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Distinct roles of putrescine and spermidine in the regulation of ornithine decarboxylase in *Neurospora crassa*

(polyamines/protein turnover/compartmentation/arginase)

ROWLAND H. DAVIS*, GARY N. KRASNER†, JOSEPH J. DIGANGI*, AND JANET L. RISTOW*

*Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717; and †Coopervision Systems, 17601 Fitch Avenue, Irvine, CA 92714

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ABSTRACT **We wished to identify metabolic signals governing changes in ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) activity in *Neurospora crassa*. By manipulations of the ornithine supply and by the use of inhibitors of the polyamine pathway, we found that spermidine negatively governs formation of active ornithine decarboxylase and that putrescine promotes inactivation of the enzyme. Direct addition of putrescine or spermidine to cycloheximide-treated cells confirmed the role of putrescine in enzyme inactivation and showed that spermidine had no effect on this process. Increases in ornithine decarboxylase activity caused by blocking spermidine synthesis occurred prior to a significant decrease in the spermidine pool. This is consistent with our prevous finding that only 10–20% of the spermidine pool is freely diffusible within *N. crassa* cells. We presume that only this small fraction of the pool is active in regulation.**

Ornithine decarboxylase (OrnDCase; L-ornithine carboxy-lyase, EC 4.1.1.17) is a key enzyme of the synthesis of polyamines (putrescine, spermidine, and spermine) in most fungi and animals (1–3). While the roles of polyamines *in vivo* are still uncertain, these compounds are indispensable in eukaryotes (2). An increase of OrnDCase activity and an increased rate of polyamine synthesis are correlated with the onset of rapid growth, of transformation to the neoplastic state, and of periods of cell differentiation in most organisms (see refs. in ref. 2). Moreover, OrnDCase is rapidly inactivated upon the cessation of growth or upon addition of polyamines to cells. The regulation of the amount of active enzyme is the major mechanism of control; feedback and product inhibition are weak or absent.

A test of polyamine pathway intermediates (Fig. 1) for their roles in regulating the OrnDCase of *Neurospora crassa* is reported here. We identify spermidine and putrescine as regulatory signals governing, respectively, the formation and inactivation of OrnDCase catalytic activity. The work is discussed in connection with our previous observations (4–6) that polyamines are highly compartmentalized in cells of *N. crassa*.

MATERIALS AND METHODS

Strains, Growth, and Sampling. The strain of *N. crassa* used was IC3, a prototrophic strain carrying the arginase-less (*aga*) allele, UM-906. Vogel's medium N (7) was used for growth. Cultures were grown exponentially in 1-liter aerated cultures (8) for determinations of growth rate, enzyme activity, and polyamine pools. Samples (20 ml) for dry weight were collected on Whatman no. 1 filter circles and dried with acetone. Mycelial samples (10 ml) for polyamine determinations were collected and washed on membrane filters (pore size, 5 μm). The pad was extracted with 1 ml of 0.4 M perchloric acid with 1 mM EDTA. As an internal standard, 100 nmol of 1,7-diaminoheptane was added before centrifugation; the supernatant was saved and frozen until use. Mycelial samples (5 ml) for enzyme assay were permeabilized with a toluene/ethanol solution, followed by freezing (9).

OrnDCase Determination. Permeabilized cells were suspended in 1 ml of 50 mM K^+ PO_4 (pH 7.2) with 1 mM EDTA. Enzyme determinations on 100 μl of this suspension were made in duplicate in 0.3-ml reaction mixtures as described (10). Two aliquots of cell suspension were used for protein determination. The cells were brought up in 1 M NaOH, allowed to dissolve for 2 days at room temperature, followed by assay of protein by the Lowry method (11). A unit of activity is 1 nmol of product per hr at 37°C. Permeabilized cells yield approximately the same specific activities as cell extracts under a variety of conditions. Most enzyme data are given in terms of units per ml of culture, so that net changes in enzyme activity can be seen readily.

