UC Irvine
UC Irvine Previously Published Works

Title
Ornithine decarboxylase from Neurospora crassa. Purification, characterization, and regulation by inactivation.

Permalink
https://escholarship.org/uc/item/0td612r4

Journal
Journal of Biological Chemistry, 262(16)

ISSN
0021-9258

Authors
DiGangi, JJ
Seyfzadeh, M
Davis, RH

Publication Date
1987-06-01

DOI
10.1016/s0021-9258(18)47651-x

Copyright Information
This work is made available under the terms of a Creative Commons Attribution License, availalbe at https://creativecommons.org/licenses/by/4.0/

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1987 by The American Society of Biological Chemists, Inc.

Vol. 262, No. 16, Issue of June 5, pp. 7889-7893, 1987
Printed in U.S.A.

Ornithine Decarboxylase from *Neurospora crassa*

PURIFICATION, CHARACTERIZATION, AND REGULATION BY INACTIVATION*

(Received for publication, November 21, 1986)

Joseph J. DiGangi‡, Manouchehr Seyfzadeh, and Rowland H. Davis§

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

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 enzyes 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 (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 *Physarum 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[1]

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[2]-purified preparation contained polypeptides of M_r = 53,000 and lesser amounts of others of M_r = 44,000-47,000, visualized after SDS-polyacrylamide gel electrophoresis (Fig. 2*A*). The lower molecular weight band(s) were labeled if the enzyme preparation was exposed to [^{14}C]difluoromethylornithine, which binds specifically and covalently to active ornithine decarboxylase molecules (Fig. 2*B*). 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. 2*C*).

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

* This work was supported in part by Research Grant BC-366 from the American Cancer Society, and United States Public Health Service Research Grant GM-35120 from the National Institute of General Medical Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Predoctoral trainee supported by Public Health Service Training Grant GM-07311. Most of this work was part of a dissertation submitted in partial fulfillment of the requirements for the degree of Ph.D. in Biological Sciences, University of California, 1985. Present address: NPI, 417 Wakara Way, Salt Lake City, UT 84108.

§ To whom reprint requests should be addressed.

[1] 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 $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; NP-40, Nonidet P-40; ODC, ornithine decarboxylase; U, unit; RIA, radioimmunoassay.

TABLE I
Purification of ornithine decarboxylase from N. crassa

Step	Total protein	Volume	Total activity	Yield	Specific activity	Purification	Purity[a]	DFMO[a]
	mg	*ml*	*units*	*%*	*units/mg protein*	*-fold*	*%*	*pmol/unit*
Crude extract	8,200	1,400	34,160	100	3.9	1.0	0.15	
Ammonium sulfate	1,105	65	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	2,138	548	81	6.9
HPLC ion exchange	1.1	1.1	3,861	11	2,610	669	99	6.8

[a] Percent purity was calculated using the average picomoles of difluoromethylornithine (DFMO) per unit of enzyme (7.3), the known subunit M_r, 53,000, and the assumption that 1 mol of DFMO binds per mol of subunit.

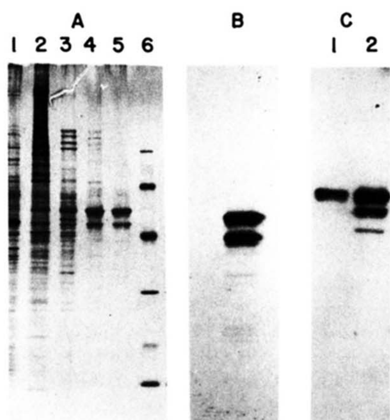

FIG. 2. *A*, purification of ornithine decarboxylase. Preparations were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis and the gel was stained with silver. The *lanes* are: *1*, crude extract; *2*, 42.5% ammonium sulfate fraction; *3*, Bio-Gel P-200 eluate; *4*, DE52 ion exchange eluate; *5*, HPLC ion exchange eluate; *6*, standard proteins (from *top*, phosphorylase *b*, M_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; α-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.

