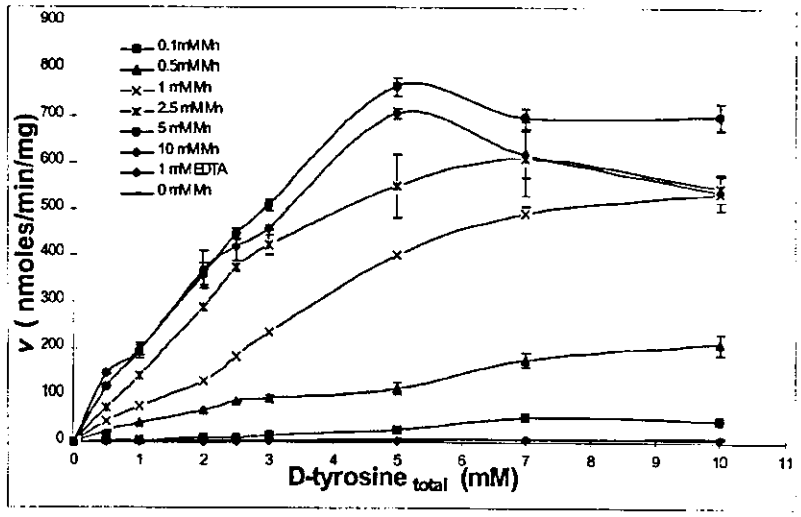


FIG. 2. **Kinetics of the sulfation of D-tyrosine by M-form PST in the presence of different concentrations of $Mn^{2+}$ or EDTA.** Concentrations of D-tyrosine and $Mn^{2+}$ are expressed in mM, and velocities ($v$) of the reactions are expressed as nmol of product formed/min/mg of protein. Each data point represents the mean value of three determinations (*error bars* are shown). The figure shows the $S_4$ profile.

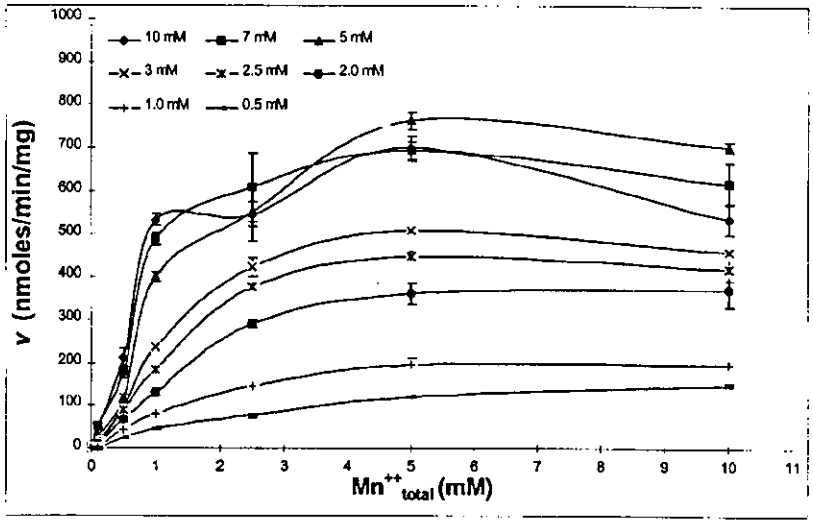


FIG. 3. **Plots of velocity ($v$) *versus* the $Mn^{2+}$ concentration in the presence of, respectively, 0.5, 1, 2.5, 3.0, and 5.0 mM D-tyrosine in kinetic studies of M-form PST with D-tyrosine as substrate in the presence of various concentrations of $Mn^{2+}$.** Concentrations of $Mn^{2+}$ are expressed in mM, and velocities of the reactions are expressed as nmol of product formed/min/mg of protein. Each data point represents the mean value of three determinations (*error bars* are shown). The data used were the same as those used in Fig. 2. The figure shows the $A_4$ profile.

The plots shown in Fig. 2 demonstrated clearly that $Mn^{2+}$ had a remarkable stimulatory effect on the sulfation of D-tyrosine by M-form PST. Fig. 2 also shows that at 5 mM $Mn^{2+}$ (and at 10 mM $Mn^{2+}$; data not shown), some inhibition started occurring in the presence of higher concentrations of D-tyrosine.

*Stereospecificity of M-form PST; Lower Activity, Affinity, and $Mn^{2+}$ Stimulation with L-Tyrosine Relative to D-Tyrosine as Substrate*—The kinetics of sulfation of L-tyrosine at various concentrations ranging from 0.5 to 10 mM by M-form PST in the presence of either 1 mM EDTA or 0 or 5 mM $Mn^{2+}$ was studied. Fig. 4 shows the corresponding velocity *versus* [S] plots. From these plots, it is clear that saturation with substrate was not reached even at 10 mM L-tyrosine in the presence of 5 mM $Mn^{2+}$. As in the case of D-tyrosine, solubility and precipitation problems made it unfeasible to extend the studies to higher concentrations of L-tyrosine or $Mn^{2+}$. However, it is clear from Fig. 4 that the affinity of M-form PST for L-tyrosine is very much lower than for D-tyrosine. With D-tyrosine as substrate, saturation was reached at ~7 mM with 2.5 mM $Mn^{2+}$ and at 5 mM with 5 mM $Mn^{2+}$, whereas with L-tyrosine as substrate, saturation was not reached even at 10 mM with 5 mM $Mn^{2+}$. The specific activities at different substrate and $Mn^{2+}$ concentrations were also found to be much lower with L-tyrosine than with D-tyrosine as substrate (*e.g.* 750 nmol/min/mg for 5 mM D-tyrosine at 5 mM $Mn^{2+}$ *versus* 6 nmol/min/mg for 5 mM L-tyrosine at 5 mM $Mn^{2+}$). Moreover, the stimulatory effect of $Mn^{2+}$ on the sulfation of L-tyrosine was much less dramatic than with D-tyrosine as substrate.

*Kinetics of Sulfation of D-Dopa by M-form PST; Stimulation by $Mn^{2+}$*—The kinetics of the sulfation of D-Dopa by M-form PST at various concentrations ranging from 1 to 1500 $\mu$M in the presence of different concentrations (0, 0.1, 0.5, 1.0, 2.5, and 5.0 mM) of $Mn^{2+}$ was studied. Fig. 5 shows the plots of velocity *versus* [S] at 0 and 2.5 mM $Mn^{2+}$. The plots demonstrated clearly the stimulatory effect of $Mn^{2+}$ on the sulfation of D-Dopa.

It is interesting to point out that the affinity of M-form PST for D-Dopa appeared to be much higher than for D-tyrosine. With D-tyrosine, saturation was reached at about 7 mM with 2.5 mM $Mn^{2+}$ and at 5 mM with 5 mM $Mn^{2+}$, whereas with D-Dopa, saturation was reached at 0.5 mM with 2.5 mM $Mn^{2+}$. The specific activities at different substrate and $Mn^{2+}$ concentrations were also much higher with D-Dopa than with D-tyrosine as substrate (*e.g.* 750 nmol/min/mg for 5 mM D-tyrosine at 5 mM $Mn^{2+}$ *versus* 1200 nmol/min/mg for 0.5 mM D-Dopa at 2.5 mM $Mn^{2+}$). However, the stimulatory effect of $Mn^{2+}$ on the sulfation of D-Dopa is less dramatic than with D-tyrosine as substrate.

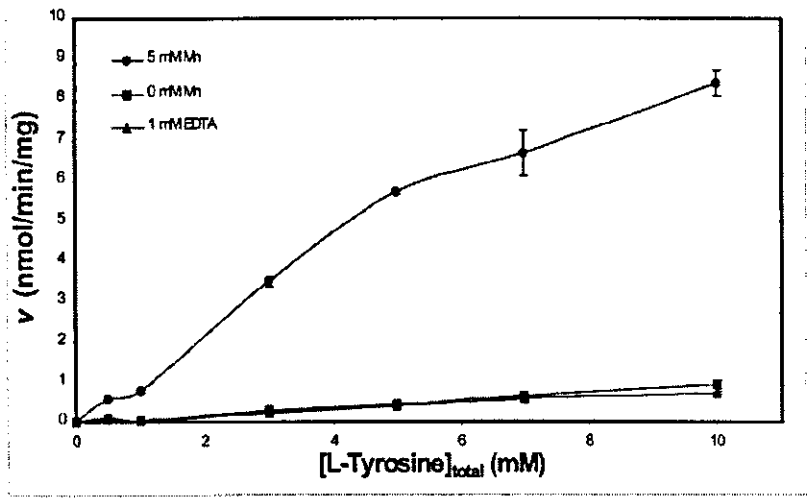


FIG. 4. **Kinetics of the sulfation of L-tyrosine by M-form PST in the presence of different concentrations of $Mn^{2+}$.** Concentrations of L-tyrosine and $Mn^{2+}$ are expressed in mM, and velocities ($v$) of the reactions are expressed as nmol of product formed/min/mg of protein. Each data point represents the mean value of three determinations (*error bars* are shown).

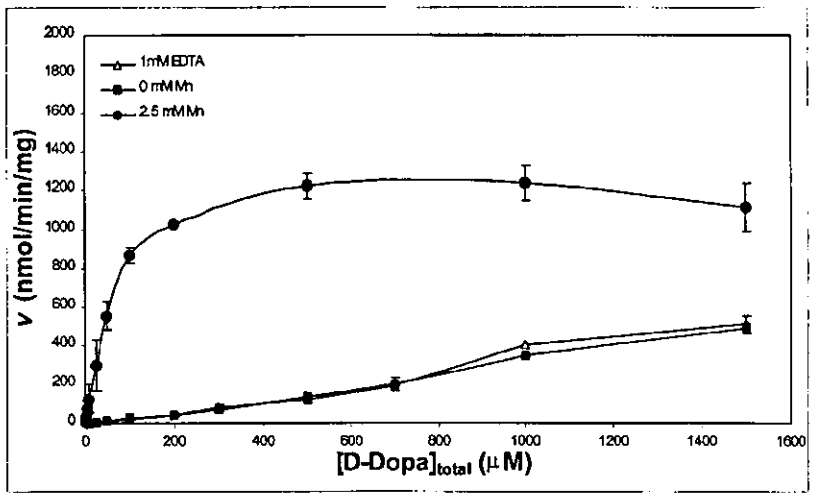


FIG. 5. **Kinetics of the sulfation of D-Dopa by M-form PST in the presence of different concentrations of $Mn^{2+}$ or EDTA.** Concentrations of D-Dopa and $Mn^{2+}$ are expressed in μM and mM, respectively, and velocities ($v$) of the reactions are expressed as nmol of product formed/min/mg of protein. Each data point represents the mean value of three determinations (*error bars* are shown).

*Kinetics of Sulfation of L-Dopa by M-form PST; Lower Affinity and $Mn^{2+}$ Stimulation Compared with the Sulfation of D-Dopa*—The kinetics of sulfation of L-Dopa at various concentrations ranging from 25 to 2500 μM by M-form PST in the presence of 0 mM $Mn^{2+}$ or 2.5 mM $Mn^{2+}$ was studied. Fig. 6 shows the corresponding v *versus* [S] plots. With or without $Mn^{2+}$ it appeared that there was no saturation with substrate even at 2500 μM L-Dopa, which approached the solubility limit for L-Dopa under the assay conditions. However, as in the case of the tyrosine enantiomers, the affinity of M-form PST for L-Dopa seemed to be very much lower than for D-Dopa, and the stimulatory effect of $Mn^{2+}$ on the sulfation of L-Dopa was much less dramatic than for D-Dopa.

## DISCUSSION

*Model to Explain the Stimulatory Effect of $Mn^{2+}$ on Dopamine Sulfation by M-form PST*—Our recent studies have established that the stimulation by $Mn^{2+}$ of the Dopa/tyrosine-sulfating activities of M-form PST is primarily mediated by residue Asp-86 in variable Region I of the molecule (11).[2] This region has been shown by x-ray crystallography (23) to be part of a mobile loop formed by residues 84–92 of one subunit that intercalates into the active site of the other subunit of this dimeric enzyme (22). Results from our current study on the sulfation of dopamine by the wild-type M-form PST (*cf.* Fig. 1) and its D86A point mutant (*cf.* Table I) indicated that the smaller stimulatory effect of $Mn^{2+}$ on the dopamine-sulfating activity of the wild-type enzyme was also mediated by the binding of $Mn^{2+}$ to the Asp-86 residue in the molecule. It is at present unclear how the binding of $Mn^{2+}$ to the Asp-86 residue at the active site stimulates the dopamine sulfation. Nevertheless, the stimulation was apparently due to an increase in $V_{max}$, without significant effect on $K_m$ (*cf.* Fig. 1). The activity toward dopamine (with or without $Mn^{2+}$) basically followed Michaelis-Menten kinetics. This suggests that the binding of $Mn^{2+}$ to the Asp-86 residue may increase the catalytic efficiency of the enzyme while not affecting (or marginally hindering) the binding of dopamine at the active site. The kinetics does not suggest any involvement of a dopamine-$Mn^{2+}$ complex, as is to be expected because dopamine contains no negatively charged group to co-ordinate to the $Mn^{2+}$. The dissociation constant for the binding of $Mn^{2+}$ to the enzyme at the Asp-86 residue appears to be in the mM range, based on the data presented. The D86A point mutant showed no stimulation by $Mn^{2+}$, probably because of the absence of the Asp-86 residue.

*Model to Explain the Stimulatory Effect of $Mn^{2+}$ on Dopa/Tyrosine Sulfation by M-form PST*—Our data demonstrated that M-form PST displayed a much greater affinity for the D-enantiomers of tyrosine and Dopa than for their L-enanti-

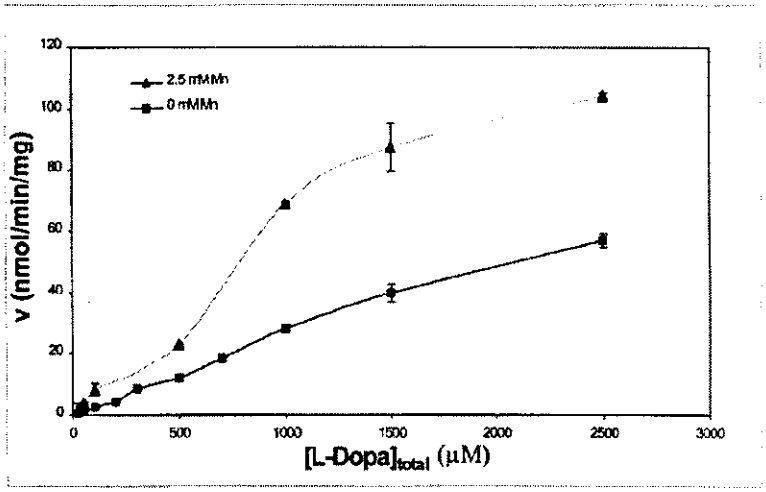


FIG. 6. **Kinetics of the sulfation of L-Dopa by M-form PST in the presence of different concentrations of $Mn^{2+}$ or EDTA.** Concentrations of L-Dopa and $Mn^{2+}$ are expressed in μM and mM, respectively, and velocities ($v$) of the reactions are expressed as nmol of product formed/min/mg of protein. Each data point represents the mean value of three determinations (*error bars* are shown).

omers. Its activity toward these compounds was dramatically stimulated by $Mn^{2+}$ in the range of $Mn^{2+}$ concentrations up to 5 mM. The stimulation was found to be much more dramatic with the D-enantiomers and was evident at $Mn^{2+}$ levels as low as 0.1 mM. The kinetic plots in Figs. 2–6 indicated that the interaction of M-form PST with Dopa or tyrosine in the presence of $Mn^{2+}$ was co-operative in nature. This could be possibly be modeled for the behavior with tyrosine by a kinetic scheme where a tyrosine-$Mn^{2+}$ or a tyrosine-$Mn^{2+}$-tyrosine complex is the real substrate (32, 33). Because the activity with tyrosine alone was found to be negligible and that with tyrosine plus $Mn^{2+}$ considerably higher, the tyrosine-$Mn^{2+}$ and tyrosine-$Mn^{2+}$-tyrosine complexes would likely be the obligatory substrates. In the case of Dopa, there was a considerable activity even without $Mn^{2+}$, indicating that the $Mn^{2+}$-Dopa complexes are not obligatory but rather better substrates compared with Dopa alone. $Mn^{2+}$ may bind to the negatively charged carboxyl group of one or two tyrosine/Dopa molecules to form the complexes. Our recent studies using point mutants suggest that a positively charged amino group of the tyrosine/Dopa-$Mn^{2+}$ complexes may interact mainly with the negatively charged Glu-146 residue at the active site of the enzyme.[2] Moreover, the positively charged $Mn^{2+}$ moiety of the complex may co-ordinate predominantly with the negatively charged Asp-86 residue of the mobile loop of the enzyme at the active site.

