Ornithine decarboxylase from *Neurospora crassa*

PURIFICATION, CHARACTERIZATION, AND REGULATION BY INACTIVATION*

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Ornithine decarboxylase, a highly regulated enzyme of the polyamine pathway, was purified 670-fold from mycelia of *Neurospora crassa* that were highly augmented for enzyme activity. The enzyme is significantly different from those reported from three other lower eucaryotic organisms: *Saccharomyces cerevisiae*, *Physarum polycephalum*, and *Tetrahymena pyriformis*. Instead, the enzyme closely resembles the enzymes from mammals. The *M*₉ = 110,000 enzyme is a dimer of 53,000 Da subunits, with a specific activity of 2,610 µmol per h per mg of protein. Antisera were raised to the purified enzyme and were rendered highly specific by cross-absorption with extracts of a mutant strain lacking ornithine decarboxylase protein. With the antisera, we show that the inactivation of the enzyme in response to polyamines is proportional to the loss of ornithine decarboxylase protein over almost 2 orders of magnitude. This is similar to the inactivation process in certain mammalian tissues, and different from the process in *S. cerevisiae* and *P. polycephalum*, in which enzyme modification, without proportional loss of antigen, accompanies enzyme inactivation. The *N. crassa* enzyme is therefore suitable as a microbial model for studies of the molecular regulation of the mammalian enzyme.

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inactivation, whereas in mammals, it is lost (11-13, 15). In *Neurospora crassa*, we have studied the loss of enzyme activity and protein after physiological manipulation of polyamine pools (16). We inferred from these preliminary results that putrescine was the signal for enzyme inactivation and that enzyme protein was lost more slowly than activity.

In this paper, the purification and properties of *N. crassa* ornithine decarboxylase are described. With immunological techniques, we show that inactivation of the enzyme in vivo is accompanied by proportional loss of protein. We compare our results with reports on ornithine decarboxylases of other eucaryotes.

**EXPERIMENTAL PROCEDURES**

**RESULTS AND DISCUSSION**

**Purification of Ornithine Decarboxylase**—Table I summarizes the purification of ornithine decarboxylase, described in detail in the Miniprint. The enzyme was purified about 670-fold over the derepressed crude extract to a final specific activity of 2,600 units/mg. Without the 75-fold augmentation of ornithine decarboxylase activity in the starting material, a 50,000-fold purification would have been required. The procedure routinely yielded 1-2 mg of pure ornithine decarboxylase from 40 g of mycelium (dry weight equivalent), with an 11% yield.

The HPLC²-purified preparation contained polypeptides of *M*₉ = 53,000 and lesser amounts of others of *M*₉ = 44,000-47,000, visualized after SDS-polyacrylamide gel electrophoresis (Fig. 2A). The lower molecular weight band(s) were labeled if the enzyme preparation was exposed to [¹⁴C]difluoromethylornithine, which binds specifically and covalently to active ornithine decarboxylase molecules (Fig. 2B). In addition, Cleveland proteolytic digests (17) of the polypeptides in the HPLC-purified preparation showed very similar peptide patterns (data not shown). Thus the polypeptide species of the purified preparation were all ornithine decarboxylase or its derivatives. The lower molecular weight polypeptides are derived by proteolysis from the *M*₉ = 53,000 polypeptide during the ammonium sulfate step of purification (Fig. 2C).

Difluoromethylornithine binding was used to determine the intrinsic specific activity of active ornithine decarboxylase
Ornithine decarboxylase is formally represented by the inverse protein of pure preparations bound was not affected by the purification procedure. All of the this value during purification (Table I) indicates that the the binding in crude materials equaled its actual specific activity obtained after purification. Immunotitration was also used to test for inactive molecules. Almost identical units of activity were precipitated per microliter of antiserum (305 and 306, respectively) in the case of crude extracts and DE52-purified ornithine decarboxylase. Thus inactive ornithine decarboxylase molecules did not accumulate during the purification procedure.

Characterization of Ornithine Decarboxylase—Pure ornithine decarboxylase eluted as an $M_r = 110,000$ protein from a Sephacryl S-200 molecular sieving column (data not shown).

Under denaturing conditions, polyacrylamide gel electrophoresis revealed that the predominant species was an $M_r = 53,000$ polypeptide (Fig. 2A), indicating that native ornithine decarboxylase is a dimer. The pH optimum of the enzyme reaction was 7.1. The $K_m$ for ornithine was 350 $\mu$M, and the $K_m$ for pyridoxal phosphate was 0.16 $\mu$M. The $K_i$ for the competitive inhibitor, $\alpha$-methylornithine, was 280 $\mu$M. Arginine, spermidine, spermine, cadaverine, and lysine at a concentration of 2 mM failed to inhibit ornithine decarboxylase. Putrescine (2 mM) inhibited ornithine decarboxylase activity only 30%. Thus it is unlikely that ornithine decarboxylase activity is controlled directly by these metabolites in vivo.

