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Stereochemistry of the Decarboxylation of L-Ornithine with Ornithine Decarboxylase from Mouse Kidney

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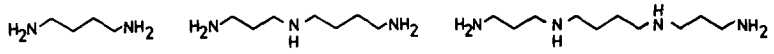
Using highly purified ornithine decarboxylase isolated from androgen-treated mice, [1*R*-^{2}H]putrescine was generated by the decarboxylation of L-ornithine in D_2O, and [1*S*-^{2}H]putrescine was generated from [2-^{2}H]ornithine by carrying out the decarboxylation in H_2O. Chirality of the putrescines was then determined from the 200-MHz ^{1}H NMR spectra of their bis-camphanamides in the presence of Eu(fod)$_3$. These results demonstrated that decarboxylation had taken place with retention of configuration. © 1984 Academic Press, Inc.

INTRODUCTION

Polyamines, such as putrescine, **1**, spermidine, **2**, and spermine, **3**, occur widely in Nature, both as free compounds and as constituents of a variety of alkaloids (*1–6*). The polyamines appear to participate in a wide range of biological processes, such as promoting cell growth (*7, 8*), stabilizing nucleic acids (*9, 10*), and modulating enzyme activity (*11–13*). In doing so, they appear to function as polycations. Furthermore, in man, increases in urinary and serum polyamine levels have been correlated with a variety of carcinomas as well as a number of noncancerous disease states (*14–18*).



1 **2** **3**

The biosynthesis of **2** and **3** in microorganisms has been well-studied; decarboxylated *S*-adenosyl-L-methionine (dcSAM) and **1** are the key intermediates. The former is derived from SAM, and the latter from L-ornithine, **4**, or L-arginine, **5** (*19*). Polyamine biosynthesis in mammals is essentially the same as in microorganisms except that ornithine is the sole source of **1** (*19*).

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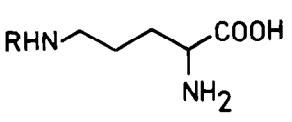

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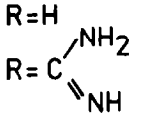

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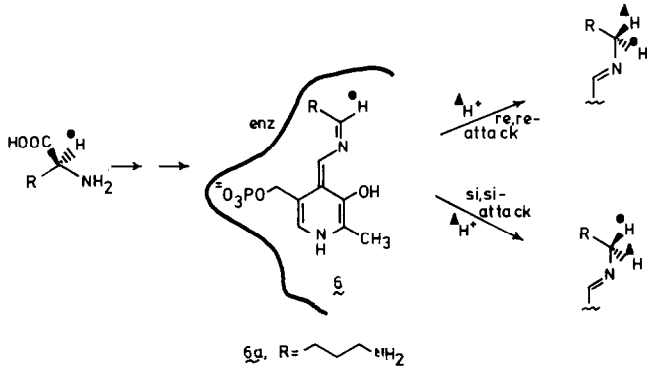
Of the four enzymes involved in mammalian polyamine biosynthesis, ornithine decarboxylase (EC 4.1.1.17) has the lowest basal activity *in vivo* (*20*), and is generally regarded as the rate-limiting enzyme in this pathway. *De novo* ornithine decarboxylase (ODC) biosynthesis can be induced by a variety of stimuli (*21–25*). Because of its central physiological role, numerous specific ODC inhibitors have been developed, including competitive (*26–27*), noncompetitive (*28*), and irreversible (*29*) inhibitors.

Despite widespread interest in this pyridoxal phosphate-dependent enzyme (*30*), direct mechanistic studies of the mammalian enzyme have been hampered by the minute quantities of ODC present in mammalian tissues and by consequent difficulties in purification of the enzyme. We recently reported the purification to homogeneity of ODC derived from the kidneys of androgen-treated mice (*31*). A homogeneous enzyme from the liver of thioacetamide-treated rats has subsequently been reported, in this case with a specific activity approximately half that of the mouse kidney enzyme (*32*).

The mechanisms of numerous pyridoxal phosphate-dependent reactions have been intensively studied (*33, 34*). In particular, the stereochemistry of decarboxylation involving enzymes from a variety of bacterial, plant, and animal sources has been reported for eight amino acids, and in all but one case the hydrogen introduced was found to occupy the same geometrical position as had the departed carboxyl group (*35, 36*). In the one anomalous case, decarboxylation of *meso*-diaminopimelic acid occurred with inversion of configuration (*37*). These studies have provided an indication of the topography of the active sites; the relative positions of catalytic and binding regions and the juxtapositions and conformations of substrates and coenzymes. The essential molecular features are the geometry of the PLP-amino acid Schiff base **6** (*38*) and the face of this adduct that is exposed on the enzyme surface to the protonating moiety, as shown in Scheme 1.



SCHEME 1

We (*39*), and others (*40*), have reported that ODC from *Escherichia coli* and from *Nicotiana tabacuum*, respectively, catalyzes the decarboxylation of L-ornithine with retention of configuration (*re–re* attack). The availability of the mouse kidney enzyme has now allowed us to determine the stereochemistry of this important mammalian enzyme reaction.

