Trichomonas vaginalis: characterization of ornithine decarboxylase

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Ornithine decarboxylase (ODC), the lead enzyme in polyamine biosynthesis, was partially purified from *Trichomonas vaginalis* and its kinetic properties were studied. The enzyme appears to be of special significance in this anaerobic parasite, since the arginine dihydrolase pathway generates ATP as well as putrescine from arginine. ODC from *T. vaginalis* had a broad substrate specificity, decarboxylating ornithine (100%), lysine (1.0%) and arginine (0.1%). The enzyme had a pH optimum of 6.5, a temperature optimum of 37 °C and was pyridoxal 5'-phosphate-dependent. Attempts to separate ornithine- from lysine-decarboxylating activity by thermal-stability and pH-optima curves were not successful. Although K_m values for ornithine and lysine were 109 and 91 μM respectively, and the $V_{max.}$ values for these substrates were 1282 and 13 nmol/min per mg of protein respectively, the most important intracellular substrate is ornithine, since intracellular ornithine levels are 3.5 times those of lysine and extracellular putrescine levels are 7.5 times those of cadaverine. Ornithine was also an effective inhibitor of lysine-decarboxylating activity (K_i 150 μM), whereas lysine was relatively ineffective as inhibitor of ornithine-decarboxylating activity (K_i 14.5 mM). Crude ODC activity was localized (86%) in the 43000 *g* supernatant and 3303-fold purification was obtained by $(NH_4)_2SO_4$ salting and DEAE-Sephacel, agarose-gel and hydroxyapatite chromatography steps. The enzyme bound difluoro[^{3}H]methylornithine ([^{3}H]DFMO) with a ratio of drug bound to activity of 2500 fmol/unit, where 1 unit corresponds to 1 nmol of CO_2 released from ornithine/min. The enzyme had a native M_r of 210000 (gel filtration), with a subunit M_r of 55000 (by SDS/PAGE), suggesting that the trichomonad enzyme is a tetramer. From the subunit M_r and binding ratio of DFMO, there is about 137 ng of ODC per mg of *T. vaginalis* protein (0.013%). The significant amount of ODC protein present supports the view that putrescine synthesis in *T. vaginalis* plays an important role in the metabolism of the parasite.

INTRODUCTION

Trichomonas vaginalis is the agent of human trichomoniasis, a sexually transmitted disease which accounts for about one-third of vaginal infections diagnosed in public clinics in the United States (Rein, 1990). There are an estimated 3 million new cases every year in the United States (McNab, 1979), which is more than double the number of cases of gonorrhoea and genital herpes and far outnumbers the annual incidence of syphilis (McNab, 1979). This anaerobic protozoan lacks mitochondria and obtains limited energy via glycolysis and acetate production, and has been thought to rely almost exclusively on glucose and stored glycogen reserves as a major source of energy (reviewed by Yarlett and Bacchi, 1991). Despite these traditional views, there is increasing evidence that metabolism of polyamines, particularly putrescine production from arginine, may provide a significant alternative to carbohydrate metabolism as an energy source in *T. vaginalis* (Linstead and Cranshaw, 1983). It has been shown previously (Chen et al., 1982; Sanderson et al., 1983) that high levels of putrescine (up to 2 mM) are present in the vaginal fluid of patients suffering from trichomoniasis, but not in healthy controls. Putrescine returns to undetectable levels after successful treatment with metronidazole (Sanderson et al., 1983). A complementary effect is found with arginine levels, which decrease by > 90% during infection and return to normal levels (200 μM) after treatment (Chen et al., 1982). Similar depletion of arginine has been found during growth of *T. vaginalis* in semi-defined medium (Linstead and Cranshaw, 1983).

In most eukaryotes putrescine is produced by the enzyme ornithine decarboxylase (ODC), which splits ornithine into putrescine and CO_2. This enzyme is the lead into the polyamine pathway, and in mammalian cells governs the rate of synthesis of polyamines because of its short half-life and rapid turnover (Pegg, 1988).

In trichomonads there is evidence that polyamine synthesis may be linked to the production of significant amounts of ATP through the arginine dihydrolase pathway (Linstead and Cranshaw, 1983; Yarlett and Bacchi, 1988). In the pathway, arginine is metabolized to putrescine, CO_2 and ATP through the activity of four enzymes: arginine deiminase, ornithine carbamoyltransferase, carbamate kinase and ODC (Linstead and Cranshaw, 1983). Putrescine appears to be the only polyamine end product in *T. vaginalis*, since, although spermine and spermidine are present in low levels in the organism, the synthesis has not been detected (Yarlett, 1988). The lack of production of higher polyamines also makes *T. vaginalis* unique among eukaryotes. *T. vaginalis* also contains lysine-decarboxylating ability, since cadaverine can be detected *in vivo* in vaginal fluid (Chen et al., 1982) and *in vitro* (Yarlett and Bacchi, 1988). A comprehensive study of polyamine metabolism and ODC in *T. vaginalis* is warranted, owing to the unique nature of the pathway in the parasite and the interest in developing drug therapies targeting polyamine production (Pegg, 1988; Pegg and McCann, 1988). The present study characterizes the metabolic regulation, substrate specificity and conditions for optimal activity of ODC in *T. vaginalis*, and examines its suitability as a target for chemotherapy.

