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Characterization of a high-affinity membrane-associated ornithine decarboxylase from the free-living nematode *Caenorhabditis elegans*

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Ornithine decarboxylase has been identified and characterized in the free-living nematode *Caenorhabditis elegans*. Unlike previously described ornithine decarboxylases, the enzyme activity is membrane-associated and remains in the membrane fraction after treatment with high salt, detergents or phosphatidylinositol-specific phospholipase C. Ornithine has an apparent K_m value of 2.7 μM for ornithine decarboxylase. The enzyme is competitively inhibited by arginine and lysine with K_i values of 4.0 and 24.4 μM respectively. None of the other naturally occurring amino acids inhibited more than 10% of the enzyme activity at concentrations up to 1 mM. Agmatine, putrescine, spermidine and spermine inhibit ornithine decarboxylase in a non-competitive manner with K_i values of 10, 53.5, 59 and 855 μM respectively. A similar ornithine decarboxylase activity was also identified in membrane preparations from the parasitic nematode *Haemonchus contortus*.

INTRODUCTION

Ornithine decarboxylase (ODC; L-ornithine carboxy-lyase, EC 4.1.1.17) catalyses the conversion of ornithine into putrescine and is a rate-limiting enzyme in polyamine biosynthesis (Tabor & Tabor, 1984; Jänne *et al.*, 1978; Pegg, 1986). ODC activity is well regulated at various levels including synthesis (Persson *et al.*, 1984), degradation (Laitinen *et al.*, 1985) and post-translational modification (Scott *et al.*, 1985). Increased ODC levels have been correlated with increased growth rates of eukaryotic cells (Seidenfeld *et al.*, 1985), and inhibition of ODC activity results in a loss of cell proliferation (Porter & Bergeron, 1983). The enzyme has been purified to homogeneity from mouse and rat tissues, and consists of dimers of approx. 54 kDa subunits (Kitani & Fujisawa, 1983). In contrast, ODC purified from various lower eukaryotes [*Saccharomyces cerevisiae* (Tyagi *et al.*, 1981) *Tetrahymena pyriformis* (Sklaviadis *et al.*, 1985) and *Physarum polycephalum* (Barnett & Kazarinoff, 1984)] are quite dissimilar from the mammalian enzyme in molecular mass and specific activity.

Caenorhabditis elegans is a free-living nematode that has been extremely well characterized and provides an important model system for the study of regulation of cellular development (Greenwald, 1985; Emmons, 1987; Kenyon, 1988). We have investigated ODC activity in *C. elegans*, and here present data demonstrating that the enzyme is membrane-associated, in contrast with other systems in which ODC is a cytoplasmic component. In addition, the K_m of ornithine for *C. elegans* ODC (2.7 μM) is 25–1000-fold lower than those for previously described ODCs.

EXPERIMENTAL

Materials

L-[1-^{14}C]Ornithine (51.6 mCi/mmol) and L-[3,4-^{3}H]ornithine (50.4 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). DL-α-Difluoromethylornithine (DFMO), DL-α-monofluoromethylornithine (MFMO) and 2-fluoroputrescine were provided by Dr. J. Kollonitsch of these Laboratories. γ-Glutamylputrescine was provided by Dr. J. Folk (National Institutes of Health, Bethesda, MD, U.S.A.). Phosphatidylinositol-specific phospholipase C (PI-PLC) isolated from *Staphylococcus aureus* was generously given by Dr. M. Low (Columbia University, New York, NY, U.S.A.). Octyl β-glucoside was from Pierce Chemical Co. (Rockford, IL, U.S.A.). *Haemonchus contortus* third-stage larvae were given to us by Dr. J. Egerton of these Laboratories. All other materials were from commercially available sources.

