

# Characterization of the biochemical and structural phenotypes of four CYP1B1 mutations observed in individuals with primary congenital glaucoma

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**Objective** The objective of this study was to examine the biochemical and physical properties of cytochrome P450 1B1 (CYP1B1) mutants, test our hypothesis that primary congenital glaucoma (PCG)-causing mutants have altered metabolic activity, and correlate these to structural changes in the molecule.

**Methods** CYP1B1.1 cDNA was mutated to four forms found in individuals with the PCG phenotype, Y81N, E229K, A330F, and R368H. Expression and stability of the mutant hemoproteins and their ability to metabolize  $\beta$ -estradiol, arachidonic acid, and retinoids, were determined. Alterations in mutant properties were related to structural changes by *in silico* examination, on the basis of the CYP1A2 crystal structure.

**Results** CYP1B1 mutations strongly affected the stability, ease of heterologous expression, and enzymatic properties of the protein. These were related to the location of the amino acid substitutions in the CYP1B1 structure. Three of the mutations involve residues located on the surface of CYP1B1, Y81N, and E229K near the distal surface, and R368H near the proximal surface. The former two substitutions, Y81N and E229K, caused greatly reduced stability at 4°C. Y81N severely inhibited all substrate turnover, but E229K only inhibited arachidonate turnover and exhibited minimal effect on efficiency of retinoid metabolism and estradiol metabolism. The R368H

mutation is relatively conservative, affecting charge-pairing with the deeper-located D374, but it severely inhibited metabolism of all substrates tested, and, like Y81N, expression of the enzyme is less facile than CYP1B1 wt. The A330F mutation replaces a small alanine by a bulky phenylalanine in the enzyme active site and had major impact on substrate binding, turnover, uncoupling, and metabolite pattern.

**Conclusion** Consistent with the hypothesis, these PCG-related mutations cause identifiable structural changes negatively impacting CYP1B1 biochemistry and stability. *Pharmacogenetics and Genomics* 18:665–676 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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## Introduction

Primary congenital glaucoma (PCG) is an eye disease that affects the anterior segment of the eye, which, if unchecked, leads to elevated intraocular pressure and blindness. PCG develops during the late fetal period and early postnatal period of eye development. Earlier studies from this group and others linked mutations in a hemoprotein, cytochrome P450 1B1 (CYP1B1), to the PCG phenotype [1–6]. The importance of CYP1B1 in eye development is at once apparent when we consider the Cyp1b1-null mouse eye demonstrating similar abnormalities in the trabecular meshwork as seen in PCG patients [7].

At least seven allelic variants, or polymorphic forms of CYP1B1, are known in the general population around the

world, carrying different combinations of five amino acid substitutions, R48G, A119S, L432V, A443G, and N453S. These variations occur in nonconserved regions of the protein and do not result in PCG. Our examination of the structure of CYP1B1, on the basis of the crystal structure of another family 1 protein, CYP1A2 [8], indicates that these individual amino acid substitutions occur well before the polyproline hinge region (R48G), in substrate recognition site 1 (SRS1; A119S), after the K' helical region (L432V), and the latter two (A443G and N453S) reside in the 'meander' region just before the K'' helix.

In an earlier study, we examined two PCG-causing CYP1B1 mutations, G61E and R469W [9]. The former is a mutation in a highly conserved part of the hinge

region and the latter is a mutation in the highly conserved heme-binding region. In the present study, four different PCG-causing point mutations have been examined and attempts made to relate the effect of the mutations on biochemical and physical properties of the hemoprotein to structural alterations, the latter based upon the crystal structure of CYP1A2 [8]. The deduced sequences of the mutations suggested synthesis of full-length proteins. These mutations are all present in highly conserved regions of the orthologous proteins in vertebrates, and include Y81N, a mutation in the A-helix; E229K, a mutation in the SRS2 in the F-helix region of the holoenzyme; A330F, a mutation that occurs in the I-helix in the SRS4; and R368H, a mutation in a residue shortly after the J-helix. The mutations in the SRS2 and SRS4 regions would suggest that these might have effects on metabolism. The influences of the other two mutations are less apparent. All forms of cytochrome P450 (CYP) are uncoupled to some degree, that is, exhibit nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) oxidase activity, producing hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in place of oxidized substrate and water. The degree of uncoupling varies with the CYP form and the substrate being metabolized [10,11], and it is possible mutations could influence this property.

As the CYP forms are monooxygenases, it would be reasonable to expect that the role of CYP1B1 in development of the eye might involve the oxidative metabolism of some conserved, endogenous lipophilic compound of intermediary metabolism, either generating some critical metabolite or eliminating some compound active in ontogeny. Using a two-species comparative approach, we have examined the metabolism of a number of known endogenous substrates, expecting the conserved critical substrate to be similarly metabolized by both the human and mouse orthologs. In this manner, we have been able to suggest that several compounds are probably not the conserved substrates, such as testosterone, progesterone and  $\beta$ -estradiol, and arachidonic acid, which were all readily metabolized by the human orthologs, but not the mouse form [12,13]. Both human and mouse CYP1B1 orthologs have the ability to metabolize retinol and retinal, but not retinoic acid [14,15], suggesting the possibility that a retinoid might be the substrate utilized by the hemoprotein for normal eye development. The purpose of this study is to examine and relate the influences of the different mutations on the biochemical and physical properties to alterations in the structure of the CYP1B1.

## Materials and methods

### Reagents

The PCR reagents (deoxynucleotide triphosphates, Platinum *Taq* DNA polymerase, and MgCl<sub>2</sub>) were

obtained from Invitrogen (Carlsbad, California, USA).  $\beta$ -estradiol, OH-estradiol standards, retinol, retinal, retinoic acid, and arachidonic acid were purchased from Sigma (St Louis, Missouri, USA). <sup>14</sup>C-labeled substrates were purchased from Perkin-Elmer (Boston, Massachusetts, USA). Complete protease inhibitor tablets were obtained from Roche (Indianapolis, Indiana, USA). Quick-change site-directed mutagenesis kit was obtained from Stratagene (La Jolla, California, USA). Arachidonate metabolite standards: 20-HETE, 15-HETE, 11-HETE, 9-HETE, 5-HETE, 8-HETE, 12-HETE, 14,15-EET, 11,12-EET, 8,9-EET, and 5,6-EET were obtained from Cayman Chemical (Ann Arbor, Michigan, USA). All other chemicals used in this study were of analytical grade.

### CYP1B1 mutant preparation

The individual CYP1B1 mutations, Y81N, E229K, A330F, and R368H were introduced into CYP1B1.1 cDNA with Site-directed Mutagenesis kit (Stratagene), using the manufacturer's protocol without modification.

