Involvement of ST1B Subfamily of Cytosolic Sulfotransferase in Kynurenine Metabolism to Form Natriuretic Xanthurenic Acid Sulfate

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ABSTRACT

Natriuretic substances are a group of molecules affecting sodium homeostasis in the body. Recently, two new molecules having natriuretic effects, xanthurenic acid 8-O-β-d-glucoside and xanthurenic acid 8-O-sulfate (XA sulfate), have been isolated from human urine. In the present study, we have investigated the sulfation of xanthurenic acid (XA) in mouse tissues to assess the contribution of specific sulfotransferases (STs) to the reaction. Cytosols from tissues of both sexes of C57BL/6N mice (liver, stomach, jejunum, colon, and kidney) were capable of forming XA sulfate, with various $K_m$ values. Jejunum cytosol showed the lowest $K_m$ value, and its $V_{max}/K_m$ value was much greater than those of other tissues. The kinetic analyses with recombinant mouse (m) STs (Sult1a1, Sult1b1, Sult1c2, and Sult1d1) showed the lowest $K_m$ value for mSult1b1, and the value was comparable with that for jejunum cytosol. The highest expression of mSult1b1 in small intestine was confirmed at the mRNA and protein levels. mSult1b1 is thus suggested as a major enzyme responsible for XA sulfation in jejunum. Similar to mSult1b1, human SULT1B1 and rat Sult1b1 mediated XA sulfation efficiently. Thus, XA is likely to be an endogenous substrate for ST1B members. In contrast to XA, an XA-related compound, kynurenic acid strongly inhibited mSult1b1-mediated sulfations, with $IC_{50}$ values at a micromolar range. These results indicate the functional role of ST1B subfamily of ST in XA sulfate formation in the body.

Various types of chemicals such as natriuretic substances are associated with sodium excretion in the body. Natriuretic peptides are well known endogenous molecules exerting the activity. These are mainly produced from heart (i.e., atrial and B-type natriuretic peptide) and intestine (i.e., guanylin and uroguanylin). The hormones modulate renal function in response to sodium load, to mediate a cardiac- and enteric-renal communication axis for the maintenance of salt and water homeostasis (Kuhn, 2005; Daniels and Maisel, 2007). Besides these peptides, several reports suggest the presence of small molecules (<1000 Da) with natriuretic activity in urine and/or serum extracts of normal humans (Clarkson et al., 1979), salt-loaded humans (Clarkson et al., 1976), or patients with chronic renal disease (Bricker et al., 1993). Although the chemical nature of such substances has not been clearly identified, it has been thought to be a nonpeptide and nondigitalis-like molecule (Bricker et al., 1993). Recently, two small molecules with natriuretic activity were isolated from human urine, and they were identified as xanthurenic acid (XA; 4,8-di-hydroxyquinoline-2-carboxylic acid) derivatives XA 8-O-β-d-glucoside and XA 8-O-sulfate (Cain et al., 2007). Both compounds showed sustained natriuresis in rats without or with minimal concomitant potassium excretion. The infusion of their parent compound, XA, however, produced neither significant natriuresis nor kaliuresis (Cain et al., 2007). These compounds may thus constitute a different class of natriuretic hormones involving sodium homeostasis.

XA is a product upon tryptophan degradation via kynurenine pathway, which is the major route of tryptophan catabolism. Kynurenine aminotransferase is the enzyme responsible for XA formation from 3-hydroxykynurenine (Peters, 1991). Previous studies have revealed that XA is excreted into urine of animals as conjugates (Baglioni et al., 1960; Sato et al., 1961). Baglioni et al. (1960) detected two metabolites in rat urine, one metabolite of which was a conjugate of XA with sulfate and glycine, and another metabolite was a derivative of XA with sulfate and glycine. In the present study, we have investigated the sulfation of xanthurenic acid (XA) in mouse tissues to assess the contribution of specific sulfotransferases (STs) to the reaction. Cytosols from tissues of both sexes of C57BL/6N mice (liver, stomach, jejunum, colon, and kidney) were capable of forming XA sulfate, with various $K_m$ values. Jejunum cytosol showed the lowest $K_m$ value, and its $V_{max}/K_m$ value was much greater than those of other tissues. The kinetic analyses with recombinant mouse (m) STs (Sult1a1, Sult1b1, Sult1c2, and Sult1d1) showed the lowest $K_m$ value for mSult1b1, and the value was comparable with that for jejunum cytosol. The highest expression of mSult1b1 in small intestine was confirmed at the mRNA and protein levels. mSult1b1 is thus suggested as a major enzyme responsible for XA sulfation in jejunum. Similar to mSult1b1, human SULT1B1 and rat Sult1b1 mediated XA sulfation efficiently. Thus, XA is likely to be an endogenous substrate for ST1B members. In contrast to XA, an XA-related compound, kynurenic acid strongly inhibited mSult1b1-mediated sulfations, with $IC_{50}$ values at a micromolar range. These results indicate the functional role of ST1B subfamily of ST in XA sulfate formation in the body.

