

# Decarboxylation of $\alpha$ -difluoromethylornithine by ornithine decarboxylase

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The mechanism of inactivation of rodent ornithine decarboxylase by  $\alpha$ -difluoromethylornithine (DFMO) was studied using the inhibitor labelled with  $^{14}\text{C}$  in both the 1 and the 5 positions.  $[1\text{-}^{14}\text{C}]\text{DFMO}$  was a substrate and was decarboxylated by the enzyme yielding  $^{14}\text{CO}_2$ . A radioactive metabolite derived from  $[5\text{-}^{14}\text{C}]\text{DFMO}$  was bound to the enzyme, and the extent of binding paralleled the irreversible inactivation of ornithine decarboxylase. The partition ratio of decarboxylation to binding was approx. 3.3. These results provide support for the postulated mechanism of action of DFMO [Metcalfe, Bey, Danzin, Jung, Casera & Vevert (1978) *J. Am. Chem. Soc.* **100**, 2551–2553], in which enzymic decarboxylation of the inhibitor leads to the generation of a conjugated imine, which then alkylates a nucleophilic residue on the enzyme.

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## INTRODUCTION

Ornithine decarboxylase (ODC) is a key enzyme in the biosynthesis of polyamines and provides the only route for putrescine production *de novo* in animals (Jänne *et al.*, 1978; Pegg & McCann, 1982; Tabor & Tabor, 1984; Pegg, 1986). The striking inducibility of ODC in response to a very wide variety of growth-promoting stimuli, and the close correlation between growth and the biosynthesis of polyamines, have led to many studies of this enzyme (review by McCann, 1980; Russell, 1980, 1983; Pegg, 1986).  $\alpha$ -Difluoromethylornithine (DFMO) is a potent irreversible inhibitor of ODC (Metcalfe *et al.*, 1978; Bey, 1978). DFMO has been used extensively in studies of the physiological function of polyamines (reviewed by Heby, 1981; Pegg & McCann, 1982; Jänne *et al.*, 1983; Tabor & Tabor, 1984; Pegg, 1986), and has considerable potential for pharmacological use against parasitic protozoa and other micro-organisms and as an anti-neoplastic agent (McCann *et al.*, 1983; Jänne *et al.*, 1983; Sjoerdsma & Schechter, 1984; Sjoerdsma *et al.*, 1984; Williamson & Tysms, 1984; Tierney *et al.*, 1985).

DFMO was conceived as an enzyme-activated irreversible inhibitor of ODC. It was postulated that it would serve as a substrate for the enzyme and that the decarboxylation would produce an intermediate carbanionic species which would readily lose a fluorine atom, generating a highly reactive imine which could alkylate the enzyme at a nucleophilic residue at or close to the active site, causing permanent inactivation (Metcalfe *et al.*, 1978; Bey, 1978; Bey & Jung, 1986). Despite the extensive use of DFMO as an inhibitor of ODC, this mechanism has not been fully proved; however, it is supported both by kinetic studies (Metcalfe *et al.*, 1978; Seely *et al.*, 1982a) and by the covalent binding of radioactive DFMO (labelled in the 5 position with  $^{14}\text{C}$  or  $^3\text{H}$ ) to ODC, which parallels the loss of activity (Pritchard *et al.*, 1981; Seely

*et al.*, 1982a; Erwin *et al.*, 1983). This binding has proved to be a useful method for the determination of the number of molecules of ODC in crude tissue extracts and for assessing the purity of ODC preparations (Seely *et al.*, 1982a,b; Erwin *et al.*, 1983; Pegg, 1986), but the method is limited in scope, because many cells contain such small amounts of ODC protein that it is not practicable to quantify the radioactivity bound.

In the present paper we provide direct evidence for the decarboxylation of DFMO by ODC and have determined the partition ratio, i.e. the average number of turnovers of the substrate by the enzyme before inactivation occurs. These results provide strong support for the postulated mechanism of action of DFMO and indicate that the use of carboxyl-labelled DFMO provides a more convenient way for titrating the number of ODC molecules.

## EXPERIMENTAL

### Materials

L-[1- $^{14}\text{C}$ ]Ornithine (57 Ci/mol) was purchased from NEN, Boston, MA, U.S.A. DL-[5- $^{14}\text{C}$ ]DFMO (60 Ci/mol) was obtained from Amersham-Searle, Arlington Heights, IL, U.S.A. DL-[1- $^{14}\text{C}$ ]DFMO (18.3 Ci/mol) was synthesized from DL-[1- $^{14}\text{C}$ ]ornithine (Amersham-Searle) by a scaled-down version of the synthesis method of Bey *et al.* (1979). The purity of this material was assessed by analysing a sample with an amino acid analyser, and was found to be more than 90%. No trace of residual labelled ornithine was detected by this procedure. Unlabelled DFMO was generously given by Merrell Dow Research Institute, Cincinnati, OH, U.S.A. Biochemical reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

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Abbreviations used: DFMO, DL- $\alpha$ -difluoromethylornithine; ODC, ornithine decarboxylase (EC 4.1.1.17).

