Kinetic properties of spermine synthase from bovine brain

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A kinetic analysis including initial-velocity and product-inhibition studies were performed with spermine synthase purified from bovine brain. The enzyme activity was assayed in the presence of 5'-methylthioadenosine phosphorylase as an auxiliary enzyme to prevent the accumulation of the inhibitory product, 5'-methylthioadenosine, and thus to obtain linearity of the reaction with time. Initial-velocity studies gave intersecting or converging linear double-reciprocal plots. No substrate inhibition by decarboxylated *S*-adenosylmethionine was observed at concentrations up to 0.4 mM. Apparent Michaelis constants were 60 μ M for spermidine and 0.1 μ M for decarboxylated *S*-adenosylmethionine. Spermine was a competitive product inhibitor with respect to decarboxylated *S*-adenosylmethionine, but a mixed one with respect to the other substrate, spermidine. 5'-Methylthioadenosine showed a mixed inhibition with both substrates, predominantly competitive with respect to decarboxylated *S*-adenosylmethionine and predominantly uncompetitive with respect to spermidine. The observed kinetic and inhibition patterns are consistent with a compulsory-order mechanism, where both substrates add to the enzyme before products can be released.

Spermine synthase (EC 2.5.1.22) catalyses the transfer of the amino propyl moiety from decarboxylated *S*-adenosylmethionine to spermidine:

Spermine synthase has been partially characterized from rat brain (Hannonen *et al.*, 1972a), rat liver (Hannonen *et al.*, 1972b), rat prostate (Hibasami *et al.*, 1980; Pegg *et al.*, 1981a,b; Pankaskie *et al.*, 1981) and bovine brain (Pajula *et al.*, 1979; Pankaskie *et al.*, 1981).

The mechanism of the spermine synthase reaction has not previously been studied in detail. The catalytic mechanism of this two-substrate reaction could be a compulsory-order or random-order mechanism (Cleland, 1970; Plowman, 1972; Dixon & Webb, 1979). Spermidine synthase (aminopropyltransferase, EC 2.5.1.16) from *Escherichia coli* has been reported to act with a two-step transfer (Ping Pong) compulsory-order mechanism (Zappia *et al.*, 1980), but an aminopropylated form of the enzyme has not been detected.

Preliminary experiments to elucidate the kinetics of mammalian spermine synthase have been reported previously (Pajula & Raina, 1979; Pajula *et al.*, 1979). The powerful product inhibition of the enzyme by 5'-methylthioadenosine made it difficult to measure the initial reaction velocity accurately

(Pajula & Raina, 1979). The present paper reports initial-velocity and product-inhibition studies for spermine synthase from bovine brain. The enzyme

activity was assayed in the presence of a 5'-methylthioadenosine phosphorylase (EC 2.4.2.28) preparation to eliminate the inhibitory product, 5'-methylthioadenosine. On the basis of kinetic data and inhibition patterns, a compulsory-order reaction mechanism involving ternary complexes is proposed.

Experimental

Chemicals

DL-[2-¹⁴C]Methionine (sp. radioactivity 5.84 Ci/mol), [1,4-¹⁴C]spermidine trihydrochloride (sp. radioactivity 10.22 Ci/mol) and [1,4-¹⁴C]spermine tetrahydrochloride (sp. radioactivity 18.75 Ci/mol) were supplied by New England Corp., Boston, MA, U.S.A. *S*-Adenosyl-L-[Me-¹⁴C]methionine (sp. radioactivity 57 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. *S*-Adenosylmethionine labelled in the aminobutylate moiety was synthesized from DL-[2-¹⁴C]-methionine essentially as described by Pegg & Williams-Ashman (1969b). Radioactive decarboxylated *S*-adenosylmethionine was prepared from

labelled *S*-adenosylmethionine by using *S*-adenosylmethionine decarboxylase from *E. coli* purified as far as step 3 by the method of Wickner *et al.* (1970). The products were purified on a Dowex 50 (H⁺ form) column and finally by preparative paper electrophoresis (Raina & Hannonen, 1971).