A search of the literature gave the log $K$ for formation of the tyrosine-$Mn^{2+}$ complex (at 25 °C and an ionic strength of 0.1; pH not specified) as 1.5 (molar concentrations used), whereas that for the formation of the tyrosine-$Mn^{2+}$-tyrosine complex was 5.0 (34, 35). We used these values as a first approximation to calculate the concentrations of the two complexes in the assay mixture with various total concentrations of $Mn^{2+}$ and tyrosine. The calculations were done using the equations for the two equilibria mentioned above together with the conditions $[\text{tyrosine}]_{free} = [\text{tyrosine}]_{total} - 2[\text{tyrosine-}Mn^{2+}\text{-tyrosine}] - [\text{tyrosine-}Mn^{2+}]$ and $[Mn^{2+}]_{free} = [Mn^{2+}]_{total} - [\text{tyrosine-}Mn^{2+}\text{-tyrosine}] - [\text{tyrosine-}Mn^{2+}]$ along with the requirement that real and positive numbers be involved. An iterative numerical procedure was used to solve these equations for the concentrations of the two complexes present when different amounts of $Mn^{2+}$ and tyrosine (total) are taken using a computer program written in visual basic. It is to be pointed out that the above equation showing the relationship between free and bound $Mn^{2+}$ concentrations does not take into consideration the possible binding of $Mn^{2+}$ to DTT, TAPS, and PAPS. An exhaustive literature search failed to find any association constants for the binding of these latter compounds to $Mn^{2+}$. In the case of DTT, the p$K_a$ values for the two sulfhydryl groups had been determined to be 9.2 and 10.1, respectively (36). Because the assays with tyrosine as substrate in the present study were performed at pH 8.25, both the sulfhydryl groups of DTT would be predominantly uncharged and may not be expected to coordinate strongly to $Mn^{2+}$ under these conditions.

In Fig. 7, the velocity (in nmol of product formed/min/mg of protein) for the sulfation of D-tyrosine by M-form PST is plotted *versus* the total concentration of the D-tyrosine-$Mn^{2+}$ and D-tyrosine-$Mn^{2+}$-D-tyrosine complexes (in moles/liter) in the assay mixture, calculated as indicated above. The data used are the same as presented in Fig. 2. It is clear that the data show Michaelis-Menten behavior in the interaction of the enzyme with these complexes. This is not the case if the concentration of only one of the two complexes is plotted on the $x$ axis, indicating that the true substrates for the reaction include both the D-tyrosine-$Mn^{2+}$ as well as the D-tyrosine-$Mn^{2+}$-D-tyrosine complexes. The $K_m$ and $V_{max}$ values of the enzyme for the two complexes, however, must differ because the residual charge on the $Mn^{2+}$ moiety in the D-tyrosine-$Mn^{2+}$ complex will be higher, and consequently, its interaction with the Asp-86 residue on the enzyme is expected to be stronger than in the case of the D-tyrosine-$Mn^{2+}$-D-tyrosine complex. However, because we are dealing with a dynamic equilibrium, it is likely that, once the D-tyrosine-$Mn^{2+}$-D-tyrosine complex enters and binds to the substrate pocket, the Asp-86 residue of the enzyme may bind to the $Mn^{2+}$ moiety, exchanging with the outer D-tyrosine residue and replacing it. Thus, this complex may also behave effectively like the D-tyrosine-$Mn^{2+}$ complex. The less than perfect fit to Michaelis-Menten behavior is to be expected considering the approximations made in using the values for the association constants for the formation of the complexes. The $K_m$ of the enzyme for these $Mn^{2+}$-D-tyrosine complexes appears to be in the range of 0.75–0.85 mM, whereas the $V_{max}$ is in the range of 750–850 nmol/min/mg. Our studies on dopamine sulfation by M-form PST as discussed above also indicated that $Mn^{2+}$ may bind to the enzyme, and it is likely that the inhibition at higher (mM) levels of $Mn^{2+}$ and tyrosine was due to their inhibition on the binding of the $Mn^{2+}$-D-tyrosine complexes.

It is likely that similar calculations can be done to show that D-Dopa-$Mn^{2+}$ and D-Dopa-$Mn^{2+}$-D-Dopa complexes are also responsible for the $Mn^{2+}$ stimulation of the D-Dopa-sulfating activity of M-form PST. However, no values of the association constants for the relevant complexes could be found in the literature, and also the analysis with D-Dopa will be compli-

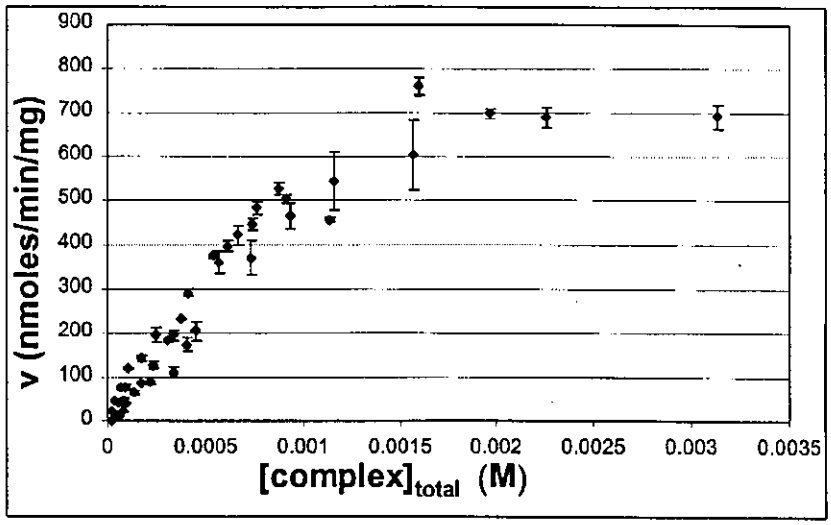


FIG. 7. **Plots of velocity ($v$) *versus* the total concentration of the D-tyrosine-$Mn^{2+}$ plus D-tyrosine-$Mn^{2+}$-D-tyrosine complexes derived from the data presented in Fig. 2.** The concentrations in mol/liter of the complexes are calculated from the concentrations of total $Mn^{2+}$ and total D-tyrosine in the assay mixture based on the procedure described under "Discussion." $v$, in units of nmol of product formed/min/mg of protein, is the velocity of the sulfation of D-tyrosine catalyzed by the M-form PST. Each data point represents the mean value of three determinations (*error bars* are shown).

cated by the fact that Dopa has a substantial activity by itself. Similar scenarios can be postulated for the interaction with the L-isomers of tyrosine and Dopa.

The involvement of a $Mn^{2+}$-PAPS complex as an obligatory or additional substrate appeared unlikely since saturation of PAPS was ensured in these experiments. Increasing the PAPS concentration (which was 15 $\mu$M in the standard assay) 10-fold at several $Mn^{2+}$ concentrations did not result in any increase in reaction velocity. Moreover, our results clearly showed that the stimulatory effect of $Mn^{2+}$ on the sulfation activity of M-form PST is a function of a particular acceptor substrate. Another argument against a $Mn^{2+}$-PAPS complex is the fact that no $Mn^{2+}$ requirement or stimulation has been reported for the activity of any of the other cytosolic STs (at least with their commonly used substrates), which all use PAPS as a co-substrate. Moreover, a comprehensive study performed in our laboratory on the effect of a variety of divalent metal ions on the activity of 10 known human cytosolic STs toward their commonly used substrates did not reveal any universal metal ion requirement or stimulation.[2]

*Stereospecificity of M-form PST and Its Relative Activity toward Dopa and Tyrosine*—In this study, we found that in the presence of 5 mM $Mn^{2+}$, the $[S]_{0.5}$ for D-tyrosine was around 1.9 mM, and the $V_{max}$ was around 750 nmol/min/mg, whereas for D-Dopa the $[S]_{0.5}$ was around 100 $\mu$M, and the $V_{max}$ was around 1200 nmol/min/mg. Part of the difference may be due to the relative values of the dissociation constants for the two substrate-$Mn^{2+}$ complexes. Additionally, D-tyrosine can only be sulfated at the 4-OH group, whereas for D-Dopa, it has been demonstrated that sulfation occurs exclusively at the 3-OH group (37). The much greater affinity for D-Dopa relative to D-tyrosine can probably be explained by QSAR (quantitative structure activity relationship) analysis (23). The $Mn^{2+}$-stimulated activity of M-form PST with, and its affinity for, the L-enantiomers of Dopa and tyrosine was far lower than with the D-enantiomers, which could probably also be explained by similar arguments and modeling studies (23). In our previous studies we had shown that the stereospecificity of M-form PST for the D-enantiomers of Dopa and tyrosine was mediated primarily by residue Glu-146 at the active site, which probably binds the positively charged amino groups of these substrates.[2] Point mutations of selected residues 84–89 that are part of the putative loop at the active site did not significantly affect this stereoselective behavior. However, a residues-84–90 deletional mutant of M-form PST showed no stereoselectivity in its sulfation activity toward tyrosine and Dopa. This suggested that although individual residues in the 84–92 loop may not be critical for the stereoselectivity, the presence of the loop as such is essential, probably as an additional steric selector. The residues-84–86 deletional mutant showed no activity toward any stereoisomer of either tyrosine or Dopa, possibly because the truncated loop may interfere with access of these substrates to the active site.

*Physiological Relevance of the Stimulation of Sulfating Activity of M-form PST by $Mn^{2+}$*—In this study, the maximum stimulatory effect of $Mn^{2+}$ was observed at concentrations of around 5 mM. However, it was evident that significant stimulatory effects already occurred at levels as low as 0.1 mM. $Mn^{2+}$ is an important element biologically and has been shown to be essential to the activity of a number of enzymes in a variety of organisms (12, 38, 39). For example, it is central to the function of superoxide dismutase, an enzyme that protects against oxidative damage in tissues (12, 8). The $Mn^{2+}$ concentrations in neuronal and brain tissue have been reported to be higher than in other tissues (12). Within the cell, $Mn^{2+}$ may be preferentially sequestered in mitochondria and endoplasmic reticulum (13). As stated previously, oxidative stress or damage, which has been implicated in neuronal apoptosis that occurs in neurodegenerative diseases, generally results in mitochondrial dysfunction (14) that may lead to the release of $Mn^{2+}$ into the cytosol. Elevated $Mn^{2+}$ concentration in the cytosol will stimulate M-form PST in its sulfating activity with dopamine and especially in its Dopa/tyrosine-sulfating activity. This may represent a detoxifying mechanism as discussed later. One such neurodegenerative disease, parkinsonism, which is believed to arise from the destruction of dopaminergic neurons, thus greatly lowering brain dopamine levels (40), may involve mitochondrial dysfunction (41). If this indeed results in a rise in the cytosolic levels of $Mn^{2+}$ in such cells, the activation of M-form PST may help to detoxify the dopamine and possibly other toxic substances (as discussed below) that could be released by such dying cells. In this connection, it may be pertinent to note that manganese poisoning (or manganism) is known to result in symptoms resembling Parkinson's disease (42, 43). One reason could be because the activation of M-form PST in dopaminergic cells by the excess $Mn^{2+}$ results in mis-guided "detoxification" of dopamine in these cells and consequently parkinsonian symptoms.

*Physiological Relevance of the Sulfation of D-Tyrosine by M-form PST*—Our study has demonstrated M-form PST to be more active toward the D-enantiomers of tyrosine and Dopa. The stimulatory effect of $Mn^{2+}$ was also much more dramatic

with these D-enantiomers. Although the L-enantiomer of amino acids is used in protein synthesis, a small percentage of the amino acid pool is present in the D-form. D-Amino acids may be formed due to spontaneous racemization in proteins with low turnover rates, such as human lens protein (44), and accumulated in aging tissues lacking D-amino acid oxidases (13). Attempts have been made to link the amount of specific D-amino acid to oxidative damage and to neurodegenerative disorders such as Alzheimer's and Parkinson's disease (14, 15). Although a clear picture has yet to emerge, the removal of D-amino acids, which cannot participate in protein synthesis and in most metabolic reactions, may be viewed as a detoxification process. Incidentally, detoxification of D-amino acids through sulfation is likely to be less deleterious than by D-amino acid oxidase, which causes oxidative stress (45). In a different perspective, the $Mn^{2+}$-stimulated activity of M-form PST toward D-Dopa and D-tyrosine may provide clues to the understanding of its stereoselective action on chiral drugs (18–21).

*Possibility of a Dual Role of M-form PST with $Mn^{2+}$ Serving as a Molecular Switch*—It is possible that M-form PST under normal circumstances acts on its physiological substrate, dopamine (of which the pH optimum is ~7.0, and the $K_m$ is ~ 2 $\mu$M) (22, 46), thereby regulating the levels of this endogenous metabolite. In the presence of elevated $Mn^{2+}$ (possibly under conditions of oxidative stress, as discussed previously), the detoxifying action of M-form PST is activated. $Mn^{2+}$ may complex with various substrates, with varying affinities, and these complexes may serve as substrates for M-form PST. $Mn^{2+}$ may thus serve as a molecular switch to increase the substrate promiscuity of M-form PST. The affinity of the enzyme for these xenobiotic substrates will depend on the dissociation constant of the substrate-metal complex, the metal ion concentration, and the affinity of the enzyme for the complex. Incidentally the different pH optimum (~8–9 for the Dopa/tyrosine-sulfating activity of M-form PST (8)) compared with the pH optimum of around 7 for dopamine sulfation may reflect the pH dependence of the formation of substrate-metal complex and offer another level of regulation. Because sulfation is an energetically expensive process (it uses up PAPS, synthesis of one molecule of which requires expenditure of three high energy phosphate bonds of ATP (1)), this proposed dual function of M-form PST may make sense from the viewpoint of cellular economy.

In conclusion, our findings on the stimulatory effect of $Mn^{2+}$ on the sulfation of D-Dopa and D-tyrosine by M-form PST through the formation of a substrate-$Mn^{2+}$ complex represent the first report of a regulatory mechanism operating in the ST enzymes. It is possible that other xenobiotic substrates may also be acted on by this enzyme in a similar fashion, in concert with $Mn^{2+}$ or other metal ions, although the affinity and concentrations involved may be quite different depending on the dissociation constant of the metal-substrate complex and other parameters. It would be interesting to see if other examples of such regulatory mechanisms, possibly involving other molecular signals, also operate among other members of this important family of enzymes.

