Kinetics of Human Soluble and Membrane-Bound Catechol *O*-Methyltransferase: A Revised Mechanism and Description of the Thermolabile Variant of the Enzyme

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ABSTRACT: Human soluble (S) and membrane-bound (MB) catechol *O*-methyltransferase (COMT, EC 2.1.1.6) enzymes have been expressed at sufficiently high levels in *Escherichia coli* and in baculovirus-infected insect cells to allow kinetic characterization of the enzyme forms. The use of tight-binding inhibitors such as entacapone enabled the estimation of actual enzyme concentrations and, thereby, comparison of velocity parameters, substrate selectivity, and regioselectivity of the methylation of both enzyme forms. Kinetics of the methylation reaction of dopamine, (−)-noradrenaline, L-dopa, and 3,4-dihydroxybenzoic acid was studied in detail. Here, the catalytic number (V_{max}) of S-COMT was somewhat higher than that of MB-COMT for all four substrates. The K_m values varied considerably, depending on both substrate and enzyme form. S-COMT showed about 15 times higher K_m values for catecholamines than MB-COMT. The distinctive difference between the enzyme forms was also the higher affinity of MB-COMT for the coenzyme *S*-adenosyl-L-methionine (AdoMet). The average dissociation constants K_s were 3.4 and 20.2 μM for MB-COMT and S-COMT, respectively. Comparison between the kinetic results and the atomic structure of S-COMT is presented, and a revised mechanism for the reaction cycle is discussed. Two recently published human COMT cDNA sequences differed in the position of S-COMT amino acid 108, the residue being either Val-108 [Lundström et al. (1991) *DNA Cell. Biol. 10*, 181−189] or Met-108 [Bertocci et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1416−1420]. The catalytic activities of these two COMT variants, expressed in *E. coli*, were similar, but the Met-108 enzyme was more thermolabile already at physiological temperature (37 °C). The reported existence of a common polymorphism of the human COMT gene coding for a thermolabile low activity, $COMT^L$, and a thermostable high activity, $COMT^H$, is discussed in light of the different thermostability of the two enzyme forms.

Catechol *O*-methyltransferase (COMT, EC 2.1.1.6) catalyzes the transfer of the methyl group from the coenzyme *S*-adenosyl-L-methionine (AdoMet) to one of the hydroxyls of catechol or substituted catechols in the presence of Mg^{2+} (Axelrod & Tomchick, 1958). COMT is a ubiquitous enzyme occuring in plants, microorganisms, and animals (Männistö et al., 1992). In mammals COMT is distributed throughout various organs (Karhunen et al., 1994). The highest activities are found in liver and kidney (Guldberg & Marsden, 1975, Rivett et al., 1983). The function of COMT is to inactivate biologically active or toxic catechols. Physiological substrates of COMT are catecholamine neurotransmitters—dopamine, noradrenaline, and adrenaline—and their metabolites. COMT inactivates catecholic steroids such as 2-hydroxyestradiol, neuroactive drugs with a catechol structure such as L-dopa, and a large number of other catechol compounds (Axelrod & Tomchick, 1958; Ball et al., 1972; Guldberg & Marsden, 1975; Borchardt, 1980). In Parkinson's disease, in which substantial destruction of dopaminergic neurons in substantia nigra takes place, L-dopa, the precursor of dopamine, has been used to replenish the depleted dopamine stores (Männistö & Kaakkola, 1990). With selective inhibitors of COMT in combination with L-dopa, a new principle has been realized in the therapy of Parkinson's disease (Männistö et al., 1992).

Two distinct forms of COMT have been found: one is soluble (S-COMT) and the other is membrane-bound (MB-COMT) (Assicot & Bohuon, 1971; Nissinen, 1984; Jeffery & Roth, 1985; Borchardt et al., 1974). Recently, rat and human soluble and membrane-bound COMT cDNAs have been cloned and characterized (Salminen et al., 1990; Ulmanen & Lundström, 1991; Malherbe et al., 1992; Bertocci et al., 1991; Lundström et al., 1991, 1992; Tilgmann & Kalkkinen, 1991; Tilgmann et al., 1992). Both soluble and membrane-bound COMT are coded by one gene using two separate promoters (Tenhunen et al., 1993). The soluble COMT enzyme contains 221 amino acids, whereas the membrane-bound form has a 50- (human) and 43- (rat) residue-long amino-terminal extension that contains the hydrophobic membrane-anchor region. The sequences of COMT enzymes from different mammalian species are highly similar (Vidgren et al., 1994). The soluble human COMT is 81% identical with the respective rat enzyme. Very recently, the atomic structure of rat S-COMT has been solved at 2.0-Å resolution, which provides new insights into the

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mechanism of the methyl transfer reaction (Vidgren et al., 1994).

The kinetic mechanism of COMT isolated from various mammalian tissues has been studied by several groups. A rapid equilibrium random order mechanism (Flohe & Schwabe, 1970; Coward et al., 1973) and a ping-pong mechanism (Borchardt, 1973) were suggested in the earlier studies. Woodard et al. (1980) studied the stereochemical course of the reaction using AdoMet carrying an isotope-labeled chiral methyl group. It was concluded that the methyl transfer from AdoMet to the catechol substrate catalyzed by COMT is a direct bimolecular transfer of the methyl group from sulfur of AdoMet to the oxygen of the catechol hydroxyl through an S_N2-like transition state (Woodard et al., 1980). Product inhibition studies established a sequential ordered mechanism (Rivett & Roth, 1982; Tunnicliff & Ngo, 1983). The coenzyme AdoMet is the first substrate to bind, and *S*-adenosyl-L-homocysteine (AdoHcy) is the last product to dissociate from the enzyme. The presence of magnesium ions is required for the catalysis. Also, other divalent cations—for instance, Cd^{2+}, Hg^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+}—promote the methylation (Axelrod & Tomchick, 1958; Senoh et al., 1962; Flohe, 1974; Boadi et al., 1991), while calcium ions inhibit it (Flohe & Schwabe, 1972). The *O*-methylation occurs primarily at the 3-hydroxyl (*meta* position), but depending on the experimental conditions and the nature of the side chain of the catechol substrate, also varying amounts of the 4-methylated (*para* position) catechols are produced (Creveling et al., 1970, 1972; Thakker et al., 1986). The kinetic behavior of soluble and membrane-bound COMT has been demonstrated to be similar. The most distinctive difference seems to be the 2 orders of magnitude lower K_m of MB-COMT for dopamine (Rivett & Roth, 1982; Nissinen, 1984).

The existence of a common polymorphism of the human COMT gene coding for a thermolabile low activity, $COMT^L$, and a thermostable high activity, $COMT^H$, has been reported (Scanlon et al., 1979; Boudikova et al., 1990; Grossman et al., 1992b). It has been proposed that the differences in the thermostability of COMT in erythrocyte lysates are due to differences in the molecular structure of COMT (Scanlon, et al., 1979). Interestingly, the two published sequences of human COMT differ in one amino acid (Bertocci et al., 1991; Lundström et al., 1992). In the sequence of human S-COMT determined by Bertocci et al. (1991), Val-108 is replaced by Met.

