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Mouse Steroid Sulfotransferases

Substrate Specificity and Preliminary X-Ray Crystallographic Analysis

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ABSTRACT. Three mouse cytosolic sulfotransferases were expressed in *Escherichia coli* cells in order to study their substrate specificities toward natural as well as synthetic steroid hormones. The K_m and V_{max} values confirmed the high substrate specificity of estrogen and hydroxysteroid sulfotransferases toward estradiol and dehydroepiandrosterone, respectively. In sharp contrast, the synthetic estrogen diethylstilbestrol was metabolized efficiently by both enzymes to its disulfate ester. These sulfotransferases display highly stereospecific sulfotransferase activity for sulfating only the *trans*-isomer of diethylstilbestrol. Crystals suitable for high-resolution structure determination of estrogen sulfotransferase were grown with polyethylene glycol. The crystals belong to the orthorhombic space group $P2_12_12$, and diffracted to 2.5 Å. BIOCHEM PHARMACOL **55**;3:313–317, 1998.

KEY WORDS. steroid sulfotransferase; bacterial expression; substrate specificity; diethylstilbestrol; stereo specificity; crystallization

Sulfotransferases comprise a family of enzymes that catalyze transfers of the sulfonate group from PAPS** to various substrates including steroids, lipids, proteins, glycoproteins, peptides, amines, and numerous xenobiotic chemicals [1–4]. Sulfonation, a major pathway for the conjugation of natural steroid hormones, is catalyzed by the cytosolic steroid sulfotransferases. Sulfonated steroids, while hormonally inactive, may serve as prohormones that can be reactivated by desulfation (steroid sulfatase). Steroid sulfotransferases are separated into two groups, using apparent substrate specificities as the criteria. EST (EC 2.8.2.4) acts on the 3-phenol group of estrogens (phenolic steroids) including estrone and E_2, whereas HST (EC 2.8.2.2) sulfates the alcohol group of androgens and progesterone. PST (EC 2.8.2.15) represents a third group of enzymes that metabolize various xenobiotic phenols and amines.

DES, one of the most characterized synthetic estrogens, has been shown to induce tumor development in mouse uteri [5, 6]. Moreover, trans-placental DES exposure during the first trimester causes genital tract abnormalities in humans [7]. Previous metabolic studies show that plasma and urinary DES is sulfated, although DES glucuronide appears to be a major metabolite [8]. Since preliminary studies in the 1970s [7], however, the understanding of DES metabolism by sulfotransferases has not progressed significantly. In this paper, we report that we have cloned mouse EST, HST, and PST with expression in bacterial cells. In addition, we have examined the substrate specificities of these enzymes toward the natural hormones E_2 and DHEA and compared their specificities with that of the synthetic estrogen DES. Mouse sulfotransferases were found to exhibit distinct substrate specificities if either natural or synthetic hormones were used as substrate. Moreover, EST has been crystallized recently and structural studies have been initiated.

MATERIALS AND METHODS

Cloning and Bacterial Expression of Sulfotransferases

The coding sequences of HST and PST were amplified from mouse liver RNAs using a Superscript Preamplification System (Life Technologies) and the specific primers. To amplify HST [9], 5′-CCGGAATTCCATGATGTCAGACTAT AATTGGTTTGAAG-3′ and 5′-CCGCTCGAGTGAC CACATCTAGGTATTCAAGATA-3′ were used as 3′-

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** *Abbreviations:* DES, diethylstilbestrol; DHEA, dehydroepiandrosterone; E_2, estradiol; EST, estrone sulfontransferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; and PST, phenol sulfotransferase.