REFERENCES


1. Lipmann, F. (1958) *Science* **128,** 575–580
2. Mulder, G. J., and Jakoby, W. B. (1990) in *Conjugation Reactions in Drug Metabolism* (Mulder, G. J., and Jakoby, W. B., eds) pp. 107–161, Taylor & Francis Ltd., London
3. Weinshilboum, R. M., Otterness, D. M., Aksoy, I. A., Wood, T. C., Her, C. T., and Raftogianis, R. B. (1997) *FASEB J.* **11,** 3–14
4. Coughtrie, M. W. H., Sharp, S., Maxwell, K., and Innes, N. P. (1998) *Chem. Biol. Interact.* **109,** 3–27
5. Duffel, M. W. (1997) in *Comprehensive Toxicology* (Guengerich, F. P., ed) pp. 365–383, Elsevier Science Ltd., Oxford
6. Matsui, M., and Homma, H. (1994) *Int. J. Biochem.* **26,** 1237–1247
7. Falany, C. N. (1997) *FASEB J.* **11,** 206–216
8. Suiko, M., Sakakibara, Y., Nakajima, H., Sakaida, H., and Liu, M.-C. (1996) *Biochem. J.* **314,** 151–158
9. Sakakibara, Y., Katafuchi, J., Takami, Y., Nakayama, T., Suiko, M., Nakajima, H., and Liu, M.-C. (1997) *Biochim. Biophys. Acta* **1355,** 102–106
10. Rubin, G. L., Sharp, S., Jones, A. L., Olatt, H., Mills, J. A., and Coughtrie, M. W. H. (1996) *Xenobiotica* **26,** 1113–1119
11. Sakakibara, Y., Takami, Y., Nakayama, T., Suiko, M., and Liu, M.-C. (1998) *J. Biol. Chem.* **273,** 6242–6247
12. Klaassen, C. D. (1996) in *Casarett and Doull's Toxicology: The Basic Science of Poisons* (Klaassen C. D., Doull, J., and Casarett, L. J., eds) 5th Ed., McGraw-Hill Inc., New York
13. Gavin, C. E., Gunter, K. K., and Gunter, T. E. (1992) *Toxicol. Appl. Pharmacol.* **115,** 1–5
14. Keller, J. N., Kindy, M. S., Holtsberg, F. W., St. Clair, D. K., Yen, H. C., Germeyer, S. M., Bruce-Keller, A. J., Hutchins, J. B., and Mattson, M. P. (1998) *J. Neurosci.* **18,** 687–697
15. D'Aniello, A., D'Onofrio, G., Pischetola, M., D'Aniello, G., Vetere, A., Petrucelli, L., and Fisher, G. H. (1993) *J. Biol. Chem.* **268,** 26941–26949
16. Fisher, G., D'Aniello, A., Vetere, A., Padula, L., Cusano, G. P., and Man, E. H. (1991) *Brain Res. Bull.* **26,** 983–985
17. Man, E. H., Fisher, G. H., Payan, I. L., Cadilla-Perezrios, R., Garcia, N. M., Chemburkar, R., Arends, G., and Frey, W. H. (1987) *J. Neurochem.* **48,** 510–515
18. Wilson, A. A., Wang, J., Koch, P., and Walle, T (1997) *Xenobiotica* **27,** 1147–1154
19. Walle, U. K., Persola, G. R., and Walle, T. (1993) *Br. J. Clin. Pharmacol.* **35,** 413–418
20. Persola, G. R., and Walle, T. (1993) *Chirality* **5,** 602–609
21. Walle, T., and Walle, U. K. (1992) *Drug Metab. Dispos.* **20,** 333–336
22. Heroux, J. A., and Roth, J. A. (1988) *Mol. Pharmacol.* **34,** 194–199
23. Dajani, R., Cleasby, A., Neu, M., Wonacott, A. J., Jhoti, H., Hood, A. M., Modi, S., Hersey, A., Taskinen, J., Cooke, R. M., Manchee, G. R., and Coughtrie, M. W. H. (1999) *J. Biol. Chem.* **274,** 37862–37868
24. Liu, M.-C., Suiko, S., and Sakakibara, Y. (2000) *J. Biol. Chem.* **275,** 13460–13464
25. Yanagisawa, K., Sakakibara, Y., Suiko, M., Takami, Y., Nakayama, T., Nakajima, H., Takayanagi, K., Natori, Y., and Liu, M.-C. (1998) *Biosci. Biotechnol. Biochem.* **62,** 1037–1040
26. Sekura, R. D., Marcus, C. J., Lyon, E. S., and Jakoby, W. B. (1979) *Anal. Biochem.* **95,** 82–86
27. Liu, M.-C., and Lipmann, F (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 3695–3698
28. Fernando, P. H. P., Karakawa, A., Sakakibara, Y., Ibuki, H., Nakajima, H., Liu, M.-C., and Suiko, M. (1993) *Biosci. Biotechnol. Biochem.* **5,** 1974–1975
29. Lin, E.-S., and Yang, Y.-S (2000) *Biochem. Biophys. Res. Commun.* **271,** 818–822
30. Laemmli, U. K. (1970) *Nature* **227,** 680–685
31. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
32. Segel, I. H. (1975) *Enzyme Kinetics*, John Wiley & Sons, Inc., New York
33. London, W. P., and Steck, T. L. (1969) *Biochemistry* **8,** 1767–1779
34. Martell, A. E., and Smith, R. M. (1974) *Critical Stability Constants: Amino Acids*, Vol. 1, Plenum Press, New York
35. Gergely, A., Nagypal, I., and Kiraly, B. (1971) *Acta Chim. Acad. Sci. Hung.* **68,** 285–294
36. Singh, R., and Whitesides, G. M. (1991) *J. Org. Chem.* **56,** 2332–2337
37. Suiko, M., Sakakibara, Y., A-Khan, R., Sakaida, H., Yoshikawa, H., Ranasinghe, J. G. S., and Liu, M.-C. (1998) *J. Biochem.* **124,** 707–711
38. Ulter, M. F. (1976) *Med. Clin. North Am.* **60,** 713–717
39. Cotzias, G. C., Miller, S. T., Papavasilion, P. S., and Tang, L. C. (1976) *Med. Clin. North Am.* **60,** 729–733
40. Chun, H. S., Gibson, G. E., DeGiorgio, L. A., Zhang, H., Kidd, V. J and Son, J. H. (2001) *J. Neurochem.* **76,** 1010–1021
41. Greenamyre, J. T., Mackenzie, G., Peng, T. I., and Stephans, S. E. (1999) *Biochem. Soc. Symp.* **66,** 85–87
42. Barbeau, A. (1984) *Neurotoxicity* **5,** 13–36
43. Corell, J., Rybick, B., Johnson, C., and Peterson, E. (1999) *Neuroepidemiology* **18,** 303–330
44. Luthra, M., Ranganathan, D., Ranganathan, S., and Balasubramanian, D. (1994) *J. Biol. Chem.* **269,** 22678–22682
45. Stegman, L. D., Zheng, H., Neal, E. R., Ben-Yoseph, O., Pollegioni, L., Pilone, M. S., and Ross, B. D. (1998) *Hum. Gene Ther.* **9,** 185–193
46. Butler, P. R., Anderson, R. J., and Venton, D. L. (1983) *J. Neurochem.* **41,** 630–639

ELSEVIER

Biochimica et Biophysica Acta 1573 (2002) 165–170

BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

# Differential xenoestrogen-sulfating activities of the human cytosolic sulfotransferases: molecular cloning, expression, and purification of human SULT2B1a and SULT2B1b sulfotransferases

T. Govind Pai, Takuya Sugahara, Masahito Suiko, Yoichi Sakakibara, Faye Xu, Ming-Cheh Liu*

*Department of Biochemistry, Biomedical Research Center, The University of Texas Health Center at Tyler, 11937 US HWY 271, Tyler, TX 75708, USA*

Received 5 April 2002; received in revised form 1 August 2002; accepted 3 September 2002

**Abstract**

Environmental xenoestrogens have been implicated in human reproductive disorders and an increased incidence of breast cancer. Sulfation, a Phase II detoxification mechanism involving the cytosolic sulfotransferases (STs), may be an important mechanism in vivo for fending off these compounds. In this study, we report on the molecular cloning, expression, and purification of two human cytosolic STs, SULT2B1a and SULT2b1b. The activities of these two enzymes, as well as the other eight known human cytosolic STs previously prepared, toward representative environmental xenoestrogens were examined. Activity data showed that P-form (SULT1A1) PST displayed the highest activity toward these compounds, while SULT1C ST #2 also showed considerable activity, indicating that these enzymes may play a more important role in detoxification of environmental xenoestrogens. SULT1C ST #1, SULT2B1a ST, SULT2B1b ST and NST showed negligible or undetectable activity toward these compounds. The other four enzymes, M-form (SULT1A3) PST, SULT1B2 ST, SULT2A1 ST and SULT1E ST showed intermediate levels of activity toward some of these compounds. Kinetic studies on the sulfation of xenoestrogens by P-form (SULT1A1) PST were performed. The results are interpreted in the context of the endocrine-disrupting nature of these xenoestrogens.


*Keywords:* 3′-Phosphoadenosine 5′-phosphosulfate; Reverse transcriptase-polymerase chain reaction, sulfation; Sulfotransferase; Xenoestrogen

## 1. Introduction

In recent years, there have been a number of reports of estrogens and estrogen-like chemicals in the environment having an adverse impact on wildlife, including reptiles and birds [1,2]. These compounds include synthetic estrogen mimics (xenoestrogens) which may exert their effects either by binding directly to the estrogen receptor with varying affinities [3] or by interfering with the action of enzymes which help regulate the level of endogenous estrogens and other hormones [4]. Among the xenoestrogens are bisphenol (which is widely used in a variety of plastics and polycarbonate resins including those used in food packaging and dental fillings), 4-*n*-octylphenol, 4-*n*-nonylphenol (alkyl phenols that are widely used as surfactants and plastic additives) and 17-α-ethynylestradiol (the synthetic estrogen component of oral contraceptives). These compounds are becoming ubiquitous in the environment and are increasingly making their way into the food chain. They not only may cause an abnormal sexual development in wildlife but are being implicated as a hazard for human health [5] and in a worldwide debate on a decline in sperm quality in men [6,7] and an increased incidence of human breast cancer [8,9].

Among the detoxification mechanisms used by mammals for the removal of xenobiotics, the Phase II detoxification pathways involving processes like glucuronidation and sulfation are meriting increased attention. Glucuronidation of bisphenol A by the rat liver UDP-glucuronosyl transferase has been reported [10]. Sulfate conjugation is a major pathway in vivo for the biotransformation and/or excretion of both xenobiotics, as well as endogenous compounds such as steroid and thyroid hormones, cate-

* Corresponding author. Tel.: +1-903-877-2862; fax: +1-903-877-2863.
*E-mail address:* ming.liu@uthct.edu (M.-C. Liu).

0304-4165/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0304-4165(02)00416-6

cholamines, and bile acids [11–13]. This is accomplished by the cytosolic sulfotransferases (STs) that catalyze the transfer of a sulfonate group from the active sulfate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), to a substrate containing either a hydroxyl or an amino group to form the respective sulfate or sulfamate ester [14]. It is generally believed that sulfation may increase the water solubility of xenobiotic or endogenous compounds and facilitate their removal from the body [11–13]. We were interested in studying the involvement of this pathway in the detoxification of xenoestrogens [4]. In this study, we report on the molecular cloning, expression, and purification of two human cytosolic STs, SULT2B1a and SULT2b1b. The activities of these two enzymes, as well as the other eight known human cytosolic STs previously prepared, toward representative environmental xenoestrogens were examined. Moreover, the kinetics of sulfation of xenoestrogens by P-form PST, the major xenoestrogen-sulfating enzyme identified in the present study, was investigated. Besides throwing light on the in vivo mechanisms for detoxification of xenoestrogens and their mode of action, such studies may assist in the design of viable strategies for bioremediation of environments containing high levels of these compounds [3].

## 2. Materials and methods

### 2.1. Materials

Dopamine, *p*-nitrophenol (pNP), bisphenol A, diethylstilbestrol, 4-*n*-octylphenol, 4-*n*-nonylphenol, 17-α-ethynylestradiol, 17-β-estradiol, dehydroepiandrosterone (DHEA), estrone, aprotinin, thrombin, adenosine 5′-triphosphate (ATP), sodium dodecyl sulfate (SDS), Trizma base, isopropyl β-D-thiogalactopyranoside (IPTG), inorganic pyrophosphatase, and dithiothreitol (DTT) were obtained from Sigma. ATP-agarose was prepared by coupling sodium periodate-oxidized ATP to adipic acid dihydrazide-agarose using the procedure of Lamed et al. [15]. LA *Taq* DNA polymerase was purchased from PanVera. Oligonucleotide primers were synthesized by Operon Technologies. pGEX-2TK glutathione *S*-transferase gene fusion vector, *Escherichia coli* BL21, First-Strand cDNA Synthesis Kit, and glutathione Sepharose 4B were products of Amersham Pharmacia Biotech. All restriction endonucleases and the pET23c prokaryotic expression vector were from New England Biolabs. QIAquick Gel Extraction Kit was a product of Qiagen. Recombinant human M-form (SULT1A3) and P-form (SULT1A1) PSTs, two SULT1C STs (designated #1 and #2), DHEA (SULT2A1) ST, thyroid hormone (SULT1B2) ST, estrogen (SULT1E1) ST, and a neuronal ST (NST), expressed using pGEX-2TK or pET23c prokaryotic expression system, were prepared as previously described [16–20]. Carrier free sodium [$^{35}$S] sulfate was from ICN Biomedicals. Chromatogram cellulose thin-layer chromatography (TLC) plates were from Eastman Kodak. All other reagents were of the highest grade commercially available.

### 2.2. Molecular cloning, expression, and purification of human cytosolic STs

We have previously prepared eight purified human cytosolic STs: the M-form (SULT1A3) and P-form (SULT1A1) PSTs, two SULT1C STs (designated #1 and #2), DHEA (SULT2A1) ST, thyroid hormone (SULT1B2) ST, estrogen (SULT1E1) ST, and a neuronal ST (NST) [16–20]. In the present study, we have cloned, expressed, and purified the other two known human cytosolic STs, the SULT2B1a ST and SULT2B1b ST.

### 2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) cloning of SULT2B1b ST cDNA

SULT2B1b ST cDNA was cloned using the RT-PCR technique. Briefly, human placenta poly A+-RNA was used as the template for the synthesis of the first-strand cDNA. Using primer pairs based on reported sequences (GenBank accession number U92314) corresponding to the 5′- and 3′-coding regions of the open reading frame of this enzyme, the SULT2B1b ST cDNA was amplified via PCR from the first-strand cDNA prepared. The PCR amplification conditions were 30 cycles of 30 s at 94 °C, 35 s at 63 °C, and 1 min 15 s at 72 °C. The final reaction mixture was applied onto a 1.0% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. The gel band containing the PCR product was cut out and the DNA was purified using the Qiagen Extraction Kit. The SULT2B1b ST cDNAs thus purified was digested with *Nde*I restriction endonuclease and ligated into the *Nde*I-restricted pET23c prokaryotic expression vector.

### 2.4. PCR cloning of SULT2B1a ST cDNA

SULT2B1a ST cDNA was directly amplified using SULT2B1b ST cDNA as the template, in conjunction with a sense primer (5′-GCGAGCTTATCACATATGGCGTCTC-CCCCACCTTTCCACAGACAGAAGTTGCCAGGT-3′; GenBank accession number U92314) specific for the SULT2B1a ST and an antisense primer (5′-GAGTCAGTCA-CATATGTTATGAGGGTCGTGGGTGCGG-3′) common to both SULT2B1a ST and SULT2B1b ST. For both primers, a *Nde*I restriction site was incorporated at the end of the sequence. The PCR amplification conditions were 30 cycles of 40 s at 94 °C, 45 s at 66 °C, and 1 min 15 s at 72 °C. The final reaction mixture was applied onto a 1.0% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. The gel band containing the PCR product was cut out and the DNA was purified using the Qiagen Extraction Kit. The SULT2B1a ST cDNA thus purified was

digested with *Nde*I restriction endonuclease and ligated into the *Nde*I-restricted pET23c prokaryotic expression vector.

### 2.5. Transformation of BL21 (DE3) cells, expression, and purification of recombinant SULT2B1a and SULT2B1b STs

The pET23c-SULT2B1a ST and pET23c-SULT2B1b ST constructs prepared were individually transformed into BL21 (DE3) cells. Transformed cells were grown in 1-l aliquots of LB broth containing 50 μg/ml ampicillin. After the cell density reached 0.8 $OD_{600\ nm}$, 0.4 mM IPTG was added to induce the production of recombinant SULT2B1a ST or SULT2B1b ST. After a 5-h induction at 37 °C, cells were collected by centrifugation and homogenized in 20 ml of a lysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) using an Aminco French press. Twenty microliters of 10 mg/ml aprotinin (a protease inhibitor) was added to the crude homogenate. The crude homogenates were subjected to ultracentrifugation at 140,000 × *g* for 2 h at 4 °C to pellet down particulate fractions. The supernatants collected were used for the purification of SULT2B1a ST or SULT2B1b ST as described below.

### 2.6. Bio-gel HTP hydroxyapatite column chromatography

Twenty-five milliliters of the supernatant collected as described above was loaded onto a Bio-Gel HTP column (4.5 cm × 25 cm). After loading, 50 ml of the lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) was first passed through the column to wash off unbound proteins. The bound proteins were then eluted with 15 ml of each of the following buffers: 20 mM Tris–HCl, 1 mM Tris–HCl, pH 8.0, and a stepwise potassium phosphate buffer (KPB), pH 7.5, gradient from 1 to 350 mM KPB. Samples taken from the eluted fractions were analyzed by 10% SDS–polyacrylamide gel electrophoresis (PAGE) for the presence of SULT2B1a or SULT2B1b ST. The fractions containing SULT2B1a or SULT2B1b ST were combined and dialyzed overnight against 10 mM Tris–HCl, pH 8.0, at 4 °C.

### 2.7. ATP-agarose affinity column chromatography

The dialyzed sample was loaded onto an ATP-agarose column (2.5 cm × 4 cm). After loading, the column was first washed with 20 ml of 20 mM Tris–HCl, pH 8.0, and the bound proteins were eluted with 15-ml fractions of 20 mM Tris–HCl, pH 8.0, containing increasing concentrations of NaCl from 0 to 400 mM in increments of 50 mM. Samples taken from the eluted fractions were analyzed by 10% SDS-PAGE for the presence of SULT2B1a or SULT2B1b ST.

