Kinetic Studies on the O-Methylation of Dopamine by Human Brain Membrane-Bound Catechol O-Methyltransferase†

A. Jennifer Rivett* and Jerome A. Roth

ABSTRACT: K_m values for dopamine and S-adenosylmethionine (AdoMet) of human brain membrane-bound catechol O-methyltransferase are 3.3 μM and 3.1 μM, respectively. S-Adenosylhomocysteine is a very potent competitive inhibitor with respect to AdoMet with a K_i value of 1 μM. Product inhibition patterns strongly support a steady-state compulsory-order ternary complex mechanism in which AdoMet binds to the enzyme before dopamine. Inhibition of membrane-bound COMT by tropolone is competitive with respect to dopamine (K_i = 5 μM) and uncompetitive with respect to AdoMet and is consistent with this type of mechanism. The mechanism proposed is different from that suggested for soluble catechol O-methyltransferases.

Two forms of catechol O-methyltransferase (COMT), a soluble and a membrane-bound species, have been demonstrated in a variety of animal tissues (Assicot & Bohuon, 1971; Creveling et al., 1972; Borchardt et al., 1974; Tong & D'Iorio, 1977; Wrenn et al., 1979). Membrane-bound COMT generally has much lower K_m values for catechol substrates than the soluble enzyme. Both forms of COMT are present in human brain (Roth, 1980), and recent studies (Roth et al., 1981) have suggested that the membrane-bound form of the enzyme may contribute significantly to the O-methylation of low concentrations of the catecholamine neurotransmitters dopamine and norepinephrine.

There are conflicting reports in the literature concerning the reaction mechanism of soluble COMT. Several kinetic studies with the enzyme isolated from rat liver have suggested that this form of COMT follows a rapid-equilibrium random-order mechanism (Flohe & Schwabe, 1970; Coward et al., 1973). However, Borchardt (1973) suggested a ping-pong mechanism from studies with dead-end inhibitors. Relatively little work has been carried out on the kinetics of the membrane-bound form of the enzyme.

In this study product and dead-end inhibition patterns have been determined in order to establish the reaction mechanism for membrane-bound COMT from human brain.

Experimental Procedures

Materials. 2-(3,4-Dihydroxyphenyl)[2-^{3}H(N)]ethylamine and Formula 963 were purchased from New England Nuclear, Boston, MA. Dopamine hydrochloride, S-adenosyl-L-methionine chloride, S-adenosyl-L-homocysteine, and pargyline hydrochloride were obtained from Sigma Chemical Co., St. Louis, MO. 3-O-Methyldopamine hydrochloride, 4-O-methyldopamine hydrochloride, and tropolone were from Aldrich Chemical Co., Inc., Milwaukee, WI. Labeled dopamine was purified over a column of Bio-Rex 70 (Bio-Rad Laboratories, Richmond, CA) equilibrated in 0.4 M sodium phosphate buffer, pH 6. Elution from the column was carried out with 0.1 M HCl.

Thawed samples of frozen human brain were homogenized in a Waring Blendor (4 × 30 s) in 5 volumes of 10 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM dithiothreitol. The homogenate was centrifuged at 600*g* for 10 min, and the supernatant was centrifuged at 15000*g* for 20 min. The resulting supernatant fraction was centrifuged at 245000*g* for 60 min. The pellet was resuspended in 10 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM dithiothreitol and centrifuged at 100000*g* for 60 min. The resulting pellet was resuspended in the same buffer and recentrifuged at 100000*g*. The final pellet was resuspended in 10 mM potassium phosphate, pH 7.4, and used for assays of membrane-bound COMT. All operations were carried out at 0–4 °C.

COMT activity was assayed by a modification of the method of Wrenn et al. (1979) using labeled dopamine. Reactions were carried out in 0.5 mL of 50 mM potassium phosphate buffer, pH 7.4, containing 2.5 mM $MgCl_2$, 1 mM pargyline, and varing concentrations of dopamine (DA)[1] and S-adenosylmethionine (AdoMet). Incubations were carried out in the dark for 30 min at 37 °C and were terminated by the addition of 1 mL of 0.5 M potassium borate, pH 10. The O-methylated products were extracted into 5 mL of toluene–isoamyl alcohol (3:2 v/v). For measurement of radioactivity, a 1-mL sample of the organic layer was mixed with 10 mL of Formula 963 scintillation cocktail. Blank values were determined in the absence of AdoMet. Recovery of the O-methylated reaction products was determined by a back-extraction procedure to be greater than 90%.

The ratio of 3-methoxytyramine to 4-methoxytyramine was determined by thin-layer chromatography with chloroform–triethylamine (5:1 v/v) as the solvent system (Creveling et al., 1972). The products were extracted into ethyl acetate, and the extract was concentrated under nitrogen and then spotted on silica gel thin-layer chromatography plates (Eastman Kodak Co., Rochester, NY) along with standard 3- and 4-methoxytyramine solutions. The plates were chromatographed twice and then sprayed with ninhydrin (0.2% in ethanol) and heated at 110 °C for 5 min. 3-Methoxytyramine appeared as a purple spot and 4-methoxytyramine as a yellowish brown spot.

