Intermolecular and intramolecular isotope effects in the deamination of putrescine catalyzed by diamine oxidase

P.S. Callery, M.S.B. Nayar, E.M. Jakubowski and M. Stogniew

Department of Medicinal Chemistry/Pharmacognosy, University of Maryland, Baltimore (Maryland 21201, USA), 6 April 1981

Summary. The enzymatic deamination of 1,4-diaminobutane (putrescine) catalyzed by hog kidney diamine oxidase was studied with the aid of deuterium labeled substrates and mass spectrometry. An intermolecular deuterium isotope effect for the deamination of putrescine labeled with deuterium in all 4 alpha positions was observed to be 1.26. 1,4-Diaminobutane-1,1-d_2 was synthesized and intramolecular isotope effects determined. The preference of diamine oxidase for the unlabeled alpha position was about 4 times greater than for the deuterated methylene. This work shows that intramolecular deuterium isotope effects are observable in enzyme systems other than cytochrome P-450.

Diamine oxidase (EC 1.4.3.6) is the enzyme primarily responsible for deamination of histamine and other physiologically important diamines[2]. Since pyridoxal phosphate is regarded as a prosthetic group at the active center of diamine oxidase[3,4], a probable mechanism for deamination involves formation of an amine-pyridoxal imine[5]. With the assistance of a base, the next step is depicted as abstraction of a hydrogen from the alpha position of the amine in order to promote a prototropic shift and conversion into an aldehyde-pyridoxamine Schiff base. Hydrolysis of this imine yields deaminated substrate. With putrescine (**1**, 1,4-diaminobutane) as substrate, the product is 4-aminobutanal which rapidly forms Δ^1-pyrroline (**2**) by dehydration[6].

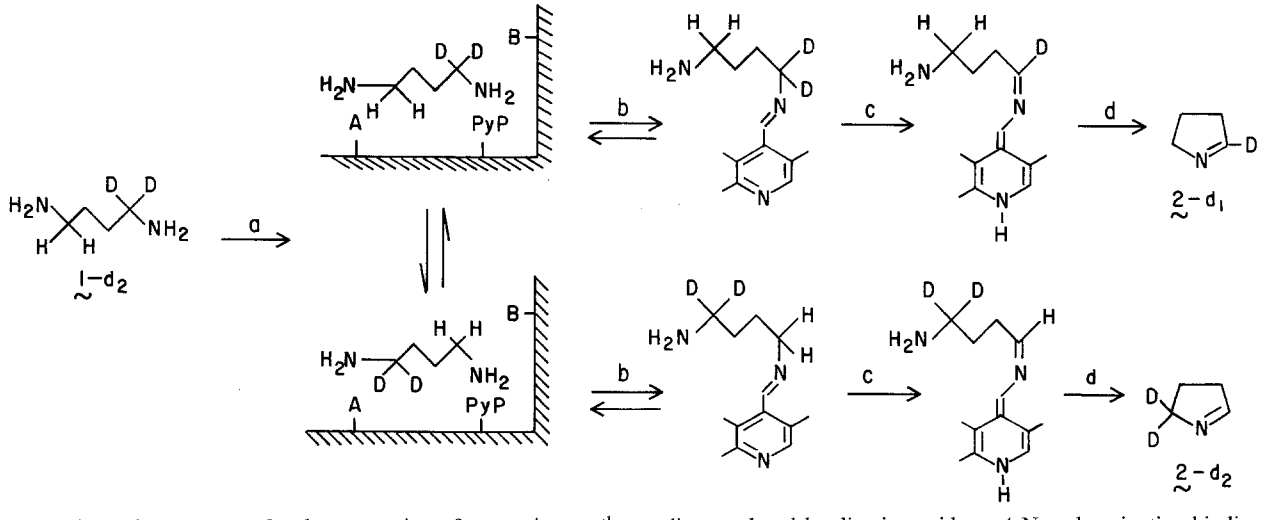
Applications of stable isotopes and mass spectrometry have yielded significant information on the nature of a number of complex biological systems[7]. For example, evaluation of differences in reaction rates resulting from deuterium incorporation has become an important tool in the understanding of mechanisms of enzymatic reactions[8]. Studies with stable isotopes on diamine oxidations catalyzed by hog kidney diamine oxidase have been reported[5,9]. Based on kinetic deuterium isotope and medium effects, Bardsley et al.[5] proposed that the rate limiting step in the oxidation of p-dimethylaminomethylbenzylamine with diamine oxidase involves breaking of a carbon-hydrogen bond.

Mass spectrometry is the method of choice for evaluating the influence of deuterium on enzyme reactions involving symmetrical substrates with 2 sites on the same molecule available for reaction, one deuterium labeled and the other unlabeled. With such a substrate, the degree of retention of label in the product provides an estimate of the intramolecular isotope effect on the relative rates of reaction of the isotope labeled site and unlabeled site.

