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Authors
Barnett, GR
Seyfzadeh, M
Davis, RH

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Putrescine and Spermidine Control Degradation and Synthesis of Ornithine Decarboxylase in *Neurospora crassa**

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Glenn R. Barnett‡, Manouchehr Seyfzadeh, and Rowland H. Davis§

From the Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717

***Neurospora crassa* mycelia, when starved for polyamines, have 50–70-fold more ornithine decarboxylase activity and enzyme protein than unstarved mycelia. Using isotopic labeling and immunoprecipitation, we determined the half-life and the synthetic rate of the enzyme in mycelia differing in the rates of synthesis of putrescine, the product of ornithine decarboxylase, and spermidine, the main end-product of the polyamine pathway. When the pathway was blocked between putrescine and spermidine, ornithine decarboxylase synthesis rose 4–5-fold, regardless of the accumulation of putrescine. This indicates that spermidine is a specific signal for the repression of enzyme synthesis. When both putrescine and spermidine synthesis were reduced, the half-life of the enzyme rapidly increased 10-fold. The presence of either putrescine or spermidine restored the normal enzyme half-life of 55 min. Tests for an ornithine decarboxylase inhibitory protein ("antizyme") were negative. The regulatory mechanisms activated by putrescine and spermidine account for most or all of the regulatory amplitude of this enzyme in *N. crassa*.**

In *Neurospora crassa*, as in other organisms, polyamines (putrescine, spermidine, and/or spermine) are indispensable for normal growth (1), although the roles of these compounds are still somewhat obscure (2, 3). A key enzyme of their synthesis, ornithine decarboxylase, varies greatly in specific activity in most organisms in response to many external factors, including trophic and hormonal stimuli, or to inhibition of polyamine synthesis (2, 3). Ornithine decarboxylase activity of *N. crassa* rises 70-fold or more upon polyamine limitation, and this is correlated with similar changes in the amount of enzyme protein (4, 5). Previous work suggests that putrescine negatively controls enzyme stability, and spermidine negatively controls enzyme synthesis (6). Here, we test this hypothesis directly by [^{35}S]methionine labeling and immunoprecipitation techniques. We also show that another regulatory mechanism, inhibition by an ornithine decarboxylase antizyme (7–9), does not prevail in conditions we have used.

Putrescine and spermidine pools are highly sequestered in cells of *N. crassa*, and the regulatory pools are small (6, 10, 11). (There is little spermine in *N. crassa*, and it has little effect on ornithine decarboxylase, even when elevated in cellular concentration (6).) Because of continuing sequestration of newly made polyamines, ornithine decarboxylase responds much more readily to the rate of polyamine synthesis than to the pools present at any given time (6). For this reason, and because putrescine and spermidine are absorbed poorly from the medium, we have manipulated the biosynthetic pathway (Fig. 1) in order to relate ornithine decarboxylase regulation to the potential metabolic signals, putrescine and spermidine. First, we use an arginase-less strain, in which ornithine limitation can be imposed by addition of arginine (12). Arginine feedback inhibits ornithine synthesis, and ornithine cannot be formed from arginine. Thus, the synthesis of putrescine and spermidine can be stopped at will. Second, we use cyclohexylamine (CHA)[1], an inhibitor of spermidine synthase (13, 14), to block spermidine synthesis. Because this block leads to putrescine accumulation (6), the effects of the two polyamines can be separately assessed. Confirmatory studies with two polyamine biosynthetic mutants are also reported.

MATERIALS AND METHODS

Strains and Growth—The *N. crassa* strains used for most of the work were IC3, a prototrophic strain carrying the arginaseless (*aga*) allele UM906 (12), and strain IC1894-53, carrying a nonsense mutation (LV10) in the ornithine decarboxylase structural gene, *spe-1* (15). The latter strain has no enzyme activity and produces no immunologically reactive ornithine decarboxylase protein. In addition strain IC1477-122a, carrying a *spe-1* allele, PE85, endowing the ornithine decarboxylase protein with very low activity and a 15-fold higher K_m for ornithine (1), and strain IC1898-72A carrying the mutation LV105 were used for confirmatory work. The LV105 mutation blocks polyamine synthesis between putrescine and spermidine, but its exact biochemical lesion is not yet known. Strain IC1898-72A is "leaky" on minimal medium, where it has a little spermidine and elevated levels of putrescine.

