Human Cytochrome P450 Oxidoreductase Deficiency Caused by the Y181D Mutation: Molecular Consequences & Rescue of Defect

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Running Title: CYPOR Y181D FMN Deficiency

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ABBREVIATIONS USED:

2AA, 2-aminoanthracene; CD, circular dichroism; CYP, cytochrome P450; CYPOR, NADPH-cytochrome P450 oxidoreductase; EROD, ethoxyresorufin-O-dealkylase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; IQ, 2-amino-3-methylimidazo(4,5-f)quinoline; NCR, NADPH-cytochrome c reductase; NFR, NADPH-ferricyanide reductase; NNK, 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone; WT, wild type.
ABSTRACT

Patients with congenital adrenal hyperplasia, exhibiting combined CYP17 and CYP21 deficiency, were shown by Arlt et al. 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 FMN and NADPH-cytochrome c reductase (NCR) activity, but retained normal FAD binding and NADPH utilization. Titration of the purified protein with FMN restored 64% of WT NCR activity in Y181D with an activation constant ~ 2 µM. As determined by FMN fluorescence quenching, Y181D had $K_d^{FMN} = 7.3$ µM. Bi-plasmid coexpression of CYPOR and CYP1A2, at the physiological ratio of ~1:10 in the engineered MK_1A2_POR E. coli strain, demonstrated the compromised capacity of Y181D to support CYP1A2-catalyzed metabolism of the procarcinogens 2AA, IQ, and NNK. Isolated MK1A2_POR membranes confirmed FMN stimulation of Y181D NCR activity with a 1.6 µM activation constant. CYP1A2 ethoxyresorufin-O-dealkylase (EROD) 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 µM FMN-activation constant. We therefore 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.
INTRODUCTION

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. 50 Type II cytochrome P450 monooxygenases (CYPs) require electrons from CYPOR to catalyze steps in xenobiotic metabolism and developmental biochemical pathways, including sterol metabolism, retinoid homeostasis and androgen/glucocorticoid synthesis. Additionally, heme oxygenases (Schacter et al., 1972), squalene monooxygenase (Ono and Bloch, 1975), and 7-dehydro-cholesterol reductase (Nishino and Ishibashi, 2000) require electrons from CYPOR.

POR deficiency (OMIM#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; Fluck et al., 2004; Fukami et al., 2005; Huang et al., 2005; Homma et al., 2006; Dhir et al., 2007; Hart et al., 2007; Agrawal et al., 2008; Hart et al., 2008; Gomes et al., 2009), it seems likely that further clinical manifestations of POR variation will be revealed in patients due to altered responses to xenobiotic exposure, including therapeutic drugs.

Reporting on three patients with congenital adrenal hyperplasia, Arlt et al. (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 in comparison to 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. (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 upon addition of FMN, with half-maximal activation achieved at [FMN] = 3.4 µM.

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), show 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.

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 [ΔT236-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, and 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 in combination 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.
METHODS

Site-Directed Mutagenesis – The construction of bacterial expression plasmids encoding OmpA3-fused, full-length human CYPOR (holo) and N-terminally truncated human CYPOR (Δ66), 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 Δ66 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) affinity-purified as described previously (Marohnic et al., 2006; Huber et al., 2009). The purity of each preparation was analyzed by SDS-PAGE. Fractions exhibiting single-band purity were pooled and concentrated (Centriprep 30, Millipore, Billerica, MA), prior to quantification by oxidized flavin absorbance (total flavin ε454 nm = 21.4 mM⁻¹cm⁻¹), and by the micro 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 µM protein samples by boiling for 5 minutes. Boiled samples were immediately chilled on ice prior to centrifugation at 14,000 x g for 10 minutes to precipitate aggregated protein. Samples were then diluted 5-fold in mobile phase prior to HPLC analysis using a Hewlett-Packard Series 1100 HPLC equipped with a G1316A diode-array detector. A Thermo Hypersil Gold column (150 x 2.1 mm, 5µ) was fitted with a guard column packed with Nova-Pak C18 Guard-Pak inserts. 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 $\varepsilon_{\text{FAD}} = 10.1 \text{ mM}^{-1}\text{cm}^{-1}$ and $\varepsilon_{\text{FMN}} = 8.0 \text{ mM}^{-1}\text{cm}^{-1}$. Extinction coefficients were determined by measurement of standards contained in the mobile phase buffer. Integration and analysis were performed using Agilent ChemStation software (Rev.B.03.02).