Both human S- and MB-COMT have been expressed recently at high levels in *Escherichia coli* and in baculovirus-infected insect cells (Lundström et al., 1992; Tilgmann et al., 1992). In the present study we have used the recombinant enzymes to study the kinetics of human S- and MB-COMT. Generally, the kinetic constants of COMT have not been well characterized. The use of tight-binding inhibitors enabled the estimation of the actual enzyme concentrations and, thereby, the comparison of velocity parameters, substrate selectivity, and regioselectivity of the methylation of these two enzyme forms. The atomic structure of COMT (Vidgren et al., 1994) is utilized in discussion of the kinetic results. Also, we used site-directed mutagenesis to examine whether the structural difference at residue 108 affects the thermostability or catalytic activity of human COMT, and we discuss the possibility that this difference explains the allelic variance of COMT activity.

MATERIALS AND METHODS

Materials. *S*-Adenosyl-L-methionine (AdoMet) and *S*-adenosyl-L-homocysteine (AdoHcy) were purchased from Sigma. Substrates of COMT—dopamine, (−)-noradrenaline, L-3,4-dihydroxyphenylalanine (L-dopa), and 3,4-dihydroxybenzoic acid (DBA)—and the respective 3- and 4-*O*-methylated products were obtained from Sigma and Aldrich (Germany). The monoamine oxidase (MAO) inhibitor pargyline (*N*-methyl-*N*-propargylbenzylamine hydrochloride) was purchased from Aldrich. The COMT inhibitors and 4-OMD (4-*O*-methyl-DL-dopa) were synthesized at the Synthetic Department of Orion-Farmos (Espoo, Finland). All other reagents were from commerical sources and of analytical grade.

Construction of the Expression Plasmids and Expression of the Recombinant COMT. The expression construct pHCX12 used for the human S-COMT (Val-108) protein production in *E. coli* has been described previously (Lundström et al., 1992). The site-directed mutagenesis Val-108 → Met to yield the clone pOGL829 was carried out using polymerase chain reaction (PCR) (Mullis & Faloona, 1987) to amplify the mutated 3′ portion of the human S-COMT coding sequence. The mutant primer was 5′-CACCCAGCGGATGGTGGATTTCGCTGGCATG-3′, and the primer containing the 3′ end of the coding sequence, followed by a double translation stop codon and *HindIII* restriction enzyme cleavage site, was 5′-TGCAAAGCTTCAGTCAGTCATCAGGGCCCTGCTTC-3′. The PCR product was digested with restriction enzymes *Pfl*MI and *Hin*dIII and ligated to the respective sites of digested pHCX12 DNA. The mutant clone was sequenced by the dideoxy chain-termination method (Sanger et al., 1977) to verify the presence of the expected mutation and the absence of unwanted mutations. The expression of the COMT proteins in *E. coli* has been described previously (Lundström et al., 1992). The recombinant COMT proteins in the induced bacteria were characterized by SDS−10% polyacrylamide gel electrophoresis (Laemmli, 1970) and Coomassie Brilliant Blue staining.

The construction of the baculoviruses used for expression of recombinant human S- and MB-COMT proteins (clones pOGL876 and pOGL814, respectively) and the procedures for the protein expression in insect Sf9 cells have been described in detail previously (Tilgmann et al., 1992).

Preparation of the recombinant Human COMT Proteins for Kinetic Studies. The isopropyl β-D-thiogalactopyranoside- (IPTG-) induced *E. coli* cells carrying the plasmids pHCX12 or pOGL829 were harvested by centrifugation and resuspended in a buffer containing 150 mM NaCl, 16 mM Na_2HPO_4, pH 7.3, 1% Triton X-100, and 0.2 mM phenylmethanesulfonyl fluoride (PMSF). The cells were disrupted by 30 cycles of sonication (30 s at 100 W/cycle followed by a 15-s incubation on ice). The lysates were clarified by centrifugation at 10000*g* at 4 °C for 15 min. Samples were kept at −20 °C.

The infected Sf9 cells were harvested by centrifugation and resuspended in 50 mM MOPS, pH 7.0, 0.25 M sucrose, and 5 mM $MgCl_2$. The cells were disrupted by repeated (5×) freezing and thawing followed by homogenization by a Potter-Elvehjelm homogenizer as described (Tilgmann et al., 1992). The cell homogenates containing recombinant S- or MB-COMT were centrifuged at 100000*g* for 1 h at 4 °C. The S-COMT-containing 100000*g* supernatant was

collected, whereas the 100000*g* pellet of the MB-COMT-expressing cells was resuspended in the homogenizing buffer and the samples were aliquotted, frozen, and kept at −20 °C until used for kinetic studies.

Preparation of Human Placental Homogenate. Human placenta (10 g) from an abortus arte provocatus (10 weeks) was homogenized with a 30-s stroke of Ultra-Turrax T-25 (Janke-Künkel, Staufen, Germany) homogenizer (9500 rpm) in 15 mL of ice-cold 10 mM sodium phosphate, pH 7.0, and 2.6 mM PMSF. The homogenate was first centrifuged for 15 min at 1000*g* at 4 °C and the supernatant at 100000*g* for 45 min at 4 °C. The 100000*g* supernatant was kept at −20 °C. An analysis with SDS−PAGE and immunoblotting with a COMT-specific antiserum indicated that the sample contained only S-COMT-immunoreactive polypeptide (not shown).

Enzyme Assay and Chromatographic Conditions. The general procedure of the COMT activity measurement was essentially the same as previously described (Nissinen & Männistö, 1984). Enzyme reactions were carried out in a mixture containing 100 mM sodium phosphate buffer (Na_2HPO_4), pH 7.4, and 5 mM $MgCl_2$. Enzyme, coenzyme AdoMet, catechol substrate, and inhibitor concentrations were varied according to the respective experiment. The mixture of compounds, except for the catechol substrate, was preincubated for 5 min at 37 °C (Type 16500 Dri-Bath by Thermolyne), and the reaction was started with the catechol substrate. The final volume of the reaction mixture was 250 μL. After a 15-min incubation, the reaction was stopped with 25 μL of 4 M perchloric acid. After removal of the protein precipitate by centrifugation, the supernatant was analyzed for *O*-methylated products by HPLC with electrochemical detection. External standards of methylated products were used for quantitation. In the case of human placental S-COMT, monoamine oxidase (MAO) was inhibited by adding 1 mM pargyline into the reaction mixture.

The HPLC system consisted of a Pharmacia LKB HPLC pump 2248 (Pharmacia, Sweden), a Pharmacia LKB autosampler 2157 (Pharmacia, Sweden), a Hewlett-Packard 3396A integrator, and a BAS amperometric detector LC-4C with a glassy carbon electrode assembly (Bioanalytical Systems). In the HPLC system the column was a 50 × 4.6 mm i.d., 3 μm ODS-2 Spherisorb (Phase Separation, U.K.). The analytical column was fitted with an RCSS C-18 Guard-Pack precolumn (Waters). The elution of *O*-methylated reaction products was carried out isocratically at ambient temperature. The mobile phase composition (buffer/methanol) differed slightly depending on the substrate. In the case of catecholamine substrates dopamine, (−)-noradrenaline, and L-dopa, the buffer part of the mobile phase consisted of 27.5 mM citric acid, 50 mM sodium acetate, 1 mM EDTA, and 1 mM 1-octanesulfonic acid. The amount of methanol (% v/v) and the final pH of the eluent varied with substrates: dopamine (13% methanol, pH 2.5), (−)-noradrenaline (3% methanol, pH 5.0), and L-dopa (13% methanol, pH 5.0). For DBA the mobile phase consisted of 86 mM sodium phosphate buffer, pH 3.2 ($Na_2HPO_4{\cdot}2H_2O$) and 0.13 mM EDTA in 14% methanol. The oxidation potential of the electrochemical detector was +0.7 V for dopamine and (−)-noradrenaline, +0.8 V for L-dopa, or +0.85 V for DBA versus the Ag/AgCl reference electrode. The sensitivity was set to 10 nA.