Cultures of 500 ml were grown exponentially with aeration in Vogel's medium N (16). To monitor growth, 25-ml samples of culture were collected by vacuum filtration on paper filter circles, rinsed, dried with acetone, and weighed.

Labeling of Cultures with [^{35}S]Methionine—In most experiments to determine ornithine decarboxylase half-life, cultures were brought to 1 μCi of [^{35}S]methionine/ml, carrier free. After 45 min unlabeled methionine was added and sampling commenced. Experiments on enzyme turnover in strains ICI477-122 and IC1898-72 were done with 10 μCi [^{35}S]methionine/ml. Determination of the rate of ornithine decarboxylase synthesis were begun by addition of carrier-free [^{35}S]methionine (2 μCi/ml culture). Samples were taken at 2-min intervals thereafter. In both kinds of studies, samples (25 ml) were collected with vacuum on Whatman No. 1 filters. Mycelia were washed with distilled water and dried with acetone before removal from the vacuum filtration apparatus.

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§ To whom reprint requests should be addressed.

[1] The abbreviations used are: CHA, cyclohexylamine; SDS, sodium dodecyl sulfate.

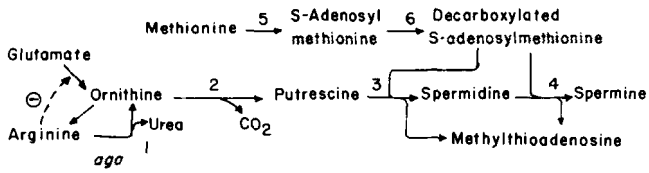

FIG. 1. **Scheme of polyamine synthesis in *N. crassa*, showing the origin of the pathway with ornithine and feedback control of ornithine synthesis by arginine.** The position of the *aga* mutation in strains IC3 is shown. The *spe-1* mutations of strains IC1894-53 and IC1477-122 block step 2, and the LV105 mutation blocks either step 3 or step 6 of the pathway. Cyclohexylamine inhibits step 3. Little spermine is synthesized in this organism. Enzymes: *1*, arginase; *2*, ornithine decarboxylase; *3*, spermidine synthase; *4*, spermine synthase; *5*, *S*-adenosylmethionine synthase; *6*, *S*-adenosylmethionine decarboxylase.

Preparation of Extracts—Acetone-dried samples were transferred to 1.5-ml Eppendorf microcentrifuge tubes containing 5–10 glass beads (3-mm diameter). The dry samples were pulverized by vigorous agitation with a Vortex mixer and then extracted in 0.25 ml of extraction buffer for 60 min on ice with occasional Vortex treatment. The extraction buffer contained 50 mM K^+ phosphate, pH 7.2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 μg each of antipain, chymostatin, leupeptin, and pepstatin/ml.

Enzyme and Protein Assay—Ornithine decarboxylase activity was determined as described previously (4). Protein was determined by the method of Bradford (17), with bovine serum albumin as a standard.

Determination of [^{35}S]Methionine Incorporation into Protein—Protein (25–100 μg) was precipitated in a 1.5-ml microcentrifuge tube by addition of 1 ml of cold 5% trichloroacetic acid. Tubes were allowed to stand in ice for 10 min and were centrifuged at 12,000 × *g* for 2 min. The supernatants were removed by aspiration; the pellets were washed by vortexing in 1 ml of 5% trichloroacetic acid and recentrifuged. The pellets were solubilized by boiling in 0.6 ml of 2% sodium dodecyl sulfate (SDS), and radioactivity was determined by counting in Ecoscint (National Diagnostics).