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

**Fluorometric Analysis of FMN Binding** – FMN fluorescence quenching upon equilibrium binding to Y181D Δ66 protein was measured as described previously (Vermilion and Coon, 1978) using a Horiba Jobin Yvon FluoroMax-3 with $\lambda_{\text{ex}} = 450$ nm and $\lambda_{\text{em}} = 530$ nm, with a 100 ms integration time in anti-bleaching 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 – 50 $\mu$M. Following measurement of OD$_{\text{ex}}$ and OD$_{\text{em}}$ at each point in the titration, inner filter effects were corrected as described by Lakowicz (Lakowicz, 2006). A further correction was applied for dilution factor prior to plotting $F_{\text{corr}}$ versus [FMN]. The difference in $F_{\text{corr}}$ in the presence and absence of enzyme, $\Delta F$, was plotted versus [FMN]. The resulting data were fitted to a single binding-site hyperbola using Sigmaplot to estimate $K_d$.

**Kinetic Analysis of Purified CYPOR Variants** – (a) 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, $\varepsilon_{420 \text{ nm}} = 1.02 \text{ mM}^{-1}\text{cm}^{-1}$ 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 MgCl₂. Reactions were monitored for 5 minutes 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, in order to determine $k_{\text{cat}}$ and $K_m$ values. (b) NADPH-cytochrome c reductase (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 *E. coli* was achieved using a bi-plasmid system described previously (Kranendonk et al., 1999). Plasmid pLCM_POR contained human WT *POR* cDNA, based upon NCBI sequence NM_000941, encoding the CYPOR consensus sequence NP_000932, lacking the first 3 amino acids. This plasmid was modified to contain the mutagenized Y181D allele, or no cDNA [plasmid pLCM (Kranendonk et al., 1998)]. The 3 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_PORWT, MK_1A2_PORY181D and MK_1A2_PORNull, 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 55477).
**MK_1A2_POR Enzyme Activities** - NADPH-cytochrome c reductase (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 ± S.D. of triplicate determinations.

CYP1A2-mediated EROD activity of membrane preparations was determined using the method described by Burke et al. (Burke et al., 1994) in microplate format. Two types of EROD assays were performed, varying the ethoxyresorufin concentration (≤ 5 μM) either in the absence or presence of 10 μM FMN or varying the FMN concentrations with fixed ethoxyresorufin concentration (2.5 μM). The solvent DMSO concentration was maintained at 0.2% (v/v) throughout. $k_{cat}$ and $K_m$ were extrapolated from a plot of the mean ± 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 previously described (Kranendonk et al., 2008). CYP1A2-mediated bioactivation is expressed in terms of mutagenic activity [L-Arginine prototrophic (revertant) colonies/nmol compound or in revertant colonies/μmol 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 $t$ test, using GraphPad software.
RESULTS

Characterization of Purified CYPOR Variants - WT human CYPOR, as well as the mutated Y181D variant, were bacterially expressed and purified in both soluble (Δ66) and membrane anchor-containing (holo) isoforms. Each of the purified enzymes exhibited the predicted molecular weight on SDS-PAGE of 72 kDa for Δ66 and 77 kDa for holo (Supplemental Fig. 1).

Both WT isoforms, when purified, were green in color due to the protein-bound content of oxidized FAD (yellow) and air-stable FMN semiquinone (blue). In contrast, both Y181D isoforms were bright yellow upon purification, suggesting either altered flavin content or flavin oxidation/reduction potentials. Representative Δ66 UV/visible spectra are shown in (Supplemental Fig. 2). The visible fingerprint of air-stable neutral blue FMN semiquinone, a broad peak around 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. Both methods yielded similar results for WT Δ66 (2.0 ± 0.2 moles flavin per mole protein) and holo (2.1 ± 0.2 moles flavin per mole 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 Δ66 and 1.1 ± 0.2 for Y181D holo. These results suggested that Y181D proteins, as purified, were deficient in flavin.

