EVIDENCE FOR THE PRESENCE OF PYRUVATE IN RAT LIVER *S*-ADENOSYLMETHIONINE DECARBOXYLASE

Anthony E. PEGG

Department of Physiology and Specialized Cancer Center, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033, USA

Received 26 August 1977
Revised version received 28 September 1977

1. Introduction

S-Adenosyl-L-methionine decarboxylase (EC 4.1.1.50) is an essential enzyme in the biosynthesis of spermidine in eukaryotes and in *Escherichia coli*. The decarboxylated S-adenosylmethionine produced by this enzyme acts as a propylamine donor for the conversion of putrescine into spermidine [1–3]. Bacterial *S*-adenosylmethionine decarboxylase from *E. coli* required Mg^{2+} for activity and did not contain pyridoxal phosphate out had a covalently bound pyruvate group which presumably acts as a prosthetic group [4]. The eukaryotic enzymes from a variety of animals and from yeast differ from the bacterial *S*-adenosylmethionine decarboxylase in that they did not require Mg^{2+} for activity but were strongly stimulated by putrescine [5,6]. Also, the putrescine-activated *S*-adenosylmethione decarboxylases were strongly inhibited by methylglyoxal bis(guanylhydrazone) (MGBG) at concentrations which did not affect the bacterial enzyme [7]. There has been controversy as to the nature of the cofactor of the eukaryotic putrescine-activated *S*-adenosylmethionine decarboxylases. On one side it has been reported that the enzyme can be stimulated by addition of pyridoxal phosphate [8,9] and is strongly inhibited by various reagents which react with carbonyl groups including NSD-1055 (4-bromo-3-hydroxybenzyloxyamine) [5,10]. This compound is a potent inhibitor of some pyridoxal phosphate-containing enzymes [11]. On the other hand, other laboratories were unable to reproduce the stimulation of activity by pyridoxal phosphate [12–15] and highly purified preparations of the rat liver enzyme did not show a spectrum characteristic of proteins containing pyridoxal phosphate [16]. In the present paper, evidence is presented for the presence of covalently bound pyruvate in rat liver *S*-adenosylmethionine decarboxylase.

2. Methods

Rat liver *S*-adenosylmethionine decarboxylase was purified by a modification of a published procedure starting from the livers of rats (male, Sprague-Dawley strain weighing 400–500 g) which had been treated with a single dose of MGBG 24 h before death in order to increase the amount of the enzyme present [12]. The livers were homogenized in 3 vol 25 mM Tris–HCl, pH 7.5, 0.1 mM disodium EDTA and 2.5 mM dithiothreitol. The homogenate was centrifuged at 100 000 × *g* for 1 h and the supernatant fractionated by addition of $(NH_4)_2SO_4$. The fraction of protein precipitating between 0.35 and 0.6 of saturation was dissolved in as small a volume of dialysis buffer as possible, dialyzed overnight against 25 mM potassium phosphate, pH 7.2, 2.5 mM putrescine and 2.5 mM dithiothreitol and then applied to a column (3 × 30 cm) of DEAE-cellulose equilibrated with the same buffer. The column was washed with a linear gradient of 0.1–0.3 M NaCl in the same buffer (total vol. 2 litres). Fractions of 20 ml were collected and made 70% saturated by addition of solid ammonium sulfate. The resulting protein precipitate was collected, dissolved in 30 ml 10 mM Tris–HCl, pH 7.5, 0.1 mM disodium EDTA, 2.5 mM putrescine and 2.5 mM dithiothreitol. The

enzyme was then isolated from this solution by affinity chromatography using columns of MGBG linked to Sepharose as previously described [12]. The fractions containing activity after elution with buffer containing 1 mM MGBG were concentrated to approx. vol. 1 ml by diafiltration in an ultrafiltration cell (Amicon Corp.). The solution was freed from residual MGBG by diluting to 10 ml with 10 mM Tris–HCl, pH 7.5, 2.5 mM putrescine, 100 mM NaCl and repeating this concentration and dilution five times. The enzyme was stored at a concentration of about 1 mg/ml at −70°C and lost activity at a rate of about 10% a week.