ABBREVIATIONS: XA, xanthurenic acid; ST, sulfotransferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; DTT, dithiothreitol; DOPAC, 3,4-dihydroxyphenylacetic acid; T₉₀, l-triiodothyronine; BSA, bovine serum albumin; Ni-NTA, nickel-nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; m, mouse; PCR, polymerase chain reaction; r, rat; h, human; RT, reverse transcription.
conjugate with glucuronic acid and glycine (Baglioni et al., 1960). Additionally, it was demonstrated in vitro that XA was sulfoconjugated with rat liver and kidney samples (Sato et al., 1961).

Cytosolic sulfotransferases (STs) are the enzymes responsible for the sulfoconjugation (i.e., sulfation or sulfonation) of low-molecular weight compounds. The enzymes catalyze the transfer of a sulfonyle group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to hydroxyl or amine groups of substrates. Sulfation has been recognized as a major phase II conjugation reaction in the biotransformation of many structurally diverse xenobiotics. Endogenous molecules including steroid and thyroid hormones and neurotransmitters are also sulfoconjugated by STs, and sulfation often alters their biological activities (Yamazoe and Kato, 1995; Nagata and Yamazoe, 2000; Nagata et al., 2005). STs constitute a gene superfamily that contains at least six different classes (i.e., ST1–6 families in mammals) based on their identities of deduced amino acid sequences (Nagata and Yamazoe, 2000; Nagata et al., 2005; Lindsay et al., 2008). ST1 family is further subdivided into five subfamilies: ST1A, ST1B, ST1C, ST1D, and ST1E. Members in ST1 family prefer phenolic compound as substrates (Yamazoe and Kato, 1995; Nagata and Yamazoe, 2000). In the present study, we found that mouse and human ST1B subfamily members have the highest affinity against XA. As a result, jejunal, which contains substantial amount of mouse Sult1b1 (mSult1b1; St1b3)1 protein, catalyzes the formation of XA sulfate with the highest efficiency among the mouse tissues tested.

Materials and Methods

Materials and Animals. Restriction endonucleases, TaKaRa Ex Taq DNA ligase kit, and RNAiso reagent were purchased from TaKaRa Bio Inc. (Ohtsu, Japan). Enterokinase was obtained from Biozyme Laboratories, Ltd. (Gwent, UK). Thermo Sequenase cycle sequencing kit was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Isopropyl-β-D-thiogalactopyranoside was purchased from Nacalai Tesque (Kyoto, Japan). XA, 3-hydroxykynurenine [2-phosphoadenosine 5'-phospho-(5')-O-(β-D-ribofuranosyl)-3'-phosphoadenosine 5'-phosphosulfate (PAPS)] was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). QiAexpress, nickel-nitrilotriacetic acid (Ni-NTA) agarose, and Enterokinase capture agarose was purchased from Sigma-Aldrich (St. Louis, MO). (5'-32P)PAPS (2000 mCi/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). QiAexpress, nickel-nitrilotriacetic acid (Ni-NTA) agarose, and Ni-NTA spin column were the products of QIAGEN (Valencia, CA). Enterokinase capture agarose was purchased from Novagen (Madison, WI). Bio-Rad protein assay kit and SDS-polyacrylamide gel electrophoresis (PAGE) molecular weight standards were purchased from Bio-Rad (Hercules, CA). 3,3'-Diaminobenzidine tetrahydrochloride was from Dojinpo (Kumamoto, Japan). All other chemicals used were of the highest grade available. Oligonucleotides

were synthesized at FASMAC Co., Ltd. (Atsugi, Japan). C57BL/6N mice (7 weeks old) were obtained from Charles River Japan (Yokohama, Japan). Structures of XA, XA-related compounds, and other phenolic compounds used for assay of ST activities are shown in Fig. 1.

