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 \(^{1}H, \, ^{2}H, \, ^{3}H\). 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 S,2 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 fundamental 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 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 dihydrate, m-amphetamine-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 2.5.1.6) 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-\(^{14}C, ^{2}H, ^{3}H\)]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 [\(^{14}C\)] and [\(^{3}H\)]toluene as an internal standard in a Beckman LS-7000 liquid scintillation counter.

An Altex model 222 MF liquid chromatograph was used to monitor progress of the enzymatic reaction. A Partisil-10 ODS-2 15% CIH reverse phase column (4.6 x 250 mm) was employed for the separations. Chromatographic conditions were as follows: mobile phase, 1 M KPO, buffer, pH 3.3; flow rate, 0.50 ml/min; UV detector (254 nm wavelength monitor) set at 0.32 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 ml of MgCl\(_2\)/Tris-HCl buffer, pH 7.9 (1 volume of 48 mM MgCl\(_2\), 3 volumes of 1.53 mM Tris-HCl), 250 ml of 50 mM epinephrine; 500 ml of adenosine deaminase; 300 ml of catechol O-methyltransferase; 250 ml of AdoMet from R-acetate (2.6 pmol/ml, 1.72 mg/ml, \(^{3}H/^{14}C = 3.09\)), and 750 ml of H\(_2\)O. After the incubation, the mixture was frozen, lyophilized, and extracted with methanol or chloroform to give the chiral labeled metanephrine. The same procedure was used for the incubation of the AdoMet from R-acetate with 250 ml of 10 mM 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 synthesis of a stereospecifically labeled substrate of known 
latter reaction was of interest because a number 
methyl group methodology (see Ref. 
studies on this enzyme have been carried out using protocate-
istry (13) of these methyl transfer reactions, we used the 
being transferred was made chiral by virtue of isotopic sub-
absolute configuration, conversion of this substrate into the 

degradation — the methylated catechols were diluted with 25 
muL of cold material in 100 μl of H2O containing 2 μl of methanol, 
and 0.2 μl of Ce(NH4)2(NO3)3 (12) was added to the mixture which 
was magnetically stirred in a 25-ml round bottom flask. After 10 
min at room temperature, 1.5 μl of glycol was added to the mixture. 
The reaction flask was connected via a vacuum U-tube to a trapping flask 
containing 1.5 μl of cyclopentane and 1 μl of methanol. The reaction 
flask was heated at 80°C for 5 days while the second flask was cooled to 
-80°C to trap the chiral methanol. The cyclopentane was removed 
from the trapping flask while still at -80°C. The aqueous layer was 
extracted with 3 μl of benzene and the extract was added to the 
cyclopentane solution. Then, 300 μl of NaH and 100 μl of benzene sulfonyl 
chioride was added to the latter and the reaction was 
sterilized at room temperature for 30 min. Excess methanol (5 to 10 μl) 
was then added to convert the sulfonyl chloride to the methyl ester. 
The reaction mixture was filtered, dried over Na2SO4, and evaporated 
to dryness. The yield was 5 to 15%, based on radioactivity. 
The chiral methylenesulfoximates were converted to acetates by 
displacement with cyanide, oxidation of the acetonirole to acetonitrile, 
and diazotation of the latter, using the same procedures described 
previously for the conversion of chiral methylditosylimines 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 
substrate 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 
metanephrine (Scheme 1, 1b) and to protocatecholic 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 protocate-
cholic acid as substrate. To unravel the chiral stereochem-
istry (13) of these methyl transfer reactions, we used the chiral 
metal group methodology (see Ref. 4), i.e. the methyl group 
being transferred was made chiral by virtue of isotopic sub-
stitutions 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 [2-14C, 2H]acetate, prepared enzymatically from 
phosphatidylethanolamine 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-[2-14C, 2H]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 
14C/13C ratios was observed throughout this reaction sequence. 

Incubations with the two stereoisomers of chirally labeled 
AdoMet were carried out using catechol O-methyltransferase 
perfusion 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 de-
amine in the assay mixture (8). High pressure liquid chromo-
matographic assay of the reaction mixtures at the end of the 
incubation period indicated the presence of only two radio-
active components, AdoMet and 2b or 2a (Scheme 1), in a 
ratio of 1.8:2.2 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 stereochem-
ically 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 
[13C2]AdoMet under those conditions does not give 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 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 benzenesulfonate under nonhydrolytic conditions. The methylenesulfonates 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 (3, 15) proceeds in almost quantitative yield and avoids the risk of racemization by $\alpha$-hydrogen exchange inherent in the hydrolytic conversion. Although the yield of methylbenzene-sulfonates is chiral. This tritium distribution can be determined by incubation with fumarase, which specifically equilibrates the pro-R hydrogen at C-3 of the acetate is chiral. This tritium distribution into acetylcoenzyme A, which is condensed with glyoxylate in the subsequent conversions, the methyl group suffers a further decrease in the configurational purity, as is evident from the F values of the acetate samples from these degradations.

The chirality of the acetate samples from these degradations was determined by the method of Cornforth (10) and Luthy et al. (11), using essentially Eggerer’s procedure (10; see Ref. 4). This method involves conversion of acetate into acetylcoenzyme A, which is condensed with glyoxylate in a reaction catalyzed by malate synthase. Due to a kinetic deuterium isotope effect in the latter reaction, the resulting acetate will show an asymmetrical distribution of tritium between the two hydrogens of the methylene group if the methyl group of the acetate is chiral. This tritium distribution can be determined by incubation with fumarase, which stereospecifically equilibrates the pro-$R$ hydrogen at C-3 of l-malate with solvent protons. Calibration of the system has shown that malate derived from acetate of $R$ configuration retains more than 50% of its tritium in the fumarase reaction, whereas malate from $S$ acetate shows less than 50% tritium retention (10, 11). The percentage of tritium retention in the fumarase reaction of this assay is referred to as the F value (see Ref. 4); configurationally pure $R$-acetate shows an F value of 79, whereas pure $S$-acetate gives an F value of 21 (16).

The results from these experiments are summarized in Table I. The starting acetate samples used for the synthesis of methionine and AdoMet were of about 60 to 75% chiral purity. It is known that partial racemization due to proton exchange is an inherent problem in the pyruvate kinase reaction, which was used to generate the chiral methyl group of the acetate (4). In the subsequent conversions, the methyl group suffers a further decrease in the configurational purity, as is evident from the F values of the acetate samples from the degradation. We have observed this decrease in chiral purity in all of our studies on transmethyleations (2–4) and 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 $^{3}H/^{14}C$ 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 methyl 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 $S$,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 Borchardt (20), using protocatechueic acid as a substrate, indicate
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 $S_{N}2$-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), and S- and N-methyltransferases involved 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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REFERENCES

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