The following mutation-specific primers were used:

Y81N/F-CTCGCCTGGCGCGGGCGCAACGG  
CGACGTTTTCCAG

Y81N/R-CTGAAAACGTCGCCGTTGCGC  
CGCGCCAGGCGAG

E229K/F-AGCTGCTCAGCCACAACAAAGA  
GTTTCGGGCGCAGC

E229K/R-CGTGCGCCCCGAACCTTTTGT  
GTGGCTGAGCAGC

A330F/F-TCACTGACATCTTCGGCGTTAG  
CCAGGACACCCTG

A330F/R-ACAGGGTGTCTGCTAACGC  
CGAAGATGTCAGTG

R368H/F-GGTCGTGGGGAGGGACCATCT  
GCCTTGATGGGTG

R368H/R-CACCCATACAAGGCAGATGGTC  
CCTCCCCACGACC

### Sequence verification

PCR of each mutant construct was performed in a total reaction volume of 50  $\mu$ l and PCR product was purified using QIAquick Gel extraction kit according to the instructions provided by the manufacturer (Qiagen Sciences, Maryland, USA). The purified product was quantified and sequencing was performed to verify the sequence as described earlier [16].

### Bacterial membrane preparation

Membrane fragments containing the different expressed CYP forms were prepared after growth at 30°C, harvesting the *Escherichia coli* (*E. coli*) cells and lysing them by French press treatments, as described earlier [9].

### SDS-polyacrylamide gel electrophoresis and western blotting

Gel separation of proteins by SDS-polyacrylamide gel electrophoresis and subsequent western blotting were performed as described earlier [9].

### P450 in membranes

Human CYP1B1.1 (wild types and mutants) holoenzyme in the membranes was detected and quantified from its reduced CO minus reduced spectrum (450 vs. 500 nm) [17] using the extinction coefficient, 91/mmol/l/cm [18]. Stability of the holoenzymes was determined by incubation at 4°C in the presence of protease inhibitors, as described earlier [9].

### Assays

#### $\beta$ -estradiol

The hydroxylation of estradiol was carried out in 1 ml assay volumes as described earlier [9]. Incubation was at 37°C for 12 min in 50 mmol/l sodium phosphate (pH 7.4), 10 mmol/l MgCl<sub>2</sub>, 0.01% Na cholate, and 0.10–0.15  $\mu$ mol/l P450 in the membrane preparation fortified with a three-fold higher content of rabbit NADPH-CYP reductase and with 33  $\mu$ mol/l 17 $\beta$ -estradiol (0.2  $\mu$ Ci 4-<sup>14</sup>C-estradiol) (except when substrate-dependence experiments were performed) and 0.5 mmol/l NADPH. Ascorbate (1.5 mmol/l) was added as an antioxidant and quantitation was as described [9].

#### Hydrogen peroxide

H<sub>2</sub>O<sub>2</sub> was measured by ferrithiocyanate formation as described by Hildebrandt and Roots [19]. Briefly, the assay conditions were the same as for estradiol metabolism, with sodium azide (0.2 mmol/l) added to prevent degradation of the H<sub>2</sub>O<sub>2</sub>. The reaction was stopped with TCA (final concentration 2%) and after centrifugation 1 ml of the supernatant was reacted with 200  $\mu$ l of 10 mmol/l ferroammonium sulfate and 100  $\mu$ l of 2.5 mol/l potassium thiocyanate. The absorbance of the Fe(SCN)<sub>3</sub> formed was measured at 480 nm and compared with an H<sub>2</sub>O<sub>2</sub> standard curve run under the same conditions. H<sub>2</sub>O<sub>2</sub> solutions were standardized spectrophotometrically at 240 nm, by using a molar extinction coefficient of  $\epsilon_{240} = 43.6/\text{mol/l/cm}$  [19]. All preparations were run with the same membrane protein concentration (27–30  $\mu$ g) as well as with a higher level of P450, up to 90  $\mu$ g of membrane protein, as amounts of membrane above 90  $\mu$ g interfered with the measurement of H<sub>2</sub>O<sub>2</sub>.

#### Retinoids

The retinoid metabolism assays containing CYP1B1wt or mutants (100 nmol/l) were performed in 1 ml volumes at 37°C, essentially as described earlier [14]. The reconstituted system containing NADPH-CYP reductase (300 nmol/l) was incubated with 35  $\mu$ mol/l all-*trans*-retinol or all-*trans*-retinal (except when substrate-dependence experiments were performed), 10 mmol/l MgCl<sub>2</sub>, 1 mmol/l

ascorbic acid, 0.01% Na cholate, and 50 mmol/l phosphate buffer (pH 7.4) for 1 min and subsequently for 15 min after the addition of 0.5 mmol/l NADPH. The reaction was terminated by the addition of 4 ml ethyl acetate (0.001% butylated hydroxytoluene). The metabolites were identified by the retention time of standards and the spectral features ( $\lambda_{\text{max}}$ ). Substrate consumption never exceeded 2%.

#### Arachidonate

For arachidonic acid metabolism, the reconstituted system containing CYP1B1wt or mutants (100 nmol/l) and NADPH-CYP reductase (300 nmol/l) was incubated in a final volume of 1 ml containing 0.01% Na cholate, 10 mmol/l MgCl<sub>2</sub>, 150 mmol/l KCl, 50 mmol/l Tris-Cl (pH 7.5), 5 mmol/l glucose 6-phosphate, 1 U glucose 6-phosphate dehydrogenase, and 50  $\mu$ mol/l arachidonic acid (0.1  $\mu$ Ci <sup>14</sup>C-arachidonate) (except when substrate-dependence experiments were performed) for 1 min at 37°C and subsequently for 30 min after the addition of 0.5 mmol/l NADPH, essentially as described [14]. The reaction was terminated by the addition of 20  $\mu$ l of 6.7% formic acid and 4 ml ethylacetate (0.001% butylated hydroxytoluene). The system used separates HETEs and EETs well, but is less effective for the identification of the bis-allylic HETEs [20].

#### Structural examination of CYP1B1

Human CYP1B1.1 primary sequence was aligned with human CYP1A2 using a CLUSTALW multiple alignment program (<http://pbil.univ-lyon1.fr>). For visualization of the impact of mutations on the three-dimensional structure of CYP1B1, the primary structure was aligned with that of CYP1A2 (2HI4) provided on the Entrez Structure web site (<http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?Dopt=s&uid=44477>) and visualized using Cn3D viewer.

## Results

### Structural regions of the CYP1B1 mutations

CYP1B1 orthologs have a very high degree of structural identity. For example, comparison of overall sequence identity between human and the zebrafish is 53%, whereas mouse and human orthologs have an overall 81% sequence identity, with sequence identity in the SRSs ranging from 90 to 100% [13]. A large number of CYP1B1 missense mutations have been reported in individuals and families in which the PCG phenotype is found (Table 1) [2,3,21–39].

