Diminished FAD Binding in the Y459H and V492E Antley-Bixler Syndrome Mutants of Human Cytochrome P450 Reductase*§

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Numerous mutations/polymorphisms of the POR gene, encoding NADPH-cytochrome P450 oxidoreductase (CYPOR), have been described in patients with Antley-Bixler syndrome (ABS), presenting with craniofacial dysmorphism, and/or disordered steroidogenesis, exhibiting ambiguous genitalia. CYPOR is the obligate electron donor to 51 microsomal cytochromes P450 that catalyze critical steroidogenic and xenobiotic reactions, and to two heme oxygenase isoforms, among other redox partners. To address the molecular basis of CYPOR dysfunction in ABS patients, the soluble catalytic domain of human CYPOR was bacterially expressed. WT enzyme was green, due to air-stable FMN semiquinone (blue) and oxidized FAD (yellow). The ABS mutant V492E was blue-gray. Flavin analysis indicated that WT had a protein:FAD:FMN ratio of ~1:1:1, whereas ~1:0.1:0.9 was observed for V492E, which retained 9% of the WT kcat/Km in NADPH:cytochrome c reductase assays. V492E was reconstituted upon addition of FAD, post-purification, as shown by flavin analysis, activity assay, and near UV-visible CD. Both Y459H and V492E were expressed as membrane anchor-containing proteins, which also exhibited FAD deficiency. CYP4A4-catalyzed ω-hydroxylation of prostaglandin E1 was supported by WT CYPOR but not by either of the ABS mutants. Hydroxylation activity was rescued for both Y459H and V492E upon addition of FAD to the reaction. Based on these findings, decreased FAD-binding affinity is proposed as the basis of the observed loss of CYPOR function in the Y459H and V492E POR mutations in ABS.

Antley-Bixler syndrome (ABS)3 is a disorder characterized by severe midface hypoplasia, humeroradial synostosis, bowing and fracture of femora, and other severe developmental malformations (1). The disease was initially attributed to fibroblast growth factor receptor 2 (FGFR2) mutations (2).

Miller’s group discovered a correlation between six allelic variants of the POR gene (encoding CYPOR, EC 1.6.2.4) and disordered steroidogenesis observed in patients with and without ABS (3). The variants were A287P, R457H, V492E, C569Y, V608F, and an intron 6 splice variant resulting in a premature stop codon. The wild-type human CYPOR cDNA and mutants, created by site-directed mutagenesis, were expressed in Escherichia coli. The resultant proteins were assayed for their respective NADPH-cytochrome c and NADPH-cytochrome P450 reductase activities. The authors concluded that deleterious POR mutations correlated well with the decreased lanosterol demethylase (CYP51) activity that had previously been observed in ABS patients, and that severe POR mutations were sufficient to cause the ABS phenotype, even in the absence of FGFR2 mutations. Retardation of somite and limb bud formation, which was observed previously in embryonic lethal CYPOR knock-out mice (4, 5), seems to substantiate this assertion. The authors also hypothesized that POR mutations were more common than the relatively low incidence of ABS suggested and that milder mutations could result in disordered steroidogenesis or increased sensitivity to drugs and environmental toxins.

By their analysis of patients with congenital adrenal hypoplasia, Arlt et al. (6) identified another POR missense mutation, Y181D, and independently verified three of the POR mutations (A287P, R457H, and C569Y), which had been identified originally by Flick et al. (3). Type I ABS patients with FGFR2 mutations, those with the most severe skeletal abnormalities but normal genitalia, were distinguished from Type II ABS patients with POR mutations who exhibited less severe skeletal abnormalities, but ambiguous genitalia. Among Japanese patients, several new POR variants were discovered, including the premature deletion mutations I446fsX449 and L565fsX574 (7), the indel, L612_W620delinsR, a silent G5G mutation that could affect splicing, and Y578C (8).

32 patients with ABS and/or disordered steroidogenesis were queried by Miller’s group to address the distribution of POR and FGFR2 mutations, which were subsequently shown to segregate completely (9). An additional 11 new POR variants were discovered: A115V, T142A, Q153R, M263V, Y459H, A503V, G539R, L565P, R616X, V631I, and F646del. Four POR variants that the authors found in the BioVentures Web data base

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3 The abbreviations used are: ABS, Antley-Bixler syndrome; CYPOR, NADPH:cytochrome P450 oxidoreductase; CYP, cytochrome P450; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; PGE1, prostaglandin E1; HPLC, high-performance liquid chromatography; MOPS, 4-morpholinopropanesulfonic acid.
(P228L, R316W, G413S, and G504R) were also analyzed. Most recently, Homma et al. (10) described the POR mutations Q201X, A462_S463insIA, and E580Q.

