Stereochemical Course of the Transmethylation Catalyzed by Catechol O-Methyltransferase*

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The steric course of the methyl group transfer catalyzed by catechol O-methyltransferase was studied using S-adenosylmethionine (AdoMet) carrying a methyl group made chiral by labeling with $^3$H, $^2$H, and $^2$H in an asymmetrical arrangement. Incubation of the two diastereomers of this substrate with catechol O-methyltransferase purified from rat liver and epinephrine or protocatechuic acid as acceptor gave the corresponding methylated catechols. These were degraded to convert the methoxy group in a series of stereochemically unambiguous reactions into the methyl group of acetate, which was then analyzed for its configuration. The results indicate that the transfer of the methyl group from AdoMet to either acceptor occurs in an inversion mode. The catechol O-methyltransferase reaction thus involves a direct transfer of the methyl group from the sulfur of AdoMet to the oxygen of the catechol in an S2 process, without a methylated enzyme intermediate.

Transmethylation reactions involving the transfer of the S-methyl group of S-adenosylmethionine (AdoMet) to a variety of nucleophiles as acceptors play an important role in many biological processes (1). Yet, their detailed mechanism is not well understood. Enzymes catalyzing this type of reaction can be divided roughly into three categories: (a) enzymes operating in "bulk" metabolic transformations, both in primary and secondary metabolism; (b) enzymes functioning in neuronal and neuroendocrine mechanisms, e.g. phenylethanolamine N-methyltransferase or catechol O-methyltransferase; and (c) enzymes involved in functional processing of informational biological macromolecules, i.e. DNA-, RNA-, and protein methylases.

In order to provide further insight into the mechanisms of enzymatic methyl group transfer, studies have recently been initiated to probe the stereochemical fate of the methyl group in such processes. Work from our laboratory (2-4) and from that of Arigoni (5) has dealt with several enzymes in the first category, and has shown that in each of these cases, the transfer of the methyl group occurs with inversion of configuration. In the present paper, we present results of a study on the stereochemical fate of the methyl group of AdoMet in the transfer reaction catalyzed by catechol O-methyltransferase, the first example of such a study on an enzyme of the second category.

EXPERIMENTAL PROCEDURES

Materials

Organic and inorganic chemicals were purchased by Aldrich Chemical Co. and Alfa-Ventron Corp., respectively. Magnesium chloride, Trizma base, epinephrine ditartrate, $\mu$-methanephrine-HCl, and ammonium reineckate were purchased from Sigma. Radioactive compounds were purchased from Amersham. All chemicals were reagent grade and were used without further purification.

S-Adenosylmethionine synthetase (ATP: methionine S-adenosyltransferase, EC 2.1.1.6) was isolated from frozen rabbit liver (Pel Freeze Biologicals, Rogers, Ark.) by the method of Cantoni (6).

Adenosine deaminase (76 units/mg of protein, 600 units/ml) was isolated from Aspergillus oryzae (Sanzyme-R from Calbiochem-Behring) by the procedure of Sharpless and Wolfenden (7). Catechol O-methyltransferase (EC 2.1.1.6) (15 units/mg of protein, 45 units/ml) was isolated and purified by a slight modification (8) of the method of Nikodejevic et al. (9).

The (methyl-$S$) and (methyl-$R$)-methyl-$^4$C,$^2$H,$^2$H$_2$ methionines were synthesized by the method of Woodard et al. (3) and converted to AdoMet as described previously (6).

Methods

Radioactivity was measured in Aquasol with $[^3$H]- and $[^2$H]toluene as an internal standard in a Beckman LS-7000 liquid scintillation counter.

An Altex model 322 MP liquid chromatograph was used to monitor progress of the enzymatic reaction. A Partisil-10 ODS-2 15% CIH$_2$O reverse phase column (4.6 x 250 mm) was employed for the separations. Chromatographic conditions were as follows: mobile phase, 1 M KPO$_4$, buffer, pH 3.3; flow rate, 0.55 ml/min; uv detector (254 nm wavelength monitor) set at 0.3 absorbance units full scale sensitivity. Retention times (in minutes) were as follows: AdoMet, 15.0; AdoHcy, 30.0; InoHcy, 40.0; 3-methoxy-4-hydroxybenzoic acid, 117.0; metanephrine, 24.