Labelled 5'-methylthioadenosine was prepared from *S*-adenosyl-L-[Me-¹⁴C]methionine by acid hydrolysis (Schlenk *et al.*, 1971), purified by adsorption chromatography on a Sephadex G-10 column (Wasternack & Reinbothe, 1972; Kajander & Raina, 1981), and stored in aqueous solution at -20°C. Radiopurity of the product was checked by paper chromatography on Whatman no. 1 paper developed with propan-2-ol/formic acid/water (7:1:2, by vol.). Unlabelled 5'-methylthioadenosine was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Putrescine dihydrochloride and spermidine trihydrochloride were obtained from Calbiochem, La Jolla, CA, U.S.A., and spermine tetrahydrochloride was from Sigma. The polyamines were purified as described previously (Pajula *et al.*, 1979).

CH-Sepharose 4B, CNBr-activated Sepharose 4B and Sephadex G-10 were products of Pharmacia Fine Chemicals, Uppsala, Sweden. Dowex 50W (X8; 100–200 mesh; H⁺ form) was purchased from Fluka, Buchs, Switzerland, hydroxyapatite (Hypatite C) was from Clarkson Chemical Co., Williamsport, PA, U.S.A., and Cellex-P was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Whatman P81 phosphocellulose paper was obtained from Reeve Angel and Co., London E.C.4, U.K. Sucrose and (NH₄)₂SO₄ (both special enzyme grade) were purchased from Schwarz/Mann, Orangeburg, NY, U.S.A., and dithiothreitol was from Calbiochem. ACS (Aqueous Counting Scintillant) was a product of Amersham Corp., Arlington Heights, IL, U.S.A.

Preparation of spermine-Sepharose has been previously described in detail (Pajula *et al.*, 1978). PCMB-Sepharose (*p*-chloromercuribenzoate-ethylenediamine-Sepharose) was prepared by the method of Manen & Russell (1974). Crystalline bovine serum albumin used in enzyme assays was purchased from Sigma (catalogue no. A4378, lot no. 41F-9300).

All other chemicals used were reagent-grade materials from various commercial sources.

Purification of spermine synthase

Spermine synthase was purified to homogeneity from bovine brain by spermine-Sepharose affinity chromatography as previously described (Pajula *et al.*, 1979).

Assay of spermidine synthase activity

Spermidine synthase activity was assayed by the method of Raina *et al.* (1976).

Assay of spermine synthase activity

Method I. The activity of spermine synthase was assayed by using propylamine-labelled decarboxylated *S*-adenosylmethionine as a substrate and Whatman P81 phosphocellulose paper to separate the radioactive spermine formed in the reaction (Raina *et al.*, 1976; Pajula *et al.*, 1979). The standard reaction mixture contained, in a total volume of 0.1 ml, 0.1 M-potassium phosphate, pH 7.4, 5 mM-dithiothreitol, 1 mM-spermidine, 20–40 μM-¹⁴C-labelled decarboxylated *S*-adenosylmethionine (sp. radioactivity 5.84 Ci/mol) and the enzyme protein.

Method II. In kinetic experiments spermine synthase activity was determined by a modification of the method of Hibasami & Pegg (1978) with the use of methyl-labelled decarboxylated *S*-adenosylmethionine (sp. radioactivity 56.9 Ci/mol). In the modified method, small phosphocellulose cation-exchange columns were used to separate the radioactive 5'-methylthioadenosine and its degradation products from the labelled substrate (Raina *et al.*, 1983b). Preincubation of the reaction mixture for 10 min was carried out with 5'-methylthioadenosine phosphorylase, which was also included in the reaction blanks. The reaction was started by adding spermine synthase, and the incubation was continued for 5 min at 37°C. The reaction was stopped by adding 0.5 ml of 25 mM-HCl to the reaction mixture (0.1 ml). A 0.5 ml portion of the acid solution (pH slightly below 3) was applied to a phosphocellulose column (containing approx. 0.5 ml of a weak cation-exchanger, Cellex-P; the void volume was about 0.8 ml), previously equilibrated with 25 mM-HCl. The effluent (0.5 ml) was discarded, and the radioactive 5'-methylthioadenosine and its degradation products were directly eluted into a scintillation vial with 1.8 ml of 25 mM-HCl. ACS scintillation solution (10 ml) was added and the vial contents were mixed thoroughly. Counting efficiency was about 79%. The column was regenerated with 2 × 2 ml of 0.1 M-NaOH and/or 2 × 2 ml of 0.5 M-HCl.

