Role of conserved Asp²⁹³ of cytochrome P450 2C9 in substrate recognition and catalytic activity

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Human cytochrome P450 2C9 (CYP2C9) is important in the metabolism of non-steroidal anti-inflammatory compounds such as diclofenac, the antidiabetic agent tolbutamide and other clinically important drugs, many of which are weakly acidic. Multiple sequence alignment of CYPs identified CYP2C9 Asp²⁹³ as corresponding to Asp³⁰¹ of CYP2D6, which has been suggested to play a role in the binding of basic substrates to the latter enzyme. Replacement of Asp²⁹³ with Ala (D293A) decreased activity by more than 90 %, and led to an approx. 3- to 10-fold increase in K_m values for the three test substrates tolbutamide, dextromethorphan and diclofenac. Conservative replacement of the carboxyl side chain in a Glu (D293E) mutant produced no significant changes in K_m values and slight increases in k_{cat} values. Changes in regiospecificity were observed for both the

Ala and Glu substitutions; low levels of both dextromethorphan O- and N-demethylation were observed in the D293A mutant, whereas increased preference for O-demethylation was observed for the D293E mutant. Expression of constructs coding for Asn (D293N) and Gln (D293Q) substitutions failed to form a P450 correctly. Our analysis suggests a structural role for the carboxyl side chain of Asp²⁹³ in CYP2C9 substrate binding and catalysis. The conservation of an Asp residue in other CYP families in a position equivalent to Asp²⁹³ indicates a common mechanism for maintaining the active-site architecture.

Key words: dextromethorphan, diclofenac, mutagenesis, P450, tolbutamide.

INTRODUCTION

The cytochrome P450 (CYP) mono-oxygenase system is involved in the initial oxidative step, or phase I, of the cellular detoxification pathway for many drugs and exogenous compounds [1]. It also functions in endogenous pathways associated with steroid hormone and prostaglandin synthesis [2]. There are currently 56 known human isoforms in the CYP superfamily [3], but five isoforms, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, are primarily responsible for drug metabolism [4–6]. Originally isolated as tolbutamide hydroxylase [7], CYP2C9 has since been shown to be involved in the metabolism of S-warfarin, phenytoin [4], the antimalarial agent 58C80 [8] and the non-steroidal anti-inflammatory drugs diclofenac [9,10], ibuprofen [10] and suprofen [11] among other drugs.

Currently, the only crystal structure for a mammalian CYP is that of rabbit CYP2C5 [12]. Homology modelling and pharmacophore modelling have become important tools for rationalizing the role of specific amino acid residues in substrate binding to CYPs. Structural models have been produced for a number of isoforms, including CYPs 1A2, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, 19 (aromatase) and 51 (14 α -hydroxylase) (reviewed in [13]). In CYP2C9, a preference has been shown for weakly acidic substrates such as non-steroidal anti-inflammatory drugs [14]. The carboxylate groups of tienilic acid and diclofenac have been shown to be responsible for substrate preference and orientation in the active site [11,15], implying the existence of a complementary active-site cationic group. Detailed CYP2C9 pharmacophores based on kinetic studies of a broad range of substrates contain a hydrophobic feature, a hydrogen bond acceptor and

either a second hydrogen bond acceptor or a donor, whereas pharmacophores based on inhibitors contain a hydrophobic feature and only one hydrogen bond acceptor or donor [16].

By contrast with CYP2C9, the preferred substrates of CYP2D6 contain basic nitrogens [17], and two negatively charged residues, Glu²¹⁶ and Asp³⁰¹, have been identified as important determinants for this preference [18–21]. Interestingly, a carboxy group equivalent to the CYP2D6 Asp³⁰¹ is conserved in approx. 25 % of CYPs [22], despite their wide range of substrate specificity. The role of this conserved active-site carboxy group in CYP families that do not metabolize basic substrates has yet to be established. Notably, an equivalent Asp residue is conserved in the CYP2C family (Figure 1), including the CYP2C9 isoform where the equivalent residue is Asp²⁹³, notwithstanding the preference of this enzyme for weakly acidic substrates.