Construction of Expression Plasmids for Recombinant Mouse Sulfotransferases. The coding regions of mSult1b1 and mSult1c2 (St1c5) were amplified by polymerase chain reaction (PCR) from mouse colon and kidney cDNA, respectively, with oligonucleotides containing BamHI/HindIII restriction sites for mSult1b1 (5'-AC-GATCCGGATCCGACATGAACAAATGATGCTCAGAAAGGT-TTGG-3' and 5'-TTAAGCTTGTAGATGCTAGCTCAGAATTCT- GTGCAG-3') or those containing HindIII restriction site for mSult1c2 (5'-GCGGATGGATGCAGATCAAAAATGCTTGGACAAACAAGGAA-GACTG-3' and 5'-GCAAGCTTCTTGATCAGATGCAATGGG-3'). The PCR reaction mixture (50 μl) contained 5 μl of the template cDNA; 10 pmol each of 5' and 3' primer; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 0.25 units of TaKaRa Ex Taq; and the Ex Taq buffer. After an initial denaturation at 94°C for 3 min, the amplification was performed for 25 cycles, with 1 min at 94°C for denaturation, 30 s at 55°C for annealing, 1 min at 72°C for extension, and a final extension period of 4 min at 72°C. The obtained cDNA fragments were ligated into a prokaryotic expression vector, pQE30 (QIAGEN). The subcloned cDNA sequences were confirmed by direct sequencing with DS2000-L DNA sequencer (Shimadzu, Kyoto, Japan). The constructed plasmids were introduced into Escherichia coli, M15[pREP4] strain (QIAGEN). The amplified cDNAs contained additional nucleotides in front of the initiation codon to include the amino acid sequence of enterokinase recognition site (DDDDK).

Bacterial Expression and Purification of Recombinant Sulfotransferases. Recombinant histidine-tagged ST, designated His6-ST, was expressed and purified from E. coli cytosols by Ni-NTA agarose. Briefly, M15[pREP4] cells were grown until optical density600 reached 0.5 to 0.7 in 1 liter of Luria Bertani medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin. After induction with 1 mM isopropyl-β-D-thiogalactopyranoside, the cultures were additionally grown at 37°C for 4 h. Cells were harvested by centrifugation and resuspended in lysis buffer (1 mM DTT, 10 mM imidazole, 300 mM NaCl, and 50 mM sodium phosphate, pH 8.0), and lysed by sonication. The soluble fraction was prepared by centrifugation at 10,000g for 30 min at 4°C and then applied to Ni-NTA agarose that had been equilibrated with lysis buffer. The agarose was washed with wash buffer (20 mM imidazole, 300 mM NaCl, and 50 mM sodium phosphate, pH 8.0). Bound protein was eluted using a stepwise gradient of 50 to 200 mM imidazole in wash buffer. The eluate was dialyzed against a buffer consisting of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250 mM sucrose, 1 mM DTT, and 50 mM NaCl. In addition to mSult1b1 and mSult1c2, mSult1a1 (St1a4; Honma et al., 2001), mSult1d1 (St1d1; Shimada et al., 2004), rat Sult1b1 (rSult1b1, ST1B1; Fujita et al., 1997), human SULT1A1 (hSULT1A1, ST1A3; Honma et al., 2001), hSULT1A3 (ST1A5; Honma et al., 2001), hSULT1B1 (ST1B2; Fujita et al., 1997), and hSULT1C2 (ST1C2; Yoshinari et al., 1998a) were also expressed as histidine-tagged proteins and purified. The histidine tag of purified His6-ST was removed by digestion with enterokinase, and the digestion reaction was stopped by enterokinase capture agarose according to the manufacturer’s instruction. The digestion reaction mixture was reapplied on Ni-NTA spin column to remove the tag and undigested proteins. The flow-through fraction was collected as digested recombinant enzyme (ΔHis6-SLT) and used as the standard of immunoblot analysis. Recombinant protein concentrations were determined by spectrophotometry and calculated based on their extinction coefficient (Gill and von Hippel, 1989). All of the purified proteins migrated as a single band corresponding to their predicted molecular weight and were at least 99% pure as evaluated by SDS-PAGE (data not shown).

