SUPPLEMENTARY MATERIAL

Subcloning and mutagenesis

The construction of bacterial expression plasmids encoding full length human POR, has been described previously [47]. Site-directed mutagenesis of the p.Pro384Leu variant was performed using the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the following mutagenic primers: FwP384L 5´-CATCACCAACCCGCTGCGTACCAACGTGC-3´, RevP384L 5´-GCACGTTGGTACGCAGCGGGTTGGTGATG-3´. PCR products were subsequently submitted to sequencing to confirm the result of mutagenesis.

Protein expression

Membrane-bound POR proteins were expressed in *E. coli* strain BL21 (Stratagene), cultured in TB containing 100 µM riboflavin and 125 µM ampicillin (Sigma-Aldrich). Cells were grown overnight at 37℃ in Terrific Broth (TB) medium containing 100 µM riboflavin and 10 µg/ml of kanamycin (Sigma-Aldrich). The cells were induced by addition of 0.4 isopropyl 1-thio-β-D-galactopyranoside (Fisher HC, Houston, TX, USA). The bacterial culture was grown under aerobic conditions for 24 hours at 28℃ and then harvested by centrifugation at 4℃ for 15 minutes at 6000 g.

Protein purification

All purification steps were carried out at 4℃. Cells were resuspended in buffer [50mM Tris-HCl; 0.5 mM EDTA, 10% glycerol and 1 mM DTT; pH 8] containing the protease inhibitors [100 mM phenylmethysulfonil fluoride; 0.1 mM aprotinin; 1 mM pepstatin and 1 mM leupeptin (all by Sigma-Aldrich)]. The cells were lysed by lysozyme (Sigma-Aldrich) with final concentration 20 µg/ml, incubating on ice for 30 minutes and then sonicated five times for two minutes. The soluble fraction and solid fractions were separated by ultracentrifugation (Beckman J25, Brea, CA, USA) for 1 h at 100 000 x g. The pellets were homogenized in buffer [50 mM Tris-HCl; 0.1 mM EDTA; 10% glycerol, 0.05 mM DTT; pH 7.7] using a dounce homogenizer. 0.1% Triton X-100 (MP Biomedicals, Cleveland, OH, USA) was added to the homogenate for solubilization and left for 12 h at 4℃. The detergent-solubilized protein fraction was cleared from cellular debris by ultracentrifugation for 1 h at 100 000 x g and the supernatant was applied to 2',5'-ADP–Sepharose 4B (GE Healthcare). The column was washed with buffer used for homogenization and then eluted with the same buffer containing in addition 5 mM 2', 3'- AMP mixed isomers (Sigma-Aldrich).

Protein purity was determined by SDS-polyacrylamide gel electrophoresis (PAGE) and the fractions exhibiting a single band on the gel were pooled and concentrated (Centriprep YM-30, Millipore Corporation, Billerica, MA, USA). Micro BSA (Pierce, Rockford, IL, USA) assay was implemented for the protein quantification according to the standard protocol and aliquots were stored under N_2.

Full length human POR expression and purification

Wild-type and p.Pro384Leu human POR molecules were bacterially expressed and purified as membrane anchor-containing (holo) proteins. For the wild-type enzyme, 11 mg of purified protein was produced per liter of culture. The purified protein had greenish-brown color. This coloration originated from spectral contributions of oxidized FAD (*yellow*) and air-stable FMN semiquinone (*blue-gray*). The purified enzyme exhibited the molecular mass of expected ~77 kDa. The same coloration and molecular mass was obtained with p.Pro384Leu variant enzyme at a concentration of 7.25 mg of purified protein per liter of culture. SDS/PAGE analysis of the proteins showed that p.Pro384Leu variant expression was indistinguishable from the wild type protein.