Polyamine Pools. Portions (100 μl) of the perchloric acid extracts were dansylated with an equal volume of dansyl chloride (5 mg per ml of acetone) in the presence of saturating Na_2CO_3. After standing overnight, the reactions were extracted with 0.2 ml of ethyl acetate, and the ethyl acetate extract was filtered through 0.45-μm membrane filters (Alpha-450, Gelman). The polyamines were determined with a high-performance liquid chromatography apparatus (Gilson), using an Altex Ultrasil ODS column (0.46 × 4.5 cm; particle size, 10 μm) and an elution program starting at 50% acetonitrile/water, and increasing linearly over 7.5 min to 95%, then decreasing to 50% over 3 min (12). Samples from cultures with dicyclohexylamine (DCHA) were eluted with a gradient of 50–70% acetonitrile over 7.5 min, 70–95% over 4 min, and a return to 50% over 3 min to resolve dansyl putrescine from dansyl DCHA. The effluent was monitored with a fluorescence detector, and the data were integrated with the Gilson Datamaster. Samples were run against calibrations of normal polyamines and internal standard dansylated at the same time as the unknowns. Polyamine data are given as nmol of polyamine per mg (dry weight), a parameter related to cellular concentration.

Immunoblots. A purified preparation of OrnDCase and a rabbit antibody to it were prepared as described in preliminary fashion (13). The antiserum used reacted to three or four other proteins of crude extracts; these could be removed by cross-absorbing the crude extract with nitrocellulose-bound proteins of a culture grown in spermidine-supplemented medium. The higher titer of the crude serum made it more favorable for counting of immunoblots. Immunoblots were prepared by standard procedures (14).

Chemicals. Most chemicals, including polyamines, were purchased from Sigma. L-[1-^{14}C]Ornithine was purchased

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Abbreviations: OrnDCase, ornithine decarboxylase; MGBG, methylglyoxal bis(guanylhydrazone); DCHA, dicyclohexylamine.

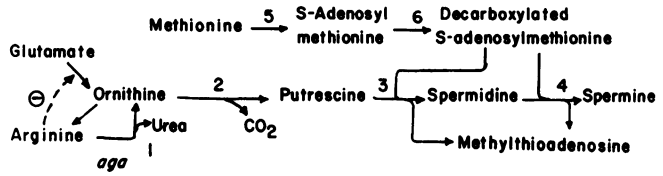

FIG. 1. Polyamine synthesis in *N. crassa*, showing enzymes and intermediates and the relationship of arginine, glutamate, and ornithine, the last initiating the polyamine pathway. In *N. crassa, de novo* synthesis of ornithine is feedback-inhibited by arginine, as indicated by a circled minus sign. The *aga* mutation blocks arginase (Enzyme 1), thus contributing to ornithine starvation of strains carrying this mutation when they are grown in the presence of arginine. Enzymes: 1, arginase; 2, OrnDCase; 3, spermidine synthase; 4, spermine synthase; 5, *S*-adenosylmethionine synthase; 6, *S*-adenosylmethionine decarboxylase. MGBG inhibits enzyme 6 and DCHA inhibits enzyme 3 in most organisms tested.

from ICN. Nitrocellulose (pore size, 0.2 μm) was obtained from Schleicher & Schuell.

RESULTS

Effects of Ornithine Deprivation. The arginase-less *aga* mutant of *N. crassa* grows well on minimal medium, but it acquires a partial polyamine requirement upon addition of arginine to the culture (15). This is due to ornithine deprivation: a genetic block in arginine catabolism to ornithine is combined with feedback inhibition by arginine of *de novo* ornithine synthesis (16, 17) (Fig. 1). Slow indefinite growth is supported by small amounts of cadaverine (1,5-diaminopentane) formed from lysine by the high OrnDCase activities in the cells. Cadaverine can satisfy the putrescine requirement to some extent (10).

