Structure−Function Modeling of the Interactions of *N*-Alkyl-*N*-hydroxyanilines with Rat Hepatic Aryl Sulfotransferase IV

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Although previous investigations have clearly shown that *N*-hydroxy arylamines and *N*-hydroxy heterocyclic amines are substrates for sulfotransferases, relatively little is known about which structural features of the *N*-hydroxy arylamines are important for sulfation to occur. The purpose of this investigation was to determine the extent to which secondary *N*-alkyl-*N*-hydroxy arylamines interact with aryl sulfotransferase (AST) IV (also known as tyrosine-ester sulfotransferase or ST1A1) and to evaluate these interactions using molecular modeling techniques. AST IV is a major cytosolic sulfotransferase in the rat, and it catalyzes the sulfation of various phenols, benzylic alcohols, arylhydroxamic acids, oximes, and primary *N*-hydroxy arylamines. In this study, three secondary *N*-hydroxy arylamines, *N*-hydroxy-*N*-methylaniline, *N*-ethyl-*N*-hydroxyaniline, and *N*-hydroxy-*N*-*n*-propylaniline, were found to be substrates for the purified rat hepatic AST IV. However, when the *N*-alkyl substituent was an *n*-butyl group (i.e., *N*-*n*-butyl-*N*-hydroxyaniline), the interaction with the enzyme changed from that of a substrate to competitive inhibition. This change in specificity was further explored through the construction and use of a model for AST IV based on mouse estrogen sulfotransferase, an enzyme whose crystal structure has been previously determined to high resolution. Molecular modeling techniques were used to dock each of the above *N*-hydroxy arylamines into the active site of the homology model of AST IV and determine optimum ligand geometries. The results of these experiments indicated that steric constraints on the orientation of binding of secondary *N*-alkyl-*N*-hydroxy arylamines at the active site of AST IV play a significant role in determining the nature of the interaction of the enzyme with these compounds.

Introduction

N-Alkyl-*N*-hydroxy arylamines are most often encountered as intermediary metabolites formed through *N*-oxidation of *N*-alkyl arylamines catalyzed by the flavin-containing monooxygenases (*1*). Although relatively few studies have been published on the sulfation of *N*-alkyl-*N*-hydroxy arylamines, it has been reported that both *N*-hydroxy-*N*-methyl-4-aminoazobenzene (*N*-OH-MAB)[1] and *N*-ethyl-*N*-hydroxy-4-aminoazobenzene (*N*-OH-EAB) were converted to reactive species by a sulfotransferase enzyme in rat liver cytosol (*2*, *3*). The limited number of investigations on the sulfation of secondary *N*-hydroxy arylamines may be due in part to the difficulty of synthesis of these compounds and in part to the instability of the sulfated metabolites. As in the sulfation of primary *N*-hydroxy arylamines and *N*-hydroxy heterocyclic amines, sulfation of *N*-alkyl-*N*-hydroxy arylamines could constitute either detoxification or metabolic activation depending upon the properties of the product of the reaction. In the case of *N*-OH-MAB, the sulfated metabolite was more reactive than *N*-OH-MAB itself (*2*, *3*).

Exposure to *N*-alkyl arylamines can occur either directly from the environment or indirectly due to intermediary metabolism of arylamines and nitroaromatic compounds. Primary, secondary, and tertiary arylamines can be interconverted by enzymatic *N*-methylation and *N*-dealkylation reactions (*4*). Such *N*-methylation reactions are catalyzed by an *S*-adenosylmethionine-dependent *N*-methyltransferase (*4*), while the *N*-dealkylation of secondary and tertiary arylamines may involve either cytochrome P450 or flavin-containing monooxygenases (*5*). The presence of these interconversion reactions increases the possibility that an *N*-hydroxy arylamine can be formed through metabolism of an arylamine.

While previously published investigations clearly show that *N*-hydroxy arylamines and *N*-hydroxy heterocyclic amines are substrates for sulfotransferases (*2*, *6*, *7*), as reviewed in ref *8*, none of these studies have attempted

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[1] Abbreviations: AST, aryl sulfotransferase; *N*-OH-EAB, *N*-hydroxy-*N*-ethyl-4-aminoazobenzene; EST, estrogen sulfotransferase; *N*-OH-MAB, *N*-hydroxy-*N*-methyl-4-aminoazobenzene; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PAP, adenosine 3′,5′-diphosphate.

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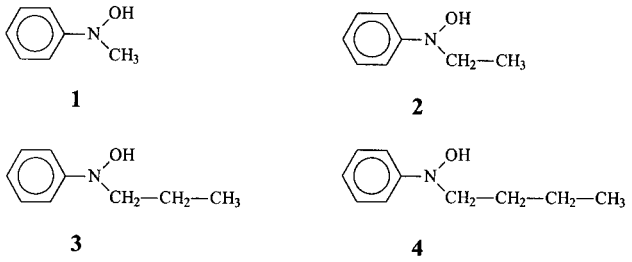

Figure 1. Summary of structures and nomenclature of the *N*-alkyl-*N*-hydroxy arylamines that have been investigated. *N*-Hydroxy-*N*-methylaniline (**1**), *N*-ethyl-*N*-hydroxyaniline (**2**), *N*-hydroxy-*N*-*n*-propylaniline (**3**), and *N*-*n*-butyl-*N*-hydroxyaniline (**4**) are shown.

to determine which structural features of the *N*-hydroxy arylamines may be important for sulfation. Furthermore, only one report has addressed the sulfation of secondary *N*-alkyl-*N*-hydroxy arylamines (*2*).

The purpose of this investigation was to explore the potential for interactions of highly purified secondary *N*-hydroxy arylamines with aryl sulfotransferase (AST) IV[2] through combination of kinetic studies on catalysis and inhibition with homology modeling and ligand docking studies. AST IV is a major cytosolic sulfotransferase in the livers of male rats and has been shown to be a critical enzyme involved in 2-acetylaminofluorene-mediated hepatocarcinogenesis (*9*). Recent studies also indicate that the enzyme isolated from rat liver also catalyzes sulfation of primary *N*-hydroxy arylamines (*7*). The studies described in this paper begin to address the hypothesis that structural features of secondary *N*-alkyl-*N*-hydroxy arylamines can influence the type of interaction that is observed. The *N*-alkyl-*N*-hydroxyaniline derivatives that were chosen for this study are seen in Figure 1. These compounds provide a systematic extension of the alkyl carbon chain on the *N*-hydroxy arylamine for investigating the possibility for steric and/or hydrophobic interactions at the active site of AST IV. The docking of the secondary *N*-alkyl-*N*-hydroxy arylamines into the active site of an AST IV homology model was used to evaluate the role of the optimum ligand geometry in the sulfation of these compounds catalyzed by AST IV.

