Carboxyl Residues in the Active Site of Human Phenol Sulfotransferase (SULT1A1)†

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ABSTRACT: The carboxyl-specific amino acid modification reagent, Woodward's reagent K (WK), was utilized to characterize carboxyl residues (Asp and Glu) in the active site of human phenol sulfotransferase (SULT1A1). SULT1A1 was purified using the pMAL-c2 expression system in *E. coli*. WK inactivated SULT1A1 activity in a time- and concentration-dependent manner. The inactivation followed first-order kinetics relative to both SULT1A1 and WK. Both phenolic substrates and adenosine 3′-phosphate 5′-phosphosulfate (PAPS) protected against the inactivation, which suggests the carboxyl residue modification causing the inactivation took place within the active site of the enzyme. With partially inactivated SULT1A1, both V_{max} and K_m changed for PAPS, while for phenolic substrates, V_{max} decreased and K_m did not change significantly. A computer model of the three-dimensional structure of SULT1A1 was constructed based on the mouse estrogen sulfotransferase (mSULT1E1) X-ray crystal structure. According to the model, Glu83, Asp134, Glu246, and Asp263 are the residues likely responsible for the inactivation of SULT1A1 by WK. According to these results, five SULT1A1 mutants, E83A, D134A, E246A, D263A, and E151A, were generated (E151A as control mutant). Specific activity determination of the mutants demonstrated that E83A and D134A lost almost 100% of the catalytic activity. E246A and D263A also decreased SULT1A1 activity, while E151A did not change SULT1A1 catalytic activity significantly. This work demonstrates that carboxyl residues are present in the active site and are important for SULT1A1 catalytic activity. Glu83 and E134 are essential amino acids for SULT1A1 catalytic activity.

Sulfation as a conjugation reaction in the biotransformation of many structurally diverse compounds has been known for more than a century (*1*). The biosynthetic mechanism for sulfation was not determined until 1956 when the structure of the cosubstrate adenosine 3′-phosphate 5′-phosphosulfate (PAPS)[1] was reported (*2*). Cytosolic sulfotransferases (SULTs) are the enzymes responsible for these biotransformation reactions. SULTs have a very broad substrate specificity. Most phenolic and alcoholic xenobiotics are substrates for one of the SULT isoforms. Endogenous compounds, such as hydroxysteroids, thyroid hormones, bile acids, and monoamine neurotransmitters, are also substrates for SULTs. The exact biological functions for the SULT-catalyzed sulfation of endogenous substrates are not very clear; however, estrogen sulfation is important in regulating estrogenic activity in estrogen-responsive tissues (*3*, *4*). Sulfation of drugs and xenobiotics is mostly associated with detoxification since biotransformation of a relative hydrophobic xenobiotic into a more water-soluble sulfuryl ester is more readily excreted (*5*, *6*). However, there are numerous important exceptions where the formation of chemically reactive sulfuryl esters is an essential step in the metabolic pathways leading to toxic or carcinogenic bioactivation (*7*−*10*). Whether sulfation results in detoxification or bioactivation is highly dependent on the electrophilic reactivity of the individual sulfuryl ester product formed. Most sulfation products are stable enough for excretion, while other sulfuryl ester products can be reactive toward nucleophilic sites on DNA, RNA, or protein and become involved in the initiation of carcinogenesis and other toxic responses.

The first SULT amino acid sequence was reported in 1989 (*11*). Due to the expansion of available SULT primary structures, there has been an increase in structure−function studies in the past decade. Protein sequence alignments of the different SULTs have revealed two highly conserved regions, one (PKSGTTW) in the N-terminal region and one (RKGXXGDWK) in the C-terminal region (*12*, *13*). Since all SULTs use the same sulfuryl donor, it was speculated that these two regions were involved in PAPS binding, and this had been supported by different structure−function studies (*12*−*19*). The X-ray crystal structure of mSULT1E1 (mouse estrogen SULT) (*20*) supported the idea that these

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[1] Abbreviations: SULT1A1, human phenol sulfotransferase P form (P-PST-1); SULT1A3, human phenol sulfotransferase M form (M-PST); mSULT1E1, mouse estrogen sulfotransferase; WK, Woodward's reagent K; SULT, sulfotransferase; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; PAP, adenosine 3′-phosphate 5′-phosphate; 4MU, 4-methylumbelliferone; PNPS, *p*-nitrophenyl sulfate.

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two regions, residues 259−265 and 45−51 for mSULT1E1, are directly involved in the PAPS binding. SULT1A3 (human M-PST) crystal structure has been partially solved independently from two laboratories (248 of the 295 residues and 223 of the 295 residues) (*21*, *22*). The crystal structure of the sulfotransferase domain of human heparan sulfate *N*-deacetylase/*N*-sulfotransferase 1 (NST1) has also been solved (*23*). Recently, the crystal structure of human hydroxysteroid sulfotransferase (SULT2A3) has been solved (*24*). All these structures support the theory that these two sequences serve as PAPS binding regions.

Very limited information is available on the structural determinants of the SULT substrate binding site. *N*-Bromoacetyl-4-hydroxyphenylamine has been used for affinity labeling of the substrate binding site in rat liver aryl SULT-IV (*25*). Sakakibara et al. (*14*) reported the localization and a functional analysis of the substrate specificity and catalytic domains of human SULT1A3 and SULT1A1. They demonstrated the differential roles of the two highly variable regions (amino acid residues 84−89 and 143−148) in substrate binding, catalysis, and sensitivity to inhibition by 2,6-dichloro-4-nitrophenol. The X-ray crystal structure of mSULT1E1 showed that residues Phe142, Ile146, and Tyr149 contribute to binding at the steroid 17β-estradiol binding site. In addition, Asn86 is believed to be in a position to form a hydrogen bond with the 17β-hydroxyl group, while Lys106 and His108 are within hydrogen bonding distance of the 3α-phenol group (*20*).

