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Putrescine-insensitive S-Adenosyl-L-methionine Decarboxylase from *Tetrahymena pyriformis*

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Extracts of *Tetrahymena pyriformis* contain a soluble *S*-adenosyl-L-methionine decarboxylase which, in contrast to the enzyme from most eukaryotic organisms, is not stimulated by putrescine or spermidine. The protozoan adenosylmethionine decarboxylase, unlike the putrescine-insensitive enzyme form *Escherichia coli*, did not require any metal ions for catalytic activity either. Adenosylmethionine decarboxylase from *Tetrahymena* resembled the prokaryotic enzyme as far the inhibition by methylglyoxal bis(guanylhydrazone) was concerned, but behaved more like putrescine-activated enzyme in regard to the inhibition by 4-bromo-3-hydroxy benzyl-oxyamine. Adesylmethionine decarboxylase from rat liver, baker's yeast, *E. coli* and *Tetrahymena* were strongly inhibited by *S*-methyladenosylhomocysteamine (decarboxylated adenosylmethionine), the product of the reaction. The function of adenosylmethionine decarboxylase from *Tetrahymena* like that of the enzymes from other organisms appears to be closely connected to the synthesis of spermidine.

The synthesis of spermidine both in prokaryotic and eukaryotic organisms is accomplished by two separate enzymes: a *S*-adenosyl-L-methionine decarboxylase (AMD; EC 4.1.1.50) catalyzing the formation of *S*-methyladenosylhomocysteamine (decarboxylated adenosylmethionine) and an aminopropyltransferase (spermidine synthase, EC 2.5.1.16) transferring the propylamine moiety from decarboxylated adenosylmethionine to putrescine.[1-4] These two enzymes appear to function very closely together in the formation of spermidine in all organisms studied. Thus crude extracts from most animal tissues catalyze a stoichiometric formation of spermidine and carbon dioxide from adenosylmethionine in the presence of putrescine.[5] A fundamental difference between AMD from prokaryotic and eukaryotic organisms seems to be the fact that the enzyme from the latter sources is intensively and specifically stimulated by minute concentrations of putrescine.[5,6,3] AMD appears to be activated by putrescine at least in all animal tissues studied so far, including livers from reptilia, crustacae, amphibia, birds, and mammals.[7] The enzyme from baker's yeast also shows an almost absolute requirement for putrescine.[7,8]. In contrast to the AMD from the eukaryotic sources mentioned, the enzyme from bacteria is totally insensitive to putrescine but instead requires Mg^{2+} ions for its activity. Thus AMD from *E.coli* [1,7] and *Azotobacter vinelandii* [7] exhibits virtually absolute requirement for divalent cations. The classification of AMD to prokaryotic putrescine-insensitive and putrescine-activated eukaryotic enzymes is, however, not universal, since AMD activity in extracts of an eukaryotic plant (mung bean sprouts) is not stimulated by putrescine but requires Mg^{2+}.[7] An even more important exception of this general rule is the AMD from the lower eukaryotic plasmodium *Physarum polycephalum*. AMD activity from the latter organism does not appear to be stimulated either by putrescine or Mg^{2+}, however, still functioning in the synthesis of spermidine.[9]

The present communication deals with some characteristics of a putrescine-insensitive AMD from extracts of *Tetrahymena pyriformis*. The enzyme did not require any metal ions

for the catalytic activity. The protozoan AMD resembled the prokaryotic enzyme in regard to the inhibition by methylglyoxal bis (guanylhydrazone), a potent inhibitor of putrescine-activated enzyme.[10]

MATERIAL AND METHODS

Chemicals. Unlabelled and labelled *S*-adenosyl-L-methionine was synthesized enzymically by the method of Pegg and Williams-Ashman.[5] Decarboxylated adenosylmethionine was prepared with the aid of AMD partially purified from *E.coli* cells. The product of the enzymic decarboxylation was purified and finally separated from adenosylmethionine by preparative paper electrophoresis.[8]

Methylglyoxal bis(guanylhydrazone) was purchased from Aldrich Chemical Co., Milwaukee, Wis., U.S.A., and 4-bromo-3-hydroxybenzyloxyamine (NSD-1055) was a product of Smith and Research Ltd., Harlow, Essex, England.

