Amino acid residue 247 in canine sulphotransferase SULT1D1: a new determinant of substrate selectivity

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The SULT (sulphotransferase) family plays a critical role in the detoxification and activation of endogenous and exogenous compounds as well as in the regulation of steroid hormone actions and neurotransmitter functions. The structure–activity relationships of the human SULTs have been investigated with focus on the amino acid 146 in hSULT1A3 and its impact on dopamine/PNP (*p*-nitrophenol) specificity. In the present study, we have generated canine SULT1D1 (cSULT1D1) variants with mutations at amino acid residues in the substrate-binding pocket [A146E (Ala-146 → Glu), A146D, A146Q, I86D or D247L]. These mutation sites were chosen with regard to their possible contribution to the marked dopamine/PNP preference of cSULT1D1. After characterization, we found that the overall sulphation efficiencies for the cSULT1D1 A146 and the I86 mutants were strongly decreased for both substrates compared with wild-type cSULT1D1 but the substrate preference was unchanged. In contrast, the D247L mutant was found to be more than 21-fold better at sulphating PNP (120-fold decrease in K_m value) but 54-fold less efficient in sulphating dopamine (8-fold increase in K_m value) and the preference was switched from dopamine to PNP, indicating the importance of this amino acid in the dopamine/PNP preference in cSULT1D1. Our results show that Asp-247 has a pronounced effect on the substrate specificity of cSULT1D1 and thus we have identified a previously unrecognized contributor to active-site selectivity.

Key words: canine, dopamine, mutagenesis, *p*-nitrophenol, SULT1D1 (sulphotransferase).

INTRODUCTION

Sulphonation, which has commonly been referred to as sulphation, implies the transfer of the sulphonate group from the PAPS (adenosine 3′-phosphate 5′-phosphosulphate) to numerous endogenous and exogenous molecules, such as steroids, neurotransmitters, bile acids, drugs and environmental chemicals. Members of the sulphotransferase (SULT) family catalyse these sulphation reactions. Cytosolic SULTs are involved in the homoeostasis and regulation of biologically active endogenous chemicals and also metabolic detoxification of therapeutic, dietary and environmental xenochemicals.

The task to identify and classify the various SULTs from a variety of tissues and species is currently proceeding at a high pace. Thus, based on their deduced amino acid sequences, cytosolic SULTs have been classified into families (i.e. SULT1, SULT2 and SULT4), with at least 45 % similarity within the family, and subfamilies (SULT1A, SULT1B, SULT1C, SULT1D, SULT1E, SULT2A, SULT2B and SULT4A) that display more than 60 % similarity within the subfamily [1]. To date, 11 human cytosolic SULTs have been identified, cloned and characterized. They represent all the different families and subfamilies of SULTs, except that no active human members of the SULT1D subfamily have been identified, although a SULT1D1 pseudogene has been discovered. This SULT1D1 pseudogene harbours mutated splice donor/acceptor sites and two in-frame stop codons [2].

To elucidate the reaction mechanism and the structural basis for substrate specificity, the X-ray crystal structures of five cytosolic SULTs have been solved: mEST (mouse oestrogen SULT), complexed with the inactive sulphate donor PAP (phosphatidic acid phosphohydrolase) and the acceptor-substrate β-oestradiol (E_2) [3]; human dopamine/catecholamine SULT (hSULT1A3), complexed with PAP [4] or complexed with sulphate [5]; human hydroxysteroid SULT (hSULT2A3) complexed with PAP [6] or with dehydroepiandrosterone ([7], but see [7a]); hEST (human oestrogen SULT), complexed with the active sulphate donor PAPS or with PAP and E_2 [8]; and human SULT1A1 (hSULT1A1), complexed with PAP and PNP (*p*-nitrophenol) [9]. The structure and function of SULTs have been reviewed recently [10,11], and the structural features appear to be well conserved within the cytosolic SULT superfamily. Thus, as demonstrated in [3–9], SULTs are single α/β globular proteins with a characteristic five-stranded parallel β-sheet surrounded by α-helices on both sides and a conserved helix running across the top of the fold [8,10]. The structural elements defined as strand-3-loop-helix 3, strand 8 and helix 6 constitute the core structure of the PAP-binding site.

