

**A Natural Variant of the Heme-Binding Signature (R441C) Resulting in Complete Loss of
Function of CYP2D6**

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Abbreviations:

CYP, cytochrome P450 monooxygenase; MR_S, sparteine oxidation metabolic ratio; SNP, single nucleotide polymorphism

ABSTRACT

A new variant allele *CYP2D6**62 (g.4044C>T; R441C) of the drug-metabolizing cytochrome P450, *CYP2D6*, was identified in a person with reduced sparteine oxidation phenotype which was unexpected based on a genetic *CYP2D6**1A/*41 background. The recombinantly expressed variant protein had no activity towards propafenone due to missing heme incorporation. Sequence alignment revealed that the positively charged R441 residue is part of the heme-binding signature but not strictly conserved among all P450s. A compilation of described P450 monooxygenase variants revealed that other enzymes can functionally tolerate even non-conservative amino acid changes at the corresponding position (i.e. the invariant cysteine -2). This suggests that heme binding in mammalian P450s depends not only on the integrity of the heme-binding signature but also on other family- and subfamily-specific sequence determinants.

INTRODUCTION

Cytochrome P450 (CYP) enzymes catalyze oxidative biotransformations of many endogenous compounds and of most xenobiotics including the majority of clinically used drugs (Nelson et al., 1996). CYP2D6 is a highly polymorphic human enzyme involved in the metabolism of about 20% of all drugs. Over 80 alleles have been defined at the CYP2D locus on chromosome 22 which lead to increased, decreased or absent 2D6 protein and/or function (Zanger et al., 2004; human CYP allele nomenclature web-site: <http://www.cypalleles.ki.se>). The poor metabolizer (PM) phenotype results from the presence of two non-functional alleles whereas the extensive metabolizer (EM) phenotype results from the presence of one or two alleles with normal function including *1 and *2 (R296C, S486T). An intermediate metabolizer (IM)-phenotype is usually observed in persons having one *CYP2D6 null* allele and one allele with impaired expression and/or function, such as *9, *10 or *41 (Raimundo et al., 2004). Here we describe a novel genetic variant that was found in an individual with unexplained low activity of CYP2D6. Structural considerations lead to a deeper insight into the role of amino acids that are part of the so-called heme-binding signature, a stretch of amino acids close to the C-terminus of cytochromes P450 and essential for incorporation and binding of the non-covalently bound heme prosthetic heme group (Li and Poulos 2004).

MATERIALS AND METHODS

Genomic DNA and sequencing.

The genomic DNA was derived from a previously characterized study population (Griese et al., 1998). The *CYP2D6* gene was completely sequenced in all exon and intron regions as described (Raimundo et al., 2004). Two DNA-sample collections were used for estimation of SNP-frequency, a liver tissue collection (n=280, Wolbold et al., 2003) and a collection of healthy Caucasian volunteers (n=200; Schaeffeler et al., 2004). All study protocols were approved by the local Ethics Committees and written informed consent was obtained from all subjects. Nucleotide position numbering was according to the guidelines available at the CYPAllele nomenclature homepage (<http://www.cypalleles.ki.se>).

Genotyping of g.4044C>T (c.1321C>T) by denaturing HPLC.

The *CYP2D6* specific PCR fragment C (1.3 kbp) was amplified from genomic DNA as previously described (Raimundo et al., 2004) followed by a nested PCR using oligonucleotide primers 2D6-5530f: 5'-AGCCGGAGGCCTTCCTGCC-3' and 2D6-5796r: 5'-ACCAGGAAAGCAAAGACACC-3', resulting in a 267 bp fragment which was analyzed by denaturing HPLC on a WAVE DNA Fragment Analysis System (Transgenomic Inc, Omaha, Neb) using a preheated reverse phase column (DNA Sep Cartridge; Transgenomic, Cheshire, UK) at 66°C. A linear acetonitrile gradient (flow rate 0.9ml/min) was applied using buffer A (0.1M triethylammonium acetate) and buffer B (0.1M triethanolammonium acetate, 25% acetonitrile; 53% to 62% in 4.5min). Heteroduplex formation was detected from melting profiles in comparison to wild type and mutant controls and after addition of equal amounts of a non-mutated sample before denaturation.