To our knowledge, studies on the identification of specific residues in the active site of SULTs by amino acid modification reagents are limited. Borchardt et al. have reported their work in this area using 2,3-butanedione and phenylglyoxal to characterize the arginyl residues in the active site of rat liver phenol SULT (*26*, *27*). *N*-Ethylmaleimide has also been used for the identification of essential sulfydryl residues on rat phenol SULT (*27*). Ribonucleotide dialdehydes (ATPDA, ADPDA, and AMPDA) have been characterized as affinity labeling reagents for rat liver phenol SULT, and it was speculated that the dialdehydes inactivated the SULT by formation of a Schiff base adduct with an active site lysine residue (*27*, *28*). More recently, ATPDA had been used as an affinity labeling reagent for the identification of a peptide sequence in the PAPS binding site (*17*).

Relatively more work has been done on the mutagenesis of SULTs. Early point mutations of guinea pig estrogen SULT demonstrated the importance for PAPS binding of the highly conserved region (GXXGXXK) (*29*). Mutational analysis of domain II of flavonol 3-SULT suggested that H118 may be involved in catalysis and that L95 is located in the substrate binding site (*30*). Site-directed mutagenesis studies on the two forms of human phenol sulfotransferases (SULT1A1 and SULT1A3) have demonstrated those residues that determine the substrate specificity of the two enzymes, especially residue 146 (Ala in 1A1, Glu in 1A3), which governs the substrate specificity of SULT1A3 (*14*, *31*−*34*). Site-directed mutagenesis of rat hydroxysteroid SULT suggested that His98 is located at a critical position in the PAPS binding site (*35*), and mutation of His256 also suggested that this residue is important for the determination of substrate specificity of this enzyme. Site-directed mutagenesis of guinea pig 3-hydroxysteroid SULT isoforms revealed that residue 51 (Asn or Ile) plays a fundamental role in determining the stereospecificity exhibited by the α- and β-isoforms of the enzyme (*36*). A mutational study of mSULT1E1, based on the crystal structure, concluded that Tyr81 contributed to the estrogen SULT substrate specificity (*37*). Mutation of K614 to A abolished the N-sulfation activity of human heparan *N*-deacetylase/*N*-sulfotransferase, indicating the importance of this residue to the catalytic activity of this enzyme (*38*). Mutagenesis studies of sulfotransferase HNK-1ST suggested that Lys128 may be in the substrate binding site and conserved residues, such as Arg189, Asp190, Pro191, and Ser197, may play a role in maintaining a functional conformation and are also directly involved in PAPS and substrate binding (*39*).

Although studies on the structure−function of SULTs have increased tremendously, the catalytic and substrate binding mechanisms are still far from clear. The purpose of this work is to identify the potential role of carboxyl residues in the active site of SULT1A1. Our results show that the modification of carboxyl residues by WK inactivates SULT1A1 and that the modification affects both substrate and cosubstrate binding. Computer modeling and site-directed mutagenesis results demonstrated that Glu83 and Asp134 are crucial for SULT1A1 catalytic activity.

MATERIALS AND METHODS

Materials. Woodward's reagent K (WK, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate), 4-methylumbelliferone (4MU), 2-naphthol, 3′-phosphoadenosine 5′-phosphate (PAP), 3′-phosphoadenosine 5′-phosphosulfate (PAPS), isopropyl-β-D-thiogalactopyranoside (IPTG), ampicillin, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Amylose affinity resin, maltose, and Factor Xa were purchased from New England Biolabs (Beverly, MA). All other chemicals and solvents were of the highest grade available.

Enzymatic Assay. 2-Naphthol was used as a substrate for SULT1A1 enzymatic activity assay. The method utilizes *p*-nitrophenyl sulfate (PNPS) to regenerate PAPS from product PAP, and at the same time, the color reagent *p*-nitrophenol (PNP) was generated for colorimetric measurement (*40*, *41*). The detailed procedure is described in our previous publication (*42*). When comparing the enzymatic activity of the mutants, 0.1 mM 2-naphthol and 0.025 mM PAPS were used. For the determination of kinetic parameters of the mutants, 4−128 μM 2-naphthol was used. For the determination of kinetic parameters of WK partially inactivated SULT1A1, 25−1600 μM 4MU was used. Kinetic constants were calculated according to the Michaelis−Menten equation.

Purification of SULT1A1. Human SULT1A1 cDNA was expressed using the pMAL-c2 expression system as previously described (*42*, *43*). In summary, the maltose binding fusion protein (MBSULT1A1) was expressed in XL1-Blue cells containing the pMAL-SULT1A1 vector. The enzymatically active fusion protein was purified on an amylose affinity column (New England Biolabs). Factor Xa was used to cleave the MBP from the SULT1A1 protein. The factor Xa cleavage site was immediately before the initial Met of SULT1A1. The digested MBSULT1A1 was applied to an amylose affinity column to separate the cleaved maltose binding protein and nondigested MBPSULT1A1 from

SULT1A1. The final purified SULT1A1 was apparently homogeneous according to SDS−PAGE analysis.

