Polyamine-stimulated alteration of the ornithine decarboxylase molecule in *Physarum polycephalum*

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The molecular mechanism for polyamine-stimulated feedback modification of ornithine decarboxylase isolated from *Physarum polycephalum* was investigated by using two-dimensional polyacrylamide-gel electrophoresis. Partially purified A-form enzyme was converted into the B-form enzyme by isolated fractions of the *Physarum* A–B-converting protein, and the substrates and products were subsequently labelled by covalent addition of α -difluoro[^{14}C]methylornithine, an enzyme-activated irreversible inhibitor. The active (A-form) and inactive (B-form) states of this enzyme were found to have the same M_r value, 52 000, yet they differed noticeably in their pI values, 5.45 and 5.65 respectively. In further experiments, the use of high-specific-radioactivity [^3H]spermidine to stimulate this enzyme modification was shown not to result in the covalent attachment of this polyamine to ornithine decarboxylase. These results demonstrate that the polyamine-induced modification of ornithine decarboxylase in *Physarum* is not due to any of the mechanisms previously suggested for ornithine decarboxylase inactivation in this and other eukaryotes, namely phosphorylation, covalent polyamine addition or the non-covalent association of a specific low- M_r protein.

The polyamines spermidine and spermine, and the diamine putrescine, are essential for eukaryotic cell growth. Variations in their cellular concentrations are intimately associated with growth-rate changes and abnormal cell stages (for references see Heby, 1981). Maintenance of precise cellular concentrations of these polyamines is frequently attributed to careful modulation of L-ornithine decarboxylase (EC 4.1.1.17), the initial enzyme in their biosynthetic pathway. Although this enzyme is not directly inhibited *in vitro* by concentrations of polyamines equivalent to cellular values, exogenous concentrations several orders of magnitude less will induce rapid losses of its activity *in vivo*. Rather indirect evidence suggests that polyamines might inhibit ornithine decarboxylase synthesis at the level of transcription or translation (Clark & Fuller, 1975; Kallio *et al.*, 1977; McCann *et al.*, 1979); however, somewhat stronger evidence supports polyamine modulation of this enzyme's activity through increasing the rate of enzyme inactivation or degradation. Several possibilities have been suggested for the mechanism of this polyamine feed-

back control. In vertebrate tissue and mammalian cells in culture, polyamines appear to induce the synthesis and/or release of an ornithine decarboxylase antienzyme that binds and conceivably aids in the inactivation of this enzyme (Heller *et al.*, 1976; McCann *et al.*, 1977; Pegg *et al.*, 1978; Kallio, 1978; McCann *et al.*, 1980; Heller & Canellakis, 1981). Alternatively, putrescine is thought to inactivate ornithine decarboxylase through its covalent addition to the enzyme by existing transglutaminases (Russell, 1981). Finally, Atmar & Kuehn (1981) have suggested that polyamines stimulate a particular protein kinase that phosphorylates and thereby inactivates ornithine decarboxylase. The extremely small cellular amount of ornithine decarboxylase has greatly hindered attempts to detect such enzyme modifications or to pinpoint exact enzyme protein changes.

We have studied feedback regulation of ornithine decarboxylase activity in the primitive eukaryote *Physarum polycephalum* (Mitchell & Carter, 1977; Mitchell *et al.*, 1978a; Mitchell & Kottas, 1979). This enzyme is subject to a reversible modification *in vivo* that interconverts it between an active state (A-form) and a state requiring unphysiologically high concentrations of coenzyme (form-B) (Mitchell

Abbreviations used: DFMO, α -difluoromethylornithine; SDS, sodium dodecyl sulphate.

et al., 1981a). We have subsequently isolated and partially purified the protein that catalyses the conversion of the A-form into the B-form and have characterized this reaction's absolute requirement for polyamines (Mitchell *et al.*, 1981b, 1982). In the present paper we report studies undertaken to elucidate the biochemical modification in ornithine decarboxylase that is catalysed by this modifying enzyme. These studies clearly demonstrate that the mechanisms previously suggested for ornithine decarboxylase inactivation, such as antienzyme complexing or the covalent addition of polyamines or phosphate, are not involved in this enzyme-inactivating process.

Experimental

Chemicals

Pyridoxal 5'-phosphate, L-ornithine, Hepps [4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid], dithiothreitol, EDTA and proteins for M_r standards were purchased from Sigma Chemical Co. L-[1- 14 C]Ornithine (50 Ci/mol), α -difluoromethyl-[5- 14 C]ornithine ([14 C]DFMO; 60 Ci/mol) and [terminal methylene- 3 H(n)]spermidine (300 Ci/mol) were from Amersham/Searle Corp. Bio-Gel P-2 and P-10 were from Bio-Rad Laboratories. Ultrogel AcA-44 and Ampholines were purchased from LKB Laboratories.