Such studies with model *N*-hydroxy arylamines begin to provide some of the fundamental structure−activity relationships necessary for improving prediction of the relative rates of formation of sulfuric acid esters from this class of xenobiotics. These investigations on the specificity of a major rat hepatic sulfotransferase are particularly relevant in light of the extensive use of the rat in studies on hepatocarcinogenesis of arylamines, *N*-hydroxy arylamines, and related compounds.

Experimental Procedures

General. NMR spectra were obtained on a Bruker WM-360 spectrometer with tetramethylsilane as the internal standard. HPLC analyses were carried out with an Econosphere C18 column (5 μm, 4.6 mm × 250 mm) obtained from Alltech Associates (Deerfield, IL). Analyses by thin-layer chromatography (TLC) were conducted on silica gel (Chromagram sheets, 2 mm, with a fluorescence indicator, Eastman Kodak Co., Rochester, NY). Preparative chromatography was conducted either with a *Chromatotron* apparatus (Harrison Research) utilizing Silica gel 60 PF-254 (EM Science, Gibbstown, NJ) or with flash column chromatography using Davisil silica gel (grade 633, 200−425 mesh, Aldrich Chemical Co., Milwaukee, WI).

Reagents and Chemicals. 2-Naphthol, 1-naphthalenemethanol, *N*-ethyl-*N*-methylaniline, *N*,*N*-diethylaniline, *N*,*N*-di-*n*-butylaniline, ethyl iodide, 1-iodopropane, *m*-chloroperoxybenzoic acid, sodium cyanoborohydride, iron(III)nitrate nonahydrate, methanol-d_4 (99.8 at. % D), and 4,7-diphenyl-1,10-phenanthroline were purchased from Aldrich Chemical Co. Aniline was obtained from Mallinckrodt Inc. (Paris, KY). L-Ascorbic acid, zinc metal dust, 37% aqueous formaldehyde, and sodium borohydride were from Fisher Scientific (Pittsburgh, PA). 2-Mercaptoethanol was obtained from Sigma Chemical Co. (St. Louis, MO). Amber Reacti-Vials (total volume of 0.1 or 0.3 mL, fitted with Teflon/silicone disks) were obtained from Pierce Chemical Co. (Rockford, IL). *N*,*N*-Di-*n*-propylaniline was prepared by alkylation of aniline with 1-iodopropane (*10*). All other assay reagents and buffer components were from commercial sources. **Caution:** *N-Alkyl-N-hydroxy arylamines should be handled with appropriate safety precautions to avoid exposure.*

Synthesis of *N*-Alkyl-*N*-hydroxyanilines. All of the *N*-alkyl-*N*-hydroxyanilines shown in Figure 1 were synthesized by Cope elimination (*11*) of the corresponding tertiary arylamine *N*-oxides under argon at 12−15 mmHg. *N*-Methyl-*N*-hydroxyaniline (**1**) and *N*-ethyl-*N*-hydroxyaniline (**2**) were isolated according to an extraction procedure described previously (*12*). *N*-Alkyl-*N*-hydroxy arylamines **3** and **4** were obtained directly from the condensate collected from the pyrolysis reaction. All four *N*-alkyl-*N*-hydroxyanilines were isolated as their oxalic acid salts (*13*) and were stable to storage under argon at −20 °C for up to 1 month. Compounds **1**−**4** were characterized by ^{1}H NMR spectroscopy as well as by their ability to reduce iron(III) to iron(II). The high concentration of oxalate in the oxalate salts made ^{1}H NMR the most desirable analytical method for characterization. In addition, it was clear from UV and NMR spectra that compounds **1**−**4** contained none of the potential oxidation products or other reaction byproducts that can be derived from *N*-hydroxy arylamines. The characterization of compounds **1**−**4** by ^{1}H NMR is summarized as follows. *N*-Hydroxy-*N*-methylaniline (**1**) oxalate (80 MHz, CD_3OD): δ 3.05 (3H, d, J = 3.75 Hz), 7.28 [5H, m, (d,d,s)]. *N*-Ethyl-*N*-hydroxyaniline (**2**) oxalate (360 MHz, CD_3OD): δ 1.23 (3H, t, J = 7.08 Hz), 3.45 (2H, q, J = 7.02 Hz), 7.10 (1H, t, J = 7.2 Hz), 7.28 (2H, d, J = 7.6 Hz), 7.34 (2H, d, J = 7.1 Hz). *N*-Hydroxy-*N*-*n*-propylaniline (**3**) oxalate (360 MHz, CD_3OD): δ 1.27 (3H, t, J = 7.45 Hz), 1.99 (2H, sextet, J = 7.46 Hz), 3.64 (2H, t, J = 7.46 Hz), 7.40 (1H, t, J = 7.13 Hz), 7.58 (2H, d, J = 7.48 Hz), 7.63 (2H, d, J = 7.14 Hz). *N*-*n*-Butyl-*N*-hydroxyaniline (**4**) oxalate (360 MHz, CD_3OD): δ 1.17 (3H, t, J = 7.04 Hz), 1.29 (2H, m), 3.60 (4H, m), 7.14 (1H, t, J = 7.4 Hz), 7.12 (2H, d, J = 7.2 Hz), 7.15 (2H, d, J = 7.5 Hz).

Synthesis of Tertiary Amine *N*-Oxides. The tertiary arylamine *N*-oxides utilized for the above preparations of secondary *N*-hydroxy arylamines were synthesized from the corresponding tertiary arylamines by oxidation with *m*-chloroperoxybenzoic acid in chloroform (*14*, *15*). These reactions were complete in 1−3 h at 4 °C, and the isolated yields were 60−88%. *N*-Ethyl-*N*-methylaniline *N*-oxide and *N*,*N*-diethylaniline *N*-oxide were extracted from the reaction mixture according to the procedure described in ref *15*, thus forming the hydrochloride salts. Since *N*,*N*-di-*n*-propylaniline *N*-oxide and *N*,*N*-di-*n*-butylaniline *N*-oxide did not partition into aqueous hydrochloric acid, the *N*-oxide products were isolated after repetitive washing with aqueous 1.0 M sodium bicarbonate to remove the benzoic acid coproduct. The chloroform layer was dried with anhydrous magnesium sulfate and filtered and the solvent removed by rotary evaporation. In the case of *N*,*N*-di-*n*-propylaniline *N*-oxide, the product was then isolated as the hydrochloride by adding ethereal HCl to an ether solution of the *N*-oxide. *N*,*N*-Di-*n*-butylaniline *N*-oxide was used as the

[2] Although the Human Genome Organization has adopted nomenclature for the human sulfotransferases, a systematic extension of that nomenclature to sulfotransferases in the rat as well as other species has not yet been accomplished.