SULT1A1 Inactivation by Woodward's Reagent K. SULT1A1 (0.1 mg/mL) was incubated in 0.1 M Tris buffer, pH 6.0, with different concentrations of Woodward's reagent K (WK) at room temperature. Aliquots (10 μL) were taken at different times for standard PNPS assay of SULT1A1 activity. Glutamic acid (20 mM) was included in the PNPS assay reaction mixture to inactivate the unreacted Woodward's reagent K. For substrate or PAPS protection experiments, phenol substrates (2-naphthol or 4-methylumbelliferone) or PAPS were added to the SULT1A1 solution before the addition of WK. To study pH effects, solutions of 0.1 M Tris/0.1 M phosphate adjusted to different pHs were used as buffers.

Modeling of the SULT1A1 Structure. The molecular coordinates of mSULT1E1 were obtained from the PDB file 1AQU. A Silicon Graphics Iris workstation was used for modeling. Since the primary sequences of SULT1A1 and mSULT1E1 could be aligned with no gaps, a significant amino acid similarity (93%) existed between the two proteins, and each had the same number of amino acids, Biosym's Homology program was used to build the backbone of SULT1A1 based on the coordinates of mSULT1E1. Side chain bumps of 0.1% overlap of the van der Waals radii were relieved manually. The structure was then minimized using steepest descents (2000 steps) followed by conjugate gradients (1000 steps) using Biosym's Discover program version 2.9. No constraints were used. The program RasMol version 2.5 was used for display of the SULT1A1 structure.

Site-Directed Mutagenesis of the cDNA Encoding SULT1A1. The cDNA encoding SULT1A1 in the pKK233-3 vector was from Dr. Charles Falany, and this construct has been described previously (*44*, *45*). All mutant cDNAs were created with the QuickChange Mutagenesis Kit (Stratagene, LaJolla, CA), and all primers for mutagenesis were obtained from Integrated DNA Technologies Inc., Coralville, IA (sequences available upon request). Mutant cDNA was generated and selected through a series of three steps consisting of primer extension/thermocycling, digestion of parental DNA, and transformation as outlined in the manufacturer's protocol. Colonies resulting from the tranformation were grown in sterile medium by standard protocols, and plasmid was isolated from cells with the QIAprep Spin Miniprep kit (Qiagen Inc., Valencia, CA). The presence of the desired mutations was confirmed by DNA sequencing which was performed by The University of Arkansas for Medical Sciences, Department of Microbiology and Immunology, Molecular Resource Laboratory.

Bacterial Expression of SULT1A1 Mutants. The cDNAs encoding mutant SULT1A1 proteins were transformed into the *Escherichia coli* strain BL21λ (DE3) (Novagen, Madison, WI) according to manufacturer's protocol. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM once the culture reached $A_{600} = 0.6$. After a 4 h induction, cultures were placed on ice for 5 min, pelleted by centrifugation, washed with 50 mM Tris-HCl, and stored as a frozen pellet at −70 °C.

Western Blot. Western blot analysis was used to determine the expression level of the mutant proteins. Primary antibodies used were rabbit anti-human phenol SULT antibody (*46*, *47*). A horseradish peroxidase-based enhanced chemiluminescence (ECL; SuperSignal West Pico, Pierce Chemical Co., Rockford, IL) method was used for detection (*48*−*50*).

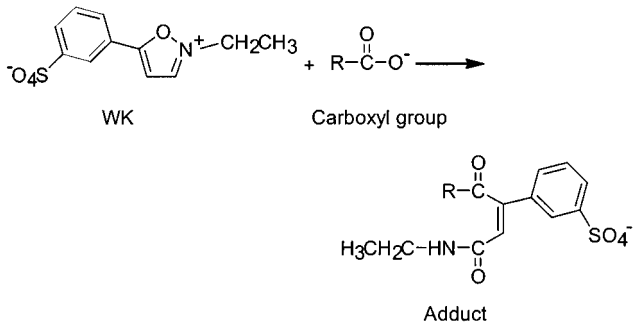

FIGURE 1: Reaction of Woodward's reagent K with a free carboxyl group.

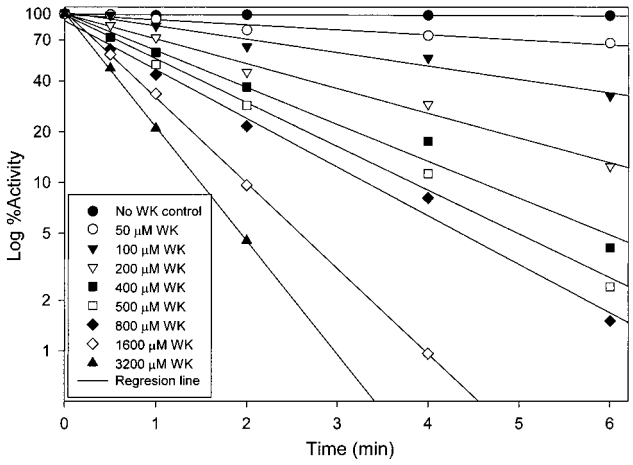

FIGURE 2: Time- and concentration-dependent inactivation of SULT1A1 by Woodward's reagent K. SULT1A1 (0.1 mg/mL, 3 μM) was incubated in 0.1 M Tris buffer, pH 6.0, with different concentrations of Woodward's reagent K (WK) at room temperature. Aliquots were taken for PNPS assay of SULT1A1 activity at the times indicated in the figure. Glutamic acid (20 mM) was included in the PNPS assay reaction mixture to remove the unreacted WK.

