using MK_1A2_POR membrane preparations. Having established that CYP/CYPOR ratios in the WT- and Y181D-containing membranes were similar and in the physiologically relevant range of ~12:1

FIG. 6. FMN dependence of NCR (A) and NADPH-CYP1A2 EROD (B) activities of MK_1A2_POR membranes containing WT or Y181D CYPOR. C, cytochrome *c* reduced; R, resorufin formed. Reaction rates of MK_1A2_POR^{WT} (—) and MK_1A2_POR^{Y181D} (---) membranes were determined in triplicate. Error bars represent + S.D. Estimates of K_d^{FMN} were made by fitting the data to a single binding site hyperbolic function using SigmaPlot (Systat Software, Inc.). Fitting parameters are reported in the inset; no fit means hyperbolic fitting did not converge such that K_d^{FMN} was not determined.

(Table 2), EROD activity was measured (Table 3). MK_1A2_POR^{null} membranes did not show any EROD activity, with or without FMN complementation. MK_1A2_POR^{WT} membranes showed a Michaelis-Menten response to increasing ethoxyresorufin concentration with $k_{\text{cat}} = 0.66 \text{ min}^{-1}$ and $K_m^{\text{ethoxyresorufin}} = 1.32 \text{ }\mu\text{M}$, typical CYP1A2 kinetic parameters for EROD (Duarte et al., 2005a). This activity is in contrast to the MK_1A2_POR^{Y181D} membranes, which showed no activity. When the same EROD assays were performed in the presence of 10 μM FMN, the activity of the WT-containing membranes was decreased by 10%. This activity was consistent with the apparent inhibitory effect of free FMN on the NFR and NCR activities of the purified WT proteins. Y181D-containing membranes exhibited a large increase in activity, recovering 41% of WT activity with $k_{\text{cat}} = 0.24 \text{ min}^{-1}$ and $K_m^{\text{ethoxyresorufin}} = 1.08 \text{ }\mu\text{M}$. To estimate the functional FMN affinity of Y181D in the EROD assay, titrations were performed over the range from 0 to 100 μM (Fig. 6B). The data again fit well to a one-site binding model with an activation constant = 1.16 μM , corroborating our values determined by the NCR assay of the MK_1A2_POR^{Y181D} membranes and purified $\Delta 66$ Y181D protein.

Effect of Y181D Allele on Whole-Cell Bioactivation of Procarcinogens. To evaluate the effects of the Y181D POR mutation on xenobiotic metabolism, we made use of a whole cell mutagenicity assay using the MK_1A2_POR strain, as described previously for BTC1A2_POR bacteria (Duarte et al., 2005b; Kranendonk et al., 2008). The frequency of mutagenic reversion of arginine auxotrophy was measured in response to exposure of the bacteria to three well characterized CYP1A2-activated procarcinogens, namely, 2-aminoanthracene (2AA), 2-amino-3-methylimidazo(4,5-f)quinoline (IQ), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), (Fig. 7). The compound 2AA was highly mutagenic to the MK_1A2_POR^{WT} cells but also had a strong effect on the MK_1A2_POR^{Y181D} and MK_1A2_POR^{null} cells. IQ was also efficiently bioactivated by MK_1A2_POR^{WT} cells, whereas MK_1A2_POR^{Y181D} and MK_1A2_POR^{null} cells showed only 23 and 7% of the mutagenicity level of the POR^{WT} for this compound, respectively. The mutagenic activity of 2AA and IQ in the null strain was observed previously (Kranendonk et al., 2008) and is thought to stem from substrate-induced differential reduction of CYP1A2 by endogenous bacterial redox partners, such as flavodoxins. The same phenomenon also occurs in strains expressing functionally deficient CYPOR variants, such as Y459H and V492E, as described previously (Kranendonk et al., 2008), and Y181D. Only MK_1A2_POR^{WT} cells were able to bioactivate NNK, the CYP1A2-mediated bioactivation of which seems to be

particularly dependent on the presence of a fully active CYPOR (Kranendonk et al., 2008).