Cytochrome *c* assay

The ability of bacterially expressed POR variants to reduce cytochrome *c* was measured as the rate of increase in absorbance at 550 nm using the extinction coefficient between reduced and oxidized cytochrome *c*, $\Delta\varepsilon$ = 21 $mM^{-1}cm^{-1}$ [48]. All measurements were carried out in triplicate in a 96-well plate format using a VERSAmax micro-plate reader (Molecular Devices, Sunnyvale, CA, USA). The reaction was performed in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, using 5nM enzyme and 80 µM cytochrome *c*, while NADPH concentration was varied from 0.1 to 200 µM. In order to maintain constant NADPH concentration, a NADPH-regenerating system (10 mM isocitrate, 0.5 units of isocitrate dehydrogenase, and 5 mM $MgCl_2$) was used. Reactions were monitored over 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.

Flavin content analysis

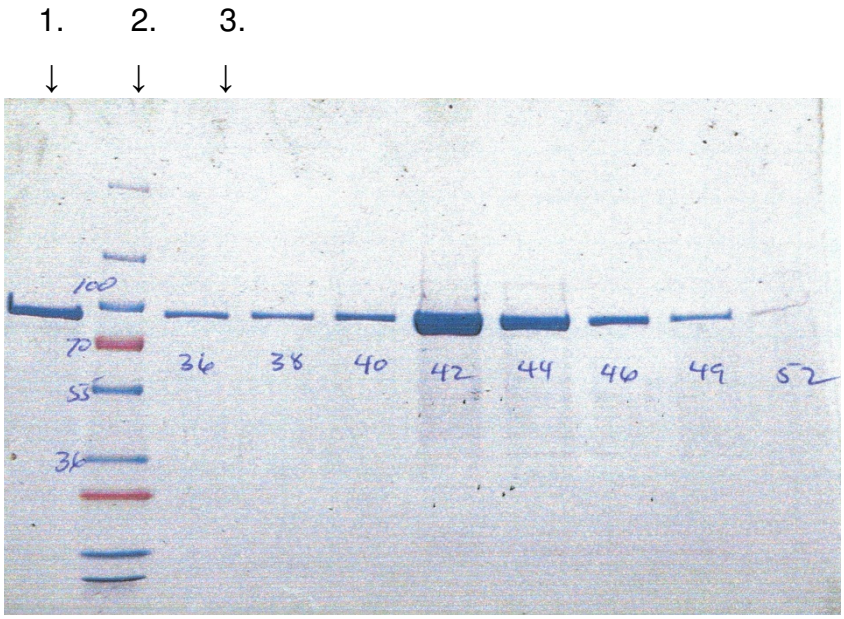
High-performance liquid chromatography (HPLC) was used to separate and quantify the flavins. Samples were diluted in water to a final concentration of 10 µM and denatured by boiling for 5 min and chilled on ice to release the flavins and precipitate the proteins effectively followed by centrifugation at 14,000 x g for 10 min to spin down the precipitated protein. 20 µl of supernatant were then used to separate FAD and FMN using a Waters Corporation analytical HPLC system, equipped with a 2487 absorbance detector (Milford, MA, USA). A Nova-Pak C_{18}, 60-Å, 4-µm (3.9 x 150mm) column was used, fitted to a guard column packed with Nova-Pak C18 Guard-Pak inserts. The column was equilibrated with the mobile phase buffer [10mM $(NH_4)_2HPO_4$, 12% acetonitrile, pH 5.5]. The solvent flow rate was 1.00 ml/min. For spectral analysis of flavins, eluent peaks were monitored at 473nm with ε_{FAD}= 10.1 $mM^{-1}cm^{-1}$ and ε_{FMN} = 8.0 $mM^{-1}cm^{-1}$. Extinction coefficients were determined by measurement of standards contained in the mobile phase buffer. Integration and analysis was evaluated with Waters Millennium 32 Chromatography Manager.