HPLC-based measurements of flavin content were therefore performed (Fig. 2). FAD and FMN standards had retention times of ~4.7 and ~7.2 minutes, respectively. WT Δ66 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 post-purification dialysis, Y181D Δ66 had FMN content ranging from <1% up to nearly 10% of protein concentration, but contained nearly the full complement of FAD (~ 95% of protein concentration). In previous
studies of FAD-deficient Δ66 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 Δ66 with 100-fold molar excess of FMN, followed by separation of protein-bound and free flavins by gel filtration, yielded no measureable increase in protein-bound FMN content (data not shown), demonstrating 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 to 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.

Circular dichroism (CD) spectra were collected for each of the Δ66 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. The absence of protein-bound FMN in the Y181D variant became apparent, however, upon comparison of near-UV/visible CD spectra of the Δ66 samples. WT CYPOR had negative CD bands centered around 360 nm and 450 nm, indicative of flavin structural conformation and microenvironment polarity, with the strongly negative character of protein-bound FMN cancelling out the positive character contributed by protein-bound FAD in the band around 360 nm. The Y181D spectrum had a strong positive band centered at 375 nm, verifying the presence of bound-FAD, but the negative band around 450 nm (attributable to bound-FMN) was weakened. Addition of 60 µM (2 x [protein]) FMN to the WT sample had a small additive effect on the spectrum, contributing positive character around 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 due to 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 due to stacking interactions with aromatic residues. Titration of buffer and Y181D Δ66 were performed with increasing concentrations of FMN. Following 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 Δ66 (Fig. 4A), the correlation between F corr and [FMN] was a smooth dose-dependent curve. The difference in F corr ± protein (FMN fluorescence quenching or ΔF) was plotted versus [FMN] (Fig. 4B), yielding a saturation curve with apparent \( K_d = 7.3 \pm 1.1 \) µM. This value represented a ~560-fold increase compared to 13 nM, the value determined by Vermilion and Coon using the same method and WT rat liver CYPOR, depleted of FMN by denaturation and refolding (Vermilion and Coon, 1978).

Having established the diminished capacity of purified Y181D Δ66 protein to maintain bound FMN, the catalytic effects of FMN deficiency were assayed. NADPH-ferricyanide reductase (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 Δ66 isoforms are reported in Table 1. Y181D Δ66 retained 80% of WT Δ66 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 Δ66 variant also exhibited a similar \( K_m^{\text{NADPH}} \) to WT Δ66, with respective values of 3.4 and 4.6 µM. Although both \( k_{cat} \) and \( K_m^{\text{NADPH}} \) of WT Δ66 were
affected by FMN, the catalytic efficiency ($k_{cat}/K_m$) was not. In the Y181D ∆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 ∆66 studies, these results suggested that FMN had an inhibitory effect that was not competitive with NADPH. FMN was therefore hypothesized to be acting as an electron accepting substrate for both WT and Y181D ∆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 $4.6 \pm 0.4$ min$^{-1}$ and Y181D at $5.9 \pm 0.6$ min$^{-1}$. When the same reactions were measured in the absence of FMN, those rates decreased 6-fold ($0.8 \pm 0.2$ min$^{-1}$) for WT and 8-fold ($0.7 \pm 0.3$ 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 semi-anaerobic 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. Upon complete reduction of FMN, NADPH consumption also stopped. Leaving FMN out of the reaction mixture had the same effect under semi-anaerobic 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 ∆66 isoforms was evaluated by measurement of residual NFR activity following incubation of aliquots for 5 minutes 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.

NADPH–cytochrome c reductase (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 Δ66 exhibited a $k_{cat}/K_m^{NADPH} = 220 \text{ min}^{-1} \mu\text{M}^{-1}$, while WT holo was nearly three times more efficient with a $k_{cat}/K_m^{NADPH} = 650 \text{ min}^{-1} \mu\text{M}^{-1}$. Reaction rates were beneath the limit of detection when either Δ66 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 prior to activity measurements, and fluorometric studies suggested that dilution would favor dissociation of FMN from the Y181D Δ66 protein. Addition of a 1000-fold molar excess of FMN to the reactions restored NCR activity to both Y181D isoforms. With a $k_{cat} = 420 \text{ min}^{-1}$ and $K_m^{NADPH} = 1.2 \mu\text{M}$, the FMN-stimulated Y181D Δ66 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 (~20%).

