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Enzymatic Synthesis of *S*-Adenosyl-L-Methionine from L-Methionine and ATP

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Abstract

S-Adenosyl-L-methionine (SAM) has been synthesized on 7-mmol scale from L-methionine and ATP. The ATP was generated *in situ* from AMP, and the synthesis of SAM was catalyzed by the enzyme ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6). Although substantial effort was required to obtain even small quantities of enzyme, it has good stability once isolated and immobilized. The SAM produced by this procedure contains 89% of the (−) diasterioisomer and 11% of the (+) diasterioisomer.

Index Entries: *S*-Adenosyl-L-methionine; ATP: L-methionine *S*-adenosyltransferase, immobilized; APT, generation from AMP; catechol-*O*-methyltransferase.

Introduction

S-Adenosyl-L-methionine [(−)-SAM] is a cofactor in various enzyme-catalyzed reactions, of which transmethylations are the most significant (*1*). SAM is usually obtained by isolation from yeast grown in media supplemented with L-methionine (*2*). Enzymatic methods for small-scale (ca. 100 μmol) syntheses of SAM from ATP and L-methionine (*3*) as well as for preparation of radioactively labeled mate-

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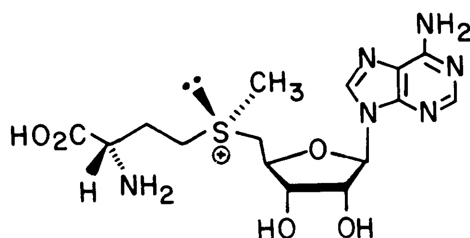


(−)-SAM

rial (*4*) have been described. A chemical synthesis that is based on methylation of *S*-adenosyl-L-homocysteine (SAH) has also been described (*5*). Although SAM is commercially available, it is too expensive to be used as a stoichiometric reagent on any significant scale; the current price of SAM is $140/g (~$60,000/mol) for 70% pure material (Sigma).

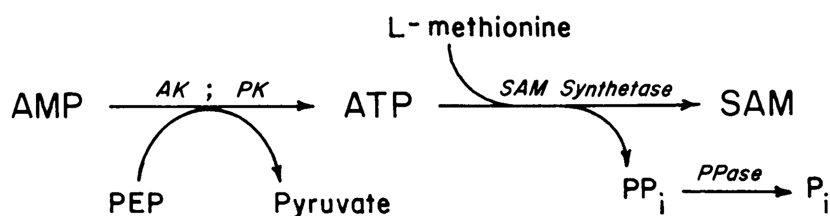
The inherent instability of SAM makes chemical synthesis of this cofactor difficult. SAM is labile to mildly alkaline conditions (with decomposition resulting from cleavage of the glycosidic bond); in acidic solutions it decomposes and gives methylthioadenosine and homoserine (*6*). Moreover, the (−) diastereoisomer, which is the substrate in enzyme-catalyzed reactions (*S*-configuration at sulfur), epimerizes at sulfur to give the inactive diastereoisomer [(+)-SAM]. This epimerization reaction has a rate constant of $8.1 \times 10^{-6}\ s^{-1}$ at pH 7.5 and 37 °C (*7*).

As part of our program in the application of enzymatic catalysis to organic synthesis, we have explored enzyme-catalyzed synthesis of SAM from ATP and L-methionine (Scheme I). The objective of this work was to determine whether SAM could be synthesized (or regenerated) enzymatically in vitro sufficiently readily that it could be considered a practical reagent for use in large-scale enzyme-catalyzed organic synthesis.

Materials and Methods

General

Adenylate kinase from rabbit muscle (EC 2.7.4.3, 2000 U/mg), pyruvate kinase from rabbit muscle (EC 2.7.1.40, 500 U/mg), inorganic pyrophosphatase from



Scheme I. Enzymatic synthesis of SAM.

