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Site-Directed Mutagenesis of the Substrate-Binding Cleft of Human Estrogen Sulfotransferase

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The sulfonation of estrogens by human estrogen sulfotransferase (humSULT1E1) plays a vital role in controlling the active levels of these hormones in the body. To understand more fully the structural and functional characteristics of humSULT1E1, we have carried out site-directed mutagenesis of critical amino acids found in the substrate-binding cleft. Three single amino acid mutations of humSULT1E1 (V145E, H107A, and K85A) were created in this study. Kinetic studies were used to provide information about the importance of these residues in substrate specificity and catalysis, using a variety of substrates. Lysine at position 85 has been proposed to be within hydrogen bonding distance to the 3α-phenol group of β-estradiol, thereby stabilising the substrate in the active site. However, substitution to a neutral alanine at this position improved substrate specificity of humSULT1E1 for β-estradiol, estrone, and dehydroepiandrosterone (DHEA). The exchange of valine 145 for negatively charged glutamic acid markedly improved the ability of humSULT1E1 to sulfonate dopamine, but caused a reduction in specificity constants toward steroids tested, in particular DHEA. The presence of a histidine residue at position 107 was shown to be essential for the production of a functional protein, as substitution of this amino acid to alanine resulted in complete loss of activity of humSULT1E1 towards all substrates tested.

Key Words: **sulfotransferase; estrogen; kinetics; human; site-directed mutagenesis; substrate-binding.**

Abbreviations used: humSULT1E1, human estrogen sulfotransferase; humSULT1A3, human dopamine sulfotransferase; mouSULT1E1, mouse estrogen sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PAP, adenosine 3′,5′-bisphosphate; IPTG, isopropyl-β-D-thiogalactoside; Ni-NTA, nickel-nitrilotriacetic acid; BSA, bovine serum albumin; DHEA, dehydroepiandrosterone; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Sulfonation is universal to most biological systems, ranging from microbes to mammals and the enzymes responsible for catalysing these reactions belong to a supergene family of proteins called sulfotransferases (1). Sulfotransferases (SULTs) utilise the sulfonate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) and have regions of homology in their amino acid sequence which are responsible for cofactor binding in the protein (2). Sulfotransferases are divided into two groups. The membrane bound sulfotransferases sulfonate large endogenous substrates and play an important role in the post-translational modification of proteins such as heparan and gastrin (3, 4). The cytosolic sulfotransferases sulfonate smaller substrates, including a range of endo- and xenobiotics. To deal with such a vast variety of compounds an organism is equipped with several cytosolic sulfotransferase isoforms. At least 9 different forms of human sulfotransferases have been cloned and sequenced (5–13). Based on amino acid sequence identity they are classified into distinct families if they show >45% identity in amino acid sequence and subfamilies if they are >60% identical in amino acid sequence. SULT1 family members are subdivided into four subfamilies: 1A, 1B, 1C, and 1E. The focus of this study was to examine the structure-function relationship of the human estrogen sulfotransferase (humSULT1E1) with respect to the importance of amino acids responsible for determining the substrate specificity of this enzyme.

One of the recognised endogenous functions of the cytosolic sulfotransferases is the sulfonation of steroids and neurotransmitters, resulting in alteration of their activity. HumSULT1E1 catalyses the transfer of a sulfonate group to the hydroxyl group of an estrogen molecule. This results in the inactivation of the steroid, as

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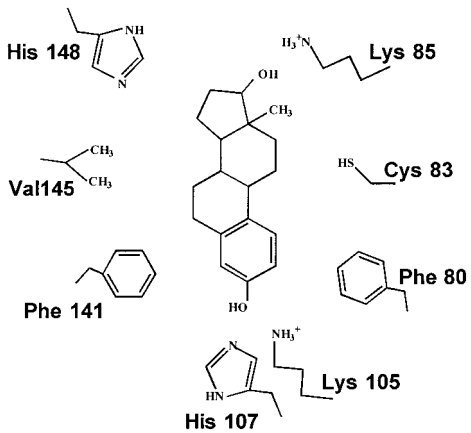