The chirality of the methyl group of acetate was determined by the method of Cornforth et al. (10) and Arigoni et al. (11) using a previously described procedure (4).

Enzyme Incubations—The following reagents were incubated at 37°C for 1 h: 250 $\mu$l of MgCl$_2$-Tris-HCl buffer, pH 7.9 (1 volume of 48 M MgCl$_2$, 3 volumes of 1.334 M Tris-HCl, 250 $\mu$l of 50 $\mu$M epinephrine; 500 $\mu$l of adenosine deaminase; 500 $\mu$l of catechol O-methyltransferase; 250 $\mu$l of AdoMet from R-acetate (2.6 pmol/ml, 1.72 $\mu$g/ml, $[^3$H/C = 3.09], and 750 $\mu$l of H$_2$O. After the incubation, the mixture was frozen, lyophilized, and extracted with methanol or chloroform to give the chirally labeled metanephrine. The same procedure was used for the incubation of the AdoMet from R-acetate with 250 $\mu$l of 10 nm protocatechuic acid (3,4-dihydroxybenzoic acid), except that the product was extracted with chloroform. The incubation mixtures using the AdoMet from S-acetate were as follows: 460

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†† The abbreviations used are: AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; InoHcy, S-inosylhomocysteine; COMT, catechol O-methyltransferase; Ad, adenosine deaminase.

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the synthesis of a stereospecifically labeled substrate of known absolute configuration, conversion of this substrate into the primary physiological substrate, the enzyme can also methylate other catechols, e.g. protocatechuc acid (Scheme 1, 1a). In the present study, we determined the stereochemical fate of the methyl group of AdoMet in the catechol O-methyltransferase-catalyzed transfer to epinephrine to produce methamphetamine (Scheme 1, 2b) and to protocatechuc acid to give 3-methoxy-4-hydroxybenzoic acid (Scheme 1, 2a).

The latter reaction was of interest because a number of kinetic studies on this enzyme have been carried along in the extraction, this would not alter the "H/14C ratios was observed throughout this reaction sequence.

The chiral methylbenzenesulphonates were converted to acetates by displacement with cyanide, oxidation of the acetoniitrile to acetamide, and diazotation of the latter, using the same procedures described previously for the conversion of chiral methyliditosylimines to acetate (3).

RESULTS

Catechol O-methyltransferase catalyzes the reaction shown at the top of Scheme 1. While epinephrine (Scheme 1, 1b) is the primary physiological substrate, the enzyme can also methylate other catechols, e.g. protocatechuic acid (Scheme 1, 1a). In the present study, we determined the stereochemical fate of the methyl group of AdoMet in the catechol O-methyltransferase-catalyzed transfer to epinephrine to produce methamphetamine (Scheme 1, 2b) and to protocatechuic acid to give 3-methoxy-4-hydroxybenzoic acid (Scheme 1, 2a). The latter reaction was of interest because a number of kinetic studies on this enzyme have been carried out using protocatechuic acid as substrate. To unravel the cryptic stereochemistry (13) of these methyl transfer reactions, we used the chiral methyl group methodology (see Ref. 4), i.e. the methyl group being transferred was made chiral by virtue of isotopic substitutions of 1 hydrogen each by deuterium and tritium.

