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Photoaffinity labeling probe for the substrate binding site of human phenol sulfotransferase (SULT1A1): 7-Azido-4-methylcoumarin

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Abstract

A novel fluorescent photoactive probe 7-azido-4-methylcoumarin (AzMC) has been characterized for use in photoaffinity labeling of the substrate binding site of human phenol sulfotransferase (SULT1A1 or P-PST-1). For the photoaffinity labeling experiments, SULT1A1 cDNA was expressed in *Escherichia coli* as a fusion protein to maltose binding protein (MBP) and purified to apparent homogeneity over an amylose column. The maltose moiety was removed by Factor Xa cleavage. Both MBSULT1A1 and SULT1A1 were efficiently photolabeled with AzMC. This labeling was concentration dependent. In the absence of light, AzMC competitively inhibited the sulfation of 4MU catalyzed by SULT1A1 ($K_i = 0.47 \pm 0.05$ mM). Moreover, enzyme activity toward 2-naphthol was inactivated in a time- and concentration-dependent manner. SULT1A1 inactivation by AzMC was protected by substrate but was not protected by cosubstrate. These results indicate that photoaffinity labeling with AzMC is highly suitable for the identification of the substrate binding site of SULT1A1. Further studies are aimed at identifying which amino acids modified by AzMC are localized in the binding site.

Keywords: fluorescent probe; phenol sulfotransferases; photoaffinity labeling; structure–function; substrate binding site

Sulfotransferases (STs) catalyze the sulfation of a large variety of structurally diverse hydroxyl- or amino-containing substrates, including drugs, carcinogens, steroid hormones, bile acids, thyroxin, glycosaminoglycans, proteins, tyrosine containing peptides, neurotransmitters, and xenobiotics (Sakakibara et al., 1998a). Different isoforms of STs have been isolated from mammals, bacteria, and plants (Sakakibara et al., 1998b). The cosubstrate for all STs is adenosine 3′-phosphate 5′-phosphosulfate (PAPS) (Weinshilboum et al., 1997).

The recent expansion of availability of sulfotransferase primary structures has led to an increase in studies examining their structure–function relationships. Protein sequence alignments of the different STs have revealed two highly conserved regions, located at the N-terminal region (PKSGTTW) and the C-terminal region (RKGXXGDWK) (Weinshilboum & Otterness, 1994; Varin et al., 1997). Because all STs use the same sulfuryl donor, it has been postulated that these two regions are involved in PAPS binding (Weinshilboum & Otterness, 1994; Duffel, 1997; Varin et al., 1997; Kakuta et al., 1998; Sakakibara et al., 1998a). Affinity labeling of aryl sulfotransferase-IV had identified a peptide sequence at the PAPS binding site that is in proximity to the conserved sequence at the N-terminal end (Zheng et al., 1994). [^{35}S] PAPS had been characterized as a photoaffinity ligand for human thermostable phenol sulfotransferase (TS-PST, SULT1A1) (Otterness et al., 1991). Site-directed mutagenesis and [^{35}S] PAPS affinity labeling studies on flavonol 3-sulfotransferase have supported that the two aforementioned regions are involved in PAPS binding (Marsolais & Varin, 1995). Point mutations and [^{35}S] PAPS affinity labeling

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Abbreviations: AzMC, 7-azido-4-methylcoumarin; EDTA, ethylenediaminetetraacetic acid; EST, estrogen sulfotransferase; IPTG, isopropyl-β-D-thiogalactopyranoside; MBP, maltose binding protein; MBSLUT1A1, human phenol sulfotransferase linked to maltose binding protein; PAP, adenosine 3′-phosphate 5′-phosphate; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; PNPS, *p*-nitrophenyl sulfate; 4MU, 4-methylumbelliferone; PST, phenol sulfotransferase; SDS, sodium dodecyl sulfate; STs, sulfotransferases; SULT1A1, human phenol sulfotransferase (P-PST-1); SULT1A3, human phenol sulfotransferase (M-PST); TCA, trichloroacetic acid; UGTs, UDP-glucuronosyltransferases; UV, ultraviolet.

studies on guinea pig estrogen sulfotransferase have also supported that the C-terminus conserved region is part of the PAPS binding site (Komatsu et al., 1994). Recently, an X-ray crystal structure of mouse estrogen sulfotransferase has been partially solved (Kakuta et al., 1997). The crystal structure supported that these two regions, located residues 45–51 and 259–265, are directly involved in PAPS binding. Besides these two regions, Thr227, Trp53, and Phe229 have also been demonstrated to be involved in PAPS binding. Mutational studies on the mouse EST also suggested that Lys48, Lys106, and His108 are crucial for the catalytic activity of this enzyme (Sakakibara et al., 1998b).