FIG. 1. Schematic diagram of the humSULT1E1 substrate binding cleft, with β-estradiol modelled in as the sulfonate acceptor substrate. This model is based on the crystal structure of mouSULT1E1 (Brookhaven Protein Data Bank ID code 1AQU; 9).

sulfonated estrogens have been shown to have no effect at the estrogen receptor (14). The sulfonation carried out by humSULT1E1 is also thought to be of importance in producing the fluctuating levels of active estrogens during the menstrual cycle (15). Furthermore, increased sulfonation of estrogens may act as a protective mechanism against estrogen responsive carcinomas such as breast cancers (16).

To further elucidate the functional properties of humSULT1E1, we investigated the influence of amino acids lining the substrate binding pocket on substrate specificity of this enzyme. The first sulfotransferase crystal structure to be reported was that of mouse estrogen sulfotransferase (mouSULT1E1; 17). This structure was solved with β-estradiol and PAP (adenosine 3′,5′-bisphosphate) modelled into the catalytic cleft, enabling identification of the regions of the enzyme primarily responsible for cofactor and substrate binding (17). Site-directed mutagenesis studies on the SULT1A subfamily of sulfotransferases and the recently published crystal structure of human dopamine sulfotransferase (humSULT1A3) by our laboratory have further emphasised the importance of distinct regions in the amino acid sequence responsible for the catalytic properties of these isoforms (18, 19).

Due to the 77% amino acid identity between mouSULT1E1 and humSULT1E1, the mouSULT1E1 crystal structure was used as a guide to estimate the approximate positions of corresponding residues in humSULT1E1, as shown in the schematic diagram of the substrate binding cleft (Fig. 1). Lys85, indicated by the mouSULT1E1 crystal structure to be within hydrogen bonding distance to the 17β-hydroxyl group of β-estradiol, was of interest in this study as it may be a prime candidate for the anchorage of steroid molecules to the enzyme (17).

HumSULT1E1 has substrate preference for aromatic steroids such as β-estradiol, estrone, and dehydroepiandrosterone (DHEA) and lacks preference for smaller or charged molecules such as dopamine (20). Previous studies identified the negatively charged glutamic acid at position 146 to be one of the determining factors of the dopamine substrate selectivity displayed by humSULT1A3 (21, 22). Substitution of the equivalent residue in humSULT1E1 (Val145) to a glutamic acid was undertaken to determine whether this would deteriorate substrate specificity of humSULT1E1 towards steroid molecules and alter the ability of this enzyme to utilise dopamine as a substrate. The third residue targeted in this study was histidine at position 107, which has been shown in previous mutagenesis studies in animal and plants to be of vital importance in catalysis (23, 24). It is thought to be involved in the transitional stage of the sulfonate group transfer to the acceptor substrate (23).

MATERIALS AND METHODS

Site-directed mutagenesis. A DNA fragment corresponding to humSULT1E1 cDNA was generated using PCR from a human liver library, which has 100% sequence identity to the sequence published by Aksoy *et al.* (11), and was subcloned into the bacterial expression vector pET28a(+) (Novagen, Madison, WI). This vector facilitates the expression of the sulfotransferase enzyme with a six histidine tag, which allows for the purification of the protein via attachment to a nickel-affinity column (Qiagen, Clifton Hill, Victoria, Australia). Three humSULT1E1 mutants (H107A, V145E, K85A) were created by applying the QuickChange site-directed mutagenesis method using *PfuTurbo* polymerase (Stratagene, La Jolla, CA). Complementary oligonucleotide primers, designed to include the required mutations, are listed below with bases in small case letters indicating point mutations introduced by the primer and underlined bases indicating the codon of the mutated amino acid: H107A, 5′-CTCCT AGAAT TGTGA AGACT gcTTT GCCAC CTGAA CTTCT TCCTG CC-3′; V145E, 5′-CTTTT ATTAT TTCTT TCTAA TGGaG GCTGG TCATC CAAAT CCTGG ATC-3′; K85A, 5′-CGAAT ACCTT TCCTG GAATG CAGAg cAGAA AACCT CATGA ATGGA G-3′.