Putrescine, which contains 2 equivalent methylene moieties that serve as sites for oxidation, is a symmetrical natural substrate for diamine oxidase. In this communication studies on the intermolecular and intramolecular isotope effects of diamine oxidase on isotopomers of putrescine are reported.

Materials and methods. Diamine oxidase (from porcine kidney) and putrescine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). 1,4-Diaminobutane-1,1,4,4-d_4(**1**-d_4) was prepared from succinonitrile following the procedure of Smith and Daves[10]. Isotopic purity of labeled putrescine was determined from electron impact mass spectra of N,N′-diheptafluorobutyryl derivatives[10] recorded on a CEC 21-110 mass spectrometer. GC-MS spectra were obtained using a Dupont 21-490 mass spectrometer coupled to a Varian 1400 gas chromatograph interfaced with a heated glass jet separator. Gas chromatography was carried out on a 1.5 m × 3 mm column of 3% OV-17 on chromosorb WHP with column temperature 100–200 °C programmed at 15 °C/min.

4-Phthalimidobutyronitrile (4.10 g, 19.2 mmole), was dissolved in 30 ml of a solution of ethanol-0-d, 10 ml D_2O and 3 ml of 20% DCl in D_2O. Platinum oxide (200 mg) was added and the reaction mixture shaken in a Paar apparatus under deuterium gas at 3 at initial pressure until deuterium gas was no longer taken up (approximately 36 h). The mixture was then filtered and the filtrate evaporated to give crude 4-(phthalimido)-1-aminobutane-1,1-d_2, which was hydrolyzed by refluxing in 20 ml of 6 N HCl for 1 h. The phthalic acid which precipitated on cooling to 0 °C was removed by filtration. The filtrate was evaporated to dryness, and the residue recrystallized from ethanol:H_2O (4:1) to yield 1.95 g (63%) of 1,4-diaminobutane-1,1-d_2



Proposed reaction sequence for the conversion of putrescine to Δ^1-pyrroline catalyzed by diamine oxidase. *A* Non-deaminating binding site, *B* base, *PyP* pyridoxal phosphate. Step a) orientations of putrescine-d_2 on active site of enzyme. Step b) imine formation with pyridoxal. Step c) tautomerization to aldehyde-pyridoxamine imine. Step d) hydrolysis and cyclization to form Δ^1-pyrroline.

dihydrochloride (**1**-d_2); melting point > 290 °C. The N,N'-diheptafluorobutyryl derivative yielded an isotopic composition of 0.9% d_0, 13.6% d_1, 80.6% d_2, and 5.0% d_3.

To a mixture of diamine oxidase (5 mg, 0.07 units/mg) in 10 ml of 0.067 M, pH 7.2 phosphate buffer was added putrescine dihydrochloride. After 2 h at 37 °C, 1 ml of 10 N hydrochloric acid was added to the solution. The denatured enzyme was removed by centrifugation and to the supernatant was added solid potassium cyanide (5 mg). After 30 min at room temperature the solution was made alkaline with solid potassium carbonate and extracted with CH_2Cl_2 (3 ml). The organic layer was dried over anh. Na_2SO_4 and then concentrated in a stream of nitrogen to a volume of 0.5 ml and treated with 0.1 ml trifluoroacetic anhydride to form N-trifluoroacetyl-2-cyanopyrrolidine[6]. After heating for 20 min at 60 °C, solvents and excess reagent were removed in a stream of nitrogen and the residue reconstituted in a small volume of CH_2Cl_2. Aliquots of this solution were analyzed by GC-MS.

Intermolecular isotope effects on product formation were calculated as the ratio of relative intensities of the ion current from the $(M\text{-}CF_3)^+$ ions of the derivatives N-trifluoroacetyl-2-cyanopyrrolidine (m/z 123) and N-trifluoroacetyl-2-cyanopyrrolidine-2,5,5-d_3 (m/z 126) corrected for trideuterated and pentadeuterated substrate. Intramolecular isotope effects were calculated as the ratio of $(M\text{-}CF_3)^+$ ions of N-trifluoroacetyl-2-cyanopyrrolidine-5,5-d_2 (m/z 125) and N-trifluoroacetyl-2-cyanopyrrolidine-2-d_1 (m/z 124) corrected for partially labeled substrate and ^{13}C natural abundance.

