On the Enzymic Preparation of Decarboxylated Adenosyl-methionine

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A method is described for enzymic preparation of S-methyladenosylhomocysteamine (decarboxylated adenosylmethionine) with the aid of magnesium-activated adenosylmethionine decarboxylase partially purified from cells of Escherichia coli. The procedure involves chromatography on Dowex 50-H+ column, followed by preparative paper electrophoresis and re-chromatography on Dowex 50-H+. The procedure yields a pure preparation which is suitable for measurements of the activity of spermidine and spermine synthases.

The synthesis of the higher polyamines, spermidine and spermine is accomplished in both eukaryotic and prokaryotic organisms by at least three separable enzymes: (i) a soluble S-adenosyl-L-methionine decarboxylase (S-adenosyl-L-methionine carboxy-lyase, EC 4.1.1.50) that catalyzes the decarboxylation of S-adenosyl-L-methionine to yield S-methylhomocysteamine (decarboxylated adenosylmethionine) and CO_2,[1-4] (ii) an aminopropyltransferase (spermidine synthase, 5'-deoxyadenosyl-(5'),3-aminopropyl-(1),methylsulfonium-salt:putrescine 3-aminopropyltransferase, EC 2.5.1.16) which transfers the propylamine group from decarboxylated adenosylmethionine to putrescine to yield spermidine, methylthioadenosine and a proton,[4-6] and (iii) a spermine synthase (5'-deoxyadenosyl-(5'), 3-aminopropyl-(1),methylsulfonium-salt:spermidine 3-aminopropyltransferase).[5]

The decarboxylation of adenosylmethionine (Ado-met) is catalyzed by a specific adenosylmethionine decarboxylase (AMDC) which in animal tissues and yeast is strongly activated by putrescine and related diamines [1,3,4,8] but in some lower eukaroytes and in prokaryotic organisms does not require any cofactors [9,10] or shows a stringent requirement for magnesium ions.[3,8,11]

The main, if not necessarily the only function of decarboxylated Ado-met appears to be to serve as the substrate for spermidine and spermine synthases. Decarboxylated Adomet can also function as methyldonor in some transmethylation reactions [12] and it occurs at high concentrations in the tapetum lucidum of the catfish (Arius felis) [13] for reasons which are not yet known.

In higher organisms, such as in animal tissues and in cells of baker's yeast AMDC is the rate controlling enzyme in the synthesis of spermidine and spermine.[14,15] The measurements of the formation of spermidine and spermine in the presence of Ado-met and the appropriate amine precursor (putrescine or spermidine) in the tissue or cell extracts thus only represents the activity of AMDC and not that of the two propylamine transferases. Accordingly, for the measurements of the actual activities of spermidine and spermine synthases decarboxylated Ado-met is needed as precursor.

In the present communication we describe a method for preparative isolation of decarboxylated Ado-met, synthesized enzymically with the aid of partially purified AMDC from E. coli. Some factors that contribute to the yield of the preparative process as well as to the purity and use of the product in the synthesis of spermidine and spermine, are likewise presented.

MATERIALS AND METHODS

Chemicals. Unlabelled *S*-adenosyl-L-methionine was synthesized by the method originally described by Cantoni *et al.*[16] and modified by Pegg and Williams-Ashman.[1]

[1,4-¹⁴C]Putrescine (sp. radioactivity 17.5 mCi/mmol), [1,4-¹⁴C]spermidine (sp. act. 12.4 mCi/mmol) and [carboxyl-¹⁴C]*S*-adenosyl-L-methionine (sp. act. 60 mCi/mmol) were purchased from New England Nuclear Corp. (Dreieichenhain, West-Germany). Radioactive putrescine was purified on a Dowex 50-H⁺ column before use.

Cultivation of Escherichia coli. *E. coli* (strain ATCC 4157) was grown aerobically in a supplemented mineral medium the composition of which has been described elsewhere.[8] The cultivation was started by inoculating 25 ml of the medium and incubating the culture at 30 °C for 12 h, after which it was transferred to 500 ml of the same medium and grown for further 12 h. The culture was transferred to 4.5 l of the medium and incubated for 10 h. This culture was finally transferred to a 400 l pilot plant fermentor (Getingeverken, Getinge, Sweden) which contained 100 l of the medium. The mixture was incubated for about 7 h. The cells were harvested in a DeLaval separator at early stationary phase and stored as frozen paste at −25 °C.

