SELECTIVE INACTIVATION OF *PHYSARUM* A-FORM ORNITHINE DECARBOXYLASE BY α-DIFLUOROMETHYLORNITHINE

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1. Introduction

The regulation of eukaryotic ornithine decarboxylase (EC 4.1.1.17), the rate-limiting enzyme in the biosynthesis of the polyamines putrescine, spermidine and spermine, is complex and incompletely understood. Rapid enzyme activity loss, especially in the presence of elevated polyamine levels, is thought to involve a small ornithine decarboxylase-regulating protein such as the 'ODC-antizyme' of mammalian cells [1,2], or the A-to-B converting factor of *Physarum polycephalum* [3,4]. In this latter, primitive eukaryotic system, the ornithine decarboxylase-modulating protein is stimulated by polyamines to modify this enzyme such that its affinity for the obligate coenzyme, pyridoxal 5′-phosphate (PLP), is severely diminished. Thus, even though total ornithine decarboxylase protein is rather stable in *Physarum*, it is rapidly and reversibly converted between the normal (A-form) and modified (B-form) states in response to external polyamines [5], altered media osmolarity [6], protein synthesis inhibitors [7], and normal progression through the mitotic cycle [8].

The enzyme modification induced by this modulating protein is therefore believed to be a critical control mechanism in normal polyamine biosynthesis. Until now, however, we have not been able to demonstrate that this in vivo enzyme alteration actually does result in an equally rapid change in the enzyme catalytic activity within the cell. In these experiments we utilized an enzyme-activated irreversible inhibitor or ornithine decarboxylase, α-difluoromethylornithine (DFMO) to specifically inactivate the catalytically active enzyme within this organism. This study not only supports the hypothesis that the B-form of this enzyme is catalytically inactive in vivo, but it greatly increases our understanding of the cellular response to this inhibitor.

In vitro studies suggest ornithine decarboxylase accepts DFMO as an alternate substrate [9]. During decarboxylation of this analog, however, a highly unstable intermediate is formed that alkylates the enzyme at or near its catalytic site resulting in inactivation. The irreversible nature of this specific polyamine synthesis inhibitor has prompted its wide use in investigations into the function of cellular polyamines and the control of their biosynthesis [10,11] as well as direct medical application in tumor [12,13] and leukemia [14] chemotherapy and eradication of parasitic protozoa [15]. Surprisingly little is actually known of DFMO's action against ornithine decarboxylase in vivo, and it is perplexing that even at high, prolonged doses this compound does not block polyamine synthesis completely [11,16,17]. These results demonstrate that cellular ornithine decarboxylase may indeed vary widely in sensitivity to DFMO depending on the state of the enzyme.

2. Experimental

Pyridoxal 5′phosphate, L-ornithine, 4-(2-hydroxyethyl)-1-piperazine-propanesulfonic acid (Hepps), dithiothreitol, EDTA, and the chloride salts of putrescine and spermidine were purchased from Sigma Chemical Co.; and L-[1-^{14}C]ornithine · HCl (50 Ci/mol) from Amersham/Searle Corp. D,L-α-Difluoromethylornithine (DFMO) was a generous gift from the Merrell-National Labs (Cincinatti OH).

Cultures of *P. polycephalum* were maintained and sampled as in [18]. Only exponentially-growing shake-flask cultures of asynchronous microplasmodia were used.

Microplasmodia (4–5 ml samples) were removed

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from their culture media by centrifugation at 200 × *g* for 30 s and immediately resuspended in 10 ml 80 mM NaCl in water at 4°C. This was centrifuged again, decanted and the pellet frozen in liquid nitrogen.

The details of the assay procedure have been reported [6]. A-form enzyme activity was determined using 0.01 M Hepps buffer (pH 8.0)/0.5 mM dithiothreitol/0.5 mM EDTA/0.5 μM pyridoxal 5′-phosphate/0.1 mM L-[1-^{14}C]ornithine (0.02 μCi). Total assayable enzyme was determined using pH 8.4 Hepps and 100 μM pyridoxal 5′-phosphate along with the EDTA and dithiothreitol. The difference between these assays was attributed to the B-form enzyme. One unit of activity was designated to be 1 nmol CO_2 released/h.