Despite the fact that crystal structures of several SULTs have been unravelled, little information is available regarding the second substrate-binding site. Several studies have been performed concerning the structural basis for the substrate specificity of hSULT1A1 and hSULT1A3. These two enzymes share > 93 % identity in their amino acid sequence and yet display markedly distinct substrate specificities, with hSULT1A1 selectively sulphating PNP at low micromolar concentrations, whereas hSULT1A3 preferentially sulphates dopamine [4,12–16]. Sakakibara et al. [17] reported two highly variable regions, spanning amino acids 84–89 and 143–148, which were shown to determine the substrate specificities of these human enzymes. Site-directed mutagenesis studies have identified residues important for substrate specificity [4,12–16], of the most important being Glu-146 in hSULT1A3, which is the determinant of selectivity of hSULT1A3 for catecholamines, through the charge interaction by the carboxylate residue with the positively charged group of the substrate. When Glu-146 of hSULT1A3 was mutated

Abbreviations used: E_2, β-oestradiol; MBP, maltose binding protein; mEST, mouse oestrogen sulphotransferase; Mx, minoxidil; PAP, phosphatidic acid phosphohydrolase; PAPS, adenosine 3′-phosphate 5′-phosphosulphate; PNP, *p*-nitrophenol; SULT, sulphotransferase.

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to the corresponding Ala-146 in hSULT1A1, the activity of the mutant was altered to that of hSULT1A1 [12,14]. Hence, the preference of hSULT1A3 for dopamine over PNP was reversed. However, when the position 146 of hSULT1A1 was mutated to the corresponding amino acid in hSULT1A3, a preference for dopamine over PNP was not seen [16]. Thus the substrate specificity of hSULT1A1 and hSULT1A3 could not be interchanged by modification of amino acid 146.

Our previously cloned and expressed canine SULT1D1 (cSULT1D1) was shown to sulphate dopamine with high efficiency [18]. Although Glu-146 seems to induce selectivity for monoamine substrates in hSULT1A3, an alanine (as in hSULT1A1) is found in cSULT1D1 in this position, despite the fact that we see a preference for dopamine over PNP.

In the present study, we have employed site-directed mutagenesis and generated several cSULT1D1 variants at amino acid residues in the substrate-binding pocket. These were chosen with regard to their possible contribution to the marked dopamine/PNP preference of cSULT1D1. After characterization, we have identified the potential role of these amino acids in the active site. Our results show that Asp-247 strongly influences the balance between sulphation of dopamine versus PNP for cSULT1D1, and thus identifies a previously unrecognized contributor to active-site specificity.

EXPERIMENTAL

Materials

DEAE-Sepharose CL-6B and Factor Xa were purchased from Amersham Biosciences (Uppsala, Sweden). Oligonucleotide primers were synthesized by Invitrogen (Leek, The Netherlands). PAPS was purchased from Dr Sanford Singer (University of Dayton, Dayton, OH, U.S.A.). PNP, minoxidil (Mx) and dopamine were purchased from Sigma–Aldrich (Stockholm, Sweden). [^{35}S]PAPS was purchased from PerkinElmer Life Sciences (Boston, MA, U.S.A.). *PfuTurbo* DNA Polymerase and *Dpn*I were purchased from Stratagene (Stockholm, Sweden). QIAprep minipreps kit was obtained from Qiagen (Hilden, Germany). Big Dye Terminator Cycle Sequencing Ready Reaction Kit was purchased from PE Applied Biosystems (Stockholm, Sweden). pMAL protein fusion and purification system were obtained from New England Biolabs (Beverly, MA, U.S.A.). All other chemicals were of reagent-grade quality.

Modelling of cSULT1D1 structure

A molecular model of cSULT1D1 was constructed through homology modelling using the program MODELLER 6v2 [19]; a primary structure alignment of cSULT1D1, hSULT1A3 and mEST was performed in ClustalX. The alignment result, together with the Protein Data Bank structure files 1AQU (mEST) and 1CJM (hSULT1A3), were fed into MODELLER for construction of the cSULT1D1 structure. The structure models for mEST and cSULT1D1 were built using InsightII (Accelrys, Burlington, MA, U.S.A.). The C-α traces were subsequently superimposed to assess the active-site topology of cSULT1D1.