Bakers' yeast (EC 3.5.1.1, 500 U/mg), and catechol-*O*-methyl transferase from porcine liver (EC 2.1.1.6, 0.03 U/mg), were purchased from Sigma Co. Samples of *S*-adenosylmethionine were obtained from Sigma (as the Cl^- salt) and from Boeringer Mannheim (as the HSO_4^- salt). Dowex AGl-X8 was from Bio-Rad, DEAE-cellulose (DE-52) from Whatman and Amberlite IRC-50 from Sigma. Fresh Bakers' yeast was bought from a local supplier. Phosphoenol pyruvate potassium salt (PEP^-K^+) (*8*) and PAN (*9*) were prepared according to published procedures. All other chemicals were of reagent grade from Sigma or Aldrich.

Spectrophotometric measurements were performed using a Perkin-Elmer Model 552 spectrophotometer equipped with a thermostated cell. TLC was conducted on Merck pre-coated silica gel plates, type 60 F254. HPLC was performed on a Waters HPLC system using Resolve C_{18} reverse-phase column (3.9 × 15 cm, 5-μm particle). ^{1}H NMR spectra were obtained on a Bruker 300 MHz instrument. Protein concentrations were determined by the method of Christian and Warburg (*10*).

Assays

Assays of the following enzymes and biochemicals were taken from Bergmeyer (*11*): Adenylate kinase (pp. 486–487), pyruvate kinase (pp. 509–510), inorganic pyrophosphatase (p. 508), phosphoenolpyruvate and pyruvate (pp. 1446–1447), and ATP (pp. 2097–2098).

Assay of SAM-Synthetase. The assay mixture contained (final volume 1 mL, pH 7.8): 100 m*M* Tris/HCl, 200 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* DTT, 5 m*M* ATP, 5 m*M* L-methionine, and 10–50 μL of enzyme solution. A blank was prepared simultaneously without methionine and both were incubated at 30°C. The reaction was stopped by the addition of 1 mL of cold 6% $HClO_4$. The mixture was centrifuged and the supernatant was neutralized with 1*N* KOH (~200 μL). The neutralized sample and the blank were applied to the Dowex AGl-X8 columns (1 × 3 cm) at pH 7–8, and both were washed with water (10 mL). The eluent from the sample was measured spectrophotometrically at 259 nm, using the blank as reference (the molar extinction coefficient of SAM at 259 nm is 15,400). When the activity of the immobilized enzyme was measured, the incubated mixtures were applied to the column after separation from the gel by centrifugation.

Isolation of SAM-Synthetase.† The isolation procedure was performed according to Chiang and Cantoni (*12*) with several modifications. Fresh Bakers' yeast was manually ground in a mortar and air-dried between two sheets of Whatman paper (No. 1) at room temperature for 6 d. The dried yeast (400 g, in 50-g portions) was ground in a Waring blender for 2 min in the presence of dry ice (~ 1 g dry ice/g yeast). The ground yeast was suspended in 1.2 L of 0.067*M* K_2HPO_4 containing 2.4 g of L-methionine. The mixture was stirred for 4 h at 37°C and centrifuged at 2400*g* for 30 min. The supernatant (640 mL) was transferred to a 2-L beaker and cooled in an ethanol–dry ice bath to 0°C. Precooled acetone (−25°C, 202 mL) was added dropwise over 45 min to the stirred solution, which

†All operations were performed at 0–4°C unless otherwise mentioned.