An exponential culture of the *aga* mutant growing on minimal medium had a low differential rate of increase of OrnDCase activity (15–24 milliunits per mg of protein); the cellular pools of polyamines were (per mg dry weight) 1.0 nmol of putrescine, 16–18 nmol of spermidine, and 0.5 nmol of spermine. Two hours after the addition of 1 mM arginine, the differential rate of increase of OrnDCase activity increased abruptly by 75-fold (Fig. 2), a rate similar to that seen at steady state in arginine-grown cultures (10). At the time OrnDCase activity increased, ornithine had been exhausted, but little change in the polyamine pools had taken place. The poor correlation of polyamine pools and the increase of OrnDCase activity is discussed below; it suffices here to note the relationship of polyamine synthetic rates (which decline to zero) (10) and the increase of OrnDCase activity. Increase of OrnDCase activity could be the result of blocking further synthesis of any or all of the polyamines, of depletion of methylthioadenosine (liberated in the spermidine synthetase reaction), or of accumulation of decarboxylated *S*-adenosylmethionine as putrescine synthesis stops.

The rapid increase in OrnDCase activity is not influenced by additions of spermine (2.5 mM) (Fig. 2), methionine (1 mM), or methylthioadenosine (0.1 mM) (data not shown). Entry of these compounds into the cells was demonstrated by the following observations: (*i*) spermine addition raised the intracellular spermine pool to 18-fold its normal level; (*ii*) methionine led to accumulation of more decarboxylated *S*-adenosylmethionine (inferred from the more rapid synthesis of spermidine when ornithine was added to the culture); and (*iii*) methylthioadenosine inhibited growth.

By contrast, putrescine (2.5 mM) and spermidine (2.5 mM) led to lower rates of increase of OrnDCase activity (Fig. 2). The effects of these compounds were variable from one experiment to the next, in keeping with their inefficient uptake from the medium. In fact, it was not possible to

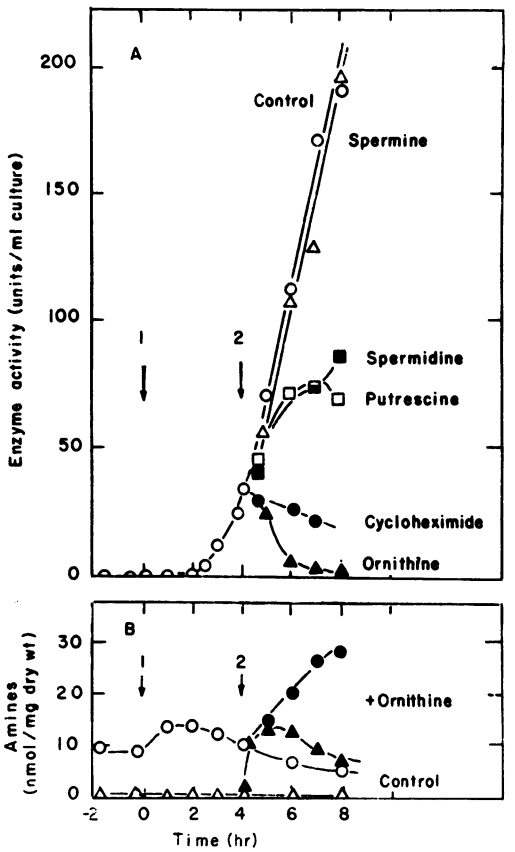

FIG. 2. (*A*) Effects on OrnDCase of 1 mM arginine addition (first arrow) to an *aga* grown in minimal medium, followed by other additions (as noted in *A* at the second arrow). ○, Control (arginine at first arrow with no further additions); △, 2.5 mM spermine at 4 hr; □, 2 mM putrescine at 4 hr; ■, 2.5 mM spermidine at 4 hr; ●, 10 μg of cycloheximide per ml of culture at 4 hr; ▲, 5 mM ornithine at 4 hr. (*B*) Putrescine (△, ▲) and spermidine (○, ●) pools of the control culture (arginine addition at first arrow with no further additions) and the culture treated at the second arrow with ornithine.

restore spermidine pools to their normal levels for some hours in the presence of high levels of this compound. When both polyamines were added simultaneously (data not shown), spermidine inhibited uptake of putrescine by the cells. Restoration of ornithine, however, led to rapid putrescine and spermidine synthesis, and to a rapid loss of OrnDCase activity (Fig. 2). The loss of enzyme activity was faster than if cycloheximide (10 μg per ml of culture) was added to the ornithine-deprived cells (Fig. 2). Other methods were needed to define individually the roles of putrescine and spermidine in the regulation of OrnDCase.