molecules at each stage of purification (see "Experimental Procedures" in Miniprint). The intrinsic specific activity of ornithine decarboxylase is formally represented by the inverse of the value of picomoles of difluoromethylornithine bound/unit of ornithine decarboxylase activity. The constancy of this value during purification (Table I) indicates that the specific activity of active ornithine decarboxylase molecules was not affected by the purification procedure. All of the protein of pure preparations bound [^{14}C]difluoromethylornithine, indicating that no inactive ornithine decarboxylase molecules were present. Moreover, the specific activity of pure ornithine decarboxylase predicted by difluoromethylornithine 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 300, 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. 2*A*), 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 [^{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

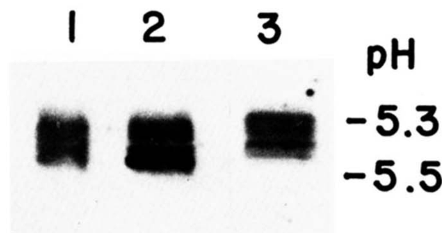

FIG. 3. **Western immunoblot of isoelectric focusing gel.** *Lanes: 1*, purified ornithine decarboxylase; *2*, crude, derepressed extract of the *aga* strain, grown in arginine; *3*, derepressed extract 2 h after the onset of enzyme inactivation (approximately one-half the initial activity had disappeared at the time of sampling).

either increased or greatly diminished putrescine content. A strain carrying the *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-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 ^{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 *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.

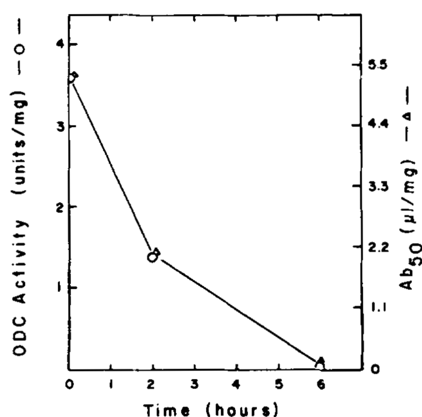

FIG. 4. **Decline of ornithine decarboxylase (*ODC*) specific activity (units/mg of protein, *left ordinate*) and immunoreactive protein (Ab_{50}, *right ordinate*) after restoration of ornithine to an ornithine- (and thus polyamine-) starved culture.**

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_{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—*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 = 86,000 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 *N. crassa* and *P. polycephalum*. In fact, the *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 *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*.[3]

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-

[3] G. 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.

Acknowledgments—We thank Dr. Daniel Knauer for advice in immunochemical techniques and for ^{125}I-protein A, Janet Ristow for excellent technical assistance, and Robert Yamamoto for help in immunizing rabbits.