Rat and human CYPORs share ~95% amino acid sequence identity. Human POR mutations were, therefore, interpreted in previous papers using the crystal structure of the soluble di-flavin catalytic domain of rat CYPOR (11). The active site of the enzyme is arranged around the interface of the FMN-, FAD-, and NADPH-binding domains. FAD is oriented with its si-face stacked against the surface of a highly conserved β-barrel structure, as observed among the ferredoxin-NADP⁺ reductase family of flavoprotein oxidoreductases (12), to which CYPOR belongs. Following displacement of the C-terminal shielding residue (Trp-677), the FAD re-face is exposed to the nicotinamide ring of NADPH, through stacking interactions that facilitate hydride ion transfer, the initial step of the catalytic cycle (13). The NADPH-binding domain, a typical Rossmann fold (14), positions the NADPH and FAD for optimal electron transfer. Following the two-electron reduction of FAD to the dihydroquinone redox state (FADH₂), electrons are sequentially transferred to FMN, which cycles between the partially reduced semiquinone (FMNH⁺) and fully reduced dihydrouquinone (FMNH₂) redox states during catalysis. The flavin isoalloxazine rings are oriented with their dimethylbenzene rings 3.85 Å apart at an angle of ~140° as dictated by the interface between the FAD- and FMN-binding domains of the protein (11). The structure of the protein influences the thermodynamics of flavin redox reactions by modulating midpoint potentials of the flavin cofactors through hydrogen bonding and π-π interactions (15, 16).

In addition to the catalytic domain of the enzyme, CYPOR also contains an N-terminal hydrophobic domain (~6 kDa) that directs membrane-binding and microsomal localization and plays a role in protein-protein interactions with cytochromes P450 and cytochrome b₅ (17–20). The site of interaction between CYPOR and the various CYPs has been studied extensively and has been shown to consist primarily of complementary charged surfaces on the component proteins (11, 21–26). With so many molecular interactions required for CYPOR functionality, it is not surprising that the various mutations/polymorphisms in the POR gene, distributed across the CYPOR structure, would result in loss of function via different mechanisms.

In this report, we have characterized the deleterious nature of the ABS-related mutations, Y459H and V492E, using bacterially expressed human CYPOR. These mutations were chosen to be studied first due to the severe phenotype described and because of their direct role(s) in FAD binding, as predicted from the structure of rat CYPOR (Fig. 1). Based on sequence alignment, residue Tyr-459 of the human enzyme corresponds to Tyr-456 in the rat sequence. The phenol side chain atoms of Tyr-456 form hydrogen bonds to the FAD ribityl moiety, through the hydroxyl group, and the benzene ring forms π-π interactions with the si-face of the FAD isoalloxazine ring. Rat Val-489, corresponding to human Val-492, forms a backbone hydrogen bond through an amide nitrogen to the FAD pyrophosphate oxygens. The extent to which FAD binding is affected by these mutations and potential rescue of function are described herein.

**EXPERIMENTAL PROCEDURES**

**Subcloning and Mutagenesis**—Standard PCR techniques were used to amplify human CYPOR cDNA for subcloning into pET28a, for Δ66 expression, and pOR263, for holoenzyme expression. Clones were mutagenized using the QuikChange kit from Stratagene to correct inherent polymorphisms (P228L and A503V) and to construct the ABS mutants, Y459H and V492E. (See supplemental material for detailed methodology).

**Protein Expression and Purification**—Soluble Δ66 CYPOR proteins were expressed in *E. coli* strain JM109(DE3) cultured in modified Terrific Broth containing 100 μM riboflavin and 10 μg/ml kanamycin. Following 24 h of induction by addition of
0.4 mM isopropyl 1-thio-β-D-galactopyranoside at 25 °C, cells were harvested by centrifugation and stored at −80 °C. Cells were lysed by sonication (5 × 2-min cycles) in the presence of the protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, pepstatin, and leupeptin. The soluble fraction was isolated by ultracentrifugation for 1 h at 100,000 × g and then applied to nickel-Sepharose (GE Healthcare, Piscataway, NJ), washed extensively, and eluted with 100 mM imidazole. The purity of the eluted protein fractions was analyzed by SDS-PAGE. Pure fractions were pooled and concentrated (Centriprep 30, Millipore, Billerica, MA), quantified spectrophotometrically when appropriate by flavin absorbance (ε454 nm = 21.4 mM−1 cm−1) (27), and stored under liquid N2.

Membrane-bound CYPOR proteins were expressed in E. coli strain BL21, cultured in modified Terrific Broth containing 100 μM riboflavin and 125 μM ampicillin. Cell harvest and lysis were the same as above for soluble proteins. Following lysis by sonification, the membrane fraction was isolated by ultracentrifugation for 1 h at 100,000 × g. The pellets were homogenized in Tris-HCl, pH 7.4, using a Dounce homogenizer. 0.1% Triton X-100 was added to solubilize the homogenate over 12 h at 4 °C, and was maintained at this concentration for all subsequent purification steps. The detergent-solubilized protein fraction was separated from the insoluble debris by ultracentrifugation for 1 h at 100,000 × g. The supernatant was then applied to 2',5'-ADP-Sepharose 4B (GE Healthcare), washed extensively with buffer containing 5 mM adenosine, and then eluted with 150 mM 2',3'-AMP mixed isomers. Protein purity and concentration were determined by SDS-PAGE. Aliquots were stored under liquid N2.