All kinetic studies were carried out at pH 7.4 with a saturating concentration of $MgCl_2$. Reactions were linear with protein concentration and with time and were carried out such that there was less than 10% conversion of either substrate. It was necessary to use washed membrane preparations to study the kinetics of membrane-bound COMT because preliminary studies have suggested that the kinetic properties may

† From the Department of Pharmacology and Therapeutics, State University of New York at Buffalo, School of Medicine, Buffalo, New York 14214. *Received October 27, 1981.* This work was supported by National Institutes of Health Grant MH29387.

[1] Abbreviations: AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosyl-L-homocysteine; DA, dopamine; 3MeOTyr, 3-methoxytyramine.

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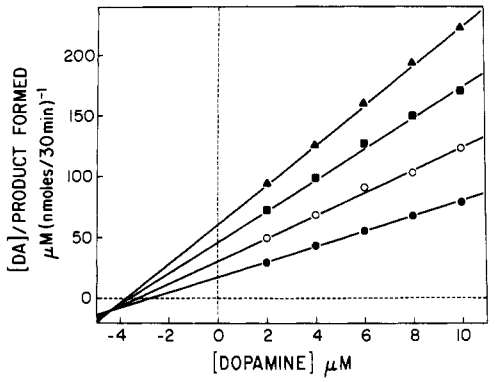

FIGURE 1: Inhibition by *S*-adenosylhomocysteine with varying concentrations of dopamine. Assays were carried out in the presence of 3 μM AdoMet, and the AdoHcy concentrations were 0 (●), 1.3 (○), 2.6 (■), and 3.9 μM (▲). Other details were as described in the text.

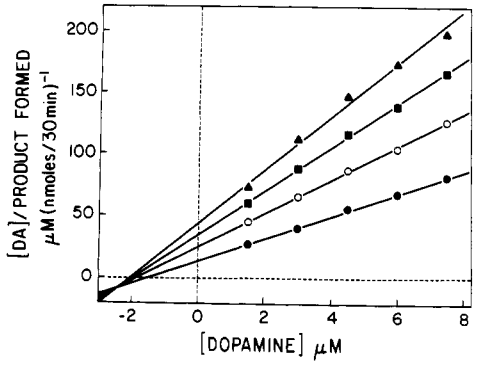

FIGURE 2: Inhibition by 3-methoxytyramine with varying concentrations of dopamine. Assays were carried out in the presence of 3 μM AdoMet, and the 3MeOTyr concentrations were 0 (●), 170 (○), 340 (■), and 510 μM (▲). Other details were as described in the text.

change upon solubilization, as has been reported for the membrane-bound transferase isolated from other tissues (Assicot & Bohuon, 1971; Borchardt et al., 1974; Tong & D'Iorio, 1977). Pargyline was used to inhibit the mitochondrial enzyme monoamine oxidase in case the membrane preparations were contaminated with outer mitochondrial fragments.

Results

Membrane-bound COMT was found to have the highest activity between pH 7.0 and pH 7.6 in potassium phosphate buffer. The enzyme is activated by magnesium ions, and maximum activity was obtained with concentrations of $MgCl_2$ above 1 mM. Addition of 1 mM dithiothreitol to the incubation medium had no effect on the COMT activity in microsomes prepared as above. The ratio of 3- to 4-methoxytyramine formed was found to be around 8.8 at pH 7.4.

Double-reciprocal plots of the data obtained by varying concentrations of DA and AdoMet within the range of 1–20 and 1–25 μM, respectively, gave converging lines which intersected at a point below the *x* axis. From multiple linear regression analysis of the data, the K_m value for DA, the K_m value for AdoMet, and K_s value for AdoMet were determined to be 3.3 μM, 3.1 μM, and 2.1 μM respectively. Substrate inhibition was not observed at saturating concentrations of DA or AdoMet.

Product inhibition studies with *S*-adenosylhomocysteine (AdoHcy) showed competitive inhibition with respect to AdoMet with a K_i value of 1 μM. Mixed inhibition was observed with respect to DA (Figure 1). Inhibition by the product, 3-methoxytyramine (3MeOTyr), was mixed with either AdoMet or DA as the variable substrate when the

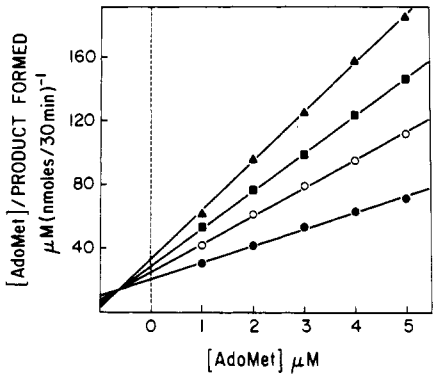

FIGURE 3: Inhibition by 3-methoxytyramine with varying concentrations of *S*-adenosylmethionine at the K_m concentration of dopamine. Assays were carried out in the presence of 3 μM DA, and the 3MeOTyr concentrations were 0 (●), 170 (○), 340 (■), and 510 μM (▲). Other details were as described in the text.