1 The nomenclature of human ST forms is based on the Human Genome Nomenclature Committee. For the rodent STs, the nomenclature system used in the National Center for Biotechnology Information is applied in this article, accompanying the names in the nomenclature system proposed by us with “ST” as a prefix (Nagata and Yamazoe, 2000; Nagata et al., 2005) for the first appearance, because rodent STs do not always have human counterparts. The accession numbers of STs are as follows: St1a4/mSult1a1, NM_133670; St1b3/mSult1b1, NM_019878; St1c5/mSult1c2, NM_029835; St1d1/mSult1d1, NM_016771; ST1A3/hSULT1A1, NM_001055; ST1A5/hSULT1A3, NM_003166; ST1B2/hSULT1B1, NM_014465; ST1C2/hSULT1C2, NM_001056; and ST1B1/rSult1b1, NM_022513.
Mouse Tissue Cytosol Preparation. Tissue cytosols (liver, kidney, stomach, jejunum, and colon) were prepared from 7-week-old male and female C57BL/6N mice. Animals were sacrificed by cervical dislocation, and tissues were quickly removed and frozen in liquid nitrogen before storage at -80°C. All subsequent procedures were undertaken at 4°C. The jejunum mucosa was dissected off by scraping with a glass slide. Tissues were homogenized with an ice-cold buffer containing 75 mM potassium phosphate, pH 7.4, 75 mM KCl, and 1 mM DTT. The homogenate was centrifuged at 9000 g for 20 min, and the supernatant was further centrifuged at 105,000 g for 60 min. The obtained supernatant was used as cytosol. Cytosols were prepared from individual livers and kidneys, whereas those from stomach, jejunum, and colon were prepared from the pooled tissues because of the limited amounts of the tissues. The protein concentration was determined by the method of Bradford (Bradford, 1976) with BSA as the standard.

RNA Preparation and RT-PCR. Total RNA was extracted from various mouse tissues, including liver, stomach, jejunum, colon, and kidney, using TaKaRa RNAiso reagent according to the manufacturer's instructions. Single-strand cDNA was synthesized using an oligo(dT) primer with Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). PCR was carried out using specific primers for mSult1b1 (5′-TACTATGAAGAATTGAAACAGAAC-3′ and 5′-TCCCAAACATCCCTCTCATTG-3′) or those for β-actin (5′-GGGAAATCTGGCTGCTG-3′ and 5′-CTGGTTCCTGATCCATC-3′) as a housekeeping control at denaturation temperature of 94°C for 15 s, annealing temperature of 56°C for 30 s, and extension temperature of 72°C for 30 s in the presence of dNTPs and Taq polymerase (ABgene, Epsom, Surrey, UK). The PCR cycle numbers were titrated to assure amplification in linear range. The reaction was completed by 7-min incubation at 72°C, and PCR products were analyzed on 1% agarose gel containing ethidium bromide for visualization.

Electrophoresis and Immunoblot Analysis. Proteins were resolved by subjecting to SDS-PAGE using 12% polyacrylamide gel. The proteins were stained by Coomassie Brilliant Blue G250. For immunoblot analysis, tissue cytosolic proteins (5 μg) separated on the gel was electrophoretically transferred to a nitrocellulose sheet. The mSult1b1 on the membrane was immunostained with the rabbit polyclonal antibody raised against purified recombinant rSult1b1 (Fujita et al., 1999), which recognizes mSult1b1 (Shimada et al., 2004), at 1:2000 dilution and with the secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000 dilution). The immunoreactive protein was visualized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.1 M Tris-HCl, pH 7.2.

Sulfotransferase Activities Assay and Kinetic Analysis. Sulfating activities were determined from the radioactivities of the metabolites obtained with [35S]PAPS as a sulfate donor after thin layer chromatography as described previously (Yoshinari et al., 1998b). A typical incubation mixture consisted of 50 mM Tris-HCl buffer, pH 7.4, 1 mM DTT, 5 mM MgCl₂, 1 mg/ml BSA, 10 μM [35S]PAPS, and 2.5 μg of cytosolic protein or 5 to 50 ng of His₆-ST protein in the final volume of 10 μl. Kinetic analyses were performed with various substrate concentrations ranging from 0.05 to 600 μM for XA, 5 to 500 μM for 3-hydroxykynurenine, 2.5 to 500 μM for p-nitrophenol, 10 to 500 μM for T₃, and 0.5 to 50 mM for DOPAC. The reaction was initiated by addition of [35S]PAPS and terminated by addition of 5 μl of ice-cold acetonitrile after incubation at 37°C for 20 min. Portions (10 μl) of the reaction mixture were spotted on a thin layer plate (thin layer chromatography aluminum plate silica gel 60 F₂₅₄, 250 μM; Merck, Darmstadt, Germany). The metabolites were developed with a solvent system of n-butanol/acetic acid/water (4:1:2). The radioactive spots were analyzed with FLA-3000 image analyzer (FujiFilm, Tokyo, Japan). The generation of XA after acid hydrolysis from XA sulfate isolated from thin layer chromatography plates was confirmed using high-performance liquid chromatography (data not shown).

To determine the IC₅₀ value of kynurenic acid on sulfating activities of mSult1b1 against various substrates, the reactions were performed as described above in the absence or presence of kynurenic acid. The substrate concentrations used were approximately 5 times above the respective apparent Kₘ values, and kynurenic acid concentrations ranged from 0.25 to 100 μM. Apparent Kₘ, Vₘₐₓ, and IC₅₀ values were determined using the Prism 5 program (GraphPad Software Inc., San Diego, CA).