Bacterial expression and purification of recombinant wild-type and mutant humSULT1E1 enzymes. *E. coli* BL21 (DE3) pLysS cells were transformed with the pET28a(+) vector containing the mutated SULT1E1 insert using 100 mM $CaCl_2$. Plasmid DNA was isolated from the bacterial cells using the Boehringer High Pure Plasmid Isolation Kit (Roche, Castle Hill, NSW, Australia) and incorporation of the mutation checked by sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA). DNA sequence analysis was undertaken by the Australian Genome Research Facility, University of Queensland, Brisbane, Australia.

A single *E. coli* BL21(DE3) pLysS colony transformed with the desired pET28a(+) SULT1E1 plasmid was used to inoculate 10 ml of SOB medium (20 g/l bactotryptone, 5 g/l bactoyeast extract, 10 mM NaCl and 10 mM $MgCl_2$) containing kanamycin (30 μg/ml) and incubated for nine hours at 37°C. A 1:100 dilution of this culture was made into fresh SOB medium (containing 30 μg/ml kanamycin) and incubated while shaking at 37°C for 4 h until the OD_{600} reached 0.8. At this point protein expression was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 100 mM and the culture left to grow shaking at 37°C for a further 14–18

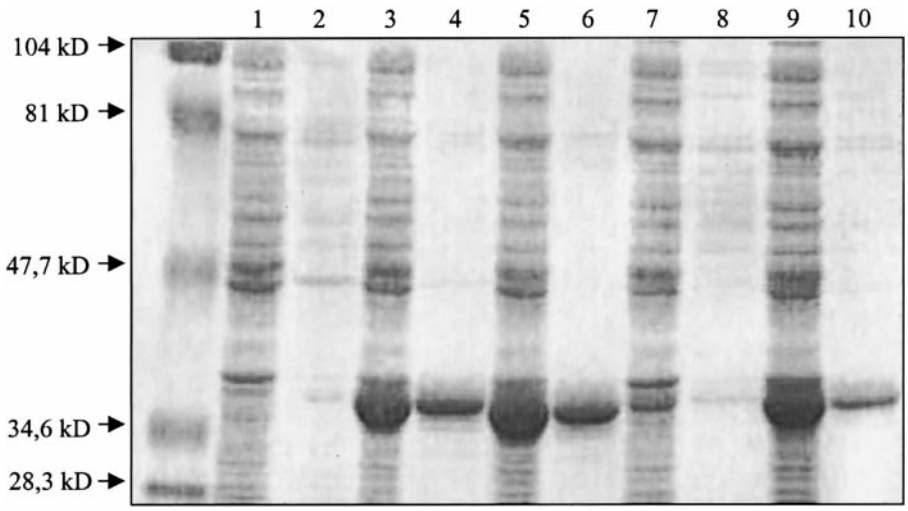

FIG. 2. SDS–PAGE of wild-type and mutant humSULT1E1 proteins. Lanes 1, 3, 5, 7, and 9 represent 20 μg of total cytosolic protein loaded, containing the empty pET28a(+) vector, wild-type humSULT1E1, humSULT1E1-V145E, humSULT1E1-H107A, and humSULT1E1-K85A mutants, respectively. Lanes 4, 6, 8, and 10 contain 4 μg of wild-type humSULT1E1, humSULT1E1-V145E, humSULT1E1-H107A, and humSULT1E1-K85A Ni-NTA spin column-purified protein, respectively. Lane 2 represents 8 μl of the Ni-NTA spin column elution of the bacterial cytosol containing the empty pET28a(+) vector. The low-range molecular weight markers are represented by arrows (BioRad).

h. The cells were harvested and protein isolated as described previously (22). Purification of protein was carried out according to manufacturer's instructions using nickel-nitrilotriacetic acid (Ni-NTA) spin columns (Qiagen, Clifton Hill, Victoria, Australia) and the purified protein was stored in 20% (v/v) glycerol at −70°C until used. Protein concentrations were determined by the method of Lowry *et al.* (24) using BSA as the protein standard.