### 2.8. Enzymatic assays using xenoestrogens as substrates

The ST activities of the recombinant human cytosolic STs were assayed using PAP[$^{35}$S] as the sulfate donor. The standard assay mixture, with a final volume of 30 μl, contained 50 mM potassium phosphate buffer, pH 7.0, 0.8 μM PAP[$^{35}$S] (15 Ci/mmol), and 50 μM of the substrate tested. For the kinetic studies on the sulfation of xenoestrogens by P-form (SULT1A1) PST, a saturating PAP[$^{35}$S] concentration of 14 μM was used, along with varying concentrations of xenoestrogens. The reaction was started by the addition of the enzyme preparation, allowed to proceed for 15 min at 37 °C and terminated by heating at 100 °C for 2 min. Since under the conditions of the assay, some of the enzymes may denature rapidly and give non-linear results with respect to time and amount of protein, the enzymes used for the assay were stabilized by the addition of 10% (w/v) glycerol and 10 mg/ml bovine serum albumin and activated by addition of DTT to a final concentration of 1.0 mM in the assay mixture. The precipitates formed were cleared by centrifugation and the supernatant was subjected to the analysis of the [$^{35}$S] sulfated product.

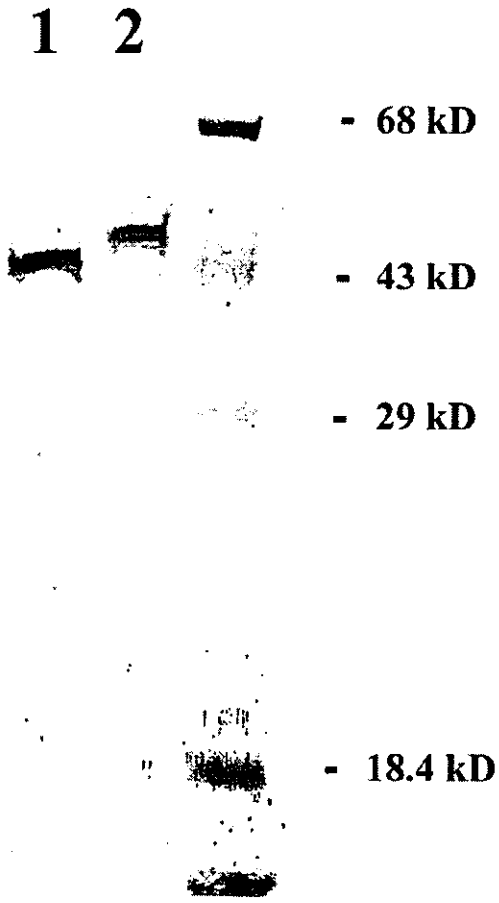


Fig. 1. SDS gel electrophoretic patterns of recombinant human SUL2B1a ST and SULT2B1b ST. Samples analyzed were SUL2B1a ST (lane 1) and SUL2B1b ST (lane 2). Protein molecular weight markers co-electrophoresed are β-lactoglobulin (Mr = 18,400), carbonic anhydrase (Mr = 29,000), ovalbumin (Mr = 43,000), and bovine serum albumin (Mr = 68,000).

### 2.9. Miscellaneous methods

PAP[$^{35}$S] (15 Ci/mmol) was synthesized from ATP and [$^{35}$S]sulfate using the sulfate-activating enzymes, ATP sulfurylase and adenosine 5′-phosphosulfate kinase, from *Bacillus stearothermophilus* as described previously [21]. Protein determination was based on the method of Bradford [22] with bovine serum albumin as the standard. The analysis of [$^{35}$S] sulfated products generated during the enzymatic assay was based on the TLC separation procedure using *n*-butanol/isopropanol/88% formic acid/water (2:1:1:2 by volume) as the solvent system [23]. SDS-PAGE was performed using the method of Laemmli [24].

## 3. Results and discussion

In view of the mounting evidence (as discussed earlier) of the hazardous effects on both wildlife and humans of xenoestrogens in the environment, we decided to investigate the sulfation of some typical xenoestrogens by all the human cytosolic STs known to date. The goal of the study was to investigate the possible relevance of the various human cytosolic STs in the inactivation/disposal of xenoestrogens.

We have previously cloned, expressed and purified eight human cytosolic STs: the M-form (SULT1A3) and P-form (SULT1A1) PSTs, two SULT1C STs (designated #1 and #2), DHEA (SULT2A1) ST, thyroid hormone (SULT1B2) ST, estrogen (SULT1E1) ST, and a neuronal ST (NST) [16–20]. In the present study, we have cloned the other two known human cytosolic STs, the SULT2B1a ST and SULT2B1b ST. As shown in Fig. 1, these two newly cloned and expressed STs appeared to be highly homogeneous upon SDS-PAGE, migrating at, respectively, 40 and 45 kDa positions.

These two STs, together with the other eight known human cytosolic STs previously prepared, were examined for enzymatic activities toward their typical substrates as well as representative xenoestrogens including bisphenol A, 4-*n*-octylphenol, 4-*n*-nonylphenol, diethylstilbestrol and 17-α-ethynylestradiol. The results are compiled in Table 1. We used a substrate concentration of 50 μM in these assays. Some of the cytosolic STs, such as EST, operate on their natural substrates in the nanomolar range [25], while others, such as the M-form and P-form PSTs have $K_m$'s in the micromolar range for their substrates [26,27]. The purpose of our study, however, was to compare the ability of the various human cytosolic STs to sulfate xenoestrogens at slightly higher substrate concentrations that may be reached when these compounds are at toxic levels in the cell. The concentration of PAPS used in this screening study was submicromolar rather than saturating to reflect the possible physiological levels when this compound in the cell becomes limited due to its rapid consumption in sulfation reactions.

Compared with the results obtained in our previous studies [16], the activities of almost all the enzymes tested were found to be somewhat higher toward almost all the xenoestrogen substrates. This may be due to the efforts to stabilize and activate the enzymes (see Materials and methods), which may denature under the conditions of the assay. It should be noted that in the case of the EST (SULT1E1) enzyme, we now used a purified recombinant enzyme rather

Table 1
Specific activities of human cytosolic sulfotransferases with their typical substrates and with environmental xenoestrogens[a]

| | M-PST | P-PST | THST | DHEA ST | EST |
|---|---|---|---|---|---|
| Bisphenol A | N.D.[b] | 1490 ± 29 | 93 ± 10 | 209 ± 6 | N.D. |
| 4-*n*-Octylphenol | 847 ± 21 | 2429 ± 53 | 110 ± 11 | 127 ± 4 | 594 ± 29 |
| 4-*n*-Nonylphenol | 98 ± 21 | 1929 ± 39 | 161 ± 15 | 210 ± 8 | 88 ± 2 |
| Diethyl stilbestrol | 85 ± 10 | 1876 ± 66 | 45 ± 4 | 246 ± 6 | 85 ± 3 |
| 17-α-Ethynylestradiol | 85 ± 7 | 1670 ± 26 | 58 ± 2 | 538 ± 18 | 1089 ± 16 |
| pNP | 1526 ± 8 | 2378 ± 30 | 1358 ± 8 | 101 ± 7 | 160 ± 7 |
| Dopamine | 2684 ± 14 | 948 ± 25 | 43 ± 4 | 134 ± 4 | N.D. |
| DHEA | N.D. | 507 ± 10 | 84 ± 10 | 1649 ± 41 | 40 ± 22 |
| Estrone | 254 ± 16 | 827 ± 31 | 80 ± 3 | 427 ± 17 | 1592 ± 103 |
| | SULT1C ST#1 | SULT1C ST#2 | SULT2B1a ST | SULT2B1b ST | NST |
| Bisphenol A | N.D.[b] | 790 ± 46 | 7 ± 1 | 13 ± 2 | 3.3 ± 0.1 |
| 4-*n*-Octylphenol | N.D. | 1294 ± 45 | 22 ± 21 | 10 ± 2 | 8.9 ± 2.9 |
| 4-*n*-Nonylphenol | N.D. | 976 ± 24 | 11 ± 6 | 9 ± 2 | 5.8 ± 2.9 |
| Diethyl stilbestrol | N.D. | 753 ± 18 | 11 ± 2 | 16 ± 4 | 3.5 ± 0.9 |
| 17-α-Ethynylestradiol | N.D. | 57 ± 4 | 17 ± 8 | 14 ± 1 | 2.7 ± 0.4 |
| pNP | 71 ± 2 | 1311 ± 35 | 163 ± 5 | 22 ± 3 | 24.9 ± 2.2 |
| Dopamine | N.D. | 307 ± 4 | N.D. | N.D. | N.D. |
| DHEA | N.D. | 86 ± 5 | 1443 ± 50 | 1018 ± 34 | N.D. |
| Estrone | N.D. | 272 ± 34 | 14 ± 2 | 22 ± 4 | N.D. |

[a] The specific activities in the table are in units of picomoles per minute per milligram enzyme. The assay mixture contained 1 mM DTT, 14 μM PAPS, 50 μM substrate and phosphate buffer, pH 7.0. The assay temperature was 37 °C. Data are the mean ± S.D. from three determinations.
[b] N.D. refers to (activity) not detected.

than the unpurified commercial preparation used in our earlier studies. As in our previous study [16], we found that P-form PST showed the highest activities toward the various xenoestrogens, as well as the broadest substrate specificity. P-form PST has been located in the intestine [28], liver [29], platelets [29], and lung [30], presumably in keeping with its generally recognized role as the major ST involved in the detoxification of xenobiotics. SULT1C ST #2 was second only to P-form PST in its ability to sulfate xenoestrogens, suggesting that it may also play an important role in xenobiotic metabolism. In contrast, SULT1C ST #1 showed no activity toward any of the compounds tested. This is consistent with studies showing that these two enzymes varied widely in their substrate specificity [31]. Similar to SULT1C ST #1, NST also showed negligible activities toward the xenoestrogens tested. Of the other six human enzymes, SULT2B1a ST and SULT2B1b ST showed high activity toward DHEA but low activity toward the xenoestrogens. M-form PST, SULT1B2 ST, DHEA ST and EST showed intermediate levels of activity toward the xenoestrogens. These enzymes are known to play a role in the metabolism and regulation of the levels of endogenous compounds (dopamine, thyroid hormones, DHEA, and estrogens, respectively). Although they may not, like P-form PST and SULT1C ST #2, be as important in detoxification of xenobiotics, the fact that they show activity toward these compounds makes them potential targets of xenoestrogens. Xenoestrogens are generally referred to as endocrine-disrupting chemicals. Though they are believed to bind to estrogen receptors and may be estrogen agonists, the binding is weak and estrogenic effects due to this pathway may not be sufficient to explain their endocrine-disrupting effect [32]. Since these compounds can serve as substrates for STs such as EST and DHEA ST, they are good candidates as competitive (or more complex) inhibitors for these enzymes. This would then be expected to upset the metabolism and turnover of the normal substrates of these enzymes such as 17-β-estradiol or DHEA, with resultant disruption of endocrine homeostatic mechanisms [4].

Table 2
Kinetic constants of the human P-form PST with xenoestrogens and 17-β-estradiol as substrates[a]

| | $K_m$ (μM) | $V_{max}$ (nmol/min/mg) | $V_{max}/K_m$ |
|---|---|---|---|
| Bisphenol A | 6.69 | 38.6 | 5.77 |
| 4-*n*-Octylphenol | 5.87 | 119 | 20.3 |
| *p*-Nonylphenol | 21.2 | 90.1 | 4.24 |
| DES | 4.53 | 47.4 | 10.5 |
| 17-α-Ethynylestradiol | 1.03 | 20.5 | 19.9 |
| 17-β-estradiol | 3.76 | 24.9 | 6.62 |

[a] The assays were performed as described with 14 μM PAPS and various concentrations of the acceptor substrates in 50 mM potassium phosphate buffer, pH 7.0, at 37 °C.

To investigate in more detail the sulfation of xenoestrogens by the human cytosolic STs, the kinetics of sulfation of these compounds by P-form PST was examined. In these experiments, a saturating concentration of PAPS, that is, 14 μM, and varying concentrations of xenoestrogens were used in the assays. Fig. 2 shows the Lineweaver–Burk double-reciprocal plot derived from the data obtained using bisphenol A as the substrate. Table 2 shows the kinetic constants determined for the sulfation of the five xenoestrogens and 17-β-estradiol, an endogenous substrate. Based on these data, the catalytic efficiency, as reflected by $V_{max}/K_m$, of P-form PST

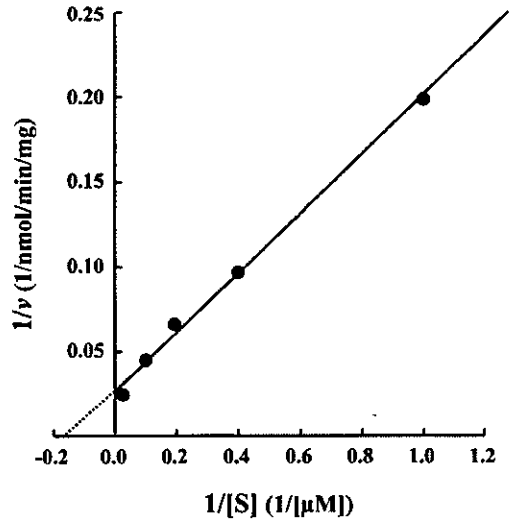


Fig. 2. Lineweaver–Burk double-reciprocal plot of the human P-form PST with bisphenol A as substrate. Concentrations of bisphenol A are expressed in micromoles, and velocities are expressed in nanomoles of product formed per minute per milligram enzyme. Each data point represents the mean value derived from three determinations.

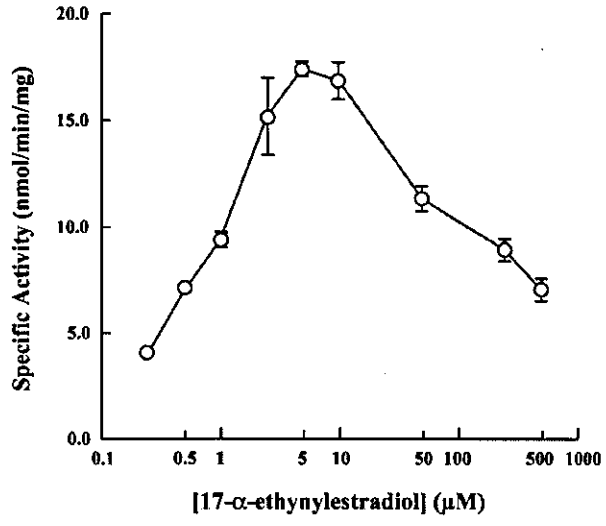


Fig. 3. Michaelis–Menten plot of the sulfation of 17-α-ethynylestradiol by the human P-form PST. Concentrations of 17-α-ethynylestradiol are expressed in micromoles, and velocities are expressed in nanomoles of product formed per minute per milligram enzyme. Each data point represents the mean value derived from three determinations.

for the sulfation of xenoestrogens appeared to be comparable to that for the sulfation of the endogenous substrate, 17-β-estradiol. It should be pointed out that, in these kinetic experiments, a substantial substrate inhibition was observed when high concentrations of xenoestrogens were used (Fig. 3 shows the example of 17-α-ethynylestradiol), and the Lineweaver–Burke plots used only lower xenoestrogen concentrations where the data are linear. Various kinetic mechanisms, including sequential Bi Bi random/ordered and ping-pong have been proposed for various ST enzymes [33]. It should also be remembered that human P-form PST is (as are most of the cytosolic STs) a dimeric enzyme and there has been speculation about the physiological importance of this dimeric structure [34]. The strong substrate inhibition exhibited in the sulfation of xenoestrogens by P-form PST provides a powerful tool to pinpoint the kinetic mechanism involved in this reaction and the importance, if any, of the dimeric enzyme structure [35]. We are currently probing these questions.

In conclusion, the results obtained in the present study indicated clearly the differential xenoestrogen-sulfating activities of different human cytosolic STs. For P-form PST, the major xenoestrogen-sulfating enzyme identified, kinetic data showed that the catalytic efficiency for the sulfation of xenoestrogens appeared to be comparable to that for the sulfation of an endogenous substrate, 17-β-estradiol. Further studies using, for example, cultured estrogen-responsive cells and laboratory animals are warranted to fully elucidate the role of sulfation and the responsible ST enzyme(s) in the inactivation/disposal of xenoestrogens in vivo.