In order to verify FMN $K_d$ determination from fluorescent methods, saturation of NCR activity by FMN titration was measured for WT and Y181D Δ66 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 $K_d^{FMN} = 2.1 \pm 0.6 \mu\text{M}$. 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 CYPs, produced using truncated constructs 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 microenvironment is considered essential (Hlavica et al., 2003). To test the effect of the Y181D POR mutation on CYP activity, we therefore 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_PORWT, MK_1A2_PORY181D and MK_1A2_PORnull strains and expression levels were evaluated (Table 3). CYP1A2 expression, measured by reduced CO difference spectra, ranged from 64 to 129 pmol per mg protein, values which 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 kD 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 non-specific, 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. Upon addition of FMN, the NCR activity of Y181D-containing membranes increased more than 20-fold, confirming that the observed FMN deficiency and activation of Y181D CYPOR was not unique to the purified enzymes.

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 (Table 2), EROD activity was measured (Table 3). MK_1A2_PORnull membranes did not show any EROD activity, with or without FMN complementation. MK_1A2_PORWT membranes demonstrated a Michaelis-Menten response to increasing ethoxyresorufin concentration with $k_{cat} = 0.66 \text{ min}^{-1}$ and $K_{m}^{\text{ethoxyresorufin}} = 1.32 \mu\text{M}$, typical CYP1A2 kinetic parameters for EROD (Duarte et al., 2005a). This is in contrast to the MK_1A2_PORY181D 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 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 $k_{cat} = 0.24 \text{ min}^{-1}$ and $K_{m}^{\text{ethoxyresorufin}} = 1.08 \mu\text{M}$. In order to estimate the functional FMN affinity of Y181D in the EROD assay, titrations were performed over the range from 0-100 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_PORY181D membranes and purified Δ66 Y181D protein.
Effect of Y181D Allele on Whole-Cell Bioactivation of Procarcinogens - In order to evaluate the effects of the Y181D POR mutation on xenobiotic metabolism, we made use of a whole cell mutagenicity assay utilizing 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_PORWT cells, but also had a strong effect on the MK_1A2_PORY181D and MK_1A2_PORnull cells. IQ was also efficiently bioactivated by MK_1A2_PORWT cells while MK_1A2_PORY181D and MK_1A2_PORnull cells demonstrated only 23% and 7% of the mutagenicity level of the PORWT 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_PORWT 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. detailing the discovery of the exon 5 POR 541T→G mutation (Arlt et al., 2004), have demonstrated 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). Prior to the discovery of POR deficiency as a cause of human disease, Shen et al. demonstrated that the orthologous Y178D variant of rat CYPOR, recombinantly expressed and purified, was unable to catalyze NCR activity due to loss of FMN (Shen et al., 1989). Since their report, the crystal structure of the rat CYPOR has demonstrated 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 employed to confirm and analyze, in more detail, compromised FMN binding as the specific molecular defect leading to multiple microsomal CYP 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 NADPH-ferricyanide reductase 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 Material).

The lack of air-stable semiquinone upon purification and low flavin:protein ratio provided indirect evidence of FMN deficiency in Y181D samples. The degree to which FMN binding was compromised was demonstrated 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 towards dissociation by the Y181D substitution, but that the process was also 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 to 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. (Shen et al., 1989) in studies in which rat Y178D function was partially restored (17% compared to WT) with an apparent $K_d^{FMN} = 3.4 \mu M$. By comparison, FMN addition restored NCR activity of the human CYPOR tested here to a greater extent: Y181D Δ66 to 64% of WT Δ66, 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 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 $K_d = 7.3 \mu M$. When restored NCR activity was the measure of FMN binding to Y181D Δ66, a $K_d = 2.1 \mu M$ was determined. The observed $K_d$
values, while 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_PORY181D 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 in order to facilitate CYP-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-CYP interaction than interactions between CYPOR and artificial electron acceptors that do not theoretically require FMN domain movement (NCR). That NCR activity of MK_1A2_PORY181D was restored by FMN addition to 74% of MK_1A2_PORWT, whereas the EROD activity of MK_1A2_PORY181D was restored only to 36% of MK_1A2_PORWT is consistent with that hypothesis, although further evidence is required to define the role of FMN in CYPOR domain rearrangement, and 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 to WT. A composite perspective of the results of FMN binding/utilization studies of Y181D suggest 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. (Kmoch et al., 1995) described successful riboflavin treatment of a patient suffering severe epileptic seizures due to 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.
ACKNOWLEDGEMENTS