Sulfotransferase kinetic assays. Radiometric sulfotransferase substrate assays with estrone (100 Ci/mmol) and β-estradiol (72 Ci/mmol) were carried out using the tritiated form of these substrates (26). The 500 μl final volume of the assay reaction mixture contained 10 mM potassium phosphate buffer (pH 7.0), [^{3}H] acceptor substrate, 1–50 μM PAPS and 0.3–2.0 μg of purified sulfotransferase protein. Estrone and β-estradiol substrate concentrations used in the assays ranged from 50 nM to 150 μM and 500 nM to 50 μM, respectively, and radiolabelled and non-radiolabelled substrates were used at a ratio of 1:9. The reaction was initiated by the addition of the enzyme and incubations were carried out in a 37°C waterbath for 20–40 min. The assay was stopped by transferring the entire reaction volume into 4 ml chloroform and vortexing. Tris–HCl buffer (500 μl of 50 mM, pH 8.7) was added to the aqueous layer to alkalise the solution, followed by vortexing (2 × 15 s). The solutions were centrifuged at 2500*g* for 5 min, after which 400 μl of the aqueous layer, containing sulfonated tritiated substrate, was transferred to a scintillation vial, containing 3 ml scintillant, and counted in a standard β-counter for 5 min. Triplicate counting standards contained 5 μl of radiolabelled substrate stock. Each assay contained negative controls which lacked either PAPS or enzyme.

Radiometric sulfotransferase substrate assays with dopamine, DHEA and *p*-nitrophenol were carried out as described previously (22) following the method of Foldes and Meek (27), using [^{35}S]PAPS (1.04 Ci/mmol). The assay conditions were kept the same as the tritiated substrate assay, with the exception of the presence of radiolabelled PAPS. Reactions contained 10 mM potassium phosphate buffer (pH 7.0), 0.3–2.0 μg sulfotransferase protein, various concentrations of acceptor substrate and 1–50 μM PAPS, where 22 nM to 1.1 μM of this was [^{35}S] PAPS. Dopamine was utilised at concentrations of 50 μM to 90 mM, *p*-nitrophenol at concentrations of 10 μM to 7 mM and DHEA at concentrations of 100–800 μM. For each assay, substrate blanks were utilised to account for background activity.

Analysis of kinetic data. Substrate assays were carried out in triplicate and corrected with the appropriate blanks. The linearity of sulfonated product formation across a range of protein concentrations and assay times was assessed to ensure that optimum conditions were utilised in the assays. The enzymatic activities toward increasing concentrations of acceptor substrate and cofactor PAPS were analysed by Michaelis-Menten kinetics using the equation $v = V_{max}[S]/(K_m + [S])$. GraphPad Prism (AMPL software, San Diego, CA) was used to fit this equation to data obtained and to provide statistical data analysis. To compare the substrate specificity of the wild-type and humSULT1E1 mutants, the specificity constants were expressed as V_{max}/K_m.

RESULTS AND DISCUSSION

Expression of recombinant wild-type and mutant humSULT1E1 protein. HumSULT1E1 has a calculated monomer molecular weight of 35,123 Da and protein of approximately this size was observed when both bacterial cytosol (20 μg) and purified protein (4 μg) of the wild-type and mutant humSULT1E1 proteins were analysed using SDS–PAGE (Fig. 2, wild-type lane 3, mutants lanes 5, 7, and 9). The recombinant wild-type humSULT1E1, humSULT1E1-V145E, and humSULT1E1-K85A proteins were present in abundance in each corresponding cytosolic fraction (representing approximately 40% of total protein), whereas the humSULT1E1-H107A mutant expressed at much lower levels (approximately 5% of total protein). A larger fraction of this protein was visible on SDS–PAGE analysis of whole cell homogenate suggesting the presence of humSULT1E1-H107A protein in inclusion bodies (results not shown).