## Acknowledgements

This work was supported in part by funds from the Research Institute of Innovative Technology for the Earth (Japan), and for Scientific Research (C) (MS #12836012) from Monbusho, Japan.

## References

[1] L.J. Guillette Jr., T.S. Gross, D.A. Gross, A.A. Rooney, H.F. Percival, Environ. Health Perspect., Suppl. 103 (1995) 31–36.
[2] D.M. Fry, Environ. Health Perspect. 103 (1995) 165–171.
[3] T.J. Ridgway, H. Wiseman, Biochem. Soc. Trans. 26 (1998) 675–680.
[4] M.H. Kester, S. Bulduk, D. Tibboel, W. Meinl, H. Glatt, C.N. Falany, M.W. Coughtrie, A. Bergman, S.H. Safe, G.G. Kuiper, A.G. Schuur, A. Brouwer, T.J. Visser, Endocrinology 141 (2000) 1897–1900.
[5] D. Roy, M. Palangat, C.-W. Chen, R.D. Thomas, J. Colerangle, A. Atkinson, Z.-J. Yan, J. Toxicol. Environ. Health 50 (1997) 1–29.
[6] E. Carlsen, A. Giwercman, N. Keiding, N.E. Skakkebaek, Br. Med. J. 305 (1992) 609–613.
[7] J. Auger, J.M. Kunstmann, F. Czyglik, P. Jouannet, N. Engl. J. Med. 332 (1995) 281–285.
[8] F. Falck Jr., A. Ricci Jr., M.S. Wolff, J. Godbold, P. Deckers, Arch. Environ. Health 47 (1992) 143–146.
[9] M.S. Wolff, P.G. Toniolo, E.W. Lee, M. Rivera, N. Dubin, J. Natl. Cancer Inst. 85 (1992) 648–652.
[10] H. Yokota, H. Iwano, M. Endo, T. Kobayashi, H. Inoue, S. Ikushiro, A. Yuasa, Biochem. J. 340 (1999) 405–409.
[11] G.J. Mulder, W.B. Jakoby, in: G.J. Mulder (Ed.), Conjugation Reactions in Drug Metabolism, Taylor and Francis, London, 1990, pp. 107–161.
[12] C. Falany, J.A. Roth, in: E.H. Jeffery (Ed.), Human Drug Metabolism: From Molecular Biology to Man, CRC Press, Boca Raton, 1993, pp. 101–115.
[13] R. Weinshilboum, D.M. Otterness, in: F.C. Kaufmann (Ed.), Conjugation–Deconjugation Reactions in Drug Metabolism and Toxicity, Springer-Verlag, Berlin, 1994, pp. 45–78.
[14] F. Lipmann, Science 128 (1958) 575–580.
[15] R. Lamed, Y. Levin, M. Wilchek, Biochim. Biophys. Acta 304 (1973) 231–235.
[16] M. Suiko, Y. Sakakibara, M.-C. Liu, Biochem. Biophys. Res. Commun. 267 (2000) 80–84.
[17] Y. Sakakibara, Y. Takami, T. Nakayama, M. Suiko, M.-C. Liu, J. Biol. Chem. 273 (1998) 6242–6247.
[18] Y. Sakakibara, K. Yanagisawa, J. Katafuchi, D.P. Ringer, Y. Takami, T. Nakayama, M. Suiko, M.-C. Liu, J. Biol. Chem. 273 (1998) 33929–33935.
[19] Y. Sakakibara, M. Suiko, T.G. Pai, T. Nakayama, Y. Takami, J. Katafuchi, M.-C. Liu, Gene 285 (2002) 39–47.
[20] T.G. Pai, M. Suiko, Y. Sakakibara, M.-C. Liu, Biochem. Biophys. Res. Commun. 285 (2001) 1175–1179.
[21] P.H.P. Fernando, A. Karakawa, Y. Sakakibara, H. Ibuki, H. Nakajima, M.-C. Liu, M. Suiko, Biosci. Biotechnol. Biochem. 5 (1993) 1974–1975.
[22] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[23] M.-C. Liu, F. Lipmann, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 3695–3698.
[24] U.K. Laemmli, Nature 227 (1970) 680–685.
[25] C.N. Falany, V. Krasnykh, J.L. Falany, J. Steroid Biochem. Mol. Biol. 52 (1995) 529–539.
[26] T.C. Ganguly, V. Krasnykh, C.N. Falany, Drug Metab. Dispos. 23 (1995) 945–950.
[27] M.E. Veronese, W. Burgess, X. Zhu, M.E. McManus, Biochem. J. 302 (1994) 497–502.
[28] R.S. Sundaram, C. Szumlanski, D. Otterness, J.A. Van Loon, R.M. Weinshilboum, Drug Metab. Dispos. 17 (1989) 255–264.
[29] C.N. Falany, M.E. Vazquez, J.A. Heroux, J.A. Roth, Arch. Biochem. Biophys. 278 (1990) 312–318.
[30] A. Baranczyk-Kuzma, T. Szymczyk, Biochem. Pharmacol. 35 (1986) 995–999.
[31] K. Yoshinara, K. Nagata, M. Shimada, Y. Yamazoe, Carcinogenesis 19 (1998) 951–953.
[32] G.G. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. Saag, B. Burg, J.-A. Gustafsson, Endocrinology 139 (1998) 4252–4263.
[33] E. Vakiani, J.G. Luz, J. Buck, J. Biol. Chem. 273 (1998) 35381–35387.
[34] E.V. Petrotchenko, L.C. Pedersen, C.H. Borchers, K.B. Tomer, M. Negishi, FEBS Lett. 490 (2001) 39–43.
[35] I.H. Segel, Enzyme Kinetics, Wiley, New York, 1975.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 3, Issue of January 17, pp. 1525–1532, 2003
Printed in U.S.A.


# Structure-Function Relationships in the Stereospecific and Manganese-dependent 3,4-Dihydroxyphenylalanine/ Tyrosine-sulfating Activity of Human Monoamine-form Phenol Sulfotransferase, SULT1A3*

Received for publication, April 1, 2002, and in revised form, November 5, 2002
Published, JBC Papers in Press, November 6, 2002, DOI 10.1074/jbc.M203108200

**T. Govind Pai, Ila Oxendine, Takuya Sugahara, Masahito Suiko, Yoichi Sakakibara, and Ming-Cheh Liu‡**

*From the Biomedical Research Center, The University of Texas Health Center, Tyler, Texas 75708*

**The human monoamine-form phenol sulfotransferase (PST), SULT1A3, has a unique 3,4-dihydroxyphenylalanine (Dopa)/tyrosine-sulfating activity that is stereospecific for their D-form enantiomers and can be stimulated dramatically by $Mn^{2+}$. This activity is not present in the simple phenol-form PST, SULT1A1, which is otherwise >93% identical to SULT1A3 in amino acid sequence. The majority of the differences between these two proteins reside in two variable regions of their sequences. Through the characterization of chimeric PSTs where these two regions were exchanged between them, it was demonstrated that variable Region II of SULT1A3 is required for the stereospecificity of its Dopa/tyrosine-sulfating activity, whereas variable Region I of SULT1A3 is required for the stimulation by $Mn^{2+}$ of this activity. Further studies using point-mutated SULT1A3s mutated at amino acid residues in these two regions and deletional mutants missing residues 84–86 and 84–90 implicate residue Glu-146 (in variable Region II of SULT1A3), as well as the presence of residues 84–90 of variable Region I, in the stereospecificity in the absence of $Mn^{2+}$. Residue Asp-86 (in variable Region I of SULT1A3), on the other hand, is critical in the $Mn^{2+}$ stimulation of the Dopa/tyrosine-sulfating activity of SULT1A3. A model is proposed, with reference to the reported x-ray crystal structure of SULT1A3, to explain how the normal role of SULT1A3 in dopamine regulation may be subverted in the presence of $Mn^{2+}$. These studies could be relevant in understanding the stereoselective action of SULT1A3 on chiral drugs.**

The sulfotransferases (STs),[1] which are ubiquitous in both plants and animals, catalyze the sulfation of hydroxyl or amino groups on a variety of target acceptor molecules (1, 2). These enzymes all use adenosine 3′-phosphate,5′-phosphosulfate (PAPS) as the sulfonyl group donor (3) and share sequences responsible for PAPS binding (4). Although the membrane-bound STs use proteins, glycolipids, and other macromolecules as acceptor substrates, the cytosolic STs sulfate smaller molecules and are part of the Phase II detoxification pathway for the biotransformation/excretion of drugs and xenobiotics (1, 2). Increasingly, the cytosolic STs have also been shown to be important in regulating the levels and/or activities of endogenous compounds such as thyroid and steroid hormones, catecholamines, and bile acids (5, 6).

Based on their sequences, the cytosolic STs have been classified into several gene families (4). Two human cytosolic STs, the monoamine-form and the simple phenol-form phenol sulfotransferases, named SULT1A3 and SULT1A1, respectively (4), show an extensive (>93%) identity in their amino acid sequences (*cf.* Fig. 1*A*) and yet vary widely in their substrate specificity and other properties (7–9). They have thus served as an ideal model system to study structure/function relationships in these proteins. Examination of their aligned sequences revealed that most of the differences between SULT1A3 and SULT1A1 occur in two variable regions, designated Region I (encompassing amino acid residues 84–89) and Region II (including residues 143–148) (*cf.* Fig. 1*A*). Based on the hypothesis that the differences in these two variable regions may account for the distinct properties of SULT1A3 and SULT1A1, we had prepared chimeric proteins (7), where these two regions were reciprocally exchanged (*cf.* Fig. 1*B*). Characterization of these chimeras indicated that both Regions I and II were indeed critical for the specificity of SULT1A3 for dopamine and of SULT1A1 for *p*-nitrophenol (7). To extend the study further, we and others (8, 10, 11) had, by site-directed mutagenesis, exchanged amino acid residues in these two regions between SULT1A3 and SULT1A1. Results from these studies implicated residue 146 in Region II and residues 86 and 89 in Region I, as important in determining the specificity of the enzymes for their respective substrates.

SULT1A3 is present in brain where it is believed to sulfate monoamine neurotransmitters (particularly dopamine) with high activity and thus to regulate their levels (5). It also serves a detoxifying function in the intestine, where it may detoxify potentially lethal dietary monoamines (12). Besides the activity toward its physiological substrate, dopamine, for which it has a $K_m$ of 2 μM, SULT1A3 has been shown recently to display a unique Dopa/tyrosine-sulfating activity at higher (millimolar and sub-millimolar) concentrations of these substrates, which can be dramatically stimulated by $Mn^{2+}$ (13).

In this study, chimeras and site-directed mutants were used to explore the structural basis for the stereospecificity and

* This work was supported in part by a grant from the American Heart Association (Texas Affiliate) (to M. C. L.), a UTHCT President's Council Research Membership Seed Grant (to M. C. L.), and an award from the Naito Foundation (to M. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Biomedical Research Center, The University of Texas Health Center, 11937 U. S. Highway 271, Tyler, TX 75708. Tel.: 903-877-2862; Fax: 903-877-2863; E-mail: ming.liu@uthct.edu.

[1] The abbreviations used are: ST, sulfotransferase; PAPS, adenosine 3′-phosphate,5′-phosphosulfate; Dopa, 3,4-dihydroxyphenylalanine; TAPS, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid; TLC, thin-layer chromatography.

$Mn^{2+}$ stimulation of the Dopa/tyrosine-sulfating activity of SULT1A3. SULT1A3 is present as a homodimer in its native state (14). Kinetic studies of the Dopa/tyrosine-sulfating activity of SULT1A3 (15) suggested that, in the case of D-tyrosine, where a 4-hydroxyphenyl group is to be sulfated, the tyrosine-$Mn^{2+}$ and tyrosine-$Mn^{2+}$-tyrosine complexes may be the real and obligatory substrates. In the case of D-Dopa, where we had shown that sulfation takes place exclusively at the 3-hydroxyphenyl group (16), the substrate-$Mn^{2+}$ complexes appeared to be better substrates than D-Dopa. These kinetic data implied that $Mn^{2+}$, while interacting with the carboxyl group of the Dopa/tyrosine substrate to form the complex, also coordinates with amino acid residues on the enzyme. Previous X-ray crystallography study on SULT1A3 (17) has suggested that residues 84–92 corresponding to the variable Region I (*cf.* Fig. 1*A*) form a "mobile" loop. Based on the amino acid residue in SULT1A3 identified as interacting with $Mn^{2+}$ in the present study, a model has been proposed, in reference to the above-mentioned mobile loop, to explain how $Mn^{2+}$ exerts its stimulatory effect on the sulfation of tyrosine and Dopa by the enzyme. This study also identified the amino acid residues and regions responsible for the unusual stereospecificity of SULT1A3 for the D-enantiomers of Dopa and tyrosine. The model developed may prove useful in understanding the stereoselective action of SULT1A3 on chiral drugs (18–21) and the possible effects of metal ions on this activity.

## EXPERIMENTAL PROCEDURES

*Materials*—L-Tyrosine, D-tyrosine, L-Dopa, D-Dopa, ATP, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid (TAPS), SDS, dithiothreitol, EDTA (tetrasodium salt), isopropyl β-D-thiogalactopyranoside, thrombin, and PAPS were from Sigma. QuikChange site-directed mutagenesis kit and XL1-Blue *Epicurian coli* competent cells were purchased from Stratagene. Oligonucleotide primers were synthesized by MWG Biotech. pGEX-2TK glutathione *S*-transferase gene fusion vector, *E. coli* BL21 host cells, and glutathione-Sepharose were products of Amersham Biosciences. Carrier-free sodium [$^{35}$S]sulfate and Ecolume liquid scintillation fluid were from ICN Biomedicals. Recombinant human bifunctional ATP sulfurylase/adenosine 5′-phosphosulfate kinase was prepared as described previously (22). Cellulose thin-layer chromatography (TLC) plates were from EM Science. All other chemicals were of the highest grades available commercially.

*Preparation of Purified Wild-type and Chimeric SULT1A3/SULT1A1 and Point-mutated SULT1A3s*—Wild-type and chimeric SULT1A3 and SULT1A1, cloned/generated and expressed using the pGEX-2TK glutathione *S*-transferase gene fusion system, were purified using glutathione-Sepharose in conjunction with thrombin cleavage to separate the fusion protein, based on the procedure established previously (7). Point-mutated SULT1A3s were prepared using the QuikChange site-directed mutagenesis kit, expressed using the pGEX-2TK glutathione S-transferase gene fusion system and purified with glutathione-Sepharose followed by thrombin cleavage to separate the fusion protein, as described previously (8).

*Generation, Expression, and Purification of Deletional Mutants of SULT1A3*—The QuikChange site-directed mutagenesis kit from Stratagene was used for the generation of deletional mutants of SULT1A3. Briefly, wild-type SULT1A3 cDNA packaged in pGEX-2TK prokaryotic expression vector was used as the template in conjunction with specific mutagenic primers. To prepare the deletional SULT1A3 mutant lacking residues 84, 85, and 86 of the mobile loop (see Introduction), the mutagenic oligonucleotide primer set, 5′-CGGGTGCCCTTCCTTGAGCC-AGGGGAACCCTCAGGG-3′ and 5′-CCCTGAGGGTTCCCCTGGCTCA-AGGAAGGGCACCCG-3′, was used. Similarly, a deletional mutant of SULT1A3 lacking residues 84 through 90 of the loop was prepared using the mutagenic primer set, 5′-CGGGTGCCCTTCCTTGAGTCAG-GGCTGGAGACTCTG-3′ and 5′-CAGAGTCTCCAGCCCTGACTCAAG-GAAGGGCACCCG-3′. The amplification conditions were 12 cycles of 30 s at 95 °C, 1 min at 55 °C, and 15 min at 68 °C. The deletional SULT1A3 mutant sequences were verified by nucleotide sequencing (23). pGEX-2TK vector harboring individual deletional SULT1A3 mutant sequence was transformed into competent XL1-Blue *E. coli* cells. The transformed cells, grown to $A_{600\ nm}$ = ~0.5 in 1 liter of LB medium supplemented with 100 μg/ml ampicillin and induced with 0.1 mM isopropyl β-D-thiogalactopyranoside overnight at room temperature, were collected by centrifugation and processed for the purification of recombinant deletional SULT1A3 mutant enzyme using the same procedure developed previously for the wild-type SULT1A3 (7).