In the present study, we examined four PCG-causing mutations, all in highly conserved regions of the CYP1B1.1 protein (Fig. 1). The deduced sequences of the CYP1B1 mutants suggested the possibility of holoenzyme synthesis. One of these mutations Y81N is in the highly conserved A-helix, adjacent to the

Table 1 List of missense mutations in CYP1B1 gene associated with PCG

Region	Mutation	Reference	Impact
<b>a. Homozygous missense mutations (same mutation on both chromosomes)</b>			
Hinge region/ $\beta$ -sheet 1–0	G61E	[2,3,21–27]	Stability <sup>a</sup> , TN, substrate specificity
A-helix	L77P	[3,28]	
	A115P	[28]	
SRS1	M132R	[28]	Substrate specificity? <sup>b</sup>
D-helix	E173K	[22,27]	
	D192V	[29,30]	
	P193L	[28,31]	Stability? <sup>b</sup>
SRS2/F'-helix	<b>E229K</b>	[22]	Stability <sup>c</sup> , TN, substrate specificity
	S239R	[28]	
	D291G	[22]	
SRS4/I-helix	G329V	[25]	TN <sup>b</sup> , substrate specificity?
J-helix	V364M	[32]	
	G365W	[23]	
	<b>R368H</b>	[3,28,22,31]	TN <sup>c</sup>
	D374N	[2,3]	TN? <sup>b</sup>
K-helix	E387K	[23,26,33]	Stability? <sup>b</sup>
K-helix	R390H	[28,22,31,23]	Stability? <sup>b</sup>
K-helix	R390S	[3]	Stability? <sup>b</sup>
K-helix	R390C	[28,31,34]	Stability? <sup>b</sup>
$\beta$ -sheet 1–4/SRS5	P400S	[35]	TN <sup>b</sup> , substrate specificity?
Meander	P437L	[28,23,33]	
Meander	R444Q	[36]	Stability? <sup>b</sup>
Heme-binding	G466D	[28]	
Heme-binding	R469W	[2,3,22,23,25]	TN <sup>a</sup> , substrate specificity, OR binding? <sup>b</sup>
<b>b. Heterozygous missense mutations (mutation on only one chromosome)</b>			
Hinge region/ $\beta$ -sheet 1–0	G61E	[22,24,25]	Stability <sup>a</sup> , TN, Substrate specificity
A-helix	<b>Y81N</b>	[37]	Stability <sup>c</sup> , TN
C-helix	Q144P	[28]	
	P193L	[21]	Stability? <sup>b</sup>
E-helix	V198I	[29]	
	S215I	[32]	
SRS2/F'-helix	<b>E229K</b>	[38,39,21,28,22,25,35]	Stability <sup>c</sup> , TN, substrate specificity
	<b>R368H</b>	[21,28,22,24,25,35]	TN <sup>c</sup>
K-helix	A388T	[24]	Stability? <sup>b</sup>
K-helix	R390H	[22]	Stability? <sup>b</sup>
Meander	P442R	[22]	
Meander	A443G	[33]	
Heme-binding	R469W	[22,25]	TN <sup>a</sup> , substrate specificity, OR binding? <sup>b</sup>
	N498D	[27]	
	E499G	[29]	
<b>c. Compound heterozygous mutations (different allelic mutations)</b>			
Hinge region/ $\beta$ -sheet 1–0	G61E	[2,3,39,28,22–24]	Stability <sup>a</sup> , TN, Substrate specificity
	R117P	[39]	
	R117W	[25]	
	D192V	[29,30]	
	D192Y	[35]	
	P193L	[31]	Stability? <sup>b</sup>
E helix	A202D	[22]	Stability? <sup>b</sup>
SRS2/F'-helix	<b>E229K</b>	[22,31]	Stability <sup>c</sup> , TN, substrate specificity
SRS2/F'-helix	G232R	[38]	TN, substrate specificity?
SRS4/I-helix	G329V	[22]	TN, substrate specificity?
SRS4/I-helix	G329D	[35]	TN, substrate specificity?
SRS4/I-helix	<b>A330F</b>	[29,30]	TN <sup>c</sup> , substrate specificity
J-helix	V364M	[29,30]	
	<b>R368C</b>	[22]	TN? <sup>b</sup>
	<b>R368H</b>	[39,28,22,31]	TN <sup>c</sup>
	D374N	[2,3]	TN? <sup>b</sup>
K-helix	E387K	[37,38,33,35]	Stability? <sup>b</sup>
K-helix	A388T	[24]	
K-helix	R390C	[31]	Stability? <sup>b</sup>
K-helix	R390H	[39,28]	Stability? <sup>b</sup>
Meander	P437L	[33]	
Meander	R444Q	[29,30,35]	Stability? <sup>b</sup>
Heme-binding	R469W	[2,3,37,22,23,25]	TN <sup>a</sup> , Substrate specificity, OR binding? <sup>b</sup>

Bold mutants are studied in this work. This list does not include mutations that result in premature truncation of CYP1B1 protein or frameshift mutations. Compound heterozygous list includes only the missense mutations present with another different missense mutation in the individual.

OR, P450 oxidoreductase; PCG, primary congenital glaucoma; SRS, substrate recognition site; TN, substrate turnover.

<sup>a</sup>Mutants characterized in [9].

<sup>b</sup>Mutation effects suggested from structural data.

<sup>c</sup>Mutants characterized in this study.

Fig. 1

Ortholog	Y81N	E229K	A330F	R368H
	A helix ↓	SRS2 F helix	SRS4 I helix	J helix ↓
mCYP1B1	RLARRYGDVFQI	SHNEEFGRITVGAGS	DIFGASQDTLS	VVGRDRRLP
rat1B1	RLARRYGDVFQI	SHNEEFGRITVGAGS	DIFGASQDTLS	VVGRDRRLP
seal1B1	RLARRYGDVFQI	SHNEEFGRITVGAGS	DIFGASQDTLS	VVGRDRRLP
Cow1B1	RLARRYGDVFQI	SHNEEFGRITVGAGS	DIFGASQDTLS	VVGRHRLP
hCYP1B1	RLARRYGDVFQI	SHNEEFGRITVGAGS	DIFGASQDTLS	VVGRDRRLP
dolf1B1	-----	SHNEEFGRITVGAGS	DIFGASQDTLS	VVGRDRRLP
Zfish1B1	RMAQKYGDVFQI	GRNDOFTKTVGAGS	DIFGASQDTLS	VVDRSRRLP
hCYP1A2	RMSQRYGDVLFQI	KNTHFEFVETASSGN	DIFGAGFDTVT	VIGRERRRF

Location of four point mutations found in CYP1B1 in highly conserved different structural regions. The mutations chosen occur in the A-helix (Y81N), SRS2 (E229K), SRS4 (A330F), and a region that is close to the J-helix (R368H), as determined in alignment of different vertebrate orthologs of CYP1B1 using CLUSTALW (<http://pbil.univ-lyon1.fr>) with human CYP1A2 [8], as indicated in Methods, and visualized using Boxshade ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)). Dashes indicate sequence not determined for the striped dolphin enzyme. h, human; m, mouse; zfish, zebra fish.

polyproline hinge region. This tyrosine is 100% conserved in all the orthologous forms of CYP1B1, as well as in CYP1A1, CYP1A2, CYP2A6, CYP2C9, and CYP3A4. To date, it has not been reported as a homozygous defect in an individual, but has been found in heterozygous individuals with the CYP1B1wt allele (Table 1b). R368H is found in PCG individuals heterozygous and homozygous for the mutation as well as individuals with compound heterozygous mutations (Table 1c). This arginine, too, is 100% conserved in the family 1 CYPs, CYP2A6 and CYP2C9. E229K and A330F are located in the SRSs, SRS2 and SRS4, respectively. Although E229 does not seem to be 100% conserved even in CYP1B1 orthologs, A330 is 100% conserved in the orthologs, human family 1 forms, and CYP2C9. A number of other missense mutations in CYP1B1 are also found in individuals with PCG (Table 1). Of the 35 CYP1B1 missense mutations shown, 22 replace residues of 100% identity in the orthologs and an additional six are identical in all, but zebra fish. Furthermore, 20 and 21 of these residues have 100% identity with human CYP1A1 and CYP1A2, respectively. Eight of the 20 CYP1B1-conserved residues (G61, Y81, R117, E387, R390, P437, P442, and G466) have 100% identity in alignment with CYP2A6 and CYP3A4, as well as with CYP1A1 and CYP1A2. If we include alignment with CYP2C9, the number of 100% conserved residues in these eight CYP forms drops to seven, G61, Y81, E387, E390, P437, P442, and G466. An additional six residues, E173, P193, D291, G365, R368, and D374 are conserved in all of these CYP forms except CYP3A4.