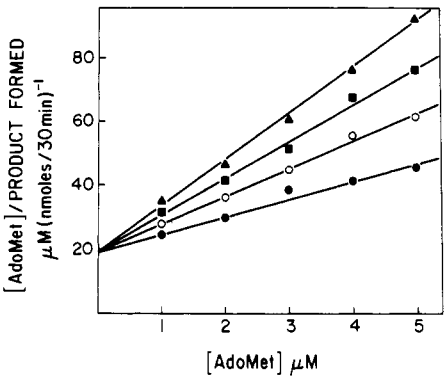

FIGURE 4: Inhibition by tropolone with varying concentrations of *S*-adenosylmethionine. Assays were carried out in the presence of 3 μM DA, and tropolone concentrations were 0 (●), 5 (○), 10 (■), and 15 μM (▲). Other details were as described in the text.

nonvaried substrate was present at its K_m concentration (Figures 2 and 3). With saturating concentrations of DA, 3MeOTyr was an uncompetitive inhibitor with respect to AdoMet with a K_i value around 250 μM. Replots of slopes and intercepts were linear in all cases.

Inhibition by tropolone with DA as the varied substrate was competitive with a K_i value of 5 μM. With varying AdoMet concentration, uncompetitive inhibition was observed (Figure 4).

Discussion

Membrane-bound COMT has a very low K_m value for dopamine compared to the value of around 280 μM for the soluble form of the enzyme (Roth et al., 1981), and linearity of double-reciprocal plots over a wide range of DA concentration shows the absence of contamination by soluble COMT in the membrane preparations used. The pH optimum for membrane-bound COMT is lower than the value of 7.9 reported for the soluble enzyme (Coward et al., 1973). Human brain membrane-bound COMT forms predominantly the 3-O-methylated product of DA as has been reported for both soluble and membrane-bound COMT from other sources (Creveling et al., 1972).

The data obtained by varying concentrations of DA and AdoMet with human brain membrane-bound COMT are consistent with the enzyme following either a rapid-equilibrium random-order or a steady-state compulsory-order ternary complex mechanism. All the results from the product inhibition studies are consistent with a compulsory-order mechanism in which AdoMet is the first substrate to bind to the enzyme.

They exclude a rapid-equilibrium random-order mechanism, and since 3MeOTyr shows mixed rather than competitive inhibition with respect to DA, they are also inconsistent with a Theorell-Chance mechanism.

Further evidence for the reaction mechanism are the inhibition patterns obtained with tropolone. Competitive inhibition with varying DA and uncompetitive inhibition with respect to AdoMet suggest that tropolone binds to the same form of the enzyme as DA after the formation of an enzyme–AdoMet complex.

The following reaction sequence is consistent with the results obtained in this study:

$$\begin{array}{ccc} E \rightleftharpoons E\cdot AdoMet \rightleftharpoons E\cdot AdoMet\cdot DA \rightleftharpoons E\cdot AdoHcy\cdot 3MeOTyr \\ \Updownarrow & & \Updownarrow \\ E\cdot AdoMet\cdot tropolone & & E\cdot AdoHcy \rightleftharpoons E \end{array}$$

The kinetic mechanism of membrane-bound COMT has not been reported previously. There have been several studies with soluble COMT, which have suggested a random mechanism for this form of the enzyme (Flohe & Schwabe, 1970; Coward et al., 1973). However, there are conflicting results in the literature. Studies with varying AdoMet and catechol substrate concentrations (Flohe & Schwabe, 1970; Ball et al., 1972) and studies on the stereochemical course of the transmethylation reaction (Woodard et al., 1980) have ruled out the ping-pong mechanism proposed by Borchardt (1973). In the latter study, tropolone was found to be an uncompetitive inhibitor with respect to AdoMet as reported here for human brain membrane-bound COMT and a competitive inhibitor with respect to catechol substrate at pH values below 7.4. These results are difficult to explain in terms of a random-order mechanism but do not exclude a compulsory-order mechanism in which AdoMet binds to the enzyme first. Flohe & Schwabe (1970) suggested a random mechanism for soluble COMT from rat liver based on the fact that varying the concentration of each substrate had no effect on the K_m values obtained. Product inhibition studies carried out at saturating levels of the nonvaried substrate rather than at their K_m concentrations (Coward et al., 1973) showed competitive inhibition by AdoHcy with respect to both AdoMet and epinephrine. However, AdoHcy has been reported to be a mixed inhibitor with respect to 2-hydroxy-17β-estradiol for soluble COMT purified from human liver (Ball et al., 1972). Mixed inhibition by the methylated reaction product with varying catechol concentration (Coward et al., 1973; Ball et al., 1972) is consistent with a random-order mechanism only if this product binds to the enzyme–AdoMet complex. Many of the results in the literature are consistent with the mechanism proposed herein for membrane-bound COMT, and it seems unlikely that soluble COMT would follow a different reaction mechanism than the membrane-bound enzyme.

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