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Phenol Ionization in Dopa Determines the Site of Methylation by Catechol-*O*-Methyltransferase

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SUMMARY

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Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) with *S*-adenosylmethionine as the methyl donor catalyzes preferentially *p*-*O*-methylation when 5-fluorodopa is the substrate. The rate of this reaction is characterized by a K_m of 0.4 ± 0.2 mmole/liter and a V_{max} of 27 ± 11 nmoles/mg of COMT per minute at pH 7.9. We conclude that these results are a consequence of fluorine-induced ionization at the *p*-hydroxyl group.

INTRODUCTION

It is generally agreed that the orientation and polarity of the side chain in catechol derivatives play the major role in determining the site at which COMT[1] (EC 2.1.1.6) acts. Furthermore, it is believed that the nucleophilicity of the phenolic groups of the catechol plays a minor role in determining the site at which COMT operates (1). By and large, catecholamines and dopa, the natural substrates for COMT, are preferentially methylated on the 3-hydroxyl group.

The purpose of this communication is to show that, when 5-fluorodopa (3,4-dihydroxy-5-fluorophenylalanine) is the substrate for COMT, the enzyme selectively methylates the 4-hydroxyl group because this has been ionized as a result of the introduction of the electronegative fluorine in position 5.

MATERIALS AND METHODS

Synthesis of DL*-5-Fluorodopa*

Because of the original synthesis for 5-fluorodopa is suitable only for milligram quantities (2), a new method was devised so that gram quantities would be available. 3-Fluorotyrosine was nitrated (3) and then hydrogenated to yield 3-amino-5-fluorotyrosine, which was diazotized and then decomposed photochemically to 5-fluorodopa (4).

3-Nitro-5-fluorotyrosine. To the ice-cold suspension of 3-fluorotyrosine (14 g) in 52 ml of water, concentrated nitric acid (37 ml) was slowly added. After the 3-fluorotyrosine had dissolved, the reaction mixture was kept below 25° overnight, during which time the yellow nitrate of 3-nitro-5-fluorotyrosine precipitated. It was filtered off and dissolved in a little hot water. This solution was neutralized with ammonia, which caused free 3-nitro-5-fluorotyrosine to precipitate (yield, 6.8 g (40%); melting point 220°; $C_9H_9N_2O_5F$; analysis C, H, N, F).

3-Amino-5-flurotyrosine. The 3-nitro-5-fluorotyrosine (7.2 g) was suspended in 150 ml of methanol, and concentrated sulfuric acid was added dropwise until it was dissolved. Palladium on charcoal, 5% (300 mg) was added and the reaction mixture was hydrogenated at 2.5 atmos at room temperature for 3 h. After filtration, the reaction mixture was evaporated. The sulfate salt of the 3-amino-5-fluorotyrosine (3.9 g) precipitated quantitatively (melting point 170°; $C_9H_{11}N_2O_3F$; analysis C, H, N, F).

5-Fluorodopa. 3-Amino-5-fluorotyrosine sulfate (6 g) was dissolved in 150 ml of water and 2.6 ml of concentrated sulfuric acid. The solution was cooled to −5°. Sodium nitrate (1.36 g), dissolved in 10 ml of water, was added. The temperature was not allowed to rise above −5°. The diazotized solution was diluted with 200 ml of ice-cold water and irradiated with ultraviolet light (Hanovia lamp, 450-watt, Pyrex filter) for 30 min. The reaction mixture was evaporated to a small volume. Fluorodopa was isolated from the reaction mixture by semipreparative high-pressure liquid chromatography (column: Waters μBondapak/C18, 0.78 × 30 cm; mobile phase, 0.1% acetic acid in water; ultraviolet-detector at 280 nm). Pure 5-fluorodopa was crystallized from ethanol-ether (yield 2 g (56%); melting point 211°; $C_9H_{10}O_4NF$ analysis C, H, N, F).

The pK_{OH} of the 4-hydroxyl group of 5-fluorodopa and dopa was determined by titration (5).

DL-5-Fluorodopa and DL-dopa were resolved into their optical isomers by the α-chymotrypsin method of Tong

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[1] The abbreviation used is: COMT, catechol-*O*-methyltransferase.