MATERIALS AND METHODS

Culture conditions and cell extracts

Trichomonas vaginalis C1 NIH (A.T.C.C. 30001) was grown at 37 °C in TYM medium (Diamond, 1957), supplemented with 10% (v/v) horse serum. Late-exponential-phase cultures were

Abbreviations used: ODC, ornithine decarboxylase; DFMO, difluoromethylornithine.
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harvested by centrifugation at 200 *g* for 5 min (8 °C). Cells were washed twice with 300 mM NaH_2PO_4/1.6 mM KCl/0.6 mM $CaCl_2$/0.074 mM NaCl, pH 6.4, and resuspended in breakage buffer [225 mM sucrose/10 mM Tris/HCl (pH 7.2)/1 mM EDTA/1 mM dithiothreitol/60 μM pyridoxal 5′-phosphate].

Fractionation studies

These were accomplished by differential centrifugation of cells resuspended in breakage buffer and broken in a Teflon/glass homogenizer fitted with a motor drive. In brief, the nuclear fraction was obtained by centrifugation at 400 *g* for 10 min. The remaining homogenate was then centrifuged at 2200 *g* for 10 min, yielding the large-particle fraction. The resulting supernatant was further centrifuged at 43000 *g* for 30 min, yielding the small-particle fraction and final non-sedimentable fraction (Lindmark et al., 1975). The non-sedimentable fraction was subjected to the following purification procedure.

Purification procedure

The non-sedimentable fraction was diluted to 3.5–5.0 mg/ml. Solid $(NH_4)_2SO_4$ was slowly added to a final saturation of 30% with stirring at 4 °C for 1 h. The precipitate was removed by centrifugation at 17500 *g* for 20 min, and the supernatant was then adjusted to 80% saturation with $(NH_4)_2SO_4$ and stirred for 1 h at 4 °C. The precipitate was removed by centrifugation at 17500 *g* for 20 min and resuspended in 0.02 M acetate buffer, pH 6.5, containing 1 mM 2-mercaptoethanol, 60 μM pyridoxal phosphate and 0.025% Brij 35 (buffer A). The resuspended 30%- and 80%-$(NH_4)_2SO_4$ precipitates together with the supernatant were dialysed against 4 litres of buffer A, with two changes of buffer. The dialysed 80%-$(NH_4)_2SO_4$ preparation was loaded on a 2 cm × 40 cm DEAE-Sephacel column previously equilibrated with 2 vol. of buffer A with a flow rate of approx. 30 ml/h. After washing the column with 2 vol. of the equilibrating buffer, protein was eluted with a linear 0–1.0 M KCl gradient and 1.3 ml fractions were collected. Fractions were assayed as described below and the active fractions were pooled.

The pooled fraction (4 mg of protein) was applied to a 1 cm × 90 cm agarose column and equilibrated in Buffer A. The column was eluted with a flow rate of 30 ml/h, and 1 ml fractions were collected. The active fractions were pooled and $(NH_4)_2SO_4$ was slowly added to a final saturation of 80% at 4 °C. The precipitate was removed by centrifugation at 17500 *g* for 20 min, resuspended in 1–2 ml of buffer A and dialysed against 4 litres of this buffer. The dialysed material (0.5–1 mg of protein) was loaded on to a hydroxyapatite column (1.0 cm × 1.0 cm) previously equilibrated with 10 mM sodium phosphate, pH 7.0, containing 10 mM 2-mercaptoethanol, 0.2 mM pyridoxal phosphate, 0.02% Brij 35 and 0.5 M NaCl. The enzyme preparation was washed on to the column with 2 column vol. of 75 mM sodium phosphate, pH 7.0, containing 10 mM 2-mercaptoethanol, 0.1 mM pyridoxal phosphate, 0.025% Brij 35 and 10% (v/v) ethylene glycol. The ODC was eluted with 20 mM sodium phosphate, pH 7.0, containing 10 mM 2-mercaptoethanol, 0.1 mM pyridoxal phosphate, 0.02% Brij 35 and 10% ethylene glycol. The enzyme could be stored at −74 °C for several months without loss of activity.

Enzyme assay

ODC activity was measured by trapping the $^{14}CO_2$ released from L-[1-^{14}C]ornithine (42.5 mCi/mmol) by using a reaction mixture containing 0.2 M acetate (pH 6.5), 60 μM pyridoxal 5′-phosphate and 3 mg/ml BSA in a final volume of 0.5 ml (Jänne and Williams-Ashman, 1971). The reaction was started by addition of 10–30 μg of protein and incubated for 30 min in a shaking water bath at 37 °C. Reactions were stopped by addition of 1 ml of 40% (w/v) trichloroacetic acid and incubated for a further 30 min. Alternative substrates were L-[1-^{14}C]lysine (309 mCi/mmol) and L-[^{14}C]arginine (327 mCi/mmol). A unit of activity released 1 nmol of CO_2 from the substrate in 1 min at 37 °C. Substrate affinities were studied with a fixed concentration of the ^{14}C-labelled amino acid and increasing concentrations of the same unlabelled amino acid. The specific radioactivity of the label was calculated for each final concentration.

