Vol. 191, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1993 Pages 295-301

cDNA EXPRESSION STUDIES OF RAT LIVER ARYL SULPHOTRANSFERASE

Debra Cruickshank[1], Lloyd N. Sansom[1], Maurice E. Veronese[2], Behnaz Mojarrabi[2], Michael E. McManus[3] and Xiaoyi Zhu[3]

[1]School of Pharmacy, University of South Australia, North Terrace, Adelaide, 5000, Australia

[2]Department of Clinical Pharmacology, School of Medicine, Flinders University of South Australia, GPO Box 2100, Adelaide, 5001, Australia

[3]Department of Physiology and Pharmacology, University of Queensland, 4072, Australia

Received January 19, 1993

SUMMARY: A cDNA encoding an isoenzyme of rat liver aryl sulphotransferase was isolated from a rat liver bacteriophage Lambda gt 11 library by the polymerase chain reaction technique. The resulting cDNA was functionally expressed in COS-7 cells and characterised by determining the sulphating capacity of the cells with a range of substrates. The COS-expressed enzyme catalysed the sulphation of both phenol and dopamine with K_ms of the same order as those obtained for the high affinity isozyme in rat liver cytosol, while low activity was observed with tyrosine methyl ester. The common food additive vanillin was also a good substrate for sulphate conjugation. The sulphation of vanillin catalysed by the COS-expressed enzyme was consistent with a single enzyme system, in contrast, the kinetics of the reaction catalysed by cytosolic sulphotransferase indicated that vanillin was sulphated by more than one isozyme.

Sulphate conjugation is an important pathway in the metabolism of a range of xenobiotics and also for a number of endogenous substances such as catecholamine neurotransmitters, steroid hormones and bile acids (1). The aryl sulphotransferases catalyse the transfer of a sulphate group from the co-substrate, 3-phosphoadenosine-5-phosphosulphate (PAPS), to the hydroxyl group of phenolic compounds or hydroxylamines. Commonly, the resulting sulphate ester is biologically inactive and is excreted in the urine (2). However, for some compounds, such as N-hydroxy-2-acetylaminofluorene, sulphate conjugation can lead to the formation of reactive or mutagenic intermediates that may result in increased toxicity or carcinogenicity (3).

Five isoenzymes of aryl sulphotransferase have been identified from rat liver and are referred to as aryl (phenol) sulphotransferases I-IV (PST I-IV) and paracetamol sulphotransferase.

ABBREVIATIONS: PAPS, 3-phosphoadenosine-5-phosphosulphate; PST, phenol sulphotransferase; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium.

0006-291X/93 $4.00

Sulphotransferases I, II, IV and paracetamol sulphotransferase have been purified by affinity chromatography and subsequently characterised (4, 5, 6). However, the purity of these proteins, that have similar molecular weights, is difficult to assess, and the purification process may adversely affect their stabilities, catalytic activities, and substrate specificity. These problems associated with purified enzymes and their characterisation can now be circumvented using the molecular biological approach of isolating cDNAs encoding individual forms of sulphotransferases and expressing them in cell culture. To date, four rat sulphotransferases have been isolated (7,8,9,10) but only one has been expressed in cell culture and functionally characterised (8).

In this report, we describe the isolation of a cDNA encoding an isoenzyme of rat liver aryl sulphotransferase in COS-7 cells and the capacity of the COS-expressed enzyme to sulphate the classic substrates phenol, dopamine, tyrosine methyl ester and the common phenolic food additive vanillin.

MATERIALS AND METHODS

Materials: Dopamine was obtained from Calbiochem (La Jolla, CA), phenol from May and Baker (Dagenham, England) and tyrosine methyl ester and adenosine 3-phosphate 5-phosphosulphate (PAPS) from Sigma (St. Louis, MO). [^{35}S]PAPS was purchased from DuPont (Wilmington, DE) and ACS II aqueous counting scintillant from Amersham (Arlington Hts., IL). Restriction enzymes and other reagents used in molecular biology techniques were obtained from New England Biolabs, Pharmacia LKB Biotechnology or IBI Biochemicals. Taq polymerase and PCR buffer were purchased from Bresatec (Adelaide, Australia). The pBluescript II plasmid and XL-1 Blue cells were from Stratagene (La Jolla, CA).