Dithiothreitol (2–5 mM) and the non-ionic detergent Brij 35 (0.01–0.1%) increased and stabilized purified ornithine decarboxylase activity, both during storage and during the enzyme reaction.

Pure ornithine decarboxylase displayed a series of isoelectric forms between pH 5.25 and 5.50 (Fig. 3). The same forms were observed in fresh crude extracts (Fig. 3), but their different proportions suggested some selectivity in the purification procedure. The quantitative results with $[^{14}C]$difluoromethylornithine binding and immunotitration indicate that most or all ionic forms are active. The several forms of the enzyme can be seen in extracts of cells grown in minimal medium (data not shown) and thus do not reflect mistranslation during the polyamine starvation of cells used as a starting material. Multiple ionic forms of the enzyme have been seen in mouse kidney (4, 20). It is not certain whether more than one active copy of the gene is present in the mouse genome or whether allelic heterogeneity among animals or in heterozygotes prevails in these diploid organisms. Because there is only one active gene for ornithine decarboxylase in N. crassa (21), the isoelectric forms seen here probably reflect post-translational modifications.

Effect of Polyamine Status on Ornithine Decarboxylase Protein—Putrescine has been implicated as a stimulus for ornithine decarboxylase inactivation in N. crassa (16). Enzyme protein and activity were therefore examined in cultures with

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**TABLE I**

Purification of ornithine decarboxylase from *N. crassa*

| Step                  | Total protein | Volume | Total activity | Yield (%) | Specific activity | Purification | Purity (%) | DFMO pmol/unit |
|-----------------------|---------------|--------|----------------|-----------|-------------------|-------------|------------|----------------|
| Crude extract         | 8,200         | 1,400  | 34,160         | 100       | 3.9               | 1.0         | 0.15       |                |
| Ammonium sulfate      | 1,105         | 43     | 16,328         | 48        | 14.8              | 3.8         | 0.56       | 7.8            |
| Bio-Gel P-200         | 42.9          | 43     | 10,542         | 31        | 244               | 63          | 9.3        | 7.6            |
| DE52-cellulose        | 3.0           | 3.0    | 6,642          | 19        | 2,136             | 548         | 81         | 6.9            |
| HPLC ion exchange     | 1.1           | 1.1    | 3,861          | 11        | 2,610             | 669         | 99         | 6.8            |

*Percent purity was calculated using the average picomoles of difluoromethylornithine (DFMO) per unit of enzyme (7.5), the known subunit $M_r$, 53,000, and the assumption that 1 mol of DFMO binds per mol of subunit.

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**FIG. 2**. A, purification of ornithine decarboxylase. Preparations were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis and the gel was stained with silver. The lanes are: 1, crude extract; 2, 42.5% ammonium sulfate fraction; 3, Bio-Gel P-200 eluate; 4, DE52 ion exchange eluate; 5, HPLC ion exchange eluate; 6, standard proteins (from top, phosphorylase b, $M_r = 94,000$; bovine serum albumin, $M_r = 68,000$; ovalbumin, $M_r = 43,000$; carbonic anhydrase, $M_r = 30,000$; soybean trypsin inhibitor, $M_r = 20,000$; $\alpha$-lactalbumin, $M_r = 14,000$). B, autoradiograph of purified ornithine decarboxylase, separated by SDS-polyacrylamide gel electrophoresis after exposure to $[^{14}C]$difluoromethylornithine. The upper band has an $M_r$ of approximately 53,000; the lower band, 47,000–49,000. C, Western immunoblot of the crude extract (lane 1) and the ammonium sulfate fraction (lane 2) after sodium dodecyl sulfate gel electrophoresis. A cross-absorbed antiserum at a dilution of 1:1,000 was used.

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**FIG. 3**. Western immunoblot of isoelectric focusing gel. Lanes: 1, purified ornithine decarboxylase; 2, crude, derepressed extract of the *aga* strain, grown in arginine; 3, derepressed extract 2 h after the onset of enzyme inactivation (approximately one-half the initial activity had disappeared at the time of sampling).
either increased or greatly diminished putrescine content. A strain carrying the aag mutation, grown in minimal medium and containing normal levels of putrescine and spermidine, had an ornithine decarboxylase activity of 0.15 units/mg of protein (Table 2 in Miniprint). Cultures grown in medium supplemented with arginine cannot synthesize ornithine and thus become depleted of both putrescine and spermidine (16, 18, 19). These cultures had a maximally augmented ornithine decarboxylase activity of 3.8 units/mg, consistent with their lack of both putrescine and spermidine (Table 2). Cultures grown in medium supplemented with the spermidine synthase inhibitor, cycloheximidine (22), accumulated putrescine (16). They had an ornithine decarboxylase specific activity of 0.72 unit/mg (Table 2). (The steady-state enzyme activity is thought to be the net result of a higher rate of synthesis, owing to the depletion of spermidine, opposed by a higher rate of turnover of the enzyme induced by putrescine (16).)