Limited information is available on the structural determinants of the sulfotransferase substrate binding site. Varin's group has proposed four regions for flavonol sulfotransferases, regions I to IV. Of these, regions I and IV are the same regions mentioned above for the PAPS binding site, while region II, which spans amino acids 92 to 194 of the flavonol 3-ST sequence, is believed to contain all the determinants for substrate binding (Varin et al., 1995, 1997). Subsequent mutational analysis of domain II of flavonol 3-ST did not identify critical amino acids for substrate binding; however, Leu95 may be in direct contact with the flavonol B ring (Marsolais & Varin, 1997). Affinity labeling with *N*-bromoacetyl-4-hydroxyphenylamine (Duffel et al., 1998) of the substrate binding site in rat liver aryl sulfotransferase-IV has been reported, and four modified amino acids, Cys232, Cys283, Lys286, and Cys289 have been identified. Recently, Sakakibara et al. (1998a) reported the localization and functional analysis of the substrate specificity and catalytic domains of the human M-form (SULT1A3) and P-form (SULT1A1) phenol sulfotransferases. By comparing the kinetic parameters of an expressed series of chimeric PSTs (SULT1A3 and SULT1A1) in which the M-form and P-form coding regions were exchanged, they concluded that amino acid residues 84–148 contain the structural determinants for substrate specificity for both SULT1A3 and SULT1A1. They also demonstrated differential roles for the two variable regions (amino acid residues 84–89 and 143–148) with regards to substrate binding, catalysis, and sensitivity to inhibition by 2,6-dichloro-4-nitrophenol. The X-ray crystal structure of mouse EST showed that residues Phe142, Ile146, and Tyr149 contribute to binding at the 17β-estradiol binding site. Asn86 is believed to be in a position to form hydrogen bonds with the 17β-hydroxyl group, while Lys106 and His108 were within hydrogen bonding distance of the steroid's 3α-phenol group (Kakuta et al., 1997).

Studies aimed at deciphering the structure–function relationships of human STs are currently limited. Affinity labeling is a powerful tool for identifying substrate binding sites. It gives direct evidence of active site amino acids at the catalytic active state of an enzyme. In this paper, we report the characterization of a novel substrate binding site photoaffinity probe, 7-azido-4-methylcoumarin, which can be used to further investigate structure–function relationships in SULT1A1 and other phenol binding proteins.

Results

Fluorescent labeling of SULT1A1 by AzMC

AzMC exhibits a fluorescence spectrum with maximum excitation and emission wavelengths of 380 and 420 nm, respectively. When exposed to long wavelength (366 nm) UV radiation, AzMC covalently linked to SULT1A1. Different concentrations of AzMC were incubated at pH 7.8 with 0.1 mg/mL SULT1A1 and exposed to UV light for 30 min. Following protein precipitation with 10% trichloroacetic acid (TCA), labeled SULT1A1 was separated on a NuPAGE Bis-Tris gel and visualized with UV light. Figure 1 shows that fluorescent labeling with AzMC is concentration dependent from 15.6 μM to 1 mM (lanes 2–5). Above 1 mM, the labeling reached saturation (lane 5, 1 mM AzMC; lane 6, 2 mM AzMC). Half saturation was reached at about 70 μM AzMC. Figure 1 clearly indicates that the labeling was light dependent (lanes 6 and 7 used the same concentration of AzMC, 2 mM; lane 6, exposed to UV light for 30 min; lane 7, without exposure to UV light). To get