Cloning, transfection and expression.

To determine which allele carried the new variant, a PCR fragment comprising the 1.3 kbp genomic region spanning exon 7 to 9 (primer F: 5'_CCATCTGGGAAACAGTGCAG_3' and R: 5'_ATTGTACATTAGAGCCTCTGGC_3') was cloned into pGEMTeasy cloning vector (Promega, Mannheim, Germany) and sequenced using standard sequencing primers (pUC/M13 forward; pUC/M13 reverse) and *CYP2D6* gene specific sequencing primers (sequences available on request). For site directed mutagenesis, a 1.5 kB *Bam*HI/*Hind*III fragment (Evert et al., 1997) comprising the *CYP2D6* cDNA was cloned into pFastBac1 (Invitrogen, Carlsbad, Germany). The resulting plasmid (pIKAT16) was subjected to site-specific mutagenesis using QuikChange Site Directed Mutagenesis Kit (Stratagene, Heidelberg) and appropriately designed oligonucleotides (available on request). The complete cDNA sequence was verified on both strands by sequencing and recloned in pFastBac1 or pCMV vectors. Bacmid preparation was done as described previously (Klein et al., 2005). Baculovirus formation and microsomal preparation from insect cells and microsomes from COS-1 cells were performed according to the manufacturer's recommendations and as described previously (Klein et al., 2005). Buffer for microsome suspension was 0.1 M sodium phosphate buffer (pH 7.4). Enzyme

activity was determined using propafenone as previously described (Toscano et al., 2006). P450 content was quantified by standard reduced CO-difference spectroscopy.

***In silico* analysis of sequences and molecular modelling**

To analyze the influence of mutations a sequence conservation analysis of the CYP2D family was performed. Sequences of 22 *CYP2D* members were compared to human CYP2D6. Sequence identity ranged from 50 to 92%. Sequence alignment was calculated using the ClustalX program with default parameters (Thompson et al., 1997). Conservation scores were calculated using the program al2co and the unweighted, not normalized sum of pairs method with the BLOSSUM62 scoring matrix (Pei and Grishin 2001). The conservation score depends on the number and kind of residues occurring at the respective position. A higher score reveals a higher degree of conservation. For calculating family specific heme-iron ligand patterns, sequences of the CYP families 1A (39), 2C (34), 2D (22), 3A (34), 7A (9), 11A+B (24) and 19A (37) were used (number of sequences in brackets). All protein sequences were derived from GenBank (Benson et al., 2006). FastA sequences and accession numbers are listed in the Supplemental data file. For molecular modelling of the structure function relationship the recently solved crystal structure of CYP2D6 was used (PDB code 2F9Q) (Rowland et al., 2006). Structure analysis and visualisation was done using the PyMOL program (<http://www.pymol.org>). PROSITE Release 20.3 (<http://www.expasy.org/prosite/>) was used for pattern search.

RESULTS AND DISCUSSION.

