Putrescine biosynthesis in *Tetrahymena thermophila*

Kwok-Ming YAO,* Wang-Fun FONG*† and Stephen F. NG‡

Departments of *Biochemistry and ‡Zoology, University of Hong Kong, Hong Kong

(Received 22 March 1984/Accepted 13 June 1984)

The putrescine-biosynthesis pathway in *Tetrahymena thermophila* was delineated by studying crude extracts prepared from exponentially growing cultures. A pyridoxal phosphate-stimulated ornithine decarboxylase activity competitively inhibited by putrescine was detected. CO₂ was also liberated from L-arginine, but analyses by t.l.c. and enzyme studies suggested that the activity was not due to arginine decarboxylase, nor could enzyme activities converting agmatine into putrescine be detected. We conclude that the decarboxylation of L-ornithine is probably the only major route for putrescine biosynthesis in this organism during exponential growth.

The biological polyamines putrescine, spermidine and spermine are ubiquitous in living organisms and are prerequisites for normal cellular proliferation and differentiation. They have been implicated in many biochemical reactions, and tissue polyamine concentrations and the activity of ornithine decarboxylase (L-ornithine carboxylase, EC 4.1.1.17), the key regulatory enzyme in polyamine biosynthesis, are often dramatically increased in response to stimuli including carcinogens, hormones, drugs and growth factors, and also during tissue hypertrophy (for references, see Heby, 1981).

Putrescine, the precursor of spermidine and spermine, is formed from L-ornithine and/or L-arginine by decarboxylation and, in the case of L-arginine, also by the removal of a molecule of urea. Decarboxylated S-adenosyl-L-methionine donates aminopropyl groups to putrescine to form spermidine and subsequently spermine (for references, see Pegg & Williams-Ashman, 1981). All the organisms studied so far are able to synthesize putrescine by the enzymic decarboxylation of L-ornithine, whereas their ability to use L-arginine as a direct precursor varies. In lower eukaryotes such as *Aspergillus* and *Neurospora*, and in mammalian tissues, the ornithine decarboxylase pathway is the only important biosynthetic route to putrescine. On the other hand, both routes are available in *Escherichia coli*. Under normal growth conditions in a minimal medium, *E. coli* derives most of its polyamines from L-ornithine (Morris & Pardee,

1966). In higher plants, there is evidence for ornithine decarboxylase activity, but the quantitative production of putrescine appears to involve arginine decarboxylase (arginine carboxylase, EC 4.1.1.19) (Montague *et al.*, 1979). However, work on plant tissues (Cohen *et al.*, 1982; Heimer *et al.*, 1979) indicates that, under certain critical physiological conditions, ornithine decarboxylase, but not arginine decarboxylase, is the enzyme responsible for the regulation of putrescine synthesis.

The decarboxylation of L-arginine produces agmatine, which forms putrescine by two routes: in certain species of bacteria the action of agmatinase (agmatine amidinohydrolase, EC 3.5.3.11) produces putrescine and urea (Morris *et al.*, 1970; Khramov, 1972; Friedrich & Magasanik, 1979); on the other hand, the action of agmatine deiminase (agmatine iminohydrolase, EC 3.5.3.12) yields ammonia and N-carbamoyl-putrescine, which is subsequently broken down to putrescine in plants (Smith, 1970) and in *Streptococcus faecalis* (Roon & Barker, 1972) by different enzymes.

The existence of polyamines in *Tetrahymena* has previously been reported, but their biosynthesis has not been demonstrated (Weller *et al.*, 1968; Holm & Heby, 1971). This organism is one of the most studied unicellular eukaryotes, and is amenable to both genetic and biochemical analyses. Furthermore, morphological features of the cell surface provide useful markers for developmental studies at the cellular and subcellular levels. This study attempts to define the putrescine-biosynthetic pathway(s) in this organism.

† To whom reprint requests should be addressed.

Experimental

Materials

L-[1-¹⁴C]Ornithine, L-[U-¹⁴C]ornithine, L-[U-¹⁴C]arginine, Protosol, Omnifluor and ethanolamine (scintillation grade) were obtained from New England Nuclear, Boston, MA, U.S.A., and DL-[1-¹⁴C]arginine was obtained from Research Products International, Mount Prospect, IL, U.S.A. Putrescine (free base), spermidine trihydrochloride, L-ornithine hydrochloride and pyridoxal 5-phosphate were purchased from Serva, Heidelberg, Germany. Aldrich, Milwaukee, WI, U.S.A., supplied agmatine sulphate. 2-Methoxyethanol, 0.1% ninhydrin spray reagent and freeze-dried urease were obtained from Merck, Darmstadt, Germany. Culture medium and supplements were from Difco, Detroit, MI, U.S.A. All other chemicals were purchased from Sigma, St. Louis, MO, U.S.A.