RESULTS

Time- and Concentration-Dependent Inactivation of SULT1A1 by Woodward's Reagent K. WK is a reagent that is specifically reactive toward the carboxyl residues of a protein (Figure 1). As shown in Figure 2, WK inactivated SULT1A1 over a concentration range from 50 μM to 3.2 mM. This inactivation was both time- and concentration-dependent. When log % activity was plotted against time, straight lines resulted as shown in Figure 2. This suggests that the inactivation is first-order relative to SULT1A1. The apparent first-order rate constants, k_{app}, can be calculated according to the equation: log % activity $= -2.3k_{app}t$ (from $d[E]/dt = k_{app}[E]$), where [E] is the concentration of active SULT1A1. To calculate the reaction order for the inactivation relative to WK, the following equation can be used: $k_{app} = k[WK]^n$ (where n is the reaction order for WK and k is the rate constant for the inactivation). From the equation $\log k_{app} = n \log [WK] + \log k$, $\log k_{app}$ was plotted versus log [WK],

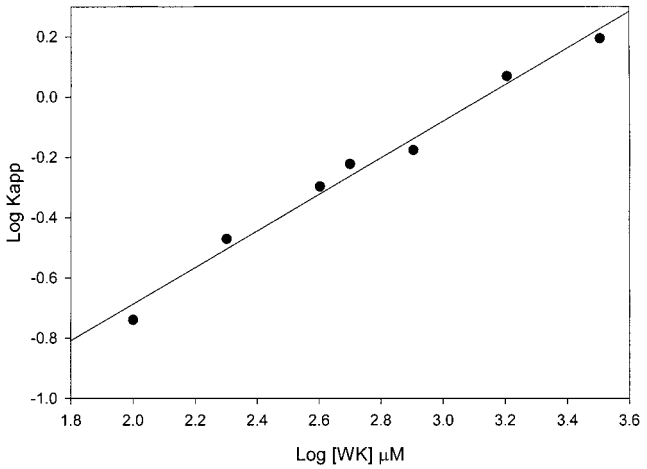

FIGURE 3: Inactivation order for WK. To calculate the inactivation order relative to Woodward's reagent K, the following equation was used: $k_{app} = k[WK]^n$ (where n is the reaction order for WK, k is the rate constant for the inactivation). According to the equation $\log k_{app} = n \log [WK] + \log k$, when $\log k_{app}$ is plotted versus $\log [WK]$, a linear relation should exist as shown in the figure. The calculated slope (n) is 0.93; the rate constant (k) is 0.07 min^{-1} M^{-1}.

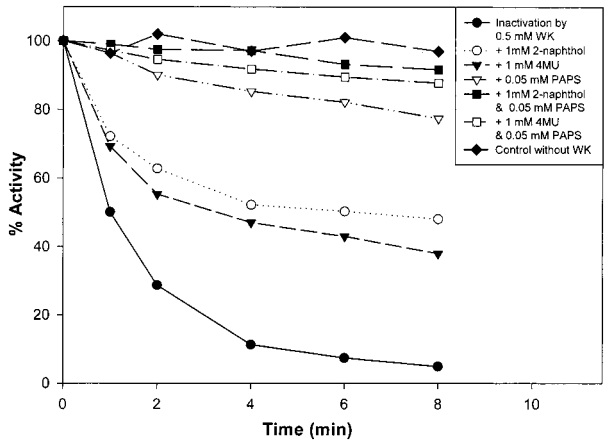

FIGURE 4: Substrate and PAPS protection of the inactivation of SULT1A1 by WK. The assay conditions were the same as described in the legend to Figure 2. 0.5 mM WK was used for all these experiments. 4MU, 2-naphthol, and PAPS were added at the concentrations as indicated before the addition of WK.

giving the straight line shown in Figure 3. From Figure 3, a second-order rate constant, $k = 0.07$ min^{-1} M^{-1}, and reaction order (slope), 0.93, were calculated. These results demonstrated that WK reacted with essential amino acids in SULT1A1 and inactivated the enzyme activity. Thus, one or more active site amino acids contain a carboxyl group.

Substrate and PAPS Protection of the Inactivation of SULT1A1 by WK. To see if the inactivation of SULT1A1 is caused by modification of residues in the substrate or PAPS binding sites, 2-naphthol, 4-methylumbelliferone (4MU), and PAPS were used to protect against WK inactivation. As seen in Figure 4, 0.5 mM WK inactivated SULT1A1 activity by 50% within 1 min, while in control reactions without WK the enzymatic activity of SULT1A1 did not change in 8 min. The addition of either substrate or PAPS or both prior to inactivation with WK blocked modification of carboxyl groups. PAPS (0.05 mM) prevented over 80% of the inactivation in 8 min, while 1 mM 2-naphthol or 4MU blocked about 50% of the inactivation in the same time. The combination of both substrate (4MU or 2-naphthol) and

