研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
榊原陽一 Ming-Cheh Liu 水光正仁	フェノール硫酸転移酵素遺伝子ファミリーの構造と機能	生化学	74 (7)	539-546	2002
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総 説

フェノール硫酸転移酵素遺伝子ファミリーの機能と構造

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硫酸転移酵素に関する我々の知識はここ十年間で急速に増加しつつある．特に分子生物学的手法の導入により，生化学中心の研究では困難であった性質の非常に似通った硫酸転移酵素の分離や諸性質の検討が可能となり，さらに新規硫酸転移酵素遺伝子が多数発見された．硫酸転移酵素に関する研究は80年代までの生化学（酵素学）の時代から90年代の分子生物学（遺伝子クローニング）の時代を経て，今再び生化学（構造生物学）の時代へと進みつつある．硫酸転移酵素研究における激動の10年間を振り返りつつ，我々の研究をたどりながらフェノール硫酸転移酵素に関する研究の過去，現在，そして未来について紹介する．

1. はじめに

我々の研究グループ（水光，榊原，Liu）が硫酸化の研究を始めたきっかけは，今をさかのぼること17年前，1985年に水光が当時ロックフェラー大学でタンパク質チロシン残基の硫酸化の研究を行っていた故フリッツ・リップマン博士の研究室に留学したことに端を発する．リップマン研究室において水光，Liuのコンビが組まれタンパク質チロシン残基の硫酸化に関する研究が始められ，タンパク質チロシン残基の硫酸化に関する興味深い研究成果が得られた[1~3]．1988年に帰国後，水光は宮崎大学において，オクラホマ大学に独立して研究室を構えたLiuと共に共同で硫酸化の研究を続けることとなった．その後，1989年に榊原が学部学生として研究に加わり，低分子化合物の硫酸化へと徐々にその研究対象が移り現在に至っている．

我々の研究グループはフェノール硫酸転移酵素として，まずウシ肝臓のフェノール硫酸転移酵素の研究よりこの酵素に関する研究を始めた．ウシ肝臓において細胞質のフェノール硫酸転移酵素とは明らかに異なるミクロソーム膜画分に存在するフェノール硫酸転移酵素活性を我々は発見し，その諸性質の検討を行った[4]．しかしながら，このミクロソーム膜画分の膜結合フェノール硫酸転移酵素の機能に関しては未だ不明である．また，ウシ以外の哺乳類からは同様なミクロソーム膜画分の低分子化合物を基質にする硫酸転移酵素は発見されていない．その後，ミクロソーム膜画分からは翻訳後修飾であるタンパク質糖鎖やチロシン残基の硫酸化に関与する硫酸転移酵素が多数発見されている[5~7]．これらミクロソーム膜画分の硫酸転移酵素に関する情報がそろってきた現在，ウシ肝臓の膜結合フェノール硫酸転移酵素との関係について再度検討を行う必要が大いに考えられる．

その後，我々は哺乳類体内に存在すると考えられる遊離チロシンの硫酸化に関与する硫酸転移酵素の研究を始めた．遊離硫酸化チロシンはヒト尿中に存在することが1955年にTallanにより報告され[8]，その後の研究から，翻訳後修飾としてチロシン残基が硫酸化されたタンパク質の分解により生じることが唯一の起源と考えられてきた．健常成人では1日当たりおよそ28 mgの遊離硫酸化チロシンが尿中に排泄されていることなどから，遊離チロシンの硫酸化に関与する硫酸転移酵素の存在が予想された[9]．我々はヒト肝腫瘍細胞由来のHepG2細胞を用いて，遊離チロシンの硫酸化が培養細胞レベルで生じていることを世界で初めて証明した[10,11]．その後の研究で，この遊離チロ

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Biochemistry and molecular biology of phenol sulfotransferases
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シンの硫酸化はマンガンイオン依存的にヒトM型フェノール硫酸転移酵素によって触媒され，チロシンのみでなくその構造類似体であるメタチロシンやドーパも同様に硫酸化されることが明らかとなった[12,13]．その後，フェノール硫酸転移酵素に関する研究はヒトフェノール硫酸転移酵素をモデルとしたキメラ硫酸転移酵素を用いた研究[14]や部位特異的変異を導入したミュータント硫酸転移酵素を用いた研究[15]へと発展し，硫酸転移酵素の基質認識に関与する領域を分子生物学的手法で明らかにしてきた．

2. 活性硫酸PAPSの合成と硫酸化

生体内での硫酸化はまず硫酸の活性化，すなわち50年代にLipmannらにより発見された哺乳類に普遍的な硫酸供与体としての活性硫酸3′-phosphoadenosine 5′-phosphosulfate (PAPS) のATPと無機硫酸塩からの合成が必要である[16]．このPAPS合成は2種の酵素活性，すなわちATP sulfurylaseとadenosine 5′-phosphosulfate kinase (APS kinase) によって触媒される（図1）．これら二つのPAPS合成に関与する酵素は，大腸菌やカビといった微生物や植物においては二つの異なる酵素タンパク質として存在している．しかしヒトやマウスといった哺乳類やショウジョウバエにおいては，二つの酵素が進化の過程で融合した酵素PAPS synthetaseとして存在し，より効率よくPAPSが合成できるようになってきたと考えられる[17,18]．1998年に我々はヒトのPAPS合成に関与するPAPS synthetase (bifunctional ATP sulfurylase/adenosine 5′-phosphosulfate kinase) をクローニングし，大腸菌で発現した．そして，bifunctionalな酵素のATP sulfurylaseとAPS kinaseの領域を単独で発現させた場合でも，それぞれの酵素活性を有していることを明らかとし報告した[20]．最近，他の研究グループから，PAPS synthetaseはPAPS synthetase 1とPAPS synthetase 2の2種がヒトおよびマウスにおいて存在することが報告されている[21]．このことから，これらの2種類のPAPS synthetaseの発現により硫酸化が生理的に機能制御されていることも考えられる．

$$ATP + SO_4^{2-} \underset{\text{ATP sulfurylase}}{\rightleftharpoons} APS + PPi$$

$$APS + ATP \xrightarrow[\text{APS kinase}]{} PAPS + ADP$$

$$PAPS + PNP \xrightarrow[\text{Sulfotransferase}]{} PAP + PNPS$$

図1 3′-phosphoadenosine 5′-phosphosulfate (PAPS) の合成と硫酸転移酵素によるフェノール性化合物の硫酸化

ATP: adenosine 5′-triphosphate, APS: adenosine 5′-phosphosulfate, PAPS: 3′-phosphoadenosine 5′-phosphosulfate, PNP: *p*-nitrophenol, PAP: 3′-phosphoadenosine 5′-phosphate, PNPS: *p*-nitrophenylsulfate.

このようにして合成されたPAPSを用いてPAPS上の硫酸基をフェノール性の水酸基の上に転移する反応が硫酸化である．図1に反応の概要をまとめた．この反応を触媒する酵素を硫酸転移酵素と呼び，現在シトクロムP-450酵素群と同様に遺伝子ファミリーを形成していることが明らかとなってきた[22]．

3. フェノール硫酸転移酵素ファミリーの多様性

現在，硫酸転移酵素の分類に関しては硫酸転移酵素ワークショップにおいて提唱された分類法により，アミノ酸配列をもとに分類することが推奨されている．分類法としては硫酸転移酵素は*SULT*という略号を用いて，その後にファミリーを表す数字を付ける．例えば，*SULT1*はフェノール硫酸転移酵素ファミリー，*SULT2*はヒドロキシステロイド硫酸転移酵素ファミリーというように分類される．さらにこれらのファミリーごとにアミノ酸配列が60%以上一致するグループをサブファミリーとし，アルファベットをAから順に付けていく．以下はこの硫酸転移酵素分類法に基づく分類名を用いることとする．

現在のところフェノール硫酸転移酵素遺伝子ファミリー (*SULT1* family) はA, B, C, D, Eの5種のサブファミリーにより形成されている．これまでの研究から，ヒト

表1 ヒトフェノール硫酸転移酵素の分類

系統名	別名，慣用名など	基　質	遺伝子座
SULT1A1	ST1A2, P-PST, TS-PST	*p*-Nitrophenol, Dopamine, Bisphenol A	16p12.1-p11.2
SULT1A2	ST1A3, P-PST, TS-PST	*p*-Nitrophenol, Dopamine	16p12.1-p11.2
SULT1A3	ST1A5, M-PST, TL-PST	Dopamine, *p*-Nitrophenol, Estradiol	16p11.2
SULT1B1	ST1B2, THST	3,3′,5-Triiodothyronine *p*-Nitrophenol, Dopamine	
SULT1C2	ST1C2	*p*-Nitrophenol 3,3′,5-Triiodothyronine *N*-Hydroxyacetylaminofluorene	2q11.1-q11.2
SULT1C3	ST1C3	*p*-Nitrophenol *N*-Hydroxyacetylaminofluorene	2q11.2
SULT1E1	ST1E4, EST	Estradiol, Estrone	

において *SULT1* ファミリーには7種の硫酸転移酵素の存在が知られている（表1）．フェノール硫酸転移酵素遺伝子ファミリーに属する硫酸転移酵素の機能としては，フェノール性の生体外異物や薬物の解毒代謝やカテコールアミン，甲状腺ホルモン，エストロゲンの代謝に関与すると考えられている．これらほとんどの硫酸転移酵素は細胞質可溶性画分に存在し，唯一ウシ肝臓の膜結合フェノール硫酸転移酵素のみがミクロソーム膜画分に存在することが報告されている[4]．