SDS/PAGE and autoradiography

The partially purified ODC (25 μg), agarose peak fraction (335 μg) or DEAE peak fraction (1.15 mg) was incubated with 50 μCi of DL-α-difluoro[^{3}H]methylornithine ([^{3}H]DFMO) for 2 h at 37 °C in ODC enzyme-assay buffer. The fractions were then dialysed for 12 h against 1 litre of buffer A with three changes of buffer. The dialysed protein was analysed by SDS/PAGE (Laemmli, 1970), with a 7%-acrylamide running gel and a 4% stacking gel. Electrophoresis was carried out for 1 h at 40 mA, with constant cooling at 10 °C (SE600E; Hoefer, San Francisco, CA, U.S.A.). After electrophoresis the gel was stained for protein with 0.01% Coomassie Blue R-250, destained, transferred to fixative (10% acetic acid/30% methanol) for 1 h and then soaked in Enhance (DuPont–New England Nuclear, Wilmington, DE, U.S.A.) for a further 1 h. The Enhance was precipitated by immersing the gel in 10% (v/v) glycerol at 4 °C for 30 min and then dried at room temperature with an air-drying system (Hoefer Easy Breeze). The gel was placed in a cassette containing X-ray film (Kodak XAR) for 2 days at −80 °C and developed with an X-OMAT apparatus (Kodak).

M_r standards (Boehringer Mannheim) were: macroglobulin, 170000, β-galactosidase, 116000; phosphorylase *b*, 97400; glutamate dehydrogenase, 55400; lactate dehydrogenase, 36500.

Calculation of data

Eadie–Hofstee and Lineweaver–Burk plots were used to analyse the kinetics of enzyme–substrate interactions (Dixon and Webb, 1964). The Eadie–Hofstee plot of the trichomonad ODC with ornithine as substrate demonstrated linear kinetics; hence the $V_{max.}$ was determined by extrapolation to the ordinate. Hill coefficients (h) were determined from the slopes of log $[Y/(1-Y)]$ versus log of concentration of ornithine, lysine or arginine as substrate, to determine co-operativity at binding sites (Dixon and Webb, 1964). The $\frac{1}{2} V_{max.}$ value was used to determine $S_{0.5}$ for substrate from the velocity-versus-substrate plot. The $V_{max.}$ values determined from the Eadie–Hofstee plots were used to calculate the percentage saturation (Y) for the Hill plot. h values were obtained from slopes of plots of [log $Y/(1-Y)$] versus log (substrate concentration). The K_i of inhibitors was determined from Lineweaver–Burk plots of varying substrate concentrations in the presence of a constant amount of inhibitor. The K_i was then determined by using the calculation: slope $= (1+[I]/K_i)$ $V_{max.}/K_m$. The K_i is defined as the amount of inhibitor which doubles the K_m of the enzyme for its substrate.

Chemicals

All radiolabelled material was from New England Nuclear. DFMO and DL-α-difluoromethyl-lysine were given by Dr. Peter

P. McCann of the Marion Merrell Dow Research Institute (Cincinnati, OH, U.S.A.). All other reagents were of the highest purity obtainable.

RESULTS

Localization of ODC

Nuclei-enriched, hydrogenosomal-enriched (large-particle fraction), lysosome-enriched (small-granular fraction) and cytosolic (non-sedimentable) fractions were obtained by differential centrifugation of whole-cell homogenates. Fractions were assayed for the following marker enzymes: malic enzyme (hydrogenosome), acid phosphatase (lysosome) and lactate dehydrogenase (cytosolic). The calculated activities and subcellular distribution of organelle marker enzymes were in good agreement with published results (Lindmark et al., 1975). ODC was non-sedimentable at 43000 ***g*** for 30 min, and was confined almost exclusively to the cytosolic fraction (86%), with a total recovery of 97% in triplicate experiments. Most of the remaining activity (11.3%) was localized within the nuclear fraction (results not shown).

Table 1 Purification of *T. vaginalis* ODC

A unit of enzyme activity = 1 nmol of CO_2 released/min.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Supernatant	3150	3197	1	4	–
80%-satd. $(NH_4)_2SO_4$	1450	1570	1	4	49
DEAE-Sephacel	28	1211	43	166	38
Agarose	6.2	1091	175	673	34
Hydroxyapatite	0.7	595	859	3303	19

Purification

(1) $(NH_4)_2SO_4$ salting

The non-sedimentable fraction from the above step was adjusted to 30% saturation with $(NH_4)_2SO_4$ which precipitated about 21% of the crude protein. The ODC was precipitated by adjusting the clarified supernatant from the 30%-satd.-$(NH_4)_2SO_4$ fraction to 80% saturation with $(NH_4)_2SO_4$. This fraction constituted approx. 40% of the starting protein and resulted in an approx. 4-fold increase in specific activity (Table 1).

(2) DEAE-Sephacel chromatography

A typical DEAE-Sephacel column elution profile for *T. vaginalis* ODC is shown in Figure 1. A single major peak of activity at 0.4 M KCl was obtained for ODC. This resulted in approx. 166-fold purification (Table 1). Active fractions 23–30, emerging at 43–52 ml, were combined (Figure 1).

(3) Agarose column

The DEAE-Sephacel combined fraction (28 mg) was applied to the 1 cm × 90 cm agarose column. The column was then eluted with 2 vol. of 0.2 M acetate buffer, 60 μM pyridoxal phosphate and 1 mM 2-mercaptoethanol (pH 6.5); approx. 1 ml fractions were collected. Under these conditions ODC was eluted as a single peak at 51–58 ml (Figure 2). The pooled fractions had an activity of 175 units/mg of protein, resulting in a 673-fold purification and 34% recovery of activity (Table 1). From this preparation, the native M_r of ODC was determined to be 210000 ± 10000 (two determinations: results not shown) by using the following standards: apoferritin, 443000; β-amylase, 200000; alcohol dehydrogenase, 150000; BSA, 66000.