The units of ornithine decarboxylase activity precipitated per microliter of antiserum were very similar in the cycloheximidine- and arginine-supplemented cultures and somewhat lower in the culture grown in minimal medium (Table 2). The last observation has little significance at this point, owing to the low ornithine decarboxylase activity and antigen in these cultures. The data, therefore, reveal no inactive ornithine decarboxylase molecules in steady-state cultures, whether the putrescine content of the cells was very high or virtually nil.

Rapid inactivation of ornithine decarboxylase follows the restoration of ornithine to ornithine-starved cells (16), such as those used to purify the enzyme. In one such experiment, ornithine decarboxylase specific activity fell rapidly from 3.6 to 0.05 units/mg in 6 h (Fig. 4). Both ornithine decarboxylase activity and enzyme protein had 2-h half-lives after correction for the dilution caused by further growth after ornithine addition (Fig. 4). The same results were obtained with extracts made from sand-ground mycelia or from acetone powders, using four different antisera. We conclude that removal of protein is simultaneous with the disappearance of activity. The conclusion differs from our preliminary report, based on crude quantification of 125I-immunoblots (16), that protein was lost somewhat more slowly than activity.

The requirement for protein synthesis during ornithine decarboxylase inactivation was examined by adding cycloheximide and ornithine simultaneously to a polyamine-starved strain. As previously seen in Neurospora (16), loss of ornithine decarboxylase activity and protein was greatly retarded under these conditions (Fig. 5 in Miniprint); in some experiments, the enzyme is entirely stable.

**Immunoblots of Ornithine Decarboxylase during Inactivation**—Immunoblots of sodium dodecyl sulfate-polyacrylamide and isoelectric focusing gels were used to reveal changes in the immunoreactive protein during inactivation. The expected augmentation of ornithine decarboxylase protein was seen in conditions of polyamine starvation, and ornithine decarboxylase protein was lost during inactivation. No antigenically active, lower molecular weight forms of ornithine decarboxylase appeared consistently during inactivation (Fig. 5), even when the autoradiographs were overexposed. Immunoblots of the isoelectric focusing gel showed multiple ionic forms before and after the onset of inactivation. The most basic form (pI = 5.5) is lost more rapidly than the others (Fig. 3). More study will show how selective the inactivation is, and whether one isoform is the actual substrate for the inactivation process.

Fig. 6 (Miniprint) summarizes the correlation between the Aboa, a measure of ornithine decarboxylase protein (See "Experimental Procedures"), and specific activity during periods of enzyme inactivation, polyamine starvation, and steady-state growth in conditions of putrescine depletion and excess. The ratio of these parameters is constant among samples that vary in specific activity by 100-fold, although deviations at low activity and protein are obscured by the scales required to include all the points. The constant ratio between ornithine decarboxylase protein and activity was also seen in immunotitration of crude extracts and partially purified ornithine decarboxylase preparations that varied in specific activity by 450-fold (see above).

**Comparison of Eucaryotic Ornithine Decarboxylases—** Neurospora ornithine decarboxylase differs markedly from the purified enzyme of other lower eucaryotes. The $M_e = 110,000$ dimer is different from the $M_e = 96,000$ monomer of yeast (5), the $M_e = 64,000$ monomer of Tetrahymena (6), or the $M_e = 80,000$ dimer of Physarum polycephalum isolated by Barnett and Kazarinoff (7). Moreover, the specific activities of the purified yeast (5) and Tetrahymena (6) enzymes (31 and 14 units/mg of protein, respectively) are 2 orders of magnitude lower than those of N. crassa and P. polycephalum. In fact, the N. crassa enzyme, with its dimeric structure, subunit molecular weight ($M_e = 53,000$), and specific activity (2,610 units/mg of protein), is unique among lower eucaryotic ornithine decarboxylases in its close resemblance to that of mammals. Mammalian ornithine decarboxylases are all dimers of about $M_e = 110,000$ and have specific activities in the range of 1,400–3,200 units/mg of protein (1–4).