Preparation of rat liver cytosol: Livers from six male Hooded Wistar rats (200g) were homogenised in 10mM triethanolamine, pH 7.5, containing 10% glycerol and 5mM 2-mercaptoethanol. The homogenate was centrifuged at 13000g for 20 min. The supernatant was re-centrifuged in a Beckman XL80 ultracentrifuge at 50000g for 50 min to sediment the microsomes. Aliquots of the resulting supernatant were stored at -70°C.

Amplification of DNA by PCR: A rat liver cDNA library constructed in bacteriophage Lambda gt 11 (a kind gift of Dr. F.J.Gonzalez, NIH, Bethesda, MD) was used as a template for PCR amplification. Two oligonucleotide primers were synthesised corresponding to the 3' and 5' flanking regions of the DNA encoding rat liver aryl sulphotransferase PST-1. The primers were selected on the basis of the previously reported cDNA sequence for this enzyme (7). The 5' and 3' primers were (5'-TCGAATTCCCAGGATCAGCAACATGG-3') and (5'-CCATCGATCACTCA-TAGTTCACAACG-3'), respectively.

The PCR reaction was performed in a reaction volume of 100µl using 100ng of Lambda gt 11 cDNA, primer concentrations of 4ng/µl, 400µM of each of the deoxynucleoside triphosphates, 2mM $MgCl_2$, 0.1 units of Taq polymerase and 1xPCR reaction buffer (Bresatec, Adelaide, Australia). A Perkin-Elmer Cetus DNA thermal cycler machine was used for the reaction at an initial heat denaturation temperature of 94°C for 5 min, then 30 cycles at an annealing temperature of 55°C for 1 min, a polymerisation temperature of 72°C for 1 min and a denaturation temperature of 94°C for 1 min. A final polymerisation temperature of 72°C for 20 min was used following completion of the 30 cycles. An aliquot (5µl) from each of the completed PCR reactions was subjected to electrophoresis in a 1% agarose gel and the product visualised following ethidium bromide staining and UV transillumination. The ~900bp product was purified from a 1.5% low melting point agarose gel, digested with EcoRI and ClaI and ligated into the corresponding EcoRI and ClaI site of pBluescript II (SK+) vector and transformed into XL-1

Blue cells. Positive clones were purified and identified by sequencing using the Taq Dye Deoxy Terminator Cycle Sequencing method (11,12) and analysed on an Applied Biosystems Model 373A DNA Sequencing System (Foster City, CA). The nucleotide sequence differed to that published by Ozawa *et al.* (7) by a single nucleotide (G instead of A at position 350 of the published sequence). However, this nucleotide difference did not alter the amino acid sequence.

Expression of PST-1 in COS-7 cells: The cDNA was excised form the pBluescript construct with EcoRI and SalI and ligated into the EcoRI and SalI digested mammalian COS expression vector, pCMV5 (13), and transformed into competent *E.coli* HB101 cells. Plasmid preparations containing the cDNA insert were then prepared by the alkaline lysis method followed by two caesium chloride gradient density centrifugations.