SULT1A サブファミリーはヒトにおいてP型フェノール硫酸転移酵素（*1A1*，*1A2*）2種とM型フェノール硫酸転移酵素（*1A3*）の3種が存在することが報告されている．生化学的手法による研究がなされていた1980年代にはヒト肝臓のフェノール硫酸転移酵素は2種存在すると考えられ，その基質特異性，熱安定性，阻害剤に対する影響によりシンプルフェノール型（P型）とモノアミン型（M型）に長い間分類されてきた[23,24]．

後に，このP型フェノール硫酸転移酵素のcDNAクローニングが報告されるに至り，二つの異なる遺伝子が存在し，P型フェノール硫酸転移酵素と考えられてきた画分に二つの異なる遺伝子産物であるSULT1A1とSULT1A2が存在することが判明した[25,26]．このことは生化学的手法のみではほとんど分離することが不可能であった性質の非常によく似た硫酸転移酵素が，分子生物学的手法の導入により初めて比較研究される対象となった典型的な例と考えられる．

SULT1A サブファミリーに属する硫酸転移酵素はヒト[25~27]以外にラット[28]，マウス[29]，ウシ[30]など多数の動物種からcDNAがクローニングされ報告されているが，ヒト以外の生物種においては1種類しかその存在は確認されていない（図2）．

SULT1B サブファミリーは我々の研究グループにより，最初ラット肝臓におけるドーパおよびチロシンとその異性体の硫酸化に関与するドーパ・チロシン硫酸転移酵素として精製され，諸性質が検討された[31]．cDNAクローニングの結果より未知の機能を持った新規硫酸転移酵素遺伝子 *ST1B1* と同一の酵素であることが判明した[32]．現在ではこれらは *SULT1B1* と分類され，ラット以外にヒト[33]，マウス[34]，イヌ[35]などからその存在が報告され，ドーパやチロシンそして甲状腺ホルモンなどの硫酸化による濃度調節に関与すると考えられている（図2）．

SULT1C サブファミリーは最初ラット肝臓のアリルアミン硫酸転移酵素 *ST1C1* として報告された[36]．この酵素の特徴として，硫酸化により変異原性が著しく活性化される変異原物質 *N*-hydroxy acetylamino fluorene の硫酸化に関与する酵素と考えられ，発がんと関係があると考えられた．

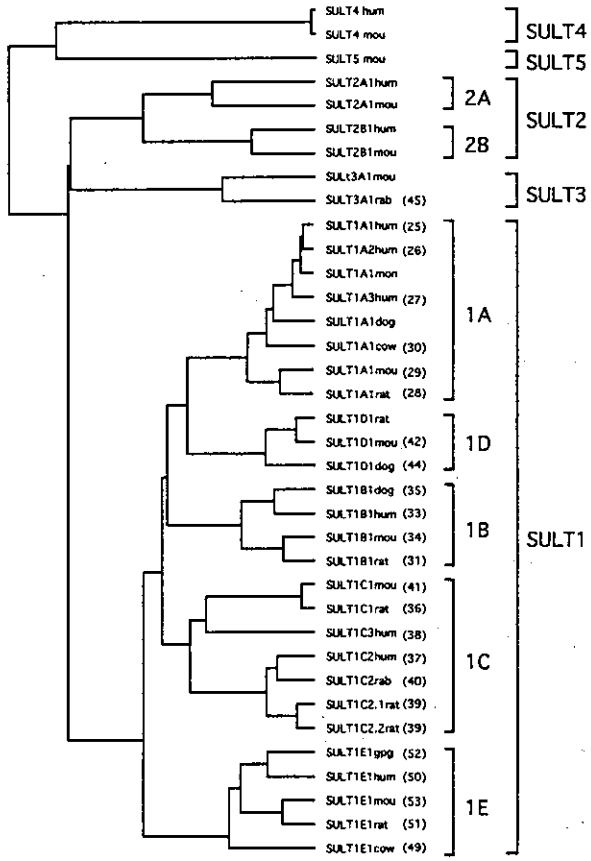

図2 硫酸転移酵素遺伝子ファミリーの分子系統樹
SULT1からSULT5はファミリーの分類を表している．1Aから2Bはサブファミリーの分類を表している．分類名の最後についているアルファベット3文字はhumがヒト，mouがマウス，ratがラット，cowがウシ，rabがウサギ，monがサル，dogがイヌ，gpgがモルモット由来であることを表している．今回，特にSULT1ファミリーを中心に系統図を作成し，それ以外に関してはヒトおよびマウスに限定した．

SULT1C1 はラットから最初に報告された後にヒト *SULT1C1* のcDNAクローニングが1998年に報告された[37]．それとは独立に我々の研究グループはヒト *SULT1C* サブファミリーに属する酵素2種をクローニングし，それらをSULT1C#1とSULT1C#2と名付け，共に *N*-hydroxy acetylamino fluorene を硫酸化することを報告した[38]．現在では，ヒト *SULT1C#1* が1998年に報告されたヒト *SULT1C1* と同一であり，*SULT1C2* と本来分類されることが妥当であると考えられる．混乱を避けるために少し説明を加えるとすれば，ヒトに関して *SULT1C* サブファミリーに属する酵素は *SULT1C2* と *SULT1C3* の2種が報告されており，*SULT1C1* に関して1998年の報告は *SULT1C2* の間違いであり，未だに報告されていないとみるのが正しいと思われる．現在までに *SULT1C* サブファミリーに属する酵素としてはラット *SULT1C1*[36]，*SULT1C2*[39]，ヒトより *SULT1C2* および

SULT1C3 の2種[37,38]，ウサギ *SULT1C2*[40]，マウス *SULT1C1*[41] などが報告されている（図2）．

SULT1D サブファミリーに関する酵素の報告は我々の研究グループによるマウスExpression Sequence Tag (EST) データベースクローン679153が最初の報告である．我々は，マウスSULT1D1がp-ニトロフェノールの他にナフチルアミンのアミノ基の硫酸化や，プロスタグランジンやロイコトリエンといった炎症性メディエーターの硫酸化を触媒することを報告している[42,43]．現在までに *SULT1D* サブファミリーに属する酵素としてはマウス[42]，イヌ[44]において報告されている（図2）．また生体内におけるアミノ基を特異的に硫酸化する酵素としては，このSULT1D1以外にSULT3という新規硫酸転移酵素ファミリーに属する酵素がウサギより報告されている[45]．

SULT1E サブファミリーはエストロゲン硫酸転移酵素と呼ばれてきた酵素（SULT1E1）がこれに該当し，非常に昔から研究されてきた硫酸転移酵素の一つであり，標的臓器におけるエストロゲンの不活性化に関与すると考えられている[46~48]．硫酸転移酵素として初めてcDNAクローニングが報告されたのはウシのエストロゲン硫酸転移酵素である[49]．ヒトにおいては1994年に酵素の単離よりも先にcDNAクローニングが報告された[50]．*SULT1E1* はそれ以外の動物種としてラット[51]，モルモット[52]，マウス[53]などからそのcDNAクローニングが報告されている（図2）．

4. フェノール硫酸転移酵素の基質認識部位に関して

我々はヒト肝臓のマンガン依存的ドーパ・チロシン硫酸転移酵素活性が，M型フェノール硫酸転移酵素によることを明らかにした[13]．ヒトフェノール硫酸転移酵素にはP型フェノール硫酸転移酵素（P-PST）とM型フェノール硫酸転移酵素（M-PST）の2種の酵素が存在することが生化学的研究よりすでに知られていた．さらにcDNAクローニングがなされるに至り，P型フェノール硫酸転移酵素が実は2種の異なる遺伝子産物からなることはすでに述べたとおりである．P-PSTとM-PSTは顕著な基質特異性の差を示し，アミノ酸配列においては93%以上一致することから，両酵素を硫酸転移酵素の基質認識部位に関する研究のモデルとして使用した．

(1) キメラフェノール硫酸転移酵素に関する研究

まず最初にヒトP型フェノール硫酸転移酵素（SULT1A1）とM型フェノール硫酸転移酵素（SULT1A3）のキメラ硫酸転移酵素を作製し，基質認識に関与する領域を決定した．*SULT1A1* と *SULT1A3* のアミノ酸配列を比較したところ，これら二つの酵素の間で変異が著しい領域が2か所存在することが明らかとなっ

図3 キメラ硫酸転移酵素とその諸性質
(A) ヒトM-PST（SULT1A3）とヒトP-PST（SULT1A1）のアミノ酸配列の比較．特にアミノ酸配列の変異の大きな領域には下線を引き，それぞれN端に近いものからIとIIとした．ミュータント硫酸転移酵素の作製時に変異を導入したアミノ酸は▼でその位置を示した．
(B) キメラ硫酸転移酵素の概要図，白抜き部分がM-PST由来，黒い部分がP-PST由来のポリペプチド鎖よりなっている．右に活性効率（V_{max}/K_m）の値をそれぞれドーパミン（DA）とp-ニトロフェノール（PNP）の順に示した．

表 2 ミュータント硫酸転移酵素の諸性質

Enzyme	Dopamine			*p*-Nitrophenol		
	K_m (mM)	V_{max} (nmol/min/mg)	V_{max}/K_m	K_m (mM)	V_{max} (nmol/min/mg)	V_{max}/K_m
Wild-type M-PST	0.44	12.1	27.4	28.2	10.5	0.37
D86A	1.33	5.00	3.76	70.4	18.2	0.26
E89I	1.11	12.5	11.3	20.6	6.06	0.29
D86A/E89I	14.3	22.2	1.55	24.1	4.44	0.18
E146A	23.8	5.88	0.25	1.27	4.12	3.24
D86A/E89I/E146A	44.9	2.74	0.06	1.21	11.4	9.39
Wild-type P-PST	47.6	2.89	0.06	0.53	8.81	16.6
I89E	45.5	6.29	0.14	0.46	6.21	15.5
A146E	90.9	7.52	0.08	1.27	4.12	3.23
A86E/I89E	59.9	3.87	0.06	0.49	5.50	11.2
I89E/A146E	61.7	4.29	0.07	N.D.	N.D.	N.D.
A86D/I89E/A146E	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