Recombinant wild-type and mutant humSULT1E1 activities towards PAPS. The kinetic constants of the wildtype and mutant humSULT1E1 proteins towards

TABLE 1

Kinetic Analysis of Wild-Type and Mutant humSULT1E1 Proteins towards PAPS[a]

	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1} protein)	V_{max}/K_m
humSULT1E1-wild-type[b]	0.46 ± 0.11	38.64 ± 1.03	84
humSULT1E1-K85A[c]	0.69 ± 0.28	23.55 ± 1.32	34.1
humSULT1E1-V145E[d]	13.18 ± 2.84	162.6 ± 13.23	12.3

[a] Values represent best fit ± standard error of the estimates obtained by fitting the Michaelis–Menten equation to the data obtained from three determinations of reaction rate studies using saturating concentrations of β-estradiol ([b] 0.75 μM, [c] 0.5 μm, [d] 30 μM) and variable PAPS concentrations.

PAPS are listed in Table 1. K_m values for PAPS remained statistically similar between the wild-type and humSULT1E1-K85A mutant, suggesting that amino acid 85 is not an important residue involved in the binding of PAPS. Interestingly the humSULT1E1-V145E mutant exhibited a decrease in PAPS affinity, as indicated by a 26-fold increase in K_m observed with this mutant compared to the wild-type enzyme. It was also necessary with the humSULT1E1-V145E mutant to use a saturating concentration of β-estradiol approximately 150-fold that of the saturating concentration used for the wild-type enzyme and humSULT1E1-K85A mutant. This result indicates that this mutation not only causes a change in the acceptor substrate binding pocket, but also in the cofactor binding cleft, affecting the binding of PAPS.

Properties of wild-type humSULT1E1. The preferred substrates of wild-type humSULT1E1 were the larger steroid structures, such as the estrogens and DHEA, as reflected by the low K_m (nM range) and high V_{max}/K_m values observed (Table 2). It suggests that the substrate binding pocket of humSULT1E1 provides a large enough physical space for the bulkier aromatic steroid molecules to enter and dock in the substrate cleft during the catalytic process. *p*-Nitrophenol is a simple phenol, which is a model substrate for the SULT1A subfamily (sulfonated by humSULT1A1 in the low μM range, 18). It has been shown in this study that this substrate is sulfonated by humSULT1E1 at higher concentrations (K_m 76.6 ± 0.4 μM), which has not been previously documented (20). It was previously thought that estrogen sulfotransferases showed no activity towards dopamine as a substrate (20). This study has essentially confirmed these results, with K_m (mM range) and V_{max} values reflecting very low substrate specificity (Table 2). Indeed the high K_m suggests that this enzyme has no physiological role in the sulfonation of dopamine.

In the present study substrate inhibition of humSULT1E1 by β-estradiol was observed at higher substrate concentrations, and an inhibition constant (K_i) of 1.11 ± 0.04 μM was obtained by applying the following equation to the kinetic constants: $v = V_1(1 + (V_2[E_2]/V_1K_i))/(1 + K_m/[E_2] + [E_2]/K_i)$ (GraphPad Prism, AMPL software, 28). It has been proposed that substrate inhibition, observed at saturating β-estradiol concentrations may be due to binding of a second estrogen molecule at an allosteric site on the enzyme (28). Substrate inhibition for the other steroids was not as profound as observed with β-estradiol, and K_i values could not be accurately determined in the substrate ranges tested. Substrate inhibition of humSULT1E1 was not observed with dopamine or *p*-nitrophenol in the concentration ranges tested. This may suggest that the structure of the steroids and their interactions with humSULT1E1 give rise to effects at allosteric sites, thus resulting in substrate inhibition.