Site-directed mutagenesis of the cDNA encoding cSULT1D1

QuikChange site-directed mutagenesis kit from Stratagene (U.S.A.) and *PfuTurbo* DNA polymerase were used for the generation of point-mutated cSULT1D1s. Wild-type cSULT1D1 cDNA inserted into pMAL-c2x fusion protein prokaryotic expression vector [18] was used as the template in conjunction with specific mutagenic primers [**A146E** (Ala-146 → Glu): 5′-TTCTACCAGATGG**AG**AAAATACACCCAAAGGCTGGTAC-C-3′; **A146D**: 5′-TTCTACCAGATGG**AC**AAAATACACCCAA-AGGCTGGTACC-3′; **A146Q**: 5′-TATTTCTACCAGATG**CA**A-AAAATACACCCAAAGGCTGGTACCTG-3′; **D247L**: 5′-CCA-CTATACCAGATTTT**CTG**ATGGATCATTCTGTATCTCCTTT-CATGAG-3′; **I86D**: 5′-CCGTTCATGGAATTGATA**GAC**CCTG-GATTCGAGAATGGTATAGAG-3′]. The amplification conditions were 16 cycles of 30 s at 95 °C, 1 min at 55 °C and 16 min at 68 °C. After digestion of parental DNA with *Dpn*I and transformation into competent Epicurian Coli™ XL1-Blue, single colonies were selected and screened for the desired mutation. The mutated cSULT1D1 sequences were verified by nucleotide sequencing employing a Big Dye Terminator Cycle Sequencing Ready Reaction Kit.

Bacterial expression and purification of cSULT1D1 and its mutants

Cultures of Epicurian Coli™ XL1-Blue containing pMAL-c2x/cSULT1D1 wild-type and mutants were grown to a $D_{600} \approx 0.5$ in 0.25 litre of Rich medium, supplemented with 0.5 g of glucose and 100 μg/ml ampicillin and induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h. The bacterial cultures were harvested by centrifugation at 3200 ***g*** for 20 min. After resuspension in bacterial lysis buffer (75 mM Tris/HCl, pH 8.0/0.25 M sucrose/0.25 mM EDTA/0.02 mg/ml lysozyme) on ice for 20 min, the bacteria were re-pelleted (3200 ***g*** for 20 min at 4 °C) and resuspended in buffer A (10 mM Tris/HCl, pH 7.4/0.1 mM dithiothreitol). After sonication on ice (3 × 10 s bursts with 30 s cooling between each burst), a final centrifugation at 100 000 ***g*** for 1 h at 4 °C was performed.

The MBP (maltose-binding protein)–cSULT1D1 fusion proteins were purified by affinity binding to amylose resins in batch-mode incubation with shaking for 2 h at 4 °C. After two washing steps with buffer A, the MBP–cSULT1D1 fusion proteins were eluted by incubation in 10 ml of buffer A containing 10 mM maltose, with shaking for 1 h at 4 °C. Factor Xa (67 units) was added to each MBP–cSULT1D1 fusion and incubated with shaking overnight at 4 °C. The Factor Xa digests were applied to a DEAE-Sepharose CL-6B column (1.6 cm × 5 cm) at a rate of 0.5 ml/min. After washing the column with 50 ml of buffer A containing 25 mM NaCl, the cSULT1D1 proteins were eluted with a linear gradient of 100 ml of 25–300 mM NaCl in buffer A. Aliquots of fractions were run on SDS/polyacrylamide (10 % gels). Fractions containing cSULT1D1 proteins were pooled. Protein concentration in the pool for each enzyme was determined by the method of Bradford [20].

SULT assays

SULT activities of cSULT1D1 wild-type and mutants were determined for dopamine and PNP. Mx was used to check the stability of the enzymes. The SULT reactions were run at pH 7.4 and final PAPS concentration was 25 μM; for Mx, the pH was 7.3 and PAPS concentration was 29 μM. In all of the reactions, the amounts of cSULT1D1 enzyme and reaction times were adjusted so that the production of sulphated products was less than 30 % of total conversion. Each measurement was repeated three times. V_{max} and K_m values were determined using non-linear regression (GraFit 4, Erithacus software). When substrate inhibition was observed, non-linear regression with a model including substrate inhibition and the equation $v = V_{max}S/(K_m + S + S^2/K_i)$ (Prism 4, GraphPad software) was used to determine

Table 1 Kinetic constants of wild-type and mutants of cSULT1D1

Results shown represent the means ± S.E.M. for three determinations.