た．そこでこれら2か所の変異の著しい部位を入れ替えたキメラ硫酸転移酵素を作製して基質認識部位に関する研究を行うこととした．キメラ硫酸転移酵素は，*SULT1A1* と *SULT1A3* の ORF 内部に共通にみられる制限酵素切断部位 *BanI* と *Bsu36I* の認識部位を使って，*SULT1A1* と *SULT1A3* の断片を入れ替えることで作製した（図3）．野生型2種とキメラ硫酸転移酵素6種を用いた実験の結果より，領域ⅠとⅡを共に入れ替えたキメラ硫酸転移酵素 PMMP は M 型の基質特異性を示し，逆のタイプである MPPM は P 型の基質特異性を示した．このことから基質の認識には領域ⅠとⅡを含む中央の部分が関与していることが判明した．さらに硫酸転移酵素の特異的な阻害剤であるジクロロニトロフェノール（DCNP）に対しても PMMP は M 型と同様な反応を示し，逆のタイプである MPPM は P 型と同様な反応を示した．領域Ⅰまたは領域Ⅱのどちらか一方のみを入れ替えたキメラ硫酸転移酵素においては，基質認識に関しては硫酸化の効率がどの場合においても悪くなり，顕著な特異性を示さなかった．さらにこれらの酵素は DCNP に対して P 型と M 型の中間の反応を示した．これらの結果より，基質の認識にはこれら二つの領域が共に関与することが示唆された[14]（図3）．

このような硫酸転移酵素のキメラを用いた研究は，我々のこの研究の他にフラボノール硫酸転移酵素のキメラを用いた研究[54]や，同じくヒトフェノール硫酸転移酵素を用いた研究[55]，ラットヒドロキシステロイド硫酸転移酵素を用いた研究[56]などが報告されている．これらの研究からも基質の認識には硫酸転移酵素の中央付近の領域が重要であると同様な結果が報告されている．

(2) フェノール硫酸転移酵素への部位特異的変異の導入

キメラ硫酸転移酵素を用いた研究より基質の認識に関与する領域が判明したので，次にこの領域内のアミノ酸に変異を導入したミュータント硫酸転移酵素を作製し，基質認識に重要なアミノ酸残基に関する研究を行った．

M 型フェノール硫酸転移酵素のミュータントに関しては，D86A/E89I，E146A，D86A/E89I/E146A がドーパミンに対する活性効率（V_{max}/K_m）を著しく低下させた．それに対して E146A，D86A/E89I/E146A は *p*-ニトロフェノールに対する活性効率を上げた．E146A は単独でも特異性を M 型から P 型へ変えたことから，M 型と P 型の特異性を決定づけるのに特に重要なアミノ酸残基であることが判明した．E146A に関しては他の研究グループからも同様な結果が報告されている[57]．また D86A/E89I/E146A でさらに活性効率が P 型により近くなっていることから，やはり基質の認識にはこれらのアミノ酸残基を含む領域ⅠとⅡが重要であることが判明した（図3，表2）．P 型フェノール硫酸転移酵素のミュータントでは，A146E，I89E/A146E，A86D/I89E/A146E に関して著しく *p*-ニトロフェノールの硫酸化の効率が悪化した．特に I89E/A146E，A86D/I89E/A146E の二つは *p*-ニトロフェノールを全く硫酸化しなくなった．これらの結果は M 型フェノール硫酸転移酵素のミュータントから得られた結果とは非常に異なっていた（表2）．今後はフェノール硫酸転移酵素の結晶構造と，これらの基質認識に関与するアミノ酸残基の3次元的な関係が解明されていくものと考えられる．エストロゲン硫酸転移酵素に関してはそのような研究がすでに報告されている[58]．

同様な部位特異的変異を導入した硫酸転移酵素の研究は，我々の研究以外にフラボノール硫酸転移酵素に関する研究[59]，ヒトフェノール硫酸転移酵素に関する研究[55,57,60]，さらに活性硫酸 PAPS の結合部位に関する研究[61]などが報告されている．

5. 硫酸転移酵素研究の未来

(1) 硫酸転移酵素遺伝子の多型とオーダーメイド医療への応用

ゲノムプロジェクトによりヒトゲノムの全塩基配列が決

定され，これからのポストゲノムの時代を迎えるにあたって，薬物代謝酵素の一つである硫酸転移酵素も格好のオーダーメイド医療の対象と考えられている．硫酸転移酵素にはシトクロムP-450や*N*-アセチルトランスフェラーゼ-2，グルタチオン*S*-トランスフェラーゼなどと同様に遺伝子多型（polymorphism）が存在することが知られている[62~65]．硫酸転移酵素の遺伝子多型に関しては現在わずかな情報が得られているのみである．しかし今後ますます新しい情報が明らかになってくることが期待される．これらの硫酸転移酵素の遺伝子多型による個体間の薬物に対する感受性の違いが明らかとなれば，投薬時のオーダーメイドのさじ加減が可能となるであろう．さらに硫酸転移酵素遺伝子多型と発がんリスクの関係や，食品機能性成分の個体間の有効性の差と硫酸化の関係など，興味の尽きない研究分野としてますます発展していくことが期待される．

(2) 今再び生化学の時代へ

遺伝情報としてほとんどすべてのヒトゲノムの配列が明らかとなった現在，ゲノムプロジェクトより明らかとなった機能未知のタンパク質の機能を推定する目的で，まずその立体構造を解析する試みがなされている．これまでに蓄積されている一次構造をもとに，機能を推定できないタンパク質でも立体構造が明らかとなれば，その機能を推定できると考えられている．このような研究も構造生物学的な技術の進歩と知識の蓄積がなされてようやく可能となってきた．

硫酸転移酵素に関しても，1997年にマウスエストロゲン硫酸転移酵素のX線結晶構造解析により初めてその立体構造が解明された[66]．その後，ヒトM型フェノール硫酸転移酵素[67]，ヒトヒドロキシステロイド硫酸転移酵素[68]，ヒトヘパラン硫酸スルフォトランスフェラーゼの硫酸転移酵素ドメイン[69]の立体構造が続いて明らかとなってきた．アミノ酸配列の情報のみでは比較することが困難であった低分子化合物の硫酸転移酵素と糖鎖（高分子）の硫酸転移酵素の間でも，PAPSと相互作用する部位で非常によく似た構造をとっていることや，共通な配列モチーフが見いだされている[69]．硫酸転移酵素の立体構造の解析などに関してはNIHの根岸正彦先生の総説に詳しく書かれているのでそちらを参考にしていただきたい[70,71]．

6. おわりに

これまで我々の研究グループの研究を紹介しつつ硫酸転移酵素に関するここ10年の研究を紹介してきた．本文中で示したように硫酸転移酵素は90年代になって遺伝子のクローニングが精力的になされ，非常に多様な遺伝子ファミリーを構成していることが明らかとなった．1980年から2001年までに発表されたタイトルに硫酸転移酵素と記された論文数と，そのうちのクローニングに関する論文数の推移を図4に示した．図から明らかなようにここ10年の硫酸転移酵素に関する研究は非常に活発である．遺伝子から発見された新規硫酸転移酵素の生理機能がこれからどんどん明らかとなり，硫酸転移酵素の多様な全貌が解明されるときも近い将来確実にくると思われる．

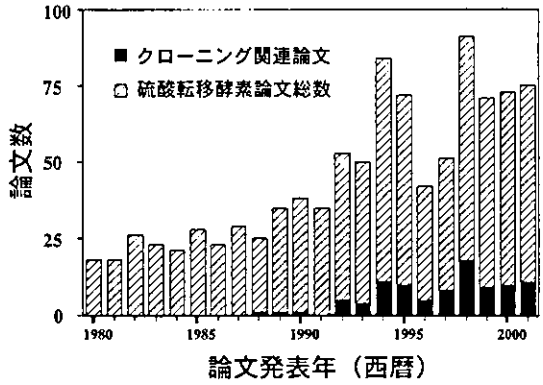

図4 硫酸転移酵素に関する論文報告数の過去20年間の推移

横軸に発表年を西暦で，縦軸に発表論文数を示した．斜線部分はタイトルに硫酸転移酵素のキーワードを持つ論文総数，黒抜き部分はそのうちでタイトルにクローニングのキーワードを持つ論文数を示す．

本文中の硫酸転移酵素の分類に関しては*SULT*の略号を用いる方法が推奨されており，大まかにこれに従った．しかしながら，非常に多様な分子種よりなる遺伝子ファミリーを形成し，研究者間でも生物種間の対応が必ずしも一致していないのが現状である．硫酸転移酵素の分類に関しては東北大学の山添康先生が提案された分類法もあり，先生の論文に詳しく記されているのでそちらを参照していただきたい[72]．

硫酸転移酵素に関する研究は，筆者（榊原，水光）が宮崎大学および共同研究先であるテキサス大学ヘルスセンターのMing-Cheh Liu博士の研究室を訪問した時に行った研究結果が中心である．研究を行うにあたりご指導とご協力をいただいた宮崎医科大学生化学第二講座の中山建男教授と高見恭成助教授，ユニチカの中島宏博士（現赤穂化学）に心よりお礼申し上げます．

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Regulatory Effects of Divalent Metal Cations on Human Cytosolic Sulfotransferases[1]

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Cytosolic sulfotransferases (STs), traditionally viewed as Phase II drug-metabolizing or detoxifying enzymes, are increasingly being implicated in the metabolism of endogenous biologically-active molecules. Except for studies on changes in their levels of expression and activity in the early stage of development in mammals, very little is known about how these enzymes are regulated. In this study, the regulatory effects of divalent metal cations on the activity of human cytosolic STs were quantitatively evaluated. Results obtained indicate that all nine human cytosolic STs examined are partially or completely inhibited/stimulated by the ten divalent metal cations tested at 10 mM concentration. Compared with the other metal cations, the inhibitory or stimulatory effect of Mg^{2+} and Ca^{2+} on the activities of the human cytosolic STs appeared to be relatively smaller. Concentration-dependent effects of the divalent metal cations were further examined. The IC_{50} or EC_{50} values determined for different divalent metal cations were mostly above their normal physiological concentration ranges. In a few cases, however, IC_{50} values close to the physiological concentrations of certain divalent metal cations were observed. Using the monoamine (M)-form phenol ST (PST) as a model, it was demonstrated that the K_m for dopamine changed only slightly with increasing concentrations of Cd^{2+}, whereas the V_{max} was dramatically decreased.