Effects of Methylglyoxal Bis(guanylhydrazone) (MGBG) and DCHA. Addition of 0.1 mM MGBG or 10 mM DCHA to the *aga* strain, growing on minimal medium, caused 10- to 20-fold higher differential rates of increase of OrnDCase activity (Table 1). Little effect on growth was seen, and the differential rates are characteristic of steady-state cultures. The increases of OrnDCase activity caused by MGBG and DCHA additions, and by ornithine deprivation, were correlated with equally severe reductions in spermidine synthesis. However, copious putrescine accumulation occurred with MGBG and DCHA additions (up to one-half of the putrescine made was excreted into the medium). MGBG inhibits *S*-adenosylmethionine decarboxylase (18), and DCHA inhibits spermidine synthase (19) in most organisms tested. The effects of these compounds upon the polyamine pools of *N. crassa* (Table 1) are consistent with these modes of action, although their specificities cannot be taken for granted. The results eliminate the accumulation of decarboxylated *S*-adenosyl-

Table 1. Polyamine pools and OrnDCase of mycelia grown in minimal medium or in medium supplemented with arginine, MGBG, or DCHA

Condition	Time, hr	Protein, mg/ml	Dry wt, mg/ml	Putrescine, nmol/mg (dry wt)	Spermidine, nmol/mg (dry wt)	OrnDCase,* Δ units/Δ mg
Minimal medium	0	0.028	0.30	1.1	7.6	
	4	0.104	0.66	0.5	18.8	15
	6	0.288	1.58	0.6	15.6	
With arginine (1 mM)	4	0.106	0.64	0	10.4	1833
	6	0.178	0.85	0	6.9	
With MGBG (0.1 mM)	4	0.138	0.67	9.5	6.7	164
	6	0.212	1.22	14.5	4.3	
With DCHA (10 mM)	4	0.154	0.72	10.3	8.3	140
	6	0.244	1.03	12.7	6.4	

*Differential rate of synthesis over the period in which protein increased from 0.1 to 0.2 mg per ml of culture.

methionine (blocked by MGBG) as an inducer of OrnDCase. The results with both inhibitors eliminate the lack of putrescine as a requirement for increases of OrnDCase activity. A remaining question is why the increase of OrnDCase activity caused by MGBG and DCHA is so much less than in conditions of ornithine deprivation. The reduced activity is not caused by the drugs directly: MGBG and DCHA do not influence the 75-fold higher rate of increase of enzyme activity resulting from ornithine deprivation of the *aga* strain (data not shown).

The Role of Putrescine in OrnDCase Inactivation. The lesser increases in OrnDCase activity caused by MGBG and DCHA additions, compared to that caused by ornithine deprivation, could be correlated with higher rates of enzyme inactivation. Cultures of the *aga* strain were grown from inoculation in minimal medium or in medium containing 0.1 mM MGBG, 10 mM DCHA, or 1 mM arginine, the last causing ornithine deprivation. At the time the cultures reached a density of ≈0.3 mg dry weight per ml, 50 μg of cycloheximide was added per ml of culture, and OrnDCase activity was monitored thereafter. The results (Fig. 3) show that the cultures with higher activity had more stable OrnDCase activity. In the first hour, the ornithine-deprived culture lost <10% of its activity; the MGBG-treated and minimal-grown cultures lost 55% and 70%, respectively. (The DCHA-treated culture, not shown on the figure, behaved almost identically to the MGBG-treated culture.) The results therefore indicate that increases of OrnDCase activity caused by MGBG and DCHA, in comparison to ornithine deprivation, were opposed by more rapid enzyme inactivation. Because inactivation was correlated with continued putrescine synthesis, the effect of putrescine additions was tested.