crystalline solid isolated directly after removal of the chloroform by rotary evaporation. The characterization of the *N*-oxide precursors of compounds **1**−**4** by ^{1}H NMR is summarized as follows. *N*-Ethyl-*N*-methylaniline *N*-oxide HCl (80 MHz, CD_3OD): δ 1.25 (3H, t, J = 7.1 Hz), 3.07 (3H, s), 4.21 (2H, q, J = 7.1 Hz), 4.95 (1H, broad s), 7.69 (3H, m), 7.95 (2H, d, J = 7.41 Hz). *N*,*N*-Diethylaniline *N*-oxide HCl (360 MHz, CD_3OD): δ 1.20 (6H, t, J = 7.08 Hz), 4.15 (2H, sextet, J = 6.93 Hz), 4.27 (2H, sextet, J = 6.90 Hz), 4.84 (1H, s), 7.68 (3H, m), 7.83 (2H, d, J = 7.53). *N*,*N*-Di-*n*-propylaniline *N*-oxide HCl (360 MHz, CD_3OD): δ 0.94 (6H, t, J = 4.46 Hz), 1.35 (2H, m), 1.82 (2H, m), 4.05 (2H, m), 4.19 (2H, m), 7.65 (3H, m), 7.86 (2H, d, J = 7.47 Hz). *N*,*N*-Di-*n*-butylaniline *N*-oxide (360 MHz, CD_3OD): δ 0.89 (6H, t, J = 7.23 Hz), 1.31 (6H, m), 1.80 (2H, m), 4.09 (2H, m), 4.24 (2H, m), 7.65 (3H, m), 7.88 (2H, d, J = 7.51 Hz).

Quantitation of *N*-Alkyl-*N*-hydroxyanilines by Iron(III)-Reducing Equivalents. Before use in the kinetic assays described below, the concentrations of *N*-alkyl-*N*-hydroxyaniline derivatives in solution were determined by a colorimetric assay that was previously developed for quantitation of *p*-chloro-*N*-methyl-*N*-hydroxyaniline (*5*). The following reagents were mixed in a glass test tube: 600 μL of 4,7-diphenyl-1,10-phenanthroline (0.5 mg/mL in ethanol), 100 μL of 1.0 M sodium acetate (pH 5.6), 20 μL of 0.1 M iron(III) nitrate, a *N*-hydroxyarylamine solution, and water to make a final volume of 800 μL. The reaction was started by addition of either *N*-hydroxy arylamine or iron reagent, and after 1 min, the reaction was stopped by adding 25 μL of 0.02 M phosphoric acid. The phosphate ions stopped the reaction by complexing with the unreacted ferric ions (*16*), thereby preventing further color formation. After the reaction was terminated, the concentration of the *N*-hydroxy arylamine in the sample was determined from the absorbance at 535 nm after subtraction of the absorbance for a blank sample containing all reagents except the *N*-hydroxy arylamine. An extinction coefficient of 19 250 M^{-1} cm^{-1} per reducing equivalent (*5*) was used, and calculations of concentrations of *N*-hydroxy arylamines were based on two reducing equivalents per molecule of *N*-hydroxy arylamine.

Purification of Hepatic Aryl Sulfotransferase IV. AST IV was purified to apparent homogeneity from the livers of male Sprague-Dawley rats (300−350 g) by a published procedure (*17*). The activity of the enzyme at pH 5.5 with 2-naphthol as a substrate (*18*) was used to follow the purification, and the specific activity of the homogeneous enzyme was 800−1000 nmol of 2-naphthyl sulfate formed min^{-1} (mg of protein)$^{-1}$. The homogeneity of the enzyme was initially assessed with SDS−PAGE (*19*), whereby the preparation exhibited one band of protein with an M_r of 33 500 (Coomassie blue staining). However, SDS−PAGE by itself will not clearly distinguish AST IV from two other sulfotransferases that have been previously characterized with the arylhydroxamic acid *N*-hydroxy-2-acetylaminofluorene and named *N*-hydroxy arylamine sulfotransferases (*20*−*23*). Despite significant differences in amino acid sequences and the masses of subunits, these enzymes are often not well separated on SDS−PAGE (*20*, *21*, *23*). Therefore, we confirmed the identity of the enzyme in these studies by using a previously described procedure (*7*) to compare HPLC tryptic peptide maps of the highly purified native AST IV with a recombinant form of the enzyme (*24*). The peptide maps (data not shown) conclusively indicated that the enzyme isolated from liver was AST IV. Protein concentrations were determined (*25*) with bovine serum albumin as the standard, and typical purifications from the liver tissue of four rats yielded 1−1.5 mg of homogeneous AST IV.

Kinetic Assay Procedure. Enzyme assays were conducted essentially as described previously for primary *N*-hydroxy arylamines (*7*). This included bubbling each component of the reaction mixture with argon and conducting the enzymatic reactions in sealed, argon-filled, amber-colored reaction vials. Reaction mixtures with a total volume of 0.03 mL contained 0.2 mM PAPS, 8.3 mM 2-mercaptoethanol, 1−2 μg of AST IV, varying concentrations of the *N*-hydroxy arylamine, and 0.25 M potassium phosphate at pH 7.0. Following incubation for 2 min at 37 °C for temperature equilibration, the reaction was initiated by addition of enzyme and conducted for 10 min at 37 °C. Because the sulfuric acid esters of the *N*-hydroxy arylamines were expected to be unstable (*2*, *3*), an assay that measures the level of formation of the coproduct, PAP, was utilized for determination of the progress of the AST IV-catalyzed reaction (*26*). Control assays were carried out with all assay components except the *N*-hydroxy arylamine to ensure that only *N*-hydroxy arylamine-dependent formation of PAP was assessed. PAPS was synthesized and purified as described previously (*7*).

Calculation of Kinetic Constants. Apparent K_m and V_{max} values were calculated from a nonlinear least-squares fit of the kinetic data to the Michaelis−Menten equation (*27*). At least four substrate concentrations with two or three repetitions at each concentration were utilized over a range of substrate concentrations 2−3-fold above and below the K_m. The K_i value for *N*-*n*-butyl-*N*-hydroxyaniline was calculated by a nonlinear least-squares fit of the data to equations modeling reversible inhibition (*27*); the best fit of the data was to the equation for competitive inhibition.

Construction of the AST IV Homology Model. The structure of AST IV was modeled using the structure of mouse estrogen sulfotransferase (EST) (*28*) as a template by fitting the AST IV sequence into the electron density map of EST. This AST IV model was refined using XPLOR 3.85 (*29*) by an initial positional refinement followed by simulated annealing using the structure factors originally obtained from the X-ray diffraction data collected on EST. The homology model also contained the coordinates of PAP and estradiol. Subsequent structural manipulations on the homology model were performed using molecular modeling package SYBYL 6.4 (Tripos Associates, St. Louis, MO). The structure of a proposed mimic of the transition state intermediate of the sulfotransferase reaction, PAP−vanadate (*30*), was extracted from PDB file 1BO6 containing coordinates of the EST−PAP−vanadate complex, and the PAP−vanadate structure was modified by replacing the vanadate with a sulfuryl group. This resulted in a PAPS molecule that retained the geometry of the adenosine 3′,5′-diphosphate present in the homology model derived from the EST−PAP complex. The PAPS molecule was then fit into the position occupied by PAP in the active site of the AST IV homology model using the Multifit option in SYBYL 6.4. The enzyme was given Kollman all-atom charges, and Gasteiger−Hückel charges were used for the ligands. The AST IV homology model with PAPS in its active site was used as the receptor in the docking studies.