Purified plasmid DNA was transfected into COS-7 cells using the DEAE-dextran protocol and chloroquine treatment as previously described (14). COS-7 cells were plated in 175cm^3 cell culture flasks and grown overnight in Dulbecco's modified Eagle's medium (DMEM) with 10% Nuserum (Collaborative Research Incorporated, Bedford, MA) (pH 7.2) until 70-80% confluent. The media was removed and 20µg of purified plasmid DNA was added in DMEM (pH 7.6) containing DEAE dextran (0.5mg/ml). Mock transfected cells were prepared by transfecting with the COS vector that did not contain any insert. After incubating for approximately 1 hour, the DEAE dextran solution was replaced with DMEM with 10% Nuserum (pH 7.2) containing 100µ M chloroquine and incubated for a further 4 hours. Cells were then grown in DMEM with 10% Nuserum for 48 hours. The cells were harvested by scraping, washed with phosphate buffered saline (pH 7.4) and resuspended in 50mM potassium phosphate buffer (pH 7.4) containing 10% glycerol and stored at -70°C until required for assay.

Protein determination: Protein concentrations for both the cytosolic and COS cell preparations were determined by the method of Lowry *et al.* (15), using bovine serum albumin as a standard.

PST activity: PST activity was measured by a modification of the radiochemical method of Foldes and Meek (16). The incubation mixture routinely consisted of 20mM potassium phosphate buffer, pH 7.4 , substrate, 0.1mg protein of either COS cell or rat liver cytosol origin and 8µM [^{35}S]PAPS in a final volume of 500µl. When tyrosine methyl ester was used as substrate, 125mM Tris HCl, pH 7.9, was substituted for the phosphate buffer. Blanks contained no substrate. All samples were incubated at 37°C for 30 min in a shaking water bath. The reaction was terminated by precipitation of protein and unreacted PAPS by the addition of 100µl of each of 0.1M barium acetate, 0.1M $Ba(OH)_2$ and finally 0.1M $ZnSO_4$. The precipitate was removed by centrifugation at 2000 rpm for 10 min. The supernatant was transferred to a clean tube prior to repeating the addition of the $Ba(OH)_2$ and $ZnSO_4$. The samples were centrifuged at 3000 rpm for 5 min and an aliquot of the supernatant was added to 3 ml of ACS II aqueous counting scintillation fluid. Radioactivity was determined in a Packard 2200CA TriCarb Liquid Scintillation analyser.

Analysis of results: Initial estimates of the apparent K_m and V_{max} for each substrate were initially determined by linear regression analysis of the Lineweaver-Burk plots (17). These initial estimates were subsequently used in Multifit 2.01, a curve fitting program (18), using a one or two enzyme Michaelis-Menten system.

RESULTS AND DISCUSSION

PST activity of transfected COS-7 cells: PST-1 transfected COS-7 cells were assessed for expression of aryl sulphotransferase by determining their sulphating capacity with a range of substrates. For all substrates studied, the reaction was found to be linear for incubation times of at least 30 min and protein concentrations ranging up to 0.1mg/0.5ml. Mock transfected COS-7 cells exhibited no detectable activity with either phenol, dopamine, tyrosine methyl ester or vanillin.

Table 1: Kinetic parameters for the sulphation of phenol, dopamine and vanillin

Substrate		PST 1 (COS-7) n=3	Pooled Rat Liver Cytosol*
Phenol	Km (μM)	0.867 ± 0.031	1.544
	Vmax (pmol/mg/min)	224 ± 47	537
Dopamine	Km (μM)	44.3 ± 10.7	19.1
	Vmax (pmol/mg/min)	199 ± 66	351
Vanillin	Km (μM)	7.75 ± 0.34	0.57 33.1
	Vmax (pmol/mg/min)	354 ± 10	119 284

* Pooled rat liver cytosol from 6 different rats. These pooled cytosols were used as positive controls when assaying the COS cell preparations.

The activity of the COS-expressed PST-1 was approximately 0.4 and 0.6 times that observed with rat liver cytosol with the substrates phenol and dopamine respectively, indicating the level of activity of the expressed PST in COS cells is comparable to that in cytosol of rat liver (Table 1).

We were able to determine the Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) for the COS-expressed PST-1 catalysed sulphation of phenol, dopamine and vanillin (Table 1). Phenol was a good substrate for the COS-expressed enzyme (Fig.1A), with an apparent K_m value of 0.867 ± 0.031μM, which is similar to the apparent K_m value obtained with pooled rat liver cytosol (1.54μM).