Micro BCA protein quantitation was performed according to the manufacturer’s protocol (Pierce, Rockford, IL). Each assay was performed in triplicate using multiple dilutions. Concentration values are reported as averages ± S.D.

Flavin Content Analysis—Samples were diluted to 10 μM in water prior to denaturation by boiling for 5 min to release protein-bound flavins. The boiled samples were quickly chilled on ice prior to centrifugation at 14,000 × g for 10 min to remove aggregated protein. 20 μl of sample was then separated by HPLC using a Waters Corp. (Milford, MA) analytical HPLC system, equipped with a 2487 absorbance detector. A Nova-Pak C18, 60-A, 4-μm (3.9 × 150 mm) column 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 mixed isomers. Protein purity and concentration were determined as described for soluble CYPOR. Aliquots were stored under liquid N2.

Micro BCA protein quantitation was performed according to the manufacturer’s protocol (Pierce, Rockford, IL). Each assay was performed in triplicate using multiple dilutions. Concentration values are reported as averages ± S.D.

Spectroscopic Analysis—UV-visible absorbance spectra (λ = 250–900 nm) were recorded using an Agilent 8453 diode-array spectrophotometer (Palo Alto, CA).

CD spectra were recorded in the far UV (λ = 180–300 nm) and near UV-visible (λ = 300–600 nm) regions using a Jasco J710 spectropolarimeter (Easton, MD). Far UV measurements were made of 7 μM protein samples contained in 50 mM KPi, pH 7.0, in a quartz cylindrical cuvette with a path length of 0.2 mm. Near UV-visible measurements were made of 50 μM protein samples contained in 50 mM MOPS, pH 7.0, in a quartz cylindrical cuvette with a path length of 1 cm.

Kinetic Analysis—NADPH-cytochrome c reduction was measured as described by Masters et al. (29) with little modification (see supplemental material for detailed methodology). In cases where FAD or FMN was added directly to the reaction mixture, each was added to a final concentration of 2.5 μM. NADPH-cytochrome P450 4A4 PGE1 ω-hydroxylation was measured as described by Ai, previously attributed to a mix of oxidized FAD (yellow) and air-stable FMN semiquinone (blue). The V492E was a distinctive blue-gray color.

FIGURE 2. UV-visible spectra of purified enzymes. The UV-visible spectra of WT (black) and V492E (blue) variants of CYPOR (Δ66) were taken from 300 to 700 nm. A broad absorption band at long wavelength (500–700 nm) of V492E spectrum (blue) shows that this protein is highly enriched in air-stable semiquinone as purified. The inset shows a photograph of WT and V492E Δ66 enzymes as purified. WT appeared green, previously attributed to a mix of oxidized FAD (yellow) and air-stable FMN semiquinone (blue). The V492E was a distinctive blue-gray color.

RESULTS

Soluble Human CYPOR Expression and Purification—Wild-type human CYPOR was bacterially expressed and purified as a soluble (Δ66) protein. The rationale behind the use of the truncated construct was first to obtain high yields of purified enzyme, independent of NADPH affinity-based 2',5'-ADP-Sepharose 4B purification, and also to use a truncated version to examine binding interactions in the catalytic domain of CYPOR. Human CYPOR mutant proteins were produced per liter of culture. The purified protein was greenish-brown in color (Fig. 2, inset), attributed to the
Human CYPOR ABS Mutants

TABLE 1
Comparative protein quantitation by flavin absorbance and micro BCA assay

Samples containing 2 μM total flavin concentration were prepared by dilution of oxidized stocks and verified spectroscopically by absorbance at 454 nm. The protein content of each of three aliquots was quantified using the micro BCA assay (Pierce). Each value represents the mean ± S.D.

| Sample               | Protein concentration of sample containing 2 μM flavin (μg) |
|----------------------|-----------------------------------------------------------|
| WT Δ66               | 1.0 ± 0.2                                                 |
| V492E Δ66            | 2.2 ± 0.3                                                 |
| V492E Δ66 FAD reconstituted | 1.1 ± 0.2                             |
| WT holo              | 1.1 ± 0.2                                                 |
| Y459H holo           | 2.0 ± 0.3                                                 |
| Y459H holo FAD reconstituted | 1.2 ± 0.2                             |
| V492E holo           | 2.2 ± 0.2                                                 |
| V492E holo FAD reconstituted | 1.2 ± 0.3                             |

Spectral contribution of oxidized FAD (yellow) and air-stable FMN semiquinone (blue-gray). The purified enzyme exhibited the predicted molecular mass on SDS-PAGE of 72 kDa (supplemental Fig. S1).