Statistical Analysis. All values are expressed as the mean ± S.D. Unpaired Student’s t test was used for statistical analysis.
Results

Sulfating Activities and Kinetic Characterization of Mouse Tissue Cytosols against XA. Sulfating activities of various tissue cytosols toward XA at concentration of 1 and 10 μM were compared (Fig. 2A). Cytosols prepared from individual livers and kidneys or those prepared from pooled stomach, jejunum, and colon of male and female C57BL/6N mice (n = 3 each) were used. All tissue cytosols studied were capable of forming XA sulfate conjugate. Jejunum cytosols mediated XA sulfation at 1 μM substrate with the highest rate in both male and female mice (21.4 ± 4.8 and 15.4 ± 1.1 pmol/min/mg protein for male and female mice, respectively). The highest sulfating activities at 10 μM XA were observed with liver cytosols in both sexes, with significantly higher activities in female mice (60.6 ± 2.9 and 88.1 ± 4.2 pmol/min/mg protein for male and female mice, respectively). In contrast to liver, jejunum cytosols of male mice showed significantly higher activities than those of female at 10 μM XA (50.8 ± 7.1 and 30.1 ± 5.8 pmol/min/mg protein for male and female mice, respectively).

To determine kinetic parameters for XA sulfation catalyzed by various mouse tissue cytosols, enzyme assays were conducted under conditions where the linearity with respect to protein concentration was obtained. Liver and kidney cytosols were pooled for kinetic analyses because there was no obvious individual difference in their XA-sulfating activities. The activities of pooled liver and kidney cytosols were comparable with those obtained in Fig. 2A at both XA concentrations (data not shown). Because of limitation in solubility, the XA concentration used in the reaction ranged from 0.05 up to 600 μM. Michaelis-Menten and Eadie-Hofstee plots are shown in Fig. 3, and apparent $K_m$, $V_{max}$, and $V_{max}/K_m$ values are summarized in Table 1. Plots of metabolite formation versus substrate concentration for XA sulfation by all tissue cytosols showed saturable hyperbolic curves. Eadie-Hofstee plots exhibited monophasic kinetics showing that the reactions were fit to a typical Michaelis-Menten equation. No significant differences were observed for $K_m$ values between both sexes, except that the values for male stomach and colon cytosols were significantly higher than those for female cytosols. Among the tissues examined, $K_m$ values were the lowest with jejunum cytosol (0.74 ± 0.08 and 0.63 ± 0.02 μM for male and female mice, respectively), followed by liver, colon, stomach, and kidney of both sexes. $V_{max}$ values varied among the tissues examined, ranging from 71 to 579 and 37 to 477 pmol/min/mg protein for male and female mice, respectively. Liver, stomach, and colon exhibited the high rate of XA sulfation, whereas the rates in jejunum and kidney were low. Comparison of the overall specificity of sulfating activities, which is given by the $V_{max}/K_m$ value, showed the highest activity for jejunum cytosols of both sexes of mice (male, 94.5 ± 4.8; female, 58.9 ± 1.5), and the values are 4 to 8-fold higher than those in the liver.

Sulfating Activities and Kinetic Characterization of Recombinant Mouse STs against XA. Based on the phenolic structure of XA, an array of recombinant mouse ST1 family enzymes (mSult1a1, mSult1b1, mSult1c2, and mSult1d1) were examined to address the role of individual form in sulfonation of XA. The enzyme activities toward 1 and 10 μM XA are shown in Fig. 2B. mSult1b1 mediated the sulfating reaction.
with the highest rate at both substrate concentrations (10.9 ± 0.5 and 17.9 ± 2.2 nmol/min/mg protein for 1 and 10 μM XA, respectively).

A range of substrate concentrations from 0.05 to 600 μM was used to determine kinetic parameters of mouse STs. The kinetics for all enzymes that exhibited the sulfating activity fell into a typical Michaelis-Menten kinetics and Eadie-Hofstee plots displayed monophasic patterns (Fig. 4). The kinetic parameters are listed in Table 2. Of the enzymes examined, mSult1b1 showed the lowest apparent $K_m$ value (0.53 ± 0.05

Fig. 3. Kinetic analysis of XA sulfation by mouse tissue cytosols. Sulfating activities of pooled mouse cytosols were determined as described in the legend to Table 1. Representative Michaelis-Menten and Eadie-Hofstee (inset) plots are shown. Each plot depicts the mean of duplicate or triplicate determinations in one typical experiment of three experiments.
muM, followed by mSult1d1, mSult1a1, and mSult1c2. These results suggested the high affinity of mSult1b1 toward XA. The results on V_max values showed that the highest activity was found for mSult1c2 (81.7 ± 5.8 nmol/min/mg protein), followed by mSult1a1, mSult1b1, and mSult1d1. Among these four STs, mSult1b1 yielded the highest value for V_max/K_m.
in both sexes of mice. The colon and stomach had also detectable levels of immunoreactive mSult1b1 protein but with lower intensities than those of jejunum. In contrast, the protein was detected in neither liver nor kidney under the condition used.