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and 5′-primers. The amplified DNA was digested with *Eco*RI and *Xho*I and ligated to the pGEX-4T3 vector (Pharmacia Biotech Inc.). Similarly, PST [10] was amplified and cloned, except that 5′-CGCGGATCCATGGCT CAGAACCCCAGCA-3′ and 5′-CCGCTCGAGTCA TATTTGACGGCGGAACGTGAAG-3′ were used for amplification and *Bam*HI and *Xho*I were used to digest the amplified DNA. EST cDNA was cloned previously from mouse testis RNAs and inserted into pGEX-4T3 [11]. These recombinant plasmids were transformed into *Escherichia coli* DH5α cells to express GST-HST, GST-PST, and GST-EST. These fusion proteins were prepared from bacterial cultures and cleaved by thrombin to obtain pure HST and PST as described for EST purification in a previous report [11].

Sulfotransferase Assay and other Analytical Methods

The reaction mixture (200 μL) was constituted by 200 mM Tris–acetate buffer, pH 7.9, containing 100 mM PAPS and the indicated concentrations of various radioactive substrates and bacterially expressed sulfotransferases. The mixture was incubated for 5 min at 37°, and the reaction was stopped by adding 2 vol. of dichloromethane. Sulfonated metabolites were extracted into the aqueous phase for quantitation by scintillation counting. DES conjugates formed by sulfotransferases were analyzed by HPLC equipped with a radioactive flow detector (FLO-One/Beta, Radiomatic Instruments & Chemical Co., Inc.) using a TSK-GEL ODS^{80TM} reverse phase column (4.6 × 25 mm) and a gradient solvent system (gradient of 50% methanol in water to 100% methanol at a flow rate of 0.5 mL/min). Protein concentration was determined using the molar coefficient [ε_{280} = 55917 $(cm \cdot mol \cdot 2mL)^{-1}$] calculated from the content of tyrosine and tryptophan in the deduced amino acid sequence of EST.

Synthesis of DES Sulfate Esters

A mixture of DES (2.6 mg, 0.01 mmol) and sulfur trioxide-pyridine (Aldrich Chemical Co.; 1.6 mg, 0.01 mmol) in 3 mL of dry pyridine was stirred at room temperature for 18 hr. Ethyl acetate (3 mL) was added to the reaction mixture, vortexed, and stored at −20° for 5 hr. The resulting precipitate was collected by centrifugation, washed five times with 2 mL each of ice-cold ethyl acetate, and dried over a stream of nitrogen. The precipitate was dissolved in water and analyzed by HPLC. The DES disulfate was further purified by preparative HPLC using a C-18 column with acetonitrile:water (1:1) as a solvent system. A negative ion mass spectrum of *m/z* 427 completed identification.

Further Purification and Crystallization of EST

The bacterially expressed EST was first purified by affinity chromatography using Glutathione-Sepharose 4B (Pharmacia). Then the EST was concentrated using an Amicon stirred cell to 50 mg/mL and applied on a Superose HR column (10 × 300 mm, Pharmacia) using PBS buffer (20 mM potassium phosphate buffer, pH 7.2, containing 100 mM NaCl). The purified protein was dialyzed against 10 mM potassium phosphate buffer, pH 7.2, containing 50 mM NaCl and was adjusted to a concentration of 15 mg/mL and 4 mM 3′-phosphoadenosine-5′-phosphate. Crystal Screening kits I and II (Hampton Research) were used to search (hanging drop method) for a crystallization condition from which EST crystals were grown at room temperature. The batch method was then used to define the final buffer conditions (crystallization buffer): 8% polyethylene glycol 6000 containing 5 mM potassium phosphate buffer, pH 7.2, 50 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer, pH 6.0, and 25 mM NaCl. Finally, streak seeding was employed to obtain a crystal that grew to 0.05 × 0.05 × 0.2 μm within 7 days. For cryocooling, crystals were first soaked in 5% ethylene glycol (antifreeze) containing the crystallization buffer. Subsequently, the concentration of ethylene glycol was increased from 5 to 10%, 10 to 15%, and finally to 20%. Diffraction data were collected at −180° on as RAXIS IV area detector system mirrors mounted on a Rigaku RV-300 rotating anode. Data were processed using DENZO and scalepack [12].