Δ66 Y459H and V492E were distinctively blue-gray while bound to Ni-Sepharose. Upon elution, the Y459H protein quickly became oxidized to yellow, while the V492E remained blue-gray (Fig. 2, inset). UV-visible spectra were immediately collected as shown in Fig. 2. The blue-gray color of the V492E sample corresponded well with the observed semiquinone character of the spectrum, with a distinct peak at 350 nm and a much broader peak centered ~590 nm. Purified protein quantities were lower for the mutants than for the wild-type Δ66 protein with the Y459H yielding 3 mg/liter and V492E, 9 mg/liter.

SDS-PAGE analysis of the Δ66 mutants showed that V492E was indistinguishable from wild type, but that Y459H was highly fragmented, with only a fraction of the total giving the predicted 72-kDa molecular mass (supplemental Fig. S1). The compromised stability of the Y459H Δ66 protein prevented further characterization but suggested that this particular mutation may have caused local misfolding that exposed the protein to degradation, despite the presence of protease inhibitors during purification.

Flavin Content of Soluble Proteins—CYPOR protein concentration was determined as a function of oxidized flavin concentration at 454 nm (ε = 21.4 mM·cm⁻¹·cm⁻¹) (27), and absolute protein concentration was measured by the micro BCA assay. Both methods yielded similar results for the wild-type Δ66 sample, suggesting complete cofactor incorporation (Table 1). Concentrations based on absorbance were 2-fold lower than the corresponding values obtained by micro BCA for V492E Δ66. This suggested that V492E, as purified, was deficient in either one or both of the two flavin cofactors. To determine which of the flavins was deficient, HPLC-based analysis of flavin content was performed (Fig. 3). FAD and FMN standards had characteristic retention times of 1.5 and 2 min, respectively. The wild-type Δ66 protein exhibited the full complement of both FAD and FMN at a 1:1 ratio. The FAD content of V492E was compromised, compared with wild-type, with a protein: FAD:FMN ratio of ~1:0.1:0.9.

CD Studies—Spectra were collected for the wild-type and V492E Δ66 samples in both the far UV and near UV-visible regions. Far UV CD spectra (supplemental Fig. S2) suggested that the V492E mutation had little effect on the protein secondary structure. This was surprising, in light of the structural role of FAD in bridging the FAD-binding β-barrel and C-terminal NADPH-binding domain of the rat enzyme through hydrogen bonds and hydrophobic interactions (11). The absence of FAD was again apparent in near UV-visible CD spectra of the V492E sample when compared with that of the wild-type CYPOR (Fig. 4). The wild-type enzyme had negative CD bands centered around 360 and 450 nm, indicative of both the formation of...
TABLE 2
NADPH:cytochrome c reductase assay-derived kinetic parameters for CYPOR variants