(4) Hydroxyapatite chromatography

The protein with peak activity after agarose-gel filtration was precipitated with 80%-satd. $(NH_4)_2SO_4$ and adjusted to 2.0 ml in

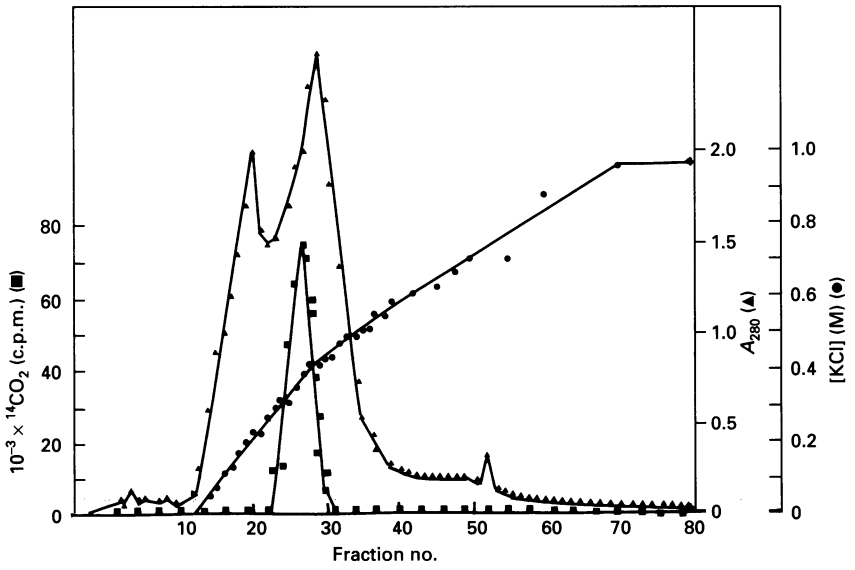

Figure 1 DEAE-cellulose column chromatography of ODC

The 80%-satd.-$(NH_4)_2SO_4$ fraction (1400 mg of protein) was layered on the column and washed with buffer A (see the Materials and methods section). The ODC was eluted with a linear gradient of 0–1 M KCl. ■, $^{14}CO_2$ released from L-[^{14}C]ornithine; ▲, A_{280}; ●, KCl gradient (M).

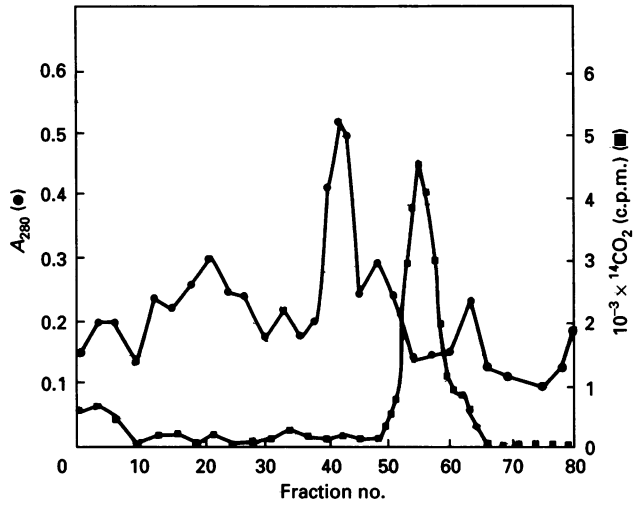

Figure 2 Agarose-gel column chromatography of ODC

Approx. 28 mg of protein containing 43 units of ODC/mg of protein was fractionated on an agarose column as described in the Materials and methods section: ■, $^{14}CO_2$ trapped from L-[^{14}C]ornithine; ●, A_{280}.

Table 2 Properties of *T. vaginalis* ODC with ornithine, lysine or arginine as substrate

A unit of enzyme activity = 1 nmol of CO_2 released/min. [a]Crude homogenate. [b]Partially purified (3303-fold) enzyme. Assays were done as described in the Materials and methods section: ND, not done. Abbreviation: DFML, DL-α-difluoromethyl-lysine; –, not applicable

	Substrate		
	Ornithine	Lysine	Arginine
$V_{max.}$ (units/mg of protein)[a]	3×10^{-1}	4×10^{-3}	4×10^{-4}
$V_{max.}$ (units/mg protein)[b]	1282	13	1.20
K_m (μM)	109	91	100
$10^{-3} \times V_{max.}/K_m$ (M)	12	0.1	0.01
Activity compared with ornithine (%)	100	1.0	0.10
K_i for DFMO (μM)	27	111	ND
K_i for DFML (μM)	111	28.6	ND
K_i for lysine (μM)	14500	–	ND
K_i for ornithine (μM)	–	150	ND
$10^{-3} \times M_r$ (agarose)	210	–	–
$10^{-3} \times M_r$ (SDS/PAGE)	55	–	–

fresh buffer A containing 0.025 % Brij 35. After dialysis, this was layered on to hydroxyapatite and eluted as described in the Materials and methods section. The eluted protein had a specific activity of 859 units/mg of protein and was purified 3303-fold.