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et al. (6). Optical rotations were measured with a Perkin Elmer Model 141 polarimeter using 10-cm tubes. All measurements were made in 1 N hydrochloric acid. Values for specific optical rotation obtained after separation were as follows: for L-dopa, $[\alpha]_D^{25^\circ} = -11.07$; for D-dopa, $[\alpha]_D^{25^\circ} = +10.98$; for L-5-fluorodopa, $[\alpha]_D^{25^\circ} = -5.32$; and for D-5-flourodopa, $[\alpha]_D^{25^\circ} = +6.10$.

Synthesis of DL-3-methoxy-4-hydroxy-5-fluorophenylalanine and DL 3-hydroxy-4-methoxy-5-fluorophenylalanine

This synthesis was achieved by partial hydrolysis (7) of diethylacetamido-(3,4-dimethoxy-5-flurobenzyl) malonate (2). Diethylacetamido-(3,4-dimethoxy-5-fluorobenzyl) malonate (500 mg) was refluxed in 100 ml of 20% hydrochloric acid for 6 hr. The acid was then evaporated and the remainder was redissolved in 5 ml of water, which was again evaporated. An aqueous solution of the hydrolysis products was chromatographed in portions with high-pressure liquid chromatography (column: Waters μBondapak/C18, 0.78 × 30 cm; mobile phase, 0.1% acetic acid in water at 2 ml/min; ultraviolet detector at 280 nm). Three compounds were eluted from the column and collected. Compound A eluted at 9.1 min and was identified as 5-fluorodopa by spiking with the authentic material. Compound B eluted at 16.7 min and contained, after evaporation of the aqueous acetic acid, 208 mg (70% yield). Compound C eluted at 22.9 min and contained 42 mg (14% yield).

The elemental analyses of Compounds B and C are consistent with methoxyhydroxyfluorophenylalanine (Table I). When the relation times and uncorrected melting points of Compounds B and C compared with those of the authentic methoxyhydroxyphenylalanine isomers, analogy suggested that Compound B might be 3-methoxy-4-hydroxy-5-fluorophenylalanine and that Compound C might be 3-hydroxy-4-methoxy-5-fluorophenylalanine (Table I). The isomers of methoxyhydroxy-5-fluorophenylalanine were identified by gas chromatography-mass spectrometry.

Gas Chromatography-Mass Spectrometry. Compounds B and C were transformed into their *N*-isothiocyanate-*O*-trimethylsilyl derivatives. Approximately 200 μg of each compound (dry) were dissolved in 45 μl of *N,O*-bis(trimethylsilyl)tri-fluoracetamide, 5 μl of trimethylchlorosilane, 50 μl of pyridine, 50 μl of ethylacetate, and 50 μl of carbon disulfide, and the mixture allowed to stand for 2 days. Before aliquots of the reaction mixture were chromatographed, the carbon disulfide was evaporated. The instrument for chromatography-mass spectrometry was a Finnigan 4021 quadruple mass spectrometer with the INCOS data system, which was run under the following conditions: column, 6 feet × 4 mm, 3% OV-101; gas flow, 15 ml of He per minute; temperature programmed from 200°–300° at 10°/min with a 1-min delay; injector and ion source at 250°; transmission line at 290°; ionization voltage 70 eV. The derivatized Compounds B and C appeared in the mass spectrometer 148 sec and 161 sec, respectively, after injection into the gas chromatograph. The relative abundances of the mass fragments at m/e 197 and m/e 209 from Compounds B and C were different. For comparison, the relative abundance of typical mass fragments of *O*-methylated isomers of dopa and dopamine are also shown in Table 2. Values for the methylated dopas were measured as described; values for the methylated dopamines were taken from Narasimhachari and Vouros (8). The higher relative abundance of the typical mass fragments was always observed with the 4-*O*-methyl catechol isomer. Compound C had a higher mass fragment ratio than did Compound B. This suggested that Compound C was 3-hydroxyl-4-methoxy-5-fluorophenylalanine and Compound B was 3-methoxy-4-hydroxy-5-fluorophenylalanine.

TABLE 1

Elemental analyses of Compounds B and C

Calculations for methoxyhydroxyfluorophenylalanine acetate $C_{10}H_{12}NFO_4 \cdot CH_3COOH$: C 49.8, H 5.54, N 4.84, F 6.58.