The purified enzyme was reduced by reaction with NaB^3H_4 in the following manner. The enzyme was dissolved in 0.5 M $NaHCO_3$ at a concentration of 0.2 mg/ml and 0.1 ml freshly prepared solution of NaB^3H_4 in 0.05 N NaOH was added. After 1 h at room temperature, the solution was dialyzed extensively against 5 changes of 2 litres each of 0.5 M $NaHCO_3$ and lyophylized. A portion of the resulting dried protein was hydrolyzed in 6 N HCl under reduced pressure at 110°C for 6 h. The hydrolyzate was then brought to dryness, dissolved in 0.1 ml H_2O and aliquots were subjected to thin-layer chromatography on cellulose and to paper electrophoresis. The solvent systems used were (1) *n*-propanol/conc. NH_4OH (4:1), (2) *n*-butanol/acetic acid/water (12:3:5), (3) *n*-pentanol/formic acid/water (20:20:1). The R_F values of lactate in these solvents were 0.27, 0.60 and 0.38, respectively. Paper electrophoresis was carried out at 3000 V for 1 h in a Savant FP30B apparatus on Whatman 3 MM paper in 1% $(NH_4)_2CO_3$, pH 8.9.

S-Adenosyl-L-[*carboxyl*-^{14}C]methionine (56 mCi/mmol), sodium [1-^{14}C]pyruvate (6.55 mCi/mmol), NaB^3H_4 (204.8 mCi/mmol) and DL-[1-^{14}C]lactate (8.3 mCi/mmol) were purchased from New England Nuclear Corporation, Boston, MA. Radioactivity was assayed using a Beckman LS-3133T liquid scintillation counter and the results corrected to dpm using an internal standard. Unlabelled *S*-adenosylmethionine and other biochemicals were products of the Sigma Chemical Company, St Louis, MI. MGBG was purchased from the Aldrich Chemical Co., Milwaukee, WI and CH-Sepharose 4B from Pharmacia Fine Chemicals, Uppsala, Sweden.

Table 1
Purification of rat liver *S*-adenosylmethionine decarboxylase

Step	Total protein (mg)	Total units	Spec. act. (units/mg)
Ultracentrifuged homogenate	30 146	3316	0.11[a]
$(NH_4)_2SO_4$ fractionation	11 152	3568	0.32[a]
DEAE-cellulose chromatography	318	2165	6.8
First Sepharose - MGBG eluate	4	1757	423
Second Sepharose - MGBG eluate	2	1069	685

[a] Measured in sample aliquots freed from residual MGBG by dialysis

S-Adenosylmethionine decarboxylase activity was measured as previously described [5,12] and 1 unit of enzyme activity was defined as that producing 1 nmol $^{14}CO_2$/min incubation at 37°C under the standard assay conditions. Protein in crude extracts was measured by the Lowry method using crystalline bovine serum albumin as standard [17]. In more purified extracts the protein concentration was calculated from the extinction at 280 nm

3. Results

S-Adenosylmethionine-L-methionine decarboxylase was prepared from rat liver by a slight modification of the previously published method (table 1). The final enzyme preparation had a specific activity some four times greater than that previously reported [12] although the earlier preparation appeared to be homogeneous on polyacrylamide gel electrophoresis [19]. In part, this may be due to the fact that the enzyme preparation loses activity at a rapid rate in the absence of putrescine. In agreement with earlier reports from several laboratories [2,8,16,18] the purified rat *S*-adenosylmethionine decarboxylase appeared to have approx. mol. wt 69 000 as measured by ultracentrifugation or by gel filtration. On polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [19], a single band corresponding to approx. mol. wt 32 000 was observed, suggesting that the enzyme may have two sub-units. In some preparations of the enzyme, large aggregate forms having mol. wts $> 100\,000$ were observed on sucrose gradient centrifugation. When the purified enzyme was reacted with NaB^3H_4 tritium was incorporated into the protein and enzymatic activity was irreversibly lost. This incorporation amounted to 2.9×10^6 dpm/mg protein. This radioactivity remained associated with the enzyme after passage through a gel filtration column and co-sediments exactly with the enzyme on sucrose gradients [6]. After hydrolysis of the enzyme in 6 N HCl, about 78% of the incorporated radioactivity was recovered in the dried hydrolyzate. This was dissolved in 0.01 N HCl and aliquots subjected to thin-layer chromatography in a number of solvents. A substantial proportion of the radioactivity migrated in the same place as authentic lactate (fig.1). Similarly, the majority of the incorporated radioactivity corresponded to a lactate marker on paper electrophoresis (data not shown). The presence of [^{3}H]lactate in the hydrolyzate, indicates that the enzyme must have contained pyruvate which was reduced by the NaB^3H_4. The radioactivity which was identified as lactate in this manner amounted to about 58% of that recovered after hydrolysis and corresponds to the presence of 0.8 molecules pyruvate/molecule enzyme. This might indicate the presence of pyruvate in only one of the sub-units but no evidence for such heterogeneity is at present available and the observed value may be a substantial underestimate. No correction was made for the possible loss of lactate during the hydrolysis and subsequent drying. When authentic [^{14}C]lactate was put through an identical procedure, the recovery was only about 73%. It is also possible that there is an