C. elegans tissue preparation

C. elegans, N2 strain, was cultivated on NG agar plates covered with a lawn of *Escherichia coli* as previously described (Brenner, 1974). Worms (all stages) were washed off the plates with ice-cold 50 mM-Tris/HCl, pH 7.3. The worms were washed twice for 2 min at 1000 *g* in order to remove the *E. coli*. The worms were then broken up by homogenization in a Braun homogenizer (Ace Scientific, New Brunswick, NJ, U.S.A.), using 0.5 mm glass beads for 30 s. The homogenate was centrifuged for 2 min at 1000 *g*. The pellet, P_1, consisted primarily of large pieces of broken cuticle and a small number (less than 0.2%) of unbroken worms. The supernatant (S_1) was centrifuged for 20 min at 28000 *g*, and the resulting pellet (P_2) was washed twice by centrifugation for 20 min at 28000 *g*, resuspended in the Tris/HCl buffer and used immediately for enzyme assays. Freezing the membranes at −20 °C resulted in loss of enzyme activity. *H. contortus* membranes were prepared in the same manner as those for *C. elegans*.

ODC

ODC was determined by measuring the rate of $^{14}CO_2$ evolution from L-[1-^{14}C]ornithine (Tabor & Tabor, 1984). 5 mM-Trizma base, adjusted to pH 7.2 with HCl (500 μl), containing 0.5 mM-dithiothreitol and 0.2 mM-pyridoxal phosphate was added to 450 μl of the tissue preparation in a 20 ml scintillation vial which was then sealed with a rubber stopper from which was suspended at 1.0 cm × 1.0 cm piece of Whatman no. 3 filter paper im-

Abbreviations used: ODC, ornithine decarboxylase; DFMO, DL-α-difluoromethylornithine; MFMO, DL-α-monofluoromethylornithine; PI-PLC, phosphatidylinositol-specific phospholipase C.

pregnated with methylbenzethonium hydroxide. The reaction was initiated by an injection of 35 nCi (in 50 μl) of [^{14}C]ornithine (23.4 mCi/mmol) with a syringe through the stopper. The reaction was carried out at 37 °C and terminated by the addition of 250 μl of 1 M-H_2SO_4. After 45 min, the filters were removed and counted in Aquasol II (New England Nuclear) with a liquid-scintillation spectrometer. In some experiments Krebs bicarbonate buffer (124 mM-NaCl/5 mM-KCl/26 mM-$NaHCO_3$/1.2 mM-KH_2PO_4/1.3 mM-$MgSO_4$, pH 7.3) was used, and no significant alteration in the ODC activity was observed.

Determination of [^{3}H]putrescine formed from [^{3}H]ornithine

C. elegans membranes were incubated with [^{3}H]ornithine, and the production of [^{3}H]putrescine was monitored as a function of time. The reaction was terminated by addition of 10 M-HCl to a final concentration of 0.1 M and then centrifuged for 2 min at 14000 *g*. An aliquot of the supernatant was applied to a Merck RP-250 thin-layer plate in the presence of a standard containing authentic ornithine, putrescine, spermine and spermidine. The solvent system was chloroform/methanol/aq. NH_3 (2:2:1, by vol.). The R_F values for ornithine, putrescine, spermine and spermidine were 0.86, 0.20, 0.13 and 0.08 respectively. The polyamines were revealed by exposure to iodine vapour scraped off the plate and quantified by liquid-scintillation spectrometry. In some experiments the polyamines were identified and measured by h.p.l.c. on a cation-exchange resin column followed by derivatization with *o*-phthalaldehyde as described by Porter *et al.* (1985).

High-salt treatment

The P_2 pellet was resuspended to a final concentration of 1.8 M-NaCl, incubated for 60 min at 37 °C and then centrifuged for 20 min at 28000 *g*. The resulting pellet (P_3) was resuspended in 5 mM-Trizma/HCl buffer, pH 7.2, and used in subsequent assays. The supernatant (S_3) was diluted in the Trizma/HCl buffer to a final NaCl concentration of 60 mM before being assayed for ODC activity.

Treatment with PI-PLC

C. elegans membranes were incubated at 30 °C for 1 h in 500 μl of the Trizma/HCl buffer containing BSA (0.1 mg/ml) with or without one of the following additions: PI-PLC, (10 μg/ml) purified from *Staphylococcus aureus* (Low, 1981) or phospholipase C (5 units/ml) from *Bacillus cereus* (Sigma). The incubation was terminated by centrifugation for 20 min at 28000 *g*. The pellet was resuspended in Tris buffer and the supernatant and pellet were then analysed for ODC activity.