	HPLC[a] retention time	Melting point	Elemental analysis
	min		
Compound B	16.7	212–214°	Found: C 48.0, H 5.48, N 5.40, F 6.49
Compound C	22.9	224°	Found: C 49.0, H 5.69 N 4.47, F 6.36
3-Methoxy-4-hydroxy-phenylalanine (authentic)	13.6	222–224°	
3-Hydroxy-4-methoxy-phenylalanine (authentic)	16.9	232°	

[a] HPLC, High-pressure liquid chromatography.

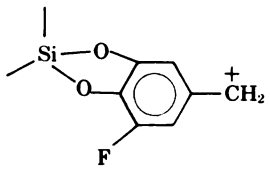
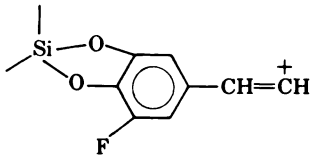
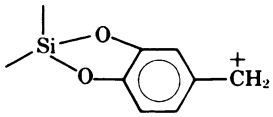
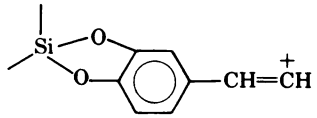
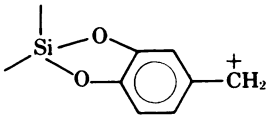
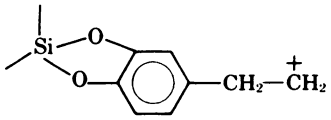
^{1}*H-Nuclear Magnetic Resonance.* Compounds B and C were dissolved in CD_3OD and their spectra were recorded with an 80-MHz Bruker FT spectrometer. Trimethylsilane was the standard. Like other *O*-methylated phenols, Compounds B and C showed a signal from the methoxy protons at δ 3.85. However, Compound B showed a singlet at 3.85 whereas Compound C showed a doublet. It is known that a ^{19}F in the position adjacent to a methoxy group on the aromatic ring couples with the methoxy protons, which gives rise to a doublet; ^{19}F in nonadjacent positions does not couple with the methoxyprotons (9, 10). The doublet observed in Compound C provided evidence for that compound's being 3-hydroxy-4-methoxy-5-fluorophenylalanine.

Reactions with Catechol-O-methyltransferase

DL-[^{14}C-*alanine*]dihydroxyphenylalanine was obtained from Amersham/Searle Corporation, Arlington Heights, Ill. (specific activity 54 mCi/mmole). DL-[^{18}F]-5-fluorodopa was synthesized (2) and purified by reverse-phase high-pressure liquid chromatography (specific activity 10 mCi/mmole). The enzymatic reaction mixture was prepared according to the method of Coward and Wu (11): [^{14}C]dopa or [^{18}F]fluorodopa, 0.025–1.0 mM; magnesium chloride, 1.4 mM; *S*-adenosylmethionine iodide (Sigma Chemical Company, St. Louis, Mo.), 1.5 mM; adenosine deaminase (EC 3.5.4.4; Sigma Chemical Company), 0.25 mg/ml; catechol-*O*-methyltransferase (Sigma Chemical Company), 0.49 mg/ml; and 0.16 M Tris buffer (pH 7.9). The volume of the incubation mixture was 1.5 ml; all

TABLE 2

Mass spectral characteristics of N-isothiocyanate-O-trimethylsilyl derivatives of methylated catecholamines

O-Methyl-catechol isomer	Typical mass fragments		Ratio of the abundance of the typical mass fragments
Compound B	m/e = 197	m/e = 209	$\frac{\text{Abundance of m/e 197}}{\text{Abundance of m/e 209}} = 1.00$
Compound C			$\frac{\text{Abundance of m/e 197}}{\text{Abundance of m/e 209}} = 19.00$
3-*O*-methyldopa	m/e = 179	m/e = 192	$\frac{\text{Abundance of m/e 179}}{\text{Abundance of m/e 192}} = 1.56$
4-*O*-methyldopa			$\frac{\text{Abundance of m/e 179}}{\text{Abundance of m/e 192}} = 5.21$
3-*O*-methyldopamine	m/e = 179	m/e = 193	$\frac{\text{Abundance of m/e 179}}{\text{Abundance of m/e 193}} = 0.92^a$
4-*O*-methyldopamine			$\frac{\text{Abundance of m/e 179}}{\text{Abundance of m/e 193}} = 10.30^a$