To investigate the role of Asp²⁹³ in substrate binding and catalysis by CYP2C9, we have examined the effects of mutating this residue on the metabolism of three test substrates: diclofenac, a carboxylate-containing CYP2C9 substrate, tolbutamide, a CYP2C9 substrate which does not contain a carboxylate group and dextromethorphan, a CYP2D6 substrate containing a basic nitrogen.

EXPERIMENTAL

Materials

Unless otherwise indicated, chemicals were purchased from Sigma (Poole, Dorset, U.K.), DNA-modification enzymes were purchased from Gibco BRL (Paisley, Renfrewshire, Scotland,

Abbreviations used: CYP, cytochrome P450; D293A, Asp²⁹³ → Ala; D293E, Asp²⁹³ → Glu; hCPR, human NADPH:CYP oxidoreductase.

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	282	*	302
1A1_macaq	LSDEKIVNVVLDLFGAGFDTV		
1A1_human	LSDEKIINIVLDLFGAGFDTV		
1A2_human	IPQEKIVNLVNDVFGAGFDTV		
1A2_macaq	IPQEKIVNLVNDIFGAGFDTI		
2B_monkey	FSHRNLIINTLSLFFAGTETT		
2B1_human	FSHQNLIIINTLSLFFAGTETT		
2B6_human	FSHQNLNLNTLSLFFAGTETT		
2C5_rabbit	FTLESIVIAVSDLFGAGTETT		
2C9_human	FTIESLENTAVDLFGAGTETT		
2C8_human	FNENLVGTVAIDLFGAGTETT		
2C10_human	FTIESLENTAVDLFGAGTETT		
2C18_human	FTVESLIATVTDMFGAGTETT		
2C19_human	FTIENLVITAADLLGAGTETT		
2D6_human	FNDENLRIVVADLFSAGMVT		
2D17_macaq	FNEENLRMVVADLFSAGMVT		
3A4_human	LSDLELVAQSIIIFIFAGYETT		
3A8_macfa	LSDLELVAQSIIIFIFAGYETT		
P450_bm3	LDDENIRYQIITFLIAGHETT		
P450_cam	ITSDEAKRMCGLLLVGGGLDVT		
		SRS 4	

Figure 1 Multiple sequence alignment of the I helix of selected mammalian CYP sequences (aligned using ClustalX [41])

Conservation of an Asp residue across many CYPs at a position corresponding to CYP2C9 Asp²⁹³ is illustrated by an asterisk. Numbering is according to the human CYP2C9 sequence.

U.K.) and Promega (Chilworth, Southampton, U.K.), and CYP metabolites were from Ultra Fine Chemicals (Manchester, U.K.). Glycerol was AnalaR grade from BDH (Poole, Dorset, U.K.). HPLC-grade acetonitrile and methanol were purchased from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland, U.K.). All spectroscopic analyses were performed on a Shimadzu MPS2000 spectrophotometer (Shimadzu Europe Ltd, Duisburg, Germany). HPLC assays were performed using an Agilent Technologies 1100 Series HPLC using Hewlett Packard Chemstation software (Agilent Technologies, Stockport, Cheshire, U.K.).

Site-directed mutagenesis

Site-directed mutants of Asp²⁹³ residue were constructed using the method described by Kunkel et al. [23] using a CYP2C9 expression construct, pQ4, as template DNA. The Asp²⁹³ → Ala (D293A), Asp²⁹³ → Asn (D293N), Asp²⁹³ → Glu (D293E) and Asp²⁹³ → Gln (D293Q) substitutions were introduced using the following oligonucleotides, the altered codon being underlined: D293A: 5'-TCCAAACAAGGCAACTGCAGT-3'; D293N: 5'-TCCAAACAAGTTAACTGCAGT-3'; D293E: 5'-TCCAAACAATTCAACTGCAGT-3'; and D293Q: 5'-TCCAAACAATTGAAGTGCAGT-3'.

A separate reporter oligonucleotide 5'-GCAGATCTGAA-TCCAGGGGCT-3' was also included in the mutagenesis reactions to generate a silent adenine (A) to thymine (T) base change at nucleotide 654 (underlined), eliminating a *Bam*H1 site. Clones were selected based on restriction mapping with *Bam*H1 and *Sph*I and confirmed by DNA sequencing.