Assay of ornithine decarboxylase activity

The assay was as previously described by Mitchell & Kottas (1979) and Mitchell (1983). A-form enzyme activity was determined with the assay buffer 10 mM-Hepps/NaOH (pH 8.0)/0.5 mM-dithiothreitol/0.5 mM-EDTA/0.5 μ M-pyridoxal 5'-phosphate/0.1 mM-L-[1- 14 C]ornithine (0.02 μ Ci). Total assayable enzyme was determined with assay medium containing Hepps buffer, pH 8.4, and 100 μ M-pyridoxal 5'-phosphate along with the EDTA and dithiothreitol. The difference between the activities observed in these assays was attributed to the B-form enzyme. One unit of activity was designated to be 1 μ mol of CO_2 released/min at 25°C.

Purification of ornithine decarboxylase

Enzyme fractions containing at least 5 units-mg of protein were prepared from *Physarum polycephalum* plasmodia as described previously by Mitchell *et al.* (1982) and Mitchell (1983).

Preparation of A-form-B-form-converting protein

Partial purification of the A-B-converting (ornithine decarboxylase-modifying) protein of *Physarum* was performed by the procedures described by Mitchell *et al.* (1982).

Two-dimensional gel electrophoresis

Samples (approx. 0.13 unit) of partially purified (5 units/mg of protein) ornithine decarboxylase were incubated for 2 h at 20°C in the presence of 50 μ M-pyridoxal 5'-phosphate and 0.042 μ mol (2.5 μ Ci) of [14 C]DFMO. Excess of labelled DFMO was removed by Bio-Gel P-10 filtration. Isoelectric focusing was performed in 12 cm thin-tube acrylamide gels with a mixture of pH 5–7 and 3–10 Ampholines in the presence of 9 M-urea and 3% (w/v) Nonidet P40. Gels were pre-electrophoresed for 1 h to establish the gradient before the application of samples, and then run for 10.5 h at 1 W (600 V). The pH gradient was determined by cutting identical gels into 1 cm sections, eluting each of these with 1 ml of boiled distilled water and measuring their resultant pH.

The isoelectric-focusing gels were laid on top of a 10%-acrylamide/0.1%-SDS vertical slab gel (Bio-Rad Laboratories, model 220) by the method of O'Farrell (1975) with 1% agarose and 1% 2-mercaptoethanol in sample buffer. M_r standards were also applied on one side of the slab: phosphorylase b (M_r 92 500); bovine serum albumin (M_r 68 000); ovalbumin (M_r 43 000); carbonic anhydrase (M_r 30 000); soya-bean trypsin inhibitor (M_r 21 500); lysozyme (M_r 14 700). Electrophoresis was at 30 mA constant current (12.5 mA/cm² gel cross-sectional area). Gels were fixed in methanol/acetic acid/water (3:1:6, by vol.) and stained with Coomassie Brilliant Blue R-250. Destained gels were dried over pastel paper (Arches no. 90, rough) and radioautographed by direct contact with X-ray film (Kodak SB) for 2–4 weeks.

Results

Our objective was to compare the physical characteristics of *Physarum* ornithine decarboxylase before and after its modification by the A-B-converting factor described previously (Mitchell *et al.*, 1981b). In order to quantify and identify the native and denatured ornithine decarboxylase accurately, it was necessary to label this enzyme through its specific covalent attachment to an enzyme-activated irreversible inhibitor, 5- 14 C-labelled DFMO, as demonstrated previously (Pritchard *et al.*, 1981; Seely *et al.*, 1982; Mitchell, 1983). The experiments summarized in Table 1 show the correlation between ornithine decarboxylase inactivation and [14 C]DFMO binding to protein in these partially purified enzyme fractions. The experiment of Fig. 1 shows that this labelling is specific, with almost all of this label attached to protein of M_r 52 000 and only a small fraction, perhaps a proteolytic product, chromatographing at about M_r 50 000. Assuming only one active site per subunit of

Table 1. *Stoichiometry of [5-¹⁴C]DFMO binding and enzyme inactivation in ornithine decarboxylase isolated from *Physarum polycephalum**

Samples of five different ornithine decarboxylase purifications were each placed in 10mM-potassium phosphate buffer, pH 8.0, containing 5mM-dithiothreitol, 10% (v/v) glycerol 10mM-KCl, 0.1–1.0mM-[5-¹⁴C]DFMO and 100μM-pyridoxal 5'-phosphate. After 3h at 26°C, samples were removed to determine the total loss of ornithine decarboxylase activity, and the remaining samples were diluted 5-fold and extensively dialysed against buffer without DFMO. Fractions of the dialysed samples were counted for radioactivity by liquid-scintillation techniques and subjected to SDS/polyacrylamide-gel electrophoresis to demonstrate the specificity of the labelling. The calculated specific activity of homogeneous ornithine decarboxylase assumes the M_r of the catalytically active subunit to be 52000.