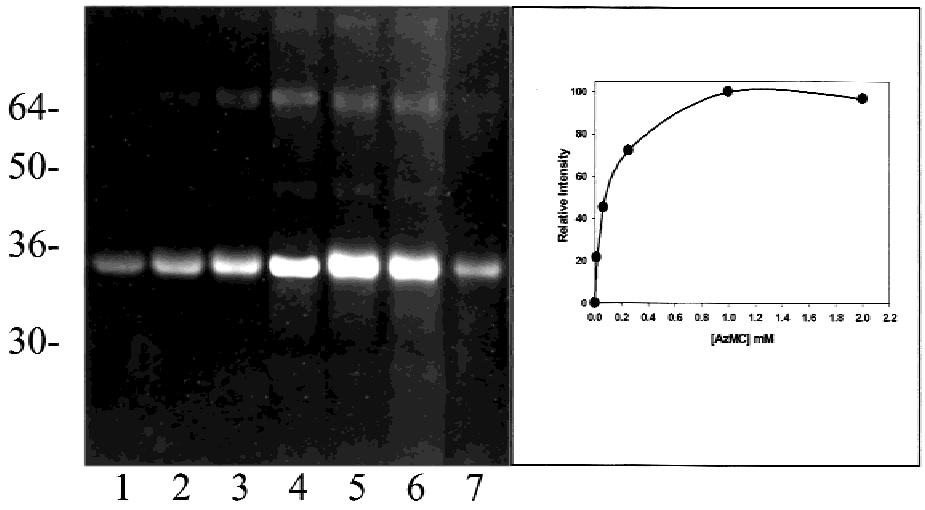


Fig. 1. Concentration-dependent photoaffinity labeling of SULT1A1 by AzMC. Ten micrograms of SULT1A1 in 250 μL of 0.25 M Tris, pH 7.8, was exposed to UV light, 366 nm, for 30 min in the presence of different concentrations of AzMC. Labeled SULT1A1 was separated on a NuPAGE Bis-Tris gel. The fluorescent picture was taken under UV light. AzMC concentration: lane 1, 0 μM; lane 2, 15.6 μM; lane 3, 62.5 μM; lane 4, 250 μM; lane 5, 1 mM; lane 6, 2 mM; and lane 7, control without UV light exposure with 2 mM AzMC.

a good fluorescent picture, 10 μg of purified SULT1A1 was used in each lane in Figure 1. Lane 1, which was a control without using AzMC, gave a weak endogenous fluorescent band. This protein's endogenous fluorescence was much lower than that of AzMC-labeled SULT1A1 bands. Figure 1 clearly shows a labeling band at approximately 68 kDa. This band is apparently a dimer of P-PST-1. Radioactive photoaffinity labeling of other proteins in our laboratory routinely produces a dimer band similar like P-PST-1.

Irreversible inactivation of SULT1A1 by AzMC labeling

Upon exposure to UV light at 366 nm at pH 7.8, AzMC irreversibly inactivated SULT1A1 when the enzyme was diluted 25-fold in the standard PNPS assay for the sulfation of 2-naphthol (Fig. 2). The inactivation was both time and concentration dependent (Fig. 2) and was not reversible by dilution. From the data in Figure 2, apparent first-order rate constants for the irreversible inactivation (K_{app}) were calculated at each concentration of AzMC. A plot of $1/K_{app}$ vs. 1/AzMC gives a straight line (inset in Fig. 2). The linear relationship between $1/K_{app}$ and 1/AzMC and the positive intercept (inset in Fig. 2) support the kinetic model described by Borchardt et al. (1982), as shown in Scheme 1. Reversible binding of AzMC to the SULT1A1 substrate binding site is followed by a first-order reaction to form an irreversible adduct. The calculated steady-state constant (K_d) for the formation of a reversible complex between AzMC and SULT1A1 was 69 μM. The first-order rate constant (k_2) for the subsequent irreversible formation of the adduct was 0.069 min^{-1}.

Substrate protection of SULT1A1 from inactivation by AzMC

Under similar conditions of photoinactivation using 0.25 mM AzMC, Figure 3 demonstrates that the SULT1A1 substrate, 4MU, significantly protected the photo-inactivation of SULT1A1 by AzMC. PAP, however, did not protect the photo-inactivation. This clearly demonstrated that AzMC label was incorporated at the substrate binding site but not the PAP binding site of SULT1A1.

$$\text{SULT1A1 + AzMC} \underset{}{\overset{K_d}{\rightleftharpoons}} \text{SULT1A1 AzMC} \xrightarrow{k_2} \text{SULT1A1-AzMC}$$

Scheme 1.

AzMC competitive inhibition of the sulfation of 4MU catalyzed by SULT1A1

Scheme 2 shows the structural relationship between AzMC and 4MU. 4MU is a substrate for SULT1A1 in Tris buffer pH 7.8 (K_m = 0.40 ± 0.07 mM, V_{max} = 239 ± 37 nmol/min/mg, Fig. 4). AzMC is not a substrate for SULT1A1 and exists as a stable compound without UV light exposure. In the absence of irradiation, AzMC competitively inhibited the sulfation of 4MU in Tris buffer, pH 7.8 (K_i = 0.47 ± 0.05 mM) (Fig. 4). This directly demonstrated that AzMC binds to the substrate binding site of SULT1A1. The competitive inhibition constant of AzMC (0.47 mM) is close to the K_m of 4MU (0.40 mM). This suggests that AzMC have approximately the same affinity as 4MU for binding to the SULT1A1 substrate binding site.

Sequence alignment of different phenol binding proteins

When different kinds of phenol binding proteins were aligned using the SIM program (Huang & Miller, 1991), we found a conserved sequence that is common among phenol STs and other phenol binding proteins (Fig. 5). These conserved residues may be responsible for the binding of phenols. Chimeric mutagenesis of human phenol STs (P-PST-1 and M-PST) has demonstrated that