Properties of the enzyme

Partially purified *T. vaginalis* ODC exhibited a broad substrate specificity, decarboxylating several amino acids, including ornithine, lysine and arginine. Activity was greatest with ornithine, followed by lysine (1 % of activity with ornithine) and arginine (0.25 % of ornithine) (Table 2). The semi-purified enzyme had an optimal pH of 6.5 and optimal temperature of 37 °C. The enzyme was pyridoxal 5′-phosphate-dependent. To derive a K_m for pyridoxal 5′-phosphate was not possible, since the cofactor is

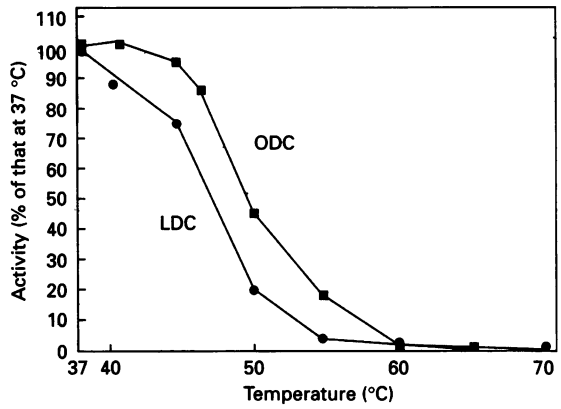

Figure 3 Thermal-stability curve for ODC

The partially purified enzyme was incubated for 30 min at each temperature point, and a sample was assayed with 2.3 mM L-[^{14}C]ornithine (ODC) or 3.2 mM L-[^{14}C]lysine (LDC) as substrate.

tightly associated with the enzyme protein, and dialysis with 10 mM cysteine to remove the cofactor inactivates the enzyme, confirming the findings of North et al. (1986).

A thermal-stability curve was generated for the partially purified protein in an attempt to separate the lysine-decarboxylating activity from the ornithine-decarboxylating activity. This was done by preincubation for 30 min at each of the temperature points. As noted from the temperature curve (Figure 3), both decarboxylating activities were simultaneously lost as the temperature was increased from 37 °C. The lysine- and ornithine-decarboxylating activities were also inseparable in the pH range 4–8 (results not shown).

Coomassie Blue staining after SDS/PAGE of the partly purified protein from the hydroxyapatite column revealed the presence of one major band (Figure 4a). When the purified ODC preparation was specifically labelled with [^{3}H]DFMO a single band of ^{3}H-labelled protein was detected at 55 kDa (Figure 4b). This band was also present in crude extracts, the cytosolic and 80 %-$(NH_4)_2SO_4$ fractions (results not shown) and also the DEAE-Sephacel and agarose fractions (Figure 4b) incubated with [^{3}H]DFMO.

Kinetics

Kinetic studies of ODC protein in both the non-sedimentable crude fraction and partially purified preparations gave similar results. It was determined from Lineweaver–Burk plots (results not shown) and Eadie–Hofstee plots (Figure 5a) that the K_m for ornithine for the partly purified enzyme was 109 μM and the $V_{max.}$ was 1282 units/mg of protein (S.D. ±35 for three determinations) (Table 2). With lysine as substrate, the K_m was determined to be 91 μM and a $V_{max.}$ of 13 units/mg of protein (S.D. ±0.9 for three determinations) was calculated (Figure 6a). Comparison of the $V_{max.}/K_m$ ratio (Table 2) for ODC with ornithine (11.8 mM) or lysine (0.14 mM) as substrate indicates that ornithine is the preferred substrate for the enzyme and there is approx. 3.5 times as much intracellular ornithine as lysine (Yarlett and Bacchi, 1988). Hill plots give a coefficient of 1.0 for ornithine (Figure 5b), suggesting that there is a single binding site for ornithine. Similar results were also obtained for lysine (Figure 6b).

Arginine also acted as a weak substrate, with an apparent affinity (K_m) of 100 μM (Table 2). An Eadie–Hofstee plot gave a

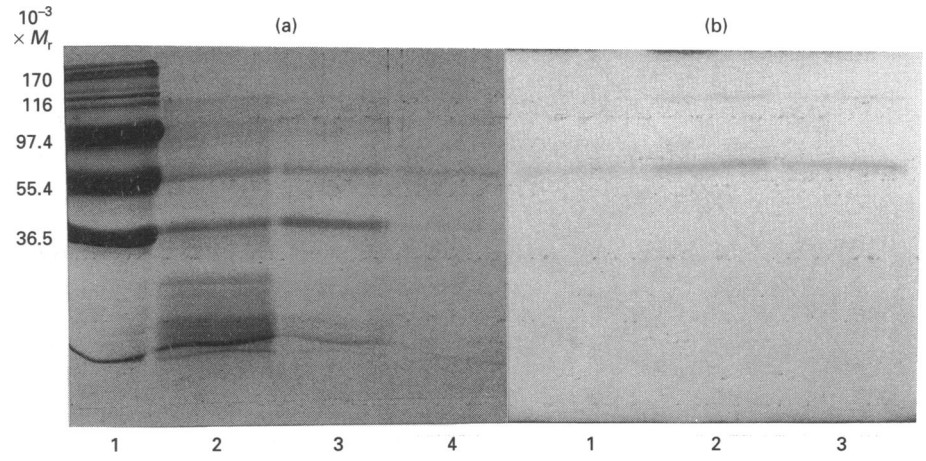

Figure 4 SDS/PAGE of [^{3}H]DFMO-labelled ODC

Samples and standards were prepared as described in the Materials and methods section. (**a**) SDS/PAGE gel. From left to right: lane 1, standards from top to bottom are macroglobulin, β-galactosidase, phosphorylase *b*, glutamate dehydrogenase and lactase dehydrogenase; lane 2, 14 μg of the DEAE-cellulose peak fraction; lane 3, 4 μg of the agarose peak fraction; lane 4, 0.3 μg of the hydroxyapatite-purified fraction. (**b**) Autoradiograph of fractions incubated with [^{3}H]DFMO. From left to right: lane 1, DEAE-cellulose peak fraction; lane 2, agarose peak fraction; lane 3, hydroxyapatite-purified fraction.