	Wild-type	A146E	A146D	A146Q	D247L	I86D
PNP						
K_m (μM)	180 ± 29	–	–	–	1.5 ± 0.17	1600 ± 140
k_{cat} (s^{-1})	0.15 ± 0.015	–	–	–	0.026 ± 1.1E-3	0.089 ± 2.6E-3
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	840*	6.3 ± 0.29†	4.8 ± 0.29†	19 ± 0.53†	18 000*	54‡
K_i (μM)	1000 ± 230	–	–	–	63 ± 8.9	–
Dopamine						
K_m (μM)	3.6 ± 0.41	–	–	–	31 ± 2.2	37 ± 5.3
k_{cat} (s^{-1})	0.18 ± 0.010	–	–	–	0.028 ± 7.0E-4	0.090 ± 4.8E-3
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	49 000*	1500 ± 39†	907 ± 18†	1100 ± 140†	910‡	2400‡
K_i (μM)	61 ± 8.7	–	–	–	–	–
$\frac{k_{cat}/K_m \text{ dopamine}}{k_{cat}/K_m \text{ PNP}}$	58	250	190	61	0.051	45

* Non-linear regression with substrate inhibition (Prism 4, GraphPad software).
† Linear regression (GraFit 4, Erithacus software).
‡ Non-linear regression (GraFit 4, Erithacus software).

V_{max}, K_m and K_i. In cases where reliable K_m and V_{max} values could not be determined, linear regression (GraFit 4, Erithacus software) was used to determine the V_{max}/K_m ratio using the ascending portion of the concentration versus velocity curves. Mx was assayed by using an extraction procedure described by Johnson and Baker [21]. PNP and dopamine were assayed using the barium precipitation procedure of Foldes and Meek [22].

RESULTS

Modelling of the cSULT1D1 structure

The constructed, unrefined model of cSULT1D1 was applied as a guide, and compared with known substrate-bound structures, in deciding residues putatively involved in defining substrate specificity. Based on the result, five cSULT1D1 mutants were engineered, A146E, A146D, A146Q, D247L and I86D.

Expression of recombinant wild-type and mutant cSULT1D1 proteins

Wild-type cSULT1D1 and five mutant cDNAs were expressed in Epicurian Coli™ XL1-Blue cells as maltose binding fusion proteins. The fusion proteins were purified by affinity binding to amylose resin, Factor Xa digestion and DEAE-Sepharose CL-6B column chromatography. The final purified pooled fractions of cSULT1D1 contained both cSULT1D1 and MBP. Protein concentrations for each enzyme were determined and the values were adjusted for the content of MBP. Similar levels of expression of each protein were observed and up to 10 mg of purified protein per litre of bacterial culture were obtained. The purified cSULT1D1 wild-type and mutants were characterized for their activity towards Mx at 4.5 mM (results not shown). Aliquots of the protein preparations were frozen in 10 % (v/v) glycerol. The stability of the wild-type and mutant enzymes were checked with the Mx assay and no loss of activity was observed during the period of our kinetic study (results not shown).

SULT activities of recombinant wild-type and mutant cSULT1D1 proteins

The K_m values, the catalytic centre activity k_{cat} and the specificity constants k_{cat}/K_m towards PNP and dopamine for wild-type and mutants of cSULT1D1 were determined and are summarized in Table 1 and Figure 1. The K_m values of the wild-type enzyme for these substrates were in accordance with our previously reported semi-purified cSULT1D1 [18].

