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, Physoarum polycephalum, and Tetrahymena pyriformis. Instead, the enzyme closely resembles the enzymes from mammals. The $M_r = 110,000$ 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.

Ornithine decarboxylase (EC 4.1.1.17) is a tightly regulated, rate-determining enzyme of polyamine biosynthesis. The enzyme has been purified to homogeneity from rat and mouse livers (1-4). These enzymes are dimers of $M_r = -54,000$ subunits, and are low-abundance proteins in most cells. In contrast, the enzymes from three lower eucaryotes, Saccharomyces cerevisiae (5), Tetrahymena pyriformis (6) and *Physoarum polycephalum* (7), vary greatly in molecular weight and specific activity, and none closely resembles the enzyme of mammals.

A prominent feature of the control of ornithine decarboxylase in all organisms is the inactivation of the enzyme (8, 9). In most organisms studied, addition of polyamines causes inactivation (10-13). In the lower eucaryotes, *P. polycephalum* (14) and *S. cerevisiae* (5), the enzyme protein persists after 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 HPLC-purified preparation contained polypeptides of $M_r = 53,000$ and lesser amounts of others of $M_r = 44,000-47,000$, visualized after SDS-polycrylamide 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 $M_r = 53,000$ polypeptide during the ammonium sulfate step of purification (Fig. 2C).

Difluromethylornithine binding was used to determine the intrinsic specific activity of active ornithine decarboxylase

† Portions of this paper (including "Experimental Procedures," Figs. 1, 5, and 6, and Table 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-4021, cite the authors, and include a check or money order for $2.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

‡ The abbreviations used are: HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; NP-40, Nonident P-40; ODC, ornithine decarboxylase; U, unit; RIA, radioimmunoassay.
ornithine decarboxylase is formally represented by the inverse protein of pure preparations bound was not affected by the purification procedure. All of the were precipitated per microliter of antiserum (305 and obtained after purification. Immunotitration was also used to test for inactive molecules. Almost identical units of activity were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis were precipitated after exposure to [14C]difluoromethylornithine. The upper band has an M, 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.

Under denaturing conditions, polyacrylamide gel electrophoresis revealed that the predominant species was an M, = 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 µM, and the K,m for pyridoxal phosphate was 0.16 µM. The K,i for the competitive inhibitor, α-methylornithine, was 280 µM. 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 [14C]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 posttranslational 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

### Table I

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<th>Step</th>
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<th>Total activity</th>
<th>Yield</th>
<th>Specific activity</th>
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<th>Purity*</th>
<th>DFMO*</th>
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<td></td>
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*Percent purity was calculated using the average picomoles of difluoromethylornithine (DFMO) per unit of enzyme (7.3), the known subunit M, 53,000, and the assumption that 1 mol of DFMO binds per mol of subunit.

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, = 94,000; bovine serum albumin, M, = 68,000; ovalbumin, M, = 43,000; carbonic anhydrase, M, = 30,000; soybean trypsin inhibitor, M, = 20,000; α-lactalbumin, M, = 14,000). B, autoradiograph of purified ornithine decarboxylase, separated by SDS-polyacrylamide gel electrophoresis after exposure to [14C]difluoromethylornithine. The upper band has an M, 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.

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 \textit{aga} 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, cyclohexylamine (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 cyclohexylamine- 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 2-1/2 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 \textsuperscript{125}I-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 \textit{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 Ab\textsubscript{50}, 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 immunotitrations of crude extracts and partially purified ornithine decarboxylase preparations that varied in specific activity by 450-fold (see above).

**Comparison of Eucaryotic Ornithine Decarboxylases—**\textit{N. crassa} ornithine decarboxylase differs markedly from the purified enzyme of other lower eucaryotes. The \( M_r = 110,000 \) dimer is different from the \( M_r = 96,000 \) monomer of yeast (5), the \( M_r = 64,000 \) monomer of Tetrahymena (6), or the \( M_r = 80,000 \) 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 \textit{N. crassa} and \textit{P. polycephalum}. In fact, the \textit{N. crassa} enzyme, with its dimeric structure, subunit molecular weight (\( M_r = 53,000 \)), 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 \( M_r = 110,000 \) and have specific activities in the range of 1,400–3,200 units/mg of protein (1–4).

The behavior of the \textit{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 \textit{P. polycephalum}, in which enzyme modification, without proportional loss of protein, has been inferred (14, 23). Again, the \textit{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 \textit{N. crassa}.

It is possible that a rate-limiting modification of the protein precedes the disappearance of the \textit{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-
tein that covalently modifies ornithine decarboxylase is not known. We are currently exploring this matter by seeking excellent technical assistance, and Robert Yamamoto for help in immunizing rabbits.

