Purification and characterization of rat liver minoxidil sulphotransferase

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Minoxidil (Mx), a pyrimidine *N*-oxide, is used therapeutically as an antihypertensive agent and to induce hair growth in patients with male pattern baldness. Mx *NO*-sulphate has been implicated as the agent active in producing these effects. This paper describes the purification of a unique sulphotransferase (ST) from rat liver cytosol that is capable of catalysing the sulphation of Mx. By using DEAE-Sepharose CL-6B chromatography, hydroxyapatite chromatography and ATP-agarose affinity chromatography, Mx-ST activity was purified 240-fold compared with the activity in cytosol. The purified enzyme was also capable of sulphating *p*-nitrophenol (PNP) at low concentrations (less than 10 μ M). Mx-ST was purified to homogeneity, as evaluated by SDS/PAGE and reverse-phase h.p.l.c. The active form of the enzyme had a molecular mass of 66 000–68 000 Da as estimated by gel exclusion chromatography and a subunit molecular mass of 35 000 Da. The apparent K_m values for Mx, 3'-phosphoadenosine 5'-phosphosulphate and PNP were 625 μ M, 5.0 μ M and 0.5 μ M respectively. However, PNP displayed potent substrate inhibition at concentrations above 1.2 μ M. Antibodies raised in rabbits to the pure enzyme detected a single band in rat liver cytosol with a subunit molecular mass of 35 000 Da, as determined by immunoblotting. The anti-(rat Mx-ST) antibodies also reacted with the phenol-sulphating form of human liver phenol sulphotransferase, suggesting some structural similarity between these proteins.

INTRODUCTION

Sulphation is one of the major conjugation reactions involved with the biotransformation of a wide variety of drugs, xenobiotics and endogenous compounds bearing a hydroxy or nitrogen functional group (Mulder, 1981). Conjugation of these compounds with sulphate is catalysed by a family of enzymes termed sulphotransferases (STs). One of the major roles of the STs in drug metabolism is the conversion of a substance into a more soluble, and therefore more readily excretable, form via formation of a sulphate or sulphamate conjugate. 3'-Phosphoadenosine 5'-phosphosulphate (PAPS) serves as the donor of the sulphate moiety for all mammalian STs (Huxtable, 1986). In addition to its role in increasing excretion, conjugation with sulphate can also serve to convert compounds into their biologically active forms (Huxtable, 1986) or into reactive toxic electrophiles (Miller & Miller, 1969; Hanna & Banks, 1985; Huxtable, 1986).

Minoxidil (Mx), a pyrimidine *N*-oxide, is an important therapeutic vasodilating agent for which ST activity has been implicated in conversion of the drug into its biologically active form. The efficacy of Mx as a vasodilating agent has been demonstrated to be dependent upon sulphation at the *N*-oxide position (McCall *et al.*, 1983). Subsequently Waldon *et al.* (1989) reported that the *NO*-sulphate of Mx is also responsible for inducing hair growth in cultured hair follicles. These results suggest that the increase in hair growth observed with patients using topical Mx is a consequence of the synthesis of Mx *NO*-sulphate and its action on hair follicles. Sulphation therefore apparently plays a vital role in the varied pharmacological activities of Mx.

Johnson *et al.* (1982) have reported the presence of Mx-ST activity in the cytosolic fraction of male rat liver. This enzyme activity serves to convert Mx only into the *NO*-sulphate metab-

olite (McCall *et al.*, 1983). Rat liver cytosol was also shown to possess enzyme activities capable of sulphating several other different pyrimidine, pyridine, triazine and imidazole *N*-oxides (McCall *et al.*, 1983). However, identification of specific forms of ST capable of catalysing the sulphation of the *N*-oxide moiety of these compounds has not been reported.

A number of different forms of ST activity capable of catalysing the sulphation of small phenols (Sekura *et al.*, 1981; Borchardt & Schasteen, 1982), steroids (Lyon *et al.*, 1981), bile acids (Chen *et al.*, 1977; Barnes *et al.*, 1989) and *N*-hydroxyarylamines (Wu & Straub, 1976; Sekura & Jakoby, 1981) have been identified and isolated from rat liver cytosol. However, only two of these ST activities have been reported to form *NO*-sulphate esters. Wu & Straub (1976) purified an ST activity from male rat liver cytosol that catalyses the sulphation of *p*-nitrophenol (PNP) and of *N*-hydroxy-2-acetamidofluorene (*N*-OH-2AAF) to the highly reactive product *N*-sulpho-oxy-AAF. Subsequently Sekura *et al.* (1981) identified four forms of aryl-ST (AST) activity, AST I, AST II, AST III and AST IV, in male rat liver cytosol. Each of the forms of AST was active with a wide variety of phenolic compounds, such as β -naphthol and PNP; however, only AST IV was capable of sulphating organic hydroxylamines, including *N*-OH-2AAF (Sekura & Jakoby, 1981). It is not known if either *N*-OH-2AAF-ST or AST IV is capable of conjugating the *N*-oxide moiety of Mx.