Detergent treatment

C. elegans membranes were incubated for 30 min at 37 °C in the presence of 1 % deoxycholate and then centrifuged for 2 h at 100000 *g*. The resulting supernatant (S_3) and pellet (P_3) were used for subsequent ODC assays. Similar experiments were performed with 0.5 % Triton X-100 and 30 mM-octyl β-glucoside.

Enzyme assays

5′-Nucleotidase (plasma-membrane marker) was measured by the method of Aronson & Touster (1974). Glucose-6-phosphate dehydrogenase (cell cytoplasm marker) activity was determined by the method of Cohen & Rosemeyer (1969). Arginase activity was measured as described by Schimke (1964).

Protein determinations

Protein concentrations were estimated with the dye-staining technique of Bradford (1976), with BSA as the standard.

RESULTS

Subcellular distribution of ODC

ODC activity was measured in whole-worm homogenates and various subcellular fractions prepared by centrifugation. As shown in Table 1, ODC activity was recovered primarily (81.5 %) in the P_2 fraction with only 2.3 % in the supernatant (S_2). The S_1 fraction contained 16 % of the total activity. However, after 12 h of dialysis, the ODC activity in the homogenate and S_1 fraction significantly increased (approx. 1.5- and 5-fold respectively). These results suggest that there is an endogenous, low-molecular-mass inhibitor of ODC activity, leading to an underestimate of soluble ODC levels in the non-dialysed preparations. The specific activity of the P_2 fraction was approx. 7.5-fold greater than the starting material (from 1.24 to 9.47 nmol of CO_2 formed/h per mg of protein). Distribution of ODC activity in these fractions paralleled the distribution of 5′-nucleotidase, an enzyme marker for plasma membranes (Aronson & Touster, 1974). Glucose-6-phosphate dehydrogenase activity was used as a cytoplasmic marker (Cohen & Rosemeyer, 1969) and was found only in very low concentrations in the P_2 pellet. These results are consistent with the association of ODC with the plasma-membrane fraction. To confirm further that *C. elegans* ODC is membrane-bound, the

Table 1. Distribution of ODC and enzyme markers

C. elegans were prepared as described in the Experimental section, and various membrane and soluble fractions were assayed for ODC, 5′-nucleotidase (5′-NT) and glucose-6-phosphate dehydrogenase (G6PDH) activity. Abbreviation: ND, not detectable.

Enzyme source	Total protein (mg)	Total activity (nmol/h)	Specific activity*	Yield (%)	Activity 5′-NT†	Activity G6PDH‡
Homogenate	26.25	32.55	1.24	100	6.7	5.9
Homogenate (dialysed)	26.10	48.75	1.87	149.8	6.9	6.1
S_1	9.4	5.14	0.55	16	ND	14.2
S_1 (dialysed)	9.2	24.25	2.64	74.5	ND	16.1
P_1	17.1	16.40	0.96	50.4	6.8	1.4
S_2	6.3	0.76	0.12	2.3	0.15	13.7
P_2	2.8	26.52	9.47	81.5	20.5	0.4

* Expressed as nmol of CO_2 released/h per mg of protein.
† Expressed as μmol of P_i released/30 min per mg of protein.
‡ Expressed as $100 \times A_{340}/2.07$ per mg of protein.

Table 2. ODC association with *C. elegans* P_2 membrane fraction

C. elegans P_2 membranes were treated under various conditions in order to dissociate ODC activity. The conditions of the various treatments are described in the Experimental section.

Treatment	Recovery (%)
P_2 (control)	100
NaCl (1.8 M)	
S_3	2
P_3	94
PI-PLC (*S. aureus*)	
S_3	4
P_3	97
PLC (*B. cereus*)	
S_3	3
P_3	99
Deoxycholate (1.0%)	
S_3	3
P_3	128
Triton X-100 (0.5%)	
S_3	2
P_3	89
Octyl β-glucoside (30 mM)	
S_3	2
P_2	98

P_2 membranes were treated with 1.8 M-NaCl. Salt treatment did not dissociate the enzyme activity from the membrane fraction (Table 2). Similarly, attempts to solubilize the ODC activity with detergents (deoxycholate, octyl β-glucoside and Triton X-100) were not successful, and deoxycholate actually enhanced the ODC activity, perhaps due to a further dilution of endogenous inhibitory factors.