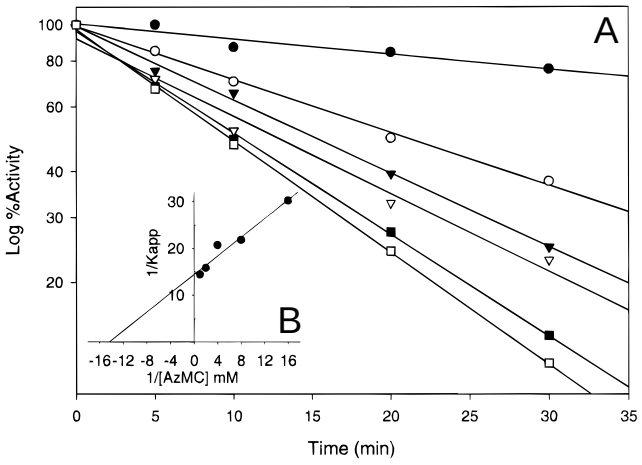


Fig. 2. Time- and concentration-dependent photo-inactivation of SULT1A1 by AzMC. Thirty micrograms of SULT1A1 in 600 μL of 0.25 M Tris, pH 7.8, was exposed to UV light, 366 nm, for 0 or 30 min in the presence of different concentrations of AzMC. Aliquots (50 μL for assay with 0.1 mM 2-naphthol as substrate, 50 μL for control assay) were taken at 0, 5, 10, 20, and 30 min for the standard PNPS assay. Percentage activities were calculated relative to zero time enzyme activity. The concentrations of AzMC, from upper to lower lines, 0 μM, 62.5 μM, 125 μM, 250 μM, 500 μM, and 1 mM. The inset is a re-plot of the data from Figure 2. K_{app} is the apparent first-order rate constant for the irreversible inactivation (the slope for ln %Activity vs. time).

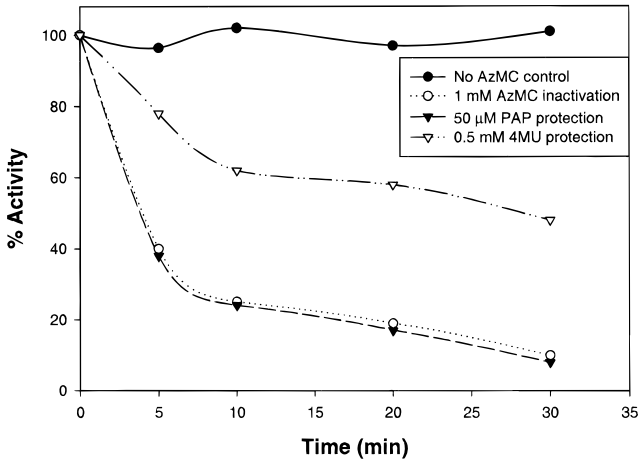
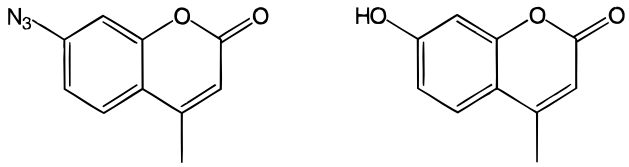


Fig. 3. Substrate and PAP protection of SULT1A1 from photo-inactivation by AzMC. SULT1A1 (0.1 mg/mL) was incubated with 250 μM AzMC in 0.25 M Tris, pH 7.8. 0.5 mM 4MU, or 50 μM PAP were added for protection. The reaction mixtures were exposed to 366 nm UV light for photo-inactivation. At the times indicated in the figure, 10 μL of the mixture was taken to do a standard PNPS assay using 0.1 mM 2-naphthol as substrate (V_t = 250 μL). Relative enzyme activities were calculated according to zero time enzyme activity.



7-azido-4-methylcoumarin (AzMC) 4-Methylumbelliferone (4MU)

Scheme 2.

the hP-PST-1 peptide sequence, YHMAKV (143-148 of P-PST-1), plays a role in determining the substrate specificity (Sakakibara et al., 1998a). Site-directed mutagenesis studies on P-PST-1 and M-PST have demonstrated that amino acid 146 (E in M-PST and A in P-PST-1) governs the substrate specificity of the two PSTs (Dajani et al., 1998). A single amino acid mutation, E146A, changed the kinetic properties of M-PST to those of P-PST-1. This result indicated that an alignment of different substrate binding proteins can produce information about the substrate binding site. A similar approach was recently used to identify common sequences based on homology within ST "families" that were apparently specific for hydroxysteroid STs, PSTs, and ESTs (Her et al., 1998).

Proteolytic digestion of AzMC labeled P-PST-1 and peptide isolation

AzMC-labeled P-PST-1 was digested by trypsin and the fluorescently labeled peptides were separated by SDS-PAGE (Fig. 6). When digested with trypsin and run on NuPAGE Bis-Tris gel, fluorescent bands were clearly visible. After 60 min digestion, the major fluorescent band had a molecular weight around 10 kDa; the two minor fluorescent bands had molecular weights of about 5 and 6 kDa. This experiment clearly demonstrated that AzMC specifically labeled amino acid residues on P-PST-1. There is a possibility that more than one amino acid residue was labeled; however, the presence of different bands does not necessarily imply different amino acid labeling because of the possibility of noncomplete proteolysis.