Properties of humSULT1E1-K85A. The mouSULT1E1 crystal structure indicates that Asp86, the corresponding residue to Lys85 in humSULT1E1, is within hydrogen bonding distance (2.8 Å) to the 17β-hydroxy group of β-estradiol, suggesting that this residue may provide an anchorage point for the steroid molecules (9). The positive charge of this residue, and resulting ability to

TABLE 2

Kinetic Analysis of Wild-Type and Mutant humSULT1E1 Proteins with Various Sulfotransferase Substrates[a]

	humSULT1E1-wild-type			humSULT1E1-K85A			humSULT1E1-V145E		
Substrate	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	V_{max}/K_m	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	V_{max}/K_m	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	V_{max}/K_m
β-Estradiol	0.3 ± 0.09	37.2 ± 2.9	124	0.1 ± 0.09	21.7 ± 2.8	217	8.2 ± 1.0	104.1 ± 3.8	12.7
Estrone	0.2 ± 0.04	16.3 ± 0.9	81.5	0.1 ± 0.07	14.7 ± 1.5	147	3.6 ± 0.7	124.4 ± 8.2	34.6
DHEA	0.2 ± 0.05	5.5 ± 0.4	27.5	0.05 ± 0.02	3.7 ± 0.2	74	60.7 ± 11.5	4.6 ± 0.2	0.08
Dopamine	32220 ± 5020	13.0 ± 0.8	0.0004	79350 ± 32500	12.0 ± 2.8	0.0002	240.7 ± 28.9	37.5 ± 1.4	0.16
p-Nitrophenol	76.6 ± 0.4	135.9 ± 2.2	1.8	624.8 ± 35.5	200.6 ± 6.2	0.3	937.2 ± 0.5	264.4 ± 4.7	0.3

[a] Values represent best fit ± standard error of the estimates obtained by fitting the Michaelis—Menten equation to the data obtained from three determinations of reaction rate studies using 20 μM PAPS and variable substrate concentrations.

form a hydrogen bond interaction with the substrate, was eliminated by substitution with alanine. Unexpectedly, humSULT1E1-K85A did not have decreased sulfonation activity towards the estrogens and DHEA. In fact, K_m values were reduced slightly, which in turn caused increases in the substrate specificity constant (V_{max}/K_m) compared to the wild-type (Table 2). It was expected that the removal of the positively charged lysine would severely compromise binding of the steroids and hence decrease the affinity of the enzyme towards these substrates. The results obtained in the present study suggest that a positive charge at residue 85 is not necessary for positioning and holding the steroid molecules in the substrate binding pocket. Petrotchenko and colleagues (29) have recently shown that an equivalent change to the mouSULT1E1 resulted in similar kinetic properties in the mouse enzyme.

The K_m of humSULT1E1-K85A towards *p*-nitrophenol was 8-fold greater than that of the wild-type humSULT1E1 (Table 2), suggesting that lysine, which is positively charged at physiological pH, may have interactions with the nitro group of *p*-nitrophenol in the wild-type enzyme. This interaction maybe abolished with the alanine mutation. Interestingly, the *p*-nitrophenol preferring sulfotransferase isoform, humSULT1A1, also has an alanine at this position (18). The ability of humSULT1E1-K85A to sulfonate dopamine was not altered significantly with this mutation, suggesting no involvement of this residue with respect to dopamine substrate specificity of humSULT1E1.