It has recently been demonstrated that numerous proteins are covalently attached to the plasma membrane via a glycosylphosphatidylinositol linkage, and treatment of these membranes with PI-PLC releases the protein from the membrane (Low & Saltiel, 1988; Ferguson & Williams, 1988). In order to determine whether ODC is attached via a glycosylphosphatidylinositol linkage, we treated the *C. elegans* P_2 membrane fraction with PI-PLC. Two different sources of phospholipase C were used; however, we were not able to dissociate ODC activity from the membrane fraction (Table 2).

Characterization of ODC

Membrane-bound ODC activity increased linearly as a function of protein concentration from 0.02 to 0.3 mg/ml. The amount of liberated $^{14}CO_2$ increased linearly for at least 90 min at 37 °C. The enzyme activity was optimal at temperatures from 37 to 42 °C, no activity was observed at temperatures below 22 °C, and the activity was sharply diminished at temperatures greater than 45 °C. The optimal pH was 7.4–8.0. The apparent K_m value was 2.7 μM (Fig. 1), which is 25–1000-fold lower than the Michaelis constants reported for soluble ODC characterized in various other tissues (Tyagi *et al.*, 1981; Isomaa *et al.*, 1983; Kitani & Fujisawa, 1983; Barnett & Kazarinoff, 1984; Sklaviadis *et al.*, 1985). Addition of 1 mM-EDTA to the incubation media resulted in a 43% decrease of ODC activity, suggesting that at least low levels of some bivalent cation(s) are required for enzyme activity. Addition of either Ca^{2+} (1 mM) or Mg^{2+} (2 mM) had no significant effect on the ODC activity (results not shown).

Ornithine metabolism

In order to measure directly the synthesis of putrescine from ornithine, the *C. elegans* membrane fraction was incubated with

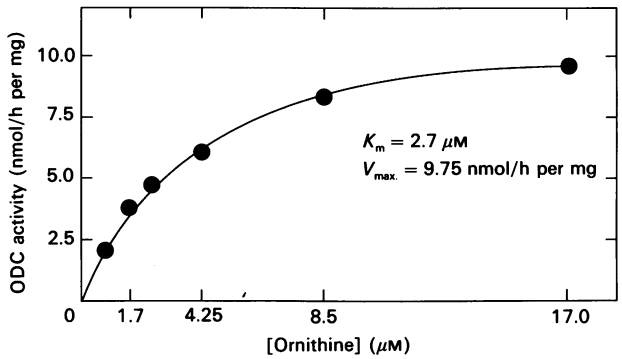

Fig. 1. ODC activity determined as a function of ornithine concentration

The enzyme activity was measured as described in the Experimental section, and the K_m and $V_{max.}$ values were estimated by using the Lineweaver–Burk (1934) plot analysis.

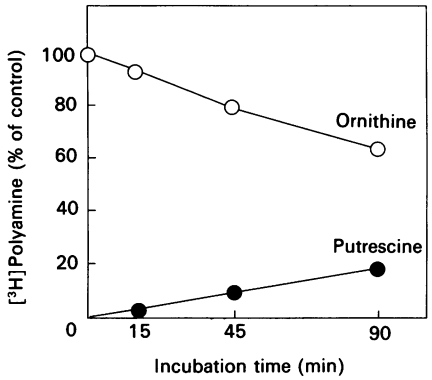

Fig. 2. Putrescine formation by *C. elegans* membranes

C. elegans membranes (250 μl) were incubated in the presence of [^{3}H]ornithine (10 μM) for various lengths of time at 37 °C in Microfuge tubes. The incubation was terminated by centrifugation for 3 min at 15000 *g*, and the amount of [^{3}H]ornithine (○) and [^{3}H]putrescine (●) in the supernatant was estimated by t.l.c. as described in the Experimental section. The experiment was repeated three times with similar results.

