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RECOVERY OF ORNITHINE DECARBOXYLASE ACTIVITY AFTER INHIBITION WITH α-DIFLUOROMETHYLORNITHINE

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SUMMARY: α-Difluoromethylornithine is an effective inhibitor of polyamine biosynthesis because of its specificity for ornithine decarboxylase and the fact that its attachment to this enzyme is considered to be irreversible. We have found, however, that ornithine decarboxylase inactivated with this inhibitor in intact cells, as well as purified enzyme inactivated in vitro, both are capable of releasing this inhibitor and recovering enzyme activity. This reactivation can be initiated by freezing of inactivated enzyme samples in the presence of reducing agents at -7 or -20 °C and can be partially induced at 37 °C. These results reveal an unexpected lability of this enzyme-inhibitor complex that needs to be considered in future experimental designs.

α-Difluoromethylornithine (DFMO) was constructed as an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC; E.C. 4.1.1.17), a key enzyme in polyamine biosynthesis (1). The effectiveness and specificity of this compound have made it both a useful tool in research on the involvement of polyamines in varied physiological functions, and a promising drug in the treatment of infections by certain protozoans and abnormal proliferation associated with cancer (2,3). The predicted mechanism of action of this compound (1,4), which has recently been affirmed experimentally (5), involves decarboxylation of DFMO by the enzyme, followed by covalent addition of a metabolite to a nucleophilic group (cys-360) on the enzyme protein. The resultant DFMO-ODC adduct is catalytically inactive.

Due to the apparent stability of this DFMO-ODC adduct it has been common procedure for investigators to utilize [^{3}H]- or [^{14}C]DFMO in this reaction to tag ODC for cytological, pharmacological and biochemical investigations. We now report evidence that this inactivation is not irreversible, as was commonly believed, rather extensive DFMO metabolite is released, with concomitant recover of ODC activity, under certain experimental conditions.

ABBREVIATIONS USED: α-difluoromethylornithine, (DFMO); ornithine decarboxylase, (ODC).

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MATERIALS AND METHODS

Chemicals. DL-α-[3,4-^{3}H]DFMO (984 GBq/mmol) was obtained from DuPont-New England Nuclear. L-[1-^{14}C]Ornithine was obtained from ICN Radiochemicals. Dithiothreitol, β-mercaptoethanol, cysteine and other biochemicals were from Sigma Chemicals, and inorganics from Fischer Scientific. DFMO was a generous gift of Merrell Dow Research Institute, Marion Merrell Dow Inc.
Cell culture. Rat hepatoma (HTC) cells were grown in monolayer and suspension cultures in Swim's-77 medium containing 10% calf serum. The ODC-overproducing variant, DH23b, had 10 mM DFMO added to this medium (6). Chinese Hamster ovary (CHO) cells were maintained in Swim's-77 medium containing 2% fetal calf serum and 6% calf serum.
ODC activity. Cell pellets were homogenized by brief sonication in 0.02 M potassium phosphate buffer (pH 7.0) containing 2.0 mM dithiothreitol and 0.2 mM EDTA. ODC activity was assayed by measuring the release of $^{14}CO_2$ from L-[^{14}C]ornithine as described previously (7). One unit is defined as the release of 1 nmol $^{14}CO_2$/h.
Preparation of ^{3}H-labeled ODC. Either pure ODC (6) or partially purified enzyme preparations (7), as indicated, were incubated at 37°C for 3 h with 50 μCi [^{3}H]DFMO in assay buffer without ornithine. Excess DFMO was removed by extensive dialysis against the same buffer.
Release of ^{3}H from [^{3}H]DFMO-labeled ODC. Labeled ODC (0.03-0.05 μCi) was placed in 1.0 mL of the indicated buffer in an 1.5 mL microfuge tube. In experiments involving pure ODC, this buffer contained 0.1% bovine serum albumin as a carrier. After the indicated experimental treatment, replicate 50 μl samples were withdrawn and either counted directly by liquid scintillation counter or the protein fraction was precipitated with 15 % trichloroacetic acid at 4 °C and centrifuged for 10 min in a microfuge, and the supernatant counted. In freshly-prepared labeled ODC the initial acid-soluble counts were less than 3% of the total.

