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Kinetic Reaction Mechanism for Magnesium Binding to Membrane-Bound and Soluble Catechol *O*-Methyltransferase†

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ABSTRACT: Catechol *O*-methyltransferase (COMT, EC 2.1.1.6) from human brain occurs in both a membrane-bound (MB-COMT) and a soluble form (SOL-COMT). While these enzymes appear to be distinct molecular entities, both catalyze the O-methylation of catecholamines through an ordered reaction mechanism in which *S*-adenosylmethionine (SAM) is the leading substrate [Rivett, A. J., & Roth, J. A. (1982) *Biochemistry 21*, 1740–1742; Jeffery, D. R., & Roth, J. A. (1985) *J. Neurochem. 44*, 881–885]. Both MB-COMT and SOL-COMT require the presence of divalent cations for catalytic activity. This series of experiments provides evidence indicating that magnesium ions bind to both MB-COMT and SOL-COMT in a rapid equilibrium sequence prior to the addition of SAM. An equation is presented that predicts the qualitative results obtained in all kinetic experiments carried out with either MB-COMT or SOL-COMT.

Catechol *O*-methyltransferase (COMT;[1] EC 2.1.1.6) catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to a ring hydroxyl group on catecholamine neurotransmitters or other xenobiotic catechols. The presence of divalent cations is essential for enzymatic activity, and magnesium ions are believed to be the physiological cofactor (Axelrod & Tomchick, 1958). While the molecular and kinetic mechanisms of the methyltransfer reaction have been studied in some detail (Hegazi et al., 1976, 1979; Rivett & Roth, 1982; Jeffrey & Roth, 1985), the role of magnesium ions in the reaction has received little attention.

Senoh et al. (1962) studied the effects of various divalent cations on the base- and COMT-catalyzed O-methylation of catecholamines and found that the total yield of O-methylated products was dependent upon the nature of the cation. Magnesium appeared to stimulate activity to the greatest extent whereas copper was the least effective cation. The order of effectiveness in promoting O-methylation was the same for the enzymatic and nonenzymatic, base-catalyzed reaction. The authors suggested that magnesium ions form a bridge between a catechol hydroxyl group and *S*-adenosylmethionine and act to align the methyl group from SAM with a ring hydroxyl group. In agreement with the conclusions reached by Senoh et al. (1962), Belleau and Burba (1963) provided evidence suggesting that tropolones (dead-end inhibitors of COMT)

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[1] Abbreviations: COMT, catechol *O*-methyltransferase; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; MB, membrane bound; SOL, soluble; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DA, dopamine.

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form a 1:1 complex between COMT and magnesium ions within the active site of the enzyme.

It should be pointed out, however, that certain divalent cations not only fail to support enzymatic O-methylation but inhibit the methyltransferase reaction even in the presence of saturating concentrations of magnesium. Quiram and Weinshilboum (1976a, b) have reported that calcium, lanthanum, europium, and neodinium are noncompetitive inhibitors of COMT when either SAM, dihydroxybenzoic acid, or magnesium is used as the varied ligand.

Human brain COMT occurs in both a soluble (SOL) and a membrane-bound (MB) form (Roth, 1980; Jeffery & Roth, 1984). Although, the MB transferase is similar to the soluble form of COMT in a variety of respects, recent evidence suggests they may be structurally distinct proteins (Grossman et al., 1985; Heydorn et al., 1986). The MB transferase is an integral membrane protein and primarily exists in neurons in the CNS whereas the soluble form of COMT is a cytosolic species and exists primarily in glial cells (Rivett et al., 1983). In addition, while the MB enzyme exhibits a K_m value for catechol substrates almost 100-fold lower than that of the soluble enzyme (3.3 and 280 μM, respectively), both SOL-COMT and MB-COMT catalyze the O-methylation of catecholamines through a sequential ordered reaction mechanism in which SAM is the leading substrate (Rivett & Roth, 1982; Jeffery & Roth, 1985).

In the present series of experiments, the kinetic reaction mechanism of magnesium binding to human MB- and SOL-COMT was studied. The results obtained are consistent with the rapid equilibrium ordered addition of magnesium ions prior to the addition of SAM and are the same for both MB-COMT and SOL-COMT.