Re-sequencing of *CYP2D6* in subjects with discrepant genotype-phenotype relationship identified a novel nucleotide variant in exon 9 (c.1321C>T, R441C) on the background of a *CYP2D6**1A/*41 genotype in an intermediate metabolizer of sparteine ($MR_S=2.09$). Molecular haplotype analysis revealed that the subject carried the R441C mutation on the *1A allele, thus defining a novel allele of *CYP2D6* which was designated as *62. The novel mutation was the only sequence difference between *62 and the reference allele *1A. The frequency of the novel mutation appears to be very rare in Caucasians (<0.1%) as no other carrier was detected among 480 DNA samples. To analyze the functional consequences of the novel SNP, the *CYP2D6.62* variant protein was expressed in two different heterologous expression systems. Although the amounts of immunodetectable mutant protein were comparable to those of the wild-type in both systems, reduced CO-difference spectroscopy of insect cell microsomes consistently failed to show absorption at 450 nm for the variant (Fig.1). In addition, enzyme activity determination using propafenone, a typical substrate of *CYP2D6*, did not reveal any detectable hydroxylated metabolite formed by the R441C variant (data not shown). The novel allele *CYP2D6**62 therefore represents a completely non-functional allele. The corrected genotype of the investigated subject can be described as heterozygous for one allele with decreased function *41 and one *null* allele *62. The *41 allele is a common low-activity allele related to *2 [R296C, S486T] but with the additional intronic 2988G>A change that causes partial erroneous splicing and decreased expression of functional protein (Toscano et al., 2006). With a MR_S of 2.09, the IM phenotype of the individual is in perfect agreement with the corrected new genotype as *41 in combination with one *null* allele predicts MR_S values between 1.2 and 15 (Raimundo et al., 2004). Analysis of the known *CYP2D6* crystal structure (Rowland et al., 2006) and amino acid sequence comparison to other P450 monooxygenases shows that residue 441 maps to the second residue N-terminal of the invariant cyteine (C443) in a region N-terminal to helix L. The heme iron is bound by C443 (Fig2A) and the heme is anchored in the heme binding site by hydrogen bonding interactions between the carboxyl groups of the two heme propionate groups and the side chains of R101, W128, R132, H376, S437 and R441 (Fig.2B). In view of our finding of R441C being a *null*-allele, this

consideration suggested an essential role of R441 within the “Cytochrome P450 cysteine heme-iron ligand signature”, a calculated consensus sequence derived from the comparison of more than 5500 P450 heme-thiolate protein sequences of various species (PROSITE identifier PS00086; documentation PDOC00081). This consensus sequence shows that the residue corresponding to R441 is not always occupied by a positively charged amino acid but different residues including H, T and P are tolerated at this position. Figure 3 shows a compilation of reported naturally occurring P450 variants. Among these, the R433W variant of CYP2C19 (*5 allele, Ibeanu et al., 1998) and the R448H variant of CYP11B1 (Curnow et al., 1993) had completely abolished S-mephenytoin and cortisol 11 β -hydroxylase activities *in vivo*, respectively. For the mutation to W in CYP2C19 a loss of activity is predicted, as W is not included in the PROSITE pattern at this position [RKHPT]. However, H is included in the pattern, thus indicating that this pattern may not be reliably predictive.

Therefore we analysed the conservation of residues in different CYP enzyme families. For this purpose, family-specific heme-iron ligand patterns for CYP1A, 2C, 3A, 7A, 11AB, 19A and the CYP2D family were calculated, which are more specific (Table 1; Supplemental data file). In general, arginine seems to be more conserved in mammalian subfamilies at the position corresponding to CYP2D6 R441. However, family CYP7A remarkably contains a conserved threonine at this position. The CYP11 family-specific pattern showed only R at this position thus explaining the loss of activity when R448 is replaced by histidine in CYP11B1. Another natural variant, human CYP1A2.8 (R456H), is also better described by the family pattern, which had normal protein amount but was functionally inactive, mainly due to missing heme incorporation (Saito et al., 2005). Consistent with the fact that cysteine is not part of the CYP19-family or PROSITE pattern, the CYP19 variant 435C had only residual but interestingly still measurable enzyme activity (1.1% of wild type) (Ito et al., 1993).

A series of amino acids were identified in rat CYP1A2 to play a role in the P450-reductase interaction (Shimizu et al., 1991). Interestingly they include the leucine and arginine which are the direct neighbours of the position investigated here. The PM variant CYP2D6.31 (R440H) also concerns one of these positions and was shown to disrupt interaction with the FMN domain of P450 reductase (Allorge et al., 2005).