Key words: divalent metal cations, human cytosolic sulfotransferases, regulatory effects.

Sulfate conjugation is a major pathway *in vivo* for the biotransformation and/or excretion of xenobiotics and endogenous compounds such as steroid and thyroid hormones, catecholamines, cholesterol, bile acids, *etc.* (*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/activation of the substrate compounds or increase their water-solubility, thereby facilitating their removal from the body (*1–3*).

Except during the early stage of development, cytosolic STs in general have been shown to be constitutive enzymes with little known about the regulation of their enzymatic activity (*1*). Although no cofactors have been shown to be required for the functioning of cytosolic STs, studies performed in our laboratory in the past several years have revealed that some divalent metal cations may exert stimulatory or inhibitory effects on cytosolic STs (*5, 6*). Using human monoamine (M)-form phenol ST (M-PST) as a model (*7*), it was shown that the addition of Mn^{2+} to the reaction mixture resulted in a dramatic increase in its Dopa/tyrosine-sulfating activity. To a lesser extent, Co^{2+} and Mg^{2+} could also stimulate the Dopa/tyrosine-sulfating activity of M-PST. In contrast, Ca^{2+} and Cd^{2+} caused an inhibition of this activity. These findings indicate that divalent metal cations may play a significant role in regulating the activity of M-PST. An important issue, therefore, is whether cytosolic STs in general are subject to regulation by divalent metal cations.

We report in this communication a systematic investigation of the effects of a variety of divalent metal cations on the activities of nine human cytosolic STs. The concentrations of different divalent metal cations causing 50% inhibition or enhancement (IC_{50} or EC_{50}) of the activities of individual enzymes were determined. Moreover, using M-PST as a model, kinetic experiments were performed to examine the mode of action of the divalent metal cation, Cd^{2+}.

MATERIALS AND METHODS

Materials—Dopamine, *p*-nitrophenol, adenosine 5′-triphosphate (ATP), Trizma base, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), 3-[*N*-tris-(hydroxymethyl)methylamino]-propanesulfonic acid (Taps), dehydroepiandrosterone (DHEA), 3,3′,5-triiodo-L-thyronine (sodium salt) (T_3), and estrone (1,3,5[10]-estratrinen-3-ol-17-one) were products of Sigma. The sulfate-activating enzymes, ATP sulfurylase and APS kinase, from *Bacillus stearothermophilus* were kindly provided by Dr. Hiroshi Nakajima of Unitika (Uji). Carrier-free sodium [^{35}S]sulfate was from ICN Biomedicals. Radioactive PAP[^{35}S] was synthesized

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Abbreviations: ST, sulfotransferase; PAPS, 3′-phosphoadenosine 5′ phosphosulfate; DHEA, dehydroepiandrosterone.

based on a previously published procedure (*8*). Cellulose thin-layer chromatography (TLC) plates were from EM Science. All other chemicals were of the highest grade commercially available.

Preparation of Purified Human Cytosolic STs—M-form (SULT1A3) and P-form (SULT1A1) PSTs, two SULT1C STs (designated #1 and #2), DHEA (SULT2A1) ST, and thyroid hormone (SULT1B2) ST (TH ST) were cloned, expressed, and purified using the pGEX-2TK Glutathione S-transferase Gene Fusion System based on the procedure previously established (*9, 10*). Estrogen (SULT1E1) ST (EST), SULT2B1a ST, and SULT2B1b ST were cloned, expressed, and purified using the pET23c protein expression system as previously described (*11*).

Determination of the Stimulatory/Inhibitory Effects of Divalent Metal Cations on Human Cytosolic STs—To determine the stimulation/inhibition patterns of divalent metal cations on purified human cytosolic STs, enzymatic assays in the presence or absence of divalent metal cations were performed based on the procedure previously established (*5–7*). The standard assay mixture, in a final volume of 30 μL, contained 10 mM of the divalent cation tested, 14 μM PAP[^{35}S], 100 mM buffer, the enzyme being assayed, and a specified concentration of the acceptor substrate (*cf.* Table I). Controls containing all the reagents, but without divalent metal cation or with 10 mM EDTA, were assayed in parallel. The reaction was started by the addition of the enzyme and allowed to proceed for 10 min at 37°C, followed by heat inactivation of the enzyme at 100°C for 2 min. Aliquots (1 μl) of the final reaction mixtures were spotted onto cellulose TLC plates and subjected to ascending TLC (*cf.* Table I for solvent systems used). After the completion of TLC, the plates were air-dried and subjected to autoradiography for 24 to 48 h. The radiolabeled sulfated products on the plates were located, cut out and eluted in 0.5 ml of water, mixed with 4 ml of Ecolume scintillation fluid, and counted for radioactivity. To determine the concentrations required for 50% inhibition or stimulation (IC_{50} or EC_{50}), a broader concentration range for each divalent metal cation was first tested. After the concentration-dependent inhibitory/stimulatory effect was observed, a narrower range of concentrations was then used for the determination of IC_{50} or EC_{50}.

Kinetic Analysis of the Inhibitory Effects of Cd^{2+} on M-PST—M-PST was chosen as a model for examining the mode of action of the metal ion Cd^{2+}, which exerts a profound inhibitory effect on its dopamine-sulfating activity. Kinetic experiments with varying substrate concentrations and fixed divalent cation concentrations were performed. Fifteen microliter assay mixtures containing a constant concentration of $CdCl_2$, 14 μM PAP[^{35}S] (15 Ci/mmol), 100 mM TAPS (pH 8.0), 0.25 μg enzyme, and specified concentrations of dopamine were prepared. The reactions were allowed to proceed at 37°C for 10 min, and terminated by heating at 100°C for 2 min. The final reaction mixtures were subjected to TLC analysis as described above. The concentrations of $CdCl_2$ tested in different sets of experiments were 0.5, 1, and 2 mM. As a control, 0.1 mM EDTA was used instead of $CdCl_2$. Data on the velocity (v) and corresponding substrate concentration were processed using the Excel program to generate the best fit for the Lineweaver-Burke double-reciprocal plot.

RESULTS AND DISCUSSION

Metal cations are known to play important roles in the function of biological molecules (*12*). Some of them, including manganese, zinc, copper, iron, and cobalt, are essential components of different enzymes, while others, such as magnesium and calcium, are required for the actions of certain enzymes/proteins. In contrast to these biologically useful metal cations, some metal cations that enter the body primarily as environmental contaminants have been shown to exert deleterious effects. For example, lead and mercury are known to act as neurotoxicants (*13, 14*). The targets of the actions of these metal cations *in vivo*, however, require further investigation. As mentioned earlier, our previous studies revealed that some divalent metal cations, including Mn^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+}, and Cd^{2+}, exert stimulatory or inhibitory effects on the Dopa/tyrosine-sulfating activity of human M-PST (*5–7*). The next logical step would be to determine if these metal cations exert similar effects on other cytosolic STs, and whether other divalent metal cations are also capable of exerting stimulatory/inhibitory effects on the activity of cytosolic STs. We therefore decided to carry out a systematic study of the regulatory effects of divalent metal cations on nine human cytosolic STs.

Effects of Divalent Metal Cations on the Activities of Human Cytosolic STs—In the first series of experiments, enzymatic assays using individual human cytosolic STs and their physiological (or preferred) substrates (*cf.* Table I) were carried out in the absence or presence of various divalent metal cations at a concentration of 10 mM. As a control for the counter ion, Cl^-, parallel assays in the presence 20 mM NaCl were also performed. Results obtained are compiled in Table II. The degrees of inhibition or stimulation were calculated by comparing the activities determined in the presence of metal cations with the activities determined in the absence of metal cations. It was noted that the NaCl controls displayed slight stimulatory or inhibitory effects on

TABLE I. **Summary of the buffers and substrates used in the sulfotransferase assays and the solvent systems used for the TLC analyses of sulfated products.**