Accurate analysis of the kinetics of the sulphate conjugation of dopamine was complicated for both COS-expressed and rat liver cytosolic systems. The velocity of the reaction catalysed by COS-expressed enzyme was markedly decreased at dopamine concentrations greater than 250μM (Fig. 2). The pattern observed was typical of that observed with substrate inhibition of the enzyme (17). Inhibition of purified rat liver aryl sulphotransferases at high substrate concentrations has been reported with a number of other substrates (4, 5). The sulphation of dopamine catalysed by rat liver cytosol was consistent with a multiple enzyme system and at high dopamine concentrations aberrant kinetics were obtained. Due to these phenomena, substrate concentrations of less than 100μM were used to generate the kinetic parameters for the sulphation of dopamine. Under these conditions, the double reciprocal plots were essentially linear for both the COS-expressed enzyme and rat liver cytosol (Fig. 1), with apparent K_m values of 44.3 ± 10.7μM and 19.1μM respectively. Substrate inhibition was not evident with the rat liver cytosolic preparation (Fig. 2), indicating that sulphotransferases other than PST-1 are involved in the sulphation of dopamine in rat liver cytosol at dopamine concentrations greater than 250μM.

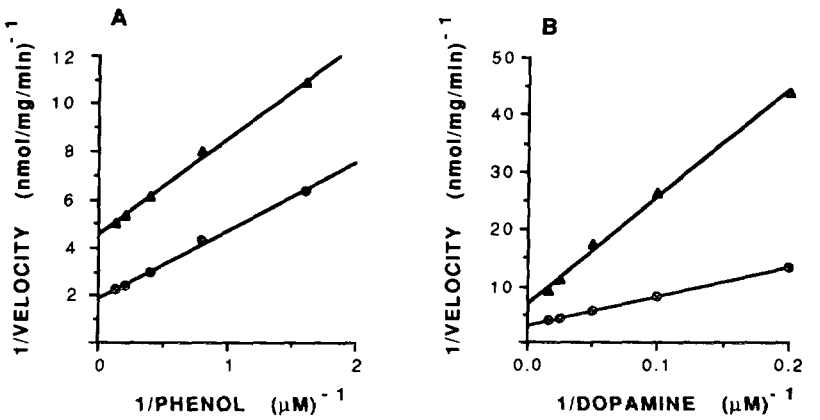

Figure 1. Double reciprocal plots of velocity versus substrate concentration for the sulphation of (A) phenol and (B) dopamine by rat liver cytosol (•) and COS-expressed PST-1 (Δ). Reactions contained 0.1mg of either cytosolic or COS cell protein and were incubated for 30 min at 37°C.

The similarities in K_m values obtained with phenol and dopamine suggests that the COS-expressed enzyme is functionally similar to phenol sulphotransferase and the high affinity form(s) of dopamine sulphotransferase in rat liver cytosol.

Classification of the rat aryl sulphotransferases has been largely based on their substrate specificities. Sekura and Jakoby reported that dopamine at a substrate concentration of 2mM failed to serve as a substrate for purified rat liver PST I, while its sulphation was catalysed by purified PST IV (4,5). It should be noted that we have observed significant inhibition of COS-expressed PST-1 at dopamine concentrations of this magnitude and it is possible that the reported inactivity of purified PST I with dopamine was actually an observation of substrate inhibition resulting from the use of a single, inappropriate substrate concentration. The activity of COS-expressed enzyme with dopamine at a concentration of 2mM was minimal (Fig. 2) and as the

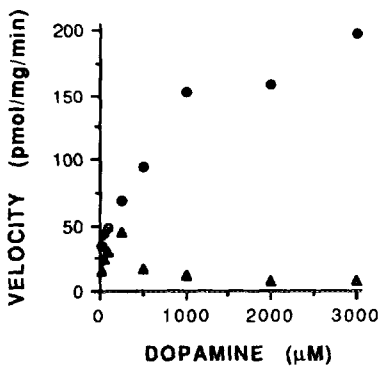

Figure 2. Sulphation of dopamine by rat liver cytosol (•) and COS-expressed PST-1 (Δ) illustrating inhibition of the expressed enzyme at high dopamine concentrations. Reactions contained 0.1mg of either cytosolic or COS cell protein and were incubated for 30 min at 37°C.