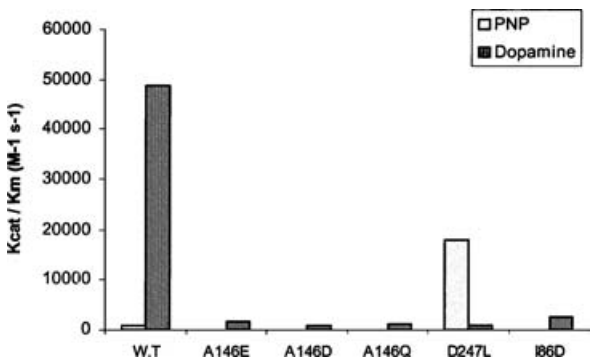

Figure 1 Sulphation of PNP and dopamine by wild-type and mutants of cSULT1D1

Regarding sulphation of dopamine, all the cSULT1D1 mutants displayed lower efficiency to different extents. Substrate inhibition was observed at approx. 500 μM for all three A146 mutants, thus making it difficult to determine reliable K_m and k_{cat} values. The k_{cat}/K_m values for dopamine of the A146 mutants were decreased from 49 000 to 1500 $M^{-1} \cdot s^{-1}$ (32-fold) for A146E, to 910 $M^{-1} \cdot s^{-1}$ (54-fold) for A146D and to 1100 $M^{-1} \cdot s^{-1}$ (42-fold) for A146Q respectively. However, the D247L and I86D mutants displayed higher K_m values for dopamine, 31 and 37 μM respectively, an 8–10-fold increase compared with wild-type cSULT1D1 ($K_m = 3.6$ μM). The k_{cat} values for these mutants were also changed. For I86D, the k_{cat} value was decreased 2-fold and for D247L, 6-fold. The decrease in k_{cat}/K_m for dopamine was 95–98 % for all the mutants.

For PNP, the activities of the A146E and A146D mutants were increased linearly up to substrate concentrations where substrate inhibition was observed at approx. 20 mM. Substrate inhibition was also observed for the A146Q mutant at 15 mM. As for dopamine, reliable K_m and k_{cat} values for these mutants could not be determined. However, the k_{cat}/K_m values of A146 mutants for PNP were dramatically decreased from 840 to 6.3 $M^{-1} \cdot s^{-1}$ (130-fold) for A146E, to 4.8 $M^{-1} \cdot s^{-1}$ (180-fold)

for A146D and to 19 $M^{-1} \cdot s^{-1}$ (44-fold) for A146Q respectively. The PNP activity for I86D mutant was also decreased. The K_m value was increased to 1.6 mM (9-fold), whereas the k_{cat} value was not affected significantly. The specificity constant was thus decreased by 15-fold. Interestingly, the D247L mutant was found to sulphate PNP much more efficiently when compared with wild-type cSULT1D1. The K_m value was found to be 1.5 μM, a decrease by more than 100-fold. Although the k_{cat} value was decreased 6-fold, the specificity constant was increased by more than 21-fold.

Examining the ratios of specificity constants for dopamine and PNP for the wild-type and mutant cSULT1D1 (Table 1), we found that the wild-type preference for dopamine over PNP was unchanged for the A146Q and I86D mutants and this preference was even enhanced for the A146E and A146D mutants. However, the overall sulphation efficiencies for these mutants were strongly decreased for both substrates. Interestingly, and in contrast with other mutants, the substrate preference was shifted for the D247L mutant. This is principally caused by the large decrease in the K_m value of this mutant for PNP.

SULTs are known to exhibit substrate inhibition. This inhibition could be analysed for the wild-type and D247L mutant. Concerning PNP sulphation, introduction of the D247L mutation resulted in a pronounced decrease in both K_m and K_i values compared with the wild-type cSULT1D1 (Table 1).