was gradually cooled to −8°C. The precipitated proteins were separated by centrifugation at 2500*g* for 10 min at −8°C. The supernatant (750 mL) was subjected to another acetone precipitation (246 mL). The precipitate was collected, suspended in 200 mL of 40 m*M* phosphate buffer (pH 6.8) containing 5 m*M* 2-mercaptoethanol and 0.5 m*M* EDTA. The solution was dialized overnight against 4 L of the same buffer and then centrifuged. The supernatant (300 mL) was divided into two portions and each was treated with 120 mL of DE-52 that had been preequilibrated with the same buffer. After stirring for 10 min at 0°C, each batch was filtered and the ion exchange resin was resuspended three times in 100-mL portions of the same buffer solution containing 5 m*M* $(NH_4)_2SO_4$. The adsorbed enzyme was eluted from the resin using a buffer containing 105 m*M* $(NH_4)_2SO_4$ (3 × 75 mL). The pH of the combined eluents (480 mL) was adjusted to 7.3 and a solution of $(NH_4)_2SO_4$ (368 g) in water (480 mL) was added. The mixture was stirred for 20 min and centrifuged at 15,000*g* for 10 min, suspended in the phosphate buffer (30 mL) and dialized against 1800 mL of the same buffer. The protein solution was applied to an equilibrated DE-52 column (2.5 × 30 cm). Phosphate buffer (100 mL, 40 m*M*, pH 6.8 containing 5 m*M* 2-mercaptoethanol and 0.5 m*M* EDTA) was passed through the column until the first protein-containing peak eluted. An ammonium sulfate linear gradient in the same buffer (pH 6.8, 30 m*M*, 400 m*M*, 500 mL) was applied. The fractions containing the two isozymes of SAM-synthetase were collected and fractionated by the ammonium sulfate procedure as described above. The enzyme precipitate was dissolved in 20 mL of 40 m*M* phosphate buffer containing 5 m*M* DTT and 0.5 m*M* EDTA (pH 6.8) and dialized overnight against 2 L of the same buffer. The solution was concentrated to a volume of 5 mL using an Amicon cell equipped with a PM-10 membrane. This solution contained 8 U of SAM-synthetase having specific activity 0.05 U/mg protein.

Immobilization of Enzymes. The enzymes were immobilized by the polymerization condensation method (*9*). Inorganic pyrophosphatase (200 U, 0.4 mg of protein) was immobilized on 1 g of PAN-1000 to afford 80 U of immobilized enzyme (40% yield). Adenylate kinase (498 U, 0.25 mg of protein) and pyruvate kinase (250 U, 0.5 mg of protein) were coimmobilized on 1.5 g of PAN-1000. The gel obtained contained 190 U of adenylate kinase (39% yield) and 68 U of pyruvate kinase (27% yield).

Immobilization of SAM-Synthetase. To the concentrated enzyme solution (160 mg of protein, 5 mL) was added 5 mL of Hepes buffer (0.3 *M*, pH 7.5) containing 2 m*M* ATP, 2 m*M* L-methionine, 10 m*M* $MgCl_2$, and 200 m*M* KCl. PAN-1000 (7 g) was dissolved with vigorous stirring in 18 mL of 0.3 *M* Hepes buffer (pH 7.5) and the enzyme solution was added. After 30 s 0.5*M* triethylene tetramine (TET) solution (5.3 mL) was added. A gel formed within 1 min. It was kept at room temperature under argon for 30 min and then ground and washed as described previously (*9*). The total activity obtained was 3 U (38% yield on immobilization).

S-Adenosyl-L-Methionine. To a 2-L flask (modified at the bottom to accept a pH electrode) was added 850 mL of a solution containing AMP (20 mmol), ATP (0.5 mmol), phosphoenol pyruvate (40 mmol, 90% pure, monopotassium salt) (*8*),