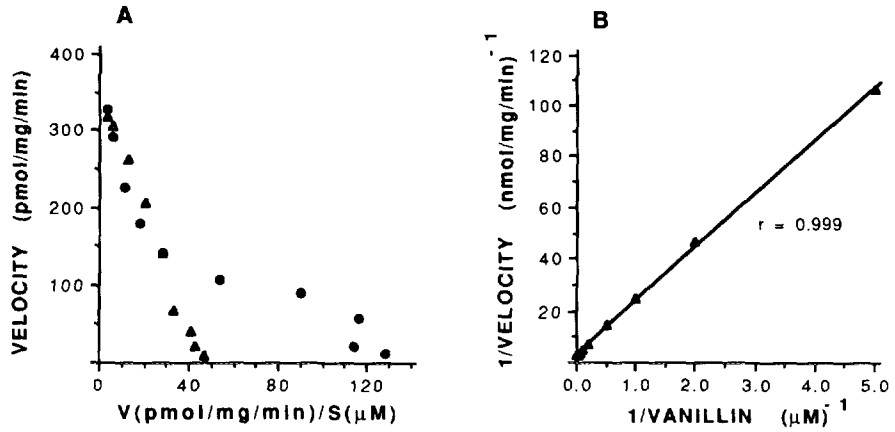

Figure 3. Kinetics of vanillin sulphation by rat liver cytosol (•) and COS-expressed PST-1 (Δ). (A) Woolf-Hofstee plot and (B) Lineweaver-Burk plot. Reactions contain 0.1mg of either cytosolic of COS cell protein and were incubated for 30 min at 37°C.

activity of purified PST I towards lower dopamine concentrations has not been reported, it is difficult to draw any comparisons between the purified and COS-expressed enzyme forms.

The purified PST I of Sekura and Jakoby was also reported to be inactive with tyrosine derivatives, including tyrosine methyl ester, while these compounds were extensively sulphated by purified enzyme PST IV (5). The activity of both rat liver cytosol and COS-expressed PST-1 with tyrosine methyl ester was assessed by the standard assay procedure both using 20mM phosphate buffer, pH7.4 and 125mM Tris HCl, pH 7.9, to reproduce the conditions used by Sekura and Jakoby (5). For both rat liver cytosol and COS-expressed enzyme, the Woolf-Hofstee plots were "atypical" (data not shown), making determination of the kinetic parameters impossible. However, the activity of COS expressed PST-1 with 2mM tyrosine methyl ester (57 pmol/mg/min) was lower than that observed with rat liver cytosol (139 pmol/mg/min).

The common food additive vanillin is a phenolic compound and is of interest as it has been shown to be a potent inhibitor of rat liver cytosolic PST activity (19). Vanillin is also a good substrate for sulphate conjugation catalysed by the rat liver cytosolic PST and the kinetics of the reaction indicated that it was sulphated by more than one isoenzyme of PST (Fig.3A) (17). Analysis of the results using a two enzyme model estimated apparent K_m values of 0.57 ± 0.13μM and 33 ± 11μM and maximum velocities (V_{max}) of 119 ± 14pmol/mg/min and 284 ± 26pmol/mg/min for the high affinity and low affinity isozymes respectively (Table 1). When vanillin was used as the sulphate acceptor for the reaction catalysed by COS-expressed PST-1, the Lineweaver-Burk plot and Woolf-Hofstee plot were consistent with a single enzyme system (Fig.3A and 3B) (17) with an apparent K_m of 7.75 ± 0.34μM and a V_{max} of 354 ± 10pmol/mg/min (Table 1).