Organism, culture condition and cell sampling

T. thermophila used was of inbred strain B 2079 obtained from J. Frankel, of Iowa University, Iowa City, IA, U.S.A. It was routinely maintained in 2% proteose/peptone/yeast extract supplemented with dextrose and iron-EDTA (Thompson, 1967). The cell density of a culture was estimated with a Coulter Counter model ZB. To initiate an experimental culture, cells obtained from a 10°C stock culture were allowed 2 days of active growth at 27°C; when the cell density reached about 20 000 cells/ml, an appropriate volume of the culture was inoculated into a 250 ml conical flask containing 50 ml of the above medium to give an initial cell density of 100 cells/ml.

After about 24 h, cells of the exponentially growing culture were pelleted by centrifugation at 460 g for 4 min at room temperature and were washed once in 0.9% NaCl. The pellet was resuspended in the assay buffer (50 mM-Tris/HCl, pH 7.3 at room temperature, 0.1 mM-EDTA, 0.075 mM-pyridoxal phosphate and 10 mM-β-mercaptoethanol) to give approx. 1×10^6 cells per 300 μl, and was then frozen quickly in liquid N₂ and stored at -70°C.

Preparation of cell extracts

After thawing, the cells suspended in assay buffer were disrupted by sonication with a B. Braun Labsonic 15/0 ultrasound generator (microprobe) at 60 W for four 15 s pulses. The preparation was centrifuged at 5°C at 37 000 g for 30 min. The cell-extract supernatant was directly assayed for enzyme activities. In the experiments performed to construct the Lineweaver-Burk plots and to study cofactor dependency, the supernatant was dialysed

overnight against the assay buffer with and without pyridoxal phosphate respectively. In the latter case the dialysis buffer also contained 0.2 mM-phenylmethanesulphonyl fluoride and 0.1% Tween 80.

Enzyme assays

Decarboxylase activities were determined by measuring the release of ¹⁴CO₂ from the labelled substrate as described by Fong *et al.* (1976), with the following modifications. The reaction mixture consisted of 45 μl of cell extract and 5 μl of substrate (0.025 μCi/31.8 nmol of L-[1-¹⁴C]-ornithine, 0.025 μCi/370 nmol of L-[U-¹⁴C]arginine or 0.125 μCi/37.1 nmol of DL-[1-¹⁴C]arginine in water), and the reaction was allowed to proceed at 37°C for 30 min and 45 min for ornithine and arginine respectively. These substrate concentrations represented approx. 2–3 times the apparent *K_m* values. The reaction was terminated by the addition of 50 μl of 10% trichloroacetic acid. The CO₂-trapping fluid was 50 μl of ethanolamine/2-methoxyethanol (1:1, v/v), and the scintillation cocktail was 6 ml of 20% (v/v) 2-methoxyethanol and 0.4% (w/v) Omnifluor in toluene. The counting efficiency was consistently 85–86%, as determined by the internal-standard method. The reaction rates were constant for the incubation periods mentioned and were linear with enzyme concentrations over the range studied. One unit of the activity was expressed as 1 nmol of CO₂ liberated/h, and protein concentrations were determined by the method of Lowry *et al.* (1951).

To detect the liberation of ammonia or urea from agmatine, 45 μl of cell extract, containing about 1 mg of protein, was mixed with 5 μl of 73.4 mM-agmatine sulphate solution and incubated at 37°C for 1 h. The reaction was stopped by heating in a boiling-water bath for 15 s, and the protein precipitate was removed by centrifugation for 5 min at top speed in a MSE clinical centrifuge. The supernatant was assayed for ammonia in the presence of urease (Wootton, 1982).

Thin-layer chromatography

T.l.c. was done on cellulose thin layers on Mylar sheets (Eastman Kodak) with an ascending-flow arrangement. The solvent was butan-1-ol/acetic acid/pyridine/water (4:1:1:2, by vol.). After development, the standards were detected with ninhydrin. With this solvent system, arginine, ornithine, agmatine, putrescine and spermidine were separated (Morris & Pardee, 1966).