Kinetics Measurements. Kinetic parameters of S-COMT and MB-COMT were determined by varying the concentrations of both coenzyme AdoMet and the catechol substrate simultaneously. The following concentratitons of AdoMet and substrate were used: (a) for S-COMT, dopamine (20−600 μM) and AdoMet (10−200 μM), L-dopa (20−900 μM) and AdoMet (10−200 μM), (−)-noradrenaline (20−600 μM) and AdoMet (10−200 (μM), and DBA (5-300 μM) and AdoMet (5−200 μM); (b) for MB-COMT, dopamine (2−100 μM) and AdoMet (5−400 μM), L-dopa (20−900 μM) and AdoMet (10−200 μM), (−)-noradrenaline (5−300 μM) and AdoMet (5−200 μM), and DBA (5−300 μM) and AdoMet (5−200 μM). The enzyme concentrations were 32 and 64 nM for S-COMT and MB-COMT, respectively.

The COMT enzyme concentrations were determined by titration with a tight-binding inhibitor. Because nitrocatechol-type COMT inhibitors are reported to be tight-binding (Schultz & Nissinen, 1989) with K_i values in the low nanomolar range, conventional experimental conditions and data analysis by using reciprocal plots were not applicable. These inhibitors are competitive with respect to the catechol substrate and uncompetitive with respect to AdoMet (Schultz & Nissinen, 1989). Therefore the following procedure was used: the enzyme at varying concentrations (S-COMT 3−34 nM, MB-COMT 3−64 nM) was incubated with the inhibitor at varying concentrations (0−45 nM) and with a saturating AdoMet concentration (200 μM). The reaction was started by addition of the catechol substrate (S-COMT, 300 μM dopamine; MB-COMT, 20 μM dopamine). Initial velocities were measured and fitted to the equation derived by Ackermann and Potter for tight-binding inhibition (Cha, 1975).

The kinetic parameters of the human (Val-108) S-COMT and the Met-108 variant expressed in *E. coli* were determined by using the enzyme assay described above. Dopamine was used as the substrate. The concentration of both enzyme preparations was evaluated by titration with the tight-binding inhibitor z-OR-611. The thermotropic behavior of COMT was studied at 37 and 40 °C. The enzyme samples [alone, with $MgCl_2$ (5 mM) and AdoMet (200 μM), with $MgCl_2$, or with AdoMet] were incubated at 40 °C for 15 min, while the control samples were kept on ice. The heated samples were returned to ice for 5 min prior to the measurements of the enzymatic activity (300 μM dopamine was used as a substrate). Thermal stabilities were expressed as heated/control (H/C) ratios. In addition, the thermotropic behavior of both enzyme preparations was examined as a function of incubation time at 37 °C. The enzyme concentrations of the Val-108 S-COMT and the Met-108 S-COMT were 15 and 13 nM, respectively. Thermal behavior of the purified rat recombinant S-COMT (Vidgren et al., 1991) at 40 °C was studied for comparison.

The binding order of magnesium was studied with the following experimental setup. The coenzyme AdoMet and dopamine were held at fixed saturating concentrations of 200 and 600 μM, respectively. Magnesium was the varied substrate (from 50 up to 5000 μM), and *S*-adenosyl-L-homocysteine (AdoHcy) from Sigma was used at increasing fixed concentrations (0, 20, 60, and 150 μM).

Data Analysis. Kinetic data were analyzed by nonlinear fitting to the respective rate equations. As the velocity data varied by more than 1 order of magnitude, weighing by $1/v$ was used. Data analysis was performed using a program

Table 1: Kinetic Parameters for 3-O-Methylation by Human COMT Expressed in Baculovirus-Infected Insect Cells

	V_{max} (min^{-1})	K_m (μM) catechol	V/K_m × 100	K_m (μM) AdoMet	K_s (μM) AdoMet
		Dopamine			
S-COMT	37.2 ± 1.3	207 ± 14	18.0	53.2 ± 4.1	23.0 ± 3.1
MB-COMT	16.9 ± 0.5	15.1 ± 0.9	111.9	37.5 ± 1.9	1.4 ± 0.8
		(−)-Noradrenaline			
S-COMT	34.9 ± 1.4	369 ± 25	9.5	47.2 ± 4.5	28.3 ± 2.9
MB-COMT	18.1 ± 0.2	24.1 ± 0.7	75.1	41.4 ± 1.2	4.1 ± 0.7
		L-Dopa			
S-COMT	42.5 ± 1.5	613 ± 35	6.9	41.2 ± 3.5	27.2 ± 2.3
MB-COMT	12.2 ± 0.2	266 ± 10	4.6	19.7 ± 1.1	3.1 ± 0.8
		DBA			
S-COMT	43.4 ± 2.2	38.9 ± 4.1	118.6	49.0 ± 2.3	21.6 ± 4.7
MB-COMT	22.2 ± 0.4	30.0 ± 1.0	74.0	48.8 ± 1.7	5.1 ± 0.9

Table 2: Kinetic Parameters for 4-O-Methylation by Human COMT Expressed in Baculovirus-Infected Insect Cells

	V_{max} (min^{-1})	K_m (μM) catechol	V/K_m × 100	K_m (μM) AdoMet	K_s(μM) AdoMet
		Dopamine			
S-COMT	8.4 ± 0.3	190 ± 11	4.4	48.4 ± 3.4	24.3 ± 2.9
		L-Dopa			
S-COMT	2.1 ± 0.1	591 ± 33	0.36	40.6 ± 3.4	29.3 ± 2.4
		DBA			
S-COMT	8.9 ± 0.2	35.1 ± 1.4	25.4	47.9 ± 1.9	20.0 ± 1.7
MB-COMT	1.3 ± 0.1	79.9 ± 5.7	1.6	107.6 ± 8.1	14.7 ± 2.5

adapted from the program by R. G. Duggleby (Duggleby, 1984). The program was written in Microsoft Quick Basic (Microsoft Inc.) for Macintosh computers (Apple Computer Inc.).

Molecular Modeling. The modeling experiments were carried out with Insight II software (Biosym Technologies, San Diego, CA). The minimum energy conformations of the substrates were calculated with the Spartan program (Wavefunction Inc., Irvine, CA).