[^{3}H]ornithine and the appearance of [^{3}H]putrescine was quantified by t.l.c. and verified by h.p.l.c. As Fig. 2 shows, the synthesis of [^{3}H]putrescine correlated with the disappearance of [^{3}H]ornithine, thus further substantiating the existence of a membrane-associated ODC. The rate of appearance of [^{3}H]putrescine (10.2 nmol/h per mg) determined in these experiments correlates well with the enzyme activity determined by measurements of $^{14}CO_2$ release (9.45 nmol/h per mg). Pretreatment of the membranes at 90 °C for 2 min completely blocked the conversion of ornithine into putrescine. Addition of cycloheximide (0.5 mg/ml) had no effect on the ODC activity nor on the rate of conversion of ornithine into putrescine (results not shown). The total amount of radiolabelled ornithine and putrescine recovered from the soluble fraction decreased as a function of time, whereas the amount of ^{3}H-labelled material associating with the membrane fraction increased. These results suggest that ornithine, or a metabolite, is being incorporated into membrane-associated proteins, perhaps via a transglutamination reaction (Beninati *et al.*, 1988).

Inhibition of ODC by amino acids and polyamines

C. elegans ODC activity was specific for the L-isomer of ornithine. L-Ornithine had a K_i value of 2.7 μM, as compared with

Table 3. Inhibition of ODC by amino acids and polyamines

C. elegans membranes were incubated with 2 μM-[^{14}C]ornithine and increasing concentrations of various structural analogues. The amount of ODC activity was determined and K_i values were calculated by using the formula:

$$K_i = IC_{50}/[1+(c/K_m)]$$

where the IC_{50} is the concentration of the drug required to inhibit 50% of the enzyme activity (determined by log–probit plots), and c is the concentration of [^{14}C]ornithine. Histamine and all of the other naturally occurring L-amino acids were tested at 500 μM, and none inhibited more than 10% of the enzyme activity.

Compound	K_i (μM)
Amino acids	
L-Ornithine	2.7
L-Arginine	4.0
L-Lysine	24.4
α-Methyl-L-ornithine	78
L-Diaminobutyric acid	220
L-Histidine	> 1000
D-Ornithine	720
D-Lysine	> 1000
D-Arginine	> 1000
Polyamines	
Agmatine	10
Putrescine	53.5
Spermidine	59
2-Fluoroputrescine	65
Cadaverine	105
1,3-Diaminopropane	465
γ-Glutamylputrescine	840
Spermine	855
Suicide inhibitors	
MFMO	15
DFMO	> 1000

720 μM for D-ornithine (Table 3). All of the naturally occurring amino acids were tested, and only L-arginine and L-lysine (K_i values were 4 and 24.4 μM respectively) inhibited more than 10% of the enzyme activity at concentrations up to 500 μM. Both arginine (Fig. 3*a*) and lysine were competitive inhibitors of ODC. These results suggest that arginine and lysine may also serve as substrates for the same decarboxylase enzyme. Verification of this hypothesis will require solubilization and purification of the enzyme. Since arginine may be enzymically converted into ornithine, we assayed for arginase activity in the *C. elegans* membrane preparation. Under the conditions of our assay we found no arginase activity (results not shown).

Several polyamines were also tested as inhibitors of ODC activity. Agmatine, putrescine, spermidine and spermine inhibited ODC activity (K_i values were 10, 53.5, 59 and 855 μM respectively). Interestingly, polyamine inhibition of ODC activity was non-competitive (Fig. 3*b*), suggesting that these compounds interact with the enzyme at a site distinct from the substrate-binding site.

DFMO and MFMO bind covalently to the pyridoxal phosphate-binding site of soluble ODC (Kollonitsch *et al.*, 1978; Mamont *et al.*, 1978) and result in irreversible inhibition of the enzyme activity. However, when *C. elegans* membranes were used, DFMO was only a very weak inhibitor of ODC activity (K_i > 1 mM), whereas MFMO had an apparent K_i value of 15 μM (Fig. 4*a*). Preincubation of the *C. elegans* membranes with MFMO for increasing lengths of time resulted in increased ODC inhibition (Fig. 4*b*). Dialysis of the inhibited enzyme preparation did not reverse the MFMO effect, suggesting that MFMO formed a covalent attachment to *C. elegans* ODC.