Docking of Ligands. The automated docking package Flexidock, a module of SYBYL 6.4, was used in the docking studies. Individual *N*-hydroxy arylamine ligands were constructed, given Gasteiger−Hückel charges, and optimized by energy minimization using the Powel algorithm. These ligands were fit into the position occupied by estradiol in the AST IV homology model using the Multifit option in SYBYL 6.4. The oxygen atom and the aromatic carbon atoms of the ligand were overlapped on the 3α oxygen and aromatic ring of estradiol, respectively. The resultant ligand structure was used as the initial conformation for the docking experiments. These experiments provided a set of solutions that represented the optimum ligand geometry in the active site of the homology model. Representations of these optimum ligand geometries in Figures 3 and 4 were obtained through the use of MOLSCRIPT (*31*) and RASTER3D (*32*).

Results

Stability of *N*-Alkyl-*N*-hydroxyanilines under Assay Conditions. We have previously reported (*7*) that primary *N*-hydroxy arylamines are susceptible to oxidative degradation during sulfotransferase assays unless steps are taken to remove dissolved oxygen from the reaction solution. Therefore, we examined the stability of a representative *N*-alkyl-*N*-hydroxyaniline under the

Table 1. Specificity of Aryl Sulfotransferase IV for *N*-Alkyl-*N*-hydroxyanilines[a]

N-alkyl-*N*-hydroxyaniline	K_m(app) (μM)	V_{max} [nmol min^{-1} (mg of protein)$^{-1}$]	k_{cat}/K_m (mM^{-1} min^{-1})	K_i (μM)
1	119 ± 15	85 ± 5	24.2 ± 3.4	–
2	650 ± 75	273 ± 12	14.2 ± 1.8	–
3	161 ± 35	98 ± 6	20.6 ± 4.7	–
4	–	–	–	66 ± 11

[a] The structures of the compounds examined are shown in Figure 1. Kinetic constants were determined in argon-saturated reaction mixtures at pH 7.0 in 0.25 M potassium phosphate with varying concentrations of the *N*-alkyl-*N*-hydroxyaniline, 0.2 mM PAPS, 8.3 mM 2-mercaptoethanol, and 1.5 μg of AST IV in a total volume of 30 μL. Reaction mixtures were incubated at 37 °C for 10 min and analyzed by HPLC as indicated in Experimental Procedures.

conditions used for enzymatic assays. Accordingly, ultraviolet spectroscopy (data not shown) revealed that *N*-*n*-butyl-*N*-hydroxyaniline was chemically stable under the conditions of argon saturation used in the assays for sulfotransferase activity with these compounds. Therefore, the anaerobic conditions previously developed for primary *N*-hydroxy arylamines were suitable for maintenance of *N*-alkyl-*N*-hydroxyanilines during enzymatic assays with AST IV.

Interaction of *N*-Alkyl-*N*-hydroxyanilines with AST IV. Each *N*-alkyl-*N*-hydroxyaniline was examined for its ability to serve as either a substrate for or an inhibitor of aryl sulfotransferase IV. *N*-Hydroxy-*N*-methylaniline (**1**), *N*-ethyl-*N*-hydroxyaniline (**2**), and *N*-hydroxy-*N*-*n*-propylaniline (**3**) were found to be substrates for the purified enzyme (Table 1 and Figure 2). The catalytic efficiency of the enzyme with these substrates, as judged by the values of k_{cat}/K_m, varied by less than 2-fold for the compounds with *N*-methyl, *N*-ethyl, and *N*-propyl substituents on *N*-hydroxyaniline. However, a dramatic change occurred upon lengthening the *N*-alkyl substituent from *N*-propyl to *N*-butyl. *N*-*n*-Butyl-*N*-hydroxyaniline (**4**) was not a substrate for aryl sulfotransferase IV, but it was instead a reversible competitive inhibitor of the AST IV-catalyzed sulfation of 1-naphthalenemethanol.

Assessment of the Potential for Irreversible Inhibition of AST IV by a Secondary *N*-Hydroxy Arylamine. Previous reports have indicated a time-dependent irreversible inactivation of AST IV with *N*-hydroxy-2-acetylaminofluorene (*33*) and *N*-hydroxyaniline (*7*). Therefore, we examined the potential for one of the secondary *N*-hydroxy arylamines to irreversibly inhibit the enzyme. AST IV (10 μg) was incubated at 37 °C in 0.25 M potassium phosphate at pH 7.0 either without or with 1 mM *N*-ethyl-*N*-hydroxyaniline and 0.2 mM PAPS for up to 21 min. At 7 min intervals, aliquots were diluted into a standard assay for sulfation of 2-naphthol at pH 5.5 (*34*). The specific activities of the aliquots of the enzyme remained within 10% of the control value throughout the preincubation, regardless of the presence or absence of PAPS (data not shown). This result indicated that neither *N*-ethyl-*N*-hydroxyaniline nor the sulfuric acid ester of *N*-ethyl-*N*-hydroxyaniline covalently inactivated the purified protein. The sulfuric acid ester of *N*-ethyl-*N*-hydroxyaniline failed to act as an irreversible inhibitor of the enzyme, yet it was nevertheless an unstable reaction product. Attempts to isolate and/or quantitate this product only led to mixtures of degradation products. Therefore, although the reaction product