The present paper describes the purification and characterization of Mx-ST from male rat liver cytosol. Mx-ST is the major ST in rat liver cytosol responsible for the sulphation of Mx, and the purified enzyme was also capable of sulphating small phenols such as PNP. During the purification process Mx-ST was also resolved from several other ST activities that were active in sulphating PNP but were not capable of conjugating Mx. The data in this paper indicate that Mx-ST represents a unique form

Abbreviations used: Mx, minoxidil; PNP, *p*-nitrophenol; *N*-OH-2AAF, *N*-hydroxy-2-acetamidofluorene; PAPS, 3'-phosphoadenosine 5'-phosphosulphate; Mx-ST, minoxidil sulphotransferase; PNP-ST, *p*-nitrophenol sulphotransferase; *N*-OH-2AAF-ST, *N*-hydroxy-2-acetamidofluorene sulphotransferase; AST, arylsulphotransferase; M-PST and P-PST, monoamine-sulphating and phenol-sulphating forms of phenol sulphotransferase respectively; DHEA-ST, dehydroepiandrosterone sulphotransferase; BAST, bile acid sulphotransferase.

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of ST and possesses different physical, kinetic and immunological properties compared with previously reported forms of rat liver STs capable of sulphating small phenols.

EXPERIMENTAL

Materials

Mx was generously given by The Upjohn Co. (Kalamazoo, MI, U.S.A.), [^{35}S]PAPS (78 $\mu\text{Ci}/\text{mmol}$) was purchased from New England Nuclear (Boston, MA, U.S.A.). PAPS was purchased from Pharmacia (Piscataway, NJ, U.S.A.). DEAE-Sepharose CL-6B, *p*-nitrophenol, ATP-agarose, hydroxyapatite and Sephacryl S-200 were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trifluoroacetic acid was obtained from Pierce Chemical Co. (Rockford, IL, U.S.A.). Acetonitrile (h.p.l.c. grade) was purchased from VWR (Rochester, NY, U.S.A.). Protein A-Sepharose 4B and the Immunoblot Assay Kit were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Isoelectric-focusing gels were purchased from FMC Bioproducts (Rockland, ME, U.S.A.). All other chemicals were of reagent-grade quality.

Purification procedure

Livers were obtained from male or female Sprague-Dawley rats (175–200 g; Charles Rivers Breeders, Portage, MI, U.S.A.) and were stored at -70°C before use. Cytosol was prepared from other male rat tissues as described for preparation of liver cytosol. The purification procedure beyond cytosol preparation was conducted with livers from only male rats, and unless otherwise noted all purification steps were carried out at 4°C .

Step 1. Preparation of rat liver cytosol. Frozen rat livers were thawed in 3 vol. of buffer A [10 mM-Tris/HCl buffer, pH 7.4, containing 0.25 M-sucrose, 1 mM-dithiothreitol and 10% (v/v) glycerol] and homogenized twice for 30 s in a Brinkman Polytron homogenizer. The homogenate was then centrifuged at 12000 *g* for 10 min, and the resulting supernatant fluid was centrifuged at 100000 *g* for 60 min. The low-density material at the top of each tube was removed by filtering the supernatant fluid through several layers of cheesecloth. The filtered liver cytosol preparations were stored at -70°C and used within 6 weeks. Protein determinations were carried out by using the Bio-Rad protein assay originally described by Bradford (1976), with BSA as a standard.

Step 2. DEAE-Sepharose chromatography. Rat liver cytosol was thawed on ice, centrifuged at 10000 *g* to remove any precipitated protein and applied to a DEAE-Sepharose CL-6B column (2.5 cm $\times$ 10 cm) that had been previously equilibrated in buffer A. The anion-exchange column was then washed with 50 ml of buffer A, followed by 130 ml of buffer A containing 50 mM-NaCl. Next, Mx-ST activity was eluted with a linear gradient established between 300 ml of buffer A containing 50 mM-NaCl and 300 ml of buffer A containing 200 mM-NaCl. Fractions (7.5 ml) were collected, and Mx-ST and PNP-ST activities were determined. Fractions containing high levels of Mx-ST activity were pooled and concentrated approximately 3-fold by ultrafiltration on an Amicon PM-30 membrane. The concentrated enzyme preparation was then dialysed overnight against two 2-litre volumes of buffer B [5 mM-sodium phosphate buffer, pH 6.8, containing 0.25 M-sucrose, 1 mM-dithiothreitol and 10% (v/v) glycerol].

Step 3. Hydroxyapatite chromatography. After dialysis, Mx-ST activity was applied to a hydroxyapatite column (2.5 cm $\times$ 3 cm) that had been previously equilibrated in buffer B without glycerol.