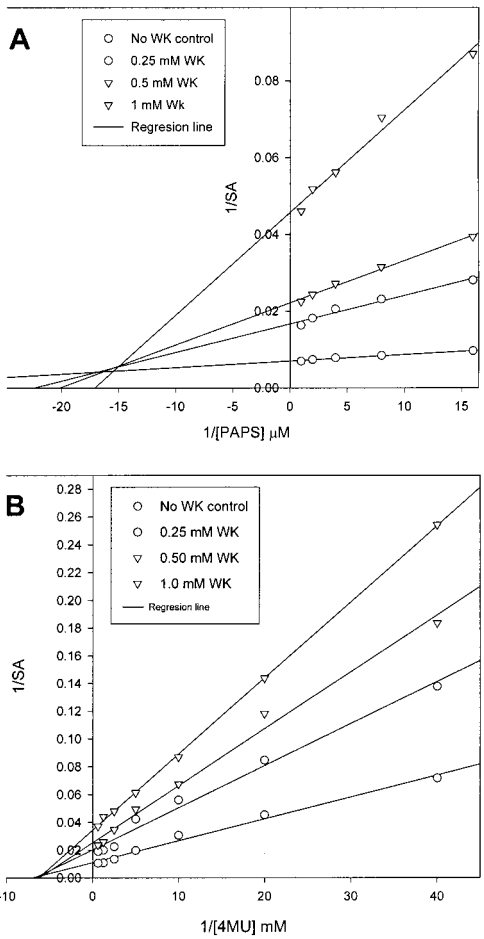

FIGURE 5: Kinetic parameters for SULT1A1 partially inactivated by WK. (A) Changes of PAPS kinetic parameters. 0.1 mg/mL SULT1A1 in 0.1 M Tris buffer, pH 6.0, was inactivated by different concentrations of Woodward's reagent K for 2 min at room temperature. The inactivation was stopped by adding an equal volume of 2 M glutamic acid. The partially inactivated SULT1A1 (including controls without WK) was used to measure the kinetic parameters by changing the PAPS concentration at the optimal 2-naphthol concentration of 0.1 mM. (B) Changes of 4MU kinetic parameters. The method used was the same as in (A). A saturating PAPS concentration of 80 μM was used.

cosubstrate (PAPS) protects more than either PAPS or substrate (4MU or 2-naphthol) alone. These results show that both substrate and cosubstrate binding can prevent the inactivation of SULT1A1 by WK.

Kinetic Parameter Changes for SULT1A1 Partially Inactivated by WK. SULT1A1 was partially inactivated by incubation with different concentrations of WK in 0.1 M Tris buffer, pH 6.0, for 2 min at room temperature. The inactivation was stopped by adding an equal volume of 2 M glutamic acid. The partially inactivated SULT1A1 was used for the determination of kinetic parameters by changing PAPS or phenol substrate concentration. Control reactions without WK were also included. The results shown in Figure 5A demonstrated that both K_m and V_{max} for PAPS changed after SULT1A1 was partially inactivated by WK. This suggests that the modification of the carboxyl residues significantly decreased the binding affinity of PAPS and the catalytic activity of SULT1A1. When substrate, 4MU, concentration was changed, the V_{max} decreased, but the K_m did not change significantly (Figure 5B). This suggested that when SULT1A1 was modified by WK, the binding affinity

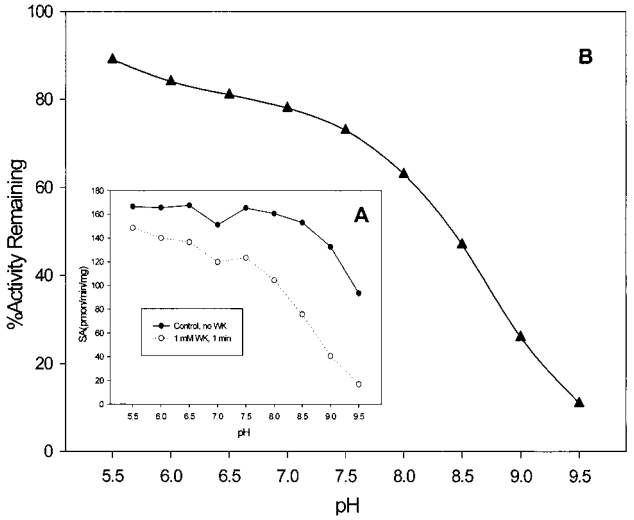

FIGURE 6: pH dependence for the inactivation of SULT1A1 by WK. Reaction mixtures containing 0.1 mg/mL SULT1A1 in 0.1 M Tris/0.1 M phosphate buffers of different pHs were incubated with 1 mM WK. At 0 and 1 min, 10 μL of reaction mixture was taken for PNPS assay of SULT1A1 activity. The specific activities, after 0 and 1 min incubation, at different pHs are shown in the inset graph (A). The ratios of the enzymatic activities at 1 and 0 min at different pHs are shown in panel B.

of 4MU to the enzyme was not much altered, whereas the catalytic activity decreased significantly.

Effect of pH on the Inactivation of SULT1A1 by WK. Figure 6 shows the inactivation of 0.1 mg/mL SULT1A1 by 1 mM WK in 1 min at different pHs (5.5−9.5). The inset graph (Figure 6A) shows the specific activity of SULT1A1 at different pH values before and after the inactivation. Figure 6B shows the percentage activity relative to the control (without WK) remaining after 1 min inactivation at that pH. WK inactivated SULT1A1 better at higher pH. This suggests that the inactivation is base-catalyzed. It was also found that the stability of WK decreased as the pH increased (data not shown). Under basic conditions, WK decomposed very quickly. All the inactivation experiments were done at pH 6.0 because WK is more stable at acidic conditions and SULT1A1 has high activity between pH 5.5 and 6.5.