Thanks to Blake A. Neiwenhuis, Aracely B. Rodriguez, and Thomas M. Shea for technical assistance. We wish to thank Dr. Markandeswar Panda for helpful discussion and critical reading of the manuscript. Thanks to Dr. C. Ainsley Davis (Bethune-Cookman University) for rendering Figure 1. The UTHSCSA Nucleic Acids Technology Core Facility was responsible for primer synthesis and DNA sequencing.
REFERENCES


This work was supported in part by the National Institutes of Health National Institute of General Medical Science [Grant GM081568] to B.S.S.M., who is The Robert A. Welch Distinguished Professor in Chemistry [AQ-0012] and by the Fundação para a Ciência e a Tecnologia (Portugal) [grant PTDC/SAU-GMG/71911/2006] to M.K.
LEGENDS FOR FIGURES

Fig. 1  Structural alignment of the FMN-binding domain of rat CYPOR [PDB = 1AMO (Wang et al., 1997), rendered in gray] with that of human CYPOR [PDB = 1B1C (Zhao et al., 1999), rendered in blue] with an r.m.s.d. = 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 using PyMOL (De Lano, 2002).

Fig. 2  Flavin Analysis: Typical HPLC elution profiles of FAD and FMN standards are shown above those of flavins extracted from CYPOR Δ66 preparations of WT and Y181D variants. Integration of elution peaks was used to calculate FAD:FMN ratios.

Fig. 3  CD Spectroscopy: Each spectrum is the average of 3 scans (see keys in insets). (A) Far UV CD: Spectra of Δ66 WT and Y181D at 0.7 µM protein. (B) Near UV/visible CD: Spectra of Δ66 WT and Y181D, as purified, at 30 µM total flavin concentration, and following 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 Δ66 protein. Measurements were made after 5 minute equilibration following each FMN addition. Each point (F_{corr}) is the average ± S.D. of 10 x 100 ms signal integrations, corrected for dilution and inner filter effects. The [FMN] range from 0 -12 µM is expanded in the inset. The solid line is the best-fit of the buffer titration, while 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 Δ66, and was plotted versus [FMN] prior to single parameter hyperbolic curve-fitting ($R^2 = 0.981$) to determine $K_d$.

**Fig. 5** FMN Dependence of NADPH-Cytochrome c Reductase Activity of Purified Δ66 CYPOR Variants: Reaction rates of Δ66 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. Fitting parameters are reported in the inset; “no fit” means hyperbolic fitting did not converge such that $K_d^{FMN}$ was not determined.

**Fig. 6** FMN Dependence of (A) NADPH-cytochrome c reductase and (B) NADPH-CYP1A2 ethoxy-resorufin-O-dealkylase 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. Fitting parameters are reported in the inset; “no fit” means hyperbolic fitting did not converge such that $K_d^{FMN}$ was not determined.

**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 $n \geq 4$. Actual values for 2AA (rev/nmol) were: WT = 11138 ± 755, Y181D = 8055 ± 1023, and null = 4010 ± 398. For IQ (rev/nmol): WT = 296 ± 33, Y181D = 67 ± 18, and null = 20 ± 4. For NNK (rev/µmol): WT = 1033 ± 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 compounds tested.
## TABLES

Table 1: Michaelis-Menten kinetic parameters for purified CYPOR variants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>NFR</th>
<th>NADPH&lt;sub&gt;cat&lt;/sub&gt; (min⁻¹)</th>
<th>NADPH&lt;sub&gt;km&lt;/sub&gt; (µM)</th>
<th>NCR</th>
<th>NADPH&lt;sub&gt;cat&lt;/sub&gt; (min⁻¹)</th>
<th>NADPH&lt;sub&gt;km&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Δ66</td>
<td>- FMN</td>
<td>1980 ± 370</td>
<td>4.6 ± 0.4</td>
<td>+ FMN</td>
<td>660 ± 10</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+ FMN</td>
<td>2660 ± 280</td>
<td>6.2 ± 1.1</td>
<td></td>
<td>660 ± 90</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>WT holo</td>
<td>- FMN</td>
<td>1890 ± 330</td>
<td>5.5 ± 0.4</td>
<td></td>
<td>780 ± 20</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+ FMN</td>
<td>1560 ± 160</td>
<td>5.0 ± 0.7</td>
<td></td>
<td>630 ± 10</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Y181D Δ66</td>
<td>- FMN</td>
<td>1580 ± 530</td>
<td>3.4 ± 0.5</td>
<td></td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Y181D holo</td>
<td>+ FMN</td>
<td>1040 ± 180</td>
<td>3.1 ± 0.9</td>
<td></td>
<td>420 ± 10</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>- FMN</td>
<td>2440 ± 470</td>
<td>4.6 ± 0.4</td>
<td></td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>+ FMN</td>
<td>1430 ± 220</td>
<td>4.4 ± 0.7</td>
<td></td>
<td>260 ± 10</td>
<td>0.9 ± 0.1</td>
</tr>
</tbody>
</table>

