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INACTIVATION OF YEAST ORNITHINE DECARBOXYLASE BY POLYAMINES IN VIVO DOES NOT RESULT FROM THE INCORPORATION OF POLYAMINES INTO ENZYME PROTEIN

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SUMMARY We have previously reported that addition of spermidine and spermine to a culture of Saccharomyces cerevisiae results in loss of ornithine decarboxylase activity even though there is no loss of enzyme protein. These findings indicate that the loss of activity is due to a post-translational modification of the enzyme. Since transglutaminase has been shown to catalyze the incorporation of putrescine into ornithine decarboxylase in vitro with resultant loss of enzymatic activity [Russell, D. H. (1981) Biochem. Biophys. Res. Commun. 99, 1167-1172], we decided to see if the inactivation we observed in vivo was caused by such incorporation. We found that this is not the case; i.e., after growth of yeast with added amines, the inactivated ornithine decarboxylase contains no covalently-bound spermidine or spermine. Thus, we conclude that transamidation by transglutaminase does not play a role in vivo in the polyamine-mediated post-translational modification of yeast ornithine decarboxylase.

The synthesis of polyamines in eukaryotic systems is controlled by the level of ornithine decarboxylase which catalyzes the first step in the biosynthetic pathway (1). The activity of this enzyme varies markedly in response to any change in growth rate or to stimulation by hormones and a variety of other agents (1-4). In several eukaryotic systems the addition of exogenous polyamines or diamines in vivo results in a decrease in the activity of ornithine decarboxylase (5-12), but the mechanism of this effect is not understood. Post-translational modification of ornithine decarboxylase has been suggested as one of the control mechanisms by several authors (10, 12-15).

Polyamines and diamines have been shown to be incorporated by transglutaminase in vitro into a variety of proteins, including ornithine decarboxylase (16, 17). Since in vitro incorporation of putrescine into ornithine decarboxylase resulted in loss of enzymatic activity, it was suggested that transamidation of ornithine decarboxylase by amines might be responsible for the post-translational modification of this enzyme in vivo (17).

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We have previously shown that addition of spermidine and spermine to the growth medium resulted in the loss of ornithine decarboxylase activity in growing Saccharomyces cerevisiae without any loss of immunologically reactive protein (15). In the present study we have investigated the possibility that post-translational modification of the ornithine decarboxylase in these experiments resulted from the incorporation of the amines into the enzyme protein by the action of transglutaminase in vivo. We report here that the in vivo loss of ornithine decarboxylase activity after addition of polyamines to a culture of S. cerevisiae does not result from incorporation of polyamines into the enzyme by a direct transamidation of enzyme protein.

MATERIALS AND METHODS

L-[1-^{14}C]-Ornithine (60 mCi/mmol), [terminal methylenes - ^{3}H]spermidine trihydrochloride (15 Ci/mmol), [3-aminopropyl-3-^{3}H]spermine tetrahydrochloride (20 Ci/mmol), and [^{35}S]methionine (400 Ci/mmol) were purchased from New England Nuclear. [Tetramethylene 1,4-^{14}C]spermine tetrahydrochloride (122 mCi/mmol) and [tetramethylene-1,4-^{14}C]-spermidine trihydrochloride (122 mCi/mmol) were from Amersham. [5-^{14}C]Difluoromethylornithine (60 mCi/mmol) was a gift from Dr. Peter McCann, Merrell Dow Pharmaceuticals.

Growth of Yeast — For these experiments we used a mutant of S. cerevisiae AN33 which cannot synthesize spermidine or spermine because it lacks S-adenosylmethionine decarboxylase (spe2-4) (18) and which is derepressed for ornithine decarboxylase when grown in minimal media. The cells were grown in SD medium containing 2% dextrose, 0.7% Difco yeast nitrogen base without amino acids, 0.005% adenine, and 0.025% threonine (18); the pH was adjusted to 7 with K_2HPO_4. The culture was grown with aeration at 28°C to the indicated optical density.