Computer Modeling Structure of SULT1A1. The crystal structure of mSULT1E1 has been reported (*20*) (PDB Accession Number 1AQU). SULT1A1 and mSULT1E1 have a high similarity in their primary sequences (49% identity, 93% similarity). Both enzymes have 295 amino acids, and the sequence alignment between the 2 enzymes has no gaps (Figure 7). Based on the coordinates of mSULT1E1, a SULT1A1 model structure was constructed (Figure 8). Our computer-modeled structure of SULT1A1 superimposed on the SULT1A3 (PDB Accession Number 1CJM) partially (223 of the 295 residues) solved crystal structure (*21*) with a root mean square deviation of 1.1 Å. The core structure of SULT1A3 is very similar to that of mSULT1E1 that we used for the modeling of SULT1A1. The core folding of the SULT domain of human heparan sulfate *N*-deacetylase/*N*-ST 1 (NST1) (*23*) (PDB Accession Number 1NST) and human hydroxysteroid SULT (SULT2A3) (*24*) is also very similar to that of mSULT1E1. According to the model structure of SULT1A1, Asp134 and Asp263 are within the binding site for 3′-phosphate of PAPS (within 5 Å). If either of these two Asp residues reacts with WK (see Figure 1 for WK

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          1                                                  50
SULT1A1   MELIQDTSRP PLEYVKGVPL IKYFAEALGP LQSFQARPDD LLISTYPKSG
mSULT1E1  METSMPEYYE VPGEFRGVLM DKRFTKYWED VEMFLARPDD LVIATYPKSG

          51                                                 100
SULT1A1   TTWVSQILDM IYQGGDLEKC HRAPIFMRVP FLEFKAPGIP SGMETLKDTP
mSULT1E1  TTWISEVVYM IYKEGDVEKC KEDAIFNRIP YLECRNEDLI NGIKQLKEKE

          101                                                150
SULT1A1   APRLLKTHLP LALLPQTLLD QKVKVVYVAR NAKDVAVSYY HFYHMAKVHP
mSULT1E1  SPRIVKTHLP PKVLPASFWE KNCKMIYLCR NAKDVAVSYY YFLLMITSYP

          151                                                200
SULT1A1   EPGTWDSFLE KFMVGEVSYG SWYQHVQEWW ELSRTHPVLY LFYEDMKENP
mSULT1E1  NPKSFSEFVE KFMQGQVPYG SWYDHVKAWW EKSKNSRVLF MFYEDMKEDI

          201                                                250
SULT1A1   KREIQKILEF VGRSLPEETV DFMVQHTSFK EMKKNPMTNY TTVPQEFMDH
mSULT1E1  RREVVKLIEF LERKPSAELV DRIIQHTSFQ EMKNNPSTNY TMMPEEMMNQ

          251                                            295
SULT1A1   SISPFMRKGM AGDWKTTFTV AQNERFDADY AKKMAGCSLT FRSEL
mSULT1E1  KVSPFMRKGI IGDWKNHFPE ALRERFDEHY KQQMKDCTVK FRMEL
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FIGURE 7: Sequence alignment of SULT1A1 and mSULT1E1. The program MultAlin (*65*) was used for sequence alignment.

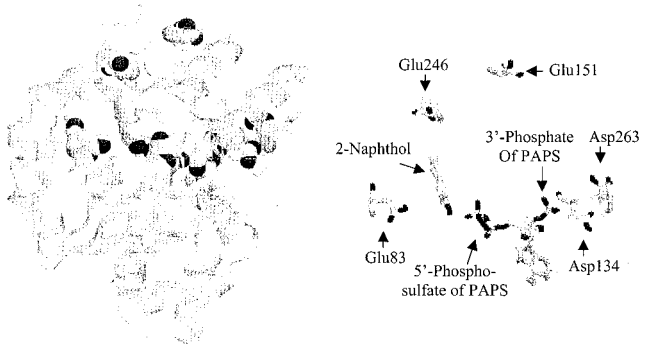

FIGURE 8: Model structure of SULT1A1. The energy-minimized model structure of SULT1A1 as depicted by RasMol version 2.5. The basic structure was represented as "Ribbons". The mutated residues and ligands were represented as "Spacefill". The picture on the right side shows the mutated residues and ligands only represented as "Sticks".

structure and the chemical reaction), the binding of PAPS will be blocked and SULT1A1 will be inactivated. This agrees with our experimental results. Glu83 is located close to the hydroxyl group of the 2-naphthol molecule where the sulfate group (SO_3) is transferred (the carbon atom of the carboxyl group of Glu83 is 7.4 Å to the oxygen of the hydroxyl group on 2-naphthol and 9.9 Å to the sulfur of 5′-phosphosulfate on PAPS). This residue may be important for the catalysis of SULT1A1. Glu246 is situated on the opening edge of the substrate binding site (15 Å to 2-naphthol). If Glu246 reacts with WK, it may block the binding of the substrate to the substrate binding site or the release of substrate from the binding site.

Site-Directed Mutagenesis of Carboxyl Residues of SULT1A1. Based on the WK inactivation and modeling results, site-directed mutation of SULT1A1 was performed to obtain E83A, D134A, E151A, E246A, and D263A mutants. E151A was selected as a negative control mutation, because it is located near, but not in the active site of, SULT1A1. It is also located on the surface of the protein.

Cytosols prepared from *E. coli* homogenates containing either the wild-type SULT1A1 or SULT1A1 mutants were evaluated using immunoblotting. The immunoblot (data not shown) demonstrates that the expression level of the mutant proteins was similar to that of the wild-type protein. Wild-type SULT1A1 and all the mutants were recognized by anti-P-PST (human SULT1A1) antibody (*46, 47*) to approximately the same extent.