Enzyme	Buffer	Substrate	TLC Solvent System (*n*-butanol:isopropanol:formic acid:water) (by volume)
M-PST (SULT1A3)	100 mM Taps, pH 8.0	10 μM dopamine	3:1:1:1
P-PST (SULT1A1)	100 mM Taps, pH 8.0	10 μM *p*-nitrophenol	3:1:1:1
DHEA ST (SULT2A1)	100 mM Taps, pH 8.0	5 μM DHEA	2:1:1:2
SULT1C ST #1	100 mM Taps, pH 8.0	25 μM *p*-nitrophenol	3:1:1:1
SULT1C ST #2	100 mM Hepes, pH 7.0	5 μM *p*-nitrophenol	3:1:1:1
EST (SULT1E1)	100 mM Taps, pH 8.0	25 μM estrone	2:1:1:2
SULT2B1a ST	100 mM Taps, pH 8.0	10 μM DHEA	2:1:1:2
SULT2B1b ST	100 mM Taps, pH 8.0	10 μM DHEA	2:1:1:2
TH ST (SULT1B2)	100 mM Taps, pH 8.0	5 μM T_3	ammonium hydroxide: n-propanol (3:2; by volume)

the nine human cytosolic STs tested. After accounting for such effects, presumably due to the counter ion (Cl^-), all nine human cytosolic STs were found to be partially or completely inhibited/stimulated by a majority of the divalent metal cations tested. The addition of equimolar concentrations of EDTA neutralized the inhibitory effects of the divalent metal cations on the dopamine-sulfating activity of M-PST, except for Hg^{2+} which is known to exhibit high-affinity interaction with the sulfhydryl group, and, therefore, causes the irreversible inhibition of proteins (*15*). (Although the data are not shown, similar results for the addition of equimolar EDTA were also found for other cytosolic STs tested.) That the cytosolic STs remained fully active in the presence of EDTA suggests that no divalent metal cations are required as a cofactor for the basal activities of these enzymes. As shown in Table II, while seven of the metal cations exerted complete inhibition in many cases and Mn^{2+} stimulated M-PST, the divalent metal ions Mg^{2+} and Ca^{2+} had relatively minor effects on the nine human cytosolic STs. These results imply that these cytosolic STs in general are less sensitive to the effects of physiologically more abundant metal cations, but are more sensitive to the detrimental effects of other metal cations, many of which may enter the body as environmental contaminants. It should be pointed out that, in contrast to the Dopa/tyrosine-sulfating activity, which is dramatically stimulated by Mn^{2+} (by a factor of two orders of magnitude) (*6, 7*), the dopamine-sulfating activity of M-PST is stimulated less than twofold by Mn^{2+}. This makes sense from the physiological standpoint, since dopamine [the physiological substrate of M-PST (*16, 17*)] plays an important role as a neurotransmitter *in vivo*. Excessive stimulation of the dopamine-sulfating activity of M-PST, leading to the sulfation of dopamine and its inactivation/elimination, may have a detrimental effect. The dopa/tyrosine-sulfating activity, on the other hand, may represent a "xenobiotic-sulfating activity" of M-PST, which may be provoked more easily by Mn^{2+}. (It is to be noted that the Dopa/tyrosine-sulfating activity of M-PST displays stereoselectivity favoring the D-form enantiomer of Dopa/tyrosine (*6, 7*)).

To examine further the inhibitory/stimulatory effects, the activities of the human cytosolic STs in the presence of different concentrations of metal cations were determined. These experiments were performed mostly in two stages, first with a broader and then a narrower concentration range of metal cation. The results compiled in Table III demonstrate that the different human cytosolic STs tested respond to the divalent metal cations differently. For example, when the Fe^{2+} concentration reached 2.0–4.0 mM, it inhibited the activities of P-PST, DHEA ST, SULT2B1a ST, SULT2B1b ST, TH ST, and EST by 50%, while it achieved the same effect on M-PST, SULT1C ST #1, and SULT1C ST #2 at much lower (0.45, 0.06, and 0.04 mM) concentrations. (It is noted that the activity of P-PST in the presence of 10 mM $FeCl_2$ was 33% of the control (Table II) and yet the IC_{50} was 3.0 mM. In contrast, no EST activity was detected in the presence of 10 mM $FeCl_2$, and the IC_{50} (4.8 mM) was higher than that for P-PST. This discrepancy might have been due to the slight yellowish precipitates observed when 10 mM $FeCl_2$ was included in the assay mixture for EST. It could have been that the purified EST was somewhat unstable at higher concentrations of $FeCl_2$ and became denatured.) Co^{2+} inhibited the activities of M-PST, DHEA ST, SULT1C ST #2, and SULT2B1a ST by 50% at concentra-

TABLE II. **Inhibitory/stimulatory effects of divalent metal cations on the activities of human cytosolic STs.**[a]

	M-PST (SULT1A3)		P-PST (SULT1A1)	DHEA ST (SULT2A1)	SULT1C ST #1	SULT1C ST #2	TH ST (SULT1B2)	EST (SULT1E1)	SULT2B1a ST	SULT2B1b ST
Control	6,702 ± 69 (100%)	6,733 ± 44[b] (101%)	3,927 ± 106 (100%)	1,364 ± 73 (100%)	7.3 ± 0.2 (100%)	1,185 ± 93 (100%)	298 ± 17 (100%)	459 ± 10 (100%)	570 ± 8 (100%)	466 ± 121 (100%)
$MgCl_2$	7,134 ± 106 (106%)	6,971 ± 50 (104%)	5,047 ± 168 (129%)	1,661 ± 65 (122%)	3.7 ± 0.2 (51%)	1,119 ± 61 (94%)	290 ± 18 (97%)	247 ± 7 (54%)	239 ± 15 (42%)	655 ± 60 (141%)
$MnCl_2$	11,606 ± 160 (173%)	6,145 ± 119 (92%)	5,069 ± 76 (129%)	683 ± 5 (50%)	3.4 ± 0.7 (47%)	1,072 ± 10 (90%)	437 ± 14 (147%)	99 ± 4 (22%)	416 ± 28 (73%)	153 ± 25 (33%)
$CaCl_2$	6,759 ± 19 (101%)	6,815 ± 25 (102%)	5,049 ± 245 (129%)	1,746 ± 76 (128%)	4.6 ± 0.4 (63%)	1,104 ± 6 (93%)	478 ± 97 (160%)	315 ± 14 (69%)	817 ± 61 (143%)	337 ± 54 (72%)
$CuCl_2$	238 ± 25 (3.6%)	6,533 ± 19 (97%)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
$ZnCl_2$	181 ± 13 (2.7%)	6,965 ± 63 (104%)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
$FeCl_2$	175 ± 13 (2.6%)	6,295 ± 25 (94%)	1,285 ± 201 (33%)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
$CoCl_2$	1,571 ± 31 (23.4%)	7,021 ± 232 (105%)	4,700 ± 117 (120%)	228 ± 15 (17%)	N.D.	171 ± 3 (14%)	66 ± 8 (22%)	90 ± 14 (20%)	56 ± 5 (10%)	39 ± 27 (8%)
$HgCl_2$	188 ± 6 (2.8%)	131 ± 13 (2.0%)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
$CdCl_2$	263 ± 44 (3.9%)	6,971 ± 131 (104%)	1,449 ± 31 (37%)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Pb acetate	801 ± 13 (12%)	6,852 ± 31 (102%)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NaCl	6,896 ± 63 (103%)	6,652 ± 38 (99%)	5,148 ± 305 (131%)	1,364 ± 73 (100%)	6.3 ± 0.3 (86%)	1,132 ± 87 (96%)	312 ± 45 (105%)	480 ± 2 (105%)	764 ± 104 (134%)	481 ± 54 (103%)

[a]The concentration of the divalent metal cations tested was 10 mM, and the concentration of NaCl tested was 20 mM. Specific activities of the STs are expressed as pmo duct produced/min/mg protein. Data shown represent means ± SD of five determinations. N.D. refers to "no activity detected" and, therefore, the complete inhibition of the enzyme. [b]The values shown in this column were derived from assays performed in the presence of 10 mM of the divalent cations tested plus 10 mM EDTA.

TABLE III. **Summary of the IC_{50} or EC_{50} of different divalent metal cations for human cytosolic STs.**[a]

	M-PST (SULT1A3)	P-PST (SULT1A1)	DHEA ST (SULT2A1)	SULT1C ST #1	SULT1C ST #2	TH ST (SULT1A2)	EST (SULT1E1)	SULT2B1a ST	SULT2B1b ST
$MgCl_2$	—[b]	—	EC_{50} = 18 mM	IC_{50} = 12.0 mM	—	—[b]	IC_{50} = 16 mM	IC_{50} = 6.0 mM	IC_{50} = 12 mM
$MnCl_2$	EC_{50} = 1 mM	—	IC_{50} = 10 mM	IC_{50} = 3.5 mM	IC_{50} = 9.5 mM	IC_{50} = 20 mM	IC_{50} = 4.0 mM	IC_{50} = 12 mM	IC_{50} = 2.0 mM
$CaCl_2$	—	—	EC_{50} = 20 mM	IC_{50} = 12 mM	—	—	IC_{50} = 14 mM	—	—
$CuCl_2$	IC_{50} = 0.4 mM	IC_{50} = 0.2 mM	IC_{50} = 0.1 mM	IC_{50} = 0.13 mM	IC_{50} = 7.0 μM	IC_{50} = 0.15 mM	IC_{50} = 0.16 mM	IC_{50} = 50 μM	IC_{50} = 0.16 mM
$ZnCl_2$	IC_{50} = 0.6 mM	IC_{50} = 0.14 mM	IC_{50} = 0.1 mM	IC_{50} = 24 μM	IC_{50} = 7.0 μM	IC_{50} = 80 μM	IC_{50} = 20 μM	IC_{50} = 2.0 μM	IC_{50} = 60 μM
$FeCl_2$	IC_{50} = 0.45 mM	IC_{50} = 3.0 mM	IC_{50} = 2.6 mM	IC_{50} = 60 μM	IC_{50} = 40 μM	IC_{50} = 2.0 mM	IC_{50} = 4.8 mM	IC_{50} = 4.0 mM	IC_{50} = 4.4 mM
$CoCl_2$	IC_{50} = 8.4 mM	—	IC_{50} = 4.5 mM	IC_{50} = 0.3 mM	IC_{50} = 3.0 mM	IC_{50} = 0.2 mM	IC_{50} = 1.2 mM	IC_{50} = 2.6 mM	IC_{50} = 70 μM
$HgCl_2$	IC_{50} = 0.4 mM	IC_{50} = 0.38 mM	IC_{50} = 0.15 mM	IC_{50} = 0.22 mM	IC_{50} = 30 μM	IC_{50} = 80 μM	IC_{50} = 3 μM	IC_{50} = 0.3 μM	IC_{50} = 0.16 mM
$CdCl_2$	IC_{50} = 0.7 mM	IC_{50} = 7.0 mM	IC_{50} = 0.14 mM	IC_{50} = 0.31 mM	IC_{50} = 0.32 mM	IC_{50} = 0.24 mM	IC_{50} = 0.18 mM	IC_{50} = 5.0 μM	IC_{50} = 0.25 mM
Pbacetate	IC_{50} = 3.7 mM	IC_{50} = 1.2 mM	IC_{50} = 0.2 mM	IC_{50} = 1.2 mM	IC_{50} = 50 μM	IC_{50} = 0.6 mM	IC_{50} = 1.2 mM	IC_{50} = 0.1 mM	IC_{50} = 0.4 mM