The cells were harvested by centrifugation and washed with distilled water. The pellet was suspended in 0.025 M Tris-chloride, pH 7.6, containing 5 mM dithiothreitol, 1 mM $MgCl_2$, and 0.1 mM EDTA, and passed through a cooled French press at 18,000 psi. The extract was immediately cooled in ice and centrifuged for 60 minutes at 20,000 x g. The supernatant was passed once through a Millipore filter (0.45 μm pore size). All steps were at 0-4°.

RESULTS AND DISCUSSION

Two procedures were used for the isolation of ornithine decarboxylase since we have found that the molecular weight (Mr) of the ornithine decarboxylase isolated depends on the conditions of the extraction. In experiment A, cell disruption was carried out in the presence of phenylmethylsulfonyl fluoride to inhibit proteolysis. Ornithine decarboxylase, isolated rapidly

by immunoprecipitation at 0°C from such extracts, migrated as an 86,000 Mr form in sodium dodecylsulfate electrophoresis (19). A small amount of an 81,000 Mr component was also present, but no 68,000 Mr form was found. In experiment B, phenylmethylsulfonyl fluoride was not added, and the ornithine decarboxylase isolated by immunoprecipitation was in the 68,000 Mr form. This 68,000 Mr form is identical in size to the homogeneous enzyme that we had obtained previously (15) [1], [2] (by a procedure that did not include phenylmethylsulfonyl fluoride). This homogeneous 68,000 Mr preparation served as the antigen for the production of the antisera (15) used for both experiment A and experiment B.

Experiment A. To test whether incorporation of polyamines into ornithine decarboxylase could explain the loss of activity noted when polyamines were added to a growing culture of S. cerevisiae, the spe2-4 mutant was grown to an $A_{650\ nm}$ of 1.5 in 40 ml of medium; [^{14}C]spermidine (122 mCi/mmol) and [^{14}C]-spermine (122 mCi/mmol) (final concentration 10 μM each) were then added to the culture, and the incubation was continued for 6 hours. (Addition of spermidine and spermine at this concentration results in a 97% decrease in enzyme activity after 6 hours.)

After the 6-hour incubation, the cells were collected and extracts prepared as described in Materials and Methods; 1 mM phenylmethylsulfonyl fluoride was present in the extracting fluid. Immunoprecipitation was then carried out, as described in reference 15, with a rabbit antiserum prepared against yeast ornithine decarboxylase. 100 μl of cell extract, containing about 500 μg of protein and 5 pmol of ornithine decarboxylase, were treated with the

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[1] Pösö and Pegg have recently reported that a partially purified ornithine decarboxylase from Saccharomyces uvarum had a molecular weight of 72,000 (20).

[2] We assume that the 68,000 Mr protein that we previously described was formed from the 86,000 Mr protein during the purification procedure. This conversion of the 86,000 Mr form to the smaller form, however, is not relevant to the modification induced by polyamines, since growth with or without polyamines does not affect the molecular weight or the quantity of the immunoreactive ornithine decarboxylase protein isolated (i.e., the molecular weight found was affected only by the method of extraction).

rabbit antibody and with an excess of protein A - Sepharose CL-4B in the presence of 1 mM phenylmethylsulfonyl fluoride. The immunoprecipitates were either counted directly or subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, followed by autoradiography. No ^{14}C was present in the immunoprecipitate, and no ^{14}C was found anywhere on the gels, even after exposure of the film to the gel for 30 days (Table 1, experiment A, and Figure 1).