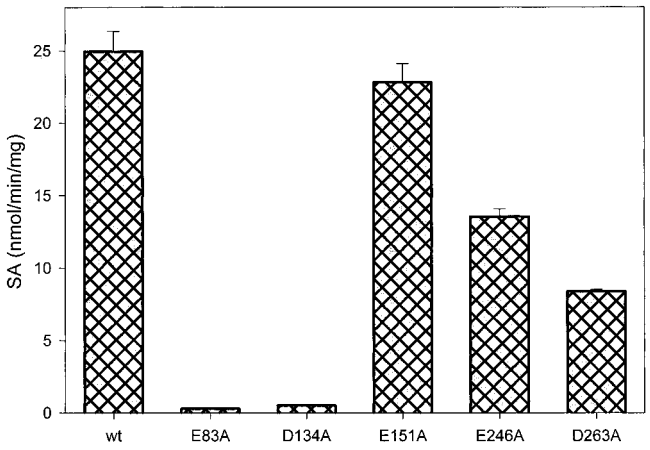

FIGURE 9: 2-Naphthol sulfation activity of SULT1A1 mutants. For the activity assay, 0.1 mM 2-naphthol and 0.025 mM PAPS were used. The detailed procedure is described under Materials and Methods.

Table 1: Calculated Distance between the Mutated Carboxyl Residues and Ligands

residue	Glu83	Glu83	Asp134	Glu151[a]	Glu246	Asp263
ligands	2-naphthol	PAPS	PAPS	2-naphthol	2-naphthol	PAPS
distance (Å)	7.4	9.9	5.0	17	15	4.9

[a] Shielded by Phe142.

Table 2: Substrate Kinetic Parameters for SULT1A1 and Mutant-Catalyzed Sulfation of 2-Naphthol[a]

mutants	V_{max} (pmol min^{-1} mg^{-1})	K_m (μM)	V_{max}/K_m	relative V_{max}/K_m
WT	59.0	200	0.295	1
E151A	51.0	165	0.309	1.05
E246A	32.5	135	0.241	0.817
D263A	10.5	38.3	0.274	0.929

[a] Assays were done at pH 6.2, PAPS final concentration 25 nM, 2-naphthol concentration changing from 4 to 128 μM. 2-Naphthol inhibited SULT1A1 at concentrations higher than 200 μM.

The specific activities of SULT1A1 wild type and mutants, using 0.1 mM 2-naphthol and 0.025 mM PAPS in the assays shown in Figure 9, demonstrated that the E83A and D134A mutants were almost completely devoid of catalytic activity. The D263A and E246A mutations also decreased catalytic activity, but the activity of the E151A mutant was not significantly affected. These mutation results demonstrate that Glu83 and Asp134 are crucial for the catalytic activity of SULT1A1. The D263A and E246A mutants also had an effect on the catalytic activity of SULT1A1, whereas the control mutation (E151A) did not significantly affect SULT1A1 catalytic activity. Kinetic evaluation of E151A, E246A, D263A, and wild-type SULT1A1, using 2-naphthol as substrate, revealed that the control mutation E151A did not significantly alter the K_m and V_{max} (Table 2). For the D263A and E246A mutations, V_{max} values were significantly decreased, but the K_m values of these mutants were also decreased. The V_{max}/K_m did not change for E151A, and was slightly decreased for the D263A and E246A mutants. Replacement of Asp or Glu with Ala in the active site increases the hydrophobicity of the substrate binding site, so the binding affinity for 2-naphthol increased for the D263A and E246A mutations. The decrease of V_{max} for the D263A and E246A mutants may be caused by a decrease in the binding of PAPS or a decrease in the catalytic ability of SULT1A1. Cosubstrate kinetic parameters determined by changing PAPS concentration are shown in Table 3. V_{max} values for E151A, E246A, and D263A mutants did not change significantly. Mutant E151A had a K_m similar to that of SULT1A1. For the D263A mutation, K_m decreased 6.1-fold compared to SULT1A1. For the E246A mutation, K_m decreased 2.6-fold. The D263A mutation decreased PAPS binding affinity significantly, and the E246A mutation also decreased PAPS binding affinity.

Table 3: Cosubstrate Kinetic Parameters for SULT1A1 and Mutant-Catalyzed Sulfation of 2-Naphthol[a]

mutants	V_{max} (pmol min^{-1} mg^{-1})	K_m (nM)	V_{max}/K_m	relative V_{max}/K_m
WT	3.16	14.8	0.214	1
E151A	2.84	17.5	0.163	0.762
E246A	2.39	39.7	0.060	0.280
D263A	2.94	89.7	0.0328	0.153

[a] Assays were done at pH 6.2, 2-naphthol final concentration 100 μM, PAPS concentration changing from 7.8 to 1000 nM.

DISCUSSION

Amino acid modification is a traditional method for the characterization of residues in the active site of enzymes (*51*). WK has been demonstrated to be a specific modification reagent for carboxyl residues of a protein and has been used for the modification of carboxyl residues in different enzymes (*52*−*58*). Combined with electrospray mass spectrometry (*59*), this method had been used recently for the identification of functional residues in different enzymes (*60*−*62*). Amino acid modification has also been used for studies of SULT active sites (*26*, *27*). 2,3-Butanedione and phenylglyoxal were used to characterize arginyl residues in the active site of rat liver phenol SULT. *N*-Ethylmaleimide had been used for the characterization of essential sulfhydryl residues in the same enzyme. When combined with other methods, like molecular modeling and site-directed mutagenesis, amino acid modification can provide detailed information on the active site of an enzyme.