Preparation of cell and tissue extracts. Acetone-dried cell powder from cells of *E. coli* was prepared essentially as described by Ellfolk and Soininen.[17] The acetone-treated cells were extracted as described earlier [8] with 25 mM Tris-HCl buffer containing 0.1 mM EDTA and 10 mM 2-mercaptoethanol (referred to below as the standard buffer). AMDC was partially purified from the extracts of *E. coli* by the method of Wickner *et al.*[11] through the ammonium sulfate fractionation and the subsequent chromatography on DEAE-cellulose. The heat step of the cited procedure, however, was omitted. One unit is defined as the amount of the enzyme catalyzing the formation of 1 nmol of CO₂ per 30 min.

Spermidine synthase was partially purified by the method of Hannonen *et al.*[18] (until ammonium sulfate fractionation) from extracts of rat liver.

Cells of baker's yeast (*Saccharomyces cerevisiae*) were obtained from Oy Alko (Helsinki, Finland) and disintegrated by the method of Pösö *et al.*[4] The proteins precipitated between 0.40−0.65 saturation of ammonium sulfate were dissolved in the standard buffer, dialyzed overnight against 100 volumes of the same buffer and used as the source of spermine synthase.

Analytical methods. The activity of AMDC from *E. coli* was assayed in the presence of 10 mM MgCl₂ essentially as described earlier.[19] The activity of spermidine synthase was assayed in the presence of 0.05 mM decarbox-

ylated Ado-met and radioactive putrescine as described elsewhere.[6,20] The activity of spermine synthase was assayed in the presence of 0.05 mM decarboxylated Ado-met and radioactive spermidine.[21] Protein was measured by the method of Lowry *et al.*[22] The concentration of Ado-met and decarboxylated Ado-met was measured assuming that the molar extinction for the compounds is 15 000 at 257 nm.[23]

Incubation conditions for enzymic preparation of decarboxylated Ado-met. The large scale incubation mixture for the preparation of decarboxylated Ado-met contained, in a total volume of 9 ml, 1 mmol of Tris-HCl buffer pH 7.4 at 37 °C, 0.5 mmol of MgSO₄, 10 μmol of dithiothreitol, 5 μmol of Ado-met and the enzyme solution (980 units). Twenty five g acetone-dried cells (equivalent to 100 g of fresh bacteria) were used for the preparation of the enzyme.

The incubation was carried out at 37 °C. The pH was maintained at 7.4 by occasional additions of 1 M Tris-base. After 3.5 h the reaction was halted with 2 ml of cold 50 % trichloroacetic acid. The supernatant solution was separated by centrifugation and the precipitate was washed once with 3.5 ml of cold 10 % trichloroacetic acid.

First Dowex 50-H⁺ chromatography. The combined supernatant solutions were applied to a Dowex 50-H⁺ column (1 cm × 5 cm) previously washed with 6 M HCl and then with water until the pH approached neutrality. After the application of the sample the column was washed with 1 M HCl until the absorbance at 257 nm in the eluate was below 0.02. The decarboxylated Ado-met and Ado-met were eluted from the column with 6 M HCl. The fractions in which the absorbance at 257 nm was more than 0.2 were pooled. The volume of a typical pool was about 35 ml.

The pooled eluate from Dowex 50 column was then evaporated to dryness (under reduced pressure at temperature below 40 °C) and dissolved in 700 μl of 0.1 M HCl. This solution was used for the paper electrophoresis.

Fig. 1 shows that when AMDC purified until ammonium sulfate fractionation was used as the source of the enzyme in the enzymic preparation of decarboxylated Ado-met, much more 1 M HCl was needed in Dowex chromatography to get the absorbance below 0.02 than when more purified preparations of the enzyme (DEAE-cellulose fraction) were used. This obviously means that the ammonium sulfate fraction contained more contaminating enzyme activities capable of degrading Ado-met than the DEAE cellulose fraction.

The yield of decarboxylated Ado-met was about 20 % greater by employing the DEAE-fraction than by using (NH₄)₂SO₄ fraction as the source of AMDC (Table 1).