Putrescine-1,4^{14}C dihydrochloride (sp. radioactivity 17.5 mCi/mmol) was purchased from New England Nuclear Corp., Dreieichenhain, West Germany, and purified before use on a column (H^+ form; 1 cm × 5 cm) of Dowex 50.[12]

Organisms and growth conditions. Tetrahymena pyriformis, strain W (obtained from Dr. G. Rølle, Department of Nutritional Biochemistry, The Royal Danish School for Educational Studies, Copenhagen, Denmark), was maintained in stock cultures of peptone-yeast medium at pH 8.4. For experimental purpose 3 – 7 day-old stock cultures were used to inoculate peptone-yeast medium (containing 2 % proteose-peptone, 0.1 % yeast extract, 0.5 % glucose and 0.1 % NaCl) in a Roux-bottle with a surface area of 200 cm^2. The cultures were incubated at 25 °C without shaking, and the cells were harvested at the early stationary phase (72 h) by centrifugation at 10 000 *g* for 10 min. The yield (wet wt.) was about 3 g/100 ml per 72 h.

Preparation of cell extracts. The cells of *Tetrahymena pyriformis* W, *E. coli* (strain ATCC 4157; harvested at the stationary phase) and *Saccharomyces cerevisiae* (obtained from Oy Alko, Helsinki, Finland) were suspended in an equal volume (wt./vol) of 25 mM Tris-HCl, pH 7.1, containing 0.1 mM EDTA and 1 mM dithiothreitol (standard buffer) and extruded twice through a chilled (– 25 °C) pressure cell (X-press, Biox AB, Nacka, Sweden) at 1500 kg/cm^2. After the disintegration of the cells. the homogenates were centrifuged at 15 000 *g* for 15 min and the supernatant fractions were used for a further purification or as the source of the AMD.

Liver cytosol fraction was prepared by homogenizing rat liver with two volumes of the standard buffer and centrifuging as above.

Analytical methods. The activity of AMD was assayed essentially as described earlier.[1,3] The activity of spermidine synthase was assayed in the presence of decarboxylated adenosylmethionine and radioactive putrescine as described elsewhere.[4,8] Protein was measured by the method of Lowry *et al.*[11]

Partial purification of adenosylmethionine decarboxylase from Tetrahymena pyriformis. A crude extract prepared from 10 g of *Tetrahymena* cells was fractionated with solid ammonium sulfate (special enzyme grade, Schwarz/ Mann, Orangeburg, N.Y., U.S.A) at 0 °C. The proteins precipitated between 0.45 – 0.70 saturation of ammonium sulfate were collected and dissolved in a small volume of the standard buffer and dialyzed for 16 h against the same buffer. The dialyzed protein fraction was subjected to chromatography on a DEAE cellulose column (1.1 cm × 17 cm; DE-52 Whatman), previously equilibrated against the standard buffer. The column was washed with 70 ml of the equilibration buffer and connected to a linear gradient of 0 to 0.4 M NaCl in the standard buffer (the total gradient volume was 200 ml). AMD activity emerged from the column at about 0.3 M NaCl. The most active fractions were pooled and concentrated in an ultrafiltration cell to 5 ml. This partial purification of the enzyme activity resulted in a 17-fold purification with an overall

Table 1. Effect of various amines, metals, pyridoxal phosphate, and nucleotides on AMD activity from *Tetrahymena pyriformis*. Dialyzed extract (0.8 mg protein) was used in Experiment I and partially purified enzyme (DEAE fraction; 0.016 mg protein) in Experiment II as the source of the enzyme. The activities are expressed as pmol of CO_2 formed per 30 min.

Compound added	Conc. (mM)	AMD activity Exp. I	Exp. II
None	–	62.0	29.2
Putrescine	2.5	70.3	33.8
Spermidine	2.5	49.0	32.7
Spermine	2.5	49.0	32.3
Diaminopropane	2.5	50.0	
Cadaverine	2.5	52.8	
Pyridoxal phosphate	0.1	39.7	19.4
Mg^{2+}	10	46.9	17.5
Mn^{2+}	10	16.3	
Na^+	100	38.0	
K^+	100	38.0	
ATP	1.0		21.1
GTP	1.0		21.8
CTP	1.0		21.4
UTP	1.0		20.7

yield not more than 0.6 %. The poor recovery of the enzyme activity was due to the extreme lability of the enzyme, so that all attempts at more extensive purifications were considered unrealistic.

RESULTS AND DISCUSSION

As shown in Table 1, the activity of AMD from cells of *Tetrahymena pyriformis* was not stimulated to any appreciable extent by putrescine, spermidine, spermine, 1,3- diaminopropane, or cadaverine. Divalent and monovalent cations appeared to be inhibitory for the enzyme. It did not matter whether dialyzed crude extract or partially purified enzyme (DEAE fraction) was used.