### Expression of mutants

The four mutants of human CYP1B1 were readily expressed in the *E. coli* heterologous expression system utilizing the pCWori plasmid vector [40], but to different extents. The Coomassie blue-stained SDS-polyacrylamide gel electrophoresis-developed protein of the bacterial membrane preparations is shown in Fig. 2. The

CYP1B1 and mutant proteins in the bacterial membrane show the same migration as the purified CYP1B1 (tracks 2 and 9). Tracks 3–8 contain equivalent amounts of membrane protein, 10 µg/track. All the membrane protein bands appear to have equal staining in the different tracks, except for those in the CYP region (53 kDa). A lower amount of the CYP1B1 protein is seen for Y81N and much less for R368H. A western blot of the gel (Fig. 2 bottom) indicates that R368H membranes had the least amount of CYP1B1 protein and the level of Y81N in the membranes was also lower than that of CYP1B1wt. The western blot, thus, agrees with the Coomassie blue-stained bands, indicating that R368H and Y81N expression using the same pCWori vector was considerably lower than the wild-type enzyme and the other mutant forms of CYP1B1. These data also agree with spectrophotometrically measured CYP holoenzyme (Table 2). The intact mutants E229K and A330F were present in the *E. coli* membranes at levels approximating the wild-type enzyme, whereas Y81N was present at about a third of that level and R368H was half of Y81N (Table 2).

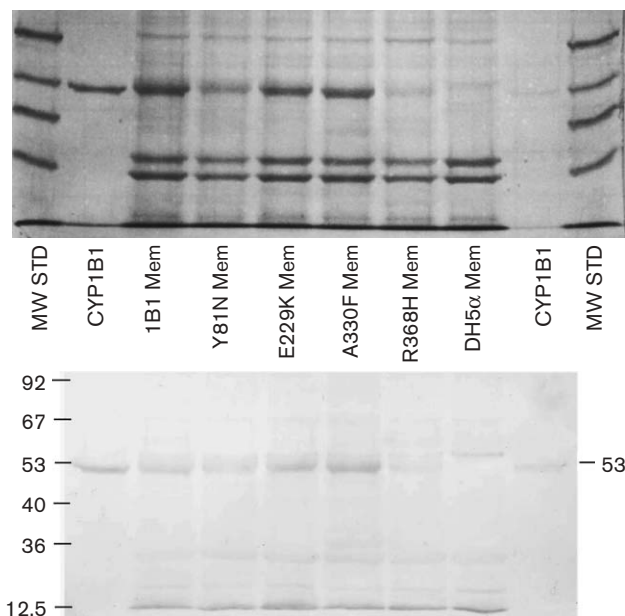
### Stability of mutants

Stability studies indicated differences in the mutant forms of CYP1B1 and the wild type. As reported previously, CYP1B1wt is quite stable in the bacterial membrane at 4°C for at least 48 h. In this study, E229K and Y81N holoenzymes were not as stable as the wild type, declining by about 25 and 50%, respectively, by 48 h (Fig. 3). In contrast, A330F content declined by 15%, whereas R368H showed the same stability as the wild-type enzyme, only declining by about 10%.

### Metabolism by mutants

CYP1B1 metabolizes 17β-estradiol to several metabolites, the major one being 4-OH-estradiol. As indicated in Table 3, the total specific activity of the wild-type enzyme is 2.42 nmol metabolites/min/nmol of CYP1B1, with an A-ring 4-hydroxy/2-hydroxy metabolite (4-OH-

Fig. 2



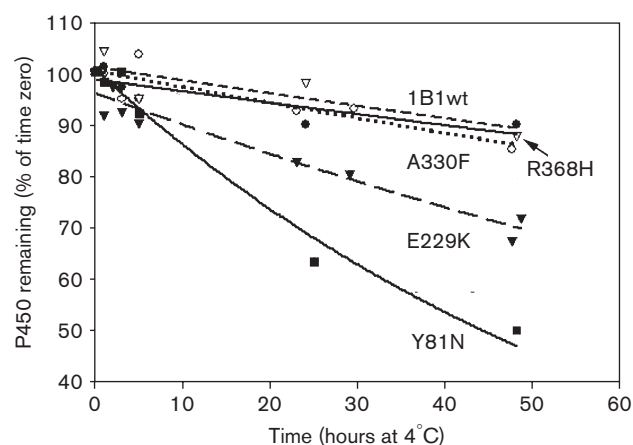
SDS-polyacrylamide gel electrophoresis and western blot of CYP1B1 mutants in bacterial cell membranes. Tracks 1 and 10 contain molecular weight standards: phosphorylase a (92 kDa), bovine serum albumin (67 kDa), glutamate dehydrogenase (53 kDa), creatine phosphokinase (40 kDa), lactate dehydrogenase (36 kDa), and cytochrome c (12.5 kDa). Tracks 2 and 9 contained purified CYP1B1, 10 and 2.5 pmol hemoprotein, respectively. Tracks 3–7 contain 10  $\mu$ g each of wild-type CYP1B1, Y81N, E229K, A330F, and R368H-containing bacterial membrane protein. Track 8 contains 10  $\mu$ g noncytochrome P450-containing DH5 $\alpha$  membrane protein. Hemoprotein levels in membranes (nmol/mg membrane protein) are indicated in Table 2. Mem, membranes; MW STD, molecular weight standards.

**Table 2** Expression levels of CYP1B1 and mutants in *Escherichia coli*

Form	Yield (nmol/l medium)	Holoenzyme nmol/mg protein
Wild type	290	1.5
E229K	250	1.24
A330F	290	1.23
Y81N	90	0.45
R368H	55	0.23

E2/2-OH-E2) ratio close to 4. The SRS2-mutant E229K had the same specific activity as the wild-type enzyme, but the isomeric specificity was altered to a ratio of about 2. Its degree of uncoupling was similar to that of CYP1B1wt, with a ratio of about 4. In contrast, the SRS4 mutant, A330F, had only 7% of the wild-type activity and showed an A-ring metabolite ratio of less than 1, indicating that its monooxygenase activity and isomeric specificity were strongly compromised. This mutant proved to be highly uncoupled, with an oxidase to monooxygenase activity ratio of over 125. The specific activity of R368H with  $\beta$ -estradiol as substrate was

Fig. 3



Stability of CYP1B1 and mutant forms at 4°C. All forms were expressed in *Escherichia coli* membranes and were stored at 4°C in medium containing added protease inhibitors for the indicated times. wt, wild type.

diminished to 46% of the wild-type activity, and that of mutant Y81N was diminished to only 30%. However, with these mutants, the ratios of 4-hydroxy metabolite to 2-hydroxy metabolite were essentially uninfluenced. The oxidase activities of these two mutants were considerably lower than that of CYP1B1wt.

