Identification of a Pyruvoyl Residue in S-Adenosylmethionine Decarboxylase from Saccharomyces cerevisiae*

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S-Adenosylmethionine decarboxylase from *Saccharomyces cerevisiae* has been purified to homogeneity. Acid hydrolysis of NaBH₄-reduced enzyme released 2.2 mol of tritiated lactate per mol of dimeric enzyme, indicating that a pyruvate moiety is present. Inhibition of enzymatic activity by NaBH₄ reduction and by carbonyl-binding reagents indicates that this pyruvyl residue is required for the activity of the enzyme. This is the first example reported of a eukaryotic enzyme containing a covalently linked pyruvoyl residue.

The biosynthesis of spermidine in both prokaryotic and eukaryotic cells requires S-adenosylmethionine decarboxylase (Fig. 1). This enzyme has been purified extensively from *Escherichia coli*, yeast, and mammalian cells (1-3). The prokaryotic and eukaryotic enzymes differ considerably, in that the prokaryotic enzyme requires Mg²⁺, whereas the eukaryotic enzyme requires putrescine for activity (4, 5). Only the eukaryotic enzyme is inhibited by methylglyoxal bis-(guanylhydrazone) (4, 5).

Inhibition studies have indicated that both the prokaryotic and eukaryotic enzymes contain a carbonyl function (1-3). Evidence for a pyruvoyl residue in the *E. coli* S-adenosylmethionine decarboxylase was previously reported from this laboratory (1). In eukaryotes, however, the cofactor has not yet been identified (2, 3). Although earlier work had suggested the possible involvement of pyridoxal 5-phosphate (5, 6), later studies failed to find evidence for this cofactor (2).

In the present study, we have shown that S-adenosylmethionine decarboxylase from *S. cerevisiae* contains covalently linked pyruvate. Since the enzyme is inhibited by carbonyl reagents (2), it seems likely that the pyruvoyl groups are required for enzymatic activity.

**Materials and Methods**

Tritiated sodium borohydride (205 μCi/μmol), S-adenosyl-L-[carboxy-¹⁴C]methionine (54.6 μCi/μmol), Na-potassium β-3-

*¹⁴C]hydroxybutyrate (3.75 μCi/μmol), and L-sodium [U-¹³C]lactate (50 μCi/μmol) were purchased from commercial sources. Methylglyoxal bis-(guanylhydrazone) dihydrochloride monohydrate was purchased from Aldrich Chemical Co., CH-Sepharose 4B from Pharmacia Fine Chemicals, and OV-17 on 3% Supelcoport from Supelco, Inc. Aminex A-27 and materials for electrophoresis and isoelectric focusing were purchased from Bio-Rad Laboratories. A protease-deficient strain of *Saccharomyces cerevisiae* (a adh₁ leu₁ pep₁), isolated by E. W. Jones (7), was the gift of R. B. Wickner of this laboratory. *Saccharomyces carlsbergensis* was obtained from the American Type Culture Collection (ATCC 9088). Molecular weight standards were purchased from Boehringer Mannheim Corp.

**Assays**—S-Adenosylmethionine decarboxylase was assayed at 37° by a modification of the method of Pegg and Williams-Ashman (5). The assay mixture contained 100 mM Tris/HCl buffer (pH 7.2), 1.0 mM dithiothreitol, 0.1 mM EDTA, 2.5 mM putrescine dihydrochloride, 100 μg/ml of bovine serum albumin, and enzyme in a total volume of 0.4 ml. The reaction was carried out in a glass scintillation vial: to trap any ¹⁴CO₂ formed, the cap contained a 1.8-cm square of Whatman No. 3 filter paper (1), impregnated with 30 μl of 1 M hyamine hydroxide (Packard). After 5 min at 37°, 1 nmol of ¹⁴CO₂ per min.

Protein was assayed by three different methods (8-10). With the purified enzyme, the values obtained from these methods agreed within 10%. The average of the three assays was used for all calculations. Bovine serum albumin was used as a standard. Aminopyrroltransferase was assayed by the isotopic method of Bowman et al. (11). Pyridoxal 5-phosphate was assayed with *S. carlsbergensis* (12) or by a fluorescence assay (cyanhydrin derivative) (13).

**Preparation of MGBG-Sepharose**—MGBG-Sepharose was prepared as described by Pegg (5). After use, the MGBG-Sepharose was transferred to a sintered glass funnel (500-ml packed volume) and was regenerated by four 1-liter washes of 1 M NaCl, followed by two 1-liter washes of distilled water.