MATERIALS AND METHODS

General. $Eu(fod)_3$ and (-)-camphanoyl chloride were purchased from Aldrich Chemical Company, and were purified immediately prior to use by sublimation at 150°C/0.05 mm and 68°C/0.05 mm, respectively. Deuterated solvents were purchased from Aldrich or from Sigma Chemical Company. Pyridine for volatile buffers was distilled from ninhydrin. ^1H NMR spectra were obtained at ambient temperature on a Varian XL-200 spectrometer at 200 MHz; samples were run in $CDCl_3$ with tetramethylsilane (TMS) or in D_2O with 3-(trimethylsilyl)tetradeutero sodium propionate (TSP) as internal reference. Mass spectra were obtained on a MAT CH-7 spectrometer at 70 eV under EI conditions; multiple scans of the molecular ion region were obtained and the deuterium enrichment was calculated by the method of Biemann (*41*).

Ornithine decarboxylase was prepared from the kidneys of androgen-treated mice (*31*). This enzyme had a specific activity of 35 μmol CO_2 mg^{-1} min^{-1}.

dl-[2-^2H]Ornithine, ***4b*** (*42*). L-Ornithine monohydrochloride (2.500 g, 14.8 mmol) was dissolved in a mixture of 10 ml deuterium oxide and 1.0 ml deuterated sulfuric acid. The resulting solution was heated in a sealed tube at 195°C for 5 h. At the end of this time the tube was cooled to room temperature, opened, and the reaction mixture was adjusted to pH 6.9 by the addition of hot, saturated barium hydroxide solution. Barium sulfate was removed by gravity filtration. The precipitate was washed once with hot water, and combined filtrate and washings were lyophilized. The resulting amorphous powder was redissolved in the minimum volume of water, filtered, and relyophilized to yield 1.913 g (98%) of **4b** monohydrochloride as a microcrystalline powder: mp 233°C (mp of **4a**: 218°C); ^1H NMR (D_2O, TSP) δ 3.60 (t, <.1H), 2.95 (m, 2H), 1.75 (m, 4H).

Putrescine biscamphanamide ***7***. Putrescine dihydrochloride (60 mg, 0.36 mmol) and potassium bicarbonate (80 mg, 0.80 mmol) were dissolved in 1 ml water. The temperature of the solution was brought to 0°C by means of an ice bath, and the solution was treated with camphanoyl chloride (198 mg, 0.92 mmol) in 1 ml toluene. The heterogenous reaction mixture was stirred at room temperature for 3 h. At the end of that time the reaction was taken up in methylene chloride and was washed sequentially with 0.1 N hydrochloric acid, water, 10% sodium bicarbonate, and water. The organic portion was then dried over sodium sulfate, filtered, and concentrated *in vacuo* to yield 140 mg (87%) biscamphanamide as a colorless crystalline solid. The product was recrystallized from methylene chloride–carbon tetrachloride: mp 208–209°C; ir 3450, 1770, 1650 cm^{-1}; ^1H NMR δ 6.47 (bs, 2H), 3.35 (m, 2H), 2.54 (2H, m), 1.93 (m, 4H), 1.70 (m, 2H), 1.60 (m, 4H), 1.10 (s, 12H),

0.90 (s, 6H); High-resolution mass spectrum: calculated for $C_{24}H_{36}N_2O_6$ 448.2575, found 448.2562.

L-Ornithine decarboxylase reactions. L-Ornithine monohydrochloride (23 mg, 138 μmol) in 30 ml of standard assay buffer made up in D_2O was treated with ODC (1 ml, 0.23 μmol/min) that had been dialyzed against D_2O buffer (experiment a), while *dl*-[2-^{2}H]ornithine monohydrate (39 mg, 300 μmol) in 30 ml of buffer prepared with H_2O was treated with an equivalent amount of enzyme that had been dialyzed against H_2O buffer (experiment b). Each mixture was incubated 3 h at 37°C, then lyophilized and purified by preparative paper high-voltage electrophoresis (0.1 M pyridinium acetate, pH 4.0; 4500 V, 30 min). The pherogram was visualized by spraying a longitudinal margin with ninhydrin, and the region containing the remaining putrescine was cut out and eluted with 0.1 M HCl in descending fashion to yield putrescine dihydrochloride. Nine milligrams was obtained from experiment a and 15 mg from experiment b.

Biscamphanamides from [1R-^{2}H]- and [1S-^{2}H]putrescines, ***7a*** *and* ***7b.*** Each diamine salt and four equivalents of sodium bicarbonate were dissolved in 1 ml H_2O and treated at 0°C with two equivalents of (-)-camphanoyl chloride in 1 ml benzene. After vigorous stirring for 3 h each at 0°C and then at room temperature, work-up afforded crude bis-camphanamide that was purified by HPLC using a 10-μm silica cartridge in a Waters Associates radial compression Z module coupled to a refractive index detector (chloroform–ethyl acetate, 60:40; 1.5 ml/min). In this manner, 0.85 mg of **7a** was obtained from experiment a and 4.0 mg of **7b** from experiment b. For complete purification, the product from experiment a was chromatographed twice.