Expt. no.	Ornithine decarboxylase inactivated (units)	[¹⁴ C]DFMO bound (nmol)	DFMO bound (nmol/unit of enzyme inactivated)	Calculated specific activity of homogeneous enzyme (units/mg of protein)
1	1.03	0.787	0.765	24.4
2	0.168	0.108	0.641	30.0
3	1.273	0.348	0.274	70.2
4	0.333	0.1305	0.392	49.1
5	0.346	0.0947	0.274	70.3
			Mean 0.469 ± 0.20	Mean 48.8 ± 19.3

ornithine decarboxylase, the theoretical specific activity of homogeneous enzyme is calculated at 48.8 units/mg of protein. This specificity, subunit M_r and specific activity are all somewhat consistent with the [¹⁴C]DFMO labelling of mammalian ornithine decarboxylase by Pritchard *et al.* (1981) and Seely *et al.* (1982). Comparison of the theoretical specific activity of homogeneous ornithine decarboxylase with the 0.004 unit/mg of protein in crude *Physarum* homogenates shows that this enzyme comprises less than 0.01% of soluble cellular protein.

Partially purified A-form enzyme (85% A-form and 15% B-form) was treated with partially purified fractions of the conversion factor in the presence of spermidine. After 2h, when the ornithine decarboxylase was 9% A-form and 91% B-form, the reaction mixture was treated with excess of [¹⁴C]-DFMO in the presence of pyridoxal 5'-phosphate. Constituents of samples taken before and after this conversion were subsequently separated by two-dimensional gel electrophoresis and detected both with protein stain and by radioautography (Fig. 1). Comparison of the ¹⁴C-labelled protein before and after this enzyme conversion (Figs. 1c and 1d) demonstrates that this modification in ornithine decarboxylase does not result in an observable change in the M_r of the SDS-denatured enzyme subunit; however, this conversion of the A-form into the B-form does appear to induce a distinct change in the isoelectric points of these two denatured enzyme states as measured in the presence of 9M-urea. When ornithine decarboxylase is catalytically in the A-form it appears to be electrically heterogeneous, with at least three different isoelectric points between pH 5.4 and 5.5. After conversion into the B-form, however, the ¹⁴C-

labelled enzyme is found in two spots, with isoelectric points between pH 5.6 and 5.7. This change in the enzyme molecule can also be seen by comparison of the respective protein spots indicated on the stained gels (Figs. 1a and 1b). The few extra proteins noted in Fig. 1b (e.g. at M_r 55000 and M_r 43000) are due to the presence, in this sample, of impurities from the partially purified conversion factor, and these are seen when this conversion-factor preparation is chromatographed alone.

The demonstration that this enzyme modification indeed induces a covalent increase in the positive charge (diminished negative) on the enzyme, and our previous demonstration that spermidine or spermine is absolutely required for this reaction (Mitchell *et al.*, 1982), seems to be consistent with the suggestion by Russell (1981) that the covalent linkage of putrescine or a polyamine to this enzyme may be a normal activity-modulating reaction. To test this possibility, partially purified A-form enzyme and modulating protein were made to react in the presence of [³H]spermidine. After the excess spermidine was removed, the proteins were separated by gel filtration on Ultrogel AcA-44 in the presence of 0.2M-NaCl. As shown in Fig. 2, the product of this conversion, B-form enzyme, was eluted at a discrete peak of M_r about 50000, coinciding exactly with the location of the remaining substrate, A-form enzyme. It should be noted that the enzyme is not catalytically active at this salt concentration; rather, the NaCl concentration must be decreased below 0.05M to show measurable activity, and at this lower ionic strength the enzyme appears to be a dimer of M_r 90000–100000. If 1 mol of [³H]spermidine had been covalently bound to each mol of ornithine decarboxylase converted from

Fig. 1. Two-dimensional gel electrophoresis of partially purified *Physarum* ornithine decarboxylase before and after conversion of the A-form into the B-form by purified modulating protein and spermidine