RESULTS

Kinetics of Methylation. Recombinant baculovirus-produced human COMT enzymes were used to study the kinetics of the methylation reactions of dopamine, noradrenaline, L-dopa, and DBA by measuring the initial rate of product formation at varying concentrations of both substrates, the catechol and the coenzyme AdoMet. Kinetic parameters were estimated by nonlinear fitting of the velocity data to

$$v = V_{max}[A][B]/([A][B] + K_{mA}[B] + K_{mB}[A] + K_{sA}K_{mB}) \quad (1)$$

where [A] and [B] are the concentrations of AdoMet and the catechol substrate, respectively, K_{mA} and K_{mB} are the corresponding Michaelis constants, and K_{sA} is the dissociation constant for the enzyme−AdoMet complex. V_{max} values were calculated taking into account the actual enzyme concentration. V_{max} is therefore given in units of $minute^{-1}$ and represents the catalytic number (k_{cat}). The parameter values for 3-O-methylation are shown in Table 1. Adequate data of 4-O-methylation for parameter estimation could only be measured in a few cases. The results are shown in Table 2. An example of the fit is shown in Figure 1.

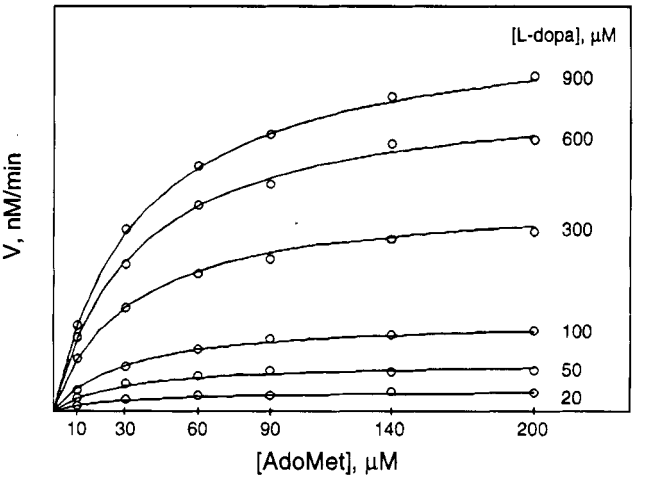

FIGURE 1: Plot of the Michaelis−Menten equation for a sequential bireactant system (eq 1): the varied substrates are L-dopa and AdoMet; a fixed concentration (32 nM) of human S-COMT expressed in baculovirus-infected insect cells is used.

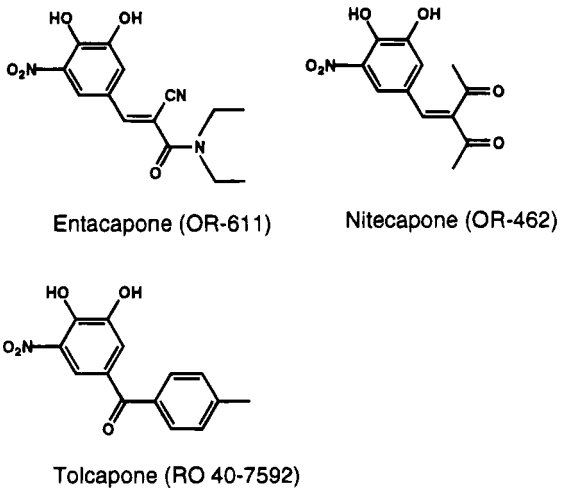

FIGURE 2: Structures of COMT inhibitors.

The actual enzyme concentrations were obtained by measuring the initial reaction rate at varying enzyme concentrations in the presence of varying concentrations of competitive tight-binding inhibitors—entacapone, nitecapone (Bäckström et al., 1989), and tolcapone (Borgulya et al., 1989), shown in Figure 2—and by fitting the data to the following equation (Morrison, 1969, Cha, 1975):

$$v = (k'/2)\{(\epsilon[E] - K'_i - [I]) + [(K'_i + [I] + \epsilon[E])^2 - 4[I]\epsilon[E]]^{1/2}\} \quad (2)$$

where [E] is the total enzyme concentration in arbitrary units and [I] is the total inhibitor concentration. Figure 3 illustrates the type of plot that is obtained when the tight-binding inhibitor nitecapone causes competitive inhibition with respect to dopamine when both dopamine and AdoMet are present at fixed concentrations. The three parameters K'_i, ϵ, and k' were esimated by nonlinear regression. K'_i is a substrate concentration dependent inhibition factor from which K_i can be evaluated in the case of competitive inhibition:

$$K_i = K'_i K_m/(K_m + [B]) \quad (3)$$

ϵ is the molar equivalency allowing the estimation of the

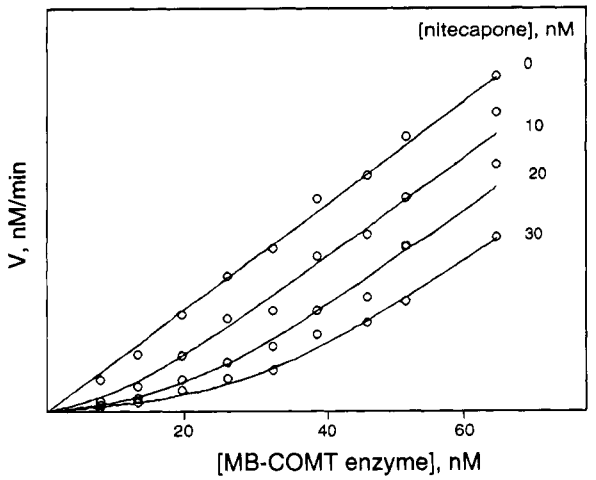

FIGURE 3: Ackermann–Potter plot of the human MB-COMT expressed in baculovirus-infected insect cells. Nitecapone is used with increasing concentrations of 10, 20, and 30 nM as a tight-binding inhibitor.

Table 3: Kinetic Parameters for the Tight-Binding Inhibitors[a]

	entacapone	nitecapone	tolcapone
	S-COMT		
K_i (nM)	0.30	1.02	0.27
k_{cat} (min^{-1})	31.1	26.2	30.9
ϵ	0.975	0.963	1.060
	MB-COMT		
K_i (nM)	2.00	1.37	0.29
k_{cat} (min^{-1})	14.1	17.8	17.4
ϵ	1.171	0.832	0.997

[a] Human COMT was expressed in baculovirus-infected insect cells.

Table 4: Parameters for Substrate Selectivity[a]

	relative V_{max}	relative K_m	relative V/K_m
	S-COMT		
DBA	1	1	1
dopamine	0.9	5.3	0.17
(−)-noradrenaline	0.8	9.5	0.08
L-dopa	1	15.8	0.06
	MB-COMT		
DBA	1	1	1
dopamine	0.8	0.5	1.5
(−)-noradrenaline	0.8	0.8	1
L-dopa	0.6	8.9	0.06

[a] Human COMT was expressed in baculovirus-infected insect cells.

real enzyme concentrations, and k' is a parameter related to the first-order rate constant k_{cat} (catalytic number) for formation of the product from enzyme–substrate complex:

$$k_{cat} = k'(K_m + [B])/[B] \quad (4)$$

Table 3 summarizes K_i, k_{cat}, and ϵ values obtained for nitecapone, entacapone, and tolcapone.

Substrate Selectivity. Relative kinetic parameters were calculated to compare the substrate selectivity in 3-*O*-methylation of the two enzyme forms (Table 4). A reference values of 1 was given to DBA, which was the best substrate on the basis of V_{max} values for both enzyme forms.