**Kinetic Characterization of Recombinant Rat and Human STs for XA Sulfation.** To assess the selectivity of XA as a probe for ST1B members, kinetic analyses were also performed with recombinant rSult1b1 and human STs (hSULT1A1, hSULT1A3, hSULT1B1, and hSULT1C2). Similar to mouse STs, the kinetics for all enzymes that exhibited the sulfating activity fell into a typical Michaelis-Menten kinetics, and Eadie-Hofstee plots displayed monophasic patterns (Fig. 4). The kinetic parameters are shown in Table 4. The $K_m$ values of rSult1b1 and mSult1b1 were comparable with each other, although the $V_{\text{max}}/K_m$ value of rSult1b1 was lower than that of mSult1b1. hSULT1B1 exhibited the lowest $K_m$ value among the human STs tested, followed by hSULT1A1 and hSULT1C2. The $V_{\text{max}}$ values of hSULT1C2 and hSULT1A1 were comparable with each other, and they were 3 to 4 times higher than that of hSULT1B1. hSULT1A3 exhibited a negligible activity for sulfocation of XA (data not shown).

**Influence of Kynurenic Acid on mSult1b1-Mediated Sulfation.** As described above, mSult1b1 had no activity toward kynurenic acid (Table 3). Because of its structural similarity to XA but lacking 8-hydroxyl group, the influence of this compound on XA sulfation catalyzed by mSult1b1 was assessed. As shown in Fig. 6, kynurenic acid exhibited strong inhibitory effects on the reaction, with an IC$_{50}$ value of 5.4 ± 0.5 μM. Consistent results were observed throughout mSult1b1-mediated sulfations of p-nitrophenol and T$_3$, showing IC$_{50}$ values of 2.7 ± 0.3 and 3.1 ± 0.6 μM, respectively (Fig. 6).

**Discussion**

In the present study, we have examined the sulfation reaction of XA occurring in mouse tissues and the contribution of STs to the reaction. All mouse tissues studied (liver, stomach, jejunum, colon, and kidney) were capable of forming XA sulfate. These data are in accordance with the previous works reporting the sulfate conjugation of XA in liver and kidney of rat in vitro (Sato et al., 1961). From kinetic analyses, jejunum cytosol shows the lowest $K_m$ value without gender preference and the value of $V_{\text{max}}/K_m$ for the reaction is much greater than those of other tissues. These indicate the most efficient catalysis for XA sulfation in jejunum.

To clarify the contribution of ST forms to XA sulfation, the reactions were analyzed with mouse recombinant enzymes. Because of the phenolic structure of XA, only the enzymes in ST1 family (phenol sulfotransferase), namely, mSult1a1, mSult1b1, mSult1c2, and mSult1d1, were used. The kinetic analysis showed that mSult1b1 catalyzes the conjugation with the lowest $K_m$ value, and this value is comparable with that for jejunum cytosol. Not surprisingly, in agreement with our present kinetic data and the previous report (Alnouti and Klaassen, 2006), the high expression of mSult1b1 in small intestine was confirmed by RT-PCR and immunoblot analyses. Taken together, mSult1b1 is supposed to be the enzyme responsible for XA sulfate formation in jejunum. It is also noteworthy that mSult1b1 catalyzes the reaction with
Materials and Methods

and recombinant proteins were subjected to SDS-PAGE with 12% gel and electrically transferred to a nitrocellulose membrane. Lane 1, His6-immunoblot analysis using rabbit anti-rSult1b1 antibody (1:2000 dilution) as described under Materials and Methods. Cytosolic proteins (5 μg/lane) and recombinant proteins were subjected to SDS-PAGE with 12% gel and electrically transferred to a nitrocellulose membrane. Lane 1, His6-mSult1b1; lane 2, male liver; lane 3, female liver; lane 4, male stomach; lane 5, female stomach; lane 6, male jejunum; lane 7, female jejunum; lane 8, male colon; lane 9, female colon; lane 10, male kidney; lane 11, female kidney; and lane 12, His6-mSult1d1/