REFERENCES

1. Kitani, T., and Fujisawa, H. (1983) *J. Biol. Chem.* **258,** 235–239
2. Kameji, T., Murakami, Y., Fujita, K., and Hayashi, S.-I. (1982) *Biochim. Biophys. Acta* **717,** 111–117
3. Seely, J. E., Poso, H., and Pegg, A. E. (1982) *Biochemistry* **21,** 3394–3399
4. Isomaa, V. V., Pajunen, A. E. I., Bardin, C. W., and Jänne, O. A. (1983) *J. Biol. Chem.* **258,** 6735–6740
5. Tyagi, A. K., Tabor, C. W., and Tabor, H. (1981) *J. Biol. Chem.* **256,** 12156–12163
6. Sklaviadis, T. K., Gerogatsos, J. G., and Kyriakidis, D. A. (1985) *Biochim. Biophys. Acta* **831,** 288–296
7. Barnett, G. R., and Kazarinoff, M. N. (1984) *J. Biol. Chem.* **259,** 179–183
8. Tabor, C. W., and Tabor, H. (1984) *Annu. Rev. Biochem.* **53,** 749–790
9. Canellakis, E. S., Viceps-Madore, D., Kyriakidis, D. A., and Heller, J. S. (1979) *Curr. Top. Cell. Regul.* **15,** 155–202
10. McCann, P. P., Tardif, C., Hornsperger, J.-M., and Bohlen, P. (1979) *J. Cell. Physiol.* **99,** 183–190
11. Seely, J. E., and Pegg, A. E. (1983) *J. Biol. Chem.* **258,** 2496–2500
12. Kanemoto, R., Utsunomiya, K., Kameji, T., and Hayashi, S.-I. (1986) *Eur. J. Biochem.* **154,** 539–544
13. Holtta, E., and Pohjanpelto, P. (1986) *J. Biol. Chem.* **261,** 9502–9508
14. Mitchell, J. L. A., Carter, D. D., and Rybski, J. A. (1978) *Eur. J. Biochem.* **92,** 325–331
15. Dircks, L., Grens, A., Slezynger, T. C., and Scheffler, I. E. (1986) *J. Cell. Physiol.* **126,** 371–378
16. Davis, R. H., Krasner, G. N., DiGangi, J. J., and Ristow, J. L. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4105–4109
17. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106
18. Davis, R. H., Lawless, M. B., and Port, L. A. (1970) *J. Bacteriol.* **102,** 299–305
19. Paulus, T. J., and Davis, R. H. (1981) *J. Bacteriol.* **145,** 14–20
20. Persson, L., Seely, J. E., and Pegg, A. E. (1984) *Biochemistry* **23,** 3777–3783
21. Eversole, P. E., DiGangi, J. J., Menees, T., and Davis, R. H. (1985) *Mol. Cell. Biol.* **5,** 1301–1306
22. Batchelor, K. W., Smith, R. A., and Watson, N. S. (1986) *Biochem. J.* **233,** 307–308
23. Mitchell, J. L. A., and Wilson, J. M. (1983) *Biochem. J.* **214,** 345–351
24. Heller, J. S., Fong, W. F., and Canellakis, E. S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 1858–1862
25. Heller, J. S., Chen, K. Y., Kyriakidis, D. A., Fong, W. F., and Canellakis, E. S. (1978) *J. Cell. Physiol.* **96,** 225–236
26. Fujita, K., Murakami, Y., and Hayashi, S. (1982) *Biochem. J.* **204,** 647–652
27. Davis, R. H., and de Serres, F. J. (1970) *Methods Enzymol.* **17A,** 79–143
28. Vogel, H. J. (1964) *Am. Nat.* **98,** 435–446
29. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
30. Laemmli, U. K. (1970) *Nature* **227,** 680–685
31. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) *Biochemistry* **10,** 2606–2617
32. Morrissey, J. H. (1981) *Anal. Biochem.* **117,** 307–310
33. Pritchard, M. L., Seely, J. E., Poso, H., Jefferson, L. S., and Pegg, A. E. (1981) *Biochem. Biophys. Res. Commun.* **100,** 1597–1603
34. Vaitukaitis, J., Robbins, J. B., Neischlaga, E., and Ross, G. T. (1971) *J. Clin. Endocrinol.* **33,** 988–991
35. Davis, R. H., Hynes, L. V., and Eversole-Cire, P. (1987) *Mol. Cell. Biol.* **7,** 1122–1128
36. Young, R. A., and Davis, R. W. (1983) *Science* **222,** 778–782
37. Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195–203
38. Obenrader, M. F., and Prouty, W. F. (1977) *J. Biol. Chem.* **252,** 2866–2872
39. Paulus, T. J., Kiyono, P., and Davis, R. H. (1982) *J. Bacteriol.* **152,** 2981–2987

Supplemental Material to

Ornithine Decarboxylase From *Neurospora crassa*. Purification, Characterization, and Regulation

Joseph J. DiGangi, Manouchehr Seyfzadeh and Rowland H. Davis

EXPERIMENTAL PROCEDURES

Materials--The arginase-less *Neurospora crassa* strain IC3, carrying the UM-906 allele of the *aga* locus, was used to allow manipulation of ornithine decarboxylase activity (16, 18, 19). Cells were grown in aerated 20-liter carboys or in 1,000-ml flasks (27) containing Vogel's medium N (28). Most chemicals were purchased from Sigma Chemical Co. L-[1-^{14}C]ornithine hydrochloride (50 mCi/mmole) was from Amersham; DL-[1-^{14}C]ornithine was purchased from ICN, and specific radioactivities of substrate were corrected to account for the difference. DL-alpha-difluoromethyl-[5-^{14}C]ornithine (56 mCi/mmole) was purchased from Amersham. ^{125}I-protein A was a gift from Daniel Knauer. Ultra pure ammonium sulfate and urea were purchased from Schwarz-Mann. Polyacrylamide, sodium dodecyl sulfate, molecular sieving molecular-weight markers, protein assay kit, Bio-Gel P-200, and the Bio-Gel TSK DEAE 5 PW HPLC ion exchange column were purchased from Bio-Rad. DE-52 was from Whatman Co.. Polyacrylamide gel markers, ampholines, Sephacryl S-200, Sepharose 4B, and Sephadex G25 were from Pharmacia Fine Chemicals. Ampholines were also purchased from L.K.B.. Freund's complete and incomplete adjuvants were purchased from Difco, and nitrocellulose from Schleicher & Schuell. Protein A adsorbent (formalin-fixed *Staphylococcus aureus* cells, Pansorbin) was purchased from Calbiochem.