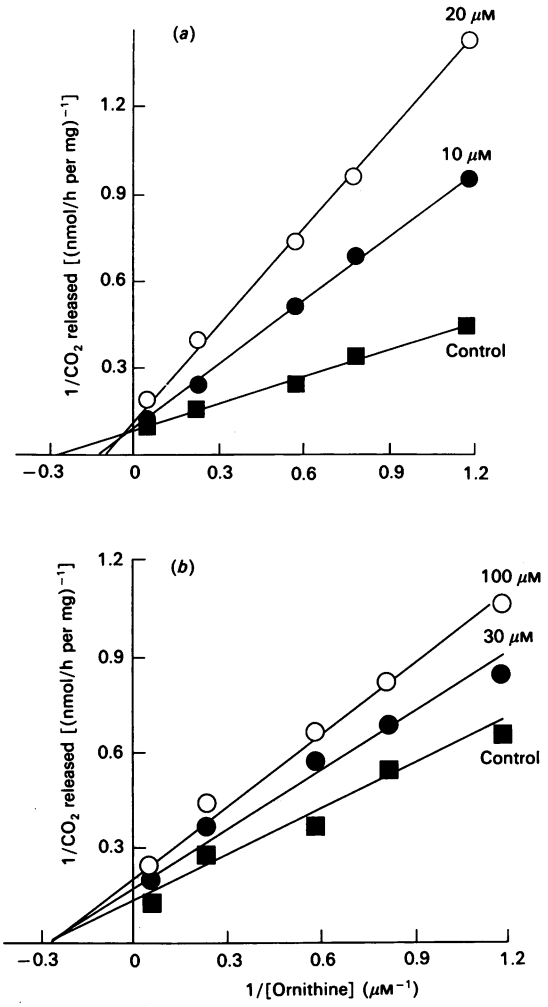

Fig. 3. (*a*) Arginine and (*b*) putrescine inhibition of ODC activity

(*a*) *C. elegans* membranes were incubated in the absence (■) or presence of a fixed concentration of arginine (10 μM, ●; 20 μM, ○) at various concentrations of [^{14}C]ornithine, and the amount of ODC activity was determined. The data were plotted by using Lineweaver–Burk (1934) analysis. (*b*) Similar experiments were performed in the absence (■) or presence of putrescine (30 μM, ●; 100 μM, ○). Each data point is the mean of at least four determinations, and the S.E.M. was less than 10%. Similar experiments were performed with lysine, cadaverine, agmatine and spermidine (results not shown).

Effects of nucleotides on ODC activity

Hölttä *et al.* (1972) reported that GTP stimulates *E. coli* ODC activity. Similarly, O'Brien *et al.* (1987) demonstrated that GTP activates mouse epidermal-tumour ODC due to a decrease in the apparent K_m of the enzyme for L-ornithine. We tested the following nucleotides for their effect on *C. elegans* ODC activity: AMP, cyclic AMP, ADP, ATP, GMP, cyclic GMP, GDP, GTP, TMP, TDP, TTP, UMP, UDP, CMP, CDP and CTP. No significant change in enzyme activity were observed in the presence of 100 μM-nucleotide.

ODC in membranes isolated from *Haemonchus contortus*

The parasitic nematode *Haemonchus contortus* was examined to determine whether the association of ODC with the membrane fraction is common to other nematodes. As Table 4 shows, ODC activity was present in the membrane fraction of *H. contortus* and had an affinity for its substrate (K_m 3.8 μM) similar to that