| Enzyme                        | $k_{cat}$ min$^{-1}$ | $K_m$ $\mu$M | $k_{cat}/K_m$ min$^{-1}$ $\mu$M$^{-1}$ |
|-------------------------------|----------------------|--------------|----------------------------------------|
| Wild-type Δ66                 | (6.6 ± 0.1) $\times 10^3$ | 3.0 ± 0.1 | (2.2 ± 0.2) $\times 10^2$ |
| Wild-type Δ66 + FAD           | (5.9 ± 0.1) $\times 10^3$ | 3.3 ± 0.1 | (1.8 ± 0.1) $\times 10^2$ |
| Wild-type Δ66 + FMN           | (6.6 ± 0.1) $\times 10^3$ | 2.9 ± 0.1 | (2.3 ± 0.6) $\times 10^2$ |
| Wild-type Δ66 + FAD + FMN     | (6.3 ± 0.2) $\times 10^3$ | 3.4 ± 0.2 | (1.9 ± 0.3) $\times 10^2$ |
| V492E Δ66                     | (5.3 ± 0.9) $\times 10^3$ | 2.8 ± 1.5 | (2.0 ± 0.2) $\times 10^2$ |
| V492E Δ66 + FAD               | (5.3 ± 0.1) $\times 10^3$ | 15.8 ± 0.5 | (3.4 ± 0.1) $\times 10^2$ |
| V492E Δ66 + FAD + FMN         | (1.6 ± 0.1) $\times 10^3$ | 9.6 ± 1.0 | (1.7 ± 0.1) $\times 10^2$ |
| V492E Δ66 + FAD + FMN         | (5.8 ± 0.1) $\times 10^3$ | 15.3 ± 0.6 | (3.8 ± 0.1) $\times 10^2$ |
| Wild-type holo                | (5.0 ± 0.1) $\times 10^3$ | 1.1 ± 0.1 | (4.6 ± 0.7) $\times 10^2$ |
| Wild-type holo + FAD          | (4.9 ± 0.1) $\times 10^3$ | 1.0 ± 0.1 | (4.9 ± 0.9) $\times 10^2$ |
| Wild-type holo + FMN          | (4.6 ± 0.1) $\times 10^3$ | 1.0 ± 0.1 | (4.6 ± 0.8) $\times 10^2$ |
| Wild-type holo + FAD + FMN    | (4.7 ± 0.1) $\times 10^3$ | 1.0 ± 0.1 | (4.7 ± 0.8) $\times 10^2$ |
| Y459H holo                    | (1.3 ± 0.1) $\times 10^3$ | 1.6 ± 0.3 | (8.1 ± 3.2) $\times 10^2$ |
| Y459H holo + FAD              | (8.0 ± 1.0) $\times 10^3$ | 5.2 ± 0.8 | (2.5 ± 1.5) $\times 10^2$ |
| Y459H holo + FMN              | (1.8 ± 0.1) $\times 10^3$ | 2.1 ± 0.1 | (8.6 ± 1.3) $\times 10^2$ |
| Y459H holo + FAD + FMN        | (6.5 ± 0.5) $\times 10^3$ | 3.6 ± 1.2 | (1.8 ± 1.2) $\times 10^2$ |
| V492E holo                    | (1.9 ± 0.1) $\times 10^3$ | 7.9 ± 1.3 | (2.4 ± 0.7) $\times 10^2$ |
| V492E holo + FAD              | (2.6 ± 0.1) $\times 10^3$ | 1.0 ± 0.1 | (2.6 ± 0.5) $\times 10^2$ |
| V492E holo + FMN              | (4.1 ± 0.2) $\times 10^3$ | 5.9 ± 1.0 | (7.0 ± 2.2) $\times 10^2$ |
| V492E holo + FAD + FMN        | (2.9 ± 0.1) $\times 10^3$ | 1.0 ± 0.1 | (2.9 ± 0.6) $\times 10^2$ |
| Wild-type Δ27*                | 300 or 292           | 0.02 ± 0.05 | > 50 |
| Y459H Δ27*                    | 13 or ND*            | > 50        | > 50 |
| V492E Δ27*                    | 28 or ND             | > 50        | > 50 |

* Values reported by Huang et al. (9).
* ND, not determined.

degree of fragmentation, and flavin content of the Δ27 proteins was not addressed but might further explain the observed variations.

**FAD Reconstitution of Soluble V492E**—Because the Δ66 V492E mutant was shown to be deficient in FAD, reconstitution experiments were performed. First, flavin cofactors were added directly to the NADPH:cytochrome c reductase assay to determine whether catalytic function could be rescued (Table 2). Indeed, Δ66 V492E showed a ~10-fold increase in $k_{cat}$ when 2.5 μM FAD was added. The increased turnover was accompanied by an increased $K_m^{NADPH}$, on the order of ~5-fold. Addition of FMN increased the $k_{cat}$ and $K_m^{NADPH}$ ~3-fold in the case of V492E, suggesting that some productive binding of FMN did occur.

FAD binding was also measured directly using spectroscopic and physical methods. A 10-fold molar excess of FAD was added to each of the purified Δ66 proteins. Following overnight incubation on ice, the mixture was separated by passing the pooled protein-containing fractions down a G-25 column twice, separating free flavins from the CYPORs. Micro BCA protein quantitation, UV-visible spectrum-based flavin quantitation (Table 1), and HPLC flavin analysis (Fig. 3) were used to determine the extent of FAD binding. Surprisingly, the V492E Δ66 protein was completely reconstituted by the addition of FAD, as indicated by the restoration of a near 1:1 ratio of protein to total flavin concentration. The reconstituted protein was also analyzed by near UV-visible CD spectroscopy (Fig. 4). V492E Δ66 reverted from the abnormal spectrum observed in the untreated state, to more closely resemble the spectrum of the wild-type Δ66 sample with distinct, negative bands present at both 360 and 450 nm following reconstitution with FAD.

**Holo CYPOR Expression and Purification**—Soluble CYPOR is insufficient to support microsomal CYPs (17), so wild-type and each of the ABS mutants were produced as membrane
anchor-containing (holo) proteins to address the extent to which a CYP-reconstituted hydroxylase reaction was affected by FAD deficiency. These experiments also addressed the role of the membrane-anchoring N-terminal residues in FAD binding. Holo wild-type, Y459H, and V492E proteins were successfully expressed and purified by 2′,5′-ADP-Sepharose 4B with yields of 8, 2, and 3 mg/liter, respectively. Like the V492E Δ66 protein, each of the holo mutant proteins purified with a high percentage of the sample in the semiquinone flavin redox state, as indicated by a vivid blue-gray appearance (photo not shown).