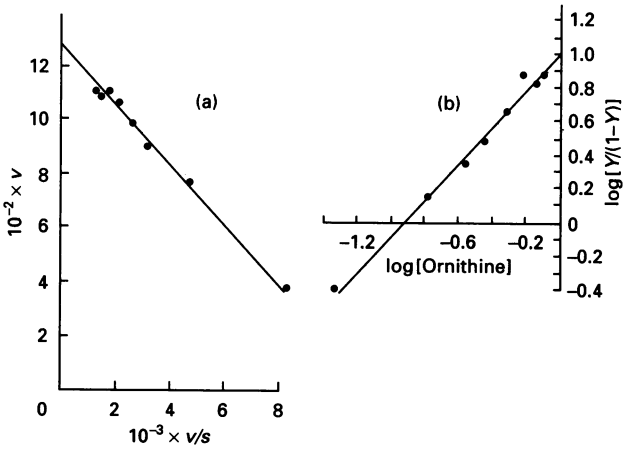

Figure 5 Kinetics of partly purified ODC with ornithine

(**a**) Eadie–Hofstee plot with varying concentrations of L-[^{14}C]ornithine as substrate. (**b**) Hill plot for L-[^{14}C]ornithine as substrate. Percentage saturation (*Y*) was determined by using the $V_{max.}$ for ornithine obtained from the Eadie–Hofstee plot. The velocity (*v*) is expressed as nmol of CO_2 released/min per mg of protein, and the concentration of ornithine (*s*) as mM.

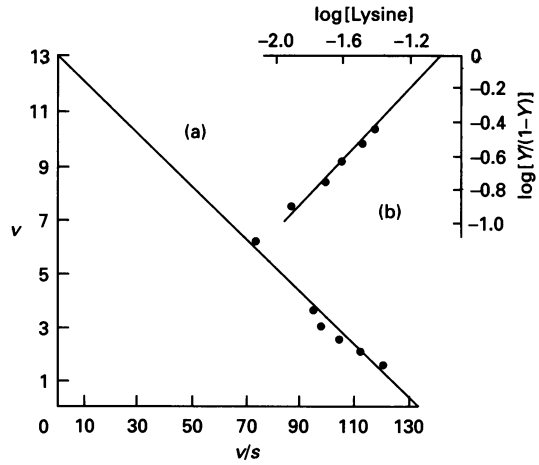

Figure 6 Kinetics of partly purified ODC with lysine

(**a**) Eadie–Hofstee plot with varying concentrations of L-[^{14}C]lysine as substrate. (**b**) Hill plot for L-[^{14}C]lysine as substrate. Percentage saturation (*Y*) was determined by using the $V_{max.}$ for lysine obtained from the Eadie–Hofstee plot. The velocity (*v*) is expressed as nmol of CO_2 released/min per mg of protein, and the concentration of lysine (*s*) as mM.

straight line with an extrapolated $V_{max.}$ of 1.2 units/mg of protein (Table 2).

Competition studies using lysine and ornithine were performed. In these experiments the substrate affinity of the enzyme for ornithine or lysine was determined in the presence of the competing amino acid and compared with controls lacking the competing substrate. Lysine was added to the ornithine-decarboxylating assay to final concentrations of 15 mM and 100 mM. Ornithine was added to the lysine-decarboxylating assay to final concentrations of 0.2 and 1.0 mM. Lineweaver–Burk analysis of the resulting plots indicated competitive inhibition of enzyme activity, with K_m values for ornithine as substrate of 333 μM and 1250 μM with 15 and 100 mM lysine respectively (Figure 7). The K_i of lysine for ornithine decarboxylation was calculated to be 14.5 mM (Table 2). The K_m for lysine as substrate was determined to be 333 μM and 667 μM with 0.2 and 1.0 mM ornithine respectively (Figure 8). The calculated K_i of ornithine for lysine decarboxylation was 150 μM (Table 2). In addition, the two activities co-purified to the same degree, 3303- and 3250-fold for ornithine and lysine respectively. From these studies we conclude that there is a single decarboxylase enzyme with broad substrate specificity, and having ornithine as the preferred substrate.

The amount of [^{3}H]DFMO causing complete inactivation of the enzyme in the final preparation was determined to be 2500 fmol/unit. The M_r of *T. vaginalis* ODC subunits was determined to be 55 kDa. By using published techniques (Seely et al., 1982) it can be calculated that there is 137 ng of ODC protein per unit of activity, based on the assumption that one

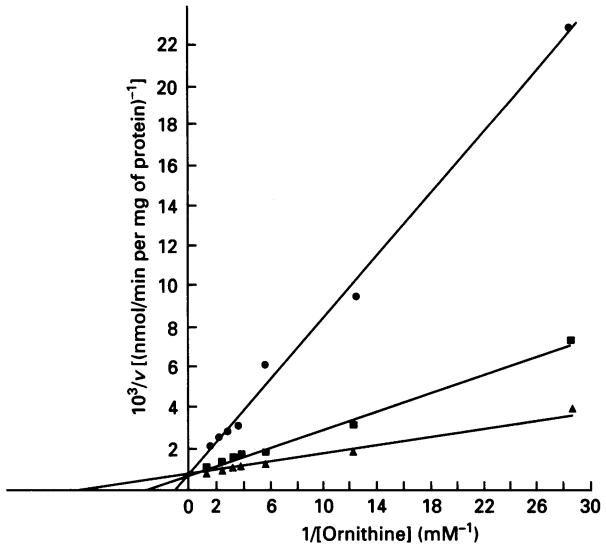

Figure 7 Effect of lysine on the decarboxylation of ornithine by partly purified ODC