$MgCl_2$ (10 mmol), KCl (150 mmol), L-methionine (20 mmol), DTT (1 mmol), and SAM^+Cl^- (30 μmol) (*30*). The solution was deoxygenated by a stream of argon introduced into the solution. The PAN-immobilized inorganic pyrophosphatase (40 U), SAM synthetase (3 U), and the coimmobilized adenylate kinase and pyruvate kinase (85 and 34 U, respectively) were added. The reaction was conducted at pH 6.8† at room temperature and monitored by assays for ATP, SAM, and phosphoenol pyruvate. After 6 d, 7.8 mmol of SAM had been formed. The gel was allowed to settle and the solution was decanted. The activities of the recovered enzymes (as percent of the starting activities) were: SAM synthetase, ~ 40; pyrophosphatase, 93; adenylate kinase, 85; pyruvate kinase, 88. Trichloroacetic acid (100 mL) was added and the mixture was filtered. The filtrate was treated with 2 L of a solution containing 1.5% w : w Reineckate ammonium salt (30 g of salt in 2 L of 5% w : v trichloroacetic acid) (*13*). The mixture obtained was left overnight at 2°C to allow complete precipitation. The pink precipitate was filtered at 2°C and dried *in vacuo*. The solid was treated with 100 mL of methyl ethyl ketone, the undissolved material was separated by centrifugation and discarded, and the dark burgundy-red solution was extracted with 150 mL of 0.1*N* H_2SO_4. The acid phase was washed with methyl ethyl ketone (50 mL portions) until no traces of pink color were visible in the aqueous phase. The water phase was extracted with ether, the pH of the solution was adjusted to pH 5.5 with 2*N* KOH and dissolved ether was removed using a rotary evaporator. Lyophilization of the solution gave a greenish powder (6.5 g). UV analysis showed that the solid contained 6.3 mmol of SAM (48% by weight, calculated as $SAM^+HSO_4^-$).

A sample of this solid was further purified by using a weakly acidic cation exchange resin (*14*). A column (1.5 × 15 cm) was packed with Amberlite IRC-50, washed with 1*N* H_2SO_4 (100 mL) and water (~400 mL). A sample of the isolated SAM (120 mg) in 10 mL of H_2O was applied to the column, and the column was washed with water (300 mL). The elution was continued with 0.1*N* H_2SO_4 and 5-mL fractions were collected. The fractions showing absorbance at 259 nm (numbers 14–24) were collected and the pH was adjusted to 3.5 with freshly prepared $BaCO_3$. The precipitate was removed by centrifugation. This solution was analyzed by TLC, using a silica gel plate which was developed in *n*-BuOH : HOAc · H_2O (60 : 15 : 25). The synthetic SAM showed the same R_f value as an authentic sample (R_f, 0.11) and gave a positive test with ninhydrin. HPLC analysis was performed using a C_{18} column. The mobile phase was 5% aqueous ethanol containing 4 m*M* Pic B-7 (1-heptane sulfonic acid), 1 m*M* EDTA, 240 n*M* acetic acid with pH adjusted to 3.8 with NaOH (flow rate 2.0 mL/min). The purified $SAM^+HSO_4^-$ had the same retention time as that of an authentic sample (R_t, 4.7 min).

The solution containing the purified SAM was concentrated by evaporation and a sample (~5 mg) was subjectd to ^1H NMR analysis. It was twice lyophylized from ^2H_2O and the pD was adjusted to 3.4 with ^2HCl. The ^1H NMR was measured vs external sodium 3-(trimethylsilyl)tetradeuteriopropionate (TSP): δ 2.40

†SAM is stable for prolonged periods of time only between pH 3.5 and 6.0. The pH optimum for SAM synthetase is 7.6. At the operating pH of 6.8, the enzyme has a rate of 0.8 of that at pH 7.6, but the product is more stable once formed.

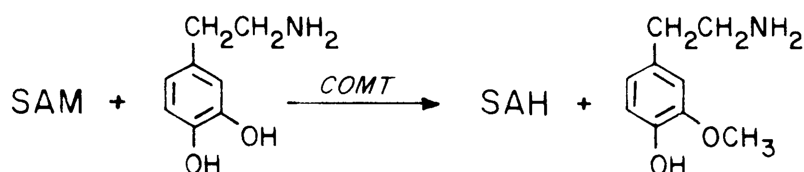
(m, 2Hβ), 3.02 and 2.98 [2 s, ratio 8 : 1, SCH_3 of (−) and (+) diastereoisomers, respectively], 3.4–4.1 (complex of *m*, 2Hα, 2Hγ, $2H_{5'}$), 4.66 (*m*, $H_{4'}$), 4.89 (*t*, $H_{3'}$, $J_{3'2'}$ = 5.1 Hz), 6.18 (*d*, $H_{1'}$, $J_{1'2'}$ = 4.1 Hz), and 8.46 (*s*, H_2, and H_8). These values are in accord with those given in ref. *15*.