*Enzymatic Assay*—Sulfotransferase activities of the purified wild-type and chimeric SULT1A3/SULT1A1, as well as point and deletional mutant SULT1A3s, were assayed using [$^{35}$S]PAPS as the sulfonate group donor. The standard assay mixture, in a final volume of 25 μl, contained 50 mM TAPS buffer, pH 8.25, 15 μM [$^{35}$S]PAPS, 5 mM D- or L-tyrosine, or 1 mM D- or L-Dopa without additions (control) or with 1 mM EDTA, 5 mM $MnCl_2$, or 5 mM EDTA plus 5 mM $MnCl_2$. The enzyme dilutions were prepared in 50 mM TAPS, pH 8.25, containing 10% glycerol and 8 mM dithiothreitol. The reaction was started by the addition of 5 μl of the enzyme preparation, allowed to proceed for 3 or 10 min (so that the reaction reached no more than 5–10% of completion) at 37 °C, and terminated by heating at 100 °C for 2 min. The precipitates were cleared by centrifugation for 1 min, and the supernatant was subjected to the analysis of [$^{35}$S]sulfated D- or L-tyrosine or D- or L-Dopa based on the TLC procedure established previously (24). In the experiments with D- or L-tyrosine as substrate, the sulfated product was separated by ascending TLC on a cellulose TLC plate in a solvent system containing *n*-butanol, isopropanol, 88% formic acid, water in a 3:1:1:1 ratio by volume. In the experiments with D- or L-Dopa as substrate, the sulfated product was first subjected to high voltage (1000 volts) thin-layer electrophoresis in one dimension before performing the ascending TLC separation in the second dimension. Each experiment was performed in triplicate, together with a control without enzyme. The results obtained were calculated and expressed in nmol sulfated product formed/min/mg protein.

*Miscellaneous Methods*—[$^{35}$S]PAPS was synthesized from ATP and carrier-free [$^{35}$S]sulfate using the bifunctional human ATP sulfurylase/ATP sulfurylase/adenosine 5′-phosphosulfate kinase, and its purity was determined as described previously (25). The [$^{35}$S]PAPS synthesized was then adjusted to the required concentration and specific activity by the addition of cold PAPS. SDS-polyacrylamide gel electrophoresis was performed on 12% polyacrylamide gels using the method of Laemmli (26). Protein determination was based on the method of Bradford with bovine serum albumin as standard (27).

## RESULTS AND DISCUSSION

Despite recent efforts from several laboratories (7–11), there is still relatively scant information concerning the structure/function relationships of cytosolic ST enzymes. As noted earlier, SULT1A3 and SULT1A1, which are >93% identical in amino acid sequence and yet display distinct substrate specificity and other properties, provide an excellent model for studies in this regard. In contrast to SULT1A1, SULT1A3 exhibits two unique activities. The sulfating activity toward dopamine, which presumably helps to regulate the levels of this endogenous compound, has a pH optimum of ~7.0 and a $K_m$ of 2 μM (in the physiological range) (14, 28). $Mn^{2+}$ stimulated this activity to a comparatively smaller extent (~2- to 3-fold) (13) but appeared to increase the $K_m$ for dopamine slightly (15). The Dopa/tyrosine-sulfating activity of SULT1A3, on the other hand, has the hallmarks of a detoxifying activity, with a pH optimum between 8 and 9 (29) and a $K_m$ (if we consider the substrate rather than substrate-$Mn^{2+}$ complex) in the millimolar or sub-millimolar range (7, 13). In contrast to the dopamine-sulfating activity, the Dopa/tyrosine-sulfating activity can be stimulated much more dramatically by $Mn^{2+}$ and, intriguingly, displays stereospecificity for the D-form Dopa/tyrosine enantiomers (13). As discussed before, our recent kinetic studies (15) suggested that Dopa and tyrosine form complexes with $Mn^{2+}$ (the pH dependence of the reaction forming the complex may explain the different pH optimum for the Dopa/tyrosine-sulfating activity compared with that for the dopamine sulfation, which does not involve such a complex) that serve either as the obligatory (in the case of tyrosine) or as a better (in the case of Dopa) substrate. The log K for the formation of the tyrosine-$Mn^{2+}$ complex is 1.5, whereas that for the tyrosine-$Mn^{2+}$-tyrosine complex is 5.0 (30, 31). $Mn^{2+}$ coordinates not only with

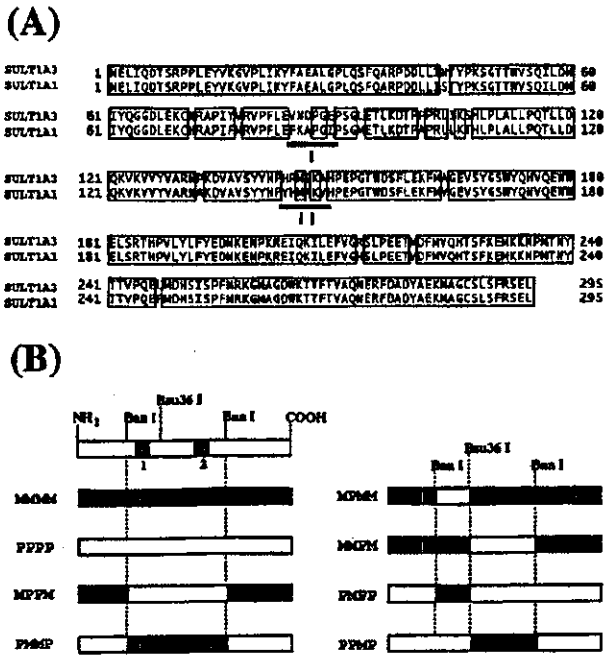


FIG. 1. *A*, amino acid sequence comparison of SULT1A3 and SULT1A1. Identical amino acid residues are *boxed*. *B*, schematic representation of the molecular architecture of wild-type SULT1A3 (labeled *MMMM*), SULT1A1 (labeled *PPPP*), and chimeras.

the carboxyl group of the substrates (to form the complexes) but also with amino acid residues on the enzyme (on formation of the enzyme-substrate complex). Our previous studies using chimeric and point-mutated SULT1A3/SULT1A1s had revealed that two variable regions (*cf.* Fig. 1*A*), and in particular residues 86, 89, and 146 therein, are important in determining the distinct substrate specificity of these two otherwise highly homologous enzymes. The current study aimed to elucidate further the structural determinants for the unique stereoselectivity and $Mn^{2+}$ dependence of the Dopa/tyrosine-sulfating activity of SULT1A3.

*Differential Roles of Variable Regions I and II of SULT1A3 in the Stereospecific Dopa/Tyrosine Sulfation and Its Stimulation by $Mn^{2+}$*—This was studied by testing wild-type SULT1A3 and SULT1A1 and chimeric SULT1A3/SULT1A1s (*cf.* Fig. 1*B*) for their sulfation activities toward the Dopa and tyrosine enantiomers, in the presence or absence of $Mn^{2+}$. Our previous kinetic studies (15) had confirmed that $Mn^{2+}$ could stimulate dramatically the sulfation activity of wild-type SULT1A3 toward the tyrosine enantiomers. $Mn^{2+}$, when added at a 5 mM concentration to the assay mixture, stimulated the sulfation activity of SULT1A3 toward D-tyrosine by two orders of magnitude in comparison with the basal activity determined without $Mn^{2+}$, whereas the activity toward L-tyrosine was stimulated about ten times (15). The same assay conditions were used to evaluate the stimulatory effect of $Mn^{2+}$ on the sulfation activities of wild-type SULT1A3 and SULT1A1 and their various chimeras. The activity data obtained (Table I) confirmed that the $Mn^{2+}$-stimulated activity with D-tyrosine as substrate for wild-type SULT1A3 was about 135 times the basal activity. When L-tyrosine was used as substrate, the stimulation was 14-fold. Therefore, as in our previous findings, wild-type SULT1A3 (designated MMMM for comparison with the chimeras; *cf.* Fig. 1*B*) showed a distinct preference for the D-form enantiomer of tyrosine relative to the L-enantiomer. Wild-type SULT1A1 (PPPP), on the other hand, showed no sulfation activity toward either tyrosine enantiomer, with or without $Mn^{2+}$. The data in Table I were analyzed to pinpoint which of the four regions (the two flanking regions and the middle variable Regions I and II) in the SULT1A3 molecule are required, respectively, for the basal tyrosine-sulfating activity, the stereospecificity, and the $Mn^{2+}$ stimulation. Interestingly, the PMMP chimera displayed essentially the same level of basal activity, extent of stimulation by $Mn^{2+}$, and stereospecificity as wild-type SULT1A3 (MMMM), whereas wild-type SULT1A1 (PPPP) showed none of these effects. These results indicated that the variable Regions I and II of the SULT1A3 can fully account for the tyrosine-sulfating activity and $Mn^{2+}$ stimulation effect. Data on the chimeras with the variable Region II derived from SULT1A1 (MPPM, MMPM, and PMPP) showed that they were all incapable of catalyzing the sulfation of tyrosine enantiomers, indicating an absolute requirement for the variable Region II of the SULT1A3 molecule for the basal tyrosine-sulfating activity. In contrast, the chimeras MPMM and PPMP showed a small basal activity with D-tyrosine as substrate but without any $Mn^{2+}$ stimulation effect. With L-tyrosine as substrate, however, no activity was detected with either of these two chimeras. These latter results suggested indirectly that the variable Region I of the SULT1A3 molecule, although not absolutely required for, may contribute substantially to its D-tyrosine sulfation activity. Moreover, the data indicated that this variable Region I is essential for the $Mn^{2+}$ stimulation effect with D-tyrosine as substrate.

A limitation in the use of tyrosine as substrate is that the basal activities detected for the wild-type and chimeric SULTs were all quite low. Although the above-mentioned data indicated that the SULT1A3 Region I is required for the $Mn^{2+}$ stimulation effect, it was difficult to determine whether it is truly required for the stereospecificity of SULT1A3 for the D-form tyrosine enantiomer. To better address this issue, Dopa enantiomers were used as substrates. Our previous kinetic studies (15) had demonstrated that wild-type SULT1A3 displayed significantly higher basal sulfation activities toward Dopa. Although much less dramatic compared with the sulfation of tyrosine, a 2- to 3-fold stimulation (at the Dopa concentrations used) of the Dopa-sulfating activity was observed in the presence of $Mn^{2+}$. Therefore, using Dopa as substrate may prove to be more useful in investigating the structural requirements for the stereospecificity and $Mn^{2+}$ stimulation effect. The sulfation activities of wild-type SULT1A3 and SULT1A1 and the chimeras toward D- or L-Dopa, in the presence or absence of 2.5 mM $Mn^{2+}$, were determined. The results obtained are compiled in Table II. Wild-type SULT1A3 showed a distinct preference for D-Dopa. The $Mn^{2+}$-stimulated activity of wild-type SULT1A3 with D-Dopa as substrate was 1.8 times the basal activity, whereas with L-Dopa it was almost 2-fold. The data obtained with the chimeras basically reinforce the conclusions reached using tyrosine as substrate. Moreover, the chimeras with the variable Region I derived from SULT1A1 and Region II from SULT1A3 (*i.e.* PPMP and MPMM), while exhibiting substantial basal sulfation activities toward both D- and L-Dopa, showed a preference for the D-enantiomer, especially with the PPMP chimera where the activities were higher. There appeared to be no significant $Mn^{2+}$ stimulation effects with these chimeras. Because the absence of SULT1A3 variable Region I abolished the $Mn^{2+}$ stimulation effect it follows that this Region I is required for the $Mn^{2+}$ stimulation effect. The stereospecificity for the D-enantiomer, however, is present regardless of whether Region I is from the SULT1A3 or SULT1A1. The variable Region II of SULT1A3 may be required for its stereospecificity for the D-enantiomers. Moreover, the variable Region II of SULT1A3 is absolutely required for the basal Dopa/tyrosine-sulfating activity, whereas this variable

TABLE I

*Specific activities of the wild-type and chimeric SULT1A3/SULT1A1s with D- or L-tyrosine in the presence or absence of $Mn^{++}$*

The values are the calculated mean ± S.D. from three determinations. The concentration of D- or L-*p*-tyrosine used was 5 mM, and the concentration of $Mn^{++}$ was also 5 mM. All other assay conditions are as described under "Experimental Procedures." ND, not detected.

| Substrate used | Specific activity, nmol sulfated product formed/min/mg protein | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MMMM | PMMP | MPMM | PPMP | MPPM | MMPM | PMPP | PPPP |
| D-*p*-Tyrosine + $Mn^{++}$ | 602 ± 63 | 619 ± 73 | ND | ND | ND | ND | ND | ND |
| D-*p*-Tyrosine | 4.5 ± 0.5 | 6.2 ± 0.6 | 0.6 ± 0.1 | 1.1 ± 0.1 | ND | ND | ND | ND |
| L-*p*-Tyrosine + $Mn^{++}$ | 14.5 ± 0.7 | 15.7 ± 0.4 | ND | ND | ND | ND | ND | ND |
| L-*p*-Tyrosine | 1.0 ± 0.1 | 1.2 ± 0.1 | ND | ND | ND | ND | ND | ND |

TABLE II

*Specific activities of the wild-type and chimeric SULT1A3/SULT1A1s with D- or L-Dopa in the presence or absence of $Mn^{++}$*

The values shown represents means ± S.D. derived from three determinations. The concentration of D- or L-Dopa used was 1 mM, and the concentration of $Mn^{++}$ was 2.5 mM. All other assay conditions are as described under "Experimental Procedures." ND, not detected.

| Substrate used | Specific activity, nmol sulfated product formed/min/mg protein | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MMMM | PMMP | MPMM | PPMP | MPPM | MMPM | PMPP | PPPP |
| D-Dopa + $Mn^{++}$ | 1545 ± 3 | 1649 ± 66 | 8.0 ± 0.4 | 52.7 ± 4.4 | ND | ND | ND | ND |
| D-Dopa | 845 ± 43 | 871 ± 46 | 7.6 ± 3.8 | 42.8 ± 4.9 | ND | ND | ND | ND |
| L-Dopa + $Mn^{++}$ | 49.9 ± 5.4 | 57.4 ± 4.8 | 1.7 ± 0.3 | 6.8 ± 1.1 | ND | ND | ND | ND |
| L-Dopa | 25.9 ± 1.3 | 25.0 ± 0.4 | 2.5 ± 0.3 | 7.8 ± 0.1 | ND | ND | ND | ND |

Region I, although not absolutely required, substantially enhances this sulfation activity.

*Identification of Specific Amino Acid Residues Required for the $Mn^{2+}$ Stimulation of the Sulfation of Tyrosine and Dopa Enantiomers*—To investigate further the structural determinants for the $Mn^{2+}$ stimulation of the sulfation of tyrosine and Dopa enantiomers, point-mutated SULT1A3s, targeted at specific amino acid residues within the variable Regions I and II, were tested. By employing the site-directed mutagenesis technique, we had generated, expressed, and purified nine point-mutated SULT1A3s (five single mutants, three double mutants, and a triple mutant) targeted at amino acid residues in the two variable regions (8). One other single mutant involved a conserved residue Lys-48 that most likely participates in catalysis (8). The sulfation activities of these point-mutated SULT1A3s toward D- or L-tyrosine, in the presence or absence of $Mn^{2+}$, were determined.

Fig. 2 shows a bar graph plotted based on the results obtained in experiments in which the $Mn^{2+}$-stimulated D-tyrosine-sulfating activities of wild-type and mutant SULT1A3s were compared with their basal activities. In the absence of $Mn^{2+}$, only a small sulfation activity was detected in all cases. It was evident that the $Mn^{2+}$-stimulated D-tyrosine-sulfating activity found with the wild-type enzyme (135 times the basal activity) was largely retained in the N85K, E89I, and H143Y mutants, as well as in the N85K/E89I double mutant. This indicates that amino acid residues Asn-85, Glu-89, and His-143 are not required for the $Mn^{2+}$-stimulated sulfation activity toward D-tyrosine, because their mutation did not abolish the stimulation by $Mn^{2+}$. In contrast, both the E146A and D86A mutants and the double or triple mutants involving one or both of these two residues (*i.e.* N85K/D86A, D86A/E89I, and D86A/E89I/E146A) showed very low sulfation activities toward D-tyrosine, with or without $Mn^{2+}$. Mutation at residue Lys-48 resulted in the virtual abolishment of the sulfation activity toward D-tyrosine, with or without $Mn^{2+}$. As revealed in our previous studies (8), however, this may be because Lys-48 is a catalytic residue (conserved in SULT1A3 and SULT1A1, as well as other cytosolic STs), and its mutation would result in the loss of virtually the entire sulfation activity.