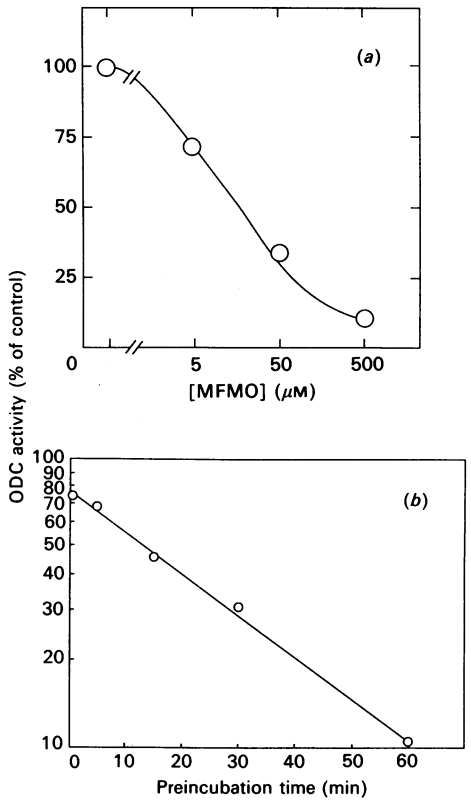

Fig. 4. MFMO inhibition of ODC activity

(*a*) *C. elegans* membranes were incubated with 2 μM-[^{14}C]ornithine in the presence of increasing concentrations of MFMO, and the ODC activity was determined. (*b*) Similar experiments were performed; however, the membranes were preincubated with 5 μM-MFMO for various lengths of time before addition of the [^{14}C]ornithine, and the ODC activity was plotted as a percentage of the activity in the absence of MFMO. In both experiments the data points are the mean of four determinations, with the S.E.M. less than 10%.

Table 4. Characterization of ODC in *C. elegans* and *H. contortus*

Membranes from *C. elegans* and *H. contortus* were prepared as described in the Experimental section.

Organism	ODC activity (nmol/h per mg)	K_D (μM)
C. elegans	9.45	2.7
H. contortus	3.33	3.8

observed for *C. elegans* ODC (K_m 2.7 μM). No ODC activity was detected in the cytosol fraction.

DISCUSSION

ODC has been extensively studied in a wide range of organisms and has been found to be localized predominantly in the cytoplasm. Here we describe the characterization of membrane-associated ODC isolated from the free-living nematode *C. elegans*. ODC activity was quantified by measuring the $^{14}CO_2$ release from [1-^{14}C]ornithine and verified by directly measuring the production of [^{3}H]putrescine from [^{3}H]ornithine. Attempts to dissociate the enzyme from the membrane with high salt, enzymes (PI-PLC) and detergents were unsuccessful. A second major difference between ODC in *C. elegans* and other organisms is the Michaelis constant for L-ornithine. In both prokaryotic and eukaryotic systems the K_m values have been reported to be between 70 and 3000 μM (Kitani & Fujisawa, 1983; Tyagi *et al.*, 1981; Sklaviadis *et al.*, 1985), whereas the *C. elegans* ODC binds ornithine with a K_m value of 2.7 μM.

Arginine and lysine are both potent competitive inhibitors of *C. elegans* ODC activity (K_i values 4 and 24.4 μM respectively; Fig. 3*a*). ODC isolated from mouse kidney will also catalyse the decarboxylation of lysine to form cadaverine; however, the K_m for this substrate is 100 times higher than that for ornithine (Seely & Pegg, 1983). In *E. coli*, specific decarboxylases for lysine, arginine and ornithine have been identified and purified (Boeker & Fisher, 1983), and these enzymes exhibit strict substrate specificity. Our results suggest that the ODC in *C. elegans* may actually be a lysine/arginine/ornithine decarboxylase; however, verification will require purification of the enzyme.

Polyamines are non-competitive inhibitors of *C. elegans* ODC activity, which suggests that the enzyme substrate and end product bind to the enzyme at distinct sites. Furthermore, the product may modulate enzyme activity via an end-product-inhibition mechanism. This is in contrast with mammalian ODC, where it has been reported that putrescine is a competitive inhibitor of L-ornithine with a K_i value of 600 μM (Kitani & Fujisawa, 1983).

The presence of ODC in the membrane fraction of the parasitic nematode *Haemonchus contortus* demonstrates that membrane-bound ODC may be common to other nematodes. This is particularly interesting in light of the observation by Wittich *et al.* (1987) that filarial nematodes contain significant levels of putrescine and other polyamines with no measurable ODC in the soluble extract of these worms (the membrane fraction was not tested for ODC activity). The authors hypothesized that the nematodes derive polyamines from their host. Our results provide an alternative hypothesis for their observation.

DFMO and MFMO are catalytic irreversible inhibitors of ODC. DFMO has been found to be efficacious in the treatment of trypanosomiasis (Bitonti *et al.*, 1985), and it is hypothesized that this is due to the lower turnover rate of ODC in trypanosomes than in mammalian tissues (Phillips *et al.*, 1987). *C. elegans* ODC is not affected by DFMO at concentrations up to 500 μM; however, MFMO is a relatively potent inhibitor of ODC in our system and warrants further study. The physiological role of membrane-bound ODC in nematodes is still unclear.

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