Regioselectivity. Relative rate parameters for 3-*O*-methylation over 4-*O*-methylation were calculated by using the K_m and V_{max} values reported in Tables 1 and 2 (Table 5). In the case of MB-COMT, adequate data for fitting to the rate equation were obtained only for DBA as the substrate. Therefore, the relative rate at the highest substrate concentration was calculated as an additional regioselectivity parameter.

Table 5: Regioselectivity Parameters for 3-*O*-Methylation over 4-*O*-Methylation[a]

	relative V_{max}	relative V/K_m	relative rate[b]
	S-COMT		
dopamine	4.4	4.0	4.2
L-dopa	20.2	19.5	15.4
DBA	4.9	4.4	5.0
	MB-COMT		
dopamine			66.3
L-dopa			88.4
DBA	17.1	45.5	21.9

[a] Human COMT was expressed in baculovirus-infected insect cells. [b] Ratio of methylation rates of the highest concentration of both substrates.

Table 6: Kinetic Parameters for Methylation of Dopamine by Human Placental S-COMT

	3-*O*-methylation	4-*O*-methylation
K_m (μM)	141.4 ± 4.6	133.5 ± 5.7
V_{max} (min^{-1})	12.2 ± 0.2	2.8 ± 0.1
$V/K_m \times 100$	8.6	2.1
V_{max} [nmol min^{-1} (g of placenta)$^{-1}$]	0.48 ± 0.01	0.11 ± 0.002

Table 7: Kinetic Parameters for Methylation of Dopamine by Human Val-108 S-COMT and the Mutated Variant Met-108 Expressed in *E. coli*

	V_{max} (min^{-1})	K_m (μM) catechol	V/K_m × 100	K_m (μM) AdoMet	K_s (μM) AdoMet
		3-*O*-Methylation			
Val-108 S-COMT	52.6 ± 1.4	199 ± 11	26.4	33.4 ± 2.5	27.1 ± 3.4
Met-108 S-COMT	34.7 ± 0.7	190.6 ± 9.9	18.2	19.3 ± 1.6	41.7 ± 3.8
		4-*O*-Methylation			
Val-108 S-COMT	12.7 ± 0.3	197.9 ± 12.2	6.4	32.1 ± 2.5	25.8 ± 3.4
Met-108 S-COMT	8.1 ± 0.2	189.2 ± 9.8	4.3	17.7 ± 1.5	39.0 ± 3.6

Kinetic Parameters of Natural COMT. To compare the recombinant proteins with natural enzymes, some kinetic parameters were determined for the soluble COMT isolated from human placenta. In this study, a saturating concentration of AdoMet was used and the dopamine concentration was varied. Data were fitted to the Michaelis–Menten equation by nonlinear regression, allowing the estimation of V_{max} and K_m for dopamine. The enzyme concentration was determined by titration with the inhibitor z-OR-611. The results are shown in Table 6.

Kinetic Parameters and Thermotropic Behavior of Two Structural Variants, Val-108 and Met-108, of Human S-COMT. Kinetic parameters for methylation of dopamine by human Val-108 S-COMT and its mutated variant Met-108 expressed in *E. coli* are presented in Table 7. Catalytic activity of the human Val-108 S-COMT was equal to that of the Met-108 variant (Table 7). The thermotropic behavior of these two structural forms of COMT was studied at two temperatures. The proportion of the enzyme activity remaining after incubation at 37 or 40 °C was expressed as the ratio H/C, that is, the activity remaining after heating, divided by activity in a control aliquot (kept on ice). This ratio was used as a measure of thermal stability. Figure 4 displays the H/C ratio as a function of incubation time at 37 °C for

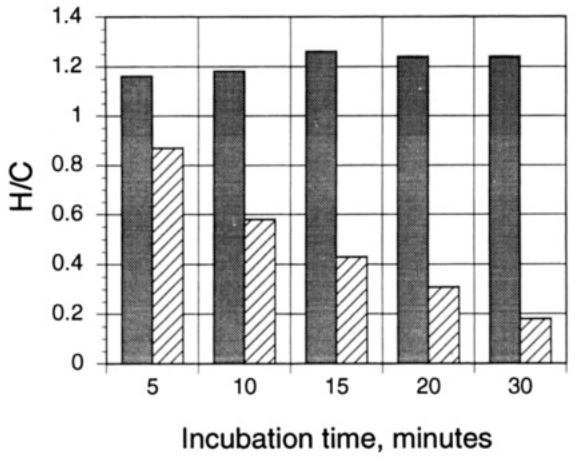

FIGURE 4: Thermotropic behavior (H/C) as a function of time at 37 °C of the human Val-108 S-COMT (shaded bars) and the mutated variant Met-108 (hatched bars) expressed in *E. coli*. Each bar is an average of four independent measurements with a standard deviation less than 0.07.

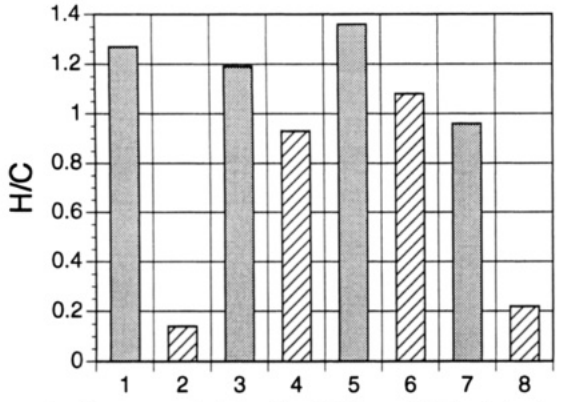

FIGURE 5: Thermotropic behavior (H/C) at 40 °C of the human Val-108 S-COMT and the mutated variant Met-108 expressed in *E. coli*. (1) Incubation of the Val-108 enzyme alone at 40 °C for 15 min; (2) incubation of the Met-108 enzyme alone at 40 °C for 15 min; (3) incubation of the Val-108 enzyme with AdoMet and Mg^{2+} at 40 °C for 15 min; (4) incubation of the Met-108 enzyme with AdoMet and Mg^{2+} at 40 °C for 15 min; (5) incubation of the Val-108 enzyme with AdoMet at 40 °C for 15 min; (6) incubation of the Met-108 enzyme with AdoMet at 40 °C for 15 min; (7) incubation of the Val-108 enzyme with Mg^{2+} at 40 °C for 15 min; (8) incubation of the Met-108 enzyme with Mg^{2+} at 40 °C for 15 min. Each bar is an average of four independent measurements with a standard deviation less than 0.05.

both Val-108 and Met-108 enzyme. The incubation did not affect the activity of human Val-108 S-COMT. However, the mutated variant Met-108 was thermolabile, and the H/C ratio decreased as a function of incubation time. Thermostability of these two S-COMT structures was studied also in an experimental setup in which the coenzyme AdoMet and magnesium were present during the incubation period (see Figure 5). Both structural variants were incubated at 40 °C for 15 min. The data shown in Figure 5 confirm the thermolability of the mutated variant Met-108 of S-COMT. However, when the Met-108 variant was incubated with AdoMet and magnesium or with AdoMet alone, the H/C ratio was equal to unity, i.e., the enzyme was thermally stable. On the other hand, incubation with Mg^{2+} alone did not stabilize the Met-108 variant. Similarly, the H/C ratio for the Met-108 variant remained unchanged as a function of the incubation time when the coenzyme AdoMet was added to the incubation mixture.