Fig. 3 shows a bar graph plotted based on the results obtained in experiments in which the $Mn^{2+}$-stimulated L-tyrosine-sulfating activities of wild-type and mutant SULT1A3s were compared with their basal activities. The results were in general similar to those obtained with D-tyrosine as substrate,

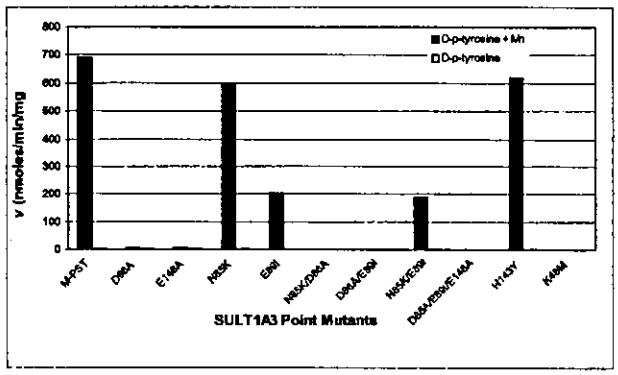


FIG. 2. **Effect of $Mn^{2+}$ on the sulfation of D-tyrosine by wild-type (labeled *M-PST*) and point-mutated SULT1A3s.** The *white bars* represent the sulfation activity of wild-type and point-mutated SULT1A3s toward 5 mM D-*p*-tyrosine in the absence of $Mn^{2+}$. The *black bars* represent this activity in the presence of 5 mM $Mn^{2+}$. The activities are expressed in nmol of sulfated product produced/min/mg enzyme.

though the extent of stimulation by $Mn^{2+}$ was considerably lower in all cases. One exception, however, is that the E146A mutant exhibited a considerable stimulation by $Mn^{2+}$ of its sulfation activity toward L-tyrosine, albeit at lower activity levels. This was in fact also the case in its sulfation activity toward D-tyrosine, which showed a four times stimulation by $Mn^{2+}$ (this $Mn^{2+}$ stimulation effect in Fig. 2, however, is overshadowed by the much more dramatic $Mn^{2+}$ stimulation effects observed with wild-type SULT1A3 and N85K, E89I, H143Y, and N85K/E89I mutant clones). These latter results therefore indicate that the Glu-146 residue is not pivotal for the $Mn^{2+}$ stimulation of the sulfation of the tyrosine enantiomers by SULT1A3.

To summarize, the data in Figs. 2 and 3 appear to rule out the requirement for residues Asn-85, Glu-89, Glu-146, and His-143 in the $Mn^{2+}$ stimulation of the tyrosine-sulfating activity of SULT1A3. The role of residue Asp-86 is not as clear. This is because the sulfation activities toward both D- and L-tyrosine, either in the presence or absence of $Mn^{2+}$, were very low for the D86A mutant, and the data were somewhat ambiguous. Whether the lowered sulfation activities were the result of a drastic loss of the basal tyrosine-sulfating activity (as appeared to be the case with the E146A mutant) or a loss of the $Mn^{2+}$ stimulation effect or both was not clear. To resolve this

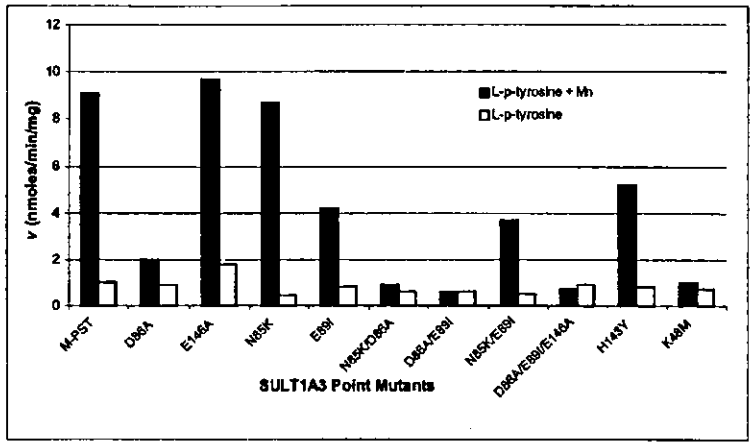


FIG. 3. **Effect of $Mn^{2+}$ on the sulfation of L-tyrosine by wild-type (labeled *M-PST*) and point-mutated SULT1A3s.** The *white bars* represent the sulfation activity of wild-type and point-mutated SULT1A3s toward 5 mM L-*p*-tyrosine in the absence of $Mn^{2+}$. The *black bars* represent this activity in the presence of 5 mM $Mn^{2+}$. The activities are expressed in nmol of sulfated product produced/min/mg enzyme.

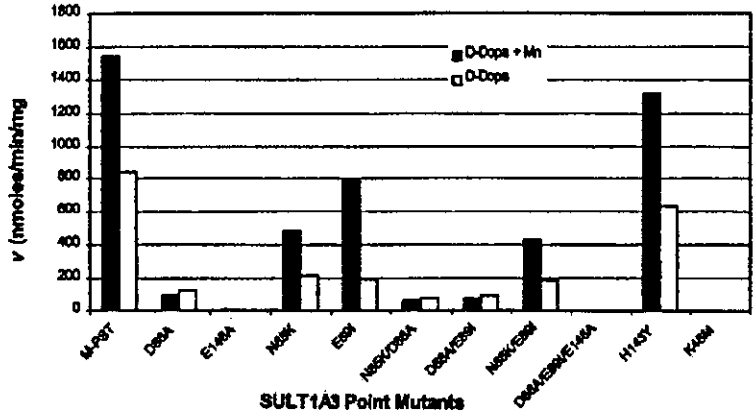


FIG. 4. **Effect of $Mn^{2+}$ on the sulfation of D-Dopa by wild-type (labeled *M-PST*) and point-mutated SULT1A3s.** The *white bars* represent the sulfation activity of wild-type and point-mutated SULT1A3s toward 1 mM D-Dopa in the absence of $Mn^{2+}$. The *black bars* represent this activity in the presence of 2.5 mM $Mn^{2+}$. The activities are expressed in nmol of sulfated product produced/min/mg enzyme.

issue, we carried out assays using the same set of wild-type and chimeric SULT1A3/SULT1A1s (*cf.* Fig. 1) with D- and L-Dopa as substrates. As revealed in Table II, wild-type SULT1A3 showed a much higher basal activity toward Dopa, as well as a clearly detectable stimulation by $Mn^{2+}$ (though not as dramatic as in the case of tyrosine). Therefore, using D- and L-Dopa as substrates, it may be possible to uncouple the Dopa/tyrosine-sulfating activity from its stimulation by $Mn^{2+}$.

Fig. 4 shows a bar graph plotted based on the results obtained in experiments in which the $Mn^{2+}$-stimulated D-Dopa-sulfating activities of wild-type and mutant SULT1A3s were compared with their basal activities. In contrast to the case with tyrosine as substrate, considerably higher basal activities for wild-type and mutant SULT1A3s were observed even in the absence of $Mn^{2+}$. It is evident from the figure that the $Mn^{2+}$-stimulated D-Dopa-sulfating activity associated with the wild-type enzyme was essentially retained in the N85K, E89I, and H143Y mutants, as well as in the N85K/E89I double mutant. It is also clear that for the D86A mutant and the double (N85K/D86A and D86A/E89I) and triple (D86A/E89I/E146A) mutants involving D86A mutation, there was no longer noticeable $Mn^{2+}$ stimulation of the basal activities toward D-Dopa. Besides reinforcing our earlier conclusion with regard to residues Asn-85, Glu-89, His-143, and Glu-146 not being required for the $Mn^{2+}$ stimulation effect, these results unequivocally confirm the primary requirement for residue Asp-86 in the $Mn^{2+}$ stimulation of the Dopa/tyrosine-sulfating activity of SULT1A3. Because residue Asp-86 is present within the variable Region I, the above-mentioned results are in line with the earlier conclusion that the presence of the variable Region I from SULT1A3 is required for the $Mn^{2+}$ stimulation of the Dopa/tyrosine-sulfating activity (*cf.* above-mentioned studies using the chimeras).

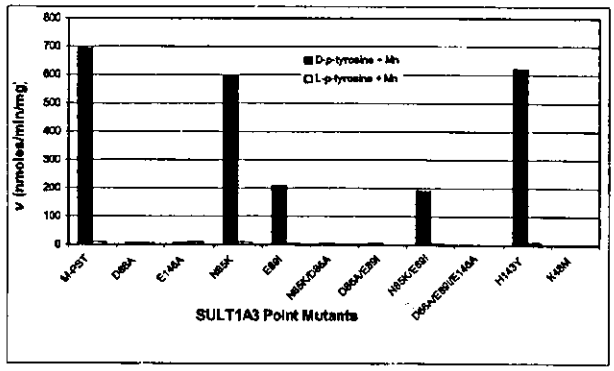


FIG. 5. **Stereospecificity of wild-type (labeled *M-PST*) and point-mutated SULT1A3s toward D- or L-enantiomers of tyrosine.** The *white bars* represent the sulfation activity of wild-type and point-mutated SULT1A3s toward 5 mM L-tyrosine, whereas the *black bars* represent the activity toward the D-enantiomer (both in the presence of 5 mM $Mn^{2+}$). The activities are expressed in nmol of sulfated product produced/min/mg enzyme.

As a negatively charged residue, Asp-86 is inherently capable of mediating the binding of $Mn^{2+}$ to SULT1A3.

*Identification of Specific Amino Acid Residues Required for the Stereospecificity of the Sulfation of Tyrosine and Dopa Enantiomers*—Fig. 5 shows a bar graph plotted based on the data in which the D-tyrosine-sulfating activities of wild-type and mutant SULT1A3s were compared with their L-tyrosine-sulfating activities (both in the presence of $Mn^{2+}$). It is clear from the figure that wild-type SULT1A3 and N85K, E89I, N85K/E89I, and H143Y mutants exhibited stereospecificity for the D-enan-

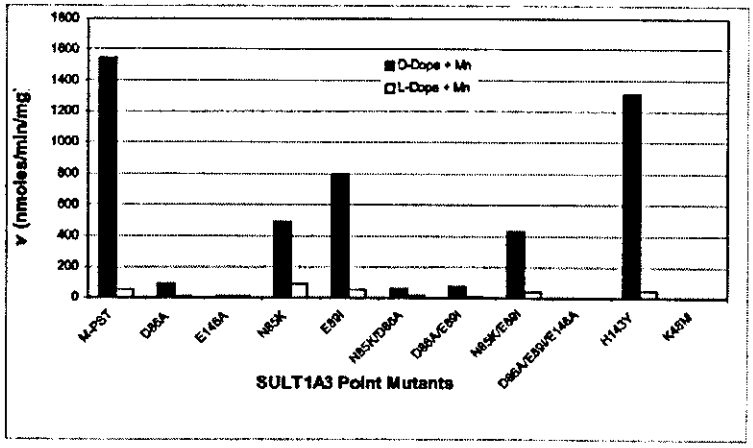


FIG. 6. **Stereospecificity of wild-type (labeled *M-PST*) and point-mutated SULT1A3s toward D- or L-enantiomers of Dopa.** The *white bars* represent the sulfation activity of the wild-type and point-mutated SULT1A3s toward 1 mM L-Dopa, whereas the *black bars* represent the activity toward the D-enantiomer (both in the presence of 2.5 mM $Mn^{2+}$). The activities are expressed in nmol of sulfated product produced/min/mg enzyme.

tiomer of tyrosine. No clearly discernable stereospecificity was observed for E146A, D86A, and double or triple mutants involving one or both of these two mutations. This is likely because of the inherently low sulfation activities of these mutants toward tyrosine, which would not allow for a clear-cut distinction of the substrate preference for D- or L-enantiomer. To better resolve the problem, the data on the comparison between the D-Dopa-sulfating activities of wild-type and mutant SULT1A3s and their L-Dopa-sulfating activities (both in the presence of $Mn^{2+}$) were analyzed. From the bar graph plotted (Fig. 6), the preference for D-Dopa over L-Dopa was clearly found for the D86A mutant and the two double mutants involving D86A mutation. Based on these results, it can be concluded that residue Asp-86 is not required (see also the following studies on deletional mutants) for the stereospecificity of SULT1A3, at least when $Mn^{2+}$ stimulation and binding to this residue is not involved. In the case of the E146A mutant, its low level of activity toward Dopa enantiomers still would not allow for a clear distinction regarding stereospecificity. However, when the data for the E146A mutant were collated and plotted together (Fig. 7), the E146A mutant no longer displayed the specificity for the D-enantiomer of tyrosine or Dopa. It is therefore clear that residue Glu-146 is the one that primarily directs the stereospecificity of SULT1A3 for the D-enantiomer of Dopa or tyrosine. This is in accordance with our earlier conclusion from the studies using chimeras that the SULT1A3 variable Region II is the one responsible for the stereospecificity of the Dopa/tyrosine-sulfating activity of SULT1A3.

*Sulfation Activities of the Residues 84–86 and 84–90 Deletional SULT1A3 Mutants toward Dopa and Tyrosine: Stereospecificity and Stimulatory Effect of $Mn^{2+}$*—The above-mentioned studies have pinpointed the residue Glu-146 of SULT1A3 as being primarily responsible for the stereospecificity for the D-enantiomers of Dopa and tyrosine. To further clarify these points, the activities of two deletional SULT1A3 mutants (lacking, respectively, residues 84–86 and residues 84–90) toward tyrosine (5 mM final concentration) and Dopa (1 mM final concentration) enantiomers were measured in the absence of $Mn^{2+}$. The residues 84–86 deletional mutant exhibited no detectable activities toward any of these substrates (or with dopamine). The residues 84–90 deletional mutant displayed detectable activity though this was much lower, as compared with that of the wild-type SULT1A3. Surprisingly, this deletional mutant seemed to have completely lost the stereospecificity for the D-enantiomers of tyrosine or Dopa as is clear from the data compiled in Table III. This seems to suggest a role for the "mobile loop" (encompassing residues 84–92; see Ref. 17 and below) *per se* as a steric selector, in the absence of

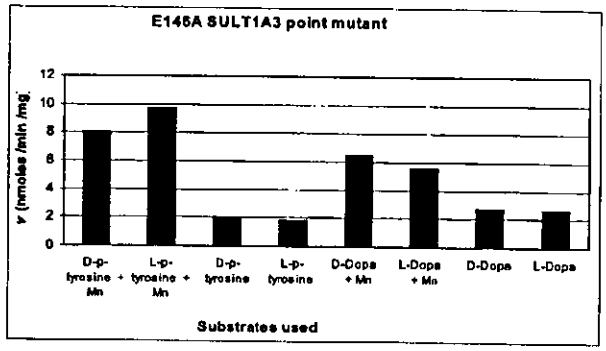


FIG. 7. **Activities of the E146A SULT1A3 mutant toward D- and L-enantiomers of tyrosine and Dopa, in the presence or absence of $Mn^{2+}$.** The activities are expressed in nmol of sulfated product produced/min/mg enzyme.

TABLE III
*Sulfation activity of the residues 84–90 deletional mutant of SULT1A3 toward various substrates*

All other conditions of the assay are as described under "Experimental Procedures."

| Substrate used | Activity |
|---|---|
| | *nmol/min/mg* |
| D-Tyrosine (5 mM) | 0.52 ± 0.13 |
| L-Tyrosine (5 mM) | 1.59 ± 0.05 |
| D-Dopa (1 mM) | 18.9 ± 0.02 |
| L-Dopa (1 mM) | 24.5 ± 0.5 |

$Mn^{2+}$, for the D-enantiomers of these substrates.

*Proposed Model Explaining the Stimulatory Effect of $Mn^{2+}$ and the Stereoselectivity for D-Enantiomers of Dopa/Tyrosine*—SULT1A3 is known to be present as a homodimer in its native state (14). Previous X-ray crystallography study (17) had shown that, in the crystal structure, residues 84–92 corresponding to the variable Region I (*cf.* Fig. 1*A*) from one monomer form a mobile loop that intercalates into the active site of the other monomer and may block the proper positioning of certain acceptor substrates. In addition to this mobile loop, the active site is also guarded by key residues (particularly Glu-146) (10, 11) from the variable Region II of the same monomer. This model had been used to explain our previous kinetic studies (15) by suggesting that the mobile loop might not hinder the positioning of the physiological substrate, dopamine, whose positively charged amino group may be stabilized by interaction with the negatively charged Glu-146 residue (8, 10, 11). It was proposed that the variable Region I loop may, however, act to prevent the

positioning of xenobiotic substrates (represented by D-Dopa or D-tyrosine) that carry, for example, an extra carboxyl group, as compared with dopamine. In this model, $Mn^{2+}$ exerts its stimulatory effect by complexing with the negatively charged carboxyl group of Dopa or tyrosine and forming a bridge with a residue in the loop. This would peg back the loop, thus allowing the proper positioning of these substrates.