RESULTS AND DISCUSSION

We have been investigating ODC regulation in a variant of a rat liver (HTC) cell line that accumulates very high levels (approx. 1% of soluble protein) of this enzyme (6). Since this variant, DH23b, only grows in the presence of high concentrations of DFMO, enzyme assays on cell homogenates that have been dialyzed to remove unbound DFMO reveal that over 97 % of the ODC is inactive. However, as depicted in Fig 1, repeated cycles of freezing and thawing associated with subsequent use of the dialyzed homogenates resulted in substantial (sometimes >25-fold), progressive increases in their catalytic activity. The activity produced by this procedure was shown to be ODC by its complete inhibition upon re-exposure to DFMO. Further, treatment of this new activity with [^{3}H]DFMO resulted in a single labeled protein band matching ODC on SDS-PAGE gels (not shown). This twist in an enzymologist's usual concern, was observed in samples stored in -7 and -20°C freezers, and not in storage conditions that precluded ice crystal formation, such as maintenance at 4°C or freezing at -7 or -20°C in 30 % glycerol. Similarly, very rapid freezing of samples in liquid N_2 or a -80°C freezer did not induce any increases in ODC activity.

The following experiments demonstrate that the observed increase in ODC activity is associated with a concomitant loss of DFMO metabolite from

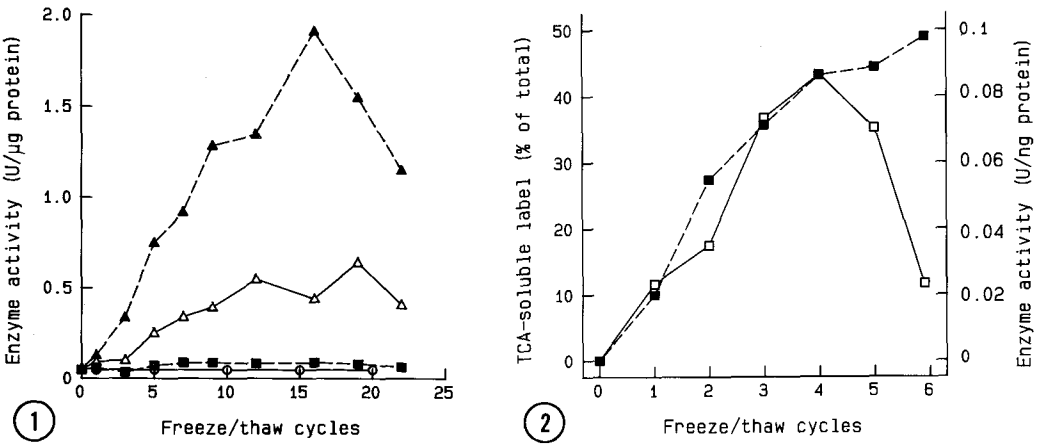

Fig. 1. Increase in ODC activity following repeated freezing and thawing of samples. Dialyzed homogenates of DH23b cells that had been cultured with 10 mM DFMO were repeatedly frozen (0.5-1.0 mL per 1.5 mL microfuge tube) at -7°C (▲), -20°C (Δ), -7°C + 30 % glycerol (■) or liquid nitrogen (O). After the indicated treatment period samples were assayed for ODC activity as indicated in the text.

Fig. 2. Release of ^3H and associated increase in enzyme activity in pure ODC preparations. ODC purified from DH23b cells was inactivated with [^3H]DFMO, dialyzed and subsequently subjected to cycles of freezing and thawing at -7°C. Samples were assayed for enzyme activity (□) and trichloroacetic acid-soluble ^3H (■).

previously inactivated enzyme. ODC purified from DH23b cells was inactivated by incubation with [^3H]DFMO and subsequently dialyzed to remove unbound inhibitor. Both FPLC (Pharmacia) ion exchange and SDS-PAGE analysis indicated that all label in this preparation was bound to ODC. During a course of 6 cycles of slow freezing (at -7°C) followed by thawing at 37 °C, a substantial portion of the ^3H-label was released to trichloroacetic acid-soluble material (Fig 2). The release of DFMO metabolite was associated with a progressive increase in ODC activity similar to that noted in the dialyzed DH23b cell homogenates. In both situations ODC activity eventually declined, as expected from the adverse affects of repeated freezing and thawing of enzyme preparations.