Preparative paper electrophoresis. Preparative paper electrophoresis by which the carboxylated Ado-met was finally separated from Ado-met
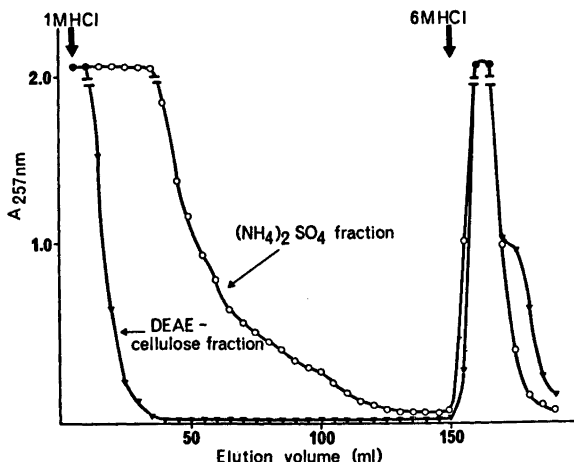
Fig. 1. Elution pattern of the incubation mixture choromatographed for the first time on Dowex 50-H$^+$. The enzymic preparation of decarboxylated Ado-met was performed as described in the text using either the ammonium sulfate fraction or the more refined DEAE-cellulose fraction as the source of the enzyme (980 units in each case). The arrows indicate the start of the elution either with 1 M HCl or 6 M HCl.

was performed as follows: Whatman No. 1 paper (20 cm × 30 cm) was loaded with 150 μl of solution from the Dowex 50-H$^+$ eluate containing both Ado-met and decarboxylated Ado-met. The paper was then subjected to electrophoresis at 300 V using 0.05 M citric acid, pH 3.6, as buffer. The running time was 2 h and the migration of both compounds was

Table 1. Effect of enzyme purification on the yield of decarboxylated Ado-met. Five μmol of Ado-met were incubated in the presence of 980 units of AMDC purified until ammonium sulfate fractionation or DEAE-cellulose chromatography as described in the text.

After incubation Ado-met and decarboxylated Ado-met were separated by paper electrophoresis and their amounts measured at 257 nm. The percentages in the parentheses indicate the relative amounts of Ado-met remaining or decarboxylated Ado-met formed from the initial 5 μmol of Ado-met.

Compound	Remaining after incubation (μmol)	
	AS-fraction [a] as enzyme	DEAE-cellulose fraction as enzyme
Ado-met	1.14 (22.7 %)	0.65 (13.0 %)
Decarboxylated Ado-met	0.70 (14.0 %)	1.63 (32.5 %)

[a] AS = ammonium sulfate.

monitored with the aid of an ultra-violet lamp at 254 nm.

The decarboxylated Ado-met fraction, migrating considerably faster than Ado-met in the paper electrophoresis, was cut into small pieces, and decarboxylated Ado-met was eluted from the paper strips with 1 M HCl overnight at 4 °C. The eluate was separated by filtration and the paper mass was washed several times with 1 M HCl.

Second Dowex 50-H$^+$ chromatography. The eluate was then applied to a Dowex 50-H$^+$ column and decarboxylated Ado-met was eluted from the column as described for the first chromatography.

The pooled 6 M HCl-eluate was evaporated to dryness (see above) and dissolved in 0.01 M HCl. The concentration of decarboxylated Ado-met was adjusted to 1 mM and the solution was stored at − 20 °C. The overall yield of the decarboxylated Ado-met was usually about 30 % from the added Ado-met (Table 1). Only one UV-absorbing spot was seen after paper electrophoresis.

The whole purification procedure is summarized in the following scheme:

Trichloroacetic acid extract → First Dowex 50-H$^+$ chromatography (wash with 1 M HCl, elute with 6 M HCl) → Evaporation of the eluate (< 40 °C) → Preparative paper electrophoresis (0.05 M citric acid pH 3.6; 300 V; 120 min) → Second Dowex 50-H$^+$ chromatography (as in the first chromatography) → Evaporation of the eluate (< 40 °C).

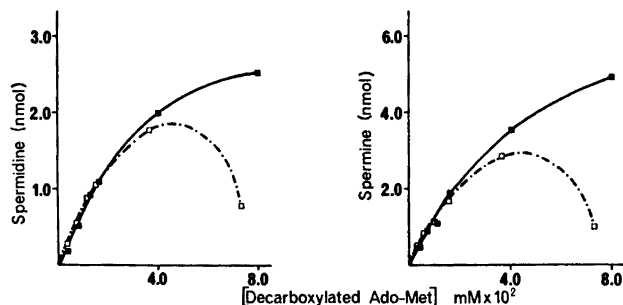*The use of decarboxylated Ado-met from different stages of the preparation as the substrate*