Identical samples of 0.13 unit of partially purified (5 units/mg of protein) ornithine decarboxylase, consisting of 85% A-form and 15% B-form, were incubated for 2 h at 30°C in 1 mM-spermidine and 20% (w/v) poly(ethylene glycol), with or without the presence of partially purified conversion factor. The sample treated with this factor was altered, to contain 9% A-form and 91% B-form. Both ornithine decarboxylase samples were then labelled with [^{14}C]DFMO as described in the text. After excess of [^{14}C]DFMO was removed, the samples were applied to 9 M-urea isoelectric-focusing tube gels with a pH gradient from 3.0 to 6.5. These gels were laid on top of SDS/10%-polyacrylamide thin-layer gels and electrophoresed along with appropriate M_r standards. After being stained for protein, the gels were dried and radioautographed. Panels (a) and (c) are pictures of part of the stained gel and its radioautograph respectively of the ornithine decarboxylase before conversion, when it was 85% A-form. Panels (b) and (d) illustrated a portion of the stained gel and its matching radioautograph of the enzyme after A-B conversion, when it was 91% B-form. Of the multiple protein spots in panels (a) and (b), only those matching the ^{14}C -labelling in panels (c) and (d) are considered to represent the catalytic unit of ornithine decarboxylase.

the A-form into the B-form, then about 8000 d.p.m. of [^3H]spermidine should have co-eluted with the ornithine decarboxylase peak. Instead the [^3H]spermidine did not show any obvious peak that was co-eluted with the catalytically active enzyme, even though some of the labelled spermidine did appear to associate with proteins of the partially purified conversion-factor preparation (M_r 20 000–35 000). In a similar experiment (results not shown) the substrate and products of this enzyme modification were

separated by isoelectric focusing in the presence of 9 M-urea. As before, there was no indication of labelled spermidine associated preferentially with B-form enzyme.

Discussion

It has frequently been suggested that the polyamines control ornithine decarboxylase activity by stimulating selective inactivation of this enzyme.

Fig. 2. Separation by gel filtration of B-form ornithine decarboxylase and $[^3\text{H}]$ spermidine after spermidine-stimulated A-B conversion

Partially purified (0.08 unit of 5 units/mg) A-form (90% A-form and 10% B-form) ornithine decarboxylase was treated for 3 h at 30°C with isolated A-B-converting factor and 1 mM - $[^3\text{H}]$ spermidine ($300\text{ }\mu\text{Ci}/\mu\text{mol}$). This catalysis resulted in 0.051 unit of A-form converted into B-form. The products of this reaction were desalted on a Bio-Gel P-2 column to remove excess of $[^3\text{H}]$ spermidine and separated by gel filtration on Ultrogel AcA-44 ($55\text{ cm} \times 0.75\text{ cm}$ column; 26 ml volume) in the presence of 0.05 M-Hepes (pH 7.2)/2 mM-dithiothreitol/0.2 M-NaCl. Fractions (0.5 ml) were eluted at 2 ml/h, and, starting with the void volume (0), 0.4 ml of each was counted for $[^3\text{H}]$ spermidine radioactivity by liquid scintillation at 40% efficiency (Δ), and 0.1 ml of each was dialysed in a multiple Microdialyzer (Bethesda Research Laboratories) to remove the NaCl, and eventually assayed for A-form ($\bullet$) and B-form ($\circ$) enzyme. This column had previously been calibrated with the following proteins: A, alcohol dehydrogenase (M_r 151 000); B (bovine serum albumin, M_r 68 000); C, ovalbumin (M_r 43 000); D, carbonic anhydrase (M_r 30 000); E, cytochrome c (M_r 12 400).

Efforts to demonstrate this modification have been hindered by (a) the unusually small amounts of this enzyme protein that exist in tissues, (b) the lack of a system *in vitro* that accurately represents the situation *in vivo*, and (c) the inability to identify the enzyme protein positively once the polyamine-induced inactivation has occurred or after denaturing separation techniques such as SDS/polyacrylamide-gel electrophoresis. In the experiments presented above, we have used isolated and

purified components of the actual polyamine-stimulated enzyme-modifying reaction *in vivo* (Mitchell *et al.*, 1981a,b), in combination with the ornithine decarboxylase-specific label $[^{14}\text{C}]\text{DFMO}$, to study this enzyme modification. This system was used to test, at least in this organism, the three current models for the mechanism of polyamine-induced inactivation of ornithine decarboxylase.