This time, however, the Y459H mutant did not rapidly reoxidize to a bright yellow color. Holo constructs had a predicted molecular mass of ~77 kDa. SDS-PAGE analysis showed that there was a small amount of N-terminally cleaved wild-type holo protein (~75 kDa) formed during purification, estimated to comprise between 1 and 2% of the total, which has previously been reported for purification of membrane-bound CYPORs (18, 19, 33). The V492E mutant was indistinguishable from wild-type, but the Y459H sample contained subpopulations that were both larger and smaller than the predicted 77-kDa product (supplemental Fig. S1B). Because the holo constructs were expressed as ompA3 fusion products, the larger band in the Y459H sample likely represents unprocessed fusion protein, whereas the smaller bands are likely fragments with intact C-terminal NADPH-binding domains. Nonetheless, these subpopulations of the Y459H holo sample represented much less of the total population than in the Y459H Δ66 sample, perhaps due to improved expression in the protease-deficient BL21 strain.

**Holo CYPOR Flavin Content**—Protein and flavin quantitative analyses were performed on the holo proteins. Wild-type enzyme had the full complement of FAD and FMN, but both Y459H and V492E were lacking in FAD, although to a lesser extent than V492E Δ66 (Table 1 and Fig. 3). The observed increases in percent FAD incorporation of the untreated mutant samples were likely representative of the population that was capable of binding 2′,5′-ADP-Sepharose 4B. Upon overnight treatment with excess FAD, followed by removal of the unbound flavin by G-25, both Y459H and V492E holo proteins were observed to contain the full complement of the cofactor (Table 1 and Fig. 3).

**NADPH-Cytochrome c Reduction by Holo CYPORs**—The $k_{cat}$ and $K_m$ values for wild-type, Y459H, and V492E holoenzymes in NADPH-cytochrome c reduction assays are reported in Table 2. The wild-type enzyme exhibited a $k_{cat}/K_m$ of ~500 min$^{-1}$ μM$^{-1}$ and was not affected by the addition of either FAD or FMN. The $K_m$ of 1 μM for wild-type holoenzyme was ~one-third of that observed for the wild-type Δ66, probably due to either the presence of the membrane-anchoring residues or purification by 2′,5′-ADP-Sepharose 4B affinity. The Y459H holoenzyme, unlike any other enzyme tested, did not produce linear kinetic traces in the absence of FAD; instead, an initial “fast” phase was observed followed by a “slow” phase (supplemental Fig. S3). The $k_{cat}$ for the initial fast phase was 27% of the wild-type turnover number, whereas the slow phase was only 16%. The $K_m$ observed in the fast phase was 60% higher than that of the wild-type enzyme, whereas the $K_m$ in the slow phase was about twice that of the fast phase. This same phase phenomenon was also observed for the Y459H holo enzyme in the presence of excess FMN but not FAD. When FAD was added, not only was the slow phase absent, but the $k_{cat}$ increased 2.5-fold over the unsupplemented enzyme to 68% of wild-type and was accompanied by a decreased $K_m$ to 1 μM, equal to that of the wild-type enzyme. The biphasic kinetics of the FAD-deficient Y459H enzyme were initially hypothesized to occur due to a turnover-induced decrease in affinity for the reduced FAD reaction intermediates compared with the oxidized state. To determine if the cofactor was being released from the enzyme during the assay, turnover conditions from the assay were replicated in a centrifugal concentrator with a 10-kDa molecular mass cutoff. Free FAD was not detected in the flow-through, suggesting another mechanism of inactivation was involved. The V492E holo enzyme was also greatly compromised compared with wild-type with a $k_{cat}$ of only 19 min$^{-1}$ (4% of wild-type) and an 8-fold increased $K_m$ of 8 μM. Monophasic kinetics were observed for the V492E mutant under all conditions. Addition of FAD to the reaction increased the $k_{cat}$ of the V492E holoenzyme mutant 14-fold (52% of wild-type) with a decrease in $K_m$ to 1 μM, equal to that of the wild-type enzyme. FMN addition caused an ~2-fold increase in $k_{cat}$ with no significant effect on $K_m$. Addition of both FAD and FMN simultaneously produced slightly better activity than addition of FAD alone, suggesting incomplete complementation of the FMN binding site.

**Holo CYPOR NADPH-CYP4A4 Activity**—CYP4A4-catalyzed ω-hydroxylation of PGE$_1$ was assayed using each of the holo CYPOR variants. A representative HPLC profile of the 10-min time point of each assay is shown in Fig. 5. Wild-type holo enzyme yielded a turnover number of 57 min$^{-1}$, whereas the Y459H and V492E mutants supported only 7 and 5 min$^{-1}$, respectively. These values were in good agreement with the data for support by ABS mutants of steroidogenic cytochromes P450 activity (9). Upon addition of excess FAD to the reaction mixture, both Y459H and V492E supported dramatically increased product formation rates of 55 and 33 min$^{-1}$, respectively. These experiments demonstrated that microsomal CYP-mediated activity was compromised in reactions containing the ABS mutants but was rescued by FAD supplementation to these reactions.