[a]Data shown were derived from three determinations. [b]IC_{50} or EC_{50} could not be determined in the concentration range of the divalent cation tested.

tions of 8.4, 4.5, 3.0, and 2.6 mM, respectively, while it inhibited the activities of SULT2B1b ST, TH ST, EST, and SULT1C ST #1 by 50% at concentrations of 0.07, 0.2, 1.2, and 0.3 mM, respectively. Interestingly, P-PST appeared to be virtually insensitive to the effect of Co^{2+}. Cd^{2+} inhibited the activity of P-PST by 50% at a concentration of 7.0 mM, but it achieved the same effect on M-PST, DHEA ST, SULT1C ST #2, SULT2B1b ST, TH ST, EST, and SULT1C ST #1 at concentrations of 0.7, 0.14, 0.32, 0.25, 0.24, 0.18, and 0.31 mM, respectively. These IC_{50} values are all above the normal physiological concentration ranges of the divalent metal cations tested (*18*), implying that human cytosolic STs, in general, are not easily influenced by these metal cations *in vivo*. In a few cases, however, IC_{50} values close to the physiological concentration ranges were ob-served. For example, SULT2B1a ST was found to be ex-tremely sensitive to Zn^{2+}, Hg^{2+}, and Cd^{2+}, with 50% of its activity being inhibited at concentrations of, respectively, 0.3, 2.0, and 5.0 μM. Similarly, 50% of the activity of SULT1C ST #2 was inhibited by Cu^{2+} or Zn^{2+} at a concentration of 7.0 μM; and 50% of the activity of EST was inhibited by Hg^{2+} at a concentration of 3.0 μM. These results indicate that these latter cytosolic STs may, in fact, be vulnerable to the inhibitory effects of certain divalent metal cations under physiological conditions. It should be cautioned that the physiological concentration ranges refer to the levels in human serum. The levels in tissues may be quite different and may differ depending on the tissue or organ, some of which tend to selectively concentrate certain metal cations (*18*). Also, in view of the fact that these are cytosolic enzymes and some of the metal ions may be sequestered in organelles such as mitochondria (*19*), any process that disrupts this sequestration (as may be caused by apoptosis or the oxidative damage that is believed to accompany many neurodegenerative disorders) (*20*) may affect the activity of these STs and the processes they regulate. In short, it is possible that relatively small increases or decreases in the serum levels of some metal ions may have important consequences for the activity of many of these STs and the processes they regulate. Moreover, some disorders (e.g., the neurodegenerative disorders) may be partly mediated via changes in the cytosolic concentrations of some metal ions, with subsequent effects on the activity of enzymes such as the cytosolic STs.

Investigation of the Mode of Action of the Divalent Metal Cation Cd^{2+} using M-PST as a Model—An important issue is the modes of action of divalent metal cations. Since M-PST is the best characterized human cytosolic ST and plays an important role in the homeostatic regulation of dopamine metabolism (*16, 17*), we decided to use it as a model in this study. The effects of Cd^{2+} on the kinetics of dopamine sulfation by M-PST were examined. Enzymatic assays using varying concentrations of the substrate, dopamine, in the presence of fixed concentrations of $CdCl_2$ were performed. Data obtained were used to generate a Lineweaver-Burk double-reciprocal plot (Fig. 1). A striking feature of the double-reciprocal plot generated is that the lines corresponding to the various concentrations of $CdCl_2$ tested, while crossing the *Y*-axis at different positions, appear to converge within a narrow region on the *X*-axis. These results indicated that the K_m value of M-PST for dopamine changes only slightly in the presence of the $CdCl_2$ concentrations tested, whereas the V_{max} decreases dramatically with increasing concentrations of Cd^{2+}. The values of K_m and V_{max}, as well as V_{max}/K_m, calculated from the Lineweaver-Burk double-reciprocal plots are compiled in Table IV. These data seem to point to a noncompetitive-type of inhibition in which the divalent cation, Cd^{2+}, and the substrate, dopamine, may bind independently at different sites on M-PST. The complex of the enzyme, Cd^{2+}, and dopamine, however, may be catalytically inactive. It is possible that Cd^{2+} may cause a change in the conformation of the M-PST-dopamine complex, and thereby prevent the proper positioning of the catalytic center, leading to the inactivation of the enzyme (*21*). It will be important to find out which amino acid residue(s) in M-PST is(are) responsible for Cd^{2+}binding.

The present study represents the first systematic investi-

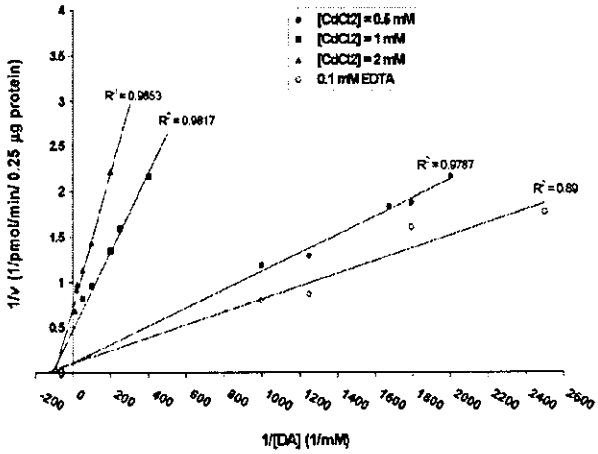

Fig. 1. **Lineweaver-Burk double-reciprocal plot of M-PST with dopamine as the substrate in the presence of different concentration of $CdCl_2$.** The concentrations of dopamine are expressed in µM and velocities are expressed as pmol of product formed/min/0.25 µg enzyme. Each data point represents the mean value derived from five determinations.

TABLE IV. **Kinetic constants of M-PST at different concentrations of $CdCl_2$ with dopamine as substrate.**[a]

$CdCl_2$ (mM)	K_m (µM)	V_{max} (pmol/min/ 0.25 µg protein)	V_{max}/K_m
0.5	4.5 ± 1.0	4.3 ± 0.9	0.96
1	6.2 ± 0.8	1.8 ± 0.1	0.29
2	11.9 ± 0.3	1.5 ± 0.1	0.13
0.1 mM EDTA	9.6 ± 3.9	15.6 ± 6.6	1.63

[a]Data shown represent means ± SD of five determinations.

gation of the role of divalent metal cations in regulating the activities of human cytosolic STs. Cytosolic STs are traditionally viewed as Phase II drug-metabolizing or detoxifying enzymes that are important in the inactivation and removal of xenobiotic compounds (*1–3*). It is becoming increasingly clear, however, that these enzymes are also involved in the metabolism of endogenous compounds that function as neurotransmitters, hormones, *etc.* (*22–24*). To some extent, the role of an enzyme may depend on its location. M-PST, for example, is found in the upper gastrointestinal (GI) tract, brain, platelets, and lung (*25*). While in the brain it may play a role in regulating the levels of dopamine, in the GI tract it may detoxify potentially lethal dietary catecholamines and help to regulate the gut-blood barrier (*24*). The presence of various metal ions of dietary, therapeutic, or environmental origin, by affecting the activity of the cytosolic STs at the various interfaces, such as gut, lung, *etc.*, is likely to disrupt the integrity of various barriers and have physiological sequelae. Fluctuations in the levels of various metal ions *in vivo* will also affect the functioning of other enzymes including the cytosolic STs, *e.g.*, brain M-PST, which play a role in the regulation of endogenous compounds. In some extreme cases, environmental or occupational heavy metal poisoning has been documented (*26–28*). Individuals intoxicated with, for example, lead or mercury have been reported to exhibit neurological symptoms (*13, 14*). In view of the fact that M-PST is involved in the homeostatic regulation of monoamine neurotransmitters (*17*), particularly dopamine, as well as in controlling the entry of toxic catecholamines in the gut (*24*), it is possible that this enzyme may be one of the targets of heavy metal contaminants producing their neurological effects. Manganese poisoning or manganism, has been documented in people working in manganese mines, and this disorder mimics some of the features of Parkinson's disease (*29, 30*). Parkinsonism is characterized by greatly lowered brain dopamine levels (31), and it is likely that the Parkinsonian symptoms of manganism are mediated through a stimulatory effect of divalent manganese ions on brain M-PST, resulting in the removal of dopamine (*5–7*).