Arachidonic acid metabolism by CYP1B1 generates a large number of metabolites, from  $\omega$ -hydroxy (CH<sub>3</sub>-terminal) HETEs to midchain HETEs to epoxides. As shown in Table 4, the mutants were all seriously impaired in the ability to metabolize this substrate. The specific activities were decreased by about 75% for E229K and A330F, without a major change in isomeric specificity for E229K, but with a significant alteration of metabolite specificity for A330F, where formation of EETs was at the expense of HETEs. The metabolism of arachidonic acid by R368H and Y81N mutants were most strongly compromised, with activities as low as 15 and 7% of the wild type, respectively, making it difficult to reliably ascertain the importance of metabolic pattern changes. The isomeric specificity of R368H was not strikingly different from that of the wild-type enzyme, but showed a trend for increased EET production, especially the 5,6-isomer. Mutant Y81N activity seemed to generate terminal HETEs, midchain HETEs, and EETs at approximately similar proportions from arachidonic acid. From Fig. 4, it is apparent that all the mutants generated a lower proportion of midchain HETEs and a higher proportion of EETs than the wild-type enzyme.

A number of forms of CYP are capable of metabolism of all-*trans*-retinol to all-*trans*-retinal and all-*trans*-retinoic

**Table 3 Estradiol metabolism and uncoupling by CYP1B1 and mutants<sup>a</sup>**

Activity	CYP1B1 wt	Y81N	E229K	A330F	R368H
<b>Total<sup>b</sup> (%)</b>	<b>2.42 ± 0.13 (100)</b>	<b>0.71 ± 0.06 (30)</b>	<b>2.33 ± 0.11 (96)</b>	<b>0.18 ± 0.02 (7)</b>	<b>1.11 ± 0.12 (46)</b>
4-OH-E2	1.61 ± 0.05	0.42 ± 0.04	1.22 ± 0.03	0.07 ± 0.01	0.72 ± 0.09
2-OH-E2	0.42 ± 0.03	0.12 ± 0.02	0.58 ± 0.05	0.08 ± 0.02	0.20 ± 0.03
B and D-ring OH-E2	0.39 ± 0.03	0.18 ± 0.01	0.53 ± 0.05	0.02 ± 0.00	0.19 ± 0.01
<b>4-OH/2-OH ratio</b>	<b>3.83</b>	<b>3.50</b>	<b>2.10</b>	<b>0.78</b>	<b>3.60</b>
$K_m$ (μmol/l) <sup>c</sup>	3.08	7.87	4.55	8.33	4.00
$k_{cat}$ (min <sup>-1</sup> ) <sup>c</sup>	3.05	1.02	3.72	0.24	1.60
$(k_{cat}/K_m)^c$ [(μmol/l) <sup>-1</sup> /min]	<b>0.99</b>	<b>0.13</b>	<b>0.82</b>	<b>0.03</b>	<b>0.40</b>
H <sub>2</sub> O <sub>2</sub> (nmol/min/nmol)	8.60 ± 0.90	1.57 ± 0.36	9.7 ± 0.41	22.98 ± 1.37	3.73 ± 0.49
<b>H<sub>2</sub>O<sub>2</sub>/OH : E2 ratio</b>	<b>3.55</b>	<b>2.18</b>	<b>4.16</b>	<b>127.67</b>	<b>3.36</b>

H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide;  $K_m$ , Michaelis constant;  $k_{cat}$ , enzyme turnover number.

<sup>a</sup>Metabolism data are means of 4–6 experiments.

<sup>b</sup>nmol/min/nmol P450 ± standard error.

<sup>c</sup>Catalytic constants data were means of two experiments with substrate concentrations varied between 0.5 and 6 μmol/l β-estradiol.

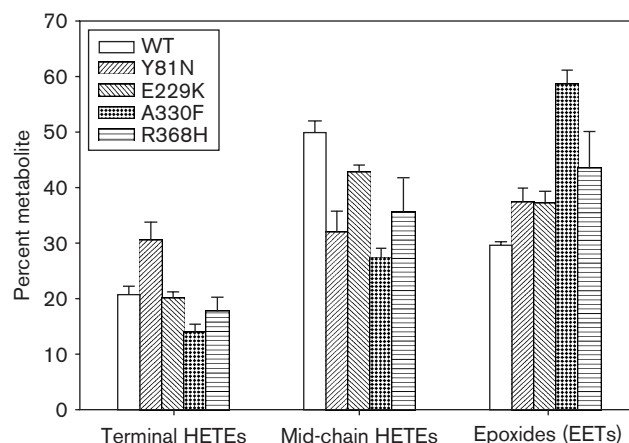
**Table 4 Arachidonic acid metabolism by CYP1B1 and mutants<sup>a</sup>**

Activity <sup>b</sup>	CYP1B1 wt	Y81N	E229K	A330F	R368H
Total activity (%)	0.371 ± 0.039 (100)	0.028 ± 0.007 (8)	0.084 ± 0.017 (23)	0.090 ± 0.006 (25)	0.057 ± 0.017 (15)
<b>Terminal HETEs (% of total metabolites)</b>	<b>20.69 ± 1.55</b>	<b>30.65 ± 3.11</b>	<b>20.15 ± 1.00</b>	<b>13.92 ± 1.37</b>	<b>17.83 ± 2.42</b>
20 and 19-HETE	5.70 ± 0.59	13.19 ± 2.50	3.93 ± 1.52	3.67 ± 2.01	7.49 ± 1.33
16, 17, and 18-HETE	14.99 ± 1.16	17.45 ± 0.61	16.22 ± 0.53	10.24 ± 0.80	10.34 ± 1.09
<b>Mid-chain HETEs (% of total metabolites)</b>	<b>49.78 ± 2.26</b>	<b>31.92 ± 3.71</b>	<b>42.72 ± 1.34</b>	<b>27.42 ± 1.57</b>	<b>35.72 ± 5.99</b>
15-HETE	8.05 ± 1.90	6.09 ± 4.16	7.58 ± 1.44	2.87 ± 0.73	11.52 ± 0.51
11-HETE	9.38 ± 0.64	9.04 ± 0.62	9.81 ± 0.82	5.28 ± 0.42	9.52 ± 1.68
12 and 8-HETE	14.89 ± 1.90	4.39 ± 0.34	12.34 ± 3.84	4.17 ± 2.06	8.39 ± 0.76
5-HETE	17.44 ± 2.21	14.56 ± 2.99	12.99 ± 2.43	15.09 ± 1.65	12.05 ± 0.69
<b>Epoxydes (EETs) (% of total metabolites)</b>	<b>29.57 ± 0.91</b>	<b>37.39 ± 2.60</b>	<b>37.16 ± 2.21</b>	<b>58.60 ± 2.57</b>	<b>43.41 ± 6.57</b>
14, 15-EET	7.69 ± 1.32	7.16 ± 2.00	11.67 ± 4.54	13.96 ± 4.13	9.35 ± 2.49
11, 12-EET	8.42 ± 0.69	3.45 ± 2.35	11.53 ± 0.46	18.01 ± 0.93	7.91 ± 5.15
8, 9-EET	1.82 ± 0.39	3.29 ± 0.13	2.27 ± 0.46	3.56 ± 0.23	1.54 ± 0.88
5, 6-EET	11.63 ± 2.79	25.13 ± 3.89	11.68 ± 4.68	23.06 ± 3.95	24.61 ± 4.81

EET, epoxyicosatrienoate; HETE, hydroxyicosatetraenoate.