**DISCUSSION**

Our data suggest that both of the ABS-related POR mutations, Y459H and V492E, result in loss of CYPOR function due to diminished affinity of the mutant proteins for the essential FAD cofactor. Early work on CYPOR FAD dependence (34–36) demonstrated that FAD was tightly bound to the wild-type enzyme, relative to FMN, which had a $K_d = ~10$ nM. FAD-deficient wild-type CYPOR, produced by treatment with low pH and high concentrations of KBr, was catalytically incompetent as a NADPH-cytochrome c reductase, but supplementation with FAD was sufficient to reconstitute the enzyme completely.

Zhang et al. (37) produced a T491V mutant of rat CYPOR (equivalent to T494V in human) with decreased affinity for FAD, but which could be reconstituted with FAD, so as to facil-
This raises the question of whether maternal administration of pharmaceutical doses of riboflavin might have an effect on the phenotypic outcome in cases where the developing embryo/fetus may possess the Y459H, V492E, or yet undiscovered/uncharacterized mutations of CYPOR.

The compromise of human CYPOR function, revealed in these studies of the mutant enzymes, Y459H and V492E, were hypothesized to be a result of anomalous binding of FAD to the C-terminal FAD-/NADPH-binding domain. This report shows that such functional defects, revealed in the specific flavin contents of these mutant proteins, do occur. Furthermore, it is also expected that such defects will result, not only in decreased or non-existent activity in the reconstruction of steriodogenic cytochromes P450 (3, 9), but in a reduced or absent ability to reconstitute hydroxylation activities catalyzed by other microsomal cytochromes P450 as well. The effects of these reduced NADPH-cytochrome P450 reductase activities would be expected to vary according to the relative affinities of interactions between CYPOR and the specific cytochrome P450, the requisite rate of electron transfer, and/or the organ in which the CYPOR is functioning. This suggests that the various polymorphisms of human NADPH-cytochrome P450 reductase will manifest themselves differently with the various cytochromes P450. Not apparent at this stage of investigation are the downstream effects of CYPOR polymorphisms with respect to the multiple embryonic developmental defects. These questions will constitute the subject of further investigation.

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REFERENCES

1. Antley, R., and Bixler, D. (1975) Birth Defects Orig. Artic Ser. 11, 397–401
2. Chun, K., Siegel-Bartelt, J., Chitayat, D., Phillips, J., and Ray, P. N. (1998) Am. J. Med. Genet. 77, 219–224
3. Flück, C. E., Tajima, T., Pandey, A. V., Arlt, W., Okuhara, K., Verge, C. F., Jabs, E. W., Mendonca, B. B., Fujiyeda, K., and Miller, W. L. (2004) Nat. Genet. 36, 228–230
4. Shen, A. L., O’Leary, K. A., and Kasper, C. B. (2002) J. Biol. Chem. 277, 6536–6541
5. Otto, D. M., Henderson, C. J., Carrie, D., Davey, M., Gundersen, T. E., Blomhoff, R., Adams, R. H.,Tickle, C., and Wolf, C. R. (2003) Mol. Cell. Biol. 23, 6103–6116
6. Arlt, W., Walker, E. A., Draper, N., Ivison, H. E., Ride, J. P., Hammer, F., Chalder, S. M., Borucka-Mankiewicz, M., Hauffa, B. P., Malunowicz, E. M., Stewart, P. M., and Shackleton, C. H. (2004) Lancet 363, 2128–2135
7. Adachi, M., Tachibana, K., Asakura, Y., Yamamoto, T., Hanaki, K., and Oka, A. (2004) Am. J. Med. Genet A 128, 333–339
8. Fukumi, M., Horikawa, R., Nagai, T., Tanaka, T., Naiki, Y., Sato, N., Okuyama, T., Nakai, H., Soneda, S., Tachibana, K., Matsuo, N., Sato, S., Homma, K., Nishimura, G., Hasegawa, T., and Ogata, T. (2005) J. Clin. Endocrinol. Metab. 90, 414–426

FIGURE 5. γ-Hydroxylation of PGE1 by CYP4A4. HPLC profiles of the 10-min time point of CYP4A4-catalyzed γ-hydroxylation of PGE1, using holo CYPOR variants. γ-Hydroxylated product had a shorter retention time (4 min) than unreacted PGE, substrate (10 min). Activity was measured ± 2.5 μM FAD for each of the mutants.