The binding order of magnesium and coenzyme AdoMet was also examined. In the experimental setup dopamine and AdoMet were held at fixed concentrations. Magnesium was the varied substrate and AdoHcy was used at increasing fixed concentrations (see Materials and Methods). Nonlinear fitting of the experimental data showed a clear noncompetitive behavior. The data did not fit the uncompetitive model. The interpretation for the uncompetitive model is that AdoHcy is competitive with respect to AdoMet and only binds to Mg−enzyme complex. Here, it is shown that magnesium does not need to bind before AdoHcy (AdoMet). The binding order is discussed below with respect to X-ray data.

In contrast to the known human COMT structures, rat and pig enzymes have Leu in position 108 instead of Val or Met. We also investigated the thermotropic behavior of purified rat liver S-COMT. The results were identical with those of the human COMT where Val is in position 108 (not shown).

DISCUSSION

Kinetic Differences of Soluble and Membrane-Bound COMT. S-COMT and MB-COMT have a similar kinetic mechanism (Männistö et al., 1992). The main difference is the lower K_m value of MB-COMT for dopamine (Rivett & Roth, 1982). The reported K_m values of dopamine for S-COMT and MB-COMT, purified from human brain, were 208 and 3.3 μM, respectively. Recently, it has been shown that recombinant human MB-COMT has a higher affinity for catechol than the soluble form. The determined K_m values were 10 μM for MB-COMT and 108 μM for S-COMT (Malherbe et al., 1992). The V_{max} values reported earlier seem to refer to the enzyme activities in various tissues rather than to basic kinetic constants of the enzyme reaction. Here, we have determined the specific amount of the COMT enzyme used for the assay, which has allowed us to determine the kinetic parameters of COMT more accurately. In the present study the catalytic number (V_{max}) of S-COMT was somewhat higher than that of MB-COMT in case of all four substrates (Tables 1 and 2). At saturating substrate levels S-COMT seems to work approximately 2 times more efficiently. Under these conditions the catalytic number for both enzyme forms was similar for all the substrates. This means that at saturating catechol concentrations neither enzyme form shows selectivity with respect to these substrates. The K_m and V/K_m values, however, varied considerably, depending on both substrate and the enzyme form. S-COMT showed about 15 times higher K_m and 5 times lower V/K_m values for catecholamines than MB-COMT. Consequently, at low concentrations catecholamines should be methylated more rapidly by MB-COMT. For the other two substrates, DBA and L-dopa, the differences were small. In the case of DBA the K_m values were practically equal for both S- and MB-COMT.

The distinctive difference between the enzyme forms was the higher affinity of AdoMet for MB-COMT. The average dissociation constants K_s were 3.4 and 20.2 μM for MB-COMT and S-COMT, respectively. K_m for AdoMet, on the other hand, was similar for both enzyme forms and for all substrates, as seen in Tables 1 and 2.

Only one of the catechol hydroxyls is methylated by COMT. Dimethylation has not been observed. It is well-known that, in the case of catecholamines, both enzyme

forms favor 3-*O*-methylation. However, MB-COMT is clearly more regioselective (Table 5).

To compare the recombinant proteins with natural enzymes, some kinetic parameters were determined for the soluble COMT isolated from human placenta. Kinetic parameters for methylation of dopamine in the case of natural human placental S-COMT differ only slightly from the parameter values determined for the recombinant human enzyme.

Comparison of the Kinetic Results with the Atomic Structure of COMT. (A) Binding Differences of the Substrates. The recently solved crystal structure of rat S-COMT (Vidgren et al., 1994) allows us to explain the observed kinetic behavior of different substrates. The active site of COMT is a shallow groove on the surface of the enzyme. The atomic structure and the sequence comparison reveal that all residues which are important for the binding of the substrates and for the catalytic reaction are conserved in human and rat proteins. Modeling of three substrates (DBA, dopamine, and L-dopa), having different binding affinities to the active site of COMT indicates that the binding of the catechol ring is identical to the inhibitor binding in the published crystal structure. The different affinities are probably caused by interactions of the substrate side chain with the enzyme residues (Figure 6). DBA has a charged carboxyl moiety. However, this structure is planar and fits well between the "gatekeeper" residues Trp 38 and Pro 174. The side chain of dopamine with its positively charged amino group has a rotational freedom, and here we evaluated only qualitatively the interactions of one minimum-energy conformation of dopamine with COMT. It can be concluded that the positively charged side chain of dopamine may have repulsive contacts with either Trp 38 or Pro 174. The last substrate, L-dopa, has the largest side chain with two charged functionalities and a moderate conformationl freedom. At least in one of its miminum-energy conformations, L-dopa has strong repulsions with the above residues and also with the hydrophobic pocket adjacent to the active site. These limited comparative modeling experiments suggest that the kinetic behavior with different substrates can be explained on a structural basis.

(B) Meta/para Methylation. The structural basis discussed above for the substrate selectivity is also applicable to the regioselectivity of the *meta/para* methylation of the substrates. Modeling reveals that, with all investigated substrates, binding with the *p*-hydroxyl group toward the methyl-donating AdoMet causes there side chains to orientate more unfavorably with the hydrophobic protein residues. The amino acid side chain of L-dopa with two charged groups, which are orientated into a hydrophobic pocket, has the strongest repulsive interactions with the COMT residues (Figure 6). This is clearly reflected in the kinetic parameters for L-dopa.

(C) Differences between S- and MB-COMT. The catalytic sites of S-COMT and MB-COMT, based on the identical sequences and the crystal structure of S-COMT, are naturally identical, but MB-COMT has a membrane-spanning extension in the amino terminus. However, the kinetic differences in the binding of the investigated substrates to the two enzyme forms are very clear. The adjacent membrane with a charged structure or an additional structural part in the amino end of MB-COMT contributes significantly to the higher binding affinity of the substrates. Thus it is probable