In general, the cytosolic STs have been considered housekeeping enzymes and, except during the early stage of development, very little is known with regard to the regulation of their expression or activity (*1–3*). That various divalent metal cations are capable of inhibiting or stimulating the activities of human cytosolic STs presents a new issue for consideration in understanding the functioning of these enzymes. More studies are warranted to fully elucidate the effects of divalent metal cations on cytosolic STs in the context of toxicology, endocrinology, neurology, *etc.*

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Manganese Stimulation and Stereospecificity of the Dopa (3,4-Dihydroxyphenylalanine)/Tyrosine-sulfating Activity of Human Monoamine-form Phenol Sulfotransferase

KINETIC STUDIES OF THE MECHANISM USING WILD-TYPE AND MUTANT ENZYMES*

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Kinetic studies were performed to dissect the mechanism underlying the remarkable Mn^{2+} stimulation of the Dopa/tyrosine-sulfating activity of the human monoamine (M)-form phenol sulfotransferase (PST). The activities and the stimulation by Mn^{2+} are highly stereospecific for the D-form enantiomers of tyrosine and Dopa. Analysis of the kinetic results strongly suggests that tyrosine-Mn^{2+} and tyrosine-Mn^{2+}-tyrosine complexes are obligatory substrates, whereas Dopa-Mn^{2+} complexes may be better substrates than Dopa alone. This activation of the Dopa/tyrosine-sulfating activity of M-form PST by Mn^{2+} via complex formation between Mn^{2+} and the substrate is the first reported case of a regulatory mechanism in this important class of enzymes. Our previous studies using point-mutated M-form PSTs established that the Mn^{2+} (in the substrate-Mn^{2+} complex) exerts its stimulatory effect by binding predominantly to the Asp-86 residue at the active site. We present here further studies using dopamine as substrate to bolster this conclusion. The possible physiological implications of this rather unusual specificity for the D-amino acid and its derivatives and the stimulation by Mn^{2+} are discussed in the context of protective and detoxification mechanisms that may operate in neurodegenerative processes in the brain. The Mn^{2+} stimulation of the activity of M-form PST toward D-enantiomers of Dopa/tyrosine may have implications for other substrates (including chiral drugs) and for the other cytosolic sulfotransferases that are involved in the regulation of endogenous metabolites as well as in detoxification.

Sulfotransferases (STs)[1] are enzymes ubiquitous in both plants and animals that catalyze the sulfation of a variety of compounds containing hydroxyl or amino groups using 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as the sulfonyl group donor (1–3). Although the membrane-bound STs use proteins, glycolipids, and other macromolecules as substrates, the cytosolic STs sulfate smaller molecules and are part of the Phase II detoxification pathway for the biotransformation/excretion of drugs and xenobiotics. This serves to both detoxify dietary, therapeutic, and environmental xenobiotics as well as regulate the levels and activities of endogenous molecules such as thyroid and steroid hormones, catecholamine hormones/neurotransmitters, and bile acids (4, 5). Except during the early stage of development, cytosolic STs in general have been shown to be constitutive enzymes with little known about the regulation of their enzymatic activities (6, 7). In the past several years, however, studies performed in our laboratory reveal that Mn^{2+} exerts a stimulatory effect on sulfation of some substrates by the human monoamine (M)-form phenol sulfotransferase (PST) (8, 9).

The human M-form PST is the only sulfotransferase that sulfates the catecholamines, in particular the neurotransmitter dopamine, with high activity (4). This enzyme is found in the upper gastrointestinal tract, brain, platelet, and lung (10). In the gastrointestinal tract it may detoxify potentially lethal dietary catecholamines. In the brain it may play a role in regulating the levels of dopamine. We had previously demonstrated that besides its activity toward catecholamines, M-form PST could uniquely sulfate the free amino acid form of tyrosine and 3,4-dihydroxyphenylalanine (Dopa) (8, 9, 11). Interestingly, it showed higher activities toward the D-enantiomers (as compared with the L-enantiomers) of these compounds and a remarkable stimulation (by more than 100-fold) of the activities by sub-millimolar and millimolar levels of Mn^{2+}, especially with the D-enantiomers. Mn^{2+} also stimulates the activity with dopamine, although only 2–3-fold (9). Mn^{2+} is known to be present at higher levels in human neuronal tissue (12) and is sequestered intracellularly in mitochondria (13). Oxidative stress or damage, which has been implicated in neuronal apoptosis that occurs in neurodegenerative diseases, generally results in mitochondrial dysfunction (14). The consequent release of Mn^{2+} into the cytosol may activate the M-form PST and, in particular, its Dopa/tyrosine-sulfating activity. It has also been observed that D-amino acids accumulate in aging tissues, especially if the levels of D-amino acid oxidases are low (15). Attempts have been made to link the amount of specific D-amino acids to oxidative damage and to neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (16, 17). A clear picture is yet to emerge. However, the removal of D-amino acids by sulfation may be viewed as a detoxification process. From a

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[1] The abbreviations used are: ST, sulfotransferase; M-form PST, monoamine-form phenol sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; Dopa, 3,4-dihydroxyphenylalanine; TAPS, 3-[*N*-tris-(hydroxymethyl)methylamino]-propanesulfonic acid; DTT, dithiothreitol.

different perspective, the sulfation of D-tyrosine could also serve as a useful model in the study of the stereospecific action of the PST enzymes on chiral drugs (18–21). It should also be emphasized that the dramatic stimulation by Mn^{2+} of the sulfation of these substrates may be part of a more general mechanism to increase the promiscuity of M-form PST toward unusual or xenobiotic substrates in the presence of a molecular trigger such as increased Mn^{2+} concentrations.

In this paper, we report kinetic studies on the sulfation of dopamine and the D- and L-enantiomers of tyrosine and Dopa by the wild-type M-form PST and its Asp-86 point mutant and the stimulation by Mn^{2+}. M-form PST is known to exist as a homodimer in its native state (22), and the reported X-ray structure of the protein (23) revealed that residues 84–92 of one subunit form a "mobile" loop that may intercalate into the active site of the other subunit. It was suggested that the presence of this mobile loop might hinder the proper positioning of some substrates (23). Our previous studies (11) have established the importance of two regions in the sequence of M-form PST, designated Region I (spanning residues 84–89) and Region II (residues 143–148), to its dopamine-sulfating activity as well as its Dopa/tyrosine-sulfating activity and the Mn^{2+} stimulation. These are the regions that vary between M-form PST and the P-form PST (that does not possess Mn^{2+}-stimulated Dopa/tyrosine-sulfating activity), which otherwise are more than 93% identical in their amino acid sequences (11). That the Region I is part of the above-mentioned mobile loop intercalating into the active site allows for the formulation of an attractive model to explain our kinetic results. Our previous studies with point mutants in Regions I and II have also underlined the importance of residues Asp-86 and Glu-89 in Region I and of residue Glu-146 in Region II in the dopamine-sulfating activity of M-form PST (24). Further studies with such point mutants and two deletional mutants (lacking residues 84–90 and 84–86, respectively, of the purported loop intercalating into the active site in the wild-type M-form PST) have revealed that both the loop as a whole (rather than the residues comprising it) as well as residue Glu-146 in Region II are important to the stereospecificity of M-form PST for the D-enantiomers of Dopa and tyrosine. Residue Asp-86 in Region I, on the other hand, is the one most important to the Mn^{2+} dependence of this activity.[2] We also present in this paper studies with the D86A point mutant to further dissect the structural basis for these activities and their activation by Mn^{2+}.

EXPERIMENTAL PROCEDURES

Materials—L-Dopa, D-Dopa, L-tyrosine, D-tyrosine, dopamine, aprotinin, thrombin, ampicillin, ATP, SDS, TAPS, HEPES, Trizma base (Tris base), dithiothreitol (DTT), EDTA (disodium salt), glycerol, bovine serum albumin, PAPS, and isopropyl β-D-thiogalactopyranoside were from Sigma. The 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 cells, and glutathione-Sepharose were products of Amersham Biosciences. Carrier-free sodium [^{35}S]sulfate and Ecolume liquid scintillation fluid were from ICN Biomedicals. Chromatogram cellulose thin-layer chromatography (TLC) plates were from EM Science. Recombinant human bifunctional ATP sulfurylase/adenosine 5′-phosphosulfate kinase was prepared as described previously (25). All other chemicals were of the highest grade commercially available.

Bacterial Expression and Purification of the Recombinant Human Wild-type and D86A Point-mutated M-form PSTs—Competent *E. coli* BL21 cells transformed with pGEX-2TK vector harboring the wild-type or D86A point-mutated M-form PST cDNA were grown to $A_{600\ nm}$ = 0.8 in 1 liter of LB medium supplemented with 50 μg/ml ampicillin. After induction with 0.1 mM isopropyl β-D-thiogalactopyranoside overnight at room temperature, the cells were collected by centrifugation and homogenized in 25 ml of an ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 10% glycerol using an Amicon French press. Ten μl of a 10 mg/ml aprotinin solution was added to the homogenate, which was then centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant collected was fractionated by equilibrating with 1.5 ml of glutathione-Sepharose for 20 min at 4 °C, and the supernatant and the washings with the lysis buffer were discarded. The bound fusion protein was treated with 3 ml of a thrombin digestion buffer (containing 5 units/ml bovine thrombin, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$, and 10% glycerol). After a 30-min incubation at room temperature, the preparation was centrifuged. The recombinant enzyme present in the supernatant was collected and analyzed by SDS-PAGE, and the protein concentration was determined and used in the enzymatic assays. Ten mg/ml of bovine serum albumin was added as a stabilizing agent to this preparation, that was then stored in small aliquots at −70 °C until use.