In a parallel set of experiments as a control, cells were grown in the presence of [^{35}S]methionine (30 μg/ml and 4 μCi/ml). When the culture reached an $A_{650\ nm}$ of 1.5, the culture was divided into two parts; to one-half, non-radioactive spermidine and spermine were added to a final concentration of 10 μM each. Incubation was continued for 6 hours. The cells were harvested, and

TABLE 1

Radioactivity found in ornithine decarboxylase after growth with labeled polyamines or with labeled methionine

Addition to the culture	% Decrease in enzyme activity	Radioactivity in immunoprecipitates (cpm)
Experiment A (with phenylmethylsulfonyl fluoride)		
[^{14}C]Spermine + [^{14}C]spermidine	> 97%	none [a]
[^{35}S]Methionine + unlabeled spermine and spermidine	> 97%	2,400
[^{35}S]Methionine alone	0%	2,430
Experiment B (without phenylmethylsulfonyl fluoride)		
[^{3}H]Spermine + [^{3}H]spermidine	> 97%	none [b]
[^{35}S]Methionine + unlabeled spermine and spermidine	> 97%	2,350

[a] Incorporation of 1 mole of either amine into 1 mole of enzyme would have resulted in ~ 1,000 cpm (see text).

[b] Radioactivity in this case was counted in the gels after dissolving them in alkaline hydrogen peroxide (23). Incorporation of 1 mole of either amine into 1 mole of enzyme would have resulted in ~ 150,000 cpm (see text).

ornithine decarboxylase was isolated by immunoprecipitation as described above. ^{35}S was found in these immunoprecipitates, and, on sodium dodecylsulfate gel electrophoresis, the radioactivity migrated principally to the 86,000 Mr position, with a small amount of radioactivity in the 81,000 Mr position (Table 1, experiment A, and Figure 1). Comparable amounts of ^{35}S were present in the immunoprecipitates from extracts of cells grown with or without the addition of polyamines (Table 1). These experiments with [^{35}S]methionine showed that the immunoreactive protein was present, even though none of the ^{14}C-labeled amines was found in the immunoprecipitates.

Experiment B. In another set of experiments the cells were labeled as described for experiment A, except that ^{3}H-labeled amines were used instead of ^{14}C-labeled amines, and no phenylmethylsulfonyl fluoride was present during the extraction. As in Experiment A, the antiserum did not precipitate any radioactivity in the experiment with [^{3}H]polyamines (Table 1, experiment B); no labeled protein was observed on the gel even after 40 days' exposure of the film to the gel (data not shown). On the other hand, in the experiment with