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hP-PST-1        142 FYHMAKVHEET  152
Bovine-PST      142 FYRMAKVHEDT  152
Dog-PST         142 FYRMAKVHEDT  152
Mouse-PST       150 FSRMNKMLEDT  160
Monkey-PST      142 FYHMAKVHEET  152
Rat-PST         141 FYNMAKLHEDT  151
hUGT 1A6         71 KYYTRKIYEVT   81
hCYP2C9         394 VLHDNKEFENT  404
hCYP2A6         223 FSSVMKHLPGP  233
hALBUMIN        356 LVEYAKRHPDY  366
phenoloxidase   114 SYCQLKINEYM  124
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Fig. 5. Sequence alignment of phenol binding proteins AIM-ALIGNMENT program was used for the sequence alignment. SIM (Huang & Miller, 1991) is a program that finds a user-defined number of best nonintersecting alignments between two protein sequences or within a sequence. All the protein sequences are from the GenBank. hP-PST (SULT1A1) was used to align with other proteins that either use 4MU as a substrate or bind 4MU. The proteins in the figure were found to have sequence similarity in this region (conserved amino acids are shadowed). The references describing each of the DNAs encoding these proteins are as follows: hP-PST (Wilborn et al., 1993) (accession number L19999); bovine-PST (Schauss et al., 1995) (accession number U35253); dog-PST (Satsukawa et al., 1994) (accession number D29807); mouse-PST (Tamura et al., 1998) (accession number AF033653); monkey-PST (Ogura et al., 1996) (accession number D85514); rat-PST (Yerokun et al., 1992) (accession number X68640); hUGT1A6 (Wooster et al., 1991) (accession number AF014112); hCYP2C9 (Goldstein et al., 1991) (accession number L16877); hCYP2A6 (Fernandez-Salguero et al., 1995) (accession number U22027); hAlbumin (Lawn et al., 1981) (accession number L00132); phenol oxidase (Jiang et al., 1997) (accession number AF003253).

Discussion

Although the primary sequence of many STs are known, and several research reports have focused on the molecular mechanism of

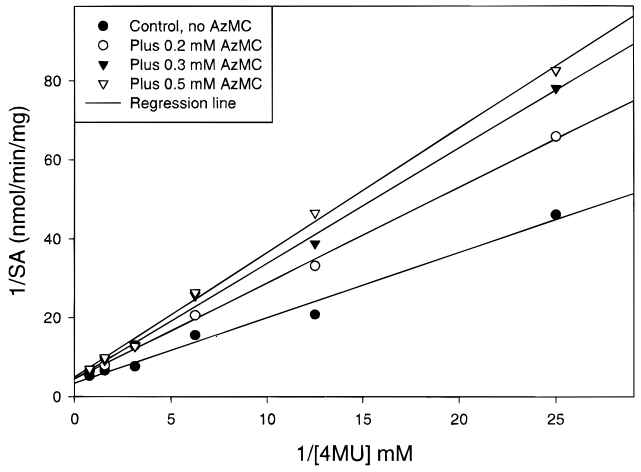


Fig. 4. Reversible Inhibition of SULT1A1-catalyzed 4MU sulfation by AzMC. SULT1A1 enzymatic activity was measured by PNPS assay (see Materials and methods). 4MU concentration used 0.04, 0.08, 0.16, 0.32, 0.64, and 1.28 mM. AzMC concentration used, from lower to upper lines, 0 (control), 0.2, 0.3, and 0.5 mM.

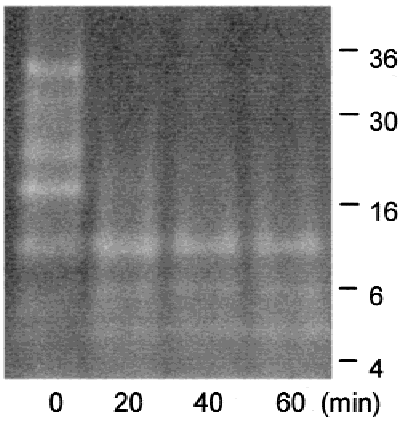


Fig. 6. Trypsin digestion of AzMC-labeled P-PST-1. Fifty micrograms of P-PST-1 was labeled with 1 mM AzMC under UV light (366 nm) for 30 min. The labeled P-PST-1 was precipitated using 10% TCA. Trypsin (1/100 of trypsin/P-PST-1 ratio) was used for the proteolysis. Novex NuPAGE Bis-Tris gel was used for peptide separation. Picture was taken under UV light.