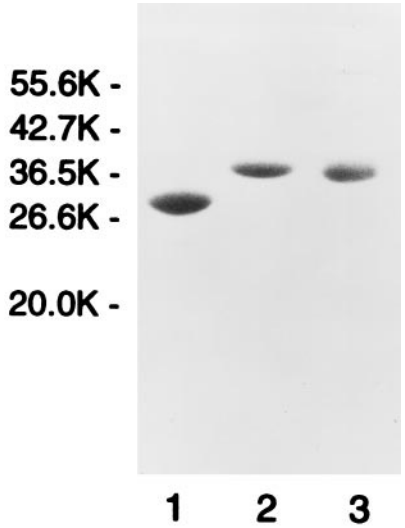

FIG. 1. SDS–PAGE of bacterially expressed mouse sulfotransferases. The purified proteins were electrophoresed on a 12% polyacrylamide gel and stained by coomassie blue. Lane 1, Lane 2, and Lane 3 were HST, EST, and PST, respectively. Molecular weight markers were obtained from New England Biolabs.

RESULTS

Expression of Sulfotransferases in E. coli

Bacterially expressed enzymes were purified by affinity chromatography and analyzed by SDS–PAGE (Fig. 1). The apparent molecular masses of EST, HST, and PST on SDS polyacrylamide gel were 38.2, 30.2, and 37.6 kDa, respectively, and were in good agreement with those estimated from their cDNA-deduced amino acid sequences [9–11].

TABLE 1. K_m **and** V_{max} **values for sulfonation of** E_2**, DHEA, and DES**

	E_2		DHEA		DES	
	K_m (μM)	V_{max} (pmol/nmol/min)	K_m (μM)	V_{max} (pmol/nmol/min)	K_m (μM)	V_{max} (pmol/nmol/min)
EST	0.28 ± 0.13	174 ± 17	34 ± 13	52 ± 27	0.08 ± 0.02	34 ± 5
HST	7.9 ± 3	216 ± 24	1.1 ± 0.2	421 ± 48	0.13 ± 0.03	53 ± 5
PST					0.46 ± 0.15	37 ± 4

Reaction mixtures contained 2 and 180 μg of bacterially expressed EST for E_2 and DHEA sulfotransferase activities, respectively. Substrate concentrations used were 0.088, 0.176, 0.264, 0.352, 0.44, 0.88, and 1.32 μM of E_2 and 0.56, 1.12, 2.24, 4.48, 5.6, 15.0, and 30.0 μM of DHEA. HST (34 and 3.4 μg, respectively) was incubated with E_2 (5.0, 10.0, 30.0, and 50.0 μM) and DHEA (0.25, 0.5, 0.75, 1.0, and 2.0 μM), respectively. The DES sulfotransferase activities were measured for EST (1.5 μg), HST (1.5 μg), and PST (5 μg). DES concentrations varied from 0.02, 0.033, 0.05, 0.08, 0.10, 0.20, to 0.40 μM when EST or HST was used as enzyme. For PST, DES concentrations used were 0.1, 0.2, 0.5, 0.8, 1.0, and 2.0 μM. Other assay conditions were described in "Materials and Methods." A non-linear regression program (created in-house and based on a program given in Ref. 13) was employed to calculate K_m and V_{max} values.

Specificity for Sulfonation Activity of Endogenous Steroids

We have shown in a previous paper that the bacterially expressed mouse EST exhibits extremely high E_2 and estrone (E_1) sulfotransferase activities compared with DHEA, progesterone, and testosterone sulfotransferase activities [11]. Consistent with this previous finding, the K_m value of EST for E_2 sulfotransferase activity was 120-fold lower than that for the corresponding DHEA sulfotransferase activity, while the V_{max} for E_2 was 3-fold higher than that of DHEA (Table 1). Conversely, for DHEA, HST displayed a 7-fold lower K_m and a 2-fold higher V_{max} value than for E_2 sulfotransferase activities. EST and HST are specific in sulfating E_2 and DHEA, respectively, although the degree of substrate specificity appears to be greater in the former. The absolute K_m values of the mouse enzymes were somewhat different from those reported previously for the corresponding EST and HST in humans and guinea pigs [14, 15]. Consistently, however, their relative substrate specificities toward E_2 and DHEA were in very good agreement.