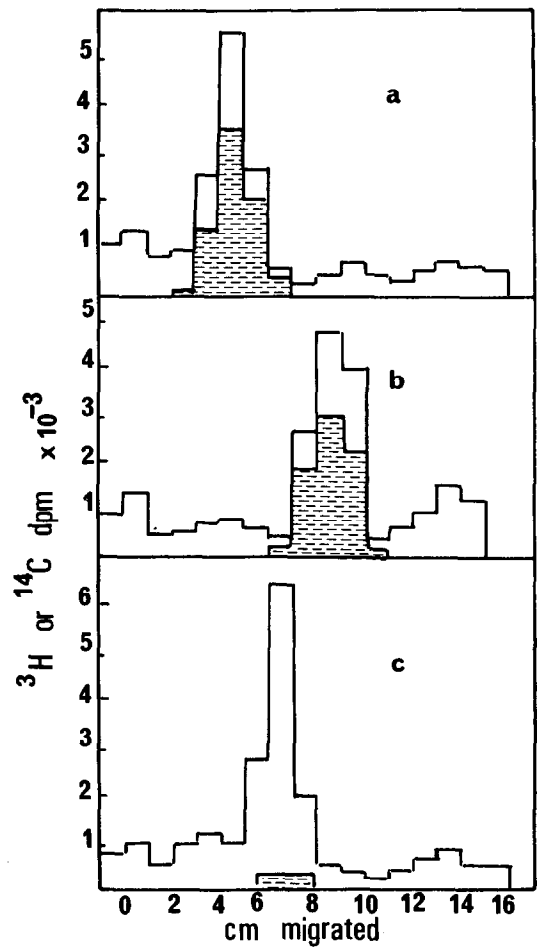

Fig.1. Thin-layer chromatography of ^{3}H-labelled products from hydrolyzate of NaB^3H_4-reduced enzyme. Aliquots of the hydrolyzate were subject to chromatography on cellulose layers using: (a) *n*-propanol/conc. NH_4OH (4:1); (b) n-butanol/acetic acid/water (12:3:5); (c) *n*-pentanol/formic acid/water (20:20:1). After drying 1 cm wide strips were scraped from the plate starting 1 cm below the origin. The cellulose from each strip was eluted with water and counted. For (a) and (b) [^{14}C]lactate was added as a marker and the ^{14}C cpm are shown in the shaded sections. For (c) the position of an unlabelled lactate marker as determined by spraying with a solution of 0.04% bromocresol green in ethanol is indicated by the shaded area.

isotope effect in reduction of the enzyme-bound pyruvate; but when [^{14}C]pyruvate was reduced with the same preparation of NaB^3H_4 lactate was obtained having a $^{14}C/^3H$ ratio within 10% of that predicted from the specific activities.

It appears unlikely that the other radioactive material which amounts to about 40% of the total incorporated and has not been identified could represent the cofactor of *S*-adenosylmethionine decarboxylase. First, part may be due to degradation of [^{3}H]lactate during the hydrolysis since increasing the time of hydrolysis to 24 h decreased the recovery of lactate and increased the amount of these other products. Second, no single component accounted for more than one-third of this radioactivity. Third, a similar peak of radioactivity to that which failed to move on thin layer chromatography was also found on reduction of other purified proteins such as ribonuclease and bovine serum albumin with the NaB^3H_4. This material might result from the non-diffusible contaminant in radioactive NaB^3H_4 preparations described by Wickner et al. [20].