The velocity of partially purified ODC was determined in the presence of ornithine concentrations in the range 35–587 μM: (▲) in the absence of lysine, or in the presence of (■) 15 mM lysine or (●) 100 mM lysine. The extrapolated K_m for ornithine is 116, 333 and 1250 μM for 0, 15 and 100 mM lysine respectively. The $V_{max.}$ was unchanged by the addition of lysine, at 1111 nmol/min per mg of protein. The plots enable the calculation of the K_i for lysine on ornithine decarboxylation by ODC (see the Materials and methods section) as 15 and 14 mM for 15 and 100 mM lysine respectively.

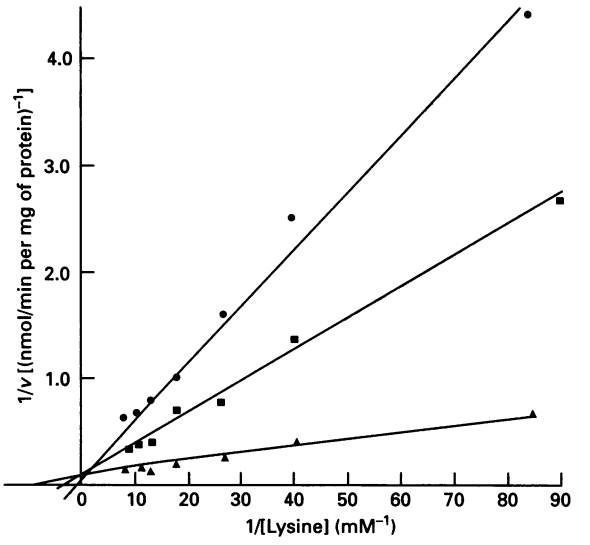

Figure 8 Effect of ornithine on the decarboxylation of lysine by partly purified ODC

The velocity of partially purified ODC with lysine (12–124 μM) as substrate was determined: (▲) in the absence of ornithine, or in the presence of (■) 0.2 mM ornithine or (●) 1 mM ornithine. The extrapolated K_m for lysine was 80, 400 and 1000 μM for 0, 0.2 and 1 mM ornithine respectively. The $V_{max.}$ was unchanged by addition of ornithine, 12.5 nmol/min per mg of protein. The calculated K_i for ornithine on lysine decarboxylation by ODC (see the Materials and methods section) was 159 and 140 μM for 0.2 and 1 mM ornithine respectively.

molecule of the drug binds to each subunit to cause complete inactivation of the enzyme. Since 595 units of ODC were recovered, the final preparation contains approx. 0.08 mg of ODC protein; this means that our final preparation is about 12% pure. The total amount of the starting material was 3197 units; hence the maximum amount of ODC protein present would be 440 μg. However much of this is lost or inactivated during the purification process. This calculation takes into account only active enzyme; hence the value expresses the minimum purification obtained.

Effect of amines and purine nucleosides on enzyme activity

Putrescine was a competitive inhibitor of ODC at high concentrations (above 1 mM). Addition of 2 mM putrescine to the ODC assay containing 87–587 μM ornithine increased the K_m for ornithine from 115 μM to 181 μM. The calculated K_i determined from the Lineweaver–Burk equation was 2.7 mM putrescine. At low concentrations (below 1.0 mM) putrescine did not inhibit ODC activity.

Addition of the polyamines spermidine and spermine and the diamines diaminopropane and cadaverine (to 5 mM) had no effect on the velocity of the ODC over the ornithine range 87–587 μM. Carbamoyl phosphate, an intermediate in the arginine dihydrolase pathway (Linstead and Cranshaw, 1983), inhibited the trichomonad ODC at high concentrations with an apparent K_i of 110 mM determined from Lineweaver–Burk plots of incubations containing 87–587 μM ornithine in the presence of 50 or 100 mM carbamoyl phosphate. However, up to 100 mM ATP, ITP or GTP had no effect on the $V_{max.}$ or K_m for ornithine of the partially purified enzyme.

DISCUSSION

T. vaginalis is an aerotolerant anaerobe which depends on glycolysis and low-redox-potential Fe–S proteins for ATP production. Hence the parasite has limited capacity to derive ATP from glucose (Müller, 1990). The arginine dihydrolase pathway as described by Linstead and Cranshaw (1983) provides an alternative mechanism for the production of ATP, which would appear to be a distinct advantage to a parasite inhabiting a site rich in amines and low in free carbohydrate (Moghissi, 1979). In this pathway, arginine is converted into putrescine, CO_2 and NH_4^+, and 1 mol of ATP is produced per mol of arginine consumed. There is ample evidence that putrescine production by *T. vaginalis* approaches dramatic proportions both *in vitro* and *in vivo*, and that arginine levels are rapidly depleted by the parasite *in situ* (Chen et al., 1982). This evidence supports the view that putrescine production by *Trichomonas* plays a vital and unusual role in the metabolism of the parasite (Yarlett and Bacchi, 1988, 1991).