The behavior of the N. crassa enzyme protein during inactivation differs from the case of yeast (5), in which no evidence of loss of the protein is found, and from P. polycephalum, in which enzyme modification, without proportional loss of protein, has been inferred (14, 23). Again, the N. crassa enzyme resembles that of some mammalian systems such as Chinese hamster ovary cells (13) and mouse kidney (4) in showing near-proportional loss of protein and activity. Certain mammalian tissues, such as rat brain, heart, and liver, however, display an antienzyme, a stoichiometrically binding protein which inhibits the enzyme (24–26). The protein may be a controlling factor in these tissues, and indeed, loss of activity without comparable loss of enzyme protein is seen in them. No antienzyme has been detected in N. crassa.²

It is possible that a rate-limiting modification of the protein precedes the disappearance of the N. crassa enzyme. This possibility is reinforced by our observation that cycloheximide interferes with polyamine-mediated enzyme inactivation (Ref. 16 and this paper). Whether this reflects a requirement for a noncovalent antienzyme-like binding agent (24–26) or a pro-

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²G. R. Barnett and R. H. Davis, unpublished results.
tein that covalently modifies ornithine decarboxylase is not known. We are currently exploring this matter by seeking mutations that affect the inactivation process.

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Supplemental material to Ornithine Decarboxylase of Neurospora: Purification, Characterization, and Immunological Studies

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Experimental PROCEDURE

Materials—the arginase-less Neurospora strain 1C3, carrying the ornithine decarboxylase allele (odcA, 1)). Cells were grown on 1% malt extract agar (MEA) or in a liquid medium (5% malt extract, 0.5% yeast extract, 2% dextrose, 0.5% peptone, 0.5% NaCl, 0.05% agar, pH 5.5). Ornithine decarboxylase activity in extracts of yeast was assayed by the method of Steck and Wallach (1970) using L-5,7-dimethylornithine as substrate.

Preparation of antigen—Two polysomal extracts, each 50 ml, were prepared as described by Burnette (1978) using antiaerun dilutions 1:500.

Antiserum preparation—Ple-immune rabbits that recognize only Onc polypeptides were used. The rabbits were immunized with 200 mg of L-ornithine decarboxylase protein in Freund's complete adjuvant; booster injections were made using antiaerun dilutions 1:500.

Preparation of antiserum—The total reaction mixture (200 ml) contained 1 mCi of 1-[14C]ornithine, 4 mCi of 1/[14C]arginine, 500 ml of 1-[14C]lysine, and 500 ml of 1-[14C]threonine to bring the specific radioactivity of L-ornithine to 75,000 cpm/mg. The mixture was incubated for 1 hour at 4°C, protein concentration was determined as previously described (19); 25% of the supernatant was retained. The supernatant was then incubated for 5 minutes at 37°C.

Preparation of antiserum—The total reaction mixture (200 ml) contained 1 mCi of 1-[14C]lysine. 1-[14C]threonine, and 1-[14C]lysine to bring the specific radioactivity of L-ornithine to 75,000 cpm/mg. The mixture was incubated for 1 hour at 4°C, protein concentration was determined as previously described (19); 25% of the supernatant was retained. The supernatant was then incubated for 5 minutes at 37°C.

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suspension in the buffer stock was added, and the mixture was incubated for 18 minutes. After centrifugation, the supernatants were assayed in duplicate for ornithine decarboxylase activity. Immunization data were calculated as the units of ornithine decarboxylase activity removed/1 ml antiserum, using linear regression to determine the slope of the regression line. Each point represents the mean of individual lines. The data were analyzed by Student's t-test to determine the significance of the difference between the means.

A positive control for recognition of inactive ornithine decarboxylase molecules was performed by immunoelectrophoresis of a wild type extract and that of a mutant carrying the pcl-1 allele T2208. This mutant lacks ornithine decarboxylase activity. The antiserum against the wild type strain of Neurospora was also used for immunoelectrophoresis, and the IgG fraction of the antiserum was isolated by precipitation with 0.5 M ammonium sulfate and dialysis. The IgG fraction was stored in aliquots at -70 °C.

RESULTS

Table 2

| Enzyme | Activity (units/ml) |
|--------|-------------------|
| None   | 1.145             |
| arginine (10 mM) | 0.32 | 3.778 (10^8) |
| cyclohexylamine (10 mM) | 1.79 | 1.95 (10^8) |

**Fig. 1.** Relation of enzyme units precipitated per ml of antiserum and the proportion of active ornithine decarboxylase molecules in the preparation. With ornithine decarboxylase protein constant, the proportion of inactive (mutant) to active (normal) molecules was varied. The slope of the immunoprecipitation curve (left ordinate) was determined for each mixture.

**Fig. 5.** Western immunoblots of extracts, separated by SDS- polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. The blots were probed with antiserum A and detected using enhanced chemiluminescence. The identity of the bands was confirmed by Western immunoblotting of extracts from wild type and mutant strains, and the bands were quantified using densitometry.

**Fig. 6.** Relation of enzyme specific activity to antigenic material (Aq) in extracts of varying levels of ornithine decarboxylase activity. All samples used were crude extracts from Neurospora spores (wild type medium) or from ornithine-starved cultures after the restoration, for various times, of ornithine (see main text).