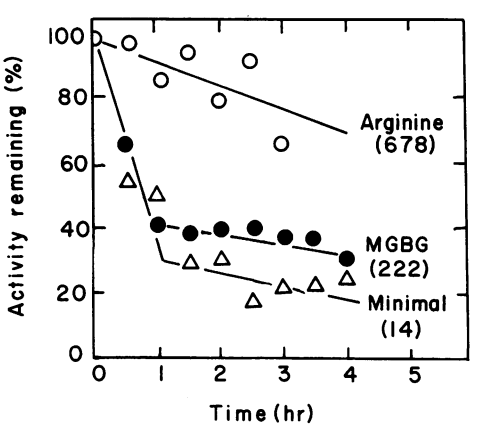

FIG. 3. Effect of cycloheximide addition (50 μg per ml of culture) on OrnDCase activity of *aga* cultures grown in minimal medium, medium supplemented with 1 mM arginine, or with 0.1 mM MGBG, as indicated. Specific activity of each culture at 0 hr is indicated below the designation of each culture.

An *aga* culture grown in arginine from inoculation was divided into four portions, each treated with cycloheximide (50 μg per ml of culture). Of the four cultures, one had no further additions; to the three others were added 2.5 mM putrescine, 2.5 mM putrescine/0.1 mM MGBG, or 2.5 mM spermidine. The results (Fig. 4) show that putrescine accumulation in cells is correlated with OrnDCase inactivation and that inactivation is independent of whether spermidine is present or absent. The results demonstrate that ornithine-deprived cells have the capacity to display OrnDCase inactivation, that putrescine suffices as a signal for inactivation, and that spermidine is not able to satisfy that role.

The period of OrnDCase inactivation in cycloheximide-treated cells is limited and somewhat variable [as are the values themselves (Fig. 3), for reasons that are not clear]. This leads to more muted responses of the system to

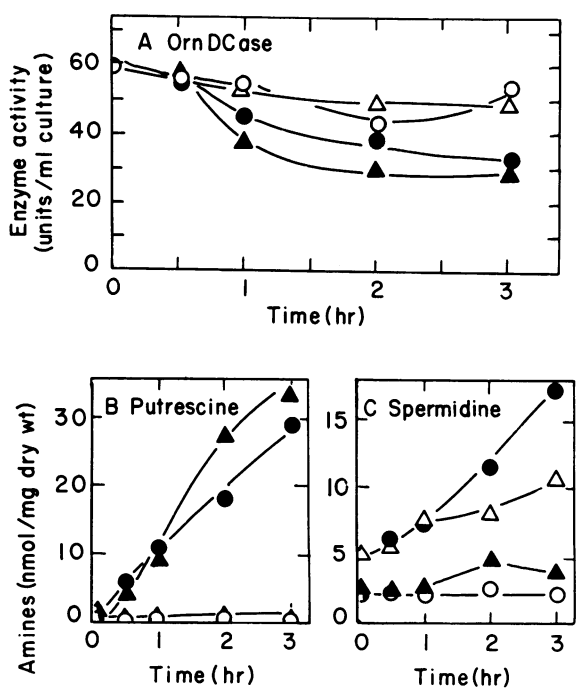

FIG. 4. Effect on OrnDCase activity (*A*) and polyamine pools (*B* and *C*) of various additions to cultures also treated with cycloheximide (50 μg per ml of culture). The *aga* strain was grown in the presence of arginine in order to starve the mycelia for ornithine and augment OrnDCase activity. Cycloheximide and other additions were made at 0 hr. ●, 2 mM putrescine; ▲, 2 mM putrescine/0.1 mM MGBG; ○, no further additions; △, 2 mM spermidine.

additions in comparison to what is seen without cycloheximide (see below). The aborted inactivation process will be discussed in a later section.