Materials and Methods

Materials. (3,4-Dihydroxyphenyl)[2-^{3}H]ethylamine ([^{3}H]dopamine, 24–36 Ci/mmol) and formula 963 liquid scintillation cocktail were obtained from New England Nuclear, Boston, MA. Labeled dopamine was purified by cation-exchange chromatography over a column of Bio-Rex 70 as previously described (Rivett et al., 1982). Dopamine hydrochloride, *S*-adenosyl-L-methionine (chloride from 85% pure), pargyline hydrochloride, and dithothreitol were obtained from Sigma Chemical Co., St. Louis, MO. All other reagents were of the highest commercial grades available.

Preparation of Purified Soluble COMT. Studies of the kinetic mechanism of magnesium binding to SOL-COMT were carried with an enzyme preparation that had been purified approximately 800-fold from the high-speed supernatant solution as described previously (Jeffery & Roth, 1984). Briefly, SOL-COMT activity was purified from the high-speed supernatant by ammonium sulfate fractionation, hydroxyapatite absorption chromatography, ion-exchange chromatography on DEAE-cellulose, and gel filtration on Sephadex G-75 superfine. The resulting enzyme preparation showed approximately ten bands on SDS gel electrophoresis when proteins were visualized by silver staining. Since blank values determined in the absence of SAM were equivalent to that obtained in the absence of magnesium, it may be concluded that the concentration of magnesium in the purified enzyme preparation was negligible.

Preparation of Membrane-Bound COMT. More than 30 samples of human frontal cortex were obtained within 12 h after death at autopsy. Samples were homogenized in 5 volumes (w/v) of an ice-cold buffer containing 100 mM potassium buffer, pH 7.4, and 5 mM β-mercaptoethanol, and the microsomal fraction was isolated by differential centrifugation as previously described (Jeffery & Roth, 1984). The 250000*g* pellet was resuspended in 100–150 mL of 10 mM potassium phosphate buffer, pH 7.4, containing 5 mM β-mercaptoethanol and centrifuged at 25000*g* for 30 min. This procedure was repeated until the supernatant solution following centrifugation was clear (about five washes). The pellet was then resuspended in the same buffer and dialyzed for 12–15 h against 1600 volumes of a buffer containing 10 mM potassium phosphate, pH 7.4, 5 mM β-mercaptoethanol, 1 mM EDTA, and 1 mM EGTA. The membrane suspension was then dialyzed against an addition 1600 volumes of the same buffer without EDTA or EGTA. Following dialysis the preparation of MB-COMT was centrifuged again at 25000*g* for 30 min and resuspended in the same buffer in the absence of chelating agents. All procedures were carried out at 4 °C. It should be noted that COMT from both human and animal brains has been shown to exhibit only negligible losses in activity during postmortem storage (Grote et al., 1974). Moreover, since blank values obtained in the absence of SAM or magnesium were equivalent, the concentration of magnesium in this preparation had no effect on COMT activity.

COMT Assay Procedures. The activity of COMT was measured as previously described with [^{3}H]dopamine as the labeled substrate (Rivett et al., 1983). Briefly, the reaction mixture contained 50 mM potassium phosphate buffer, pH 7.4, 10 mM dithiothreitol, 1 mM pargyline (to inhibit monoamine oxidase in assays of MB-COMT), and varying concentrations of SAM, $MgCl_2$, and dopamine as indicated in the figure legends. Assays were carried out at 37 °C for 30 min (MB-COMT) or 45 min (SOL-COMT) in a final volume of 0.4 mL and stopped by the addition of 0.8 mL of 0.5 M borate buffer, pH 10.0. Products were extracted into toluene–isoamyl alcohol (3:2 v/v), and 1-mL aliquots were transferred to scintillation vials for measurement of radioactivity. Reaction velocity was linear with time and protein concentration throughout the duration of the assay.