Results and discussion. Deuterium labeled putrescine was found to be oxidized with diamine oxidase to Δ^1-pyrroline at a slower rate than unlabeled putrescine. A more detailed study of the reaction was undertaken in order to evaluate the influence of enzyme catalyzed carbon-deuterium bond breaking on the overall reaction rate. The calculated ratio of the amounts of Δ^1-pyrroline formed from putrescine-d_4 (1,4-diaminobutane-1,1,4,4-d_4, **1**-d_4) and from unlabeled putrescine (**1**-d_0) yielded a deuterium isotope effect of 1.26. The results are summarized in table 1. Similar values were obtained in experiments in which **1**-d_4 and **1**-d_0 were incubated separately and then combined at the end of the incubation period or in which an equimolar mixture of **1**-d_4 and **1**-d_0 was incubated. In either case, the ratio of non-deuterated to deuterated Δ^1-pyrroline produced provided an estimate of the isotope effect. Since these values were derived from measurements obtained from products of 2 distinct substrates, **1**-d_4 and **1**-d_0, the results are appropriately termed intermolecular isotope effects. Although **1**-d_4 is clearly metabolized more slowly than unlabeled substrate, the low intermolecular isotope effect of 1.26 leaves some doubt regarding assignment of the isotope effect as primary or of involvement of carbon-deuterium bond breaking in the rate determining step in the reaction[11]. An intermolecular deuterium kinetic isotope effect of 2.2 for diamine oxidase has been determined by Bardsley et al.[5] employing p-dimethylaminomethylbenzylamine as substrate. Values for intermolecular isotope effects for other deaminating enzyme systems such as monoamine oxidase[12], plasma benzylamine oxidase[13], and cytochrome P-450 mediated oxidative deamination[14], typically fall in the range of 1–2.

Intramolecular isotope effects have been observed for a number of symmetrical substrates undergoing oxidation by the cytochrome P-450 system[15–18]. In studies where both intermolecular and intramolecular deuterium isotope effects were determined for the same substrates, calculated intramolecular effects were substantially larger than values derived from intermolecular studies. Foster et al.[15] calculated an intermolecular isotope effect of about 2 and an intramolecular effect of 10 for the 0-demethylation of deuterium labeled p-dimethoxybenzene. For the N-demethylation of N,N-dimethylphentermine, Miwa et al.[16] detected no intermolecular deuterium isotope effect. When the reaction was carried out with substrate labeled on one N-methyl group, intramolecular isotope effects of 1.6–2 were observed.

In order to determine if an intramolecular deuterium isotope effect is operative in a system other than cytochrome P-450, the deamination by diamine oxidase of putrescine labeled with 2 deuterium atoms in one of the alpha positions was monitored. Putrescine-d_2 (**1**-d_2) was prepared by catalytic reduction with deuterium gas of 4-phthalimidobutyronitrile followed by hydrolysis. Incubation of putrescine-d_2 with diamine oxidase produced Δ^1-pyrroline-5,5-d_2(**2**-d_2), formed from attack on the unlabeled alpha position of putrescine-d_2, and Δ^1-pyrroline-2-d_1 (**2**-d_1), which arose from deamination at the labeled alpha position (fig.). The intramolecular isotope effect in the deamination of putrescine-d_2 was calculated from a ratio of the products **2**-d_2 and **2**-d_1 corrected for partially labeled substrate. The results are summarized in table 2. The preference of diamine oxidase for the unlabeled side of putrescine-d_2 was about 4-fold that for the labeled methylene. Varying incubation time, substrate concentration and pH yielded intramolecular isotope effects ranging from 3.91 to 4.75.

As a means of explaining the observation of an intramolecular isotope effect, two interconvertible enzyme-substrate complexes are proposed in the figure. These structures are consistent with known details of the catalytic mechanism of diamine oxidase in that pyridoxal phosphate (PyP) appears to be included as part of the active center and a second binding site not directly associated with the deaminating site makes up part of the catalytic surface[3]. These interme-

Table 1. Intermolecular isotope effects in the oxidation of putrescine with diamine oxidase

Substrate	Intermolecular isotope effect
1-d_0 / **1**-d_4	1.26[a]
1-d_0 and **1**-d_4	1.27[b]

[a] Calculated as the product ratio of pyrrolines **2**-d_0/**2**-d_3 formed from putrescine-d_0 and putrescine-d_4 incubated separately. Total putrescine substrate concentration 5.8×10^{-3} M. [b] Calculated as the product ratio of pyrrolines **2**-d_0/**2**-d_3 formed from incubation of an equimolar mixture of putrescine-d_0 and putrescine-d_4. Total putrescine substrate concentration 5.8×10^{-3} M.