sulfation, the identities of specific amino acids that play a role in substrate binding and sulfotransferase catalytic activity are still unknown. Recent reports focusing on the active sites of STs have centered on identifying the PAPS binding site (Varin et al., 1992; Falany et al., 1994; Komatsu et al., 1994; Lee et al., 1994; Zheng et al., 1994; Driscoll et al., 1995; Marsolais & Varin, 1995, 1998; Radominska et al., 1996). These studies revealed two conserved regions that serve this purpose. The solution of the mouse EST crystal structure further confirmed the role of these two regions as PAPS binding sites (Kakuta et al., 1997). Despite these advances, studies aimed at localizing the substrate binding amino acids within the binding site are less complete. The limited information that exists on substrate binding comes from mutagenesis studies and an analysis of the EST crystal structure. This structure demonstrated that Phe142, Ile146, and Tyr149 contribute to 17β-estradiol binding. This is in agreement with recent mutagenesis studies in which amino acids 143–148 of SULT1A1 were implicated as structural determinants for substrate binding (Sakakibara et al., 1998a). A different mutagenesis study involving SULT1A1 and SULT1A3 found that a single amino acid located at position 146 (E in SULT1A1, A in SULT1A3) governed the substrate specificity between the two enzymes. Also, by changing this amino acid from glutamate to alanine, the researchers altered the kinetic properties and substrate specificity of SULT1A3 to match that of SULT1A1 (Dajani et al., 1998). Collectively, the above findings suggest that amino acids 140–150 comprise a region of substrate binding common to all sulfotransferases.

In the present study, photoaffinity labeling was used to characterize the substrate binding site of human SULT1A1. A novel labeling reagent, AzMC, had been synthesized in our laboratory and successfully used to photolabel human phenol UDP-glucuronosyltransferase (UGT1A6) (unpublished data). This same photoaffinity probe was utilized to identify the substrate binding site of human SULT1A1. AzMC competitively inhibited SULT1A1-catalyzed sulfation of 4MU (Fig. 4). This provided direct evidence that AzMC can bind to the substrate binding site of SULT1A1. Also, the kinetics of inactivation of SULT1A1 by AzMC support specific labeling (Fig. 2). This irreversible, time- and concentration-dependent inactivation suggests that AzMC first reversibly then covalently binds to SULT1A1 and inactivates the enzyme. Interestingly, PAPS did not protect against photo-inactivation. However, the photo-inactivation was partially protected by 4MU, a substrate of SULT1A1 (Fig. 3). These results suggested that AzMC labeled SULT1A1 at the substrate binding site rather than the PAPS binding site. This demonstrated that AzMC can be employed as a specific, substrate binding site probe for phenol STs. A photoaffinity probe will also enhance our ability to identify which amino acids are involved in phenol binding. By determining which amino acids are involved in phenol binding, we will be in a better position to elucidate the molecular mechanism of sulfation reactions.

A radioactive photoaffinity probe, 2-iodo-4-azidophenol, had been previously characterized as a photoaffinity ligand for human thermostable phenol sulfotransferase (TS-PST, SULT1A1) (Otterness et al., 1989). The photo-incorporation of this probe was protected by both PAP and substrates. This suggested that this probe interacted with both substrate and PAPS binding sites. This was different from our probe, AzMC. The labeling with AzMC was protected only by substrates but not by PAP. The authors also stated in their Discussion that the extent of photoaffinity labeling with 2-iodo-4-azidophenol was greatly enhanced in the presence of PAPS. On the other hand, our results demonstrated that PAPS did not enhance photoaffinity labeling of AzMC (data not included). AzMC is obviously different from 2-iodo-4-azidophenol as a photoaffinity labeling probe. AzMC is not a substrate for SULT1A1 while 2-iodo-4-azidophenol is still a substrate for SULT1A1.

As an alternate approach, we utilized database searches to compare SULT1A1 with other proteins involved in phenol-directed reactions. STs, UGTs, and cytochromes P450 that contain potential phenol binding sites are identified in Figure 5. Alignments revealed a consensus sequence localized in the putative substrate binding site of these enzymes. STs and UGTs contained the most extensive motif, YXXXKXXPXP. In UGT1A6, this conserved domain is localized between amino acids 71–81, a region postulated as part of the aglycon binding site of all UGTs (Battaglia et al., 1997; Meech & Mackenzie, 1997). In STs, the conserved motif was found between amino acids 141(142)–151(152). This is in agreement with the mouse EST crystal structure, which indicated that Phe142, Ile146, and Tyr149 contribute to substrate binding. Additional confirmation that amino acids 141(142)–151(152) participate in substrate binding is provided by mutagenesis studies carried out by Sakakibara et al. (1998a), in which region II for SULT1A1 (143–148) was postulated to be the structural determinant for substrate binding. Also, mutagenesis studies concerning SULT1A1 and SULT1A3 demonstrated that a single amino acid at position 146 (glutamate in SULT1A3 and alanine in SULT1A1) governs substrate specificity between the two enzymes (Dajani et al., 1998).