Expression of CYP2C9 and human NADPH:CYP oxidoreductase (hCPR) in *Escherichia coli* strain DH5α

A CYP2C9 expression construct, pQ4, was created by the addition of an N-terminal ompA leader sequence and C-terminal

His tag. CYP2C9 and hCPR were co-expressed in *E. coli* strain DH5α, from pQ4 and pJR7 [24] respectively. Bacterial membranes were prepared by the method of Blake et al. [25]. Single colonies of co-transformants were selected on Luria–Bertani broth plates, supplemented with 50 µg/ml ampicillin and 25 µg/ml chloramphenicol. For protein expression, 250 ml of Terrific broth, supplemented with 50 µg/ml ampicillin contained in a 2 litre flask was inoculated at 1:50 with an overnight culture grown at 37 °C in Luria–Bertani broth supplemented with 50 µg/ml ampicillin and 25 µg/ml chloramphenicol, and grown at 30 °C. When the absorbance (A_{600}) reached 0.45–0.5, δ -amino-laevulinic acid was added to a final concentration of 0.5 mM, and protein expression was induced at A_{600} 0.9–1.0 by the addition of isopropyl β -D-thiogalactoside to a final concentration of 1 mM. Cells were harvested by centrifugation at 4500 *g* for 25 min at 4 °C using a H6000A rotor in a Sorvall RC3C centrifuge. The pellet was resuspended in ice-cold 1 × TSE [50 mM Tris/acetate (pH 7.6), 250 mM sucrose, 0.25 mM EDTA] [26] at 100 ml/l of starting culture, treated with 50 µg/ml lysozyme for 60 min at 4 °C, repelleted and resuspended in ice-cold 1 × TSE for storage at –70 °C. Bacterial membranes were isolated by the method of Renaud et al. [26]. Whole cells were thawed in the presence of the protease inhibitors, aprotinin and leupeptin, at a final concentration of 1 µg/ml, and PMSF to 1 mM final concentration. The cells were subsequently sonicated and centrifuged at 12000 *g* for 20 min at 4 °C using an SS-34 rotor in a Sorvall RC-5B centrifuge. Bacterial membranes were isolated from the supernatant by centrifugation at 180000 *g* for 50 min at 4 °C using a T-1250 rotor in an OTD 65B Sorvall Ultracentrifuge. The membrane pellet was resuspended in ice-cold 1 × TSE and stored frozen at –70 °C. Before use, membranes were characterized based on recombinant CYP content as estimated by Fe²⁺–CO versus Fe²⁺ difference spectra [27] and the hCPR content, as estimated by cytochrome *c* reductase activity.

Determination of recombinant hCPR content using cytochrome *c* reduction

Assays were performed in 0.3 M potassium phosphate (pH 7.7) at 25 °C. Stock solutions of 50 mM cytochrome *c* and 5 mM NADPH made in 0.3 M potassium phosphate (pH 7.7) were used at final concentrations of 50 µM cytochrome *c* and 50 µM NADPH.

Steady-state kinetic assays

CYP content for all enzyme assays was standardized at 10 pmol. Routinely, an NADPH-generating system was used to maintain a continual source of NADPH throughout the course of the reaction, and consisted of NADP⁺, glucose 6-phosphate and glucose-6-phosphate dehydrogenase at final concentrations of 0.001 mM, 0.025 mM and 1 unit/ml respectively. Reactions were performed in 50 mM potassium phosphate (pH 7.4) for tolbutamide and dextromethorphan, whereas 100 mM Tris/HCl (pH 7.4) was used for diclofenac. Reaction mixtures were incubated at 37 °C in opaque Eppendorf tubes. Stock solutions of substrate consisted of 500 mM tolbutamide in 100% methanol, 50 mM diclofenac in methanol/1 M HCl (8:1, v/v), and 50 mM dextromethorphan in 50 mM potassium phosphate (pH 7.4); subsequent dilutions were such that the final concentration of solvent present in the reaction did not exceed 2.5% (v/v). The tolbutamide reactions were quenched using 50 µl of 10% (w/v) trichloroacetic acid, whereas diclofenac was quenched by the addition of ice-cold methanol to a final concentration of 33%,