DISCUSSION

We have previously cloned and characterized three different canine SULTs in the SULT1 family: cSULT1D1 [18], cSULT1B1 [23] and cSULT1A1 [24]. PNP is a substrate for all of these canine enzymes, but only cSULT1A1 and cSULT1D1 sulphate dopamine, with cSULT1D1 displaying the highest efficiency. As for hSULT1A1, cSULT1A1 has a strong preference for PNP and an orthologue of the dopamine, preferring SULT1A3 has not been found in dog. Dousa and Tyce [25] found that human, dog, bear and African green monkey are particularly good catecholamine conjugators. In fact, up to 95 and 90% of circulating dopamine in human and dog exists in the sulphated form [25,26], indicating the importance of sulphation in the deactivation of this neurotransmitter in dog. The important role of Glu-146 in the ability of hSULT1A3 to accept physiologically important biogenic amines and in the selectivity of catecholamines (e.g. dopamine) over phenols (e.g. PNP) is well established [4,12–16]. Since our previously cloned cSULT1D1 [27] sulphates dopamine with high efficiency and prefers dopamine over PNP as substrate, we chose to study the substrate binding site of this enzyme. In addition to residue 146, we selected with guidance by the constructed, unrefined model of cSULT1D1, two additional amino acids in the substrate-binding pocket of cSULT1D1 for site-directed mutagenesis to elucidate their possible influence on substrate specificity. Five different mutants, A146E, A146D, A146Q, I86D and D246L, were engineered and characterized for their capacity to sulphate dopamine and PNP. The K_m, k_{cat}, k_{cat}/K_m and K_i values towards PNP and dopamine are summarized in Table 1.

Amino acid 146

Dajani et al. [4] compared the kinetic properties of hSULT1A3 and hSULT1A1 with a series of paired catechols and phenols. They found that hSULT1A3 has significantly lower K_m values for the catechol forms when compared with the corresponding phenols, although in general there was little difference in V_{max} value. On the contrary, hSULT1A1 showed much less discrimination in terms of K_m values between catechols and phenols, but the V_{max} values were consistently higher for the phenol form. In addition, compounds with an amine nitrogen at the β-carbon of the 4-substituent are highly selective substrates for hSULT1A3 by interaction with Glu-146, although the substrate specificity of these human enzymes cannot be interchanged by modification of this residue [12,14,16]. Thus additional determinants are indicated.

The specificity constants of the cSULT1D1 A146 mutants for PNP and dopamine were decreased by more than 96%, indicating that introduction of a negatively charged amino acid (as in A146E and A146D) or an amino acid with more steric constraints (as in A146Q) at position 146 strongly influence the efficiency of cSULT1D1 to sulphate these substrates. As for the hSULT1A1, introducing a charge at position 146 did not enhance dopamine sulphation. On the contrary, the sulphation efficiency for this substrate was strongly decreased. However, the substrate preference towards dopamine over PNP of the various cSULT1D1 A146 mutants was unchanged and even enhanced for A146E or A146D mutation. Thus the cSULT1D1 [13,16] A146E mutant sulphates both PNP and dopamine less efficiently in analogy with hSULT1A1 [14].

Amino acid 86

The involvement of residue 86 in the substrate specificity of hSULT1A3 has previously been investigated by others. Brix et al. [14] observed a small decrease in affinity for PNP and a total loss of dopamine activity for the A86D mutant compared with wild-type hSULT1A1, indicating that amino acids at position 86 influence the binding of PNP and dopamine. Liu et al. [16] found that mutation of residue Asp-86 of hSULT1A3 to an alanine resulted in a dramatic decrease in the V_{max}/K_m value for dopamine. The V_{max}/K_m value of this D86A mutant for PNP was not affected significantly, as both the K_m and the V_{max} values were doubled. A previous X-ray crystallography study had shown that, in the unligated hSULT1A3 structure, residues 84–92 form a mobile loop that intercalates into the active site and may block proper positioning of certain acceptor substrates [4,5]. Although not hindering the entry of the endogenous substrate dopamine, this loop may block the entry of other substrates such as dopa and tyrosine that contain an extra CO_2H group. Pai et al. [27] found that residue Asp-86 is critical in the Mn^{2+} stimulation of the dopa/tyrosine-sulphating activity of hSULT1A3. Mn^{2+} appears to form a bridge between the CO_2H group of the substrate with which it is complexed and the Asp-86 of the enzyme molecule, thus pegging back this mobile loop. Furthermore, they observed that besides the E146A mutant, the D86A and E89I mutants also showed decreased basal activities towards dopamine, dopa and tyrosine.