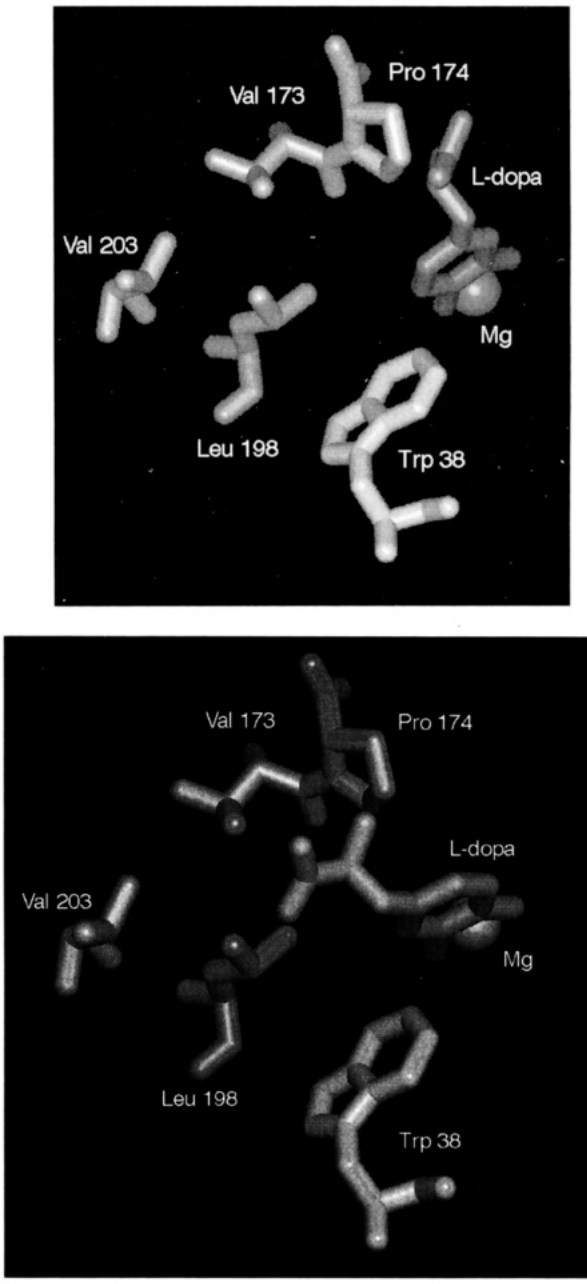

FIGURE 6: Binding of L-dopa to COMT. Protein residues contributing to the substrate selectivity are shown. (Top) Orientation of the substrate corresponds to the methylation at the *m*-hydroxyl group. (Bottom) L-Dopa positioned for the methylation at the *p*-hydroxyl group.

that there is no conformational change in the basic enzyme structure of MB-COMT. In the case of an extended charged side chain of the substrate, the membrane anchor region of COMT (a possible helix) or the membrane itself causes the more favorable binding interactions.

COMT Variants with Different Thermostability. Analysis of COMT activities from red blood cells (Scanlon et al., 1979) and other human tissues (Weinshilboum, 1978; Sladek-Chelgren & Weinshilboum, 1981; Boudikova et al., 1990) has shown that a common polymorphism of two codominant alleles of the COMT gene determines the level and thermostability of COMT activity. Different human populations seem to have different frequencies of the $COMT^{L}$ and $COMT^{H}$ alleles (Rivera-Calimlim & Reilly, 1984), and it has been suggested that the variation in individual response to L-dopa therapy is genetically determined by the COMT

alleles (Reilly et al., 1980; Rivera-Calimlim & Reilly, 1984; Klemetsdal et al., 1994). We compared the two published human COMT cDNA sequences and found that they differed in the position of S-COMT amino acid 108, the residue being either Val (Lundström et al., 1991) or Met (Bertocci et al., 1991). As Grossman et al., (1992a) had reported that Val-108 and Met-108 would correlate with the high- and low-activity phenotypes of COMT, we decided to express the two enzyme forms in *E. coli* and compare their kinetic properties and thermostability. Interestingly, the catalytic activities of the two enzyme forms were similar, but the Met-108 enzyme was more thermolabile already at physiological temperature (37 °C). However, the enzyme was fully stabilized by bound AdoMet. Mg^{2+} alone did not influence the thermal stability. Thus the activity of the thermolabile COMT form may be physiologically controlled by the availability of AdoMet. Our results also imply that the high- and low-activity COMT polymorphism may actually result from the different thermostabilities of the two enzyme forms and not from their kinetic properties. This confirms that residue 108 does not contribute to the active site of the S-COMT enzyme. Residue Val (Met) 108 is located about 15−20 Å from the binding site of AdoMet on the opposite site of the COMT molecule. However, between residue 108 and the binding groove for AdoMet there is a direct connection along an α-helix and a β-strand. It is also possible that the COMT structure has a different conformation before AdoMet binds (Vidgren et al., 1994). The Val/Met variance probably alters the physicochemical properties of the protein.

Two different inbred rat strains have different genetically determined COMT activity levels (Weinshilboum et al., 1979), suggesting that also other mammals may carry polymorphic COMT alleles. The rat and pig S-COMT sequences have Leu in the amino acid position 108 (Salminen et al., 1990, Malherbe et al., 1992), showing that the human Val/Met variation is not conserved. We found that the thermostability of the *E. coli*-expressed rat enzyme was identical with the human Val-108 enzyme. As it is unlikely that rat COMT would have variation in thermostability (Weinshilboum et al., 1979), it is possible that rat COMT polymorphism has a different structural basis from human COMT.

Implications for the Kinetic Mechanism. The results described here give evidence for the binding order of ligands. First, our kinetic experiments show that magnesium does not need to bind before AdoMet. Second, AdoMet is binding alone without magnesium into the Met variant of COMT and is stabilizing it. Finally, crystallographic studies on COMT reveal that the bound AdoMet has no interactions with Mg^{2+}. The binding pocket for the methionine part of AdoMet is deeper in the protein behind the Mg^{2+} binding site and it would be impossible for AdoMet to bind after magnesium (Vidgren et al., 1994). These results suggest that the coenzyme AdoMet has to bind first, then magnesium, and as the last ligand the catechol substrate (Figure 7). This kinetic reaction cycle differs from that of earlier studies (Jeffery & Roth, 1987) in which it was suggested that magnesium ions bind to both MB-COMT and S-COMT in a rapid equilibrium prior to the addition of AdoMet.

Physiological Importance of Soluble and Membrane-Bound Enzymes. The kinetic observations of the present study with recombinant COMT enzymes confirmed the suggestion that under physiological conditions MB-COMT would have an important role in the methylation of catecholamines in man (Roth, 1992). Further, the role of MB-COMT may be more important in man than, for example, in rat because its relative amount, compared with S-COMT, is significantly higher in human than in rat tissues (Tenhunen & Ulmanen, 1993; Tenhunen et al., 1994). Recent subcellular localization studies have shown that MB-COMT occurs intracellularly, on the rough endoplasmic reticulum, whereas S-COMT is mainly cytoplasmic and to some extent also nuclear (Peränen et al., personal communications). This distribution of COMT enzymes in several cellular compartments suggests that the contribution of S- and MB-COMT to the methylation of different substrates may depend not only on the kinetic properties of the enzymes but also on the intracellular distribution of the substrates.

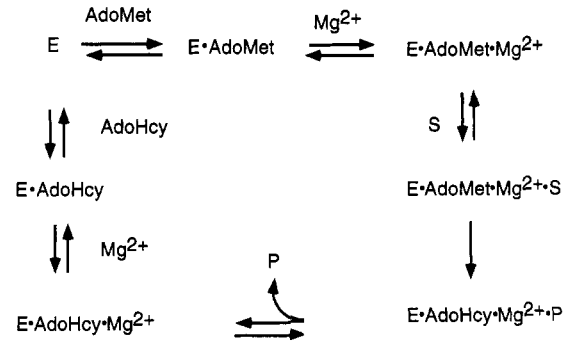

FIGURE 7: Proposed kinetic reaction mechanism of COMT (E = enzyme; S = catechol substrate; P = methylated catechol product).