Table 2. Intramolecular isotope effects in the oxidation of putrescine with diamine oxidase

Concentration putrescine-d_2 (mM)	pH	Incubation time (min)	Intramolecular isotope effect[a]
5.8	7.2	120	4.44 ± 0.05[b]
5.8	7.2	30	3.91
5.8	7.2	60	4.34
1.5	7.2	120	4.02
2.9	7.2	120	4.14
5.8	6.0	120	4.17
5.8	8.0	120	4.75

[a] Intramolecular isotope effects were calculated as the product ratio of pyrrolines **2**-d_2/**2**-d_1 formed from putrescine-d_2. [b] Mean ± SD of 4 experiments.

diates represent a step closely preceding a prototropic shift which subsequently leads to deaminated putrescine. A rapid exchange between the 2 orientations on the enzyme surface would account for the observed intramolecular isotope effect.

Irrespective of the actual mechanism, it remains that in every experiment performed, intramolecular effects were greater than values obtained for intermolecular effects. The intermolecular isotope effect is probably a measure of the influence of deuterium on the overall reaction, while intramolecular effects, which are less influenced by events prior to interaction with the catalytic surface, are an indicator of the process closely associated with bond cleavage.

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Inducing effect of clofibrate on alkaline phosphatase and histidine-glyoxylate aminotransferase in rat liver

L.F. Panchenko, S.V. Popova and V.D. Antonenkov

Department of Biochemistry, All-Union Research Institute of Psychiatry, Kropotkinsky per. 23, Moscow 119034 (USSR), 29 June 1981

Summary. The activity of plasma membrane alkaline phosphatase and both mitochondrial and peroxisomal histidine-glyoxylate aminotransferase was significantly increased in the livers of male rats following treatment with the hypolipidemic drug clofibrate. Cycloheximide or puromycin administration to rats inhibited the effects of clofibrate.

The hypolipidemic drug clofibrate (ethyl-p-chlorphenoxy-isobutyrate) induces hepatomegaly and marked proliferation of peroxisomes and smooth endoplasmic reticulum[1]. Treatment with this drug results also in an increase in the activities of some peroxisome-associated enzymes; catalase, carnitine acetyltransferase and the peroxisomal fatty acyl-CoA oxidizing system[1-3]. However, there are few data concerning the effect of clofibrate on the enzyme activity in the other cell compartments[4]. In the present communication we study the effects of clofibrate administration to rats on the activity of liver alkaline phosphatase and histidine-glyoxylate aminotransferase.

Materials and methods. Male Wistar rats (250–300 g) were injected i.p. with saline (control) or clofibrate in a dose 250 or 800 mg/kg once daily in the course of 4–16 days as indicated in the text. Animals were anesthetized with light ether, and killed by decapitation after 16–18 h starvation. Livers were immediately removed, homogenized and fractionated by differential centrifugation as described previously[5,6]. Plasma membranes were isolated from perfused livers by the method of Toda et al.[7]. In some experiments liver subcellular particles (peroxisomes and mitochondria) were fractionated by isopycnic sucrose density gradient centrifugation[5]. Alkaline phosphatase; EC 3.1.3.1[7], D-amino acid oxidase; EC 1.4.3.3[8], acid phosphatase; EC 3.1.3.2[9], glucose-6-phosphatase; EC 3.1.3.9[10] and histidine-glyoxylate aminotransferase; EC 1.6.1[11] were determined at 37 °C. Catalase; EC 1.11.1.6[8], and carnitine acetyltransferase; EC 2.3.1.7[7] were determined at 25 °C. Chlorophenoxy isobutyric acid (sodium salt) was prepared by alkaline hydrolysis of clofibrate[12]. Protein was measured by the method of Lowry et al.[13].

Results and discussion. It was found that clofibrate treatment changes some peroxisomal enzyme activities (table). Thus, the activity of carnitine acetyltransferase was increased more than 20-fold, whereas the activity of D-amino acid oxidase was decreased to 10–20% of the control level. There are no pronounced changes in catalase activity after such treatment. However, the total activity of rat liver catalase in animals treated with clofibrate at a dose of

Effect of clofibrate treatment on the activities of hepatic enzymes

Enzyme		Activity (nmole/min/mg protein) Control	Clofibrate
Catalase, units*	(9)	0.298 ± 0.045	0.299 ± 0.058
D-amino acid oxidase	(12)	1.4 ± 0.4	0.4 ± 0.1**
Carnitine acetyltransferase	(5)	2 ± 1	48 ± 12**
Histidine-glyoxylate aminotransferase	(4)	1.0 ± 0.1	2.2 ± 0.2**
Glucose-6-phosphatase	(5)	68 ± 12	53 ± 8
Alkaline phosphatase	(4)	4.8 ± 1.2	16.3 ± 3.0**
Acid phosphatase	(12)	156 ± 18	147 ± 18

* Catalase activity was expressed as described[10]. Mean values ± SE are shown for specific activities, and the number of observations is given in parentheses. Clofibrate (250 mg/kg) was injected for 16 days. ** Increase statistically significant - $p \leq 0.001$.