Fig. 2. Effect of the concentration of decarboxylated Ado-met, with or without the final chromatography on Dowex 50-H^{+}, on the activity of spermidine or spermine synthases. Decarboxylated Ado-met was prepared as described in the text and used before (open symbols, dotted curves) or after (closed symbols, solid curves) the second chromatography on Dowex 50-H^{+} as the substrate for spermidine (left) or spermine (right) synthases.

in the synthesis of spermidine and spermine. Although only one fraction was seen after paper electrophoresis prior to the second chromatography on Dowex 50-H^{+}, it was necessary to subject the preparation to a further ion exchange chromatography prior to use. This was because the decarboxylated Ado-met without the final chromatography on Dowex 50-H^{+} caused an apparent substrate inhibition when used in spermidine and spermine synthase reactions, as illustrated in Fig. 2.

The inhibition was not due to citric acid, the latter being used as the buffer in the paper electrophoresis, since citric acid (5 mM) added directly into the incubation mixture of spermidine synthase did not inhibit the enzyme activity to any appreciable extent, and a concentration of 10 mM citric acid was only slightly inhibitory (20 %). It appears that the impurities inhibiting the synthases were derived from the paper which was used in paper electrophoresis; these impurities, however, were removed by using the second chromatography on Dowex 50-H^{+}.

DISCUSSION

There are a few reports in the literature describing either enzymic or chemical preparation of decarboxylated Ado-met. The main difficulty in the preparation of pure decarboxylated Ado-met is to separate the decarboxylated compound from Ado-met, especially when enzymic methods have been employed. A separation of the two compounds has been achieved with chromatography on XE-64 columns [23] or employing HCl gradient elution on Dowex 50-H^{+} columns.[12] Furthermore, the heat stability of the compounds has been reported to be different so that upon heating at 100 °C Ado-met is preferentially decomposed and the remaining decarboxylated Ado-met can easily be separated from the decomposition products of Ado-met.[24] A chemical synthesis of decarboxylated Ado-met has also been described.[25]

The reaction with *E. coli* AMDC stops at about 60 − 90 % completion [1,23] thus necessitating a separation of the decarboxylated product from the substrate. Although reported in literature, we have not been able to achieve proper separation of decarboxylated Ado-met from Ado-met on XE-64 or Dowex 50-H^{+} columns by employing the conditions described.[12,23]

The chemical synthesis, albeit established, is very complicated containing 14 steps and yielding a racemic mixture that might not be suitable for use in biological systems.

The electrophoretic separation of decarboxylated Ado-met from Ado-met, which already has been used a few times before,[6,30] appears to yield relatively pure decarboxylated Ado-met with reasonable yields and is highly reproducible. As seen in the present paper there are some precautions to be taken into consideration when the preparation is performed with the aid of AMDC from *E. coli*. It is necessary to refine the decarboxylase to some extent, since without previous DEAE cellulose chromatography the enzyme appears to contain all kinds of contaminating enzyme activities including protein methylases.[26,27] Non-enzymic

methylation of macromolecules has also been reported.[28] An extensive methylase activity in the decarboxylase preparation will cause, of course, a rapid breakdown of Ado-met thus lowering the yield and seriously hampering the purification of decarboxylated Ado-met (Fig. 1).

The main function of decarboxylated Ado-met in mammalian tissues appears to be to serve as the precursor of spermidine and spermine molecules, a reaction catalyzed by two specific propylaminotransferases; spermidine and spermine synthases. In most animal tissues the rate limiting reaction in the enzymic synthesis of higher polyamines is the decarboxylation of Ado-met by AMDC.[5,14] The activities of the polyamine synthases markedly exceed that of AMDC.[15,18] Furthermore, the affinity of both spermidine and spermine synthase for decarboxylated Ado-met is very high; the apparent K_m value being less than 10 μM for spermidine synthase from rat ventral prostate (Jänne and Williams-Ashman, unpublished) and 3 − 5 μM for spermine synthase from rat brain.[21]

The high total activity of the polyamine synthases in comparison with AMDC in most tissues together with remarkably high affinity of the transferases for decarboxylated Ado-met explain, at least partly, the earlier misinterpretations that only one enzyme or an inseparable enzyme complex would be responsible for the decarboxylation of Ado-met and the subsequent transfer of the propylamino group to appropriate amine acceptor to yield spermidine or spermine.[29,30] Thus the assay of spermidine and spermine synthase activities in most animal tissues is only possible by employing decarboxylated Ado-met and appropriate amine (putrescine or spermidine, respectively) as substrates.

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