In conclusion, using cDNA expression in COS-7 cells, we have demonstrated that PST-1 can catalyse the sulphation of phenol, dopamine, tyrosine methyl ester and vanillin. The similarity in the kinetics towards phenol and dopamine of the COS-expressed enzyme to that of the cytosolic form(s) in rat liver indicates that PST-1 may be responsible for the sulphation of these compounds

in rat liver. It is not possible to identify this enzyme as one of the previously purified forms of PST. The role of other sulphotransferases involved in the sulphation of phenol and dopamine cannot be excluded until cDNAs encoding other aryl sulphotransferases have been cloned, expressed and functionally characterised. These studies are currently under investigation in our laboratory.

REFERENCES

1. Mulder, G.J., and Jakoby, W.B. (1990) Sulfation. In Conjugation Reactions in Drug Metabolism (G.J. Mulder, Ed.), pp.107-161, Taylor and Francis Ltd., London.
2. Jakoby, W.B., Sekura, R.D., Lyon, E.S., Marcus, C.J., and Wang, J-L. (1980) Sulfotransferases. In Enzymatic Basis of Detoxification (W.B. Jakoby, Ed.), vol. 2, pp.199-228, Academic Press, New York.
3. DeBaun, J.R., Miller, E.C., and Miller, J.A. (1970) Cancer Res. 30, 577-595.
4. Sekura, R.D., and Jakoby, W.B. (1979) J. Biol. Chem. 254, 5658-5663.
5. Sekura, R.D., and Jakoby, W.B. (1981) Arch. Biochem. Biophys. 211, 352-359.
6. Coughtrie, M.W.H. and Sharp, S. (1990) Biochem. Pharmacol. 40, 2305-2313.
7. Ozawa, S., Nagata, K., Gong, D., Yamazoe, Y., and Kato, R. (1990) Nucleic Acids Res. 18, 4001.
8. Demyan, W.F., Song, C.S., Kim, D.S., Her, S., Gallwitz, W., Rao, T.R., Slomczynska, M., Chatterjee, B., and Roy, A.K. (1992) Mol. Endo. 6, 589-597.
9. Ogura, K., Kajita, J., Narihata, H., Watabe, T., Ozawa, S. , Nagata, K., Yamazoe, Y., and Kato, R. (1990) Biochem. Biophys. Res. Commun. 166, 1494-1500.
10. Hashimoto, Y., Orellana, A., Gil, G., and Hirschberg, C.B. (1992) J. Biol. Chem. 267, 15744-15750.
11. Prober, J.M., Trainor, G.L., Dam, R.J., Hobbs, F.W., Robertson, C.W., Zagursky, R.J., Cocuzza, A.J., Jensen, M.A., and Baumeister, K. (1987) Science 238, 336-341.
12. Connell, C., Fung, S., Heiner, C., Bridgham, J., Chakerian, V., Heron, E., Jones, B., Menchen, S., Mordan, W., Raff, M., Recknor, M., Smith, L., Springer, J., Woo, S., and Hunkapiller, M. (1987) Biotechniques 5, 343.
13. Andersson, S., Davis, D.L., Dahlbäck, H., Jörnvall, H., and Russell, D.W. (1989) J. Biol. Chem. 264, 8222-8229.
14. Guan, J-L, and Rose, J.K. (1984) Cell 37, 779-787.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
16. Foldes, A., and Meek, J.L. (1973) Biochim. Biophys. Acta 327, 365-373.
17. Dixon, M., and Webb, E.C. (1979) Enzymes, 3rd ed., Academic Press, New York.
18. Multifit 2.0: Curve Fitting for Apple Macintosh (1990) Day Computing, Cambridge.
19. Cruickshank, D, and Sansom, L.N. European J. Pharmaceutical Sciences (in press).