In conclusion, *CYP2D6**62 [R441C] is a rare, naturally occurring allele of CYP2D6 that leads to the PM phenotype if inherited together with another null-allele. Whereas R441 appears to be essential for heme binding and enzymatic function of CYP2D6, the role of this residue in other P450 monooxygenases appears to be dependent on the sequence context and may also include disturbance of the interaction with P450-reductase. Family-specific signatures of the heme binding region may be more predictive than the overall PROSITE pattern.

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FOOTNOTES

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LEGENDS TO FIGURES AND TABLES

Figure 1. Recombinant protein expression of CYP2D6 variant and wild type:

A: representative Western Blot is shown for COS1-cell microsomes (10 μ g per lane); CYP2D6 standard protein: stably transfected lymphoblast microsomes (25, 10 fmol per lane) B: CO-difference spectra of CYP2D6.1 (dashed line) and CYP2D6.62 (solid line) recombinantly expressed in insect cells.

Figure 2. Structure of the heme binding region of CYP2D6 (PDB code 2F9Q).

The heme group is coordinated with C443 as fifth ligand (A) and anchored in the binding site by hydrogen bonding interactions (B). The side chain of R441 is interacting with the carboxyl group of the heme propionate group of heme ring D.

Figure 3. Sequence alignment of natural human cytochrome P450 monooxygenase variants. The region surrounding residue R441 of CYP2D6.1 (boxed) is shown. Residues are highlighted as follows: identical amino acids, white letters on black background; block of similar, white letters on dark grey background; conservative residues, black letters on grey background.

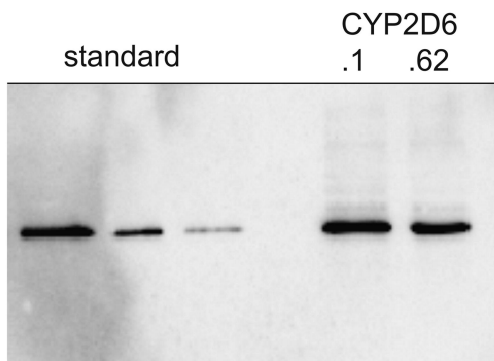
Protein accession numbers: P05177 (CYP1A2), P10635 (CYP2D6), P33261 (CYP2C19), P22680 (CYP7A1), P15538 (CYP11B1), J04127 (CYP19)

Table 1: Cytochrome P450 cysteine heme-iron ligand signature (PROSITE: PS00086) of the P450 superfamily and family specific patterns of CYP families.
 x: any amino acid; square parenthesis: those allowed at this position; curly brackets: those forbidden at this position.

PS00086	[FW]	[SGNH]	X	[GD]	{F}	[RKHPT]	{P}	C	[LIVMFAP]	[GAD]
Position	436	437	438	439	440	441	442	443	444	445
CYP2D	F	S	A	G	[RHP]	R	[ASVI]	C	L	G
conservation score (CYP2D)	6.0	4.0	4.0	6.0	3.4	5.0	1.6	9.0	4.0	6.0
CYP1A	F	G	[ML]	G	[KR]	R	[KRQ]	C	[IV]	G
CYP2C	F	S	[ATISL]	G	[KR]	R	[IAMVSK]	C	[VAMLP]	G
CYP3A	F	G	[NTASLID]	G	P	R	N	C	[ILV]	[GD]
CYP7A	F	G	S	G	[AV]	T	[IK]	C	P	G
CYP11A+B	F	G	F	G	[VMAS]	R	Q	C	[LVI]	G
CYP19A	F	G	[FSC]	G	P	R	[SAG]	C	[VAI]	G