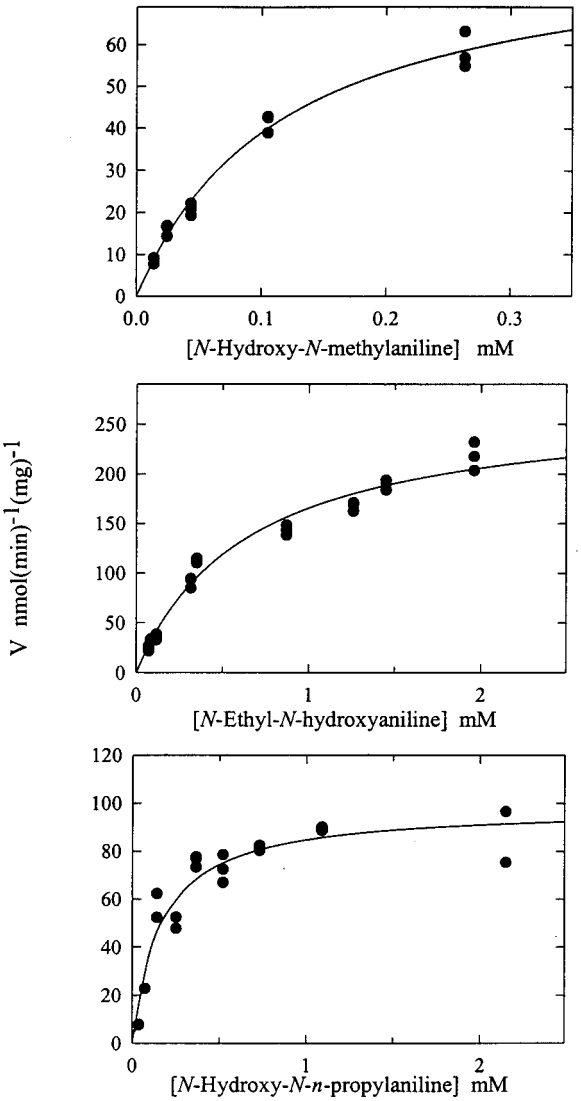

Figure 2. Initial velocity data for AST IV-catalyzed sulfation of *N*-hydroxy-*N*-methylaniline, *N*-ethyl-*N*-hydroxyaniline, and *N*-hydroxy-*N*-*n*-propylaniline. Assays were carried out as described in Table 1. Data points are observed values, and the lines represent the results of nonlinear curve fitting as described in Experimental Procedures.

was unstable in our attempts at its isolation, it did not react with the AST IV in such a way as to impair the catalytic function of the enzyme.

Modeling AST IV by Homology to Mouse Estrogen Sulfotransferase (EST). The dramatic change in the interaction of *N*-*n*-alkyl-*N*-hydroxyarylamines with AST IV as the *n*-alkyl group was extended could not be explained on the basis of changes in hydrophobic characteristics, and it suggested that steric and/or electrostatic interactions at the active site of the enzyme were critical. In the absence of a crystal structure for AST IV, we developed a model for the enzyme based upon its strong homology to EST. As described in detail in Experimental Procedures, the homology model was produced by manual alignment of the amino acid sequence of AST IV with the electron density map of EST-PAP, followed by structural refinement and energy minimization of the model. The structure of a proposed transition state mimic, an EST−PAP−vanadate complex, was used as a template for construction of PAPS and its placement at the active site of the AST IV homology model.

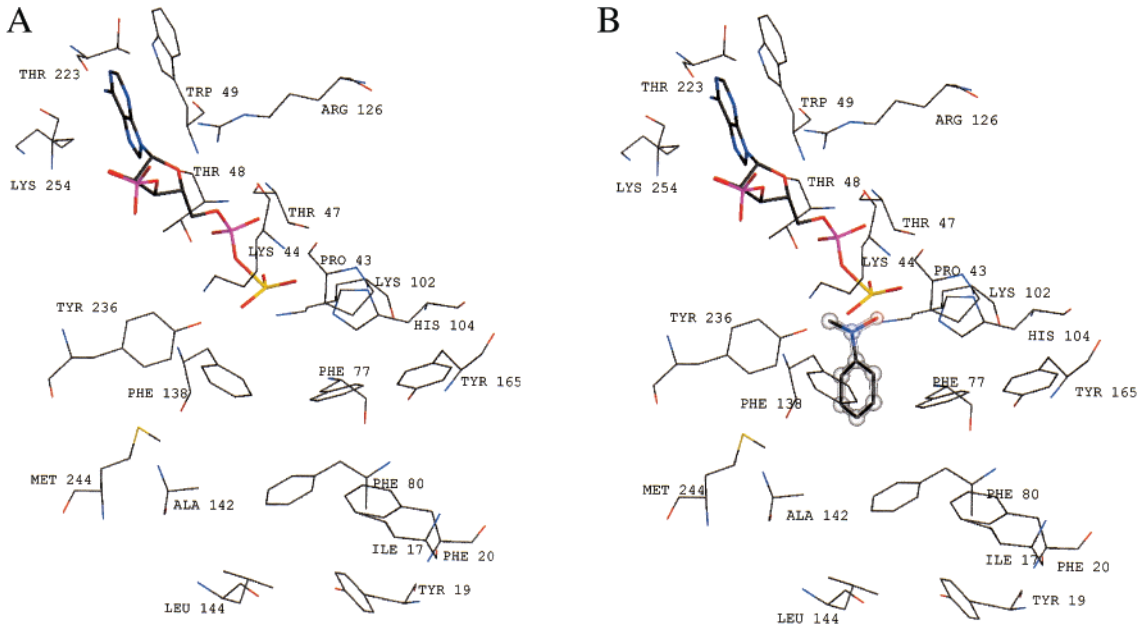

Figure 3. Active site region of a model of AST IV constructed by homology to EST. (A) The homology model of AST with estradiol coordinates removed and a sulfuryl group replacing the PAP−vanadate complex as described in Experimental Procedures. (B) Starting conformation of *N*-methyl-*N*-hydroxyaniline for energy minimization with Flexidock. The oxygen atom and aromatic carbons of *N*-methyl-*N*-hydroxyaniline were aligned with coordinates corresponding to the positions of the 3α oxygen and aromatic carbons of estradiol in the crystal structure of the EST−PAP complex.

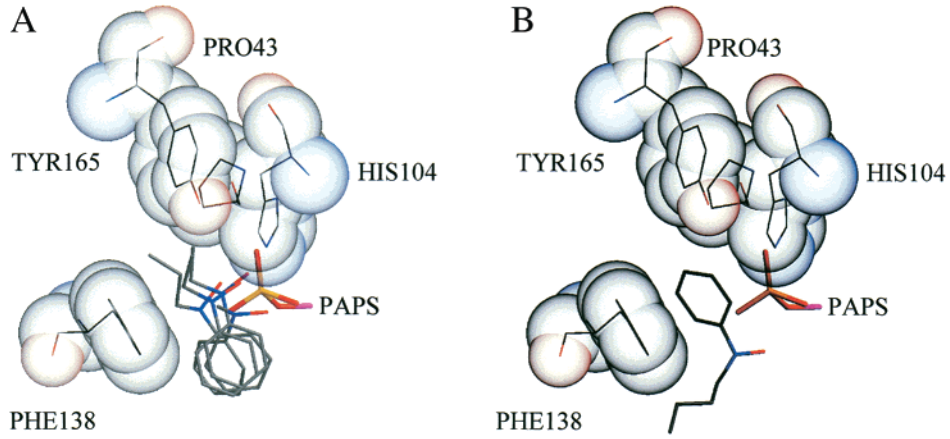

Figure 4. Optimum geometries of *N*-alkyl-*N*-hydroxyanilines calculated for ligand docking within the active site of the homology model for AST IV. (A) *N*-Hydroxy-*N*-methylaniline, *N*-ethyl-*N*-hydroxyaniline, and *N*-hydroxy-*N*-*n*-propylaniline are shown. (B) *N*-*n*-Butyl-*N*-hydroxyaniline is shown.

Likewise, the crystal structure of the EST−PAP−vanadate complex and an EST−PAP−estradiol complex were utilized to assign a starting orientation of the oxygen atom and the aromatic ring of each *N*-hydroxy arylamine for ligand docking computations. These calculations, carried out with Flexidock as described below, were then used to determine an optimum ligand geometry for the *N*-hydroxy arylamine in relation to the amino acid residues at the active site of the homology model of AST IV. A representation of the active site region of the model of AST IV based on the crystal structure of EST is seen in Figure 3.