Protein concentrations were determined on 50 μl samples of the crude enzyme homogenates using the Bio-Rad protein assay, which follows [19].

^{14}C-Labeled DFMO was obtained by special contract with Amersham/Scarle Corp. and the Merrell-National Labs. Labeled DFMO (1 mCi/mmol) was added to the media of exponentially-growing *Physarum* cultures to a final concentration of 0.1 mM. Samples (5 ml) were withdrawn immediately after label addition, and again 1 h later, and rapidly washed 6 times in 80 mM NaCl in water at 4°C. After each wash the cellular material was pelleted by centrifugation for 20 s at 1000 × *g*. The washed pellets were oven-dried in glass scintillation vials and counted with 1 ml H_2O and 5 ml scintillation cocktail (Research Products International 3a70B) at ~40% efficiency.

3. Results

Since the irreversible inhibition by DFMO is contingent upon ornithine decarboxylase action on this inhibitor, it follows that conditions which limit this enzyme's activity will also prevent the inactivation of this enzyme. Thus the A-form of *Physarum* ornithine decarboxylase (which has a K_m^{PLP} = 0.05 μM under these conditions) is readily inactivated by this inhibitor in vitro when catalyzed in the presence of either 0.25 or 20 μM PLP, yet the B-form (which has a K_m^{PLP} = 12.7 μM) is quite insensitive to DFMO at the lower coenzyme level (fig.1).

This correlation between functioning enzyme and inactivation by DFMO was then used in an attempt to determine the relative activities of these two enzyme forms in vivo. We anticipated that either both forms

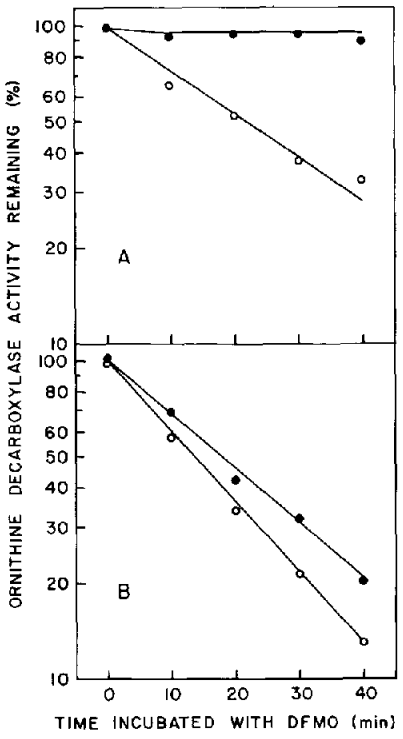

Fig.1. DFMO-inactivation of ornithine decarboxylase in crude homogenates containing two different levels of coenzyme. DFMO (0.1 mM) was added to samples of freshly homogenized *Physarum* microplasmodia that were identical except one contained 0.25 μM PLP (A) and the other 20 μM PLP (B). These crude enzyme fractions were subsequently incubated at 25°C, with samples extracted at the indicated times and assayed for A-form (○) and B-form (●) activity.

of this enzyme would be equally sensitive to DFMO in vivo, or else only the A-form would be active and therefore DFMO sensitive. The addition of 5 mM DFMO to cultures of *Physarum* resulted in a loss of >95% of the total ornithine decarboxylase activity in 3 h (fig.2). Since these cultures initially contained a portion of their enzyme in the B-form (~45% in the experiment of fig.2) it appeared at first that this form must also be sensitive to DFMO in vivo. However, if both forms of this enzyme were equally sensitive to cellular DFMO then the rate of enzyme inactivation should have been unchanged by conditions which induce modification of the enzyme to the B-form. Yet the addition of 0.1 mM spermidine, which rapidly promotes A-to-B form conversion in vivo, quickly blocked any additional ornithine decarboxylase inactivation, even in culture pre-exposed to this inhibitor for 90 min (fig.2).