Small-scale extracts--Crude extracts from small cultures (500 - 1000 ml), grown as described earlier (16) were made by grinding moist mycelial pads in buffer with sand (21), or by extracting acetone powders (27). In the latter case, a weighed quantity of powder was extracted twice in an Eppendorf tube by suspension in the extraction buffer used in enzyme purification (see below).

Ornithine Decarboxylase Assay--The decarboxylation of [1-^{14}C]ornithine was determined as previously described (39) in the following, modified reaction mixture: 50 mM Tris-acetate pH 7.4, 0.1 mM EDTA, 5.0 mM dithiothreitol, 0.01% Brij 35, 50 μM pyridoxal phosphate, 2 mM L-ornithine, and [1-^{14}C]ornithine to bring the specific radioactivity of L-ornithine to 100-500 cpm/nmole. One unit of ornithine decarboxylase activity is an amount of enzyme that releases one μmole of CO_2 per hour at 37° C. Protein concentration was determined by the method of Bradford (29), using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard.

Sephacryl S-200 Chromatography--A Sephacryl S-200 column (2.6 x 90.0 cm) was used to estimate the molecular weight of native ornithine decarboxylase. It was equilibrated in 50 mM Tris-acetate, 0.1 mM EDTA, 5 mM DTT, and 0.01% Brij 35, pH 7.4 and calibrated with gamma globulin (M_r = 150,000), ovalbumin (M_r = 43,000), myoglobin (M_r = 16,500), and vitamin B_{12} (M_r = 1,300).

Polyacrylamide Gel Electrophoresis--SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (30), using 7.5-15% gradient gels. Gels were stained with Coomassie Brilliant Blue (31) or silver (32). For isoelectric focussing, gels (0.8 mm) contained 3.5% acrylamide, 9.0 M urea, 0.1% NP-40, and 3.6% ampholines (80% pH 4-6.5, 20% pH 3.5-10). Urea was added to samples (0.5 mg/ul extract), which were then combined with an equal volume of sample buffer (0.2% NP-40, 20 mM DTT, and 4% ampholines [80% pH 4-6.5, 20% pH 3.5-10], saturated with urea) and centrifuged in a 1.5 ml Eppendorf tube for 10 minutes. The gel was placed on a LKB flat-bed isoelectric focussing apparatus at 15°C on a layer of kerosene, and wicks soaked in 0.01 M phosphoric acid or 0.02 M NaOH were placed on the gel. The gel was pre-focussed for 5 min at settings of 25 watts, 2000 volts, and 10 mA. After loading, the samples were run at an initial setting of 5 watts, 2000 volts, and 5 mA. They were run for four hours; the gel was blotted onto nitrocellulose paper for two hours at 50 volts. To determine the pH gradient, 0.5 cm slices of gel were cut out and soaked in 2 ml water overnight. These were heated at 100 C for five minutes and flushed with nitrogen before pH was measured.

Difluoromethylornithine Binding-- [^{14}C]Difluoromethylornithine with a specific radioactivity of 56 mCi/mmole was bound to 10-100 units of ornithine decarboxylase as described by Pritchard et al (33). With the specific radioactivity (cpm/pmole) of difluoromethylornithine, the bound radioactivity (cpm) was converted to pmole difluoromethylornithine bound per mg of sample protein, and, with the specific activity of the enzyme, to pmole/unit enzyme. Assuming one difluoromethylornithine molecule bound per 53,000 M_r enzyme subunit, the mg ornithine decarboxylase protein/unit enzyme was determined. This allowed a calculation of the purity (mg ornithine decarboxylase protein/mg total protein) of any preparation. Radioactive difluoromethylornithine was used to identify ornithine decarboxylase on gels by labeling the native preparation, removing unbound difluoromethylornithine by exhaustive dialysis, and subjecting the sample to SDS-polyacrylamide gel electrophoresis and autoradiography.