The column was washed with 20 ml of the same buffer, and Mx-ST activity was eluted with a linear gradient established between 150 ml of buffer B without glycerol and 150 ml of 150 mM-potassium phosphate buffer, pH 6.8, both containing 0.25 M-sucrose and 1 mM-dithiothreitol. Fractions (3.75 ml) were collected and Mx-ST and PNP-ST activities were determined.

Step 4. ATP-agarose affinity chromatography. Fractions from the hydroxyapatite column containing Mx-ST activity were combined and concentrated approximately 8-fold by ultrafiltration on an Amicon PM-30 membrane. Mx-ST activity was then applied at 25°C to an ATP-agarose column (1.3 cm $\times$ 3.5 cm) that had been previously equilibrated in buffer C [10 mM-sodium phosphate buffer, pH 6.8, containing 0.25 M-sucrose, 1 mM-dithiothreitol and 10% (v/v) glycerol]. The affinity column was washed with 25 ml of buffer C. Mx-ST activity was then specifically eluted with a linear gradient established between 10 ml of buffer C and 10 ml of buffer C containing 10 μM -PAPS. Fractions (approx. 1.3 ml) were collected and assayed for Mx-ST and PNP-ST activities.

Enzyme assays

Mx-ST activity was assayed by using the extraction procedure for Mx *NO*-sulphate described by Johnson & Baker (1987). The Mx-ST reaction mixture contained 240 mM-Hepes/NaOH buffer, pH 7.3, 1 mM-Mx and 7 mM-dithiothreitol. Reactions were started by the addition of [^{35}S]PAPS to a final concentration of 29 μM and the mixture was incubated for 10 min at 37°C . The optimum pH for Mx-ST activity was 7.3 in Hepes buffer. Reactions were terminated by the addition of 1 ml of ethyl acetate to the reaction mixture, which was immediately placed on ice, and this was followed by 0.4 ml of 1 M- NH_3 to alkalize the solution. The reaction mixture was vortex-mixed for 15 s, then centrifuged at 800 *g* to separate the organic and aqueous phases, and the radioactivity in 0.5 ml of the ethyl acetate phase was quantified by scintillation spectrometry. This extraction takes advantage of the unusual hydrophobicity of Mx *NO*-sulphate, which is more soluble in organic solvents than in water (McCall *et al.*, 1983).

PNP-ST activity was assayed by using the barium precipitation method of Foldes & Meek (1973). The optimum pH for PNP sulphation catalysed by Mx-ST was 6.5–6.8 in triethanolamine buffer. The reaction mixture contained 50 mM-triethanolamine/HCl buffer, pH 6.8, 1.2 μM -PNP and enzyme. Reactions were initiated by the addition of [^{35}S]PAPS to a final concentration of 20 μM , in a final reaction volume of 0.25 ml. Reactions were terminated by the addition of 50 μl each of 0.1 M solutions of barium acetate, $\text{Ba}(\text{OH})_2$ and ZnSO_4 . The resulting precipitate was removed by centrifugation at 1500 *g* for 5 min, and the supernatant was transferred to new microcentrifuge tubes, whereupon the reaction mixture was reprecipitated with barium acetate, $\text{Ba}(\text{OH})_2$ and ZnSO_4 . The resulting precipitate was then removed by centrifugation at 12000 *g* for 3 min and the radioactivity in 250 μl of the supernatant fluid was determined by scintillation spectrometry. ST assays with both Mx and PNP were linear with respect to time and protein concentration.

SDS/PAGE

SDS/PAGE was performed in the presence of SDS with the use of a Bio-Rad Protean II unit and the buffer system described by Laemmli (1970). Samples were pretreated with the addition of 2-mercaptoethanol and SDS to final concentrations of 5% (v/v) and 1% (w/v) respectively, and then heated at 100°C for 10 min. After electrophoresis, the gels were stained with Coomassie Blue R-250 as previously described by Falany & Tephly (1983). Minimum subunit molecular masses were estimated by

comparison with commercial molecular-mass standards (Sigma Chemical Co.).

Sephacryl S-200 chromatography

Gel exclusion chromatography was used to estimate the molecular mass of the active form of Mx-ST. Concentrated Mx-ST activity obtained after the hydroxyapatite chromatography procedure was applied to a Sephacryl S-200 gel-filtration column (2.5 cm $\times$ 48 cm) that had been previously equilibrated in buffer A. Mx-ST was then eluted from the column with buffer A, and 1.5 ml fractions were collected. The elution of Mx-ST was compared with the elution of commercial molecular-mass standards (Sigma Chemical Co.).

Immunization protocol

Polyclonal antibodies were raised against purified Mx-ST in a female New Zealand White rabbit. The initial challenge consisted of 50 μ g of pure Mx-ST in complete Freund's adjuvant injected into eight subcutaneous sites along the shaved back of the animal. The rabbit was boosted 2 weeks later with another 50 μ g of Mx-ST in incomplete Freund's adjuvant. The rabbit was then injected with 30 μ g of Mx-ST 6 weeks later, followed by an additional 30 μ g of protein 4 weeks later. The rabbit was bled 2 weeks after each injection and the sera were tested for immunoreactivity by using an immunoblotting protocol.