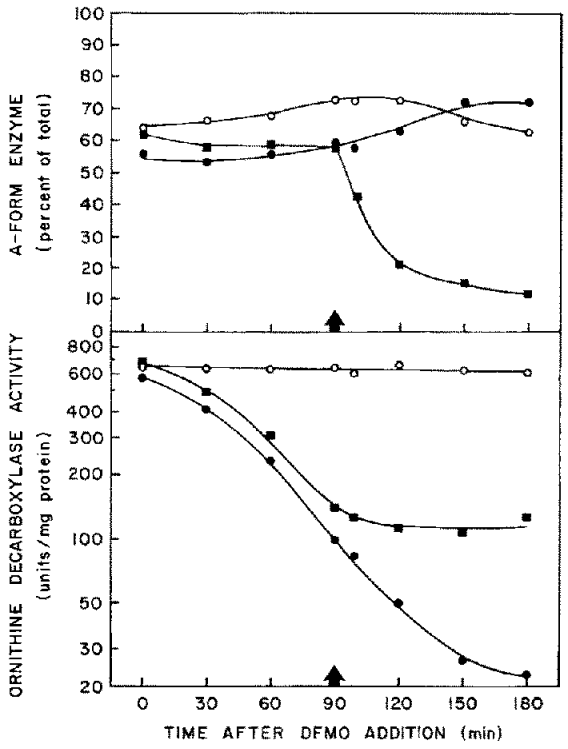

Fig.2. In vivo DFMO-inactivation of ornithine decarboxylase. DFMO (5.0 mM) was added to the media of two culture flasks of exponential-phase *Physarum* microplasmodia (●,■). A third culture (○) served as a control. After 90 min DFMO exposure, 0.1 mM spermidine was also added to one of the cultures (■). At the designated times samples were extracted, washed, frozen and eventually assayed for both the A- and B-forms of this enzyme. The top panel shows the variation in A-form enzyme as % of total. The lower panel illustrates the loss in total (A + B-form) ornithine decarboxylase activity due to DFMO addition.

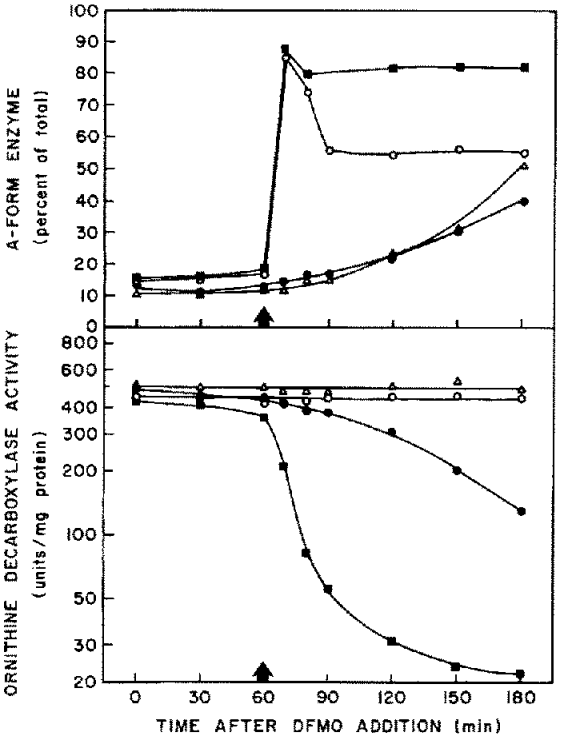

Fig.3. Altered in vivo DFMO sensitivity with respect to the form of ornithine decarboxylase. Four exponentially growing cultures of *Physarum* were treated with 0.1 mM spermidine to induce the modification of their ornithine decarboxylase to predominantly the B-form. After 90 min 5 mM DFMO was added to 2 of these cultures (●,■). After 60 min of this DFMO treatment, when the cultures were still relatively insensitive to this inhibitor, one of the DFMO-treated cultures (■) and one of the controls (○) were centrifuged and their cellular material resuspended in culture flasks containing distilled water. Samples were assayed and data presented as in fig.2.

Spermidine does not directly inhibit cellular uptake of this inhibitor. ^{14}C-Labeled DFMO presented at 0.1 mM was found to be incorporated at the rate of 632 ± 53 (11) pmol . mg protein^{-1} . h^{-1}, compared to 636 ± 32 (5) pmol . mg protein^{-1} . h^{-1}, in the presence of 1 mM spermidine. Rather, the induction of DFMO insensitivity appeared to be directly related to the conversion of A-form enzyme to the B-form since the elevation of media osmolarity (following [6]) or the addition of cycloheximide [7] also immediately blocked enzyme sensitivity to DFMO (not shown).