Preparation of Antisera--Two polyclonal antisera, sera X and Z, were raised using 150 μg of native, HPLC-purified ornithine decarboxylase for two initial injections and 50 μg for all other injections. Two other antisera, A and B, were generated using about 50 ug of denatured ornithine decarboxylase eluted from SDS gels in a solution of 50 mM Tris-HCl, pH 7.9, 0.1% sodium dodecyl sulfate, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride and 150 mM NaCl. Initial injections used Freund's complete adjuvant; booster injections used Freund's incomplete adjuvant. Pre-immune serum was collected from all rabbits before beginning the injection schedule. Multiple site injections (34) were performed two weeks apart on the backs of four rabbits. Every two weeks for two months, subcutaneous injections into the skin folds on each side of the neck and above the back of the hind legs were performed. Rabbits were boosted once a month and bled ten days after each injection. Blood was collected by heart puncture or bleeding from the ear vein and serum separated by low speed centrifugation. Sodium azide (in phosphate buffer) was added to 0.1% and the serum was stored in aliquots at -70° C, or at 4° C for several months.

Non-specific antibodies were removed by cross-absorption with an extract of a mutant strain carrying the LV10 nonsense mutation, which contains no detectable ODC antigen (35). The extract was immobilized on Sepharose 4B and used as described by Young and Davis (36). The resulting antisera recognized only ODC polypeptides in normal Neurospora extracts, and none in extracts of the LV10-bearing strain.

Immunoblotting--Immunoblotting was performed essentially as described by Burnette (37) using antiserum dilutions of 1:500 - 1:1000. Antiserum incubation times were sometimes increased to amplify the signal.

Immunotitration--The total reaction mixture (200 ul) contained antiserum, cell extract, and buffer (50 mM sodium phosphate, 150 mM NaCl, 0.03% Brij 35, 2.0% RIA grade BSA, 0.02% sodium azide, pH 7.4). After an hour incubation at 4°C, protein A adsorbent (50 μl of a 4% Pansorbin

suspension in the buffer above) was added, and the mixture was incubated for 30 minutes. After centrifugation, the supernatants were assayed in duplicate for remaining ornithine decarboxylase activity. Immunotitration data were calculated as the units of ornithine decarboxylase activity removed/ul antiserum, using linear regression to determine the slope. Confidence limits of 95% were calculated to determine the slope variation of individual lines. The data were also transformed to antibody-50 (Ab_{50}) values (38) by calculating the amounts of antisera required to precipitate half of the enzyme activity in samples containing 1 mg protein.

A positive control for recognition of inactive ornithine decarboxylase molecules was performed by immunotitrating a mixture of a wild type-extract and that of a mutant carrying the spe-1 allele LV206. This mutant lacks ornithine decarboxylase activity, but contains a normal amount of (defective) ornithine decarboxylase protein (33b). Extracts of this 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 immunotitration. The result (Fig. 1) showed a linear decrease in the effectiveness of the antiserum to precipitate enzyme units as the fraction of inactive protein in the mixture increased, indicating that the mutant ornithine decarboxylase protein was recognized by the antiserum.

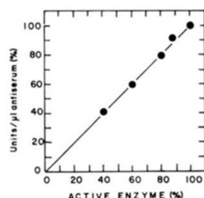

Fig. 1. Relation of enzyme units precipitated per ul of antiserum and the proportion of active ornithine decarboxylase molecules in the preparation. With ornithine decarboxylase protein constant, the proportion of inactive (mutant) to active (normal) molecules was varied. The slope of the immunotitration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

Purification of Ornithine Decarboxylase--Growth. The IC-3 strain, carrying the aga mutation, was grown in 20-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 filter circles, were washed with distilled water. All other steps were carried out at 4° C.

Crude Extract. About 40 g (dry weight equivalent) of mycelia were homogenized in buffer A (50 mM potassium phosphate, 0.1 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 mM dithiothreitol (DTT), 1 µg/ml each antipain, chymostatin, leupeptin, and pepstatin, and 0.03 trypsin inhibitor units of aprotinin/ml at pH 7.4) with 0.3-0.5 mm diameter glass beads, using a Bead-Beater (BioSpec Products). Mycelia were broken with three one-minute pulses, separated by one-minute rests to control heating. The supernatant was centrifuged at 27,500 x g for 40 minutes, followed by filtration through Whatman 934-AH glass fiber filters.