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REFERENCES


Supplemental material to Ornithine Decarboxylase of Neurospora: Purification, Characterization, and the Effect of Ornitcarboxylase 1. Introduction

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EXPERIMENTAL PROCEDURES

Materials—The arginase-less Neurospora strain IC3, carrying the ornithine decarboxylase allele I(4), was obtained from the American Type Culture Collection (ATCC), Rockville, Maryland. The strain was maintained on a 2% maltose-soluble casein medium at a pH of 7.8. Inoculum was prepared by culturing the strain in liquid maltose medium at 30°C for 5 days. The resulting mycelium was harvested and used for all experiments.

Preparation of mycelia—Two-polyethylene paper, 4 x 2, was raised using 175 ml of water, pH 7.5, purified ornithine decarboxylase, for two initial injections and 8.2 ml for each injection. The two injections contained 0.02% sodium azide, 0.018 ug/ml of pyridoxal phosphate, 200 ng/ml of bovine serum albumin, and 0.02% sodium azide. The resulting mycelium was used for all experiments.

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suspension in the buffer (s) was added, and the mixture was incubated for 18 minutes. After centrifugation, the supernatants were assayed in duplicate for ornithine decarboxylase activity. Immunodiffusion data were calculated as the units of ornithine decarboxylase activity recovered per ml of reaction mixture. Immunodiffusion data were calculated using a linear regression to determine the slope of the line and the intercept, which gives the relative proportions of individual lines. The data were also transformed to antibody-50 (Abs/0) values (10) by calculating the amounts of antibody required to precipitate half of the enzyme activity in samples containing 1 mg protein.

A positive control for recognition of inactive ornithine decarboxylase was performed by immunodiffusion of a mixture of a wild-type-extract and that of a mutant carrying the sac 5 allele US460. This mutant lacks ornithine decarboxylase activity. O drought contained a normal amount of ornithine decarboxylase activity. A second preparation of the strain and of the IC-3 (standard) strain having similar amounts of ornithine decarboxylase protein, judged by autoradiographs of dilution series of each, were prepared, with ornithine decarboxylase protein constant, the proportions of normal and mutant protein were varied and tested by immunodiffusion. The result (Fig. 1) showed linear decreases in the effectiveness of the antisera to precipitate enzyme units as the fraction of inactive or normal units increased. The determination of the mutant ornithine decarboxylase protein was recognized by the antisera.

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

 purification of Ornithine Decarboxylase—Growth. The IC-3 strain, carrying the sac 5 mutation, was grown in 1 liter lots at 25°C in arginine-supplemented medium to starve the cells for polyamines and to cause elevation of ornithine decarboxylase activity. Mycelia, collected on Whatman 3A filter paper, were washed with distilled water. All other steps were carried out at 4°C.

Crude Extract. About 48 g (dry weight equivalent) of mycelia were homogenized in buffer A (0.6 M potassium phosphate, 8.1 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 mM dithiothreitol (DTT), 1 ps/ml each antipain, leupeptin, and pepstatin, and 0.83 Kppan inhibitor units of aprotinin/ml) at pH 7.4) with a 250-A, 1.5-mm diameter glass beater, using a Dean-starter (Ritter Products). Mycelia were broken with 140 strokes, with a breakage of 10%. Homogenates were filtered through four layers of cheesecloth. The filtrates were centrifuged at 27,000 g for 45 minutes, followed by filtration through Whatman 3A filter paper.

Ammonium Sulfate Precipitation. A saturated ammonium sulfate solution in buffer A was added over 45 min with stirring to the supernatant. The mixture was stirred for 20 minutes after addition and centrifuged at 12,000 g for 10 minutes. The supernatant (500 ml) was adjusted to pH 7.4 with sodium hydroxide and centrifuged at 100,000 g for 1 hour. The supernatant was treated with sephadex G25 columns equilibrated with buffer B (same as buffer A except that 0.6 M Tris-acetate pH 7.4 was used in place of potassium phosphate, and 8.15 and 0.5 g were added). The yield varied between 50 and 75.

Bio-Gel P-200 filtration. The preparation (ca. 60 ml) was applied to a Bio-Gel P-200 (50 m) filtration column (5 x 58 cm) equilibrated with buffer B. The column was eluted at the rate of 1 ml/min. The fractions, emulgated in about 40 hours, were pooled and concentrated by ultrafiltration on an Amicon UM-10 membrane. The salt concentration was adjusted to 0.2 M sodium acetate, using a 200 ml solution buffer B (50 M Tris-acetate, 0.6 M EDTA, 0.5 M DTT, and 5.13 lit [35] pH 7.4).