Antiserum and Immunoprecipitation—The preparation, titering, and specificity of the ornithine decarboxylase-specific antiserum used has been described previously (5, 15). All immunoprecipitations of a particular series contained the same amount of antiserum. The amount used was twice that needed to precipitate all the ornithine decarboxylase activity in the most active sample of the series. Blanks were prepared by adding excess extract of unlabeled strain IC3, grown in arginine to assure high ornithine decarboxylase activity, to duplicate immunoprecipitations. The amount of IC3 extract was 10 times that needed to saturate the antiserum present. The experimental incubations contained the same amount of protein of an unlabeled extract of the ornithine decarboxylase-deficient strain IC1894-53. (It was grown in arginine and limiting putrescine to make it comparable to the IC3 extract.) The IC1894-53 extract has the virtue of blocking the precipitation of ^{35}S-labeled, nonspecific antigens that might be recognized by the antiserum but does not block the precipitation of ornithine decarboxylase protein.

Extracts (35–70 μl) of ^{35}S-labeled samples were preincubated for 30 min with 30 μl of a 50% suspension of the protein A adsorbent, Pansorbin (Behring Diagnostics), previously washed by boiling in 2% SDS. The suspension was in radioimmunoassay buffer (50 mM Na^+ phosphate, pH 7.4, 150 mM NaCl, 0.03% Brij 35, 2% radioimmunoassay grade bovine serum albumin, 0.02% NaN_3). Pansorbin was removed by centrifugation and the supernatants transferred quantitatively to tubes containing antiserum and unlabeled extract (of either IC1894-53 or IC3). The tubes were incubated 3 h on ice. Pansorbin was then added (20 μg/μl antiserum), and the volume of each tube was brought to 1 ml with radioimmunoassay buffer. After 30 min the adsorbed immune complexes were collected by centrifugation and washed 6–8 times with wash buffer (50 mM K^+ phosphate, pH 7.2, 200 mM NaCl, 5 mM EDTA, 5 mM L-methionine, 1% Triton X-100, 0.25% SDS, 0.02% NaN_3). Each wash was followed by gentle centrifugation (2 min, 4500 × *g*). Immune complexes were solubilized by boiling for 5 min in SDS-electrophoresis sample buffer (18), centrifuged to remove Pansorbin, and saved for electrophoresis. The turnover rate of total protein was determined by measuring the conservation of radioactivity of the trichloroacetic acid-precipitated samples after solubilizing them in sodium dodecyl sulfate.

Electrophoresis and Densitometric Scanning—The samples were resolved by SDS-polyacrylamide gel electrophoresis, as described by Laemmli (18), using 10% acrylamide separating gels. Gels were fixed in 30% methanol, 10% trichloroacetic acid, soaked in EN^{3}HANCE (Du Pont-New England Nuclear) radioautographic enhancer, and dried before exposure to Kodak XAR film at −70 °C. Radioactivity of the bands was quantified by densitometric scanning of developed films.

RESULTS

Ornithine Decarboxylase Degradation—The half-life of the ornithine decarboxylase protein was determined in three conditions: (i) growth in minimal medium, in which putrescine and spermidine synthesis are normal; (ii) growth in the presence of 10 mM CHA, in which putrescine accumulates, but in which spermidine synthesis is severely limited; and (iii) growth in the presence of 1 mM arginine, in which neither putrescine nor spermidine is synthesized (6) (Table I).

All cultures grew exponentially with a doubling time of 3.3 h. Half-life determinations began when cultures were 0.3–0.4 mg, dry weight, per ml of culture, approximately 10 h after inoculation. In the case of arginine-grown cultures, determinations began 3 h after the addition of arginine to a culture grown in minimal medium. At this time, polyamine depletion had begun, but no change in the growth rate had taken place. (Half-life determinations in this case were the same as in steady-state growth in arginine, in which growth at a slower rate was maintained by small amounts of cadaverine synthesis (4).) For growth in cyclohexylamine the inhibitor was added at the time of inoculation. Putrescine accumulated in this culture; spermidine synthesis was limited but sufficient to sustain a normal growth rate (6).