Results

Since both MB-COMT and SOL-COMT catalyze the methyltransferase reaction through an ordered mechanism in which SAM is the leading substrate (Rivett & Roth, 1982; Jeffery & Roth, 1985), the order of magnesium binding was determined by holding one ligand constant at a saturating concentation while varying the concentration of the other two ligands. In the first experiment the concentration of SAM (0.1 mM) was held constant while magnesium was used as the varied ligand and dopamine was used at increasing fixed concentrations. For a simple steady-state kinetic system, the binding of magnesium prior to the addition of SAM would be evidenced by the absence of a slope effect on double-reciprocal plots. However, the data generated for MB-COMT revealed that the individual lines intersected to the left of the $1/v$ axis, suggesting that magnesium may not bind prior to the addition of SAM. Similarly, when the same data were plotted with dopamine as the varied ligand, the lines also intersected to the left of the $1/v$ axis. In addition, comparable results were obtained when the same experiment was carried out with the purified soluble form of COMT.

When the alternative initial velocity experiments were performed with SAM as the varied substrate and magnesium as the increasing fixed ligand while the concentration of dopamine was held constant, the series of lines intersected on the $1/v$ axis in the double-reciprocal plots. These results are diagnostic for rapid equilibrium ordered mechanisms in which the increasing fixed ligand is the leading ligand (Segel, 1975; Morrison & Ebner, 1970). Identical results were obtained with

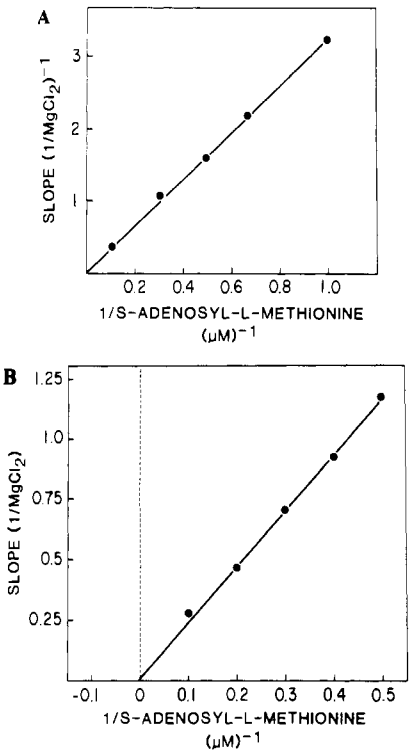


FIGURE 1: Slope replot of initial velocity of MB-COMT (A) and SOL-COMT (B). The data shown were obtained from the slopes of lines of double-reciprocal plots in which $MgCl_2$ was varied ligand and *S*-adenosylmethionine was the fixed ligand.

Table I: Kinetic Constants for MB-COMT and SOL-COMT

kinetic constant	MB-COMT	SOL-COMT
$K_m(Mg^{2+})$ (mM)	1.6	2.1
K_m(SAM) (μM)	3.9	9.0
K_m(DA) (μM)	3.3	280
K_I(SAH) (μM)	3.0	3.0

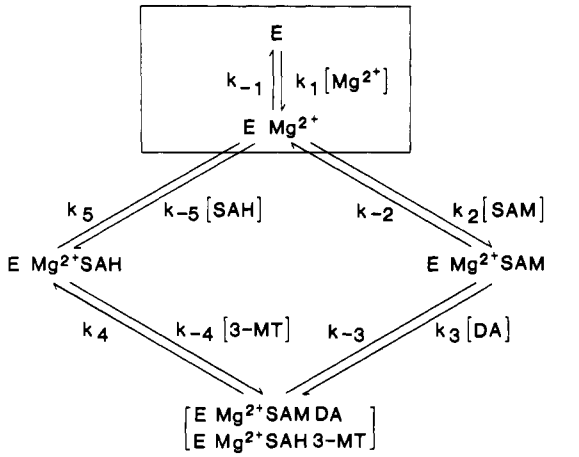
the purified soluble form of COMT, suggesting that a similar mechanism is operative for this enzyme.

When the data decribed in the preceding paragraph were plotted with magnesium as the varied ligand and SAM as the increasing fixed substrate, a series of lines that intersected to the left of the $1/v$ axis was obtained. As above, similar results were obtained with the soluble form of COMT. If magnesium binding to soluble and membrane-bound COMT takes placed in a rapid equilibrium sequence, replots of the slopes of these lines when plotted against the reciprocal of the SAM concentration should intersect through the origin (Segel, 1975). As illustrated in panels A and B of Figure 1 for MB-COMT and SOL-COMT, respectively, the slope replots indeed intersect the origin, providing further evidence that magnesium binds to MB- and SOL-COMT in a rapid equilibrium sequence prior to the addition of SAM.