Metabolism of DES by Sulfotransferases

DES metabolites formed by the sulfotransferases were subjected to HPLC analysis (Fig. 2). Radioactive DES used as substrate contained both *trans-* and *cis*-isomers that were separated by HPLC. No nonenzymatic product was formed from DES even in the presence of the co-factor PAPS (Fig. 2A). A short incubation of DES with EST decreased specifically the peak height of the *trans*-isomer and two peaks of DES metabolites increased (Fig. 2B). The fast-eluting peak was co-chromatographed with chemically synthesized DES disulfate ester, while the late-eluting peak had the same retention time as DES monosulfate ester. Monosulfate ester appeared to be formed first, since more of this intermediate was produced than the disulfate ester in the 6-min incubation (Fig. 2B), while only the disulfate ester appeared in the 25-min incubation (Fig. 2C). HST also sulfated DES first to monosulfate and then to disulfate esters (data not shown). Interestingly, the *cis*-isomer of DES remained unsulfated even in the 25-min incubation with either sulfotransferase (Fig. 2C). These results, therefore, indicate that DES sulfotransferase activity of both EST and HST is stereospecific to the *trans*-isomer.

Kinetics of DES Sulfotransferase Activity

To further compare the DES sulfotransferase activities of EST, HST, and PST, we measured their K_m and V_{max} values. A typical Michaelis–Menten kinetics plot is shown in Fig. 3, and these values are summarized in Table 1. The K_m value (0.08 μM) of EST for DES sulfotransferase activity was 3.5-fold lower than that of the corresponding E_2 sulfotransferase activity. Surprisingly, HST efficiently catalyzed DES sulfotransferase activity: the K_m value for DES sulfotransferase activity was 10-fold lower than that for the original DHEA sulfotransferase activity. The V_{max}/K_m ratios (405 and 382, respectively) showed that HST sulfated DES and DHEA with the same turnover rates. These rates were very similar to the ratio (425) of EST for DES sulfotransferase activity. PST also sulfated DES, although its V_{max}/K_m ratio for DES sulfotransferase activity was $>$ 5-fold higher than those for EST and HST. DES sulfonation has also been reported with human PST and EST [14]. Consistent with our present findings, the human enzymes exhibit a much poorer substrate specificity toward DES sulfonation than toward E_2 sulfonation. The reported absolute K_m values differ significantly between the human and mouse ESTs, which remains to be investigated in the future. However, it is not surprising to observe these differences, since substrate specificity of enzymes that metabolize xenobiotics (e.g. cytochrome P450) can be altered by only a minor amino acid substitution [16].

EST Crystals

Crystals of EST diffracted X-rays to a resolution of 2.5 Å. (Fig. 4). Redundancy and completeness were 5.5 and 97.5%, respectively. The space group was $P2_12_12$ with unit cell dimensions a = 96.4 Å, b = 80.4 Å, and c = 80.8 Å. There were two molecules in the asymmetric unit giving a V_M of 2.24 $Å^3$/Da, which corresponded to a solvent content of 46% [17]. A search for heavy atom derivatives is currently in progress.