Conversely, conditions which promoted the conversion of ornithine decarboxylase B-to-A form in vivo resulted in the rapid inactivation of this enzyme by DFMO (fig.3). The ornithine decarboxylase of *Physarum* cultures exposed to 1 mM spermidine remained predominantly in the B-form and subsequently insensitive to DFMO for many hours. However, at 0.1 mM spermidine A-form enzyme began to reappear after only ~2.5 h. As shown in fig.3 the increase in A-form was closely matched by a decrease in total enzyme activity in the presence of DFMO.

We had shown that very rapid conversion of B-form ornithine decarboxylase to A can be induced by suspending cells in low ionic strength media [6]. As shown in fig.3 the placement of *Physarum* microplasmodia that were pre-exposed to 0.1 mM spermidine and DFMO into distilled water, with neither spermidine nor DFMO, resulted in a sudden increase in

the A-form of the enzyme. This B-to-A form conversion was followed closely by unusually rapid inactivation of total ornithine decarboxylase enzyme activity in these cells.

4. Discussion

These experiments suggest that only the unmodified, A-form of *Physarum* ornithine decarboxylase is directly inactivated by DFMO in vivo, while the B-form of this enzyme must first be converted to A to be sensitive to this inhibitor. The relative insensitivity of B-form enzyme to DFMO strongly supports our previously untested contention that the post-translational enzyme modification, induced by the A-to-B form converting factor, does render this enzyme catalytically inactive in vivo.

Since the A-form appears to be much more sensitive to DFMO inactivation one might have expected to observe the proportion of total enzyme in this form to diminish sharply during this inactivation, yet this was not observed here. An explanation fully consistent with our observations is that as the DFMO reacted with, and reduced the levels of, catalytically active A enzyme form it lowered local, free cellular polyamine levels. This is likely to have stimulated additional conversion of the B-form of the enzyme to the A-form, thereby maintaining, or even increasing (as in fig.2), the fraction of total remaining enzyme in the A-form. For example, placement of *Physarum* microplasmodia in low ionic strength media, as in the control culture of fig.3, rapidly stimulated almost complete activation (B-to-A form conversion) of this enzyme, followed by a gradual re-establishment of 60% A-form ratio that is normal for a culture of this age in the absence of added polyamines. By contrast the DFMO-treated culture, which was similarly resuspended and had almost all its enzyme converted to the A-form, was greatly limited in the amount of putrescine it could synthesize. Therefore, the polyamine-stimulated A-to-B form converting factor was not activated in this culture and the percent A-form enzyme remained very high.

It was noted, as in the first 60 min of the experiment in fig.3, that a small fraction of A-form activity remained insensitive to in vivo A-to-B form modification and was similarly not inactivated by DFMO. We are now investigating whether this represents a refractory state of a fraction of the A-form enzyme due to a constraint within the cell, or conversely, a small portion of cellular B-form enzyme that was activated to A-form during tissue homogenization and enzyme isolation.

Direct extrapolation of these results to mammalian tissues is somewhat hindered by the current dearth of information on the disposition and reversibility of modified ornithine decarboxylase forms in these cells. If, as it currently appears, antizyme-bound enzyme or perhaps the 'cryptic' form [20] indeed are found to represent reversibly inactivated enzyme states similar to *Physarum* B-form, then we may observe that the close correlation between the state of the enzyme and its sensitivity to DFMO is a general eukaryotic phenomenon. Agents which stimulate modification of ornithine decarboxylase and temporarily reduce its cellular activity would make these enzyme molecules relatively insensitive to short DFMO exposure or levels of this inhibitor that could be quickly reduced by cellular degradation. Conversely, maximal sensitivity to low concentrations of this inhibitor should accompany enzyme-activating conditions. Indeed, such variable sensitivity to DFMO has been reported in human cells [17]. Further work along these lines is needed to attain greater understanding of the in vivo sensitivity of ornithine decarboxylase to DFMO in order to maximize the usefullness of this drug in medicine and research.

Acknowledgements

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