Orientation of Docked Ligands in the Active Site Model. The optimum geometry for the binding of each of the four *N*-alkyl-*N*-hydroxy arylamines to the homology model of AST IV was determined. The algorithm that was used for this optimization, Flexidock, took into account van der Waals, electrostatic, and torsional energy terms to calculate a single energy term describing the ligand−receptor interaction. The bond lengths and bond angles were kept constant during the docking procedure. As seen in Figure 4A, the orientations of the *N*-hydroxyarylamine molecules that served as substrates were similar. Namely, the alkyl substituent groups occupied a pocket formed by Pro 43, His 104, Phe 138, and Tyr 165 in the homology model. However, in the case of *N*-*n*-butyl-*N*-hydroxyaniline (Figure 4B), this pocket was unable to accommodate the *n*-butyl substituent, and the orientation of the inhibitor changed so that the aromatic ring of the inhibitor was accommodated in this region rather than the *N*-alkyl substituent. As noted below, one result of this dramatic change in orientation was to place the oxygen of the N−OH group a much greater distance from both the sulfuryl group and the critical His 104.

Table 2. Calculated Energies and Bond Distances for *N*-Alkyl-*N*-hydroxyanilines Docked into the Active Site Model for AST IV

N-alkyl-*N*-hydroxyaniline	binding energy term[a] (kcal/mol)	distance from the oxygen of the ligand to the sulfur of PAPS (Å)	distance from the oxygen of the ligand to the nitrogen of His 104 (Å)
1	−1636.7	1.886	3.204
	−1636.3	1.886	3.205
2	−1441.0	2.142	1.911
	−1434.0	2.021	2.414
3	−1237.0	2.095	3.282
4	−944.0	2.710	5.089

[a] All binding energies and bond distances for binding of *N*-alkyl-*N*-hydroxyanilines to the homology model for AST IV were calculated as described in Experimental Procedures. Two very similar docking positions were found for compounds **1** and **2**, while single solutions to the docking procedure were obtained for compounds **3** and **4**.

The calculated values of the binding energy for each of the Flexidock solutions and of the corresponding distances between the oxygen atom of the docked ligand and both the sulfur atom of PAPS and the nitrogen atom of His 104 are shown in Table 2. The binding energy terms calculated for compounds **1**−**3** were lower in energy (−1447 ± 240 kcal/mol) than that calculated for *N*-*n*-butyl-*N*-hydroxyaniline (−944 kcal/mol). Although this represents a difference between substrates **1**−**3** and the inhibitor, **4**, the distances from the oxygen atom of the *N*-hydroxy arylamine to the sulfur atom of PAPS and the closest nitrogen of the key histidine residue are also critical parameters for use of the model in predicting catalytic function. The oxygen−sulfur distance is 2.006 ± 0.118 Å for the calculated geometries of *N*-methyl-, *N*-ethyl-, and *N*-*n*-propyl-*N*-hydroxyaniline (compounds **1**−**3**). In contrast, the distance from the oxygen of *N*-*n*-butyl-*N*-hydroxyaniline to the sulfur of PAPS (2.710 Å) is significantly greater than that observed for the three substrates. Likewise, the distance between the nitrogen of His 104 and the oxygen of the *N*-alkyl-*N*-hydroxyaniline is much greater for the inhibitor (5.089 Å) than for substrates **1**−**3** (2.803 ± 0.612 Å).

Discussion

Drugs, carcinogens, and other xenobiotics containing various organic functional groups are known to interact with aryl (phenol) sulfotransferases such as AST IV, yet within each class of similar substrates or inhibitors, enzyme activity is affected to varying degrees by electronic, steric, and lipophilic characteristics (*35*−*39*). For example, the sulfation of phenols and aryl oximes catalyzed by AST IV is affected by the electronic contribution of aromatic ring substituents; however, the magnitude and direction of this effect are not consistent between the two different structural classes (*35*, *38*). The AST IV-catalyzed sulfation of benzylic alcohols is affected by the lipophilicity of the substrate (*37*) and by steric effects related to the configurations of chiral benzylic alcohols (*39*). In addition, aldehydes and carboxylic acids corresponding to the higher oxidation states of benzylic alcohols act as inhibitors of the enzyme (*39*).

In the studies presented here, AST IV was found to catalyze the sulfation of secondary *N*-hydroxy arylamines. *N*-Alkyl-*N*-hydroxy arylamines **1**−**4** were chosen for investigation of the possibility of steric and hydrophobic interactions at the active site of AST IV. On the basis of previous work with other substrate classes, one might expect a consistent increase in the catalytic efficiency of the enzyme as the alkyl chain length on the *N*-hydroxy nitrogen was increased. However, this was not observed. The *N*-methyl, *N*-ethyl, and *N*-*n*-propyl derivatives of *N*-hydroxyaniline (compounds **1**−**3** in Table 1) were substrates for AST IV, while the *N*-butyl derivative was found to be a reversible competitive inhibitor of the purified enzyme. It is also noteworthy that previously published kinetic constants (*7*) for *N*-hydroxyaniline [i.e., $K_m = 230 \pm 18$ μM, $V_{max} = 43 \pm 1$ nmol min^{-1} (mg of protein)$^{-1}$, and $k_{cat}/K_m = 6.3$ mM^{-1} min^{-1}] are also consistent with the results in Table 1. That is, AST IV can catalyze sulfation of an unsubstituted *N*-hydroxy arylamine as well as an *N*-hydroxy arylamine bearing a methyl, ethyl, or *n*-propyl substituent on the nitrogen with relatively small effects on the overall catalytic efficiency of the reaction. The similarities in catalytic efficiencies observed for the enzyme with these substrates may in part be due to the ability of the active site to accommodate multiple binding orientations of these *N*-hydroxy arylamines, of which some orientations are not catalytically productive. Nevertheless, for those *N*-hydroxy arylamines that are substrates, these noncatalytic orientations are in equilibrium with an orientation that is suitable for sulfuryl transfer.

While compounds **1**−**3** are capable of binding to the active site of AST IV in an orientation that is appropriate for catalysis, the *n*-butyl substituent seen in compound **4** imparts a specificity of binding that precludes any orientation of the molecule at the active site that is favorable for sulfuryl transfer to the oxygen of the *N*-hydroxy arylamine. The fact that the K_i of **4** (66 μM) is 2−10-fold lower than the K_m's of **1**−**3** (119−650 μM) suggests that the steric effects primarily involve orientation of the molecule in relation to catalysis rather than simply the hydrophobic characteristics of substrate binding. Thus, the lack of a strong trend in kinetic parameters among compounds **1**−**3** as substrates, as well as the observation that the *n*-butyl derivative became an inhibitor of the enzyme, points to a complex relationship between steric and hydrophobic factors in this class of molecules.