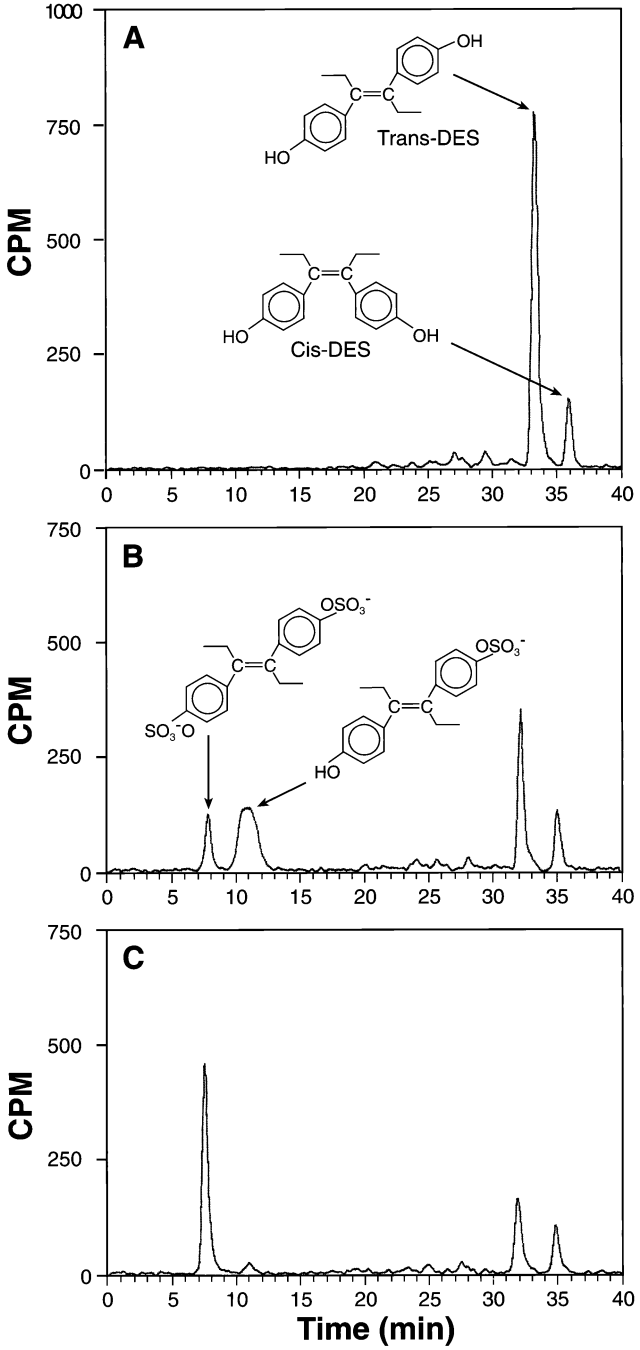

FIG. 2. HPLC analysis of DES metabolites. EST (6 μg) and DES (1.6 μM) were incubated under the conditions described in Materials and Methods. Reaction was stopped at three time points (0, 6, and 25 min in panels A, B, and C, respectively), and DES metabolites were extracted and subjected to HPLC analysis.

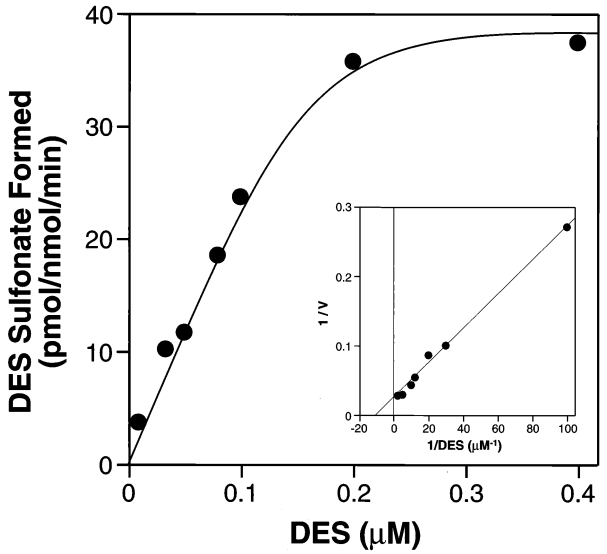

FIG. 3. Michaelis–Menten kinetics of DES sulfotransfease activity in HST. DES concentrations varied from 0.02, 0.033, 0.05, 0.08, 0.10, 0.20 to 0.40 μM and the enzyme amount of HST was 1.5 μg. Inset: Double-reciprocal plot.