Fig. 5. Tissue distribution of mSult1b1. A, mSult1b1 mRNA levels were measured by RT-PCR as described under Materials and Methods. β-Actin was used as an internal control. Lane 1, male liver; lane 2, female liver; lane 3, male stomach; lane 4, female stomach; lane 5, male jejunum; lane 6, female jejunum; lane 7, male colon; lane 8, female colon; lane 9, male kidney; and lane 10, female kidney. B, mSult1b1 protein levels were determined by immunoblot analysis using rabbit anti-rSult1b1 antibody (1:2000 dilution) as described under Materials and Methods. Cytosolic proteins (5 μg/lane) and recombinant proteins were subjected to SDS-PAGE with 12% gel and electrically transferred to a nitrocellulose membrane. Lane 1, His6-mSult1b1; lane 2, male liver; lane 3, female liver; lane 4, male stomach; lane 5, female stomach; lane 6, male jejunum; lane 7, female jejunum; lane 8, male colon; lane 9, female colon; lane 10, male kidney; lane 11, female kidney; and lane 12, His6-mSult1d1/

TABLE 4
Apparent kinetic parameters for sulfation of XA by recombinant rat and human STs

<table>
<thead>
<tr>
<th>ST</th>
<th>K_m (μM)</th>
<th>V_max (nmol/min/mg protein)</th>
<th>V_max/K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>rSult1b1</td>
<td>0.52 ± 0.07</td>
<td>1.05 ± 0.04</td>
<td>2.0 ± 0.02</td>
</tr>
<tr>
<td>hSULT1B1</td>
<td>6.3 ± 0.3</td>
<td>14.5 ± 1.1</td>
<td>2.3 ± 0.03</td>
</tr>
<tr>
<td>hSULT1A1</td>
<td>43.3 ± 2.7</td>
<td>51.1 ± 1.5</td>
<td>1.2 ± 0.02</td>
</tr>
<tr>
<td>hSULT1C2</td>
<td>205.7 ± 16.6</td>
<td>57.2 ± 3.5</td>
<td>0.3 ± 0.00</td>
</tr>
</tbody>
</table>

The highest efficiency among STs examined as measured by V_max/K_m value, suggesting that XA is a selective endogenous substrate for mSult1b1. Apparent kinetic parameters for sulfation of 3-hydroxykynurenine, kynurenic acid, p-nitrophenol, T3, and DOPAC support the substrate preference of mSult1b1 for XA. Consistent data were observed for the reaction catalyzed by rSult1b1 and hSULT1B1. We have reported that the K_m values for T3 and p-nitrophenol sulfations by rSult1b1 are 40.2 and 115.9 μM, respectively, and those by hSULT1B1 are 63.5 and 24.1 μM, respectively (Fujita et al., 1997). These values are much higher than the K_m values for XA sulfation by these two enzymes, indicating that XA is an excellent substrate for rSult1b1 and hSULT1B1 as well as mSult1b1.

Apart from jejunum, we have investigated the formation of XA sulfate in other tissues. The tissue cytosols examined formed XA sulfate with various K_m values. The sulfocation of XA in liver and colon yielded K_m values similar to that obtained with mSult1a1. In accordance with the K_m value, the abundant expression of mSult1a1 is observed in these two tissues (Shimada et al., 2004; Alnouti and Klausen, 2006), suggesting that mSult1a1 is predominantly involved in XA sulfate formation in liver and large intestine. A different ST enzyme may mediate the sulfation of XA in stomach and kidney. The K_m values of XA sulfation obtained for these tissues are similar to that obtained with mSult1c2. The substantial amount of mSult1c2 found in mouse stomach and kidney (Alnouti and Klausen, 2006) is in agreement with our kinetic data. These data indicate that mSult1c2 is possible to be the main enzyme for XA sulfation in stomach and kidney. Thus, distinct ST1 family enzymes may have a role in XA sulfation in tissues other than jejunum.

ST1C subfamily members have been isolated from a variety of species such as rSult1c1 from rat (Nagata et al., 1993) and hSULT1C2 from human (Her et al., 1997; Yoshinari et al., 1998a). hSULT1C2 and their orthologs are abundant in stomach and kidney in rat (Sugimura et al., 2002), rabbit (Hehonah et al., 1999) as well as human (Her et al., 1997). Their capability for sulfation of p-nitrophenol, a typical substrate for phenol STs, is very poor, with the K_m value in a millimolar range (Hehonah et al., 1999; Sugimura et al., 2002). To date, no endogenous substrates have been identified for members of ST1C subfamily. In this study, we have for the first time demonstrated the endogenous role of mSult1c2 catalyzing sulfation reaction of endogenously produced XA.