In minimal and CHA-grown cultures, with putrescine at normal or high levels, the enzyme half-life was 50–55 min (Table I and Fig. 2). This was true despite the spermidine limitation in the latter culture. In the arginine-grown culture, producing neither putrescine nor spermidine, the half-life of the enzyme was 450 min (Table I and Fig. 2). Total trichloroacetic acid-insoluble protein turned over with a half-life of greater than 18 h, and this was unaffected by cyclohexylamine or arginine in the medium. No ornithine decarboxylase antigen of altered M_r was consistently observed in SDS-gel electrophoresis in the conditions used. Our data suggest that the rapid degradation of the enzyme was correlated with the presence of putrescine and only when this was stopped did the enzyme become stable.

The use of CHA poses the question of whether it is directly affecting the turnover of ornithine decarboxylase. In addition it could be argued that turnover is actually mediated by

TABLE I

Ornithine decarboxylase relative specific activity, half-life, and differential rate of synthesis in relation to polyamine synthesis in N. crassa. Enzyme activity was 35–50 nmol of CO_2 evolved/h/mg of protein with no additions in the control cells, grown in minimal medium

Additions	Polyamine synthesis		Ornithine decarboxylase		
	Putrescine	Spermidine	Specific activity	$t_{1/2}$	Relative synthesis rate
			fold[a]	*min*	*fold*
None	+	+	1	50–55	1
Cyclohexamine (10 mM)	+	−	4–10	50–55	4–5
Arginine (1 mM)	−	−	40–70	450–500	4–5

[a] From Refs. 5 and 6.