Interestingly, all STs contain an acidic amino acid, either glutamate or aspartate, at the position between the conserved prolines. The presence of carboxyl residues in the active site of human phenol STs was first indicated in separate studies (unpublished data from our laboratory). Carboxyl group-directed reagents, such as Woodward's reagent K, inhibited the sulfation reaction. Protection experiments carried out with PAPS and phenol derivatives clearly demonstrated that both the PAPS binding site and the substrate binding site of SULT1A1 contained acidic amino acids. A less extensive motif, KXXPXP, was present in the cytochromes P450. As is the case with the human UGT1A6, the two cytochromes P450 failed to contain an acidic amino acid between the conserved prolines. Two proteins that contain phenol binding pockets, such as human albumin and phenoloxidase, displayed a similar motif, YXXXRXXP. In conclusion, we have found a motif that closely corresponds to the domain revealed by the recent X-ray crystal structure of the mouse EST and recent site-directed mutagenesis studies. Only a combination of complementary approaches, such as X-ray crystal structure analysis, mutagenesis, and photoaffinity labeling with active site-directed probes, will result in unambiguous identification of the substrate binding site.

Materials and methods

Material

4-Methylumbelliferone (4MU), 2-naphthol, 3′-phosphoadenosine-5′-phosphate (PAP), 3′-phosphoadenosine-5′-phosphosulphate (PAPS), isopropyl-β-D-thiogalactopyranoside (IPTG), ampicillin, and dithiothreitol (DTT) were purchased from Sigma Chemical Co (St. Louis, Missouri). Amylose, maltose, and Factor Xa were purchased from New England Biolabs (Beverly, Massachusetts). Endoproteinase Lys-C, endoproteinase Glu C, and trypsin were supplied by Promega (Madison, Wisconsin). NuPAGE Bis-Tris gels and tricine gels were purchased from Novex (San Diego,

California). All other chemicals and solvents were of the highest grade commercially available.

Synthesis of 7-azido-4-methylcoumarin (AzMC)

Three grams of 7-amino-4-methylcoumarin were dissolved in 125 mL of 12 N HCl at 4 °C in the dark; 5.5 mL of 25% $NaNO_2$ was added and the mixture allowed to incubate for 10 min; 6.5 mL of 20% NaN_3 was added, and the reaction was stirred on ice for 48 h. The mixture was neutralized with 12 N NaOH and extracted with chloroform. The final product was a yellow powder, m.p. 110 °C. The yield was generally over 90%. The identity of the product was further validated by proton-NMR, infrared, and UV spectroscopy.

Purification of SULT1A1

The human SULT1A1 cDNA was expressed using the pMAL-c2 expression system as previously described for the expression of the human EST (Falany et al., 1995). To express the MBSULT1A1 fusion protein, XL1-Blue cells containing the pMAL-SULT1A1 vector were cultured in Luria broth, 4 × 250 mL, containing 50 μg/mL ampicillin, to an $O.D._{550}$ of 2.0 at 37.0 °C. IPTG was added to a final concentration of 0.3 mM, and the incubation was continued for an additional 14 h. Cells were pelleted and resuspended in 100 mL bacterial lysis buffer (75 mM Tris, pH 8.0, 0.25 M sucrose, 0.25 mM EDTA, and 0.1 mg/mL lysozyme) and incubated on ice for 30 min. Cells were again collected and resuspended in 100 mL 5 mM phosphate buffer, pH 7.4, containing 1.5 mM dithiothreitol (DTT), and 10 μg/mL phenylmethylsulfonylfluoride. The solution was sonicated for three 1 min cycles, with 2 min cooling between each cycle. The cytosolic fraction was recovered following centrifugation at 100,000 *g* for 1 h. The enzymatically active fusion protein was purified on a prewashed 2.5 × 8 cm amylose (New England Biolabs) affinity column. After washing with 250 mL of 5 mM phosphate buffer, pH 7.4, the MBSULT1A1 was eluted with 100 mL of 10 mM maltose in the same buffer. The eluted enzyme was analyzed using the PNPS enzymatic assay and SDS-PAGE. The fractions from the affinity column that contained only one protein band on SDS-PAGE (MW ~ 67 kDa) and had 2-naphthol sulfation activity were collected and concentrated using Centricon concentrators from Amicon (Beverly, Massachusetts). The yield of enzymatically active MBSULT1A1 was 40–60 mg protein from 1 L of bacterial culture. To isolate pure SULT1A1, 2.5 mg of MBSULT1A1 was added to 2.5 mL digestion buffer (20 mM Tris, pH 8.0, containing 100 mM NaCl and 2 mM $CaCl_2$), followed by the addition of 0.05 mg of Factor Xa. This mixture was incubated at room temperature for 5 h. The digested enzyme was dialyzed twice into 2 L of 5 mM phosphate buffer, pH 7.4. After dialysis, the enzyme was applied to a DEAE-Sepharose CL-6B column (1 × 7 cm), prewashed with 5 mM phosphate buffer, pH 7.4, containing 1 mM DTT. The column was washed with 40 mL of the same buffer followed by 40 mL of 0.1 M NaCl, 5 mM phosphate buffer, pH 7.4. The enzyme was eluted using a linear gradient formed from 100 mL 0.1 M NaCl to 100 mL 0.225 M NaCl. The collected fractions (5 mL) were analyzed by SDS-PAGE (12%) and an enzymatic activity assay. The fractions containing only one protein band (around 35 kDa) were collected and concentrated again by centrifugation. The yield was approximately 1 mg SULT1A1 from 2.5 mg MBSULT1A1.