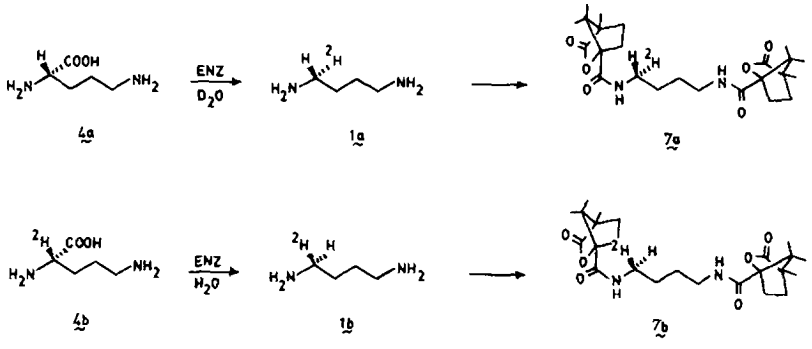
Mass spectrometric analysis indicated the *R* isomer, **7a**, was 12% d_0 and 87.1% d_1, while the *S* isomer, **7b**, was 7.9% d_0 and 92.1% d_1.

RESULTS AND DISCUSSION

Our approach to determining the stereochemistry of the mammalian ODC reaction involved the preparation of chirally deuterated putrescines as a consequence of the enzymatic decarboxylation. The relevant enantiotopic hydrogens adjacent to nitrogen were rendered diasterotopic by formation of the bis-camphanamide with (-)-camphanoyl chloride. These hydrogens were then distinguishable by ^{1}H NMR in the presence of europium shift reagent (*36*).

As shown in Scheme 2, one of the two possible enantiomers of [1-^{2}H]putrescine, **1a**, was produced by incubation of L-ornithine **4a** with purified mouse kidney ODC and pyridoxal phosphate (PLP) in buffer made up in D_2O, and the other enantiomer, **1b**, was produced by incubation of *dl*-[2-^{2}H]ornithine **4b** with enzyme and coenzyme in buffer prepared in unenriched water. The product of each decarboxylation was purified and each converted directly to the biscamphanamide, **7a** and **7b**, respectively.

The 200-MHz proton NMR spectrum of unlabeled **7** was obtained first in the presence of increasing amounts of the shift reagent $Eu(fod)_3$ (*36*), and it was determined that, at 32.5 mol% shift reagent, the resonances of the diastereotopic



SCHEME 2

hydrogens of the amido-methylene (corresponding to C-1 of putrescine) were cleanly separated (Fig. 1a). Because of the symmetrical nature of **7**, each of these resonances corresponds to two hydrogens. However, each enzymatically derived putrescine would contain, at most, only one deuterium. The spectra of **7a** and **7b** were then obtained under the same conditions. Integrations indicated 2.0 and 1.1 hydrogens in the downfield and upfield resonances, respectively, for **7a**, while for **7b** these resonances integrated for 1.0 and 2.0 hydrogens (Figs. 1b and c).

The presence of only one deuterium atom per molecule was confirmed by mass spectrometry. From the NMR experiments described above, it follows that the hydrogens introduced upon decarboxylation, deuterium in the case of **7a** and protium in the case of **7b**, correspond to that which produces the upfield resonance in the NMR spectrum. We have shown that this is due to the pro*R* hydrogen (*36*). Therefore, in the mammalian ODC reaction protonation takes place while still enzyme bound, and attack occurs on the *re* face of the imine intermediate **6a.** This is consistent with the bacterial and plant enzymes, and the overall reaction occurs with retention of configuration. *meso*-Diaminopimelic acid decarboxylase remains the only exception to the stereochemical homology observed for pyridoxal phosphate-dependent amino acid decarboxylases (*37*). The pyruvate-dependent amino acid decarboxylases also catalyze reactions that proceed with retention of configuration (*43, 44*).

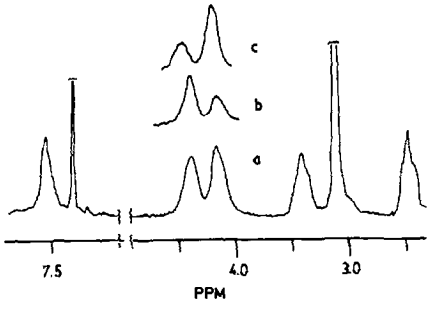


FIG. 1. 200-MHz ^{1}H NMR spectra. (a) **7** in $CDCl_3$, 32.5 mol% $Eu(fod)_3$. (b) **7a** in $CDCl_3$, 32.5 mol% $Eu(fod)_3$. (c) **7b** in $CDCl_3$, 32.5 mol% $Eu(fod)_3$.

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