AMD from the protozoan was extremely labile. The partially purified enzyme was inactivated with an apparent half-life of about 2 h when dialyzed at 3 °C against the standard buffer. A dialysis of the crude extract for 16 h resulted in a loss of AMD activity up to 80 %. It was unlikely that the rapid inactivation was caused by the removal of a dialyzable small molecule since the combination of the partially purified enzyme with undialyzed crude extracts from *Tetrahymena* only resulted in a simple addition of the enzyme activity. Pyridoxal-5'-phosphate was slightly inhibitory, and neutralized nucleotides were without any effect whatsoever (Table 1). The enzyme thus resembled AMD from dialyzed extracts of the plasmodium *Physarum polycephalum* in being insensitive to putrescine (and other polyamines) as well as to divalent cations.[9]

Table 2. Inhibition of AMD activity from *Tetrahymena pyriformis* by carbonyl reagents. Dialyzed extract (0.8 mg protein) was used as the source of the enzyme. The enzyme activity is expressed as pmol of CO_2 formed per 30 min.

Inhibitor	Conc. (mM)	Enzyme activity	Inhibition %
None	–	41.8	0
KCN	5	4.6	89
Isonicotinic acid hydrazide	5	38.8	7
Semicarbazide	5	16.1	61
$NaBH_4$	10	16.6	60

The activity of AMD from extracts of *Tetrahymena* was inhibited by a number of carbonyl reagents like KCN, semicarbazide and borohydride but not by isonicotinic acid hydrazide (Table 2). The latter finding together with the apparent ineffectiveness of added pyridoxal phosphate might indicate that carbonyl groups other than pyridoxal phosphate are required for the catalytic activity of AMD from *Tetrahymena pyriformis*. The nature of the prosthetic group of mammalian putrescine-activated adenosylmethionine decarboxylase still remains open, whereas the magnesium-activated enzyme from *E. coli* contains covalently bound pyruvate probably functioning as the prosthetic group.[1]

Methylglyoxal bis(guanylhydrazone) is known to be a specific and powerful inhibitor of putrescine-activated AMD from rat ventral prostate,[10] baker's yeast,[10] and rat liver.[13] It also inhibits the putrescine-insensitive adenosylmethionine decarboxylase from the plasmodium *Physarum polycephalum*,[9] whereas relatively high concentrations are needed for a similar inhibition of the putrescine-insensitive AMD from *E. coli*.[10] The mechanism of the inhibition could involve interactions with the putrescine binding site since the inhibitor appears to diminish the magnitude of the stimulation by putrescine.[10,13] The finding that diamine oxidase from rat tissues (using putrescine as the substrate) is also extremely sensitive to methylglyoxal bis(guanylhydrazone) also points in this direction.[13]

As seen in Fig. 1, the activity of AMD from *Tetrahymena* was also inhibited by methyl-

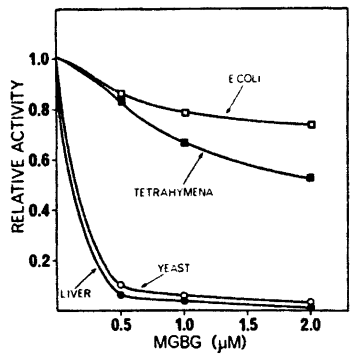

Fig. 1. Inhibition of AMD activity from various organisms by methylglyoxal bis(guanylhydrazone) (MGBG). Dialyzed crude extracts were used as the source of the enzyme in each case.

glyoxal bis(guanylhydrozone) but in this respect the enzyme resembled AMD from *E. coli* being considerably less sensitive than the enzyme from extracts of rat liver or baker's yeast.

4-Bromo-3-hydroxy benzyloxyamine (NSD-1055) is known to inhibit some pyridoxal phosphate requiring enzymes like histidine decarboxylase from rat tissues.[14,15] The compound apparently forms an oxime with pyridoxal phosphate.[15] Pegg and Williams-Ashman [5] reported that AMD from rat ventral prostate was powerfully inhibited by the drug, whereas the enzyme from *E. coli* cells was relatively insensitive. The inhibition of AMD from rat ventral prostate could be reversed by a prior incubation of the enzyme preparation in the presence of pyridoxal phosphate but hardly in the presence of free pyridoxal.[5] The latter finding has been taken as a suggestive evidence that the prosthetic group of mammalian AMD would be pyridoxal phosphate. However, no direct evidence has been obtained to prove or disprove this hypothesis. If anything, the data available at present rather suggest that the carbonyl group obviously needed for the decarboxylation of adenosylmethionine by eukaryotic AMD would not be pyridoxal phosphate.[16] As also seen in the present study, AMD from *Tetrahymena* was not affected by the inclusion of pyridoxal phosphate in the incubation mixture (Table 1), neither was the enzyme activity inhibited by 5 mM isonicotinic acid hydrazide (Table 2).