Immunoblot procedure

Proteins were resolved by SDS/PAGE in a 12.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose paper by means of a Bio-Rad Transblot apparatus and the buffer system described by Towbin *et al.* (1979). Gelatin (3%, w/v) in Tris-buffered saline (500 mM-NaCl/20 mM-Tris/HCl buffer, pH 7.5) was used for blocking of the nitrocellulose, and incubations in primary antibody were carried out overnight at 28 °C with gentle shaking in Tris-buffered saline containing 0.1% Tween-20 and 1% gelatin. A 1:5000 dilution of rabbit anti-(rat Mx-ST) IgG fraction (0.5 μ g of protein/ μ l before dilution) was normally used as primary antibody. The IgG fraction of the antiserum was prepared by Protein A-Sepharose 4B chromatography by the method of Harlow & Lane (1988). The Bio-Rad Immunoblot Assay Kit containing goat anti-(rabbit IgG) antibody-alkaline phosphatase conjugate as the secondary antibody was used for detection of the immunoconjugates.

Amino acid analysis

Mx-ST was further purified by h.p.l.c. in preparation for amino acid analysis. The affinity-purified enzyme was applied to a Vydac C₁₈ column (46 mm $\times$ 250 mm) and eluted with a linear gradient of 0–80% (v/v) acetonitrile in the presence of 0.1% (v/v) trifluoroacetic acid. Elution of protein from the C₁₈ column was monitored by use of a Perkin-Elmer diode-array detector at wavelengths of 215 nm and 280 nm. After h.p.l.c., Mx-ST was frozen at –70 °C and the amino acid composition analyses were performed by the Protein Structure Facility of the University of Iowa with a Beckman 6300 amino acid analyser.

Isoelectric focusing

Isoelectric focusing of affinity-purified Mx-ST was carried out with Isogel Agarose pH 3–7 isoelectric-focusing plates (FMC Bioproducts). Isoelectric focusing was performed for 45 min at 10 W constant power and the pI of Mx-ST was determined by comparison with commercial pI 2.5–6.5 markers (Pharmacia). The presence of electrofocused Mx-ST protein was detected by staining of the isoelectric-focusing plates with Coomassie Blue R-250.

Table 1. Mx-ST and PNP-ST activities in liver cytosol from male and female rats

Cytosol was prepared from livers of Sprague-Dawley rats (175–200 g) as described in the Experimental section. ST activity with assay concentrations of 1 mM-Mx and 1.2 μ M-PNP was determined in the individual preparations. Specific activity is reported as the mean ST activity $\pm$ S.E.M. from five different preparations, assayed in triplicate. One unit of activity is defined as 1 nmol of substrate converted into product/min.

Source of liver cytosol	Mx-ST activity (units/mg of protein)	PNP-ST activity (units/mg of protein)
Male rats	0.63 $\pm$ 0.09	0.56 $\pm$ 0.06
Female rats	0.29 $\pm$ 0.07	0.26 $\pm$ 0.03

RESULTS

Purification of Mx-ST

Table 1 shows the comparison of the rates of sulphation of Mx and PNP in cytosol prepared from male and female rat livers. The specific activities in male rat liver cytosol for the sulphation of Mx and PNP with final reaction concentrations of 1 mM and 1.2 μ M respectively are approx. 2-fold greater than those observed in female rat liver cytosol. Mx and PNP reaction concentrations were the concentrations giving maximal sulphation rates with affinity-purified Mx-ST. When Mx-ST and PNP-ST activities were determined in cytosol prepared from male rat liver, kidney, intestine and brain, detectable levels of PNP-ST activity were present in all the tissues (results not shown). However, measurable levels of Mx-ST activity were detectable only in liver. Consequently Mx-ST was purified from male rat liver cytosol.

Fig. 1 illustrates the resolution of Mx-ST and PNP-ST activities during the DEAE-Sepharose CL-6B chromatography procedure. Mx-ST activity was eluted during the NaCl gradient at approx.

Fig. 1. DEAE-Sepharose chromatography of Mx-ST and PNP-ST activities in rat liver cytosol

Male rat liver cytosol (1200 mg) was applied to a DEAE-Sepharose CL-6B column (2.5 cm $\times$ 10 cm) that had been equilibrated in buffer A. The column was then washed with 50 ml of buffer A, followed by 150 ml of buffer A containing 50 mM-NaCl. Mx-ST activity was subsequently eluted with a linear gradient (—) consisting of 300 ml of buffer A and 300 ml of buffer A containing 200 mM-NaCl. Fractions (7.5 ml) were collected and Mx-ST activity (■) and PNP-ST activity (○) were determined using assay concentrations of 1 mM-Mx and 1.2 μ M-PNP respectively.