Enzymatic Assay—The sulfotransferase assays were performed using [^{35}S]PAPS as the sulfate donor. The standard assay mixture for the Dopa/tyrosine sulfation assays contained, in a final volume of 25 μl, 50 mM TAPS, pH 8.25, 0.8 mM DTT, 15 μM [^{35}S]PAPS, substrate (*i.e.* D-Dopa, L-Dopa, D-tyrosine, or L-tyrosine), $MnCl_2$ or 1 mM EDTA, and 2.5 μl of enzyme solution, with the amount of enzyme ranging from 5 ng to 5 μg. The assay mixture with dopamine as substrate differed only in that 50 mM HEPES, pH 7.0, instead of 50 mM TAPS, pH 8.25, was used. The enzyme dilutions were prepared in 50 mM TAPS, pH 8.25, or 50 mM HEPES, pH 7.0, containing 10% glycerol. The $MnCl_2$ and enzyme solutions were added last to the reaction mixture, which was immediately incubated for 3 min at 37 °C. The reaction was stopped by the addition of 5 μl of 2.5 M acetic acid, vortexed, and centrifuged to clear any precipitates (26). The amount of enzyme chosen was such as to ensure that there was not more than 5% reaction so that the reaction was linear with time and amount of enzyme. The final reaction mixture was subjected to the analysis of ^{35}S-sulfated product by spotting a 3-μl aliquot onto the cellulose TLC plate, which was then subjected to ascending TLC using a solvent system containing *n*-butanol, isopropanol, 88% formic acid, H_2O in a 3:1:1:1 ratio by volume. In the case of Dopa and dopamine, where the sulfated product migrated too close to unused [^{35}S]PAPS for efficient separation or whenever the background was too strong, a two-dimensional separation was performed on the samples spotted on the TLC plate by first running a high voltage (1000 volts) thin-layer electrophoresis in the first dimension followed by the above-mentioned ascending TLC in the second dimension (27). Afterward, the plates were air-dried and autoradiographed. The radioactive spots on the TLC plates due to ^{35}S-sulfated products were cut out and eluted by shaking in 0.5 ml of H_2O in glass vials. Four ml of scintillation fluid was then added to each vial and thoroughly mixed, and the radioactivity was counted using a liquid scintillation counter. The counts obtained were used to calculate the specific activity of the enzyme under the particular reaction conditions in units of nmol of sulfated product formed/min/mg of enzyme. Assays were performed in triplicate, and a control without enzyme was installed to correct for any background counts.

Miscellaneous Methods—[^{35}S]PAPS (carrier-free) was synthesized from ATP and carrier-free [^{35}S]sulfate using the human bifunctional ATP sulfurylase/adenosine 5′-phosphosulfate kinase, and its purity was determined as described previously (28). The [^{35}S]PAPS synthesized was then adjusted to the required concentration and specific activity by the addition of cold PAPS. The concentration of PAPS was confirmed by measuring its absorbance at 260 nm (29). SDS-PAGE was performed on 12% polyacrylamide gels using the method of Laemmli (30). Protein determination was based on the method of Bradford with bovine serum albumin as the standard (31).

RESULTS

Our previous studies demonstrate that M-form PST, besides sulfating dopamine, could also sulfate tyrosine and Dopa (8, 9, 11). Some interesting features of these latter activities were (i) that these activities can be dramatically stimulated by Mn^{2+} and (ii) that the enzyme shows higher activities toward the D- rather than the L-enantiomers of tyrosine and Dopa. By taking a kinetic approach along with studies using mutated M-form PSTs, the current study was aimed at investigating the underlying mechanism for the Mn^{2+} stimulation and stereospecificity.

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

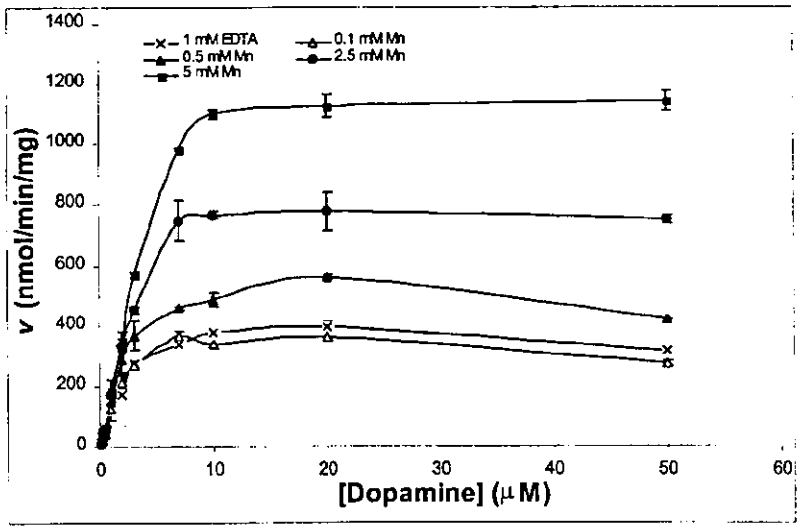

FIG. 1. **Kinetics of the sulfation of dopamine by M-form PST in the presence of different concentrations of Mn^{2+} or EDTA.** Concentrations of dopamine are expressed in μM, and concentrations of Mn^{2+} are expressed in mM. The velocities (v) of the reactions are expressed as nmol of product formed/min/mg of protein. Each data point represents the mean value of three determinations (*error bars* are shown).

Kinetics of Sulfation of Dopamine by M-form PST in the Presence of Varying Concentrations of Mn^{2+}—We first studied the modest stimulation by Mn^{2+} of the sulfation of dopamine by M-form PST (8, 9). Dopamine is believed to be the physiological substrate for M-form PST. Because it has no optical isomers and contains no carboxyl group as in Dopa and tyrosine, it was interesting to investigate the extent and mechanism of the stimulation by Mn^{2+} of dopamine sulfation by M-form PST. The kinetics of the sulfation of dopamine by M-form PST was studied using varying concentrations (ranging from 0.5 μM to 50 μM) of dopamine, in the presence of different concentrations (0, 0.1, 0.5, 1.0, 2.5, 5.0 mM) of Mn^{2+} or in the presence of 1 mM EDTA (as a control). It had been demonstrated that the dopamine-sulfating activity of M-form PST was maximal at pH 7.0 (22). HEPES buffer at pH 7.0 was therefore used in this study along with a saturating PAPS concentration of 15 μM. Fig. 1 shows the plots of the velocity (v) *versus* substrate (dopamine) concentration ([S]) in the presence of different concentrations of Mn^{2+}. It is clear from these plots that regardless of the Mn^{2+} concentration, V_{max} was reached with 10–20 μM dopamine concentrations. Mn^{2+} appeared to increase the V_{max} while changing K_m for dopamine only slightly. In the presence of 1 mM EDTA, the V_{max} was 370 nmol/min/mg, and the K_m was 2.4 μM, whereas with 5 mM Mn^{2+}, the V_{max} was 1000 nmol/min/mg, and the K_m was 4.5 μM. The kinetics appeared to be of the Michaelis-Menten type.

Effects of Mn^{2+} on the Sulfation of Dopamine by the D86A Point Mutant of M-form PST—Our recent studies show that, although Mn^{2+} has a remarkable stimulatory effect on the sulfation, especially of the D-enantiomers of Dopa and tyrosine (Refs. 8 and 10) by M-form PST, the D86A point mutant of this enzyme showed none or just a marginal Mn^{2+} stimulation of sulfation of these substrates.[2] This was interpreted to imply that the stimulatory effect of Mn^{2+} is exerted predominantly via its binding to the negatively charged residue Asp-86, which from the X-ray diffraction studies is believed to be part of the mobile loop intercalating into the active site of M-form PST (23). To find out whether residue Asp-86 also mediates the modest Mn^{2+} stimulation of the sulfation of dopamine, the sulfation of dopamine (at 20 μM) by the wild-type M-form PST and the D86A point mutant in the absence or presence of 5 mM Mn^{2+} was studied. The results tabulated in Table I showed clearly that not only were the activity levels of the D86A point mutant lower, but the stimulatory effect of Mn^{2+} seemed to have been lost.

TABLE I
Stimulation by Mu^{2+} of the activity of wild-type and mutant M-form PSTs with dopamine as substrate

The final concentration of dopamine used was 20 μM, whereas that of Mn^{2+} was 5 mM. All other conditions are as described under "Experimental Procedures."

Substrate used	Wild type	D86A point mutant
	nm:/min/mg	
Dopamine	499 ± 45	59 ± 4
Dopamine + Mn^{2+}	1125 ± 37	63 ± 6

Kinetics of Sulfation of D-Tyrosine by M-form PST in the Presence of Varying Concentrations of Mn^{2+}—The kinetics of the sulfation of D-tyrosine by M-form PST was studied using varying concentrations (ranging from 0.1 to 10 mM) of D-tyrosine in the presence of different concentrations (0, 0.1, 0.5, 1.0, 2.5, 5.0, and 10 mM) of Mn^{2+} or in the presence of 1 mM EDTA (as a control). We had previously demonstrated that this activity was maximal between pH 8.0 and 9.0 (8). TAPS buffer at pH 8.25 was therefore used in this study. It was first established that the PAPS concentration used (15 μM) was saturating, since there was no appreciable increase in the velocity of the reaction even when a 10-fold higher concentration of PAPS was used at several different D-tyrosine and Mn^{2+} concentrations (such studies were also repeated with the other substrates subsequently used). Fig. 2 shows the plots of the velocity (v) *versus* total substrate (D-tyrosine) concentration ($[S]_t$) in the presence of different concentrations of Mn^{2+} (the S_t profiles). It is evident from these plots that at 10 mM D-tyrosine, although saturation with substrate was reached in the presence of 1, 2.5, or 5 mM Mn^{2+}, the curves still reached maximal velocity in the presence of lower concentrations of Mn^{2+} (0, 0.1, and 0.5 mM). However, D-tyrosine has a limited solubility in water, and it was difficult to prepare stable solutions containing greater than 10 mM D-tyrosine under the assay conditions to show the extended curves at lower concentrations of Mn^{2+}. On the other hand, concentrations of Mn^{2+} higher than 5 mM resulted in precipitation and, consequently, inconsistent results at higher concentrations of D-tyrosine. Therefore, with 10 mM Mn^{2+}, only the data obtained at D-tyrosine concentrations of 5 mM or less were analyzed. This is clear from the plots of velocity (v) *versus* total Mn^{2+} ($[A]_t$) concentration in the presence of different concentrations of substrate D-tyrosine shown in Fig. 3 (the A_t profiles).