Consistent with this mechanism are the patterns of inhibition obtained with SAH (a mixed product and dead-end inhibitor). In this case, SAH showed a noncompetitive pattern of inhibition for MB-COMT when magnesium was the varied substrate (data not shown). Similar results were also observed for SOL-COMT. The kinetic constants for the reaction caculated from these experiments are presented in Table I.

Discussion

As mentioned earlier, few investigations have been concerned with a role of divalent cations in promoting the methyltransfer reaction catalyzed by COMT. A a result, both the mechanism by which divalent cations promote the reaction



$$\frac{V}{VMAX} = \frac{[A][B][C]}{Kia\ Kib\ Kmc + Kib\ Kmc\,[A] + Kia\ Kmb\,[C] + Kmc\,[A][B] + Kmb\,[A][C] + [A][B][C]}$$

$$E = Kia\ Kmb\,[C] + Kia\ Kib\ Kmc$$
$$EA = Kmb\,[A][C] + Kia\ Kmc\,[A]$$
$$EAB = Kmc\,[A][B]$$

FIGURE 2: Proposed kinetic reaction mechanism for MB-COMT and SOL-COMT. Magnesium binds to the free enzyme in an ordered rapid equilibrium sequence prior to the addition of SAM. Once magnesium is bound, SAM and dopamine are bound in an ordered sequence and methyltransfer takes place. Methoxytyramine is released from the enzyme prior to the release SAH, which regenerates the enzyme–magnesium complex. Magnesium may dissociate to leave the free enzyme or the enzyme–magnesium complex may bind another molecule of SAM to initiate the next catalytic cycle.

and their kinetic relationship to SAM and dopamine are poorly understood. In the present series of studies the kinetic relationship of magnesium binding to MB-COMT and SOL-COMT have been examined. The results are characteristic of a rapid equilibrium reaction mechanism (Morrison & Ebner, 1970) in which magnesium binds to COMT in a rapid equilibrium sequence prior to the addition of SAM as illustrted in Figure 2.

Following the release of 3-methoxytyramine and *S*-adenosyl-L-homocysteine (SAH), the enzyme–magnesium complex is regenerated. The subsequent release and rebinding of magnesium take place faster than the actual conversion of substrates to products, and the proportion of the total enzyme with magnesium bound to it will depend on the concentration of SAM and dopamine. The equation for this reaction is presented in Figure 2.

Although the soluble and membrane-bound forms of COMT are localized in specific cell types in the central nervous system, it is interesting to find, as revealed in this paper, that the kinetic mechanism of both reactions is similar. This is not totally surprising in light of the evidence demonstrating that many of the other bochemical properties of the enzyme are comparable. The primary difference between the two enzyme species is the apparent higher affinity of the acceptor substrates for MB-COMT.

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CORRECTION

Magnitude and Direction of the Change in Dipole Moment Associated with Excitation of the Primary Electron Donor in *Rhodopseudomonas sphaeroides* Reaction Centers, by David J. Lockhart and Steven G. Boxer*, Volume 26, Number 3, February 10, 1987, pages 664–668.

In the analysis of the dependence of the ratio $\Delta A(\chi)/\Delta A(\chi = 90°)$ on χ (Figure 1), we neglected to correct for the increase in path length of the probing light as χ is decreased [multiplication of $\Delta A(\chi)/\Delta A(\chi = 90°)$ by cos $(90 - \chi)$]. The following corrected values are obtained: Q_y transition of the special pair, $\zeta = 40 \pm 2°$ and $|\Delta\mu| = f^{-1}(9.3 \pm 0.7)$ D; Q_y transition of bacteriochlorophyll *a* and bacteriopheophytin *a* in polystyrene, $\zeta = 17 \pm 3°$ and $|\Delta\mu| = f^{-1}(2–2.5)$ D. These corrections do not change the central conclusions of the paper.