Methylation of Dopamine. An incubation mixture contained the following components (total volume, 1 mL): 50 μ*M* phosphate buffer (pH 8.0), 10 μmol $MgCl_2$, 0.3 μmol dopamine, and the synthetic $SAM^+HSO_4^-$ (1.2 mg, after chromatography on Amberlite). An aliquot (2 μL) of this solution was analyzed by HPLC using the conditions described above. Dopamine eluted with R_t 4.3 min and SAM with R_t 4.7 min. The reaction was initiated by addition of COMT (0.005 U) and the mixture was incubated at 30°C for 1 h. The reaction was stopped with 6% $HClO_4$, neutralized and centrifuged. A 10-μL aliquot of the supernatant was analyzed by HPLC and revealed two new peaks at R_t 3.35 min and R_t 10.2 min. These peaks correspond to SAH and 3-methoxytyramine respectively.

Results and Discussion

Formation of SAM in vivo from ATP and L-methionine is catalyzed by the enzyme ATP: L-methionine *S*-adenosyltransferase (SAM-synthetase, EC 2.5.1.6). This enzyme has been isolated from mammalian (*16–19*) and microbial (*20–23*) sources. The SAM-synthetase employed in our work was isolated from Bakers' yeast. This SAM synthetase, an allosteric enzyme with two subunits, is composed of two isozymes. This isolation proved to be the major difficulty in the synthesis of SAM. The isolation is not intrinsically difficult or lengthy (~4 d) and the enzyme is quite stable and easily manipulated once immobilized, but it is present in yeast in low concentrations, and large quantities of yeast yield only small quantities of enzyme. The enzyme was partially purified to a specific activity of 0.05 U/mg of protein and was immobilized in PAN-gel (*9*). The ATP used in the synthesis was generated *in situ* from AMP (*8*) using phosphoenol pyruvate (PEP) as the source of phosphate and pyruvate kinase (PK) and adenylate kinase (AK) as catalysts. Since SAM-synthetase seemed to be contaminated (even after five steps of purification) with one or more enzymes having ATPase activity, the ATP regeneration system was included to regenerate unproductively hydrolyzed ATP. Inorganic pyrophosphatase (PPase) was added to drive the synthesis of SAM by hydrolyzing pyrophosphate (Scheme I).

A demonstration synthesis carried out with this enzymatic system afforded 7.8 mmol of SAM. This material was isolated as $SAM^+HSO_4^-$ using a procedure based on purification of an intermediate Reineckate salt. The product thus obtained had a purity of 48%. Further purification wa performed on a 0.3-mmol scale by column chromatography (Amberlite IRC-50). The synthetic material showed TLC, HPLC, and ^1H NMR characteristics indistinguishable from those of authentic samples. It methylated dopamine to give 3-methoxytyramine and SAH when incubated with catechol-*O*-methyltransferase (COMT; Scheme II) (*24*). The synthetic $SAM^+HSO_4^-$ showed a major singlet at δ 3.02 in the ^1H NMR [corresponding to the sulfonium methyl group of (−)SAM] and a minor singlet at δ 2.98



Scheme II. Methylation of dopamine.

with lower intensity [11% of the major one; (+)-SAM] (*15*). Higher proportions of (+)-SAM are found in commercial samples (18–32%).

Conclusions

The procedure described here establishes that synthesis of SAM on a scale of 5–10 mmol is practical. This procedure should be useful in synthetizing isotopically labeled SAM. It may also be useful in preparations of (−)-SAM containing relatively small amounts of (+)-SAM. It is, however, clear that the present difficulties in obtaining SAM-synthetase are such that *in situ* use and regeneration of SAM as part of procedures for large-scale enzyme-catalyzed organic synthesis is not practical. Although immobilized SAM-synthetase is relatively sturdy, and it could probably be generated in quantities by recombinant DNA methods (*23*), the importance of SAM in enzyme-catalyzed synthesis is not presently sufficient that a major effort to obtain this enzyme would be worthwhile.

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