[a] Ref. 8.

concentrations are final concentrations. All incubations were carried out at 37°. The reaction was started by the addition of substrate. When fluorodopa was the substrate, the incubation lasted 4 min; when dopa was the substrate, it lasted 60 min. The reaction was stopped by the addition of 0.1 ml of 2 N hydrochloric acid. The *O*-methylated dopas were separated by high-pressure liquid chromatography (column: Whatman, Partisil PXS $^{10}/_{25}$ ODS, 0.46 × 25 cm; mobile phase, 0.05 M sodium phosphate buffer, pH 3.5, 0.5 ml/min). The ^{18}F radioactivity in the effluent was recorded continuously by a NaI scintillation detector. When [^{14}C]dopa was the substrate, the effluent was collected into 0.5-ml fractions and the ^{14}C content of each fraction was measured by liquid scintillation spectrometry. The ^{18}F and ^{14}C in the peaks due to the *O*-methylated dopas were used to derive the amounts of *O*-methylated dopa. The short half-life of ^{18}F (110 min) necessitated a correction for decay. The K_m and V_{max} values for each substrate were estimated by a weighted least-squares fit to a Lineweaver-Burke plot (12).

The amounts of *m*- and *p*-methylated dopas formed by catechol-*O*-methyltransferase were determined as follows: L- or D-dopa and L- or D-5-fluorodopa (0.8 mM) were incubated at 37°, for 2 hr when the dopas were the substrates and for 1 hr when the fluorodopas were the substrates. In both instances the incubation mixtures contained magnesium chloride 1.4 mM, *S*-adenosylmethionine iodide (Sigma Chemical Company) 1.5 mM, COMT (Sigma Chemical Company) 0.4 mg/ml, and adenosine deaminase (Sigma Chemical Company) 0.25 mg/ml in 0.16 M Tris buffer (pH 7.9). The reactions were stopped by the addition of 0.1 ml of a solution of phosphotungstic acid 5% in 2 N HCl. After a reaction had been stopped, the mixture was allowed to stand for 30 min at 0° before it was centrifuged. A 0.1-ml aliquot of the supernatant was analyzed by high-pressure liquid chromatography [columns: two Whatman, Partisil, PXS 10/25 ODS, 0.46 × 25 cm, in sequence; mobile phase, 0.05 M sodium phosphate buffer (pH 2.5) at 0.5 ml/min; ultraviolet detector at 280 nm]. A typical separation of the *O*-methylated fluorodopas is shown in Fig. 1. The *O*-methylated dopas and fluorodopas in the chromatograms were identified by spiking with the authentic materials: 3-*O*-methyldopa (Sigma Chemical Company), 4-*O*-methyldopa (donated by Dr. C. R. Creveling, National Institutes of Health, Bethesda, Md.), 3-*O*-methylfluorodopa, and 4-*O*-methylfluorodopa. On the basis of the height of the peak of the *m*- and *p*-methylated dopas and fluorodopas and a calibration factor, the amount of each isomer produced during the enzymatic reaction was determined.

RESULTS AND DISCUSSION

The pK_{OH} of dopa (8.93) obtained in the present study (Table 3) is in accord with that reported by Martin (5). The lower pK_{OH} of 5-fluorodopa (7.23), derived by the same method, was expected because Vlasov and Yakobson (13) have shown that monofluorinated phenols have lower pK_{OH} values than does phenol itself. Vlasov and Yakobson (13) have also shown that the influence of the fluorine is maximal when fluorine is ortho to the hydroxyl group. It can therefore be concluded that the difference in the pK_{OH} of 5-fluorodopa and dopa is due primarily to a change in the ionization of the 4-hydroxyl group.

At pH 7.9, the pH at which the COMT reactions were allowed to occur, few of the hydroxyl groups of dopa are ionized (monocatechol anion:un-ionized catechol = 0.085:1). In contrast, at the same pH, 7.9, 5-fluorodopa is strongly ionized at its 4-hydroxyl group (monocatechol anion:un-ionized catechol = 4.68:1).