Ile-86 is conserved in the SULT1D1 enzymes in dog, rat and mouse. By replacing this amino acid with an aspartate, i.e. introducing a charged residue which might interact with the NH_2 group of dopamine, the dopamine-sulphating efficiency might be enhanced. Similarly to the hSULT1A1 and hSULT1A3, mutation of residue 86 in cSULT1D1 also affects sulphation activities. However, the K_m values for dopamine and PNP were increased 10- and 9-fold respectively, indicating that the hypothesis was wrong. The specificity constants were decreased by more than 93% of the wild-type values, demonstrating the involvement of Ile-86 in the substrate specificity of cSULT1D1. However, the selectivity towards dopamine over PNP is unchanged.

Amino acid 247

Recently, Gamage et al. [9] have solved the crystal structure of hSULT1A1 in the presence of PAP and PNP. Unexpectedly, they

found two molecules of PNP in the active site of this enzyme, whereas one PNP molecule is bound in a catalytically competent manner. The nitro group of this PNP molecule interacts with a water molecule and forms van der Waals contacts with the side chains of Val-148, Phe-247 and Met-248. The other PNP molecule does not interact with catalytic residues of hSULT1A1. The nitro group of this second PNP also interacts with a water molecule, and van der Waals interactions are formed between the molecule and side chains of Ile-89 and Phe-247. Thus Phe-247 seems to be an important residue in orientating both the PNP molecules in the substrate-binding pocket of hSULT1A1. Brix et al. [13] conducted site-directed mutagenesis studies on residue Leu-247 of hSULT1A3. However, no effect on dopamine/PNP preference was observed.

In cSULT1D1, an aspartate residue is found at position 247. By replacing this amino acid with the uncharged and larger amino acid leucine, we found a 21-fold increased efficiency in PNP sulphation. Asp-247 might thus prohibit productive van der Waals interactions to PNP molecules either due to charge or the smaller size of the CO_2H side chain.

The hSULT1A1 active site is extremely hydrophobic. In hSULT1A3, two of the hydrophobic residues are replaced with negatively charged residues (Glu-146 and Glu-89) and these would favour binding of substrates with positively charged groups such as dopamine [9]. As for the hSULT1A3, two of the hydrophobic residues in the active site of cSULT1D1 have been replaced with negatively charged residues (Asp-247 and Glu-90), and in addition, one positively charged residue (Lys-77). Since the important Glu-146 is missing in cSULT1D1 and yet this enzyme shows strong preference for dopamine over PNP [18], we speculated that negatively charged amino acids in the proximity of position 146 might provide the crucial negative charge interaction with positively charged monoamines. Asp-247 is positioned close to Ala-146 in the active site of cSULT1D1 (as deduced from a homology model) and might thus provide an additional negative charge interaction. A charged amino acid (Glu) at position 247 is also found for both the rat and mouse orthologue of SULT1D1. Indeed, the D247L mutant sulphates dopamine less efficiently when compared with the wild-type cSULT1D1 as a decrease in the specificity constant of 98 % was observed. Comparing the ratio of specificity constants for dopamine and PNP with the wild-type and the D247L mutant, the substrate preference was completely reversed for this mutant. This is principally caused by the decrease in the K_m value of this mutant for PNP. As K_i values displayed a clear-cut decrease, an increased affinity for PNP is indicated. Our results clearly demonstrate the importance of Asp-247 in determining the dopamine and PNP preference for cSULT1D1.

The substrate inhibition could be evaluated only in some cases, as the substrate inhibition observed with the alanine mutants could not be fitted to the simple equation given in the Experimental section. Gamage et al. [9] have recently described an alternative analysis based on their observation that two PNP substrate molecules can bind in file in the substrate-binding site. Although this analysis was not performed in the present study, it is possible that this situation could also apply. The lack of substrate inhibition with some of the mutant/substrate combinations indicates that the active site is altered in such a way that two substrate molecules cannot be accommodated or that binding of a second substrate molecule does not make a kinetically significant inhibitory contribution (in the concentration range tested).

In summary, by employing site-directed mutagenesis, we have generated, expressed and characterized five cSULT1D1 mutants. The sulphation capacities of these mutants were dramatically changed compared with wild-type cSULT1D1. Most interesting is the D247L mutant, which was found to be more than 21-fold better in sulphating PNP and 54-fold less efficient in sulphating dopamine and, thus, the preference is switched from dopamine to PNP, indicating the importance of this amino acid in this preference.

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