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**Table 1. Partition ratio and binding of DFMO to ODC from mouse kidney, thioacetamide-treated rat liver and L1210 cells**

Results are means  $\pm$  S.D. for at least four determinations.

Source of ODC	Partition ratio	Binding of [5- <sup>14</sup> C]DFMO (fmol/unit inactivated)
Rat liver	3.5 $\pm$ 0.6	21.0 $\pm$ 4.2
Mouse kidney	3.3 $\pm$ 0.3	12.2 $\pm$ 1.4
L1210	3.5 $\pm$ 0.4	13.4 $\pm$ 2.8

### Preparation and assay of ODC

ODC was purified from the kidneys of mice pretreated with androgens as described by Seely *et al.* (1982b) or from rat liver after induction with thioacetamide as described by Pritchard *et al.* (1981). Extracts of L1210 cells containing high ODC activity were obtained by serum stimulation as described by Pera *et al.* (1986). ODC activity was measured by the following release of <sup>14</sup>CO<sub>2</sub> from L-[1-<sup>14</sup>C]ornithine (Pritchard *et al.*, 1981) with an assay medium which contained 0.4 mM-L-[1-<sup>14</sup>C]ornithine (5 Ci/mol), 40  $\mu$ M-pyridoxal phosphate, 2.5 mM-dithiothreitol and 50 mM-Tris/HCl, pH 7.5. One unit of enzyme activity released 1 nmol of CO<sub>2</sub> in a 30 min incubation at 37 °C.

### Interaction of ODC with DFMO

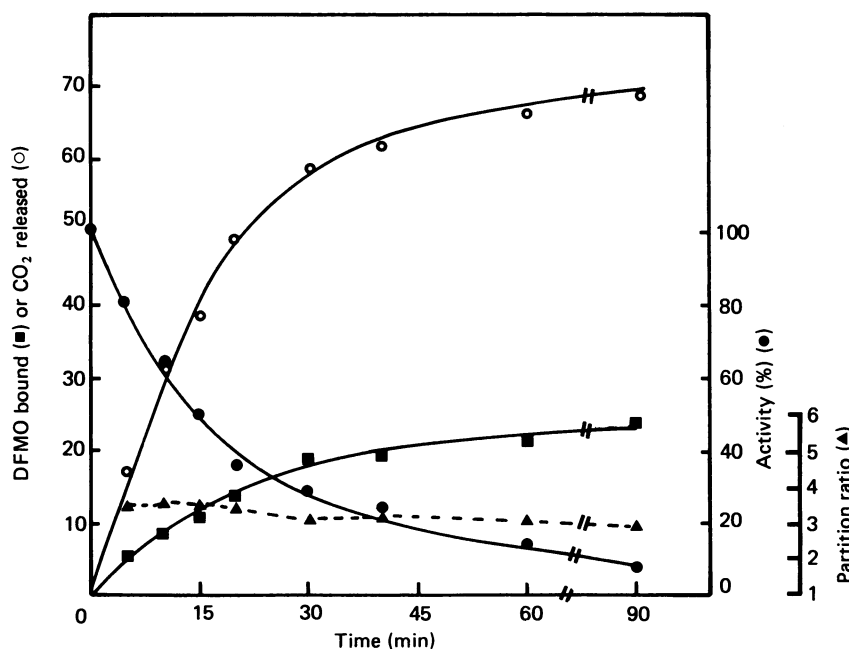
The decarboxylation of DFMO was measured in a similar way to the assay of ODC by replacing the labelled ornithine in the assay medium with 5  $\mu$ M-DL-

[1-<sup>14</sup>C]DFMO (18.3 Ci/mol). The binding of [5-<sup>14</sup>C]DFMO to ODC protein was determined as described by Seely *et al.* (1982a) after incubation of the ODC with 5  $\mu$ M-DL-[5-<sup>14</sup>C]DFMO (60 Ci/mol), 40  $\mu$ M-pyridoxal phosphate, 2.5 mM-dithiothreitol and 50 mM-Tris/HCl, pH 7.5.