The essential features of this model could also be used to explain the results obtained in the current study. However, one important modification has to be made. The previously reported x-ray structure of SULT1A3 (17, 32) demonstrated that the interaction between the subunits of the homodimer, in the crystal structure, takes place at the surface near the substrate binding sites of the subunits, which is consistent with the Region I loop from one monomer intercalating into the substrate binding site of the other monomer. This idea seemed to be supported by buried surface area calculations and data on the x-ray structure of other SULT enzymes (33). However, more recent work using cross-linking and limited proteolysis, in conjunction with mass spectrometry (34), suggested that the physiological dimer interface in solution (as opposed to the crystal dimer interface) may be near the C-terminal region of the subunit, away from the surface of the substrate binding site. Site-directed mutagenesis, along with gel filtration data, pinpointed a stretch of 10 amino acid residues near the C-terminal end of the subunit (in SULT1A3 this occurs in the region between Lys-265 and Glu-274) with the sequence K*XXX*-TV*XXX*E, conserved in all the dimeric SULTs, dubbed the "dimerization motif." This dimerization motif forms a hydrophobic "zipper" with ion pairs at the ends, at the interface between the dimers (34). This finding basically ruled out the possibility of the variable Region I mobile loop, which lies at the other end of the subunits, intercalating into the active site of the other subunit in solution. But because the evidence from our laboratory and others (see Refs. 7–10 and 15 and the present study) implicates the residues 84–89 in the variable Region I of SULT1A3 in interactions with the acceptor substrate at the active site, and because the x-ray crystallographic evidence indicates that residues 84–92 of SULT1A3 form a mobile loop, we must conclude that this loop, in solution, rather than intercalating into the substrate binding site of the other subunit of the dimer, by the same free energy minimization criteria, tucks back into the substrate binding site of the same subunit. This loop formed by the variable Region I therefore would still exert the same effects, albeit in the same subunit.

This model suggests that the loop, although not hindering the entry of the physiological substrate dopamine, may block the entry of other substrates such as Dopa and tyrosine with an extra carboxyl group. $Mn^{2+}$ stimulates the activity with these latter substrates by complexing on the one hand with the carboxyl group of these substrates and on the other hand specifically with the Asp-86 residue, thus pegging back the loop. This model explains why the D86A SULT1A3 mutant failed to show any $Mn^{2+}$-stimulated sulfation activity with D-Dopa as substrate (*cf.* Fig. 4). Data from our studies on the kinetics of the sulfation of dopamine and of the tyrosine and Dopa enantiomers by SULT1A3 and the effects of $Mn^{2+}$ on the kinetics of sulfation seem to support this model (15). The interaction of SULT1A3 with dopamine appears to follow typical Michaelis-Menten-type kinetics, and $Mn^{2+}$ seems to exert a smaller stimulatory effect (increasing $V_{max}$ by 2–3-fold but increasing $K_m$ only slightly) by binding directly to the Asp-86 residue of SULT1A3, and no complex with dopamine seems to be involved (15). In contrast, the interaction of SULT1A3 with D-tyrosine and D-Dopa involves a tyrosine-$Mn^{2+}$ or a tyrosine-$Mn^{2+}$-tyrosine complex as an obligate substrate and a Dopa-$Mn^{2+}$ or a Dopa-$Mn^{2+}$-Dopa complex as a better substrate than Dopa (15). With these substrates, $Mn^{2+}$ appears to form a bridge between the carboxyl group of the substrate with which it is complexed and the Asp-86 residue of the enzyme molecule.

The stereospecificity of SULT1A3 for the D-enantiomers of Dopa and tyrosine and the importance of the Glu-146 residue in this effect will require more detailed modeling studies and perhaps quantitative structure activity relationship analysis (17). However, it is clear that the interaction of the negatively charged Glu-146 residue with the positively charged amino groups of Dopa and tyrosine is an important factor in the stereospecific interaction between SULT1A3 and these substrates (8, 10, 11). Our studies with the 84–90 deletional mutant also revealed that, in the absence of $Mn^{2+}$, the presence of the variable Region I loop as a whole may serve as an important steric selector, because, in its absence, the stereoselectivity of the SULT1A3 for the D-enantiomers of Dopa and tyrosine appears to be lost. Our previous studies with dopamine as substrate (8) and the present study with Dopa and tyrosine as substrates revealed that besides the E146A mutant, the D86A and E89I mutants also showed reduced basal activities toward these substrates. In the case of the E146A mutant, the loss of activity could be because of the loss of an energetically favorable electrostatic interaction. With the D86A and E89I mutants, although the mechanism remains unclear, it is possible that the lower basal activity could result from altered structural interactions making the variable Region I loop even more restrictive. Some support for this possibility comes from our recent studies on the extent of inhibition of the activity of SULT1A3 and its mutants by 2,6-dichloro-4-nitrophenol (DCNP).[2]

Our model suggests that SULT1A3, under normal circumstances, acts only on its physiological substrate dopamine, thereby regulating its levels. This makes sense from the viewpoint of cellular economy, because sulfation is an energetically expensive process that uses PAPS, the synthesis of one molecule of which requires the expenditure of three high energy phosphate bonds of ATP (3). Under conditions of oxidative stress (which may result in the release of $Mn^{2+}$ from mitochondria into the cytosol), however, the constraining loop in the active site may be pinned back by a substrate-$Mn^{2+}$ complex, allowing the proper positioning, and therefore the sulfation, of non-physiological xenobiotic molecules present at higher levels (15). It is possible that other candidate molecules serving as substrates in this way may form complexes with $Mn^{2+}$ with much higher log K values.

The physiological significance, if any, of the $Mn^{2+}$ stimulation of the Dopa/tyrosine-sulfating activity of SULT1A3, and the specificity for the D-enantiomers of these substrates, which has also been demonstrated in cell culture and in cell-free extracts (13, 29), is somewhat speculative and has been considered in a previous report (15). From a more practical perspective, however, the stimulation of the detoxifying activity of SULT1A3 by $Mn^{2+}$ and its stereoselective action may have implications for the detoxifying activity of the SULTs toward chiral drugs (18–21). We are currently investigating the stereoselective action of SULT1A3 and its mutants on some chiral dopamine analogs widely used as drugs.[3] Besides throwing further light on the structure-function relationships we have considered here, the principles emerging may enable some engineering of the protein with regard to its stereospecific action.

[2] T. Sugahara, I. Oxendine, T. G. Pai, and M.-C. Liu, unpublished data.

[3] M. C. Liu, I. Oxendine, and T. G. Pai, unpublished results.

REFERENCES


1. Mulder, G. J., and Jakoby, W. B. (1990) in *Conjugation Reactions in Drug Metabolism* (Mulder, G. J., and Jakoby, W., eds) pp. 107–161, Taylor and Francis, Ltd., London
2. Falany, C., and Roth, J. A. (1993) in *Human Drug Metabolism: From Molecular Biology to Man* (Jeffery, E. H., Ed) pp. 101–115, CRC Press, Inc., Boca Raton, FL
3. Lipmann, F. (1958) *Science* **128,** 575–580
4. Weinshilboum, R. M., Otterness, D. M., Aksoy, I. A., Wood, T. C., Her, C. T., and Raftogianis, R. B. (1997) *FASEB J.* **11,** 3–14
5. Coughtrie, M. W. H., Sharp, S., Maxwell, K., and Innes, N. P. (1998) *Chem. Biol. Interact.* **109,** 3–27
6. Duffel, M. W. (1997) in *Comprehensive Toxicology* (Guengerich, F. P., Ed) pp. 365–383, Elsevier Science, Ltd., Oxford
7. Sakakibara, Y., Takami, Y., Nakayama, T., Suiko, M., and Liu, M.-C. (1998) *J. Biol. Chem.* **273,** 6242–6247
8. Liu, M.-C., Suiko, M., and Sakakibara, Y. (2000) *J. Biol. Chem.* **275,** 13460–13464
9. Brix, L. A., Duggleby, R. G., Gaedigk, A., and McManus, M. E. (1999) *Biochem. J.* **337,** 337–343
10. Brix, L. A., Barnett, A. C., Duggleby, R. G., Leggett, B., and McManus, M. E. (1999) *Biochemistry* **38,** 10474–10479
11. Dajani, R., Hood, A. M., and Coughtrie, M. W. H. (1998) *Mol. Pharmacol.* **54,** 942–948
12. Rubin, G. L., Sharp, S., Jones, A. L., Glatt, H., Mills, J. A., and Coughtrie, M. W. H. (1996) *Xenobiotica* **26,** 1113–1119
13. Sakakibara, Y., Katafuchi, J., Takami, Y., Nakayama, T., Suiko, M., Nakajima, H., and Liu, M.-C. (1997) *Biochim. Biophys. Acta* **1355,** 102–106
14. Heroux, J. A., and Roth, J. A. (1988) *Mol. Pharmacol.* **34,** 194–199
15. Pai, T. G., Ohkimoto, K., Sakakibara, Y., Suiko, M., Sugahara, T., and Liu, M.-C. (2002) *J. Biol. Chem.* **277,** 43813–43820
16. Suiko, M., Sakakibara, Y., Awan-Khan, R., Sakaida, H., Yoshikawa, H., Ranasinghe, J. G. S., and Liu, M.-C. (1998) *J. Biochem.* **124,** 707–711
17. Dajani, R., Cleasby, A., Neu, M., Wonacott, A. J., Jhoti, H., Hood, A. M., Modi, S., Hersey, A., Taskinen, J., Cooke, R. M., Manchee, G. R., and Coughtrie, M. W. H. (1999) *J. Biol. Chem.* **274,** 37862–37868
18. Wilson, A. A., Wang, J., Koch, P., and Walle, T. (1997) *Xenobiotica* **27,** 1147–1154
19. Walle, U. K., Persola, G. R., and Walle, T. (1993) *Br. J. Clin. Pharmacol.* **35,** 413–418
20. Persola, G. R., and Walle, T. (1993) *Chirality* **5,** 602–609
21. Walle, T., and Walle, U. K. (1992) *Drug Metab. Dispos.* **20,** 333–336
22. Yanagisawa, K., Sakakibara, Y., Suiko, M., Takami, Y., Nakayama, T., Nakajima, H., Takayanagi, K., Natori, Y., and Liu, M.-C. (1998) *Biosci. Biotechnol. Biochem.* **62,** 1037–1040
23. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
24. Liu, M.-C., and Lipmann, F. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 3695–3698
25. Fernando, P. H. P., Karakawa, A., Sakakibara, Y., Ibuki, H., Nakajima, H., Liu, M.-C., and Suiko, M. (1993) *Biosci. Biotechnol. Biochem.* **5,** 1974–1975
26. Laemmli, U. K. (1970) *Nature* **227,** 680–685
27. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
28. Butler, P. R., Anderson, R. J., and Venton, D. L. (1983) *J. Neurochem.* **41,** 630–639
29. Suiko, M., Sakakibara, Y., Nakajima, H., Sakaida, H., and Liu, M.-C. (1996) *Biochem. J.* **314,** 151–158
30. Martell, A. E., and Smith, R. M. (1974) *Critical Stability Constants*, Vol. 1, Plenum Press, New York
31. Gergely, A., Nagypal, I., and Kiraly, B. (1971) *Acta Chim. Acad. Sci. Hung.* **68,** 285
32. Bidwell, L. M., McManus, M. E., Gaedigk, A., Kakuta, Y., Negishi, M., Pedersen, L. C., and Martin, J. F. (1999) *J. Mol. Biol.* **293,** 521–530
33. Pedersen, L. C., Petrotchenko, E. V., and Negishi, M. (2000) *FEBS Lett.* **475,** 61–64
34. Petrotchenko, E. V., Pedersen, L. C., Borchers, C. H., Tomer, K. B., and Negishi, M. (2001) *FEBS Lett.* **490,** 39–43

*J. Biochem.* **133**, 259–262 (2003)
DOI: 10.1093/jb/mvg033

# Differential Roles of Human Monoamine (M)-Form and Simple Phenol (P)-Form Phenol Sulfotransferases in Drug Metabolism

**Takuya Sugahara, T. Govind Pai, Masahito Suiko, Yoichi Sakakibara and Ming-Cheh Liu***

*Biomedical Research Center, The University of Texas Health Center, 11937 US HWY 271, Tyler, TX 75708, USA*

Received October 28, 2002; accepted December 25, 2002

**Cytosolic sulfotransferases (STs) are traditionally known as Phase II drug-metabolizing or detoxifying enzymes that facilitate the removal of drugs and other xenobiotic compounds. In this study, we carried out a systematic investigation on the sulfation of drug compounds by two major human phenol STs (PSTs), the monoamine (M)-form and simple phenol (P)-form PSTs. Activity data obtained showed the differential substrate specificity of the two enzymes for the thirteen drug compounds tested. Kinetic studies revealed that the M-form PST displayed stereoselectivity for the chiral drug, isoproterenol. The effects of divalent metal cations on the activity of the M-form and P-form PSTs toward representative drug compounds were quantitatively evaluated. Results obtained indicated that the drug-sulfating activities of the two human PSTs were partially or completely inhibited or stimulated by the ten divalent metal cations tested at a 5 mM concentration. The two enzymes appeared to be less sensitive to the effects of physiologically more abundant metal cations such as $Mg^{2+}$ and $Ca^{2+}$, but more sensitive to the detrimental effects of other metal cations that may enter the body as environmental contaminants.**

**Key words: drug metabolism, phenol sulfotransferase, stereoselectivity, substrate specificity, sulfation.**

Abbreviations: ST, sulfotransferase; PST, phenol sulfotransferase; PAPS, 3′-phosphoadenosine 5′ phosphosulfate.

Sulfate conjugation is a major pathway *in vivo* for the biotransformation and excretion of drugs and xenobiotics, as well as endogenous compounds such as steroid and thyroid hormones, catecholamines, cholesterol, and bile acids (*1–3*). The responsible enzymes, called the "cytosolic sulfotransferases (STs)", catalyze the transfer of a sulfonate group from the active sulfate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), to an acceptor substrate compound containing either a hydroxyl or an amine group (*4*). Sulfate conjugation may result in the inactivation or activation of the substrate compounds or increase their water-solubility, thereby facilitating their removal from the body (*1–3*).

In searching for the cytosolic ST enzymes involved in the sulfation of drugs, earlier studies revealed two distinct forms of the "phenol ST" (PST) in human platelets (*5, 6*). The monoamine (M) (thermolabile)-form PST catalyzes more effectively the sulfation of monoamines such as dopamine and epinephrine, and the simple phenol (P) (thermostable)-form PST preferentially sulfates neutral phenols such as p-nitrophenol and α-naphthol (*7*). It is now clear that, in addition to their presence in platelets, these two PSTs have a somewhat widespread tissue distribution in the human body. In particular, the M-form PST has been found in the upper gastro-intestinal tract and brain (*8*), and the P-form PST in the adrenal gland, lung, and liver (*9*). Both forms are thought to be constitutive enzymes though little is known about the regulation of their enzymatic activity (*1*). In the past several years, however, studies performed in our laboratory have revealed that some divalent metal cations may exert stimulatory or inhibitory effects on these enzymes (*10, 11*). For example, the addition of $Mn^{2+}$ to the reaction mixture resulted in a dramatic increase in the Dopa/tyrosine-sulfating activity of the M-form PST, while $Cd^{2+}$ inhibited this activity. These findings indicate that divalent metal cations may play a significant role in regulating the activity of the M-form PST and perhaps cytosolic STs in general. It may therefore be important to investigate whether divalent metal cations that enter the body in food or as environmental contaminants exert significant stimulatory or inhibitory effects on the sulfation of drugs. Another interesting aspect of the metabolism of drugs through sulfation is the stereoselective sulfate conjugation of some of these compounds. A study using rat hydroxysteroid ST (STa) has revealed the stereoselective sulfation of chiral secondary alcohols (*12*). Studies using homogenates of, or intact, human liver, intestine, or platelets have also demonstrated the stereoselective sulfation of some chiral drugs including isoproterenol and albuterol (*13, 14*). In view of our previous studies showing that the M-form PST displayed stereoselectivity for the D-enantiomers of Dopa and tyrosine (*11*), it is interesting to investigate whether the M-form PST also exhibits stereoselectivity toward chiral drugs.

*To whom correspondence should be addressed. Tel: +1-903-877-2862, Fax: +1-903-877-2863, E-mail: ming.liu@uthct.edu

 