**Electrophoresis**—Disc polyacrylamide gel electrophoresis at pH 8.9 in 7.5% gels and sodium dodecyl sulfate-disc polyacrylamide gel electrophoresis were performed by published methods (14-16). Proteins to be subjected to electrophoresis under denaturing conditions were first placed in boiling water for 90 s (16). Isoelectric focusing was carried out according to the method supplied by Bio-Rad for their Bio-Lyte carrier ampholytes.

*¹⁴C]hydroxybutyrate (3.75 μCi/μmol), and L-sodium [U-¹³C]lactate (50 μCi/μmol) were purchased from commercial sources. Methylglyoxal bis-(guanylhydrazone).
Adenine + CH₃CH₂CHOHCH₃ → adenosine + CH₃CH₂CH₂NH₃

S-adenosylmethionine

\[ 5^+ \text{[aminopropyl]-methylsulfonio]-5'-deoxyadenosine (decarboxylated S-adenosylmethionine)} \]

Reduction of Proteins with Trinitiated Sodium Borohydride (Procedure I) - The protein samples (0.1 to 0.5 ml) were dialyzed against water and then heated in boiling water for 2 min. An equal volume of 50 mM sodium borate/boric acid (pH 8.0). The tritiated lactate formed was isolated by Dowex 1-X8 chromatography (17), and quantitated spectrophotometrically with lactate dehydrogenase-NAD+ (18). The specific radioactivity of the lactate was found to be 0.12 mCi/μmol.

The tritiated lactate, and tritiated α-hydroxybutyrate made similarly by reduction of α-keto butyrate, were used as standards for chromatographic experiments.

Fig. 1. The biosynthesis of spermidine: S-adenosylmethionine decarboxylase (Reaction 1) and aminopropyltransferase (Reaction 2).

Determination of the Specific Radioactivity of Tritiated Sodium Borohydride - Five microliters of 1 M NaBH₄ were added to 0.1 ml of a solution of 50 mM pyruvate in 50 mM sodium borate/boric acid (pH 8.0). The tritiated lactate formed was isolated by Dowex 1-X8 chromatography (17), and quantitated spectrophotometrically with lactate dehydrogenase-NAD+ (18). The specific radioactivity of the lactate was found to be 0.12 mCi/μmol.

The tritiated lactate, and tritiated α-hydroxybutyrate made similarly by reduction of α-keto butyrate, were used as standards for chromatographic experiments.

Reduction of Proteins with Trinitiated Sodium Borohydride (Procedure I) - The protein samples (0.1 to 0.5 ml) were dialyzed against 50 mM sodium borate/boric acid (pH 8.0) containing 0.1 mM EDTA and 1.0 mM dithiothreitol. NaBH₄ (1 M) was added to a final concentration of 50 mM, and the mixture was periodically shaken during a 1-1.5 hour incubation at room temperature. The pH remained between 8.0 and 9.0 during the reduction. The sample was then applied to a Sephadex G-10 column (0.9 x 30 cm) equilibrated with 50 mM NaHCO₃ (pH 8.0). The column was washed with 50 mM NaHCO₃ fractions containing both radioactivity and protein (assayed by absorption at 280 nm) were pooled and hydrolyzed in 6 N HCl (metal-free Utrex, J. T. Baker) at 110°C under vacuum for 24 h. The hydrolysate was adjusted to pH 8.0 with 4 N NaOH and lyophilized to dryness. The tritiated material was extracted from the residual salts with methanol; the methanol was then evaporated under vacuum. The residue containing the tritiated material was stored at -20°C. No radioactivity was found in the recovered methanol.

Reduction of Proteins with Trinitiated Sodium Borohydride (Procedure II) - The sample (0.1 to 0.5 ml) was dialyzed against water and then heated in boiling water for 2 min. An equal volume of 50 mM NaHCO₃ (pH 8.0) containing trypsin (0.5 mg/ml) was added, and the mixture was incubated at 37°C for 16 h. An equal volume of 100 mM NaHCO₃ (pH 8.0) was then added, and the sample was reduced with NaBH₄. Following the reduction, a known quantity of [3H]lactate, which served as an internal standard, was added to the sample, which was then subjected to acid hydrolysis. Conditions for NaBH₄ reduction, hydrolysis, and subsequent preparation of the sample were identical to those described in the preceding section.