Loss of OrnDCase Activity Upon Restoration of Ornithine to Ornithine-Starved Cultures. Addition of ornithine to arginine-grown *aga* cultures was followed by rapid putrescine and spermidine synthesis. A rapid decay of OrnDCase activity ($t_{1/2}$ = 1.5 hr; Fig. 2) took place at the same time. After 5 hr, the specific activity of OrnDCase and the rates of polyamine synthesis were restored to normal. If MGBG is added with ornithine, a transient or only slow decay of OrnDCase activity is seen (data not shown). In the latter cultures, putrescine accumulated copiously, and only a small amount of spermidine synthesis takes place early in the experiment. Such results are consistent with the more direct observations described above. The rapid loss of OrnDCase activity upon addition of ornithine alone can be attributed to putrescine-mediated enzyme inactivation and the spermidine-mediated block in the formation of more active enzyme. When MGBG is also added, only enzyme inactivation would occur, but active enzyme formation continues, owing to the continued lack of spermidine. The kinetics of increase and decrease of enzyme activity varies in such experiments. This is to be expected, because enzyme activity here is determined by the rapid and opposing processes of formation and inactivation.

Regulation of the Amount of OrnDCase Protein. Using an antibody that recognizes the OrnDCase protein, we visualized OrnDCase on immunoblots (Fig. 5). The large variation of activity was correlated with variation in the amount of protein. Counting the ^{125}I-labeled bands corresponding to OrnDCase, however, showed that the amount of OrnDCase protein in cultures deprived of ornithine was only 7.7-fold that in cultures grown in minimal medium, while the enzyme activities differed by a factor of 43. Similarly, ornithine addition to the ornithine-deprived culture caused a 75% decrease in OrnDCase protein after 6 hr, as the activity decreased 98%. (Both parameters are given on the basis of culture volume.) The data suggest that both modification and loss of the OrnDCase protein take place in the inactivation process.

DISCUSSION

Our results indicate that spermidine negatively governs formation of active OrnDCase and that putrescine promotes inactivation of the enzyme. The manipulations of the pathway with inhibitors, with ornithine deprivation, and with additions of pathway intermediates appear to exclude methionine, decarboxylated *S*-adenosylmethionine, methylthioadenosine, and spermine from prominent roles in OrnDCase formation and inactivation. While data regarding the methionine-related intermediates must be verified by direct measurement, addition of inhibitors and certain intermediates themselves had the expected metabolic effects and support the major conclusions. The use of cycloheximide allowed independent assessment of the inactivation process, although variation of its extent and duration compromised quantitative correlations.

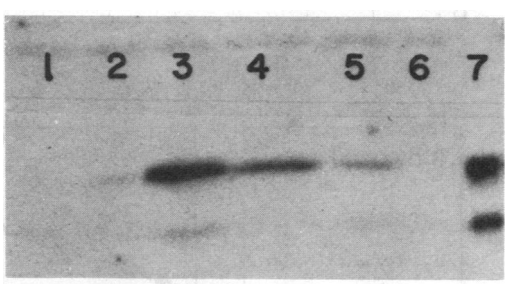

FIG. 5. Immunoblot of crude mycelial extracts and of purified OrnDCase of *N. crassa*. The amount of protein loaded in each lane was constant. Lanes 1–3, *aga* strain grown on minimal medium with 1 mM spermidine, no additions, and 1 mM arginine, respectively. Lanes 4–6, arginine-grown strain 2, 4, and 6 hr, respectively, after addition of ornithine to the culture. Lane 7, purified OrnDCase. (The lower band in lane 7 is a proteolytic product of the native enzyme, which becomes prominent during purification.) The units of enzyme applied and the ^{125}I counts in the bands corresponding to OrnDCase were, respectively, 0.25 and 117 (lane 1), 0.71 and 143 (lane 2), 30.7 and 1098 (lane 3), 9.5 and 788 (lane 4), 4.6 and 631 (lane 5), 0.5 and 273 (lane 6). The last three were corrected for the growth of the culture that occurred after ornithine addition.