4. Discussion

Recently, an abstract indicating that yeast *S*-adenosylmethionine decarboxylase also contains pyruvate has been published [21]. Therefore, despite other differences, the putrescine-activated enzymes from eukaryotes resemble the bacterial *S*-adenosylmethionine decarboxylase in having a covalently-bound pyruvate cofactor. The reason that the mammalian and yeast enzymes were so much more sensitive to NSD-1055 [5,10] remains unclear but may be related to the interaction of the putrescine activator with the prosthetic group [18]. A number of other enzymes are known in which a bound keto acid serves as cofactor including a bacterial histidine decarboxylase [22] and proline reductase [23] which also contain pyruvate and sheep liver serine–threonine dehydratase which contains α-ketobutyrate [24]. (The latter finding may have to be re-examined in the light of the recent retraction [25] that urocanase from *Pseudomonas putidae* contained α-ketobutyrate.) However, adenosylmethionine decarboxylase apparently represents the first mammalian amino acid decarboxylase having a bound pyruvate as a prosthetic group.

Acknowledgements

This research was supported by an Established Investigatorship from the American Heart Association and by grants CA-18138 and 1P30 CA19450 from the National Cancer Institute.

References

[1] Morris, D. R. and Fillingame, R. H. (1974) Annu. Rev. Biochem. 43, 303–325.
[2] Raina, A. and Jänne, J. (1975) Med. Biol. 53, 121–147.
[3] Tabor, C. W. and Tabor, H. (1976) Annu. Rev. Biochem. 45, 285–306.
[4] Wickner, R. B., Tabor, C. W. and Tabor, H. (1970) J. Biol. Chem. 245, 2132–2139.
[5] Pegg, A. E. and Williams-Ashman, H. G. (1969) J. Biol. Chem. 244, 682–693.
[6] Coppoc, G. L., Kallio, P. and Williams-Ashman, H. G. (1971) Int. J. Biochem. 2, 673–681.
[7] Williams-Ashman, H. G. and Schenone, A. (1972) Biochem. Biophys. Res. Commun. 46, 288–295.
[8] Feldman, M. J., Levy, C. C. and Russel, D. H. (1972) Biochemistry 11, 671–677.
[9] Sturman, J. A. and Kremzner, L. T. (1974) Biochim. Biophys Acta 372, 162–170.
[10] Pösö, H., Sinervirta, R., Himberg, J. J. and Jänne, J. (1975) Acta Chem. Scand. B 29, 932–936.
[11] Leinweber, F. J. (1968) Molec. Pharmacol. 4, 337–348.
[12] Pegg, A. E. (1974) Biochem. J. 141, 581–583.
[13] Williams-Ashman, H. G., Jänne, J., Coppoc, G. L., Geroch, M. E. and Schenone, A. (1972) Adv. Enz. Regul. 10, 225–245.
[14] Hannonen, P. (1976) Acta Chem. Scand. B30, 121–124.
[15] Eloranta, T. O., Kajander, E. O. and Raina, A. M. (1976) Biochem. J. 160, 287–294.
[16] Pegg, A. E. (1977) Biochem. J. 166, 81–88.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[18] Hannonen, P. (1975) Acta Chem. Scand. B 29, 295–299.
[19] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[20] Wickner, R. B., Tabor, C. W. and Tabor, H. (1971) Adv. Enzymol. 17, 647–651.
[21] Cohn, M. S., Tabor, C. W. and Tabor, H. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. 36, 714.
[22] Riley, W. D. and Snell, E. E. (1968) Biochemistry 7, 3520–3528.
[23] Hodgins, D. and Abeles, R. H. (1967) J. Biol. Chem. 242, 5158–5159.
[24] Kapke, G. and Davis, L. (1975) Biochemistry 14, 4273–4276.
[25] Phillips, A. T. and Egan, R. M. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. 36, 714.