<sup>a</sup>Data are means of three experiments ± standard error.

<sup>b</sup>nmol/min/nmol P450. The metabolism assay was performed with 50 μmol/l arachidonic acid as a substrate for 100 nmol/l CYP1B1 wt and the respective mutant enzyme forms.

**Fig. 4**

Metabolite production from arachidonate by CYP1B1 wt and mutants. Pictorial representation of metabolites by type as percentage of total products. Data are means of three experiments ± standard error. In general, EET formation increased in mutants at the expense of HETEs.

acid. CYP1B1 is one of these forms. As seen in Table 5, the wild type has an almost five-fold higher specific activity for conversion of retinal to retinoic acid than for the first step of vitamin A metabolism, the conversion of retinol to retinal. This ratio is retained by the two most severely inhibited mutants, Y81N and R368H. However, the two SRS mutant forms have strongly altered metabolite ratios. The SRS2 mutant E229K is only slightly impaired in retinol metabolism and, if anything, demonstrates a small stimulation of retinal oxidation. The SRS4 mutant A330F had a much greater inhibition of retinol oxidation than retinal oxidation.

## Discussion

In the present study, we have focused on four mutations of CYP1B1.1 that have been implicated as responsible for the disease phenotype in individuals with PCG, and have analyzed their stabilities and abilities to carry out metabolic functions with several substrates. Of these mutations, all but one, E229K, are in residues normally highly conserved in the CYP structure of vertebrates.

**Table 5 Retinoid metabolism by human CYP1B1 and mutants<sup>a</sup>**

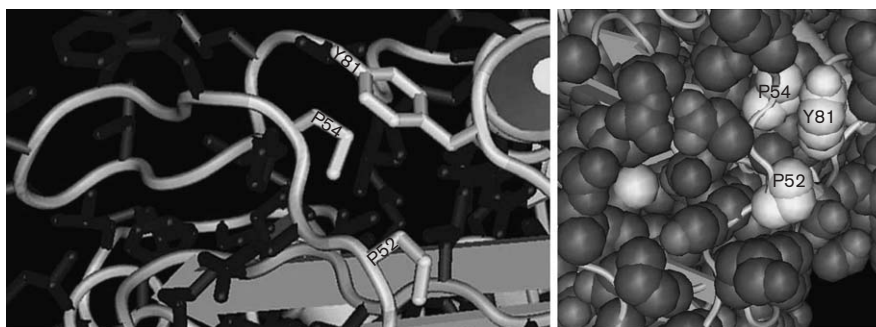
Reactions	CYP1B1wt	Y81N	E229K	A330F	R368H
Metabolic activity of retinol to retinal <sup>b</sup> (%)	0.162 ± 0.008 (100)	0.035 ± 0.010 (22)	0.116 ± 0.015 (72)	0.045 ± 0.003 (28)	0.011 ± 0.003 (7)
$K_m$ (μmol/l) <sup>c</sup>	17.85	16.66	34.48	20.83	43.47
$k_{cat}$ (min <sup>-1</sup> ) <sup>c</sup>	0.212	0.050	0.228	0.075	0.054
$[(k_{cat}/K_m) \times 10^{-3}]^c$	<b>11.87</b>	<b>3.00</b>	<b>6.62</b>	<b>3.60</b>	<b>1.25</b>
Metabolic activity of retinal to retinoic acid <sup>b</sup> (%)	0.783 ± 0.093 (100)	0.188 ± 0.042 (24)	0.872 ± 0.114 (111)	0.486 ± 0.007 (62)	0.065 ± 0.00 (8)
$K_m$ (μmol/l) <sup>c</sup>	12.19	20.83	18.51	18.21	43.47
$k_{cat}$ (min <sup>-1</sup> ) <sup>c</sup>	0.909	0.303	1.16	0.819	0.150
$[(k_{cat}/K_m) \times 10^{-3}]^c$	<b>74.55</b>	<b>14.56</b>	<b>62.66</b>	<b>44.97</b>	<b>3.54</b>
<b>Ratio -retinal/-retinol</b>	<b>4.83</b>	<b>5.37</b>	<b>7.5</b>	<b>10.8</b>	<b>5.9</b>

$K_m$ , Michaelis constant;  $k_{cat}$ , enzyme turnover number.

<sup>a</sup>Metabolism data are means of three experiments.

<sup>b</sup>nmol/min/nmol P450 ± SE.

<sup>c</sup>Catalytic constants data were from single experiments with substrate concentrations varied between 10 and 80 μmol/l substrate.

**Fig. 5**

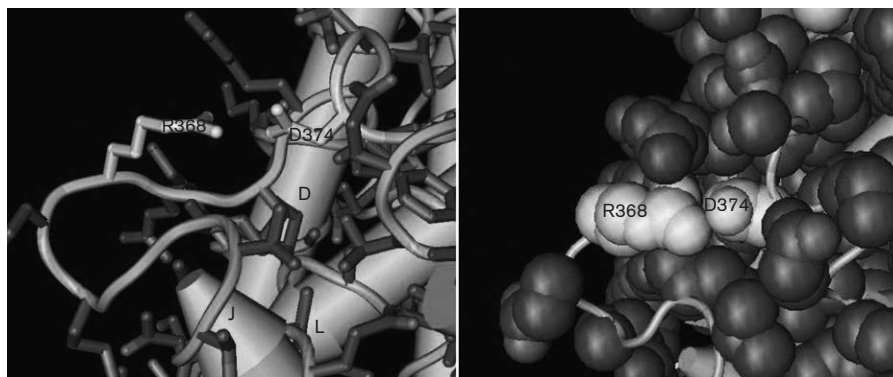
Association of Tyrosine 81 with Proline 54 and Proline 52 in CYP1B1. These CYP1B1 residues align with Y72, P45, and P43, respectively, in the crystal structure of CYP1A2 [8]. Left, tube model; Right, space-filling model. In Figures 5–8, the structure of CYP1A2 was from the Protein Data Base (PDB: 2H14), and was visualized with NCBI Cn3D viewer.

A tyrosine corresponding to Y81 is present in alignments with CYP1B1, CYP1A1, CYP1A2, CYP2A6, CYP2C9, and CYP3A4. Y81N is located at the end of the A-helix and its aromatic ring is surface-oriented, adjacent to P52 and abuts on proline P54 of the hinge region (Fig. 5), based on the alignment with another family 1 member, CYP1A2, crystal structure [8]; and the way this affects enzyme function can only be speculated.