The behavior of OrnDCase activity in cycloheximide-treated cultures suggests that the inactivation system itself may depend on protein synthesis for its maintenance. One obvious model for the system is that a stoichiometrically binding OrnDCase "antizyme" is available in cells (20). If it is consumed without replenishment in the inactivation process, the extent of OrnDCase inactivation will be limited by the initial antizyme content. To date, we have no evidence for an OrnDCase antizyme in *N. crassa*, but critical tests are lacking. The other model is that an OrnDCase-modifying system is responsible for inactivation (e.g., see refs. 21 and 22) and that this system itself turns over rapidly. Again, there is little critical evidence on the point. The behavior of the protein itself shows that the increases and loss of OrnDCase activity are correlated with increases and decreases of the amount of OrnDCase protein. Because changes in the protein levels are considerably less pronounced than changes in activity, modification of the protein may prevail, particularly during inactivation and before its disappearance.

The poor correlation of changes in OrnDCase activity and the much slower changes in cellular polyamines (ref. 4; Fig. 2) are paradoxical and are similar to findings in other systems. The enzyme appears to respond to variation of polyamine synthetic rates rather than to the cellular polyamine content. The data are reconciled by our previous findings that only 10–20% of the cellular putrescine and spermidine in *N. crassa* are freely accessible as pathway intermediates, the rest being sequestered (4–6, 23). This being the case, the cessation of polyamine synthesis would lead to a rapid depletion (by sequestration) of the freely accessible polyamines. Conversely, the restoration of abnormally rapid polyamine synthesis would lead to abnormally rapid accumulation of the freely accessible fraction. We therefore presume that the fraction of the pools accessible to the biosynthetic enzymes are those that are active in the regulation of OrnDCase.

Studies in other organisms (24–27) have been unable to identify metabolic signals for OrnDCase regulation with precision. Some studies similar to those reported here have been done with the yeast *Saccharomyces cerevisiae* (26). Spermidine and spermine caused OrnDCase inactivation when added to growing cultures of wild type or of the *spe2* mutant, the latter blocked in *S*-adenosylmethionine decarboxylase activity. Putrescine had no effect. Moreover, cycloheximide blocked the effects of spermidine and spermine. These data indicate, although not definitively, a real difference in the metabolic signal for inactivation between *S. cerevisiae* and *N. crassa*. In most systems, putrescine synthesis cannot be blocked without inhibiting OrnDCase itself. In the present work, the *aga* mutation allowed us to block synthesis of all polyamines through ornithine starvation, and to use this regime to distinguish the roles of putrescine and spermidine.

In the slime mold *Physarum polycephalum*, Atmar and Kuehn report that a nucleolar form of OrnDCase is inactivated by phosphorylation by a spermidine/spermine-activated protein kinase, which putrescine actually inhibits (22). This is another indication that the roles of the polyamines in *N. crassa* may not be universal.

The mammalian systems that have been investigated, however, show that active OrnDCase decreases in response to additions of putrescine (27) and the putrescine analog, 1,3-diaminopropane (28, 29). Because 1,3-diaminopropane is not readily metabolized to the corresponding spermidine analog (30), the data suggest that putrescine has a negative effect independent of its conversion to spermidine. Both OrnDCase antizyme formation (20) and loss of OrnDCase protein (31) have been reported in cells treated with 1,3-diaminopropane.

The major contributions of the present study are to assign to putrescine and spermidine different and specific roles in OrnDCase regulation in *N. crassa* and to legitimize the idea of small "free" putrescine and spermidine pools as regulators. These are points that have not been demonstrated clearly in other organisms, owing to limitations of methodology or constraints of the biological systems. If conditions allowing comparable experiments with mammalian cells are found, it will be important to define the roles of putrescine and spermidine in OrnDCase regulation. Only then will it be possible to determine whether OrnDCase regulation is always mediated by these intermediates or whether the enzyme responds separately to other signals, such as might be seen at the onset of growth and differentiation. Because of the substantial compartmentation of spermidine and putrescine that may prevail, some of the regulatory responses of OrnDCase to growth-related stimuli in other organisms may be provoked by redistribution of polyamines between the freely diffusible and sequestered states.

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