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REFERENCES

Assicot, M., & Bohuon, C. (1971) *Biochimie 53*, 871−874.
Axelrod, J., & Tomchick, R. (1958) *J. Biol. Chem. 233*, 702−705.
Bäckström, R., Honkanen, E., Pippuri, A., Kairisalo, P., Pystynen, J., Heinola, K., Nissinen, E., Linden, I.-B., Männistö, P. T., & Kaakkola, S. (1989) *J. Med. Chem. 32*, 841−846.
Ball, P., Knuppen, R., Haupt, M., & Breuer, H. (1972) *J. Clin. Endocrinol. 34*, 736−746.
Bertocci, B., Miggiano, V., Da Prada, M., Dembic, Z., Lahm, H.-W., & Malherbe, P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1416−1420.
Boadi, W. Y., Urbach, J., Barnea, E. R., Brandes, J. M., & Yannai, S. (1991) *Pharmacol. Toxicol. 68*, 317−321.
Borchardt, R. T. (1973) *J. Med. Chem. 16*, 387−391.
Borchardt, R. T. (1980) in *N- and O-Methylation. Enzymatic Basis of Detoxification*, pp 43−62, Academic Press, New York.
Borchardt, R. T., Cheng, C. F., & Cooke, P. H. (1974) *Life Sci. 14*, 1089−1100.
Borgulya, J., Bruderer, H., Bernauer, K., Zurcher, G., & Da Prada, M. (1989) *Helv. Chim. Acta 72*, 952−968.
Boudikova, B., Szumlanski, C., Maidak, B., & Weinshilboum, R. (1990) *Clin. Pharmacol. Ther. 48*, 381−389.
Cha, S. (1975) *Biochem. Pharmacol. 24*, 2177−2185.
Coward, J., Slixz, E., & Wu, F. (1973) *Biochemistry 12*, 2291−2297.
Creveling, C., Dalgard, N., Shimizu, H., & Daly, J. (1970) *Mol. Pharmacol. 6*, 691−696.
Creveling, C. R., Morris, N., Shimizu, H., Ong, H. H., & Daly, J. (1972) *Mol. Pharmacol. 8*, 398−409.
Duggleby, R. (1984) *Comput. Biol. Med. 14*, 447−455.
Flohe, L. (1974) *Int. Pharmacopsychiat. 9*, 52−60.

Flohe, L., & Schwabe, K. (1970) *Biochim. Biophys. Acta 220,* 469−476.
Flohe, L., & Schwabe, K.-P. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353,* 463−475.
Grossman, M. H., Littrell, J. B., Weinstein, R., Szumlanski, C. L., & Weinshilboum, R. M. (1992a) *Soc. Neurosci. 18,* 70.
Grossman, M. H., Szumlanski, C., Littrell, J. B., Weinstein, R., & Weinshilboum, R. M. (1992b) *Life Sci. 50,* 473−480.
Guldberg, H., & Marsden, C. (1975) *Pharmacol. Rev. 27,* 135−206.
Jeffery, D. R., & Roth, J. A. (1985) *J. Neurochem. 44,* 881−885.
Jeffery, D., & Roth, J. (1987) *Biochemistry 26,* 2955−2958.
Karhunen, T., Tilgmann, C., Ulmanen, I., Julkunen, I., & Panula, P. (1994) *J. Histochem. Cytochem. 42,* 1079−1090.
Klemetsdal, B., Straume, B., Giverhaug, T., & Aarbakke, J. (1994) *Eur. J. Pharmacol. 46,* 231−235.
Laemmli, U. K. (1970) *Nature 227,* 680−685.
Lundström, K., Salminen, M., Jalanko, A., Savolainen, R., & Ulmanen, I. (1991) *DNA Cell Biol. 10,* 181−189.
Lundström, K., Tilgmann, C., Peränen, J., Kalkkinen, N., & Ulmanen, I. (1992) *Biochim. Biophys. Acta 1129,* 149−154.
Malherbe, P., Bertocci, B., Caspers, P., Zürcher, G., & Da Prada, M. (1992) *J. Neurochem. 58,* 1782−1789.
Männistö, P., & Kaakkola, S. (1990) *Pharmacol. Toxicol. 10,* 54−56.
Männistö, P. T., Ulmanen, I., Lundström, K., Taskinen, J., Tenhunen, J., Tilgmann, C., & Kaakkola, S. (1992) in *Progress in Drug Research* (Junker, E., Ed.) pp 291−350, Birkhäuser Verlag, Basel, Switzerland.
Morrison, J. F. (1969) *Biochim. Biophys. Acta 185,* 269−286.
Mullis, K. B., & Faloona, F. A. (1987) *Methods Enzymol. 155,* 335−350.
Nissinen, E. (1984) *Biochem. Pharmacol. 33,* 3105−3108.
Nissinen, E., & Männistö, P. (1984) *Anal. Biochem. 137,* 69−73.
Reilly, D. K., Rivera-Calimlim, L., & Van Dyke, D. (1980) *Clin. Pharmacol. Ther. 28,* 278−286.
Rivera-Calimlim, L., & Reilly, D. K. (1984) *Clin. Pharmacol. Ther. 35,* 804−809.
Rivett, A., & Roth, J. (1982) *Biochemistry 21,* 1740−1742.
Rivett, A. J., Francis, A., & Roth, J. A. (1983) *J. Neurochem. 40,* 1494−1496.
Roth, J. A. (1992) *Rev. Physiol. Biochem. Pharmacol. 120,* 1−29.
Salminen, M., Lundström, K., Tilgmann, C., Savolainen, R., Kalkkinen, N., & Ulmanen, I. (1990) *Gene 93,* 241−247.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 5463−5467.
Scanlon, P. D., Raymond, F. A., & Weinshilboum, R. M. (1979) *Science 203,* 63−65.
Schultz, E., & Nissinen, E. (1989) *Biochem. Pharmacol. 38,* 3953−3956.
Senoh, S., Tokuyama, Y., & Witkop, B. (1962) *J. Am. Chem. Soc. 84,* 1719−1724.
Sladek-Chelgren, S., & Weinshilboum, R. M. (1981) *Biochem. Genet. 19,* 1037−1053.
Tenhunen, J., & Ulmanen, I. (1993) *Biochem. J. 296,* 595−600.
Tenhunen, J., Salminen, M., Jalanko, A., Ukkonen, S., & Ulmanen, I. (1993) *DNA Cell Biol. 12,* 253−263.
Tenhunen, J., Salminen, M., Lundström, K., Kiviluoto, T., Savolainen, R., & Ulmanen, I. (1994) *Eur. J. Biochem. 223,* 1049−1054.
Thakker, D., Boehlert, C., Kirk, K., Antkowiak, R., & Creveling, C. (1986) *J. Biol. Chem. 261,* 178−184.
Tilgmann, C., Melen, K., Lundström, K., Jalanko, A., Julkunen, I., Kalkkinen, N., & Ulmanen, I. (1992) *Eur. J. Biochem. 207,* 813−821.
Tunnicliff, G., & Ngo, T. (1983) *Int. J. Biochem. 15,* 733−738.
Vidgren, J., Tilgmann, C., Lundström, K., & Liljas, A. (1991) *Proteins: Struct., Funct., Genet. 11,* 233−236.
Vidgren, J., Svensson, L. A., & Liljas, A. (1994) *Nature 368,* 354−358.
Weinshilboum, R. M. (1978) *Life Sci. 22,* 625−630.
Weinshilboum, R. M., Raymond, F. A., & Frohnauer, M. (1979) *Biochem. Pharmacol. 28,* 1239−1247.
Woodard, R., Tsai, M., Floss, H., Crooks, P., & Coward, J. (1980) *J. Biol. Chem. 255,* 9124−9127.

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