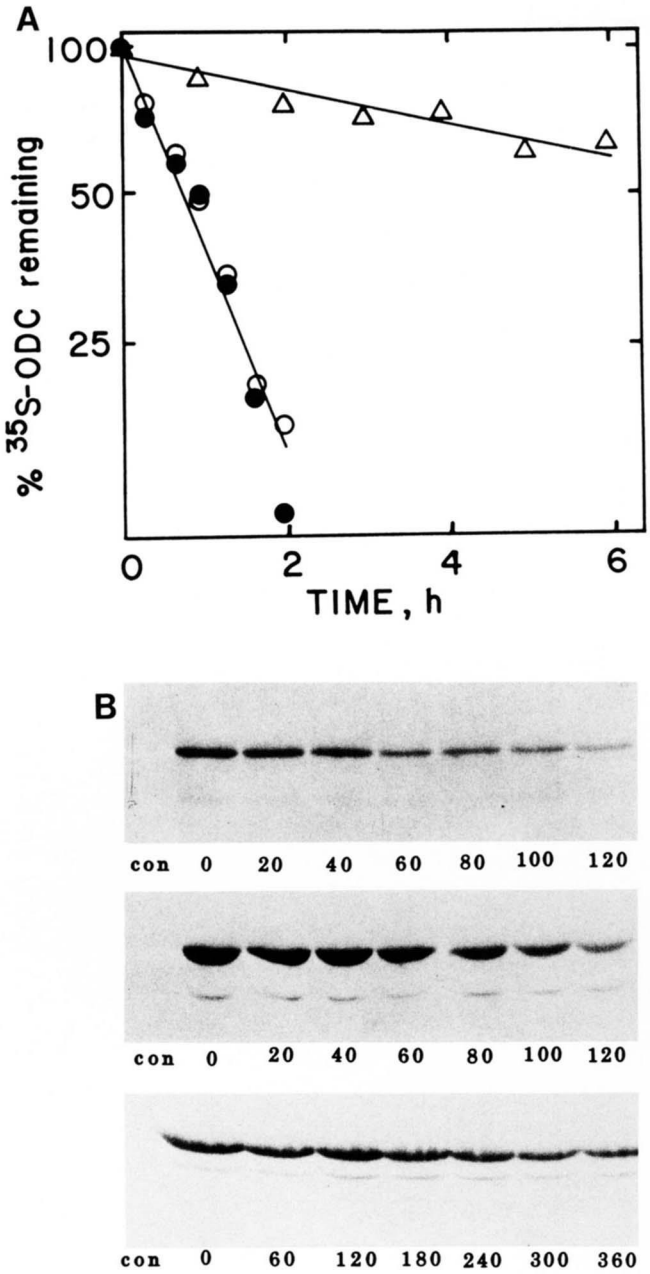

FIG. 2. **Loss of prelabeled ^{35}S-ornithine decarboxylase (*ODC*) with time in three cultures.** *A*, plots of densitometric values for the immunoprecipitated ornithine decarboxylase band in SDS gels. *Open circles*, minimal medium; *closed circles*, CHA-supplemented culture; *open triangles*, arginine-supplemented (polyamine-starved) culture. *B*, autoradiographs of the gels used for densitometry. The *left lane* in each case is a control, in which the immunoprecipitation was done in the presence of saturating amounts of an unlabeled preparation of the derepressed strain IC3. The remaining lanes were experimentals, in which the competing material was from an unlabeled preparation of the ornithine decarboxylase nonsense mutant, IC1894-53. *Numbers* are time in minutes from the beginning of the methionine chase. *Top*, minimal medium; *middle*, CHA-supplemented culture; *bottom*, arginine-supplemented culture.

spermidine, and CHA did not sufficiently reduce the rate of spermidine synthesis to interfere with its action. (The spermidine pool was about half-normal, and the growth rate was not impaired.) These questions were answered with strain IC1898-72A, carrying mutation LV105. This strain, in minimal medium, has a generation time 1.3–1.4 times normal; it has more than 3-fold the putrescine and less than 1/10 the spermidine pool of strain IC3 in the same conditions. The half-life of ornithine decarboxylase in this strain was 55 min (data not shown). The data show that neither CHA nor spermidine is necessary, and putrescine is sufficient, for fast enzyme turnover.

Another question is whether spermidine alone can induce rapid turnover of the enzyme. To answer this a mutant strain with a catalytically impaired ornithine decarboxylase protein was used so that endogenous putrescine synthesis would be blocked. The strain was grown to steady state in medium supplemented with 1 mM spermidine; the resulting internal pool of spermidine was slightly elevated, but putrescine was not detectable. The half-life of ornithine decarboxylase protein was 60 min. This indicates that spermidine alone can induce rapid turnover of the enzyme.

A mechanism of ornithine decarboxylase inactivation found in other organisms involves an ornithine decarboxylase-inhibitory protein called ornithine decarboxylase antizyme (7–9). This protein is produced when putrescine is plentiful and leads to an increased rate of ornithine decarboxylase turnover when the ornithine decarboxylase-antizyme complex forms. While no evidence for this mechanism was obtained in an earlier study of *N. crassa* (5), two more critical tests were applied here. First, the IC3 strain was derepressed for ornithine decarboxylase synthesis by growth for 4 h in arginine. Ornithine (2.5 mM) was added, leading to putrescine overproduction (19). Extracts were made, and ornithine decarboxylase (and putative ornithine decarboxylase-antizyme complexes) were immunoprecipitated with antiserum and Pansorbin. No antizyme activity was detected in supernatants after washing the immunoprecipitate with 0.25 M NaCl (20). This method would have detected antizyme-ornithine decarboxylase complexes at the level of 0.2% of the active ornithine decarboxylase.

A second method of detecting ornithine decarboxylase antizyme made use of strain IC1894-53, having no detectable ornithine decarboxylase protein. The strain was grown in 1 mM putrescine and 10 mM CHA, conditions which would promote the accumulation of free antizyme. Ornithine decarboxylase was not inhibited by extracts of this culture.