In this report, we utilized the carboxyl-specific reagent WK to modify the carboxyl residues in the active site of human SULT1A1. Although all the surface carboxylic amino acids can react with WK, reaction between WK and carboxyl residues that are not essential for enzymatic activity should not inactivate SULT1A1 significantly (*51*). Additionally, any enzyme inactivation by the reaction of WK with non active site carboxyl residues should not be protected by the addition of substrate or cosubstrate (*51*). Figures 1 and 2 demonstrated that WK inactivated SULT1A1 very effectively. SULT1A1 activity was decreased over 50% by incubation with 0.5 mM WK within 1 min. Plots of log % activity versus time gave a straight line, demonstrating that the inactivation is first-order relative to SULT1A1. A plot of the reciprocal of the apparent first-order rate constant, K_{app}, at each concentration of WK versus the reciprocal of the concentration of WK was linear ($r = 0.98$) (Figure 3). These results are consistent with enzyme inactivation by formation of a covalent adduct in the active site (*27*). Figure 4 demonstrates that both substrate and cosubstrate protected against the inactivation of SULT1A1 by WK. This suggests that the modification

causing the inactivation took place in the active site. These results directly demonstrate that there are essential carboxyl residues within the active site of SULT1A1. Furthermore, both V_{max} and K_m for PAPS changed for SULT1A1 partially inactivated by WK (Figure 5A). This suggests that the modification of certain carboxyl residues hindered the binding of PAPS to the PAPS binding site. For the substrate 4MU, the K_m for partially inactivated SULT1A1 did not change, while the V_{max} decreased (Figure 5B). This suggests that the modified SULT1A1 did not change the affinity of SULT1A1 for 4MU, but decreased the catalytic activity of the enzyme.

A computer model of the three-dimensional structure of SULT1A1 was constructed based on the mSULT1E1 crystal structure and sequence alignment. According to the model structure, Asp134 and Asp263 (conserved for both enzymes) are located within the PAPS 3′-phosphate binding site (Figure 8 and Table 1). Glu83 is located within the substrate binding site, and the carboxyl group on Glu83 is close to the hydroxyl group of 2-naphthol, where SO_3 transfer occurs. Glu246 is located on the edge of the substrate binding pocket; its modification could physically block the substrate from the binding pocket. A sequence alignment of 12 mammalian SULTs (4 phenol SULTs, 4 estrogen SULTs, and 4 DHEA-SULTs; data not shown) demonstrated that both Asp134 and Asp263 are conserved among the 12 SULTs and are also located in a highly conserved region. On the other hand, Glu246 is not conserved among the 12 SULTs and is located in the variable region. Because different SULTs have different substrate specificities, the substrate binding site should be in the areas of variable amino acids. Glu83 is also conserved among the 12 mammalian SULTs, although it is not located in a highly conserved region.

Site-directed mutagenesis of these residues confirmed these conclusions. Activity assays of these mutants demonstrated that Glu83 and Asp134 are crucial amino acids for SULT1A1. Combining WK inactivation and computer modeling results, Glu83 may play a crucial role in the catalytic reaction. Glu83 is situated near the site where the sulfuryl group is transferred (Table 1 and Figure 8). The carboxyl group on Glu83 may contribute in some way to the transfer of the sulfuryl group from PAPS to the phenolic substrate. Two positively charged amino acids, His175 and Lys106, are also within this site, and may also contribute to the catalysis of SULT1A1. Asp134 may be crucial for PAPS binding or maintenance of the crucial active site structure. E134 and Asp263 are located within the binding site of the 3′-phosphate of PAPS. Close to Glu134, Asp263, and 3′-phosphate, there are four amino acids carrying positive charge, Arg130, Lys197, Arg257, and Lys258 (within 5 Å). Glu134 and Asp263, together with these positively charged amino acids, may have functional roles in maintaining the structure of the PAPS binding site and/or the catalysis of SULT1A1. The E134A mutation resulted in an almost complete loss of activity, and the D263A mutation resulted in an 83% decrease in specific activity (V_{max} values in Table 2). Mutational studies on mSULT1E1 based on its crystal structure demonstrated the importance of Lys48, Thr45, Thr51, Thr52, Lys106, His108, and Try240 in catalysis (*63*). All these amino acids are conserved between the two enzymes. Studies on the positively charged amino acids (Lys and Arg) and histidine of SULT1A1 are in progress in our laboratory to further identify the important active site amino acids that contribute to the catalytic activity of SULT1A1.

The E246A mutation decreased the 2-naphthol V_{max} of SULT1A1 significantly, but increased the affinity of the enzyme for the substrate. This suggests that the decreased catalytic activity is not caused by a decrease in the binding of substrates. Glu246 is located on the surface of the protein, close to the edge of the substrate binding site. According to the crystal structures of other SULTs (*22*, *24*), Glu246 is also located in the area where dimerization occurs. It has been reported that SULT1A1 exists as dimer in solution (*22*, *46*, *64*) and an effect on the formation of dimers may also affect the catalytic activity of SULT1A1. The control mutation, E151A, did not significantly change the catalytic activity of SULT1A1, further demonstrating the possible important roles for Glu83, Asp134, and other active site carboxyl residues in the catalytic reaction of SULT1A1. The fact that the D263A and E246A mutants exhibited a K_m for PAPS significantly different from the wild type suggests that SULT1A1 has a very defined specific binding site for PAPS, and any structure change will significantly decrease PAPS binding affinity. The D263A and E246A mutants did not change the K_m for 2-naphthol significantly, suggesting that the substrate binding site of SULT1A1 is not very well-defined; a slight structure change will not change the binding affinity for the substrate. These data agree with the fact that SULT1A1 has a very broad substrate specificity for different phenols.

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