Cell extracts were subjected to the decarboxylase assay procedures, except that L-[U-¹⁴C]ornithine (0.050 μCi/16.7 nmol in 5 μl) was used instead of L-[1-¹⁴C]ornithine. After the removal of acid precipitate from the reaction mixture by centrifugation,

10 μ l of the supernatant was applied directly on to the t.l.c. plate. The standards were 10 μ l (5–10 nmol) of each of L-arginine, L-ornithine, agmatine, putrescine and spermidine.

For the determination of radioactivity on t.l.c. plates, the plate was cut into rectangular strips, placed in scintillation-counting vials and wetted with 100 μ l of water and 450 μ l of Protosol. After 5 h, 6 ml of 0.4% Omnifluor in toluene was added and the radioactivity determined.

Results and discussion

Ornithine decarboxylase of *T. thermophila*

Activity liberating $^{14}\text{CO}_2$ from L-[1- ^{14}C]-ornithine of up to 50 nmol of CO_2/h per mg of protein has been detected in cell extracts prepared from exponentially growing cultures. That this activity was due to decarboxylase was supported by evidence obtained from t.l.c. analysis, which showed the conversion of L-[U- ^{14}C]ornithine into a substance co-migrating with putrescine (results not shown).

Putrescine at concentrations above 10 μM substantially inhibited the ornithine decarboxylase activity in these cell extracts, and 50% inhibition was obtained with 1 mM-putrescine. Spermidine at concentrations up to 10 mM showed no appreciable

effect. Lineweaver–Burk plots with and without the addition of 0.9 mM-putrescine indicated that putrescine was a competitive inhibitor with respect to L-ornithine, with a K_i of 1.37 mM (Fig. 1). The K_m for L-ornithine was estimated to be 0.187 mM. These values are higher than those found for purified rat liver ornithine decarboxylase (K_m for L-ornithine = 0.06 mM; K_i for putrescine as a competitive inhibitor of L-ornithine = 0.6 mM) (Kitani & Fujisawa, 1983), but lower than that found for *E. coli* ornithine decarboxylase in crude cell extract (K_m for L-ornithine = 1 mM) (Morris & Pardee, 1966). Purified enzyme preparations usually show lower K_m values. Rat liver ornithine decarboxylase is inhibited by putrescine, but not by spermidine, whereas the *E. coli* enzyme is inhibited by both. In this respect, *T. thermophila* ornithine decarboxylase is more similar to the rat liver ornithine decarboxylase than to the *E. coli* ornithine decarboxylase.

Pyridoxal phosphate at concentrations above 0.1 μM substantially enhanced the ornithine decarboxylase activity (Fig. 2). However, we failed to demonstrate a complete pyridoxal phosphate de-

Fig. 1. Lineweaver–Burk plot for ornithine decarboxylase activity

Enzyme activity in a dialysed extract of an exponentially growing *T. thermophila* culture was measured at various L-ornithine concentrations without (●) and with (▲) 0.9 mM-putrescine. Lines were fitted by least-squares linear regression.

Fig. 2. Stimulation of the ornithine decarboxylase activity by pyridoxal phosphate

A cell extract was dialysed against pyridoxal phosphate-free buffer containing phenylmethanesulphonyl fluoride and Tween 80, and enzyme activity was measured by adding various amounts of pyridoxal phosphate. Each assay contained approx. 0.12 mg of protein.

pendence of the enzyme, for a residual amount of enzyme activity remained after overnight dialysis. Extensive dialysis resulted in the complete irreversible loss of enzyme activity, even in the presence of phenylmethanesulphonyl fluoride and Tween 80. The shape of the curve in Fig. 2 may be interpreted as being due to two different enzyme activities, and this possibility requires further investigation.

Liberation of CO_2 from L-arginine

Activity liberating $^{14}\text{CO}_2$ from DL-[1- ^{14}C]-arginine was detected in extracts of exponentially growing cells. The specific activity was comparable with that of ornithine decarboxylase (17.56 and 19.63 nmol of CO_2/h per mg of protein respectively). Although the activity was inhibited by agmatine, the reaction product of arginine decarboxylase, the inhibition was non-competitive with reference to DL-arginine, as judged from the Lineweaver-Burk plot (Fig. 3). We also failed to detect the production of labelled agmatine from L-[U- ^{14}C]arginine by t.l.c. (see below).