The concomitant release of the DFMO metabolite and increase in ODC activity was not restricted to enzyme prepared from the DH23b variant cells. ODC was prepared from both the parental HTC cell line and Chinese hamster ovary (CHO) cells, and inactivated with labeled DFMO. During the course of freezing and thawing as described above up to 90 % of the ^3H-DFMO metabolite was released with recovery of 25-35% of the original ODC activity.

The release of DFMO metabolite from inactivated ODC is greatly stimulated by dithiothreitol, cysteine or 2-mercaptoethanol, but not reduced glutathione (Table I). Poulin et al. (5) suggested that DFMO inactivation of ODC is mainly through the alkylation of cysteine-360. It is conceivable that,

Table I. Effect of reducing agents on removal of DFMO from ODC. [^{3}H]DFMO was used to inactivate partially purified ODC from DH23b cells. The amount of ^{3}H appearing in the acid-soluble fraction was evaluated following 6 freeze/thaw cycles (-7°C), experiment #1; 6h at 4°C, experiment #2a; or 6h at 37°C, experiment #2b. In experiment #2 the buffer was 0.5 M potassium phosphate (pH 7.0).

Experiment	Reducing agent	^{3}H released (% of total)
1	< 0.1 mM dithiothreitol	7.2
	5 mM dithiothreitol	74.3
	5 mM β-mercaptoethanol	21.8
	10 mM reduced glutathione	1.7
2a(4°C)	5 mM dithiothreitol	0.3
	250 mM dithiothreitol	5.4
	250 mM cysteine	4.7
2b(37°C)	5 mM dithiothreitol	21.3
	250 mM dithiothreitol	48.7
	250 mM cysteine	43.4

under appropriate conditions, this DFMO metabolite can be subsequently exchanged to other small sulfhydrals such as 2-mercaptoethanol or dithiothreitol. By this extension of the mechanistic scheme proposed by Poulin et al. (5) we might expect to observe the respective S-((2-(1-pyrroline))methyl) derivative as the soluble product once the remaining DFMO-ODC adduct has been precipitated by 10% TCA. We examined the dithiothreitol-released product from [^{3}H]DFMO-ODC adduct using anion-exchange chromatography (Mono-S; Pharmacia) with a 0.02 M PO_4 (pH 5.5) buffer and a salt gradient from 0 to 0.5 M NaCl. Consistent with the basic nature of the expected product we found the ^{3}H-labeled compound to bind to this column and elute in a single peak at a higher NaCl concentration than ornithine or DFMO, and well before putrescine or methylputrescine.

It is not immediately obvious why this sulfhydryl exchange should be facilitated by slow freezing of the enzyme samples. The DFMO-ODC adduct was found to be stable in this buffer under more extreme, and even denaturing, conditions including ammonium sulfate precipitation, heating to 60 °C for 3 h and NaCl to 2 M. Slow freezing of buffers can result in solute concentration as water molecules are selectively withdrawn during the formation of ice crystals. Possibly a substantial elevation in buffer salts or reducing agent is responsible for the release of DFMO metabolite from ODC. Consistent with this model, elevated concentrations of reducing agents did stimulate at least partial release of the ^{3}H-labeled DFMO metabolite during incubation at 37 °C. Inactivation of ODC by DFMO involves covalent attachment of the DFMO metabolite, consequently, it was not anticipated that this inhibitor could be removed without destroying the catalytic activity of the enzyme. That this reactivation occurs readily under certain sample storage conditions, and in

elevated levels of reducing agents, makes this a potentially confounding reaction that must be considered in planning DFMO inhibition studies and in handling of DFMO-inhibited tissues. Although the in vitro release of DFMO metabolite could have devastating effects on experiments involving storage of samples before autoradiography or biochemical and enzymatic analyses, it is not clear to what extent this reversal could affect DFMO-inhibition studies in intact tissue. Since reactivation can proceed to a certain extent at physiological temperatures, it is possible that a similar sulfhydryl substitution reaction could affect DFMO action in vivo; however, such reversal of DFMO inhibition may be rather minor in comparison to the normally rapid turnover of ODC protein.

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