Characterization of Lactate by Ammonex A-27 Chromatography - Chromatography was performed at 54°C with the anion exchanger Ammonex A-27 (0.9 x 10 cm column). The column was equilibrated and developed with either pH 5.4 buffer (0.4 M sodium acetate containing 0.08 M acetic acid) or pH 3.6 buffer (0.4 M acetic acid titrated to pH 3.6 with 0.4 M sodium acetate). Samples were applied after being dissolved in 1.0 ml of pH 5.4 or pH 3.6 buffer. The eluate was collected from the bottom of the column with a fraction collector at a flow rate of 1.0 ml/min; the pump system of an amino acid analyzer was used.

Gas Chromatography - Samples were derivatized by reaction with a solution of 100 mM phenylboronic acid and 10% trifluoroacetic acid in ethyl acetate (19). The phenylboronate derivatives were chromatographed on OV-17 (3% on Supelcoport, 6-foot column, 2-mm column internal diameter) at 120°C in a Finnigan model 9500 gas chromatograph equipped with a flame ionization detector, stream splitter, and tritium monitor (Nuclear Chicago Corp.). Flow rates were: argon carrier, 56 ml/min; for the tritium monitor; quench gas (propane), 6 ml/min; and hydrogen carrier, 4 ml/min. The flow rate of argon in the tritium monitor branch of the stream splitter was 40 ml/min.

RESULTS

Purification of S-Adenosylmethionine Decarboxylase - S-Adenosylmethionine decarboxylase was purified 12,000-fold from homogeneity from Saccharomyces cerevisiae with the use of the MGBG-Sepharose affinity column introduced by Pegg (3) for the final steps (Table I). The details of the purification, which differs from the previously published one (2), are given in the miniprint supplement.²

Electrophoresis (pH 8.9) of 25 μg of enzyme on nondenaturing polyacrylamide gels and 10 μg in the presence of sodium dodecyl sulfate each gave a single band upon staining with Coomassie blue (20). Enzymatic activity of slices from the nondenaturing gel coincided with the protein band (Fig. 1). Sedimentation velocity and equilibrium data also indicated that the purified enzyme was homogeneous. The purified enzyme contained no detectable aminopropyltransferase (i.e., <0.1%).

Properties - The specific activity of our most purified preparation was 106 nmol of CO₂ released/mg/min. To compare this preparation with that described by Pöösö et al. (2), the specific activity was also measured with their assay, which used a higher concentration of substrate. A value of 610 nmol of CO₂ released/mg/min was found for our preparation, compared with their value of 137 (2). The value for our yeast preparation was 5 times that found for the Escherichia coli enzyme (1), using comparable conditions for each assay (i.e., the same concentration of S-adenosylmethionine).

Storage of the purified enzyme at 2-4°C in the buffer used for the final dialysis (see miniprint) resulted in no loss of activity over a 3-month period. Storage at -70°C inactivated the enzyme. The preparation rapidly lost activity at 37°C unless protected by the addition of 100 μg/ml of bovine serum albumin. Treatment of the enzyme with 50 mM sodium borohydride completely inactivated the enzyme in agreement with Pöösö et al. (2) who showed that the enzyme is inhibited by reagents which react with carbonyl groups.

The enzyme had a sedimentation coefficient of 5.44 S and the molecular weight was 84,000 (determined by sedimentation equilibrium ultracentrifugation) (21). A molecular weight of 88,000 was determined by chromatography on Sephadex G-200 (22). A single band with a molecular weight of 41,000 was found after electrophoresis in sodium dodecyl sulfate gels. An isoelectric point of 5.3 was obtained from narrow range (pH 5 to 7) isoelectric focusing.

Evidence for the Lack of Pyridoxal 5-Phosphate - Since pyridoxal 5-phosphate is the usual co-factor for this type of enzymatic catalysis, we looked for evidence of pyridoxal 5-phosphate in our pure enzyme. The spectrum of the purified enzyme (Fig. 2) contains no chromophores in the region where pyridoxal phosphate would normally absorb (340 to 450 nm), although a low absorption is present in the region 300 to 340 nm; this has previously been observed in several other enzymes with cofactors of either the carbonyl type or dehydroalanine, or with metal ions (reviewed in Ref. 23).

Pyridoxal 5-phosphate could not be detected in 1 to 4 μg of the protein in the tritium monitor branch of the stream splitter was 40 ml/min.