* ND = activity not detected

20 µM added to +FMN reactions
Table 2: MK_1A2_POR Membrane Contents

<table>
<thead>
<tr>
<th>Model</th>
<th>CYP Content (pmol/mg)</th>
<th>CYTOR Content(^a) (pmol/mg)</th>
<th>CYP:CYPOR molar ratio</th>
<th>Cytochrome c reduction(^a) (nmol reduced/[min.mg])</th>
</tr>
</thead>
<tbody>
<tr>
<td>null</td>
<td>64 ± 12</td>
<td>-(^b)</td>
<td>~11</td>
<td>0.9 ± 0.8</td>
</tr>
<tr>
<td>WT</td>
<td>113 ± 3</td>
<td>10.6 ± 0.4</td>
<td>~11</td>
<td>33.9 ± 1.1</td>
</tr>
<tr>
<td>Y181D</td>
<td>129 ± 18</td>
<td>~10.6(^c)</td>
<td>~12</td>
<td>2.1 ± 0.7</td>
</tr>
</tbody>
</table>

(a): n > 3
(b): not detectable
(c): values estimated by comparison of Western blot band intensity to CYTOR_WT
(d): 10 µM FMN added to reaction
Table 3: MK_1A2_POR EROD Kinetic Parameters

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Y181D</th>
<th>null</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>- FMN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (min$^{-1}$)</td>
<td>0.66 ± 0.02</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>$K_m$ ethoxyresorufin (µM)</td>
<td>1.32 ± 0.09</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>+ FMN (10 µM)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (min$^{-1}$)</td>
<td>0.58 ± 0.03</td>
<td>0.24 ± 0.01</td>
<td>nd</td>
</tr>
<tr>
<td>$K_m$ ethoxyresorufin (µM)</td>
<td>1.23 ± 0.14</td>
<td>1.08 ± 0.05</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd = EROD activity < limit of detection
Figure 3

A

\[\text{CD (mdeg)}\]

\[
\begin{array}{c}
\text{Wavelength (nm)} \\
200 & 220 & 240 & 260 & 280 & 300
\end{array}
\]

\[\begin{array}{c}
\text{WT} \\
\text{Y181D}
\end{array}\]

B

\[\text{CD (mdeg)}\]

\[
\begin{array}{c}
\text{Wavelength (nm)} \\
300 & 350 & 400 & 450 & 500 & 550
\end{array}
\]

\[\begin{array}{c}
\text{Free FMN} \\
\text{WT} \\
\text{WT + FMN} \\
\text{Y181D} \\
\text{Y181D + FMN}
\end{array}\]
Figure 4

The diagram shows two sets of data points plotted against the concentration of FMN in μM. The upper graph represents the corrected fluorescence intensity ($F_{corr}$) in counts per second ($\times 10^5$), while the lower graph shows the change in fluorescence ($\Delta F$, $\times 10^4$) as a function of FMN concentration.

- **Buffer Only**: Represented by solid black circles.
- **Y181D Δ66**: Represented by open white circles.

The $K_d$ value for the Y181D Δ66 protein is given as $7.3 \pm 1.1 \mu M$.
Figure 5

WT $k_{\text{cat}} = 660 \pm 90 \text{ min}^{-1}$

WT $K_{d}^{\text{FMN}} = \text{no fit}$

Y181D $k_{\text{cat}} = 450 \pm 30 \text{ min}^{-1}$

Y181D $K_{d}^{\text{FMN}} = 2.1 \pm 0.6 \mu\text{M}$
Figure 7

Relative Mutagenicity (% MK_1A2_POR<sup>WT</sup>)

- **2AA**
- **IQ**
- **NNK**

- **WT**
- **Y181D**
- **null**

**Significance Levels:**
- **** p < 0.05
- *** p < 0.001