and dextromethorphan with ice-cold methanol and 60% HClO₄ to final concentrations of 33 and 1% respectively. Particulate material was removed by subsequent centrifugation at 16000 *g* for 5 min in a bench top Microfuge (Eppendorf) at room temperature (25 °C). Routinely, 100 μ l of the reaction supernatant was assayed for the presence of CYP metabolites using reversed-phase HPLC. Metabolism of the CYP substrates was monitored using the metabolites 4-methylhydroxytolbutamide, 4'-hydroxydiclofenac, dextrorphan and 3-methoxymorphinan (the O- and N-demethylation products of dextromethorphan respectively).

Quantification of metabolites

Separation of the 4-methylhydroxytolbutamide metabolite was performed using a 5 μ m (4.0 mm $\times$ 250 mm) Hypersil ODS column (Hewlett Packard) maintained at constant temperature of 25 °C. The mobile phase contained acetonitrile (A) and 10 mM sodium acetate, pH 4.3 (B) forming a gradient (0 min: 32% A, 68% B; 3 min: 32% A, 68% B; 11.5 min: 49% A, 51% B; and 12.5 min: 32% A, 68% B) run at a flow rate of 1 ml/min. Measurement of 4'-hydroxydiclofenac used a 5 μ m (4.0 mm $\times$ 250 mm) Hypersil ODS column (Hewlett Packard) maintained at 30 °C, with a mobile phase consisting of acetonitrile (23%) and 20 mM potassium phosphate buffer, pH 7.0 (77%), run isocratically at a flow rate of 1 ml/min. In both cases, metabolites were detected by measuring the absorbance at 280 nm.

The dextromethorphan O-demethylation product, dextrorphan, was separated using a 5 μ m (4.6 mm $\times$ 250 mm) Hypersil BDS-C18 column (Agilent Technologies) at room temperature with acetonitrile (22%) and 0.1 M ammonium acetate, pH 5.0 (78%), run isocratically at a flow rate of 1 ml/min. The 3-methoxymorphinan product was separated using a mobile phase of acetonitrile (32%) and 0.1 M ammonium acetate, pH 5.0 (68%), run isocratically at 1 ml/min using the same column matrix. Both metabolites were detected by measuring the emission at 312 nm after excitation at 270 nm. In all cases, metabolites were quantified against commercially available authentic standards. The data were analysed using the Michaelis–Menten equation implemented in Prism 3.0 (GraphPad).

RESULTS

To determine the role of the carboxy group of Asp²⁹³ residue in CYP2C9 substrate selectivity, the residue was replaced by alanine, asparagine, glutamine or glutamate. The wild-type and mutant enzymes were co-expressed in *E. coli* with hCPR, and isolated membrane fractions were used to analyse the catalytic activity. Levels of expression of wild-type, D293A and D293E enzymes, estimated from carbon monoxide difference spectra, were 90, 280 and 120 nmol of CYP/mg of membrane protein respectively. These preparations had hCPR activities of 178, 176 and 377 nmol of cytochrome *c* reduced $\cdot$ min⁻¹ $\cdot$ (mg of membrane protein)⁻¹ respectively, producing estimates of 290, 286 and 610 nmol of hCPR/mg of membrane protein by comparison with a purified hCPR standard. Thus the CYP/hCPR ratios were approx. 1:3, 1:1 and 1:5 for wild-type, D293A and D293E mutants respectively. The asparagine and glutamine substitutions did not form spectroscopically detectable CYP, although protein expression was detected by Western-blot analysis (results not shown). This suggests that these mutations may have an effect on the structural stability of CYP2C9; they were not analysed further.

Figure 2 Structures of the three test substrates used: (a) tolbutamide, (b) diclofenac and (c) dextromethorphan

The arrows indicate the sites of CYP2C9-mediated metabolism examined in the present study.