Table 2. Purification of Mx-ST from liver cytosol from male rats

Cytosol was prepared from male Sprague-Dawley rat livers and the purification was conducted as described in the Experimental section. One unit of enzyme activity represents 1 nmol of substrate conjugated/min.

Step	Protein (mg)	Total activity (units)		Specific activity (units/mg)		Mx-ST/PNP-ST specific activity ratio	Purification of Mx-ST activity (fold)
		Mx-ST	PNP-ST	Mx-ST	PNP-ST		
Cytosol	1240	645	1761	0.52	1.42	0.37	1.00
DEAE-Sepharose chromatography	43.2	174	74.7	4.02	1.73	2.32	7.73
Hydroxyapatite chromatography	9.17	19.7	11.9	2.15	1.30	1.65	4.13
ATP-agarose chromatography	0.25	30.7*	1.02†	123	40.6	3.03	237

* Represents 4.76 % recovery of Mx-ST total activity after the final step in the purification procedure.

† Represents 0.06 % recovery of PNP-ST total activity after the final step in the purification procedure.

Fig. 2. Resolution of Mx-ST and PNP-ST activities during hydroxyapatite chromatography

Mx-ST and PNP-ST activities obtained from the DEAE-Sepharose CL-6B procedure were concentrated by ultrafiltration, dialysed and applied to a hydroxyapatite column (2.5 cm × 3 cm) that had been equilibrated in buffer B without glycerol. The column was then washed with 20 ml of the same buffer, and the ST activities were eluted with a linear gradient (300 ml total volume; —) between this buffer and 150 mM-potassium phosphate buffer, pH 6.8, containing 0.25 M-sucrose and 1 mM-dithiothreitol. Fractions (approx. 3.8 ml) were collected and Mx-ST (■) and PNP-ST (○) activities were assayed as described for Fig. 1.

115 mM-NaCl. PNP-sulphation activity was eluted during the anion-exchange procedure as two major peaks at about 75 mM-NaCl and 105 mM-NaCl and a minor peak at 55 mM-NaCl. Fractions containing high levels of Mx-ST activity and relatively low levels of PNP-ST activity were combined. With respect to the two enzyme activities in rat liver cytosol, the DEAE-Sepharose chromatography procedure gave a 7.7-fold purification of Mx-ST activity in comparison with only a 1.2-fold purification of PNP-ST activity (Table 2).

Fig. 2 shows the elution of Mx-ST activity during hydroxyapatite chromatography. All of the Mx-ST activity applied to the hydroxyapatite column was adsorbed on the resin and it was eluted with approx. 115 mM-potassium phosphate buffer. Fig. 2 also illustrates that hydroxyapatite chromatography was effective in resolving other PNP-ST activities from the Mx-ST activity; however, some PNP-ST activity was still co-eluted with Mx-ST activity. Although the procedure resulted in a decrease in the purification of Mx-ST and PNP-ST activities as compared with cytosol (Table 2), hydroxyapatite chromatography was essential for obtaining pure Mx-ST.

Fig. 3. Elution of Mx-ST and PNP-ST activities during ATP-agarose chromatography

Mx-ST activity from the hydroxyapatite procedure was concentrated by ultrafiltration and applied to a column of ATP-agarose (1.3 cm × 3.5 cm) that had been equilibrated with buffer C. The column was then washed with 25 ml of the same buffer, and Mx-ST activity was eluted with a 20 ml linear gradient of 0–10 μ M-PAPS in the same buffer. The arrow indicates the beginning of the PAPS gradient. Fractions (1.3 ml) were collected and ST activity was measured with 1.0 mM-Mx (■) and 1.2 μ M-PNP (○) as described in the Experimental section.

Mx-ST was subsequently purified to homogeneity by using ATP-agarose affinity chromatography (Fig. 3). Most of the applied Mx-ST activity was retained on the affinity column and was specifically eluted as a sharp peak of activity at approx. 5 μ M-PAPS during a 0–10 μ M-PAPS gradient. Fig. 3 also shows that some PNP-ST activity was co-eluted with Mx-ST activity during the affinity-chromatography procedure. The results of a typical purification procedure are presented in Table 2. After the affinity-chromatography procedure, Mx-ST activity and its associated PNP-sulphation activity were purified 237-fold and 28-fold respectively, and with a recovery of 4.8 % of the initial Mx-ST activity. Also, more Mx-ST activity was obtained after the affinity-chromatography procedure than was obtained after hydroxyapatite chromatography. These results suggest that Mx-ST activity was inhibited under the conditions used to perform the hydroxyapatite chromatography, and this inhibition may be the reason for the decrease in purification observed after this procedure (Table 2).