A study of this kind involves three distinct tasks. These are the synthesis of a stereospecifically labeled substrate of known absolute configuration, conversion of this substrate into the enzyme reaction product, followed finally by degradation and analysis of the latter to determine the configuration of the stereospecifically labeled center in the product. The synthesis of AdoMet carrying a chiral methyl group started from chiral sodium [2-'H, 13C, 3H]acetate, prepared enzymatically from phosphoglyceric acid labeled stereospecifically at C-3 with deuterium and/or tritium (2-4), and involved the reaction sequence shown in Scheme 2. Chemical conversion of acetate into methionine in a 15 to 25% yield by our previously published procedures (2-4) was followed by enzymatic activation of methionine as described by Cantoni (6) to give AdoMet in a 24 to 30% yield (based on methionine). It will be noted that the conversion of acetate into AdoMet involves one inversion of configuration of the methyl group; hence the AdoMet from R-[2-'H, 13C, 3H]acetate will carry a chiral methyl group of S configuration and that from S-acetate will have a methyl group of R configuration. No significant change in the 'H/14C ratios was observed throughout this reaction sequence.

Incubations with the two stereoisomers of chirally labeled AdoMet were carried out using catechol O-methyltransferase purified from rat liver. Limitations of the yield of conversion of AdoMet due to the well known product inhibition by AdoHcy (8, 14) were overcome by including adenosine deaminase in the assay mixture (8). High pressure liquid chromatographic assay of the reaction mixtures at the end of the incubation period indicated the presence of only two radioactive components, AdoMet and 1b or 2a (Scheme 1), in a ratio of 1.8:8.2 in the case of 2b, and 2.4:7.6 in the case of 2a. The reaction products were extracted from the lyophilized reaction mixtures with methanol or chloroform and diluted with small amounts of carrier material for degradation.

The third task involved conversion of the O-methyl group of the enzyme reaction products in a sequence of stereochemically unambiguous reactions into the methyl group of acetate for subsequent chirality analysis. This was accomplished by the reaction sequence shown in Scheme 1. The crucial step is the oxidation of 2a or 2b with the Ce4+ ion to give methanol. This reaction is known to proceed with cleavage of the bond between the oxygen and the aromatic carbon (12); hence, the methanol will have the same configuration as the methoxy group in 2a or 2b. A control experiment showed that [13CH3]AdoMet under those conditions does not give any methanol; thus, even if any unreacted AdoMet should have been carried along in the extraction, this would not alter the results. The reaction requires water; however, in view of the need for anhydrous conditions in the next step and the difficulty of recovering traces of methanol from large volumes of water, the reaction was conducted in the presence of a limited amount of water, followed by addition of glycol to aid in the separation of methanol and water by slow distillation. The
methanol was then converted into its benzenesulphonate under nonhydrolytic conditions. The methylbenzenesulphonates were subjected to cyanide displacement to give acetonitrile with inversion of configuration at the methyl group. The conversion of acetonitrile into acetate by alkaline hydrogen peroxide oxidation followed by diazotation of the resulting acetamide into acetate by alkaline hydrogen peroxide has been a matter of some controversy. Studies by Flohe and Schwabe (18, 19) and by Coward et al. (8) strongly support a random Bi Bi mechanism. On the other hand, inhibition studies with tropolones and 8-hydroxyquinolines by Bor-chaude (20), using protocatechueic acid as a substrate, indicate

attribute it, for the most part, to a partial racemization in one of the steps of the methionine synthesis, most likely the Schmidt reaction converting acetate into methylamine. As evidenced by the low $^{1}H/^{14}C$ ratio of the acetate obtained from the 3-methoxy-4-hydroxybenzoic acid from methionine of $S$ configuration, the methyl group in this case may have undergone some additional racemization during the degradation. However, this decrease in chiral purity does not obscure the results of this study. As is evident from Schemes 1 and 2, both the synthesis of AdoMet from acetate and the conversion of the methoxy group of 2a and 2b into acetate each involve one inversion of configuration at the methyl group. Thus, the starting acetate and that derived from the degradation will have the same configuration if the enzymatic methyl group transfer proceeds in a retention mode; they will have opposite configurations if it proceeds in an inversion mode. In all four analyses, the acetate derived from the degradation of the enzyme reaction product has the opposite configuration as the starting acetate. Hence, the transfer of the methyl group of AdoMet to either substrate catalyzed by catechol O-methyltransferase occurs in an inversion mode, as shown in Scheme 1.