The Y81N mutant form is not so common in PCG, being reported as a heterozygous mutation (Table 1b) in individuals of German origin. It has also been reported in Spanish [41] and French patients [42] with primary open-angle glaucoma. In all the cases, the reported CYP1B1 defect was a heterozygous mutation. Incomplete penetrance has been noted with this mutation [41]. Y81N has considerable instability, based on the rate of denaturation at 4°C in *E. coli* membranes. Half of the holoenzyme spectrum is lost within 48 h. In this respect Y81N is like G61E, the hinge region mutant that we characterized earlier [9]. The Y81 mutation may negatively impact a structurally important proline, suggesting that this region is important for stability of the enzyme.

G61 (Table 1) is also a very highly conserved residue, aligning with G52 in CYP1A2, which is adjacent to and affects interactions between residue P49 and L51 on the surface of that molecule (P58 and I59 on CYP1B1). The increased instability of these CYP1B1 mutants, as well as E229K, probably results in the increased degradation of the apoproteins via cellular proteosomal pathways, as observed with CYP1B1.4 [43], one of the polymorphic forms of the enzyme. Unlike G61E, which demonstrated a marked alteration of isomeric specificity with β-estradiol, Y81N demonstrates a very large diminution in efficiency toward this substrate owing to an increase in  $K_m$  and decrease in  $k_{cat}$ . Large decreases in metabolic efficiencies were also observed with the retinoids. Its metabolite pattern with arachidonate was not too dissimilar from that of the CYP1B1wt, but total activity was diminished by 92%, making conclusions about its isomeric specificity difficult and causing an inability to determine kinetic constants with this substrate. The severely depressed activity of this mutant might explain its ability to cause PCG when present in individuals heterozygous for the mutation (Table 1b). Its diminished monooxygenase activity is not because of uncoupling,

Fig. 6



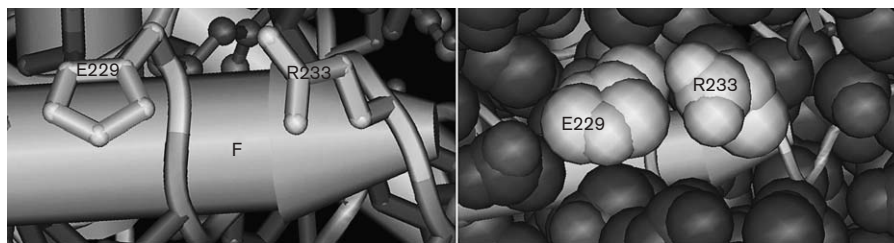
Charge-pairing surface couple, R368 and D374. The identified CYP1B1 residues align with CYP1A2 residues R355 and D361, and are shown in tube (left) and space filling (right) models of the latter protein crystal structure.

as its rate of  $H_2O_2$  formation was similarly affected, indicating overall decreased Y81N activity.

The R368H mutation, which might be considered as a more conservative mutation, has been shown to be capable of causing PCG in individuals homozygous for the mutation as well as heterozygous with the wild-type allele (Table 1a and b). It is a fully conserved residue in family 1 CYPs and the two above-mentioned family 2 CYPs, but not CYP3A4. This mutation is also seen in individuals with juvenile open angle glaucoma (JOAG) in the heterozygous condition [44,45], and in Peter's anomaly [46], and in a JOAG individual with a MYOC mutation as well [45]. The heterozygous mutation has also been reported in an individual with Reiger anomaly [37]. In contrast to Y81N, this mutation does not lead to instability of the protein and its impact on the ability of the protein to metabolize substrates seems to depend on the substrate, with inhibition greater than 90% with retinoids, about 85% with arachidonate and about 50% with  $\beta$ -estradiol, without an apparent influence on isomeric specificity. This mutation occurs just after the J-helix, based on structural alignment with CYP1A2 (Fig. 1), and seems to affect a charge-pairing between R368 and D374 on the edge of the proximal surface (Fig. 6). Expression of this stable mutant protein in *E. coli* was much lower than that of the other forms (Table 2). If its expression in humans is also less facile, this and a severely constrained activity might explain its PCG-causing effect, as well as the other mentioned eye abnormalities, in individuals heterozygous for the mutant allele. In support to this conclusion, the homozygous and compound heterozygous missense mutations in D374, with which R368 charge-pairs (e.g. D374N, Table 1a), also cause PCG [2]. Metabolic efficiency with all substrates tested was impaired with the R368H mutant. The diminished product formation with estradiol was not because of uncoupling, which was similarly diminished.

The E229K mutation in CYP1B1 is found mainly in the heterozygous condition in PCG [38,39,41,42,], as well as in JOAG and primary open angle glaucoma individuals [21,28]. Homozygous E229K mutation has also been observed [22]. It involves a change from an acidic glutamate to a basic lysyl residue in the SRS2 [47] region of the molecule. E229 is located on the F'-helix and aligns with a histidine (H224) in CYP1A2. Although E229 is considered as a part of SRS2, it is located at the periphery of the active site and is exposed to the distal surface of the molecule, rather than being in the active site (Fig. 7). E229 forms a charge-pair with R233. In CYP1A2, the corresponding residues are H224, charge-pairing with residue E228, which aligns with R233 on CYP1B1. Replacing E229 with K229 results in charge repulsion with R233 in CYP1B1, which would be expected to impact the deeper-located F231 in the SRS2 active site region of the F'-helix. The net effect of the mutation would, thus, be structural changes that might be felt in the substrate-binding region and influencing metabolite formation. The E229K mutation had a moderate influence on the stability of the protein and its ability to be expressed by *E. coli*, in that it was not as deleterious as the Y81N mutation. Its impact on the ability to metabolize substrates depended more strongly on the substrate in question. It did not impair the turnover number with  $\beta$ -estradiol as substrate and at assay levels, although surprisingly it did influence the isomeric specificity, diminishing the 4-hydroxylation and increasing the 2-hydroxylation activities. In contrast, with arachidonic acid as substrate only the turnover number, but not the metabolite pattern, was altered. With retinol as substrate, the turnover number was diminished by 28%, but with retinal as substrate, if anything, the turnover number was slightly increased. Interestingly, the structural importance of this residue is unclear, as it is not 100% conserved in the CYP1B1 orthologs examined, differing in zebrafish, and is not a conserved residue in other CYP forms examined.