### RESULTS AND DISCUSSION

When purified mouse kidney ODC was incubated with [1-<sup>14</sup>C]DFMO, <sup>14</sup>CO<sub>2</sub> was released in a time-dependent manner (Fig. 1), but after 60–90 min the production of <sup>14</sup>CO<sub>2</sub> ceased. This time period coincided with the time at which all of the ODC activity had been lost. Fig. 1 shows that there was a clear reciprocal relationship between the release of <sup>14</sup>CO<sub>2</sub> and the amount of ODC activity remaining. There was no release of <sup>14</sup>CO<sub>2</sub> from [1-<sup>14</sup>C]DFMO when albumin or *S*-adenosylmethionine decarboxylase was substituted for the ODC, and the maximal amount of <sup>14</sup>CO<sub>2</sub> released was proportional to the amount of ODC added (results not shown).

Parallel experiments were carried out with the same enzyme preparation, and the binding of [5-<sup>14</sup>C]DFMO to ODC protein was measured. As previously reported (Pritchard *et al.*, 1981), the binding of [5-<sup>14</sup>C]DFMO to ODC was proportional to the amount of enzyme inactivated. In the present experiments, the ratio of binding to units of enzyme inactivated in the experiment was 12.2 fmol/unit (Table 1), which is in reasonable agreement with the previously found value of 14 (Seely *et al.*, 1982a,b). As shown in Fig. 1, the time courses of release of <sup>14</sup>CO<sub>2</sub> from [1-<sup>14</sup>C]DFMO and of binding of [5-<sup>14</sup>C]DFMO to ODC were similar, and at all times the partition ratio between them was just over 3.



**Fig. 1. Time course of interaction of DFMO with ODC**

Mouse kidney ODC (2000 units) was incubated with 5  $\mu$ M-DL-[1-<sup>14</sup>C]DFMO or DL-[5-<sup>14</sup>C]DFMO as described in the Experimental section. At the times indicated, the remaining ODC activity (●), pmol of <sup>14</sup>CO<sub>2</sub> released from [1-<sup>14</sup>C]DFMO (○) and pmol of [5-<sup>14</sup>C]DFMO bound (■) were measured. The partition ratio of <sup>14</sup>CO<sub>2</sub> released to [5-<sup>14</sup>C]DFMO bound was also calculated as shown (▲).

A more precise estimation of this partition ratio was carried out by incubating the mouse kidney ODC for 90 min with a mixture of [5- $^{14}$ C]DFMO and [1- $^{14}$ C]DFMO. The partition ratio for eight separate estimations was  $3.3 \pm 0.3$  for mouse kidney ODC (Table 1). Similar experiments were carried out with ODC which had been partially purified from thioacetamide-treated rat livers and from L1210 cells in culture. As shown in Table 1, the partition ratio was between 3 and 4 for all sources of ODC tested, although for the rat liver enzyme the amount of [5- $^{14}$ C]DFMO bound per unit inactivated was somewhat greater than that found with the mouse ODC, in agreement with previous estimations (Seely *et al.*, 1982a).

Our results support the proposed mechanism for the inactivation of ODC by DFMO and show clearly that DFMO is a substrate for the enzyme. However, it should be noted that the structure of the ODC-DFMO adduct has not yet been determined, and that the last steps in the inactivation scheme proposed by Metcalf *et al.* (1978) remain unproven. Alternative pathways leading to the formation of a stable enzyme-inhibitor adduct after decarboxylation of the DFMO are discussed by Bey & Jung (1986).

Although the titration of the number of ODC molecules in a crude tissue extract by reaction with radiolabelled DFMO provides a convenient method to determine the amount of active ODC protein in a cell (Seely *et al.*, 1982a,b; Erwin *et al.*, 1983), this method has an inherent limitation, owing to the very low amount of ODC in many tissues. Even though DFMO can be labelled with  $^3$ H at very high specific radioactivity, samples containing little ODC activity can only be quantified if very large amounts of protein are processed for radioactivity determination and the efficiency of counting and washing of such samples is very poor. As the amount of protein per sample increases, consistent washing of such samples becomes difficult, leading to errors owing to non-specific binding, and the counting efficiency decreases drastically as well. Our results suggest that the same information could be obtained much more conveniently by determining the total release of  $^{14}$ CO $_2$  from [1- $^{14}$ C]DFMO and using the partition ratio to determine the number of molecules of ODC. This method has the advantage that the  $^{14}$ CO $_2$  that is released can be trapped in alkali and the radioactivity present determined with very high efficiency, irrespective of the amount of protein in the sample.

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