**DISCUSSION**

Recent isotope effect studies by Hegazi et al. (17) have shown that the transfer of the methyl group in the catechol O-methyltransferase reaction, or more precisely every transfer of the methyl group in the overall process, occurs through a tight, symmetrical $S_{N}2$ transition state. Therefore, no matter how many methylated species are involved in the overall process, every single transfer of the methyl group in this reaction must occur with inversion of configuration. The finding, in the present study, that the transfer of the methyl group of AdoMet to the catechol oxygen catalyzed by catechol O-methyltransferase proceeds with net inversion of configuration therefore indicates that the overall process involves an uneven number of transfers of the methyl group, most likely a single transfer.

The kinetic mechanism of catechol O-methyltransferase has been a matter of some controversy. Studies by Flohe and Schwabe (18, 19) and by Coward et al. (8) strongly support a random Bi Bi mechanism. On the other hand, inhibition studies with tropolones and 8-hydroxyquinolines by Borchar (220), using protocatechueic acid as a substrate, indicate

### TABLE I

| Compound                              | $^{1}H/^{14}C$ ratio (total µCi $^3$H) | F value found (theoretical) | Methyl group configuration | $^{1}H/^{14}C$ ratio (total µCi $^3$H) | F value found (theoretical) | Methyl group configuration |
|---------------------------------------|---------------------------------------|----------------------------|---------------------------|---------------------------------------|----------------------------|---------------------------|
| Acetate from metanephrine             | (2.3)                                 | (68)                       | R                         | (2.3)                                 | (68)                       | R                         |
| 3-Methoxy-4-hydroxybenzoic acid (2a) | (2.25)                                | (1.3)                      | R                         | (2.25)                                | (1.3)                      | R                         |
| Acetate from 3-methoxy-4-hydroxybenzoic acid | (2.4)                                | (67)                       | R                         | (2.4)                                 | (67)                       | R                         |
| 3-Methoxy-4-hydroxybenzoic acid methyl group | (2.4)                                | (67)                       | R                         | (2.4)                                 | (67)                       | R                         |

*Theoretical F values of the acetate, if no racemization occurred throughout the entire reaction sequence.
a ping-pong mechanism, with AdoMet binding to the enzyme first. Such a mechanism would involve a methylated enzyme as an intermediate and would thus require two transfers of the methyl group, one from AdoMet to a nucleophilic site on the enzyme and a second from there to the catechol oxygen. The stereochemical result of net inversion of the methyl group configuration in the transfer, seen both with epinephrine and with protocatechuc acid as substrate, clearly rules out such a ping-pong mechanism, unless one wants to make the extremely unlikely assumption that it involves not only one, but in succession, two methylated enzyme intermediates. Our results are best compatible with a random Bi Bi mechanism, involving a direct bimolecular transfer of the methyl group from the sulfur of AdoMet to the oxygen of the catechol, in which precise alignment of the two reactants (21) and compression of the \( S_{N2} \)-like transition state (17) are major factors contributing to the rate enhancement brought about by the enzyme.

Finally, the stereochemical results reported here for catechol O-methyltransferase indicate that this enzyme, a member of the second class of methyltransferases, which function in neuronal and neuroendocrine processes, conforms to the pattern seen with enzymes of the first category, which are involved in bulk metabolic transformations, i.e. C-, N-, O-, and S-methyltransferases involved in the biosynthesis of the antibiotic indolmycin (2, 3), the iridoid loganin (4, 5), or vitamin B_{12} (4, 5), and in the transfer of the methyl group of AdoMet to homocysteine (4, 5). So far, without exception, all transfers of an \( sp^3 \) carbon catalyzed by methyltransferases which have been examined have been found to proceed with inversion of configuration at the migrating carbon, and all transmethylations from AdoMet to nucleophilic carbon, nitrogen, oxygen, and sulfur atoms studied to date appear to involve a direct transfer of the methyl group from the donor to the acceptor substrate. It will be of interest to examine whether this uniform pattern extends to further examples and particularly, whether it also holds for members of the third category of methyltransferases, the enzymes involved in the processing and modification of informational biological macromolecules.

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