² Portions of this paper (including Fig. 1s and Tables Is and IIs) are presented in miniprint following the references. Full size photocopies are available from The Journal of Biological Chemistry, 9050 Rockville Pike, Bethesda, Md. 20014. Request Document No. 77M-854, cite authors, and include a check or money order for $1.00 per set of photocopies.
purified enzyme by the microbiological assay using Saccharomyces carlsbergensis, or by a fluorescence assay of the cyanohydrin derivative (less than 0.05 mol of pyridoxal phosphate per mol of enzyme subunit would have been detected).

Identification of Pyruvate—The best proof for the presence of pyruvate in the enzyme was the recovery of tritiated lactate from the acid hydrolysate of the purified enzyme after it had been reduced by NaB³H₃ (Procedure I). Thirty-eight per cent (1500 cpm) of the counts applied to Aminex A-27 at pH 5.4 co-chromatographed with a standard of [¹⁴C]lactate (eluting at 19 to 21 ml) (Fig. 3A). [³H]lactate, prepared by reduction of pyruvate with NaB³H₃, also chromatographed with the lactate standard (Fig. 3B). We could not recover [³H]lactate from hydrolysates of NaB³H₃-reduced bovine serum albumin (Fig. 3C), ribonuclease A, trypsin, or lactate dehydrogenase (data not shown). The hydrolysates of each of these proteins and purified S-adenosylmethionine decarboxylase contained some tritiated material which was not retained by the column. A possible explanation of this incorporation of tritium is the cleavage of peptide bonds by sodium borohydride with the resultant formation of amino alcohols (25, 26).

The [³H]lactate peak obtained from the experiment presented in Fig. 3A was rechromatographed at pH 3.6 since lactate separates from α-hydroxybutyrate and β-hydroxybutyrate at this pH (but not at pH 5.4). This material (i.e. from the purified enzyme) co-chromatographed with [¹⁴C]lactate (Fig. 4).

Evidence for the formation of [³H]lactate upon reduction of the enzyme was also obtained by identification of the phenylboronate derivative of the lactate phenylboronate. When the sample was assayed on OV-17 using a gas chromatograph coupled to a tritium monitor, a tritium peak appeared at 260 s, which is the known response time for the phenylboronate derivative of lactate (Fig. 5). The material migrating with the solvent front is presumably due to the tritium that was incorporated into the enzyme, but not as lactate (Fig. 3A).

Determination of the Stoichiometry of Pyruvate in S-Adenosylmethionine Decarboxylase—The characterization of lactate from the purified enzyme indicates that pyruvate was present in the enzyme at the time of the reduction. A yield of 2.1 nmol of tritiated lactate was obtained from the 120 μg (2.9 nmol of the subunit, molecular weight 41,000) of S-adenosylmethionine decarboxylase. After correction for an 80% recovery of standard tritiated lactate (determined by samples of pyruvate carried through the procedure), we concluded that

0.91 nmol of tritium was incorporated into pyruvate per nmol of subunit of S-adenosylmethionine decarboxylase (Table IIa).

To ensure optimum reduction of all carboxyl groups, the reduction was carried out on another sample of the enzyme (175 μg) that had been previously denatured in boiling water and digested with trypsin (Procedure II). Also, to allow a direct calculation of recovery, 3,400 cpm of [¹⁴C]lactate was added to the reduced enzyme before hydrolysis.

The preparation of the material by Procedure II was immediately followed by chromatography on Aminex A-27 at pH 3.6 to purify the tritiated lactate. All tritium co-chromatographing with the [¹⁴C]lactate was pooled, and the ratio of tritium to [¹⁴C]lactate was measured. It was calculated that 4.9 nmol of lactate were recovered from the original 4.3 nmol...
of enzyme subunit, indicating that 1.1 nmol of pyruvate were present per nmol of enzyme subunit (Table II).

The [3H]lactate formed after reduction of S-adenosylmethionine decarboxylase by NaB4H4, remained associated with the protein after dialysis or Sephadex G-25 chromatography. All of the radioactivity associated with the protein after dialysis was precipitated by the addition of 10% trichloroacetic acid (in the presence of bovine serum albumin as carrier). The pellet, after being boiled in 0.2 N NaOH, was reprecipitated with 10% trichloroacetic acid, and again all of the radioactivity was precipitated. We conclude that the lactate (and therefore pyruvate) is covalently linked to the enzyme.

DISCUSSION

In this study, we have identified [3H]lactate from acid hydrolysates of yeast S-adenosylmethionine decarboxylase reduced with NaB4H4, indicating the presence of covalently bound pyruvate in the enzyme before reduction. Two separate determinations indicate the presence of 1 pyruvoyl residue per subunit of dimeric enzyme. As this enzyme is inhibited by NaBH4 and other reagents which attack the carbonyl group (2), it seems likely that these pyruvoyl residues are required for enzymatic activity. Pyridoxal 5-phosphate was not present in the enzyme.