Atmar & Kuehn (1981) reported that polyamines control *Physarum* ornithine decarboxylase by

stimulating the phosphorylation and inactivation of this enzyme. Their study, however, used a purified protein preparation where the main protein, presumably ornithine decarboxylase, had M_r 70 000 and pI 4.2, containing ornithine decarboxylase activity of at most 0.0024 unit/mg of protein. By comparison, the enzyme that we have purified from this same organism contains 19.8 units/mg of protein (Mitchell, 1983), and we have calculated the theoretical specific activity of pure ornithine decarboxylase to be 48.8 units/mg, on the basis of [^{14}C]DFMO binding. Similar calculations for mammalian enzyme suggest a theoretical specific activity of 20–50 units/mg for pure enzyme (Pritchard *et al.*, 1981; Seely *et al.*, 1982). Thus the preparation used by Atmar & Kuehn (1981) was at most 0.005% active ornithine decarboxylase. We furthermore have demonstrated that native *Physarum* enzyme has a minimal M_r in the presence of 0.2 M-NaCl of about 50 000, with pI 5.5 and 5.9 (Mitchell *et al.*, 1978b) for the A-form and the B-form respectively. These values are in good agreement with our estimates of M_r 52 000 and pI 5.4 and 5.7 based on two-dimensional electrophoresis of [^{14}C]DFMO-labelled ornithine decarboxylase in the present study. Clearly, then, the phosphorylation of the 70 000 M_r protein observed by Atmar & Kuehn (1981) was not a demonstration of the phosphorylation of ornithine decarboxylase. The coincidental loss of enzyme activity that they observed may well have been due to the polyamine-stimulated increased sensitivity of this enzyme to mild oxidizing conditions, as we have previously reported (Mitchell *et al.*, 1978a).

These data do not exclude phosphorylation as a possible modification of ornithine decarboxylase. In addition to the slight charge diversity noted within each form of the *Physarum* enzyme, Fig. 1 demonstrates a definite charge difference between the A-form and the B-form of this enzyme. The increased acidity of the A-form could indeed be due to the presence of one or more phosphate groups, and likewise A–B-converting protein could be a specific phosphatase. Unfortunately, we have not been able to support this possibility through attempts to alter either form of the enzyme by exposure to either alkaline phosphatase or protein kinase-stimulating conditions (Mg^{2+} , ATP and cyclic AMP) in crude homogenates or purified enzyme (J. L. A. Mitchell & D. D. Carter, unpublished work).

Russell (1981) suggested that putrescine may be covalently linked to this enzyme by the action of transglutaminase, and that this is the key step in the product feedback control of this enzyme. To support this claim she demonstrated that preparations of transglutaminase attach about 4 mol of putrescine to each mol of purified ornithine decarboxylase, with a coincident loss in the enzyme's activity. We have

previously demonstrated that spermidine and not putrescine is responsible for the inactivating feedback modification of this enzyme in *Physarum* (Mitchell *et al.*, 1982). Our present results (Fig. 2) confirm the fact that, even though spermidine or a similar polyamine is essential for the modification of ornithine decarboxylase, this polyamine does not end up covalently linked to the inactivated (B-form) enzyme. This observation agrees with that of Tyagi *et al.* (1982), who reported that the inactivation of yeast ornithine decarboxylase by polyamines *in vivo* also did not involve the direct incorporation of polyamines into enzyme protein.

A third mechanism for the polyamine-induced inactivation of ornithine decarboxylase has been presented involving the polyamine-stimulated release or synthesis of a small protein (M_r 26 000) that specifically binds and thereby inactivates this enzyme. Such an ornithine decarboxylase antienzyme has been reported to occur in several diverse tissues (Heller *et al.*, 1976; Pegg *et al.*, 1978; Branca & Herbst, 1980; Heller & Canellakis, 1981). By analogy, one might expect that the A–B-converting protein of *Physarum* is indeed another form of antienzyme that is stimulated by polyamines to bind to A-form enzyme, altering its catalytic properties, resulting in the B-form. That this is not the case is demonstrated first by the observation in Fig. 1 that the A-form and the B-form are distinct in charge under conditions that would easily have dissociated such a protein–protein interaction (9 M-urea), and secondly by the observation in Fig. 2 that kinetically active A-form and B-form enzymes are both eluted in the same fractions (M_r about 50 000) from a molecular-sieving column in the presence of high-ionic-strength buffer.

This direct demonstration of a polyamine-induced modification of ornithine decarboxylase has thus been useful in discounting several currently proposed mechanisms of this control; unfortunately, we still do not know the actual chemical mechanism of this polyamine-induced modification.

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