The increased hydrophobicity of **4** relative to those of **1**−**3** most likely enhances the ability of the xenobiotic to bind to the active site of AST IV, while steric effects of the longer alkyl substituent alter the orientation of the binding so that catalysis does not occur. Molecular docking studies also support this hypothesis. It is seen that in the case of AST IV substrates **1**−**3**, the alkyl chain on the *N*-hydroxy nitrogen of the docked ligands is accommodated in a pocket of four amino acids comprising Pro 43, His 104, Phe 138, and Tyr 165. Due to the steric constraints on this pocket, it is unable to accommodate the *n*-butyl chain of the *N*-*n*-butyl-*N*-hydroxyaniline.

On the basis of studies performed on mouse estrogen sulfotransferase, it has been suggested that after the proton on the oxygen atom of a substrate has been abstracted by catalytic His 108, there is an in-line attack by this oxygen on the sulfur atom of PAPS (*30*). The residue corresponding to His 108 of mouse estrogen sulfotransferase is His 104 in AST IV, and the sequence of catalytic events outlined for mouse estrogen sulfotransferase based on its crystal structure (*30*) is consistent with the kinetic mechanism proposed earlier for AST IV based on initial velocity studies in solution (*35*). From the crystal structure of a PAP−vanadate−

estrogen sulfotransferase complex, the distance from the position of the oxygen of estradiol to the vanadium atom in the PAP−vanadate complex (analogous to the sulfur atom in PAPS) is 2.3 Å, and the distance from the oxygen to the nitrogen of His 108 is 2.9 Å (*30*). In the AST IV homology model (Table 2), the computed distances between the oxygen atom of the docked ligand and both the sulfur atom of PAPS (1.9−2.1 Å) and the nitrogen atom of His 104 (1.9−3.3 Å) are consistent with a catalytic role for His 104, and they also suggest distinctions between favorable and unfavorable binding orientations for catalysis, with a calculated O−S distance of 2.7 Å and an O−N distance of 5.1 Å for the inhibitor *N*-*n*-butyl-*N*-hydroxyaniline (Table 2). According to this model, the change in the docked ligand geometry of *N*-*n*-butyl-*N*-hydroxyaniline results in an unfavorable orientation for catalysis. Therefore, although *N*-*n*-butyl-*N*-hydroxyaniline binds well to the active site of AST IV as seen by the K_i value, its inability to accept a sulfuryl group from PAPS is predicted by the homology model and docking experiments.

Our experimental data on sulfation of secondary *N*-hydroxyanilines are explained well by a model of the active site of AST IV based upon homology to the crystal structure of mouse estrogen sulfotransferase. The sequences of the two enzymes are 50% identical, and as noted above, there is evidence suggesting that the catalytic mechanisms of sulfuryl transfer are essentially the same in the mouse estrogen sulfotransferase and the rat hepatic AST IV. β-Estradiol serves as a substrate for both enzymes, although the apparent K_m with mouse estrogen sulfotransferase (*40*) is approximately 100-fold less than the apparent K_m obtained with AST IV under similar reaction conditions (J. Sheng and M. Duffel, unpublished results); maximal velocities for sulfation of β-estradiol catalyzed by the two enzymes are comparable under the same conditions of buffer, pH, and temperature. These similarities provide a degree of confidence that the homology model is relevant to catalytic function. Nonetheless, there are differences between the two enzymes that warrant further investigation with respect to their potential role in catalytic function. For example, the mouse estrogen sulfotransferase exists as a monomer in solution, while AST IV is a dimer. The potential for this structural difference to be manifested in alterations in catalytic specificity remains to be determined. Thus, even though this homology model predicts the complex behavior of the secondary *N*-hydroxy arylamines presented here, it may undergo further refinement as other substrate classes and structural aspects of the enzyme are explored. Such investigations are in progress.

In conclusion, our finding that AST IV is able to catalyze the sulfation of *N*-alkyl-*N*-hydroxy arylamines contributes to a more complete understanding of the metabolic pathways available to these intermediary metabolites. Moreover, these results provide an indication that steric factors relating to the substituent on the *N*-hydroxy nitrogen can dramatically influence the nature of the interaction with the aryl sulfotransferase. Although these observations provide the first quantitative exploration of the role of these structural characteristics in the sulfation of secondary *N*-hydroxy arylamines, further studies with larger sets of compounds and other hepatic sulfotransferases will undoubtedly be necessary to provide a more complete understanding of the catalytic process and more detailed predictions of the specificity of aryl sulfotransferases for this class of xenobiotic metabolites.

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References

(1) Ziegler, D. M. (1984) in *Drug Metabolism and Drug Toxicity* (Mitchell, J. R., and Horning, M. G., Eds.) pp 33−53, Raven Press, New York.

(2) Kadlubar, F. F., Miller, J. A., and Miller, E. C. (1976) Hepatic metabolism of *N*-hydroxy-*N*-methyl-4-aminoazobenzene and other *N*-hydroxy arylamines to reactive sulfuric acid esters. *Cancer Res.* **36**, 2350−2359.

(3) Kadlubar, F. F., Ketterer, B., Flammang, T. J., and Christodoulides, L. (1980) Formation of 3-glutathione-5-yl-*N*-methyl-4-aminoazobenzene and inhibition of aminoazo dye-nucleic acid binding *in vitro* by reaction of glutathione with metabolically-generated *N*-methyl-4-aminoazobenzene-*N*-sulfate. *Chem.-Biol. Interact.* **31**, 265−278.

(4) Ziegler, D. M., Ansher, S. S., Nagata, T., Kadlubar, F. F., and Jakoby, W. B. (1988) *N*-Methylation: Potential mechanism for metabolic activation of carcinogenic primary arylamines. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2514−2517.

(5) Prough, R. A., and Ziegler, D. M. (1977) The relative participation of liver microsomal amine oxidase and cytochrome P-450 in *N*-demethylation reactions. *Arch. Biochem. Biophys.* **180**, 363−373.

(6) Duffel, M. W., Modi, R. B., and King, R. (1992) Interactions of a Primary *N*-Hydroxy Arylamine with Rat Hepatic Aryl Sulfotransferase IV. *Drug Metab. Dispos.* **20**, 339−342.

(7) King, R. S., and Duffel, M. W. (1997) Oxidation-dependent inactivation of aryl sulfotransferase IV by primary N-hydroxy arylamines during in vitro assays. *Carcinogenesis* **18**, 843−849.

(8) Glatt, H. (1997) Sulfation and sulfotransferases 4: bioactivation of mutagens via sulfation. *FASEB J.* **11**, 314−321.

(9) Ringer, D. P., Norton, T. R., and Howell, B. A. (1990) 2-Acetylaminofluorene-mediated alteration in the level of liver aryl sulfotransferase IV during rat hepatocarcinogenesis. *Cancer Res.* **50**, 5301−5307.