It was not surprising that COMT methylated fluoro-

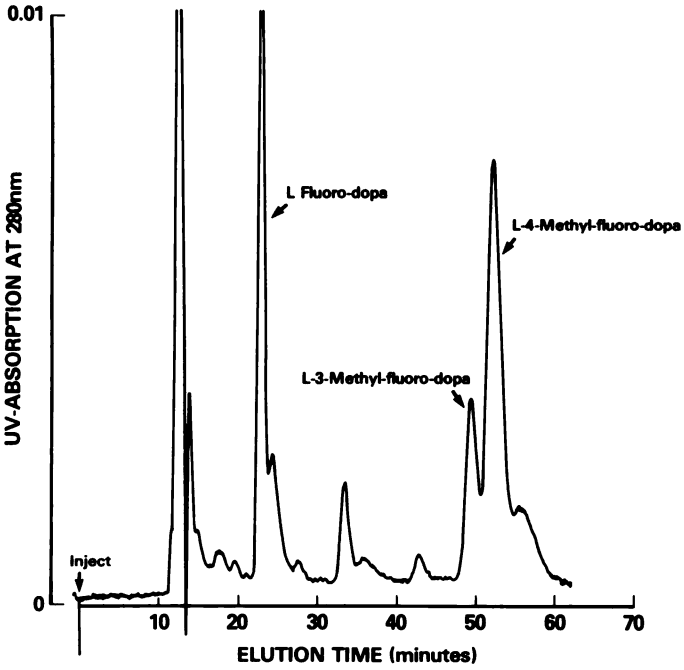


FIG. 1. *High-pressure liquid chromatogram of the reaction products after the action of COMT on* L-*5-fluorodopa*

dopa because the enzyme is known to methylate a variety of endogenous and foreign catechol compounds (1). We have previously found O-methylated derivatives of DL-fluorodopa in monkeys within 7 min of giving them DL-5 fluorodopa (14). The K_m and V_{max} values (Table 3) show that although the affinities of COMT and dopa are similar, the capacity of the enzyme for 5-fluorodopa exceeds that for dopa. Since, at pH 7.9, 5-fluorodopa is more ionized than is dopa, it is the better nuceophile; therefore it follows from the suggestion of Axelrod (15) and that of Senoh *et al.* (16) that the better nucleophile will be the preferred site of methyl transfer. These authors considered that *O*-methylation was a nucleophilic displacement of a methyl group from the sulfonium of *S*-adenosylmethionine.

Table 4 shows that the amounts of *m*-methylated dopa produced by COMT are similar when either dopa or 5-fluorodopa is the substrate. In contrast, the amounts of *p*-methylated dopa are increased 50-fold by the addition of fluorine in position 5. We conclude that the increased methylation at the *para* position is a direct consequence of its ionization and hence increased nucleophilicity.

Our results with 5-fluorodopa differ from those of Creveling *et al.* (17), who studied the related compounds 5-fluorodopamine and 5-fluoronoradrenaline and concluded that the 3-hydroxyl group was preferentially

TABLE 3

Kinetic data on the action of COMT on dopa and 5-fluorodopa

Catechol	pK^a_{OH}	K_m[a]	$V^{a,b}_{max}$
		mM	*nmoles mg/min*
DL-Dopa	8.93 ± 0.10	0.4 ± 0.3	2.3 ± 2
DL-5-Fluorodopa	7.23 ± 0.17	0.4 ± 0.2	27 ± 11

[a] Values are means ± 1 SD, $n = 6$.

[b] The difference between V_{max} of DL-dopa and V_{max} of DL-5-fluorodopa is significant ($p < 0.01$).

TABLE 4

The influence of fluorine in position 5 on the production of m- and p-O-methylated dopas

Amino acid	*m*-Methylated dopa[a]	*p*-Methylated dopa[a]
	nmoles/2 hr	
L-Dopa	10.4 ± 0.4	0.30 ± 0.08
D-Dopa	5.2 ± 0.2	0.44 ± 0.04
L-5-Fluorodopa	11.8 ± 1.0	27.2 ± 1.4
D-5-Fluorodopa	6.6 ± 0.2	23.0 ± 0.6

[a] Values are means ± 1 SD, $n = 6$.

methylated. However, the conclusion of Creveling *et al.* was based on the Gibbs reaction, which, according to Dacre (18), is nonspecific for halogenated phenols.

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