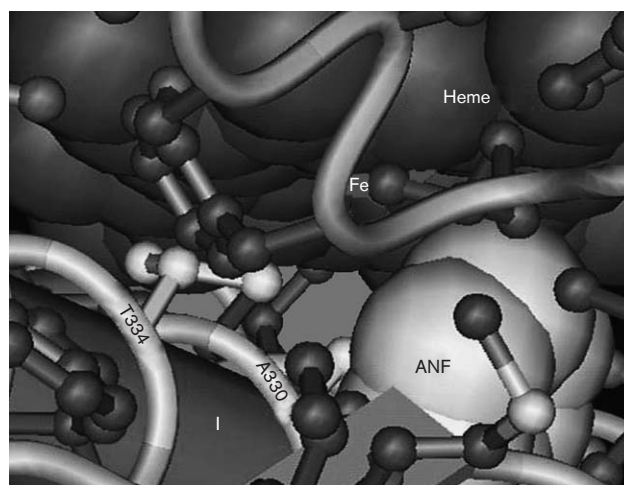
Fig. 7



Charge-pairing interactions between surface residues E229 and R233 of CYP1B1. The identified CYP1B1 residues align with CYP1A2 residues, H224 and E228, respectively. Charge pairing occurs in both the hemoproteins, but anionic and cationic charges are reversed in CYP1A2 relative to CYP1B1. These two highly conserved charged residues bracket very highly conserved, inner EF residues on the F'-helix, which place phenylalanine 226 (F231 of CYP1A2) as one boundary of the active site. Left, tube model; right, space filling model.

The A330F mutation resides in the SRS4 region of the active site. It is located on the I-helix and is a part of a highly conserved AGXDT region (<http://drnelson.utmem.edu/>), which in CYP1A2 and CYP1B1 translates to AGFDT and ASQDT, respectively. It is not a common mutation found in PCG individuals and, to date, has not been seen in the homozygous or heterozygous state, but only in the compound heterozygous state in Japanese families [29,30], where its effect is combined with that of some other mutation. Using the crystal structure of CYP1A2 as a template, we observe that the A330 on the I-helix is close to and oriented toward the heme iron (Fig. 8). The adjacent glutamine is oriented away from the heme, as is aspartate, but threonine (T334), like alanine, is close to and orients toward the heme iron. Mutation of the alanine to phenylalanine (A330F) might be expected to have a strong influence on substrate oxidation, as the bulky aromatic phenylalanine structure on the distal side and close to the heme iron would be expected to interfere with substrate interaction with the heme iron. This mutation caused a high degree of uncoupling owing to impaired approach of the substrate toward the heme iron-bound active oxygen (Table 3). The uncoupling causes release of large amounts of H<sub>2</sub>O<sub>2</sub>, which may result in an increased rate of degradation of this mutant protein. The importance of this residue in the CYP structure is determined by its 100% retention in all the family 1 CYPs and CYP2C9, but not CYP3A4, which, notably, has a much larger substrate-binding cavity [48], or CYP2A6. The A330F mutation differed in effect from the SRS2 mutation (E229K) in that the turnover numbers with  $\beta$ -estradiol and arachidonate were severely reduced with strong impact on metabolite patterns. The impact of this mutation on retinoid metabolism was likewise influenced, the first oxidative step in retinoic acid formation, oxidation of retinol, being impaired by about 73%, with the major effect being on  $k_{cat}$  rather than  $K_m$  and the second step, oxidation of retinal, being impaired by 38%. Retinal metabolism showed a small increase in  $K_m$  with negligible impact on  $k_{cat}$ . The availability of the small

Fig. 8



Orientation of A330 of CYP1B1 with heme and substrate in the active site. The identified CYP1B1 residues (A330 and T334) aligning with CYP1A2 residues A317 and T321 are oriented between the substrate, alpha naphthoflavone (ANF), and the heme in the substrate-binding region of the active site.

retinoid molecules in the active site seemed less impacted than the larger substrate molecules.

In most of the PCG cases in which the four described mutations are present, the affected individuals carry a single mutated allele and the wild-type allele. This might suggest that an adequate level of CYP1B1 activity is required for normal development of the eyes, necessitating utilization of both allelic CYP1B1 gene products, with little room for flexibility. The data provided in this study indicate that a mutation in just one of the two cellular CYP1B1 genes (Table 1b) to a form with diminished stability or strongly impaired substrate turnover number or with altered isomeric specificity toward an endogenous substrate, might explain why such a mutation may cause

the PCG phenotype. In, at least some of these individuals, there might be other factors contributing to the PCG phenotype, such as an additional unidentified mutation in the promoter region of the CYP1B1 or in the noncoding region of CYP1B1 that modifies the effect of the reading frame mutation. The possibility of modifier influences of other interacting gene products also exists. In addition, seven polymorphic forms of CYP1B1 are known. These forms of CYP1B1 have been shown to differ in *in vitro* rates of metabolism of some substrates [49–52]. The polymorphic forms, with defined differences in metabolic activities, might also have an influence when combined with the mutations, either protecting against or exacerbating the effects of the mutations, and perhaps explaining the frequently observed incomplete penetrance of the PCG phenotype [3,41]. In recent studies, PCG-causing mutations have been shown in individuals with different polymorphic forms of CYP1B1 [22,31], and this may modulate the PCG-causing effects of CYP1B1 mutations. In addition, a preferential selection of allelic variants for enhanced proteolytic degradation, as observed for CYP1B1.4 overexpressed in COS-1 cells [43], could also lower tissue content of a mutant enzyme to deleterious levels.

Our results indicate that the metabolite pattern and/or specific activity of the individual mutants may be strongly altered toward different substrates, rendering the individual susceptible to developmental eye abnormalities that result in PCG. Examination of the structural regions containing mutations in CYP1B1 (Table 1) helps in understanding the mechanistic basis for the resultant impairment of the enzyme function. For example, the R469W mutation (Table 1) occurs in the highly conserved heme-binding region, the FXXGXRXCXG motif, where substitution of tryptophan for arginine greatly stabilizes the hemoprotein, but impairs catalytic activity [9]. R469 in CYP1B1 corresponds to the charged R457 residue on CYP1A2 adjacent to and located on the surface of the molecule in a depression above the cysteine-heme ligand. R457 was suggested by Fujii-Kuriyama's group to be involved in interaction of NADPH-CYP reductase with CYP1A2 [53]. Substitution by tryptophan would be a change to a hydrophobic residue that might impair reductase binding or electron flow and, thus, catalytic activity of the hemoprotein. Two other CYP1B1 mutations E387K and R390H (Table 1a) occur in the highly conserved EXXR motif in the K-helix of the protein and stabilize that helix by charge-pairing with surrounding residues like R444 and D436, respectively. Eighteen of these residues are conserved in the family 1 CYPs and a number of these are also conserved in CYP2A6, CYP2C9, and CYP3A4. One might predict that some of these mutants would also have diminished stability. Interestingly, none of the listed PCG-causing mutations are in the basic residues that ring the proximal surface depression above the thionyl bond of C470. These

include C-helix residues R146 and R145 (that align with R138 and R137 of CYP1A2), B-helix residue H104 (that aligns with R95), L-helix residue K477 (that aligns with K465), K467 and R469 (that align with K455 and R457, respectively), and the K' helix residue, K454 (that aligns with K442) [48]. These residues contribute a cationic proximal surface to the molecule and possibly serve as a charge-pairing surface for interaction with redox partners. The results discussed in this study showing the influence of mutations on the stability and function of CYP1B1 are consistent with our earlier suggestion [5,14,54,55] that the role of CYP1B1 in eye ontogeny involves its capacity to oxidatively metabolize a compound of intermediary metabolism critical for the normal development of that tissue.

## Acknowledgements

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