Figure 1

A



B

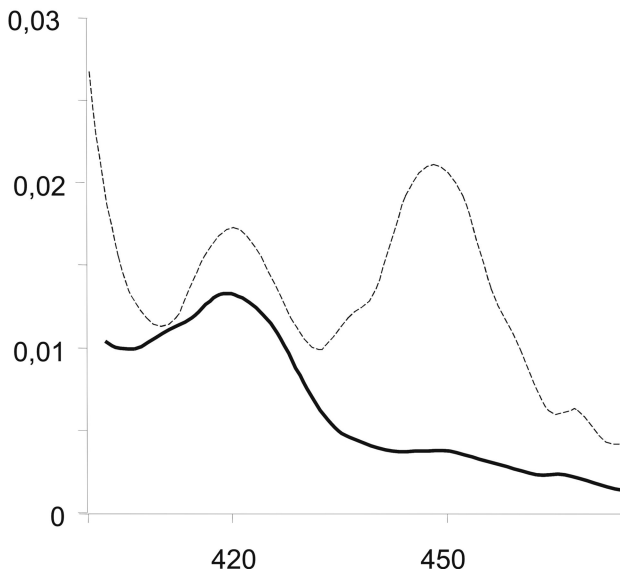


Figure 2

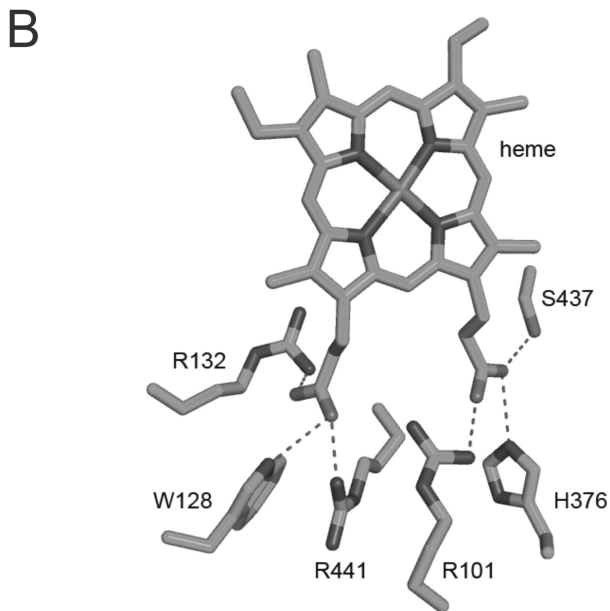
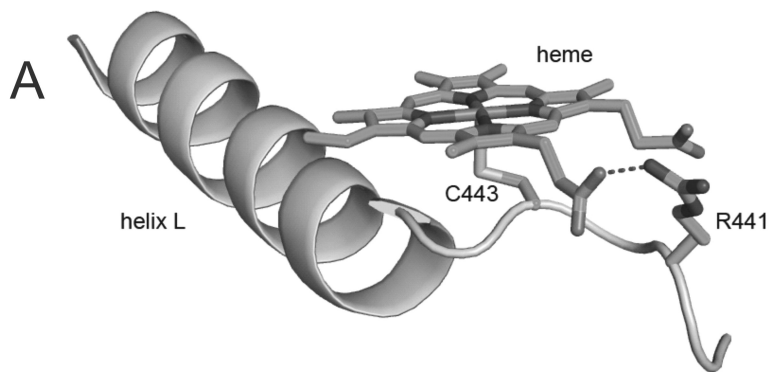


Figure 3

CYP1A2.8 (R456H)	451....	F	G	M	G	K	H	R	C	I	G
CYP2C19.5 (R433W)	428....	F	S	A	G	K	W	I	C	V	G
CYP2D6.1 (R441)	436....	F	S	A	G	R	R	A	C	L	G
CYP2D6.62 (R441C)	436....	F	S	A	G	R	C	A	C	L	G
CYP7A1 (T442)	437....	F	G	S	G	A	T	I	C	P	G
CYP11B1 (R448H)	443....	F	G	F	G	M	H	Q	C	L	G
CYP19 (R435C)	430....	F	G	F	G	P	C	G	C	A	G