Although purified Mx-ST was capable of sulphating PNP, the ATP-agarose chromatography procedure was also capable of resolving another form of PNP-ST activity from Mx-ST. This is shown by the increase achieved in the ratio of the specific activity of Mx-ST to that of PNP-ST activity after affinity chromatography (Table 2). Furthermore, when PNP-ST activity was

Fig. 4. Effect of PNP concentration on PNP-ST activity after hydroxyapatite and affinity chromatography

PNP-ST activity was assayed with various concentrations of PNP in fractions from the hydroxyapatite (○) and ATP-agarose (■) chromatography procedures that contained high levels of Mx-ST activity.

assayed in fractions from the hydroxyapatite column that contained Mx-ST activity, a biphasic pattern of activity was observed with increasing PNP concentrations (Fig. 4). However, when affinity-purified Mx-ST was assayed for activity with increasing concentrations of PNP, substrate inhibition was noted above micromolar PNP concentrations, and increasing PNP-ST activity was not observed at the higher PNP concentrations. Nakamura *et al.* (1987) have also observed a similar biphasic pattern of PNP-ST activity with increasing PNP concentrations in impure preparations of rat liver AST activity.

Fig. 5 illustrates the stepwise purification of Mx-ST as demonstrated by SDS/PAGE of samples obtained during the purification procedure. Analysis of affinity-purified Mx-ST by

Fig. 5. SDS/PAGE of Mx-ST

(a) Samples of Mx-ST activity from each stage of the purification procedure were resolved by SDS/PAGE in a 10.5% polyacrylamide gel and stained with Coomassie Blue as described in the Experimental section. The lanes contain: STD, glyceraldehyde-3-phosphate dehydrogenase (36000 Da), 5 μg; RLC, rat liver cytosol, 50 μg; DEAE, DEAE-Sepharose fraction, 100 μg; HA, hydroxyapatite fraction, 1 μg; AFF, ATP-agarose fraction, 3 μg. (b) A 1 μg portion of affinity-purified Mx-ST (lane A) and commercial molecular-mass standards (lane B) were resolved by SDS/PAGE in a 12.5% polyacrylamide gel and stained with Coomassie Blue. The standards included: phosphorylase *b*, 97400 Da; BSA, 66200 Da; ovalbumin, 45000 Da; carbonic anhydrase, 31000 Da; soya-bean trypsin inhibitor, 21500 Da; lysozyme, 14400 Da.

SDS/PAGE revealed that Mx-sulphation activity co-purified with a single protein that migrated with a subunit molecular mass of approx. 35000 Da. The molecular mass of active Mx-ST was also estimated by gel exclusion chromatography. Mx-ST activity was eluted during a Sephacryl S-200 gel exclusion chromatographic procedure with a molecular mass between 66000 and 68000 Da as compared with elution of molecular-mass standards (results not shown). Comparison of the subunit size to the active form of the enzyme suggests that Mx-ST exists as a homodimer in its active form. Several other rat liver STs have also been reported to exist as homodimers in their active forms (Wu & Straub, 1976; Sekura *et al.*, 1981).

Analysis of affinity-purified Mx-ST by h.p.l.c. using a reverse-phase C_{18} column (Vydac) revealed that the protein was eluted as a single peak at approx. 53% acetonitrile in the presence of 0.1% trifluoroacetic acid with absorbance at both 280 nm and 215 nm. No other major peaks absorbing at both wavelengths were observed to be eluted during the h.p.l.c. procedure, nor were any proteins other than Mx-ST detected by SDS/PAGE of the h.p.l.c. fractions. In addition, when subjected to analytical isoelectric focusing between pH 2.5 and 6.5, affinity-purified Mx-ST was found to migrate as a single distinct band with a pI of 4.8 (result not shown).

Immunological characterization

Immunoblot analysis of male and female rat liver cytosol with rabbit anti-(rat Mx-ST) IgG detected a single species of immuno-

Fig. 6. Immunoblot analysis of rat liver Mx-ST

Rabbit anti-Mx-ST IgG was used to detect Mx-ST in different rat tissues. Affinity-purified Mx-ST (2 μg) and cytosol (100 μg) prepared from male rat liver (mRLC), female rat liver (fRLC), kidney, small intestine lung and brain were resolved by SDS/PAGE in a 12.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose with the use of the buffer system described by Towbin *et al.* (1979). A 1:5000 dilution of rabbit anti-Mx-ST IgG was used as primary antibody and the immunoconjugates were detected with goat anti-(rabbit IgG) antibody-alkaline phosphatase conjugate second antibody as described in the Experimental section.

Fig. 7. Immunoreactivity of Mx-ST with anti-(human PST) serum

Mx-ST (2 μ g; lane A) and male rat liver cytosol (100 μ g; lane B) were resolved by SDS/PAGE in a 12.5% polyacrylamide gel and electrophoretically transferred to a nitrocellulose filter. Immuno-reactive proteins were detected with a 1:10000 dilution of rabbit anti-(human PST) serum and the immunoconjugates were detected with goat anti-(rabbit IgG) antibody-alkaline phosphatase conjugate second antibody as described in the Experimental section.

reactive protein with the same molecular mass as Mx-ST in both cytosolic preparations (Fig. 6). The relative level of immunoreactivity was greater in male cytosol than in female cytosol, and this result was consistent with the observation that male rat liver possessed about 2-fold greater Mx-ST activity than female liver (Table 1). In comparison with liver, Fig. 6 also shows no detectable immunoreactivity in cytosolic preparations of several male rat tissues that did not possess detectable Mx-ST activity.