We observed that hydrazine, a strong inhibitor of pyridoxal phosphate-requiring amino acid decarboxylases (Clark, 1963), although strongly inhibiting the ornithine decarboxylase activity (80%,

97% and 100% inhibition at 0.09 mM, 0.9 mM and 9 mM respectively), affected $^{14}\text{CO}_2$ liberation from [U- ^{14}C]arginine only to a small extent (10% inhibition at 9 mM-hydrazine). When DL-[1- ^{14}C]-arginine was used as substrate, the activity became highly sensitive to hydrazine (96% inhibition at 10 mM-hydrazine). Furthermore, the addition of unlabelled ornithine to reaction mixtures to a final concentration of 8 mM caused a 75% decrease in the liberation of $^{14}\text{CO}_2$ from L-[U- ^{14}C]arginine. These observations are consistent with the interpretation that the liberation of $^{14}\text{CO}_2$ from [1- ^{14}C]arginine is due not to arginine decarboxylase, but probably to breakdown of L-arginine by arginine deiminase and citrullinase, which produce ornithine and also release CO_2 (see below). Ornithine produced by this pathway is further converted into CO_2 and putrescine by ornithine decarboxylase.

Lack of agmatinase and agmatine deiminase activities

In this study the production of ammonia from agmatine was monitored in the presence of urease. The sensitivity of the assay was such that the production of a few nmol of ammonia per assay would have been detected. A cell extract with a $^{14}\text{CO}_2$ -liberating activity from L-[U- ^{14}C]arginine of 357.8 nmol of CO_2/h per 45 μl of cell extract was used, but no agmatine-derived urea or ammonia could be detected after incubation for 1 h. We conclude that agmatinase and agmatine deiminase activities either are absent or play an insignificant role in this system.

Conversion of L-arginine into L-ornithine

When L-[U- ^{14}C]arginine was used as the substrate and reaction products were subjected to analysis by t.l.c., most of the radioactivity was shifted to a position corresponding to L-ornithine, and no appreciable radioactivity was found at the agmatine position (Fig. 4).

The conversion of L-arginine into L-ornithine in *Tetrahymena* is not due to arginase (L-arginine amidinohydrolase, EC 3.5.3.1), since there is overwhelming evidence showing the absence of an active urea cycle and that the ciliate is ammonotelic (Dewey *et al.*, 1957; Hill & van Eys, 1965). The conversion has been ascribed to the L-proline-biosynthetic pathway, involving the successive action of two enzymes, arginine deiminase (L-arginine iminohydrolase, EC 3.5.3.6) and citrullinase (L-citrulline N^5 -carbamoyldihydrolase, EC 3.5.1.20) (Hill & Chambers, 1967). Arginine deiminase, which catalyses the conversion of L-arginine to L-citrulline and ammonia, has been purified 75-fold (Hill & Chambers, 1967). It is located in the soluble fraction of the cell, and L-ornithine is a competitive inhibitor, with a K_i

Fig. 3. Lineweaver-Burk plot for CO_2 liberation from DL-[1- ^{14}C]arginine

CO_2 -liberating activity in an extract of an exponentially growing *T. thermophila* culture was measured at various DL-arginine concentrations without (●) and with (▲) 9 mM-agmatine. Lines were fitted by least-squares linear regression. Each assay contained approx. 0.16 mg of protein.

Fig. 4. T.l.c. of L-[U- 14 C]arginine treated with samples of a cell extract

L-[U- 14 C]Arginine was incubated with samples of a cell extract and reactions were stopped either at zero time (●) or after 45 min (▲). After chromatography the standards (Orn = L-ornithine, Arg = L-arginine, Put = putrescine, Agm = agmatine) were detected with ninhydrin, and their positions are indicated with arrows. Lanes containing radioactivity were cut into strips and counted.

of 0.0108 mM, compared with a K_m for L-arginine of 2.84 mM. Citrullinase catalyses the formation of L-ornithine, ammonia and CO_2 from L-citrulline.