Discussion

A number of studies, including the original from Arlt et al. (2004) detailing the discovery of the exon 5 *POR* 541T→G mutation, have shown the catalytic incompetence of Y181D-substituted CYPOR using various assays and means of recombinant protein expression (Huang et al., 2005; Wang et al., 2007; Agrawal et al., 2008). Before the discovery of *POR* deficiency as a cause of human disease, Shen et al. (1989) showed that the orthologous Y178D variant of rat CYPOR, recombinantly expressed and purified, was unable to catalyze NCR activity because of loss of FMN. Since their report, the crystal structure of the rat CYPOR has shown the role of Y178 (corresponding to human Y181) as a stacking residue in the binding of FMN (Wang et al., 1997). Here, both purified and membrane-associated Y181D models were used to confirm and analyze, in more detail, compromised FMN binding as the specific molecular defect leading to multiple microsomal P450 deficiencies.

Data reported here show that FMN binding by human CYPOR is severely compromised as a result of Y181D substitution. Y181D protein, as purified, retained NFR activity because NADPH and FAD binding sites were unperturbed. The far-UV CD spectrum of the Y181D protein was consistent with proper folding. Thermal stability was likewise unaffected by the Y181D substitution (supplemental data).

The lack of air-stable semiquinone on purification and low flavin/protein ratio provided indirect evidence of FMN deficiency in Y181D samples. The degree to which FMN binding was compromised was shown directly by HPLC. Depending on the stringency of column washes and dialysis, the FMN content of the purified samples ranged from <1% up to 10%. These observations provided evidence that the equilibrium of FMN association/dissociation was not only shifted toward dissociation by the Y181D substitution but also that the process was highly dependent on the concentration of FMN present in solution. Accounting for extensive dilutions, such as those typically used to achieve catalytic enzyme concentrations for steady-state assays, it was not surprising that FMN association would diminish, along with FMN-dependent activity, in Y181D preparations. This weak FMN binding was consistent with the total lack of measurable NCR activity in previous studies and in the purified Y181D protein samples, as well as the very low levels in membranes prepared from MK_1A2_POR^{Y181D} cells.

In the context of living bacterial cells, the membrane-bound holo Y181D variant again exhibited diminished activity. Support of CYP1A2-mediated procarcinogen metabolism in the MK_1A2_POR model varied depending on the compound being tested. In all cases, a significant decrease was observed for the Y181D containing strain compared with WT.

Beyond confirmation that FMN binding was compromised by the mutation, these studies were designed to address restoration of CYPOR enzymatic function by reconstitution with FMN, as was previously observed by Shen et al. (1989) in studies in which rat Y178D function was partially restored (17% compared with WT) with an apparent $K_d^{\text{FMN}} = 3.4 \text{ }\mu\text{M}$. By comparison, FMN addition restored NCR activity of the human CYPOR tested here to a greater extent: Y181D $\Delta 66$ to 64% of WT $\Delta 66$, Y181D holo to 33% of WT holo, and MK_1A2_POR^{Y181D} membranes to 74% of MK_1A2_POR^{WT} membranes. The structural basis for the observed difference in FMN functional restoration of NCR activity between rat and human CYPORs is not apparent, considering the high degree of similarity between the two structures (Fig. 1). In the CYP1A2-mediated EROD assay, ~37% of the activity of

Fig. 7. Relative mutagenic activities of procarcinogens 2AA, IQ, and NNK on MK_1A2_POR WT, Y181D, and null strains. Data for each compound are reported relative to mutagenicity (reversion frequency) with respect to MK_1A2_POR^{WT} with $n > 4$. Actual values for 2AA (rev/nmol) were WT = 11,138 $\pm$ 755, Y181D = 8055 $\pm$ 1023, and null = 4010 $\pm$ 398. For IQ (rev/nmol): WT = 296 $\pm$ 33, Y181D = 67 $\pm$ 18, and null = 20 $\pm$ 4. For NNK (rev/ μmol): WT = 1033 $\pm$ 188, Y181D = none detected, and null = none detected. Corresponding P values for WT versus Y181D were 2AA <0.001, IQ <0.0001, and NNK <0.0001. P values for WT versus null were <0.0001 for all the compounds tested.