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9. Huang, N., Pandey, A. V., Agrawal, V., Reardon, W., Lapunzina, P. D., Mowat, D., Jabs, E. W., Van Vliet, G., Sack, J., Flück, C. E., and Miller, W. L. (2005) *Am. J. Hum. Genet.* **76**, 729–749
10. Homma, K., Hasegawa, T., Nagai, T., Adachi, M., Horikawa, R., Fujiwara, I., Tajima, T., Takeda, R., Fukami, M., and Ogata, T. (2006) *J. Clin. Endocrinol. Metab.* **91**, 2643–2649
11. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S., and Kim, J. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 8411–8416
12. Karplus, P. A., Daniels, M. J., and Herriott, J. R. (1991) *Science* **251**, 60–66
13. Hubbard, P. A., Shen, A. L., Paschke, R., Kasper, C. B., and Kim, J. J. (2001) *J. Biol. Chem.* **276**, 29163–29170
14. Rossmann, M. G., Moras, D., and Olsen, K. W. (1974) *Nature* **250**, 194–199
15. Iyanagi, T. (1977) *Biochemistry* **16**, 2725–2730
16. Munro, A. W., Noble, M. A., Robledo, L., Daff, S. N., and Chapman, S. K. (2001) *Biochemistry* **40**, 1956–1963
17. Black, S. D., French, J. S., Williams, C. H., Jr., and Coon, M. J. (1979) *Biochem. Biophys. Res. Commun.* **91**, 1528–1535
18. Black, S. D., and Coon, M. J. (1982) *J. Biol. Chem.* **257**, 5929–5938
19. Bonina, T. A., Gilep, A. A., Estabrook, R. W., and Usanov, S. A. (2005) *Biochemistry* (Mosc) **70**, 357–365
20. Porter, T. D., and Kasper, C. B. (1986) *Biochemistry* **25**, 1682–1687
21. Shen, A. L., and Kasper, C. B. (1995) *J. Biol. Chem.* **270**, 27475–27480
22. Hasemann, C. A., Kurumbail, R. G., Boddupalli, S. S., Peterson, J. A., and Deisenhofer, J. (1995) *Structure* **3**, 41–62
23. Geller, D. H., Auchus, R. J., Mendonca, B. B., and Miller, W. L. (1997) *Nat. Genet.* **17**, 201–205
24. Auchus, R. J., and Miller, W. L. (1999) *Mol. Endocrinol.* **13**, 1169–1182
25. Kondo, S., Sakaki, T., Ohkawa, H., and Inouye, K. (1999) *Biochem. Biophys. Res. Commun.* **257**, 273–278
26. Davydov, D. R., Kariakin, A. A., Petushkova, N. A., and Peterson, J. A. (2000) *Biochemistry* **39**, 6489–6497
27. Oprian, D. D., and Coon, M. J. (1982) *J. Biol. Chem.* **257**, 8935–8944
28. Pietta, P., Calatroni, A., and Rava, A. (1982) *J. Chromatogr.* **229**, 445–449
29. Masters, B. S. S., Williams, C. H., Jr., and Kamin, H. (1967) *Methods Enzymol.* **10**, 565–573
30. Aitken, A. E., Roman, L. J., Loughran, P. A., de la Garza, M., and Masters, B. S. (2001) *Arch. Biochem. Biophys.* **393**, 329–338
31. Bewley, M. C., Davis, C. A., Marohnic, C. C., Taormina, D., and Barber, M. J. (2003) *Biochemistry* **42**, 13145–13151
32. Edmondson, D. E., and Tollin, G. (1971) *Biochemistry* **358**, 296–301
33. Kurzban, G. P., Howarth, J., Palmer, G., and Strobel, H. W. (1990) *J. Biol. Chem.* **265**, 12272–12279
34. Zhang, H., Gruenke, L., Saribas, A. S., Im, S. C., Shen, A. L., Kasper, C. B., and Waskell, L. (2003) *Biochemistry* **42**, 6804–6813
35. Black, S. D., Gaul, G. E., Beratis, N. G., Gillam, B. M., Tallan, H. H., and Hirschhorn, K. (1975) *Science* **190**, 1209–1211
36. Blass, J. P., and Gibson, G. E. (1977) *N. Engl. J. Med.* **297**, 1209–1211
37. Ogle, R. F., Christodoulou, J., Fagan, E., Blok, R. B., Kirby, D. M., Seller, K. L., Dahl, H. H., and Thorburn, D. R. (1997) *J. Pediatr.* **130**, 138–145
38. Schole, H. R., Busch, H. F., and Luyn-Houwen, I. E. (1992) *J. Inherit. Metab. Dis.* **15**, 331–334
39. Bell, R. B., Brownell, A. K., Roe, C. R., Engel, A. G., Goodman, S. I., Freeman, F. E., Seccombe, D. W., and Snyder, F. F. (1990) *Neurology* **40**, 1779–1782
40. Knox, S., Zeman, J., Hrebicek, M., Ryba, L., Kristensen, M. J., and Gregersen, N. (1995) *J. Inherit. Metab. Dis.* **18**, 227–229
41. Vergani, L., Barile, M., Angelini, C., Burlina, A. B., Nijtmans, L., Freda, M. P., Brizzi, C., Zerbetto, E., and Dabbeni-Sala, F. (1999) *Brain* **122**, 2401–2411
42. Wallace, A. C., Laskowski, R. A., and Thornton, J. M. (1995) *Protein Eng.* **8**, 127–134