Our laboratory has previously reported the characterization of an antibody raised against the monoamine-sulphating form of human platelet phenol ST, M-PST (Heroux *et al.*, 1989). This antibody reacts with both the monoamine (M)- and phenol (P)-sulphating forms of PST, but not with DHEA-ST, a steroid ST purified from human liver (Falany *et al.*, 1989). To investigate the immunological relationship between rat Mx-ST and human liver STs, Mx-ST was tested for cross-reactivity with the rabbit anti-(human PST) antibody. Fig. 7 shows that affinity-purified Mx-ST was recognized by the anti-(human PST) antibody during an immunoblotting procedure. However, several other proteins in male rat liver cytosol were also reactive with the anti-(human PST) serum. The identities of the other cross-reactive proteins in rat liver cytosol are not known but may represent other forms of ST. Conversely, a 1:5000 dilution of rabbit anti-(rat Mx-ST) IgG was also capable of reacting with the purified human liver P-PST. However, the rabbit anti-Mx-ST antibody also recognized purified human DHEA-ST during the immunoblotting procedure (result not shown).

Kinetics

Substrate inhibition of Mx-ST activity was observed when reaction concentrations above 1.2 μ M-PNP (Fig. 4) or 1 mM-Mx were used. Therefore assays were conducted at substrate concentrations that gave maximal rates of sulphation of Mx and PNP. The apparent K_m values for Mx and PNP sulphation catalysed by purified Mx-ST were 625 μ M and 0.5 μ M respectively, as estimated by the method of Cleland (1979) for enzymes demonstrating substrate inhibition. The apparent K_m for PAPS with

Fig. 8. Inhibition of Mx-ST activity by PNP

Affinity-purified Mx-ST was assayed for Mx-sulphation activity with 1 mM-Mx in the presence of increasing concentrations of PNP as described in the Experimental section.

1 mM-Mx as sulphate acceptor was approx. 5 μ M, and inhibition of Mx-ST activity with concentrations of PAPS up to 30 μ M was not observed. The PNP-sulphation activity of Mx-ST exhibited a pH maximum of 6.5–6.8, whereas only 43% of the maximum activity was observed at pH 5.5. Sekura *et al.* (1981) reported a pH maximum of 5.5 for β -naphthol sulphation catalysed by rat liver AST IV, another ST capable of sulphating *N*-hydroxy compounds.

Fig. 8 shows that the addition of PNP to Mx-ST reaction mixtures resulted in a potent inhibition of Mx sulphation. Mx sulphation catalysed by affinity-purified Mx-ST was inhibited by

Table 3. Amino acid compositions of Mx-ST, AST IV, AST I and BAST I

For Mx-ST 24 h, 48 h and 72 h HCl hydrolyses were performed and the amino acid composition was calculated from a subunit molecular mass of 35000 Da. Performic acid oxidation was carried out to determine cysteine and methionine residues. Abbreviation: N.D., not determined.

Amino acid	Amino acid composition (mol of residue/mol)			
	Mx-ST	AST IV*	AST I†	BAST I‡
Cys	5	N.D.	N.D.	N.D.
Asx	28	31.5	32.5	27
Glx	31	28.5	25	23
Ser	16	13	15	19
Gly	20	20	16.5	21
His	9	7.5	10	5
Arg	13	11	10	8
Thr	22	12	16	17
Ala	13	16.5	15.5	10
Pro	23	23	17	15
Tyr	14	10	13	11
Val	16	15.5	17	13
Met	13	8	8	11
Ile	15	15	12	9
Leu	28	21.5	23.5	30
Phe	17	13	14	20
Lys	25	24.5	32	27
Trp	N.D.	N.D.	5.5	N.D.
Total	308	271.5	282.5	266

* From Sekura & Jakoby (1981).

† From Sekura & Jakoby (1979).

‡ From Barnes *et al.* (1989).

approx. 55 % by the presence of 0.5 μ M-PNP. These results are consistent with the apparent sulphation of PNP by Mx-ST. However, the exact mechanism by which PNP inhibits Mx-ST activity was not determined because of the substrate inhibition observed with both PNP and Mx.

Amino acid composition

The amino acid composition of Mx-ST was determined for comparison with the reported compositions of other rat liver STs. Table 3 shows the number of residues estimated for each amino acid present in Mx-ST in comparison with the reported amino acid compositions of several other rat STs. The composition of Mx-ST appears to be most similar to that reported for AST IV; however, significant differences are observed in the numbers of methionine, threonine and leucine residues between these enzymes. The composition of Mx-ST differs the most from that for the rat liver bile acid ST (BAST), the one ST that does not sulphate small phenolic compounds.