Ornithine Decarboxylase Synthesis—The increased enzyme activity seen in the arginine-grown (polyamine-deprived) IC3 culture (Table I) is associated with increased enzyme stability, but the increased enzyme activity of the CHA-grown culture (deprived only of spermidine) is not. We therefore measured differential rates of ornithine decarboxylase synthesis in the

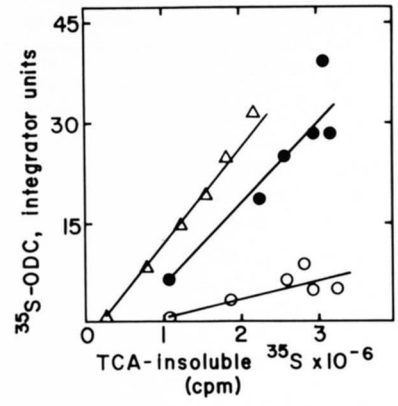

FIG. 3. **Differential rates of ornithine decarboxylase (*ODC*) synthesis.** Incorporation of [^{35}S]methionine into immunoprecipitated ornithine decarboxylase is plotted against isotope incorporation into trichloroacetic acid- (*TCA*) insoluble material. The slopes of the curves are the differential rates of the synthesis of ornithine decarboxylase protein. *Open circles*, minimal medium; *closed circles*, CHA-supplemented culture; *open triangles*, arginine-supplemented culture.

three experimental conditions. To do this we determined the rate of [^{35}S]methionine incorporation into the enzyme protein relative to isotope incorporation into total protein. The data are thus not confounded by alterations of specific activity of the methionine pool. Isotope incorporation was determined at 2-min intervals over a 12-min period, corresponding to the midpoint of the time during which the enzyme half-lives had been measured.

The data indicate that the increased enzyme activities of the CHA-grown (spermidine-deprived) and arginine-grown (spermidine- and putrescine-deprived) culture are associated with a 4–5-fold increase in the differential rate of enzyme synthesis (Table I and Fig. 3). The rate of enzyme synthesis is correlated to the limitation of spermidine synthesis and is not influenced by the the 20–40-fold excess accumulation of putrescine in the CHA-grown culture.

DISCUSSION

The results show that ornithine decarboxylase is controlled at the level of the synthesis and the degradation of enzyme protein. These processes differ in their response to the polyamines as metabolic signals: only spermidine controls synthesis, while both putrescine and spermidine control degradation.

Spermidine negatively affects the synthetic rate of the enzyme 4–5-fold and can account for a similar difference in specific activity between minimal and spermidine-starved mycelia. Putrescine appears to have no effect upon the synthetic rate of ornithine decarboxylase. The phase of gene expression affected by spermidine (transcription, mRNA stability, translation, etc.) is not yet known. Prototropic strains grown in the presence of spermidine have enzyme-specific activities about one-third to two-thirds normal. This must be explained by further repression, because spermidine, like putrescine, maintains rapid turnover of the enzyme.

Putrescine and spermidine induce a 10-fold faster turnover of the ornithine decarboxylase protein, compared to mycelia in which all polyamine synthesis is impeded. The same rate constant of inactivation was seen when putrescine and spermidine were being synthesized at normal levels and when the polyamines accumulated to greater than normal levels.

Taken together the mechanisms described account for the 50–70-fold difference in ornithine decarboxylase-specific activity in normal and polyamine-starved cells (6). Further, the mechanisms are separable, suggesting that repression and inactivation are not mediated by a single, polyamine-activated entity. The identification of the roles of the two polyamines in these mechanisms is clearer here than in mammalian systems, where further metabolism and turnover of the polyamines have obscured their individual roles in enzyme control. Evidence for the involvement of both putrescine (21, 22) and spermidine (23, 24) in promoting ornithine decarboxylase turnover has accumulated in mammalian systems, but it is not clear that both are effective in all systems.

In other eucaryotes polyamines appear to regulate ornithine decarboxylase activity by many mechanisms, including control of mRNA transcription (25, 26) and translatability (27), control of enzyme degradation (21–23, 28, 29), and antizyme action (9). Often, as in the case of *N. crassa*, these work in concert. However, several systems of enzyme inactivation appear to work through inactivation without proportionate loss of the enzyme protein (9, 30). Some of these mechanisms may involve an antizyme, others a covalent modification (30). In *N. crassa* isoelectric isoforms of the enzyme have been detected after polyamine depletion, and inactivation of the enzyme may be selective for one or more of these forms (5). We now seek the details of the inactivation process and the relative contributions of translational and transcriptional control to the rate of enzyme synthesis.

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