Properties of humSULT1E1-V145E. The change of this neutral amino acid to a negative residue resulted in drastic changes towards steroid substrate specificity of the enzyme. Substrate specificity constants for β-estradiol and estrone were reduced 9.8-fold and 2.4-fold, respectively with this mutant (Table 2). Higher K_m values observed for the estrogens, suggest that binding has become more difficult for these steroids, possibly due to structural and charge interference by glutamic acid. A much more dramatic decrease in substrate specificity (340-fold decrease in V_{max}/K_m) was observed when the sulfonation of DHEA by humSULT1E1-V145E was investigated (Table 2). One hypothesis to account for this decrease may be the interference of the long protruding carbon chain of glutamic acid with the methyl group found between phenolic rings A and B of DHEA.

One of the reasons for targeting Val145 was to investigate the inability of humSULT1E1 to sulfonate dopamine. The corresponding amino acid in humSULT1A3 (Glu146) has previously been shown to be of critical importance in the binding of the positively charged dopamine by this sulfotransferase isoform (22). The substitution of Val145 to glutamic acid resulted in a dramatic 400-fold increase in the ability of humSULT1E1 to sulfonate dopamine (V_{max}/K_m, Table 2). However, the affinity of humSULT1E1-V145E for this substrate was 65-fold less than that of humSULT1A3 which displays a K_m for dopamine of 3.7 μM (22). The V145E mutation in humSULT1E1 provides a negative binding site for the positively charged dopamine, which was previously not present in the wild-type. Although this mutation suggests that a negative amino acid at this position is favourable for this substrate binding, there is clearly a combination of amino acids involved in determining the dopamine specificity of humSULT1A3. Binding of dopamine to the humSULT1E1-V145E mutant may be affected by the positively charged lysine at position 85 interfering with the positively charged substrate entering the binding pocket.

The sulfonation of *p*-nitrophenol by humSULT1E1-V145E compared to the wild-type was also markedly affected, with the former exhibiting a six-fold decrease in substrate specificity (K_m/V_{max}, Table 2). These values reflected humSULT1A3 activity towards *p*-nitrophenol (K_m: 1024 μM; 22), again suggesting that the binding pocket has been altered in this mutant to more closely resemble that of humSULT1A3. Here the negatively charged glutamic acid would decrease binding of *p*-nitrophenol, due to interference with the nitro group of this substrate.

Properties of humSULT1E1-H107A. The histidine at position 107 is conserved in all cytosolic sulfotransferases known to date and has been implicated in playing a vital role in catalysis, by stabilising the transitional state of the sulfonation reaction (23). Replacement of histidine 107 by alanine, a small and neutral amino acid, resulted in complete loss of activity of humSULT1E1 towards all substrates tested. This is the first time it has been shown that histidine at this position also plays a vital role in the catalytic function of a human cytosolic sulfotransferase. Limited protein expression was observed with this mutant, as well as the formation of inclusion bodies. To assess if this mutation causes the protein to become structurally unstable and thus a non-functioning enzyme, a simple dialysis experiment was carried out, which suggested that humSULT1E1-H107A was unable to bind the radiolabelled substrate estrone (results not shown). Kakuta and coworkers (23) found that a similar mutation in the mouSULT1E1 enzyme resulted in an equally structurally unstable protein. However the construction of a glutathione S-transferase H108K fusion enzyme of mouSULT1E1 by this group, resulted in an enzyme which had catalytic activity 100-fold less than the wild-type enzyme (23).

In conclusion, this study represents the first investigation of the properties of humSULT1E1 with re-

spect to substrate specificity. The roles of three amino acids proposed to line the substrate binding pocket of humSULT1E1 are reported. Lysine at position 85 has previously been implicated to interact with the estrogen substrate (9), however substitution to an alanine suggests the proposed hydrogen bonding of this residue to the substrate is not necessary for sulfonation of the steroids tested. From this work it would appear that a negative charge in the substrate binding pocket compromised the binding of aromatic steroids by humSULT1E1. However substitution of valine 145 to the negatively charged glutamic acid significantly increased substrate specificity for dopamine. The mutation of the highly conserved histidine at position 107 to alanine resulted in complete loss of activity of the enzyme suggesting a possible importance of this residue in the production of a structurally stable protein.

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