As illustrated in Fig. 2, AMD from rat liver most markedly inhibited by NSD-1055 whereas the enzyme from *E. coli* was hardly affected by the compound at concentrations up to 1 mM. The degree of inhibition of putrescine activated AMD from baker's yeast and that of the putrescine-insensitive enzyme from protozoan *Tetrahymena* was somewhere between the two extremities.

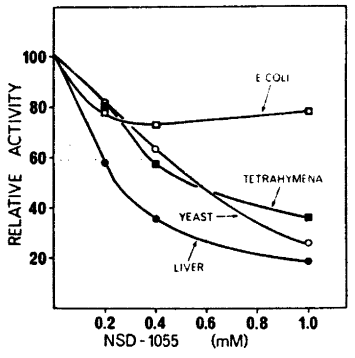

Fig. 2. Inhibition of AMD activity from various organisms by 4-bromo-3-hydroxy benzyloxyamine (NSD-1055). Dialyzed crude extracts were used as the source of the enzyme in each case.

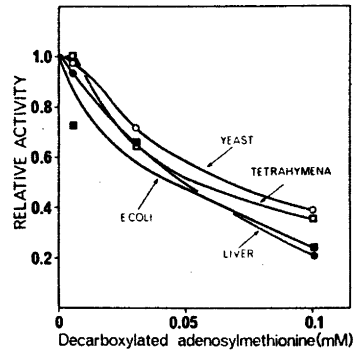

Fig. 3. Inhibition of AMD activity from various organisms by *S*-methyladenosylhomocysteamine (decarboxylated adenosylmethionine). The source of the enzymes as in Figs. 1 and 2.

The product of the decarboxylation reaction, decarboxylated adenosylmethionine, S-methyladenosylhomocysteamine, is a potent inhibitor for both eukaryotic and prokaryotic AMD.[8] In fact, it has been reported that putrescine-activated enzyme from rat ventral prostate or baker's yeast would be more sensitive to the product inhibition than the enzyme from *E. coli*.[8] As shown in Fig. 3, AMD from all four organisms was inhibited by the product of the reaction in a similar fashion with an half-maximal inhibition occurring at a concentration of approximately 50 μM. The reason for this apparent discrepancy with the earlier report is not known. This might conceivably be due to slightly different experimental conditions, different rat tissue (liver *versus* ventral prostate) and a different strain of *E. coli* used in the present study.

When the formation of spermidine from adenosylmethionine was followed simultaneously with the formation of carbon dioxide from the latter compound a close stoichiometry was found in the presence of putrescine (not tabulated).

It appears that the stimulation of AMD activity by putrescine can be no longer consid-

ered as a general property of eukaryotic organisms. There exist at least three eukaryotic AMD's which are not stimulated by putrescine or by any other amine: (i) AMD from bean sprouts,[7] (ii) that of the plasmodium *Physarum polycephalum*,[9] and (iii) that from protozoan *Tetrahymena pyriformis*. The enzyme from the plasmodium and protozoan also is insensitive to divalent cations (which might even be inhibitory) whereas the activity of AMD from bean sprouts is markedly enhanced by Mg^{2+}.[7]

It is quite impossible to decide what really determines the sensitivity of AMD to putrescine. However, it also appears somewhat unlikely that the presence of real nuclear membrane should change the properties of a cytosolic enzyme. Nevertheless, there seems to exist an interesting difference between the two types of AMD. All rat tissues [17] and baker's yeast [18] contain substantial amounts of spermine and, at least rat tissues, also contain relatively active spermine synthase activity [19,20] that catalyzes the transfer of the propylamine group of decarboxylated adenosylmethionine to spermidine. On the contrary, *E. coli* cells do not contain any spermine, the only polyamines in this organism are putrescine and spermidine.[18] The cells of *Physarum polycephalum* [9] and those of *Tetrahymena pyriformis* [21] only contain almost unmeasurable traces of spermine as compared to the levels of putrescine and spermidine. It is thus possible that the stimulation of AMD by putrescine is an evolutionary change in the enzyme appearing at the time when the organism starts to synthesize spermine. The stimulation of AMD by putrescine would thus assure that sufficient amounts of decarboxylated adenosylmethionine are available for the synthesis of spermidine by spermidine synthase.

However, more extensive systematic studies obviously are needed to elucidate the possible relations between the stimulations of AMD activity by putrescine and the ability of the organism to synthesize and accumulate spermine.

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