From the above evidence, it is likely that the only important pathway for putrescine biosynthesis in exponentially growing *T. thermophila* is the decarboxylation of L-ornithine, and there is an active production of L-ornithine from L-arginine. Although at this time the substrate concentrations in the exponentially growing cells are not known, the $V_{\max}$ of $^{14}\text{CO}_2$ liberation from L-[U- 14 C]arginine was an order of magnitude higher than that from DL-[1- 14 C]arginine or L-[1- 14 C]ornithine, thus it is likely that a significant portion of the L-ornithine generated from L-arginine is shunted for L-proline biosynthesis or for energy production via the tricarboxylic acid cycle (Hill & Chambers, 1967). This notion is further supported by t.l.c., which shows that the main product from L-[U- 14 C]arginine is not putrescine. Ornithine decarboxylase, being feedback-inhibited by putrescine, probably controls the flux of

L-ornithine into the polyamine-biosynthetic pathway. The flux of L-ornithine into the L-proline-biosynthetic pathway or for energy production is probably regulated by ornithine-oxo-acid aminotransferase (L-ornithine:2-oxo-acid aminotransferase, EC 2.6.1.13); the latter produces glutamate γ -semialdehyde and is moderately inhibited by L-valine (Hill & Chambers, 1967). Moreover, arginine deiminase, which is competitively inhibited by L-ornithine, the metabolite at the branching point, may control the total flux of L-arginine through both polyamine- and L-proline-biosynthetic pathways.

It must be pointed out, however, that we have studied cells from only one stage of growth. We have not excluded the possibility that, under restrictive growth conditions, such as when decarboxylation of L-ornithine is impeded, an alternative putrescine-biosynthesis pathway may be turned on. We also do not have any evidence suggesting that the decarboxylation of L-ornithine is the only source of the intracellular putrescine pool. It has been reported that in heat-synchronized *Tetrahymena* cultures the increase in intracellular putrescine parallels that of the uptake rate of the diamine from the medium (Holm & Emanuelsson, 1971). We have observed, however, that there is an increase in putrescine in exponentially growing cells over that of stationary cells, and this increase can be totally accounted for by the increase in ornithine decarboxylase activity, assuming that there is an ample supply of L-ornithine (results not shown).

This research was supported by University of Hong Kong research grants awarded to W.-F. F. and S. F. N.

References

- Clark, W. G. (1963) in *Metabolic Inhibitors* (Hochster, R. M. & Quastel, J. H., eds.), vol. 1, pp. 316–346, Academic Press, New York and London
- Cohen, E., Heimer, Y. M. & Mizrahi, Y. (1982) *Plant Physiol.* **70**, 544–546
- Dewey, V. C., Heinrich, M. R. & Kidder, G. W. (1957) *J. Protozool.* **4**, 211–219
- Fong, W. F., Heller, J. S. & Canellakis, E. S. (1976) *Biochim. Biophys. Acta* **428**, 456–465
- Friedrich, B. & Magasanik, B. (1979) *J. Bacteriol.* **137**, 1127–1133
- Heby, O. (1981) *Differentiation* **19**, 1–20
- Heimer, Y. M., Mizrahi, Y. & Bachrach, V. (1979) *FEBS Lett.* **104**, 146–148
- Hill, D. L. & Chambers, P. (1967) *Biochim. Biophys. Acta* **148**, 435–447
- Hill, D. L. & van Eys, J. (1965) *J. Protozool.* **12**, 259–265
- Holm, B. & Emanuelsson, H. (1971) *Z. Zellforsch. Mikrosk. Anat.* **115**, 593–597

- Holm, B. & Heby, O. (1971) *Z. Naturforsch. B* **26**, 604–606
- Khramov, V. A. (1972) *Biokhimiya* **37**, 922–924
- Kitani, T. & Fujisawa, H. (1983) *J. Biol. Chem.* **258**, 235–239
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Montague, M. J., Armstrong, T. A. & Jaworski, E. G. (1979) *Plant Physiol.* **63**, 341–345
- Morris, D. R. & Pardee, A. B. (1966) *J. Biol. Chem.* **241**, 3129–3135
- Morris, D. R., Wu, W. H., Applebaum, D. & Koffron, K. L. (1970) *Ann. N.Y. Acad. Sci.* **171**, 968–976
- Pegg, A. E. & Williams-Ashman, H. G. (1981) in *Polyamines in Biology and Medicine* (Morris, D. R. & Marton, L. J., eds.), pp. 3–42, Marcel Dekker, New York
- Roon, R. J. & Barker, H. A. (1972) *J. Bacteriol.* **109**, 44–50
- Smith, T. A. (1970) *Ann. N.Y. Acad. Sci.* **171**, 988–1001
- Thompson, G. A., Jr. (1967) *Biochemistry* **6**, 2015–2022
- Weller, D. L., Raina, A. & Johnstone, D. B. (1968) *Biochim. Biophys. Acta* **157**, 558–565
- Wootton, I. D. T. (1982) *Microanalysis in Medical Biochemistry*, pp. 64–65, Churchill Livingstone, Edinburgh