To investigate the effects of mutations on catalytic activity, membranes from *E. coli* cells co-expressing CYP2C9 and hCPR were incubated with three substrates, the acidic CYP2C9 substrates diclofenac and tolbutamide, and the CYP2D6 substrate dextromethorphan, which contains a basic nitrogen (Figure 2). The contrasting physicochemical properties of each of the substrates [14] allow us to probe different aspects of CYP2C9 substrate binding and catalysis.

The kinetic parameters obtained for tolbutamide, diclofenac and dextromethorphan metabolism by the wild-type and mutant enzymes are shown in Table 1. For the wild-type enzyme, the observed k_{cat} value of 4.1 min⁻¹ for tolbutamide 4-methyl hydroxylation is comparable with the range of 5.5–10.7 min⁻¹ reported for human liver microsomes, although the K_{m} value is rather higher when compared with the reported range of 97–202 μ M [28]. For diclofenac 4'-hydroxylation, both kinetic parameters for the recombinant enzyme fall within the reported range (k_{cat} = 14–41 min⁻¹, K_{m} = 1.8–25 μ M [29–32]). Interestingly, wild-type 2C9 showed clear activity towards the basic nitrogen-containing compound, dextromethorphan, catalysing its O-demethylation to dextrorphan with k_{cat} = 1.8 min⁻¹ and K_{m} = 290 μ M, values quite comparable with those for the classic 2C9 substrate tolbutamide.

The conservative replacement D293E had only slight effects on the kinetic parameters for the three test substrates. The k_{cat} values for diclofenac and tolbutamide were increased by approx. 1.4-fold only, with a slightly larger increase (1.8-fold) for dextromethorphan; changes in K_{m} values were not significant.

By contrast, the D293A substitution produced much larger effects, showing large decreases in k_{cat} values and increases in K_{m} values for tolbutamide, diclofenac and dextromethorphan. These effects are greater for the carboxylate-containing diclofenac (500-fold decrease in k_{cat} value; 11-fold increase in K_{m} value) than for the sulphonamide tolbutamide (11-fold decrease in k_{cat} value; 5-fold increase in K_{m} value), whereas the O-demethylation of

Table 1 Kinetic parameters of wild-type CYP2C9, and the D293E and D293A mutants

Enzyme	Tolbutamide 1'-hydroxylation		Diclofenac 1'-hydroxylation		Dextromethorphan O-demethylation	
	k_{cat} (min ⁻¹)	K_m (μM)	k_{cat} (min ⁻¹)	K_m (μM)	k_{cat} (min ⁻¹)	K_m (μM)
Wild-type	4.12 ± 0.30	410 ± 70	21.2 ± 0.5	8.1 ± 0.8	1.84 ± 0.24	290 ± 80
D293E	5.96 ± 0.18	330 ± 30	31.1 ± 1.0	11.2 ± 1.1	3.21 ± 0.95	310 ± 190
D293A	0.36 ± 0.03	2100 ± 44	0.04 ± 0.001	87.4 ± 5.4	0.032 ± 0.002	848 ± 120

Table 2 Dextromethorphan regioselectivity

Enzyme	[Dextromethorphan] (mM) ...	O-Demethylase activity (min ⁻¹) (dextrorphan production)			N-Demethylase activity (min ⁻¹) (3-methoxymorphinan production)		
		0.01	0.10	1.00	0.01	0.10	1.00
Wild-type		0.090 ± 0.010	0.590 ± 0.010	1.540 ± 0.030	n.d.	0.120 ± 0.010	0.580 ± 0.020
D293E		0.120 ± 0.010	1.130 ± 0.060	3.100 ± 0.150	n.d.	0.170 ± 0.020	0.960 ± 0.020
D293A		0.001 ± 0.0002	0.004 ± 0.0002	0.017 ± 0.0002	n.d.	0.002 ± 0.0004	0.013 ± 0.003

n.d., not detectable.

dextromethorphan was less affected (6-fold decrease in k_{cat} value; 3-fold increase in K_m value).