DISCUSSION

HST displays a paradoxical substrate specificity: although the enzyme preferentially targets the alcohol group of natural steroid hormones such as DHEA, testosterone, and progesterone, it catalyzes sulfonation of the hydroxy groups of the synthetic estrogen DES as efficiently as the estrogen sulfotransferase EST. EST is the enzyme specific for sulfating the hydroxy group of natural steroids such as E_2. Apparently, the sulfotransferases have broader substrate specificity for DES as a substrate. Despite lower substrate specificity, however, both EST and HST display high stereospecificity, sulfating only the *trans*-DES isomer. These paradoxical substrate specificities are often observed for enzymes such as cytochromes P450, which are capable of metabolizing endogenous as well as exogenous chemicals [15, 18]. The structural basis for these paradoxical characteristics, however, is not available at the present time. The three-dimensional structures of the sulfotransferases may

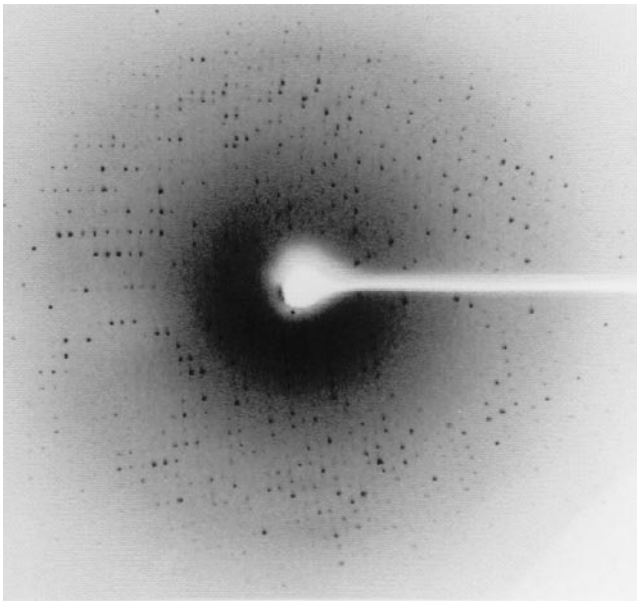

FIG. 4. Diffraction pattern of an EST crystal. This picture represents a 2° oscillation of a crystal of EST with a 30-min exposure.

provide us with a structural clue for understanding these types of paradoxical substrate specificities.

This stereospecificity of EST for DES sulfonation is reminiscent of DES binding to the estrogen receptor [19]. Structurally, *trans*-DES can be superimposed on the E_2 molecule more closely than the *cis*-isomer, and the binding affinity of *trans*-DES to the estrogen receptor is 100-fold greater than that of the *cis*-isomer. Future study will be warranted to see whether a common structural constraint is shared by the substrate binding pocket of EST and the hormone binding domain of the estrogen receptor, and may provide a structural insight into understanding the potentially important roles of EST in endocrine action [1–4]. Sulfated steroids are apparently inactive and, while secreted into urine, are also stored in blood as precursors reactivated by sulfatases. The sulfotransferase-sulfatase system, therefore, is thought to be a mechanism that regulates the levels of steroid hormones. Consequently, disruption of the steroid sulfotransferase activities by drugs and/or synthetic chemicals may elicit serious adverse effects on normal physiology, e.g. altering EST activity by estrogenic xenobiotics such as DES. It is reasonable that these interferences with EST activity may occur at the level of enzyme activity as well as at the level of direct binding competition with the estrogen receptor in target tissues [20].

In conclusion, our present study on the substrate specificities of the sulfotransferases using natural and synthetic steroid hormones has provided the functional insight to understand the role of each sulfotransferase in endocrine action, as well as the alterations of activity that might be caused by xenobiotics. The anticipated three-dimensional structure of EST should provide a structural basis for understanding the paradoxical nature of the substrate specificities of this interesting family of enzymes.

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