Ammonium Sulfate Precipitation. A saturated ammonium sulfate solution in buffer A was added over 40 min with stirring to the supernatant. The mixture was stirred for 20 minutes after addition and centrifuged at 18,500 x g for 20 minutes. The precipitate was resuspended in buffer A plus 1 mg/ml heparin and recentrifuged at 100,000 x g for one hour. The supernatant (ca. 30-40 ml) was passed through Sephadex G25 columns equilibrated with buffer B (same as buffer A except that 50 mM Tris-acetate pH 7.4 was used in place of potassium phosphate, and 0.01% Brij 35 was 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 gel filtration column (5 x 90 cm) equilibrated with buffer B. The column was eluted in 7.5-ml fractions at a rate of 15 ml/hr. Active fractions, emerging in about 48 hours, were pooled and concentrated by ultrafiltration on an Amicon YM10 membrane. The salt concentration was adjusted to 50 mM sodium acetate, using a 200 mM solution in buffer C (50 mM Tris-acetate, 0.1 mM EDTA, 5 mM DTT, and 0.01% Brij 35, pH 7.4).

DE-52 Cellulose Chromatography. The concentrated preparation from the Bio-Gel P-200 step (ca. 12-15 ml) was applied to a DE-52 column (1.5 x 9.7 cm) equilibrated with 50 mM sodium acetate in Buffer C. After washing the column with ca. 90 ml of Buffer C, ornithine decarboxylase was eluted with a linear gradient of 50 to 700 mM sodium acetate in buffer C (150 ml of each concentration). Active fractions, emerging halfway through the gradient, were pooled and concentrated by ultrafiltration. During concentration, buffer C was added to decrease the sodium acetate concentration to about 200 mM.

High Performance Liquid Chromatography (HPLC)-Ion Exchange. The concentrated DE-52 eluate (1.0 ml) was applied to a Bio-Gel TSK DEAE 5 PW ion exchange HPLC column (75 x 7.5 mm) connected to a Gilson HPLC apparatus and equilibrated with 200 mM sodium acetate in buffer C. The column was washed with 320 mM sodium acetate in buffer C, and ornithine decarboxylase was eluted with 450 mM sodium acetate in buffer C. The active fractions were pooled and concentrated as before. Buffer C was added to reduce the sodium acetate concentration to less than 200 mM. The pure preparation was stored in aliquots at -70° C.

RESULTS

Table 2

Ornithine decarboxylase activity and protein in IC-3 (aga) cultures grown in the presence or absence of cyclohexylamine or arginine

Additions to medium	Enzyme specific activity		Ab_{50}		Enzyme U/ul antiserum	
	U/mg protein	%	ul/mg protein	%	serum Z	serum X
None	0.145	3.8	0.32	5.7	209	45
cyclohexylamine (10 mM)	0.724	19	0.82	15	345	68
arginine (1 mM)	3.778	(100)	5.63	(100)	353	68

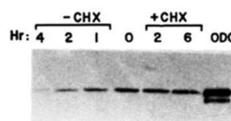

Fig. 5. Western immunoblot of extracts, separated by SDS-made from culture grown in the addition of ornithine or ornithine + cycloheximide (CHX) (50 µg/ml culture medium). Samples represent the same volume of culture medium to correct for growth. Control: purified preparation of ornithine decarboxylase (ODC). Antiserum A was used at a dilution of 1:500.

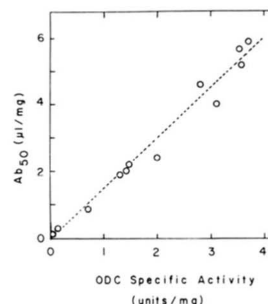

Fig. 6. Relation of enzyme specific activity to antigenic material (Ab_{50}) in extracts of varying ornithine decarboxylase activity. All samples used were crude extracts from from cultures grown in minimal medium; in the presence of cyclohexylamine; in the presence of arginine (ornithine-starved); or from ornithine-starved cultures after the restoration, for various times, of ornithine (see main text).