In addition to O-demethylation, CYPs can also catalyse the N-demethylation of dextromethorphan to produce 3-methoxymorphinan, a reaction normally associated with CYP3A4, although it is also a minor pathway in CYP2D6-mediated metabolism of dextromethorphan [33]. The O- and N-demethylation of dextromethorphan by the wild-type and mutant CYP2C9 was examined over a wide range of dextromethorphan concentrations, since in CYP2D6 the relative rates of these two reactions are dependent on the substrate concentration [34]; the results are shown in Table 2. Wild-type CYP2C9 was found to carry out both O- and N-demethylation of dextromethorphan, the rate of N-demethylation being slower than that of O-demethylation (and undetectable at the lowest substrate concentration used). The conservative D293E mutant similarly performed both reactions. As noted above, the rate of O-demethylation by this mutant was somewhat faster than that by the wild-type, whereas the rate of N-demethylation was more similar; as a result, the ratio of N- to O-demethylation by this mutant is slightly higher than that for the wild-type (3.2–6.6 compared with 2.7–4.9). In the D293A mutant, although O-demethylation activity was detectable, slow N-demethylation was also detected at $\geq 100 \mu\text{M}$ dextromethorphan, the differences in activity reducing the N- to O-demethylation ratio when compared with the wild-type (1.3–2.1 compared with 2.7–4.9).

DISCUSSION

The results from the present study show clearly that Asp²⁹³ plays a significant role in the catalytic activity of CYP2C9; replacement of this residue by an alanine leads to marked decreases in k_{cat} values and increases in K_m values for several substrates. This is in accordance with observations on mutants of the homologous Asp³⁰¹ in CYP2D6 [18,20], although in the latter case, the effects are primarily on K_m values [18]. In CYP2D6, which shows a preference for substrates containing a basic nitrogen, a clear role for this residue in forming an ion pair with the nitrogen of the substrate has been proposed [20,21], although subsequent work has also demonstrated a substantial structural role [18,19,35].

Figure 3 Ribbon diagram of the rabbit CYP2C5 structure [12] (PDB accession code 1dt6) with Ala¹¹³ replaced by its CYP2C9 equivalent, Val¹¹³

Relative locations of the haem, the I helix, and the B'-C loop are illustrated. The hydrogen bonds observed in the CYP2C5 crystal structure connecting the B'-C loop to the I helix via the OD2 of Asp²⁹³ and the backbone amide between Val¹¹³ and Ile¹¹² is shown by a blue broken line.

The role of this conserved carboxylate in the active site of CYP2C9, an enzyme which prefers weakly acidic substrates, is much less apparent. The observation that the D293A mutant shows a marked increase in K_m values for diclofenac and tolbutamide would seem to rule out the possibility of an unfavourable interaction between the carboxylate of Asp²⁹³ and the negatively charged centres of these two substrates.

Examination of the structure of a modified rabbit CYP2C5 isoform [12] indicates that the carboxy group of the homologous

residue (Asp²⁹⁰) does not extend directly into the active site. This residue is located in the I helix, with its side chain forming a hydrogen bond with the backbone amide between Ile¹¹² and Ala¹¹³ (Val¹¹³ in CYP2C9) in the B'-C loop (Figure 3). This loop, along with the loop between helices F and G, defines a substrate-access channel [12,36,37] and forms part of 'substrate recognition sequence 1' as defined by Gotoh [38]; several residues in this loop have been shown to affect activity, substrate binding and/or regiospecificity in both mammalian and bacterial CYPs [31,37,39,40]. It is probable that removal of the carboxy group and the resulting loss of the interactions with the B'-C loop in the D293A mutant may alter the position or mobility of this loop, having an indirect effect on the binding of the substrate.

The idea that Asp²⁹³ has a structural role is supported by the fact that the D293N and D293Q mutants were unable to correctly incorporate haem and form the characteristic spectroscopically detectable haem-thiolate interaction, although it is not clear why the structural consequences of these mutations should apparently be greater than those of the alanine substitution. Mutations at the corresponding position 301 in CYP2D6 have also been reported to decrease protein stability [19,20]. The fact that an aspartate residue, equivalent to Asp²⁹³, is conserved in CYPs from other families (cf. Figure 1), which have a wide range of substrate preferences, indicates that this residue has a key role in maintaining the structural integrity of the active site, specifically the position of the B'-C loop, in many members of this important family of enzymes.

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