Discussion

This paper describes the purification and characterization of Mx-ST from male rat liver cytosol. The enzyme preparation was homogeneous as evaluated by SDS/PAGE, isoelectric focusing and h.p.l.c. analysis. An overall purification of approx. 240-fold was achieved and the pure enzyme had a specific activity of approx. 120 nmol of Mx and 41 nmol of PNP sulphated/min per mg of protein. Mx-ST therefore represents approx. 0.4 % of rat cytosolic protein and the PNP-ST activity of the pure enzyme represents about 11 % of the cytosolic PNP-ST activity. The fold purification obtained for purified Mx-ST is slightly lower than the purifications (280–610-fold) reported for other male rat liver STs (Sekura *et al.*, 1981; Borchardt & Schasteen, 1982), and the specific activity of purified Mx-ST for PNP sulphation was lower than that reported for AST I, AST II and AST IV, but higher than for the PST purified by Borchardt & Schasteen (1982).

In rat liver Mx-ST appears to be related to a family of STs that are capable of sulphating small phenols such as PNP. Several rat STs that have been reported to catalyse PNP sulphation have been purified and characterized. These include AST I, AST II, AST III and AST IV (Sekura *et al.*, 1981), *N*-OH-2AAF-ST (Wu & Straub, 1976) and a different rat liver PST isolated by Borchardt & Schasteen (1982). Of these enzymes the only STs reported to be active with *N*-hydroxy compounds are *N*-OH-2AAF-ST and AST IV. It is not known if either of these enzymes can sulphate compounds at an *N*-oxide moiety. The possibility that AST IV and *N*-OH-2AAF-ST are the same enzyme as Mx-ST is unlikely, since the differences in kinetic properties, subunit molecular masses, isoelectric points, amino acid compositions and immunological properties between these enzymes and Mx-ST indicate that Mx-ST is a unique form of ST. Therefore multiple forms of ST are present in rat liver that are capable of forming *NO*-sulphates.

The phenol-sulphating activity of Mx-ST may not have been detected previously because the potent substrate inhibition observed at relatively low PNP concentrations may have masked its PNP-ST activity if relatively high assay concentrations of PNP (above 10 μ M) were used. Previous investigators have used PNP concentrations greater than 20 μ M to assay PNP-sulphation activity in ST preparations (Wu & Straub, 1976; Sekura & Jakoby, 1979, 1981; Borchardt & Schasteen, 1982). Also, the PNP concentration for maximal activity with Mx-ST is less than the K_m values for PNP sulphation reported for previously purified rat STs (Sekura & Jakoby, 1979, 1981; Borchardt *et al.*, 1982).

Johnson *et al.* (1982) reported that a number of *N*-oxide compounds are capable of being sulphated by rat liver cytosol. It

is not known how many STs are involved in the conjugation of these compounds; however, the data in the present paper demonstrate that Mx-ST is responsible for most of the sulphation of the *N*-oxide moiety of Mx in rat liver cytosol. An interesting feature of Mx is that the *NO*-sulphate is more lipophilic than the parent compound (McCall *et al.*, 1983). This suggests that sulphation of minoxidil may increase the half-life of the active form of Mx in the body. Since Mx-ST activity was only detected in liver, the liver may serve as a source for maintaining circulating and tissue concentrations of Mx *NO*-sulphate. It is not known if tissues other than liver that are responsive to Mx *NO*-sulphate, such as smooth muscle (Meisner & Cipkus, 1987), possess Mx-ST activity or respond to circulating concentrations of Mx *NO*-sulphate. Also, it is not known if Mx-ST has a physiological role in the activation via sulphation of endogenous compounds similar to Mx.

Mx is sulphated in human liver by the phenol-sulphating form of phenol ST, P-PST (Falany & Kerl, 1990). Rat liver Mx-ST is similar to P-PST in that both enzymes sulphate PNP in the micromolar range, as well as Mx. P-PST and Mx-ST also share immunological similarity, since anti-Mx-ST antibodies recognize P-PST and rabbit anti-(human P-PST) antibodies recognize Mx-ST. These similarities suggest that sulphation of Mx in rats may provide a model system for Mx sulphation in humans. However, in contrast with rats, P-PST is present in many different human tissues, including liver (Falany *et al.*, 1989), platelets (Hart *et al.*, 1979; Anderson *et al.*, 1981), intestine (Bostrum & Wengle, 1967; Wong, 1976) and brain (Rein *et al.*, 1984). Furthermore, the ability to sulphate an *N*-oxide suggests the possibility that Mx-ST may be capable of sulphating other *N*-hydroxy compounds or carcinogenic substrates such as 1'-hydroxysafrole and *N*-OH-2AAF. Studies on the further characterization and localization of Mx-ST will aid in our understanding of the role of sulphation in the pharmacology of *N*-oxide compounds.

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