PNPS enzyme activity assay

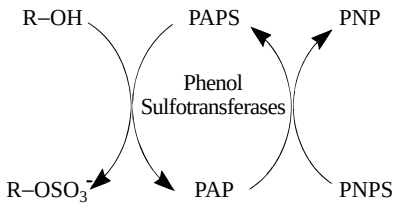
The PNPS (*p*-nitrophenyl sulfate) assay takes advantage of the fact that phenol sulfotransferases also catalyze a reverse reaction that transfers a sulfuryl group from PNPS to PAP to regenerate PAPS (Frame et al., 1997; Mulder & Scholtens, 1977). This produces a colored product, *p*-nitrophenol (Scheme 3). At high concentrations of PNPS, PAP formed in the mixture will react with PNPS to regenerate PAPS and *p*-nitrophenol. The latter has a very high absorbance at 401 nm, when the pH is above 8 (millimolar extinction coefficient: 18.4). The detailed procedure is as follows. The reaction mixture, totaling 250 μL, contains 50 mM sodium phosphate at pH 6.2, 5 mM $MgCl_2$, 5 mM PNPS, 0.02 mM PAPS, and 1–2 μg purified SULT1A1 or an appropriate amount of cytosol. Following incubation at 37.0 °C for 2 min, the reaction was initiated by adding 5 μL of various substrates in ethanol (for the enzyme purification and cytosolic PST activity assay, 5 mM 2-naphthol was used) or 5 μL ethanol as a control. After a 45 min incubation at 37.0 °C, the reaction was stopped by the addition of 250 μL of 0.25 M Tris buffer, pH 8.7. The absorbance at 401 nm was measured within 30 min. The assay was done in triplicate, and the average of the measurements minus the controls was used to calculate the enzymatic activity.

Photoaffinity labeling and photo-inactivation of SULT1A1 with 7-azido-4-methylcoumarin (AzMC)

Photoaffinity labeling of SULT1A1 was performed in 0.2 M Tris-HCl buffer, pH 7.8, containing 0.1 mg/mL SULT1A1 (or MBSULT1A1) and different concentrations of AzMC (AzMC stock solutions were made in DMSO, the final concentration of DMSO in the reaction mixture was kept under 2%). The total volume of the reaction mixture was adjusted in accordance to the total protein used (from 20 to 500 μL). The reaction mixture was incubated in microcentrifuge tubes at 25 °C for 2 min under yellow light. AzMC was then added and incubation continued for 2 min more. The tubes were exposed to a hand-held UV light (Spectroline, Westbury, New York), with a maximal radiation of 366 nm for 30 min with a 2.5-cm distance between the surface of the light and the surface of the solution. SULT1A1 was separated by NuPAGE Bis-Tris gel (NuPAGE, Novex). Protein labeled by AzMC was detected by UV fluorescence. For studies of photo-inactivation of SULT1A1 by AzMC and its protection by substrate and PAP, the incubation mixture was directly used for SULT1A1 enzymatic assay (PNPS assay, 25-fold dilution).

Proteolytic hydrolysis of AzMC labeled P-PST-1 and peptide separation on SDS-PAGE

Fifty micrograms of P-PST-1 was labeled with 1 mM AzMC under UV light for 30 min. The labeled P-PST-1 was precipitated using



Scheme 3.

10% TCA. The pellet was washed with ice-cold ethanol and dissolved in 50 μL of resuspension buffer (100 mM Tris-HCl, pH 8.0, 0.1% SDS) and then 0.5 μg of trypsin was added to the mixture. Immediately after the addition of trypsin, 10 μL of the mixture was taken to 10 μL of 2× SDS-PAGE sample buffer and boiled for 5 min. Aliquots of 10 μL were taken in the same way at 20, 40, and 60 min. After running a 4–12% Bis-Tris SDS NuPAGE (Novex), the gel was washed overnight with 10% acetic acid, 10% ethanol solution. The labeled fluorescent bands were visualized under UV light. The fluorescent pictures were taken in a dark room with a special UV box as light source.

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