MK_1A2_POR^{WT} membranes was restored to MK_1A2_POR^{Y181D} membranes by FMN addition. These data suggest that a high level of electron transfer activity may be gained when FMN is delivered to the Y181D variant of human CYPOR. It remains to be tested whether a dietary or pharmaceutical regimen could achieve that desired effect in vivo.

FMN binding to purified Y181D Δ66, when measured as a function of FMN fluorescence quenching, was found to occur with an apparent $K_d = 7.3 \mu\text{M}$. When restored NCR activity was the measure of FMN binding to Y181D Δ66, a $K_d = 2.1 \mu\text{M}$ was determined. The observed K_d values, although in relative agreement, can be attributed to the differences in the requirement for stable versus catalytically productive FMN binding. A short-lived interaction with FMN is apparently sufficient to restore NCR and CYP1A2-mediated EROD function to the enzyme but is apparently insufficient to achieve quenched fluorescence, where more sustained binding interactions are required to achieve measurable saturation.

When the FMN dependence of NCR and CYP1A2-catalyzed EROD activities of MK_1A2_POR^{Y181D} were measured, K_d values of 1.6 and 1.16 μM were determined, respectively. These lower activation/binding constants likely reflect a role for membrane incorporation of CYPOR in FMN binding and utilization. It would also be consistent to hypothesize that as the number of slower steps in the catalytic mechanism increases (EROD turnover being much slower than NCR), the half-life of the FMN-CYPOR complex makes less of a contribution to the rate of reaction. On the other hand, if FMN binding is required to sense or initiate domain rearrangement of CYPOR from the closed to open conformation to facilitate P450 binding and electron transfer, as described recently (Aigrain et al., 2009; Hamdane et al., 2009), then Y181D substitution and the subsequent lack of FMN affinity would be expected to influence more negatively the structurally dynamic CYPOR-P450 interaction than interactions between CYPOR and artificial electron acceptors that do not theoretically require FMN domain movement (NCR). That NCR activity of MK_1A2_POR^{Y181D} was restored by FMN addition to 74% of MK_1A2_POR^{WT}, whereas the EROD activity of MK_1A2_POR^{Y181D} was restored only to 36% of MK_1A2_POR^{WT} is consistent with that hypothesis, although further evidence is required to define the role of FMN in CYPOR domain rearrangement, as well as the role of domain rearrangement in the overall catalytic mechanism of the enzyme.

Whether purified or left intact in a membrane environment, the Y181D variant of CYPOR exhibited several hundred-fold reduced affinity for FMN compared with WT. A composite perspective of the results of FMN binding/utilization studies of Y181D suggests a K_d between 1 and 8 μM , a range that is incompatible with in vivo flavinylation, where estimates of cytosolic FMN concentration vary by tissue and cell type but, under normal conditions, are not thought to exceed ~50 nM (that of circulating erythrocytes) (Hustad et al., 2002).

Relationships between riboflavin, FMN, and FAD in health and nutrition have been reviewed extensively (Powers, 2003). Riboflavin and FAD, and FMN to a lesser extent, are normally absorbed from the diet, where in the serum, flavins have been shown to associate with albumin and globulins. Pregnancy-specific flavin-binding proteins, involved in delivery of flavins across the placenta, are essential to fetal development (Seshagiri and Adiga, 1987). Low-dose riboflavin administration has been shown to elevate significantly the intracellular pools of FAD and FMN in humans (Hustad et al., 2002). High-dose riboflavin administration has been used previously, often with great success, to treat patients with conditions caused by genetic defects in a number of flavin-dependent enzymes, including ubiquinone oxidoreductase, pyruvate dehydrogenase, and mitochondrial

electron transport complex I. Kmoch et al. (1995) described successful riboflavin treatment of a patient suffering severe epileptic seizures caused by short-chain acyl-CoA dehydrogenase deficiency. Taken together, these studies suggest that the difference between naturally occurring intracellular FMN concentrations and those required to restore function to flavin-deficient CYPOR molecules in patients with certain *POR* mutations (i.e., Y181D, Y459H, or V492E) could be bridged by therapeutic riboflavin administration, provided genetic predictions were made.

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Address correspondence to: Christopher C. Marohnic, Department of Biochemistry, 7760, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900. E-mail: marohnic@uthscsa.edu