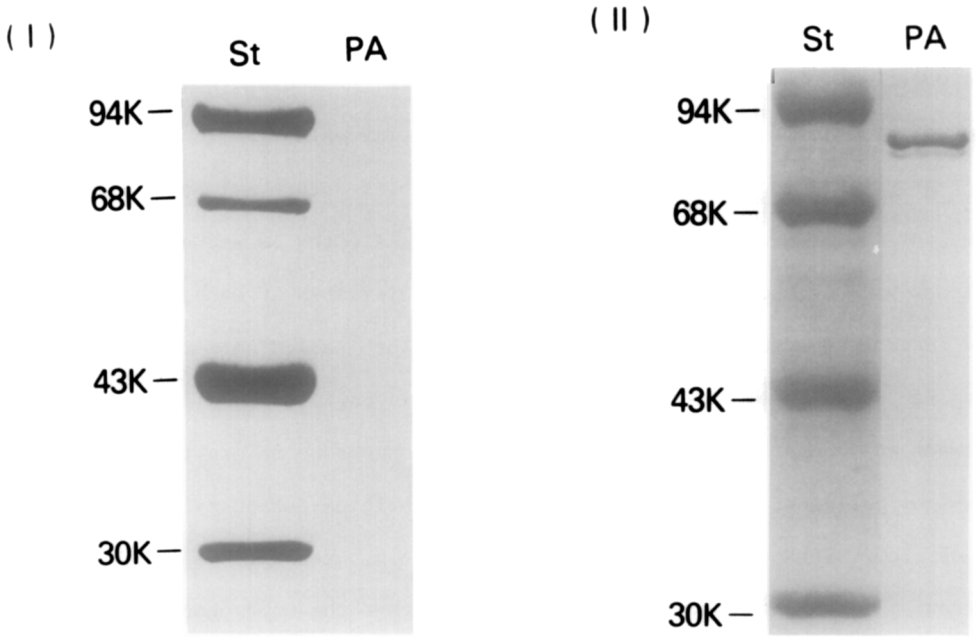

FIG. 1. Autoradiography of ornithine decarboxylase isolated by Method A after growth in the presence of added polyamines. (I) St, ^{14}C-labeled standards; PA, immunoprecipitates from extracts of cells grown with [^{14}C]polyamines. (II) St, ^{14}C-labeled standards; PA, immunoprecipitates from extracts of cells grown with [^{35}S]methionine and nonradioactive polyamines.

[^{35}S]methionine and unlabeled amines, a band of 68,000 Mr was observed after immunoprecipitation and electrophoresis. In comparable experiments we showed that the amount of antiserum used was sufficient for complete precipitation. These studies show that ^{3}H-labeled polyamines were not incorporated into ornithine decarboxylase, confirming the results in experiment A.

Amine Uptake — To be certain that the labeled amines had been taken up by the yeast cells, we next measured the uptake of labeled amines by yeast, which had been grown to an $A_{650\ nm}$ of 1.5 in amine-free medium. The addition of [^{14}C]spermine or [^{14}C]spermidine to a final concentration of 10 μM to the medium resulted in the uptake of 7.6 nmol and 2.3 nmol of labeled spermidine or labeled spermine, respectively, per ml of culture (containing 0.9 mg cells, wet weight) in 2 hours at 28°C. We also determined the amount of residual endogenous spermidine and spermine after growing the cells to an $A_{650\ nm}$ of 1.5 (before the labeled amines had been added) in order to determine if any significant dilution of the specific activity of the labeled amines could have occurred. Analysis of these cells with the assay procedure described in reference 21 revealed no detectable endogenous spermidine and spermine. Thus the specific activity of the polyamines added to the medium could not have been diluted intracellularly by endogenous polyamines.

We determined the number of nmol of ornithine decarboxylase in the sample used for immunoprecipitations in order to calculate the amount of radioactivity which would have been present in the immunoprecipitates if each enzyme molecule had indeed contained at least one molecule of the labeled amine. Since we had obtained a homogeneous enzyme after a 1,500-fold purification (15), the crude extract must have contained approximately 670 nanograms of ornithine decarboxylase per mg of protein, i.e., 5 pmol of ornithine decarboxylase in the 100 μl of crude extract (containing 500 μg of protein) used for the immunoprecipitation. Since it was important to be certain that the homogeneous protein isolated after the 1,500-fold purification was indeed active ornithine decarboxylase, we quantitated the amount of ornithine decarboxylase in the 1,500-fold purified preparation by allowing it to react with [^{14}C]difluoromethyl-

ornithine according to the method of Pritchard et al. (22). This compound is a suicide inhibitor of ornithine decarboxylase and reacts stoichiometrically at the active site to form an irreversible covalently-linked derivative with active enzyme protein. We found that 0.75 nmol of labeled inhibitor reacted with 1.0 nmol of the purified protein, indicating that at least 75% of the purified preparation consisted of active ornithine decarboxylase.

With the above data (i.e., 5 pmol of enzyme in the 100 µl of crude extract) and the known specific activities of the added tritiated or [^{14}C]-polyamines one can calculate that, even if only one mole of either amine had reacted with each mole of enzyme, the immunoprecipitates would have had ~ 150,000 cpm of [^{3}H]spermidine and [^{3}H]spermine or more than 1,000 cpm when [^{14}C]spermine and [^{14}C]spermidine were used (Table 1). However, no radioactivity was found in the immunoprecipitates from the cell extracts labeled with either [^{14}C]polyamines or [^{3}H]polyamines.

These studies show that post-translational modification of ornithine decarboxylase *in vivo* leading to its complete inactivation after growth in the presence of radioactive spermine and spermidine does not result from the covalent binding of these amines to the enzyme protein.

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