In general, the biotransformation of endogenous substrates by sulfocation has two possible consequences; inactivation or activation of their biological activities. The examples of the former include the sulfation of thyroid hormone (Spaulding et al., 1992) and catecholamines (Onasch et al., 2000). In the latter cases, sulfoconjugation confers biological activities to parent compounds: pregnenolone sulfate but not pregnenolone shows potent neuroexcitatory properties (Paul and Purdy, 1992), and cholesterol sulfate plays a crucial role in keratinocyte differentiation and development of the skin barrier (Shimada et al., 2002; Strott and Higashi, 2003). The sulfation of XA is thus another example for sulfoconjugation as metabolic activation to physiologically active endogenous compounds.

Kynurenic acid has similar structure to XA except that it lacks 8-hydroxyl group. Both substances are endogenous metabolites of tryptophan via kynurenine pathway (Peters, 1991). In the present study, we have also examined the sulfoconjugation of kynurenic acid. As expected from the chemical structure of kynurenic acid, mSult1b1 has no activity toward this compound. Similar to mSult1b1, the other mouse and human STs examined show no sulfating activity to kynurenic acid (data not shown). These data support the idea that XA is conjugated with a sulfate moiety at 8-hydroxyl position, which is in agreement with previous reports indicating XA is sulfated at the position (Baglioni et al., 1960; Sato et al., 1961). In fact, 4-hydroxyxoyli moiety of XA molecule is in a para-position to the nitrogen atom and has weak phenolic properties, suggesting that it seems unlikely to be sulfated. The nuclear magnetic resonance spectroscopic analyses of XA sulfate that was recently purified from urine also clearly demonstrated the sulfoconjugation occurred at 8-hydroxyl position (Cain et al., 2007).
kynurenic acid and XA are present in intestine in a similar level (89.7 and 42.9 pmol/g tissue, respectively) (Pawlak et al., 2003). Although kynurenic acid inhibits mSult1b1-mediated XA sulfation, its amount in jejunum does not seem to be high enough to exert its inhibitory effect in vivo. Therefore, it is reasonable to assume that the sulfoconjugation of XA in jejunum is mediated by mSult1b1 in vivo despite of the copresence of XA and kynurenic acid.

Besides sulfoconjugation, XA is also conjugated with glucuronate and secreted into bile (Sato et al., 1961). Because it is known that glucuronide conjugates can be subsequently hydrolyzed by β-glucuronidases located in the lumen of gastrointestinal tract, the interplay between deconjugating enzymes of bacteria and STs responsible for XA sulfation may happen in the gut lumen. Several reports have demonstrated that XA itself is a toxic metabolite. For example, it binds covalently to proteins, leading to their unfolding and cell death (Malina, 1999). XA also causes nuclear DNA damages and induces apoptosis of vascular smooth muscle and retinal pigment epithelium cells (Malina et al., 2001). Moreover, XA is suggested as an endogenous molecule exerting diabetogenic properties (Kotake and Murakami, 1971). Therefore, the process of sulfation occurring in the intestine may be essential for the excretion of an excessive amount of toxic XA from the body.

In conclusion, we have demonstrated that XA sulfate formation occurs in various mouse tissues, with jejunal enzymes showing the highest affinity. With recombinant STs, XA is found to be an excellent endogenous substrate for ST1B subfamily enzymes. Because XA sulfate has natriuretic activity in vivo, these results suggest a possible role of these enzymes in sodium homeostasis in the body.

References


Fig. 6. Inhibitory effects of kynurenic acid on mSult1b1-mediated sulfation. Assays were performed at pH 7.4 in the absence or presence of 0.25 to 100 μM kynurenic acid and 5 ng of mSult1b1, with various concentrations of substrates (3 μM XA, 250 μM p-nitrophenol, and 400 μM T3). The sulfating activities were given as percentage of control (the activity without kynurenic acid). Each plot depicts the mean of duplicate determinations in one typical experiment of three experiments.

As mentioned above, kynurenic acid is not a substrate of mSult1b1 enzyme. In contrast, it exhibits inhibitory effects on the mSult1b1-catalyzed sulfation of XA as well as p-nitrophenol and T3. The IC₅₀ values obtained for the inhibition of mSult1b1-mediated sulfation of these compounds are within a low micromolar range. The value for XA sulfation inhibition is 5.4 μM. Previous studies have shown the presence of enzymes involved in kynurenine pathway in various tissues such as liver, kidney, lung, intestine, spleen, and muscle (Pawlak et al., 2003). Consequently, the accumulation of tryptophan metabolites under kynurenine pathway is observed in these tissues.


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798 Senggunprai et al.