This is the first eukaryotic enzyme which has been shown to contain a pyruvoyl residue, although it has been found in the S-adenosylmethionine decarboxylase of Escherichia coli (1), as well as in three other prokaryotic enzymes (from Lactobacillus 30a, Clostridium sticklandii, and E. coli (27-30), reviewed in Ref. 31). It is of particular interest that pyruvate has been found in the S-adenosylmethionine decarboxylase from both yeast and E. coli, since these two enzymes appear different otherwise: the yeast enzyme has a requirement for putrescine while the E. coli enzyme needs Mg2+ for activity. Furthermore, the yeast enzyme contains one pyruvate per subunit (molecular weight, 41,000), while the E. coli enzyme has one pyruvate per molecule of native enzyme (molecular weight 113,000, subunit molecular weight 15,000) (1).

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REFERENCES

Identification of a Pyruvoyl Residue in Yeast S-Adenosylmethionine Decarboxylase

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Preparation of crude extract — A culture, a 50 ml flask, was grown in a 500 ml fermentor with airflow 1500 ml/min. The cells were harvested by centrifugation at 9000 x g. The pellets were suspended in buffer A (50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1 M NaCl, 0.1 M EDTA) at a ratio of 1:10 (v/v). The suspension was homogenized by ten 30-s bursts with a Virtis homogenizer. The homogenate was centrifuged at 15,000 g for 30 minutes, and the supernatant fluid was collected (Procedure I).

Pyruvoyl substrate — 4 mg of 1-pyrroline methyl ester in buffer A was added to 3 ml of the crude extract. The homogenate was divided into two equal parts to be used over a period of 15 minutes with constant stirring. Buffer A was maintained for 20 minutes, the reaction mixture was then centrifuged at 15,000 g for 30 minutes, and the supernatant fluid was collected (Procedure II).

Pyruvoyl-sulfadiazine chromatography — Fraction II was applied to a 2.5 x 35 cm column of aminoephelous cellulose with 100 ml buffer A and eluted with a solution of 50 ml of buffer A containing 50 ml of 1 M NaCl. The activity was detected by monitoring the absorbance at 280 nm. The active fractions were pooled and concentrated to 10 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure III).

Pyruvoyl-sulfadiazine II — The active fractions were pooled and concentrated to 2 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure IV).

Pyruvoyl-sulfadiazine III — The active fractions were pooled and concentrated to 1 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure V).

Pyruvoyl-sulfadiazine IV — The active fractions were pooled and concentrated to 0.5 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure VI).

Pyruvoyl-sulfadiazine V — The active fractions were pooled and concentrated to 0.2 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure VII).

Pyruvoyl-sulfadiazine VI — The active fractions were pooled and concentrated to 0.1 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure VIII).

Pyruvoyl-sulfadiazine VII — The active fractions were pooled and concentrated to 0.05 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure IX).

Pyruvoyl-sulfadiazine VIII — The active fractions were pooled and concentrated to 0.025 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure X).

Pyruvoyl-sulfadiazine IX — The active fractions were pooled and concentrated to 0.01 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure XI).

Pyruvoyl-sulfadiazine X — The active fractions were pooled and concentrated to 0.005 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure XII).

Pyruvoyl-sulfadiazine XI — The active fractions were pooled and concentrated to 0.0025 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure XIII).

Pyruvoyl-sulfadiazine XII — The active fractions were pooled and concentrated to 0.001 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure XIV).

Pyruvoyl-sulfadiazine XIII — The active fractions were pooled and concentrated to 0.0005 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure XV).

Pyruvoyl-sulfadiazine XIV — The active fractions were pooled and concentrated to 0.00025 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure XVI).

Pyruvoyl-sulfadiazine XV — The active fractions were pooled and concentrated to 0.0001 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure XVII).

Pyruvoyl-sulfadiazine XVI — The active fractions were pooled and concentrated to 0.00005 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure XVIII).

Pyruvoyl-sulfadiazine XVII — The active fractions were pooled and concentrated to 0.000025 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure XIX).

Pyruvoyl-sulfadiazine XVIII — The active fractions were pooled and concentrated to 0.00001 ml with an Amicon concentrator containing a PM-10 membrane. The concentrated sample was dialyzed against 1 liter of buffer A overnight (Procedure XX).
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