Kinetic analysis of purified full length variant

To understand the enzymatic consequences of p.Pro384Leu amino acid replacement mutations, we assayed wild-type and p.Pro384Leu full length proteins for the ability to catalyze CYP-mediated reaction. CYP activity was measured using mouse microsomes in which POR has been conditionally knocked out and supplemented with purified WT or p.Pro384Leu in a ratio of 1:5 of Cyp to Por. Cyp2e1 activity was measured as the rate of conversion of p-nitrophenol (pNP) to 4-nitrocatechol as the rate of change of absorbance at 480nm (ε = 4.08 mM). Metabolism of 7-benzyloxy-4-trifluoromethyl-coumarin (BFC), a substrate for both Cyp3a11 and Cyp1a2, was measured using a spectroflurometer (λ_{ex} = 400nm, λ_{em} = 530nm). Arachidonic acid (AA) metabolism, which is metabolized by both Cyp4a and Cyp4f, was measured as the hydroxylated product that can be separated using HPLC. All Cyp-mediated reactions contained an NADPH regenerating system (mixture of 60mU isocitrate dehydrogenase and 10 mM isocitrate). Both pNP and BFC metabolism were measured continuously, whereas AA metabolism was an endpoint measurement in the HPLC.

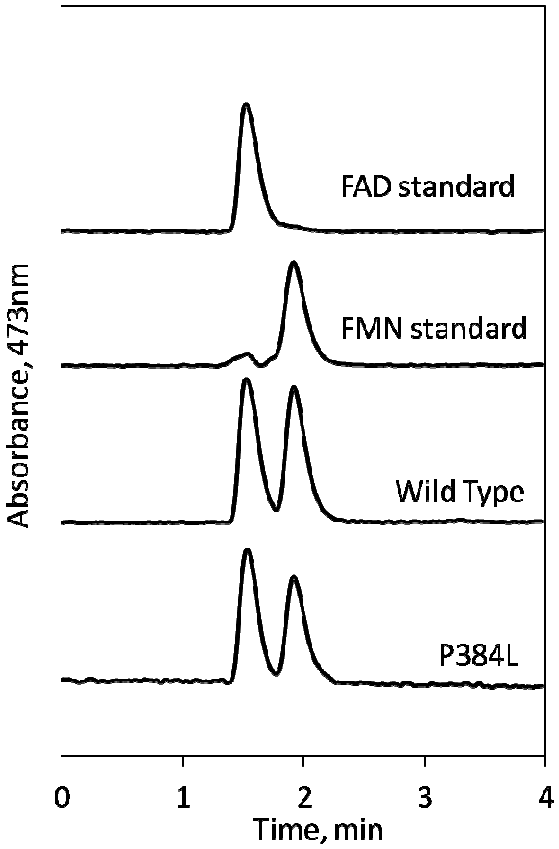
Suplementary Figure1. Purification of POR variants. Fractions of p.Pro384Leu holo enzyme preparation

1. POR control, 2. PageRulerPrestained Protein Ladder (Fermentas), 3. Full-length p.Pro384Leu POR preparation fractions (36-52)