Analysis of amine end products *in vitro* in *T. vaginalis* indicated that putrescine was present at 10–20 times the amount of other polyamines, and that *T. vaginalis* produces and exports cadaverine as well (Yarlett and Bacchi, 1988). Extra- and intracellular levels of both putrescine and cadaverine were sensitive to inhibition by DFMO. In other eukaryotes it was found that the entire polyamine pool is susceptible to decrease by DFMO (Pfaller et al., 1987; Glass and Gerner, 1986). These results imply that the roles of putrescine are different in *T. vaginalis* compared with other eukaryotes, and from these findings it is apparent that the regulation of ODC and its sensitivity to other agents differ from that in other eukaryotes. The results of this study demonstrate that cadaverine production by *T. vaginalis* occurs via decarboxylation of lysine by ODC, and therefore explain the simultaneous disappearance of putrescine and cadaverine in DFMO-treated cells (Yarlett and Bacchi, 1988). Hence the trichomonad ODC, like that of some other eukaryotes (Pegg and

McGill, 1979; Seely and Pegg, 1983), also decarboxylates lysine at a slow rate. The ODC isolated from mouse kidney decarboxylates lysine to form cadaverine; however, the K_m for this substrate is 100-fold higher than that for ornithine (Seely and Pegg, 1983). Similarly, the K_i for lysine as a competitive inhibitor of *T. vaginalis* is approx. 100-fold higher than the K_m for ornithine (14.5 mM versus 0.11 mM). It appears that the ODC from eukaryotes has a broader substrate specificity than the enzyme of prokaryotes, and that the latter have separate enzymes to decarboxylate ornithine, lysine and arginine, which exhibit strict substrate specificity (Boeker and Fischer, 1983). The partially purified ODC from *T. vaginalis* decarboxylated ornithine, lysine and arginine with decreasing activity.

In a wide variety of both eukaryotes and prokaryotes the K_m values of ODC for ornithine range from 70 to 3000 μM (Kitani and Fujisawa, 1988; Sklaviadis et al., 1985; Tyagi et al., 1981); hence in this respect the trichomonad enzyme is similar to ODC from other organisms. Of the polyamines, only putrescine in high concentration had any inhibitory effect. This is in contrast with mammalian ODC, where putrescine is a competitive inhibitor of L-ornithine, with a K_i of 0.6 mM (Kitani and Fujisawa, 1988).

In many plants and mammals ODC has been shown to occur in several subcellular fractions, including mitochondria, cytosol and nuclei (Schaeffer and Donatelli, 1990). However, in *T. vaginalis*, which lacks mitochondria and a urea cycle, most of the enzyme was recovered in the cytosol. About 11 % of the activity was associated with the nuclear fraction, and this could reflect an underestimate, as the possibility that the enzyme leaks out into the cytosol during processing cannot be ruled out (Seely and Pegg, 1983).

The ODC from *T. vaginalis* has an apparent native M_r of approx. 210000, which compares favourably with the finding of North et al. (1986), who obtained a native M_r of 230000. The *T. vaginalis* ODC is therefore large by comparison with the enzyme from other sources. A subunit M_r of 55000 was determined by SDS/PAGE and autoradiography of [^{3}H]DFMO-bound enzyme. A subunit M_r of 53000 was reported for the enzyme from mouse and rat tissues (Seely et al., 1982). In contrast, ODC purified from various lower eukaryotes [*Saccharomyces cerevisiae* (Tyagi et al., 1981); *Tetrahymena pyriformis* (Sklaviadis et al., 1985) and *Physarum polycephalium* (Barnett and Kazarinoff, 1984)] is dissimilar to the mammalian enzyme in molecular mass and specific activity. The enzyme from *Trypanosoma brucei brucei* has a M_r of 90000 with M_r-45000 subunits (Phillips et al., 1988). This is smaller than the enzyme from *Leishmania donovani*, which was found to be a dimer of subunit M_r 76 kDa (Coons et al., 1990), and is closer to the results obtained for *T. vaginalis*. The subunit M_r of the trichomonad enzyme is not dissimilar from that published for a range of eukaryotes; it is, however, the first reported example of a tetramer. For the mammalian enzyme, it is assumed that one molecule of DFMO is required to bind to each of the subunits to inactivate the enzyme (Seely et al., 1982). This is also assumed for *T. vaginalis* by us in the present study, hence it can be calculated that the ODC represents 0.013 % of the soluble protein. Androgen-stimulated mouse kidney is reported to contain 0.017% ODC protein (Seely et al., 1982), which is considerably higher than in androgen-simulated rat liver, 0.00014 % (Pritchard et al., 1981).

In addition to the higher M_r of the *T. vaginalis* ODC, the enzyme has a half-life of > 15 h (Yarlett et al., 1992), which is far greater than for mammalian enzymes (Pegg, 1988) and *T. b. brucei* (2–5 h: Phillips et al., 1988; Bacchi et al., 1989). The *T. b. brucei* ODC was considered to have a long half-life, and this is attributed to the lack of a PEST sequence (Phillips et al., 1988).

The exact role of ODC and polyamine metabolism in *T. vaginalis* remains to be elucidated. It is clear that production of putrescine far exceeds levels required for cell division in the parasite (Yarlett and Bacchi, 1988). The exact contribution of the pathway to energy metabolism is at present unclear; however, the significant and rapid turnover of all available arginine to putrescine argues that this energy factor is considerable. The large intracellular and extracellular levels of putrescine also leave open to question whether putrescine serves as an internal or external pH buffer, or serves to decrease the numbers of competing micro-organisms in the environment. Further studies examining the conditions governing putrescine production may clarify the role of this metabolite in the parasite.

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