(10) Zander, A. (1883) Ueber die specifischen volumina einiger allyl-, propyl- und verwandter verbindungen. *Liebigs Ann. Chem.* **214**, 138−171.

(11) Cope, A. C., LeBel, N. A., Lee, H.-H., and Moore, W. R. (1957) Amine oxides. III. Selective formation of olefins from unsymmetrical amine oxides and quaternary ammonium hydroxides. *J. Am. Chem. Soc.* **79**, 4720−4729.

(12) Kadlubar, F. F., McKee, E. M., and Ziegler, D. M. (1973) Reduced pyridine nucleotide-dependent *N*-hydroxy amine oxidase and reductase activities of hepatic microsomes. *Arch. Biochem. Biophys.* **156**, 46−57.

(13) Rogers, M. A. (1955) Aliphatic hydroxylamines. Part I. Preparation. *J. Chem. Soc.*, 769−772.

(14) Craig, J. C., and Purushothaman, K. K. (1970) An improved preparation of teriary amine *N*-oxides. *J. Org. Chem.* **35**, 1721−1722.

(15) Muntz, R. L., Pirkle, W. H., and Paul, I. C. (1972) Determination of absolute configuration of (−)-*N*-ethyl-*N*-methylaniline oxide by X-ray analysis: the molecular geometry of an *N*-oxide salt. *J. Chem. Soc., Perkin Trans. 2*, 483−488.

(16) Tsen, C. C. (1961) An improved spectrophotometric method for the detection of tocopherols using 4,7-diphenyl-1,10-phenanthroline. *Anal. Chem.* **33**, 849−851.

(17) Duffel, M. W., Binder, T. P., Hosie, L., Baden, H., Sanders, J. A., Knapp, S. A., and Baron, J. (1991) Purification, immunochemical characterization, and immunohistochemical localization of rat hepatic aryl sulfotransferase IV. *Mol. Pharmacol.* **40**, 36−44.

(18) Sekura, R. D., and Jakoby, W. B. (1981) Aryl sulfotransferase IV from rat liver. *Arch. Biochem. Biophys.* **211**, 352−359.

(19) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**, 680−685.

(20) Gong, D. W., Ozawa, S., Yamazoe, Y., and Kato, R. (1991) Purification of hepatic N-hydroxyarylamine sulfotransferases and their regulation by growth hormone and thyroid hormone in rats. *J. Biol. Chem.* **110**, 226−231.

(21) Nagata, K., Ozawa, S., Yamazoe, Y., and Kato, R. (1993) Isolation and expression of a cDNA encoding a male-specific rat sulfotransferase that catalyzes activation of *N*-hydroxy-2-acetylaminofluorene. *J. Biol. Chem.* **268**, 24720−24725.

(22) Yamazoe, Y., Nagata, K., Ozawa, S., and Kato, R. (1994) Structural similarity and diversity of sulfotransferases. *Chem.-Biol. Interact.* **92**, 107−117.

(23) Kiehlbauch, C. C., Lam, Y. F., and Ringer, D. P. (1995) Homodimeric and heterodimeric aryl sulfotransferases catalyze the sulfuric acid esterification of *N*-hydroxy-2-acetylaminofluorene. *J. Biol. Chem.* **270**, 18941−18947.

(24) Chen, X., Yang, Y.-S., Zheng, Y., Martin, B. M., Duffel, M. W., and Jakoby, W. B. (1992) Tyrosine-ester sulfotransferase from rat liver: bacterial expression and identification. *Protein Expression Purif.* **3**, 421−426.

(25) Bensadoun, A., and Weinstein, D. (1976) Assay of proteins in the presence of interfering materials. *Anal. Biochem.* **70**, 241−250.

(26) Duffel, M. W., Binder, T. P., and Rao, S. I. (1989) Assay of purified aryl sulfotransferase suitable for reactions yielding unstable sulfuric acid esters. *Anal. Biochem.* **183**, 320−324.

(27) Perella, F. W. (1988) EZ-FIT: A practical curve-fitting microcomputer program for the analysis of enzyme kinetic data on IBM-PC compatible computers. *Anal. Biochem.* **174**, 437−447.

(28) Kakuta, Y., Pedersen, L. G., Carter, C. W., Negishi, M., and Pedersen, L. C. (1997) Crystal structure of estrogen sulphotransferase. *Nat. Struct. Biol.* **4**, 904−908.

(29) Brunger, A. T., Kuriyan, J., and Karplus, M. (1987) Crystallographic R-factor refinement by molecular dynamics. *Science* **235**, 458−460.

(30) Kakuta, Y., Petrotchenko, E. V., Pedersen, L. C., and Negishi, M. (1998) The sulfuryl transfer mechanism. Crystal structure of a vanadate complex of estrogen sulfotransferase and mutational analysis. *J. Biol. Chem.* **273**, 27325−27330.

(31) Kraulis, P. J. (1991) MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**, 946−950.

(32) Merritt, E. A., and Bacon, D. J. (1997) Raster3D: Photorealistic molecular graphics. *Methods Enzymol.* **277**, 505−524.

(33) Ringer, D. P., Norton, T. R., and Self, R. R. (1992) Reaction product inactivation of aryl sulfotransferase IV following electrophilic substitution by the sulfuric acid ester of *N*-hydroxy-2-acetylaminofluorene. *Carcinogenesis* **13**, 107−112.

(34) Sekura, R. D., Duffel, M. W., and Jakoby, W. B. (1981) Aryl sulfotransferases. *Methods Enzymol.* **77**, 197−206.

(35) Duffel, M. W., and Jakoby, W. B. (1981) On the mechanism of aryl sulfotransferase. *J. Biol. Chem.* **256**, 11123−11127.

(36) Campbell, N. R., Van Loon, J. A., Sundaram, R. S., Ames, M. M., and Hansch, C. (1987) Human and rat liver phenol sulfotransferase: structure−activity relationships for phenolic substrates. *Mol. Pharmacol.* **32**, 813−819.

(37) Binder, T. P., and Duffel, M. W. (1988) Sulfation of benzylic alcohols catalyzed by aryl sulfotransferase IV. *Mol. Pharmacol.* **33**, 477−479.

(38) Mangold, J. B., McCann, D. J., and Spina, A. (1993) Aryl sulfotransferase IV-catalyzed sulfation of aryl oximes: steric and substituent effects. *Biochim. Biophys. Acta* **1163**, 217−222.

(39) Duffel, M. W. (1994) Molecular specificity of aryl sulfotransferase IV (tyrosine-ester sulfotransferase) for xenobiotic substrates and inhibitors. *Chem.-Biol. Interact.* **92**, 3−14.

(40) Kakuta, Y., Pedersen, L. C., Chae, K., Song, W.-C., Leblanc, D., London, R., Carter, C. W., and Negishi, M. (1998) Mouse steroid sulfotransferases: substrate specificity and preliminary X-ray crystallographic analysis. *Biochem. Pharmacol.* **55**, 313−317.

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