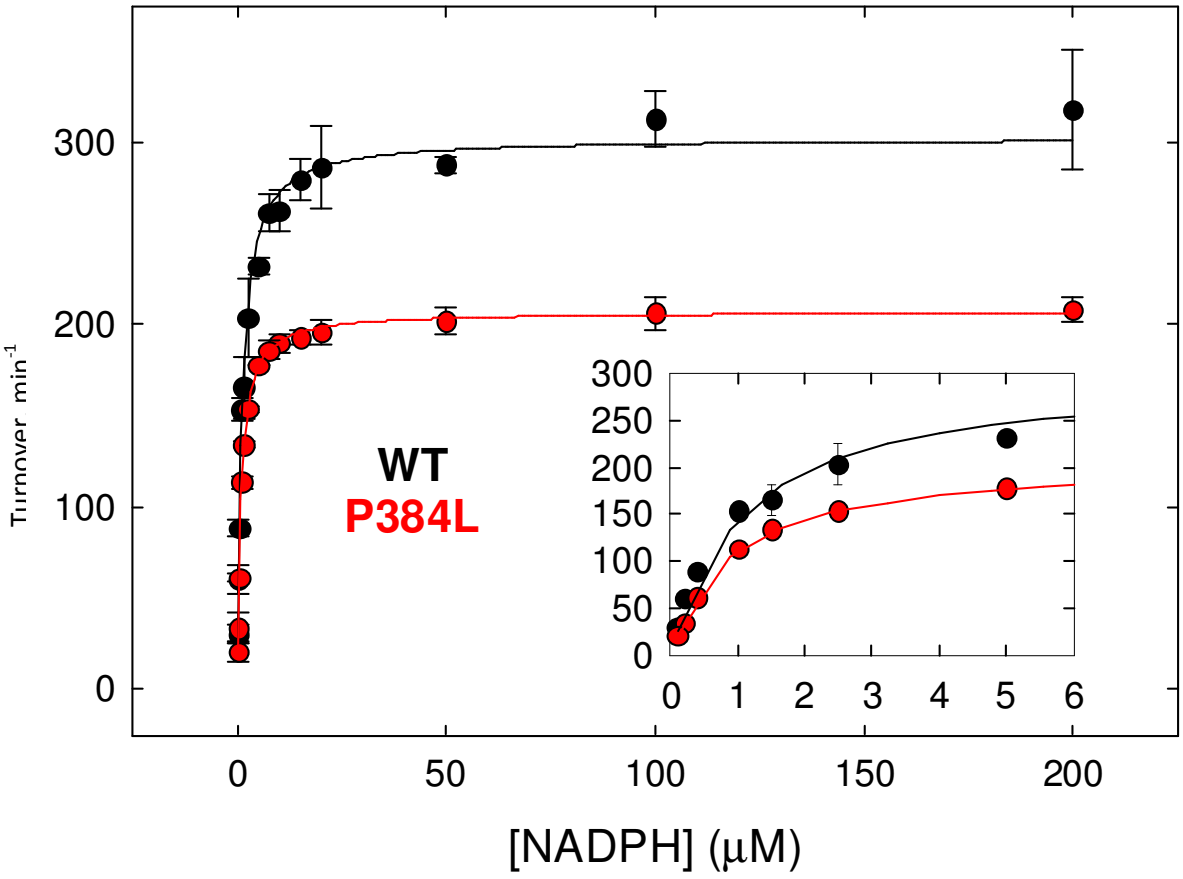
Supplemetary Figure 2. Flavin analysis.

First and second tracings show elution profiles of FAD and FMN standards. The remaining three graphs show flavin content of full-lenght proteins of WT and POR p.Pro384Leu variant. No significant difference in the flavin content was observed between the WT and POR p.Pro384Leu variant.



Supplementary Figure 3.

POR function was quantified by measuring NADPH-cytochrome *c* reduction. The p.Pro384Leu variant retained ~60% of WT turnover in the cytochrome *c* reductase assay with no significant effect on K_m^{NADPH} (WT = 1 µM and p.Pro384Leu = 0.9 µM).



Supplementary Table 1. Different P450 isoform specific activities in full-length POR control and full-length p.Pro384Leu POR, a novel variant found in the Czech Slavic control samples (expressed in per cent of control turnover).

These assays were conducted by reconstitution with pooled mouse liver microsomes in which POR has been conditionally knocked out (to be published), thereby providing the milieu of the endoplasmic reticulum in which to measure the activities of purified reductase (mutant and WT).

Cytochrome P450 partner	**Assay Substrate**	**% Activity compared w/ WT control**	**Method**
2e1	*p*-Nitrophenol	93.7	Spectrophotometric
3a11, 1a2	BFC (7-Benzyloxy4-trifluoro-methylcoumarin)	61	Spectrofluorometric
4a's, 4f's	Arachidonic Acid	86	HPLC