One unit of enzyme activity represents the formation of 1 nmol of spermine or 5'-methylthioadenosine in 1 min under standard assay conditions.

Purification of 5'-methylthioadenosine phosphorylase

5'-Methylthioadenosine phosphorylase was partially purified from human placenta by a modification of the method of Cacciapuoti *et al.* (1978). Human full-term placenta was exsanguinated and cooled on ice, freed of membranes and homogenized in 2.5 vol. of 0.25 M-sucrose solution containing 0.1 mM-disodium EDTA and

2 mM-2-mercaptoethanol, with an Ultra-Turrax (Janke und Kunkel, Staufen, West Germany) homogenizer. The homogenate was centrifuged at 10000 *g* for 60 min. All subsequent operations were performed at 0–4°C. The crude supernatant (1980 ml) was fractionated with solid $(\text{NH}_4)_2\text{SO}_4$. The proteins that were precipitated between 55% and 75% saturation with $(\text{NH}_4)_2\text{SO}_4$ were collected by centrifugation. One-third of the precipitate was dissolved in 35 ml of 50 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-2-mercaptoethanol, and dialysed overnight against 5 × 2 litres of the same buffer. The dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was centrifuged at 10000 *g* for 10 min.

The dialysed fraction (50 ml, 1600–2000 mg of protein) was applied to a hydroxyapatite column (Hypatite C; 3 cm × 9 cm) equilibrated with 50 mM-potassium phosphate buffer, pH 7.4. The column was washed with 100 ml of 50 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-dithiothreitol. Practically all of the spermidine synthase and most of the spermine synthase activity was eluted unadsorbed at this step (Raina & Hannonen, 1971). The column was then connected to a linear gradient of 50–500 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-dithiothreitol (total gradient volume was 300 ml, flow rate 1 ml/min). Fractions of volume 4 ml were collected. 5'-Methylthioadenosine phosphorylase activity (the main peak) was eluted at 130–260 mM-phosphate concentration. The active fractions were pooled and adjusted to 80% saturation with $(\text{NH}_4)_2\text{SO}_4$ to concentrate the enzyme. One-half of the precipitate was dissolved in 15 ml of 50 mM-Tris/HCl buffer, pH 7.1 (the pH of Tris/HCl buffers refers to that determined at 20°C) containing 0.15 M-NaCl, 0.1 mM-disodium EDTA and 0.2 mM-2-mercaptoethanol, and dialysed overnight against 4 × 2 litres of the same buffer.

The dialysed fraction (20 ml, 100–120 mg of protein) was centrifuged at 10000 *g* for 10 min. Dithiothreitol was added up to 1 mM and the fraction was passed through a spermine-Sepharose column (1.6 cm × 10 cm) to remove the residual spermine synthase activity. The column was washed with 40 ml of equilibration buffer containing 1 mM-dithiothreitol. The main protein peak with the 5'-methylthioadenosine phosphorylase activity was removed from the column with the washing buffer, whereas the residual spermine synthase was retained by the column (Pajula *et al.*, 1979). The active fractions were pooled and the proteins were collected by precipitation with $(\text{NH}_4)_2\text{SO}_4$.

The precipitate was dissolved in 4 ml of 50 mM-potassium phosphate buffer, pH 7.4, containing 0.5 M-KCl, and dialysed twice. The first dialysis was overnight against 4 × 1 litre of the above buffer containing 1 mM-2-mercaptoethanol, and the second dialysis for 2 h against 4 × 1 litre of the same buffer

without 2-mercaptoethanol to remove the thiol compound.

The dialysed enzyme solution (5 ml, 36–45 mg of protein) was applied to a column (1.5 cm × 9 cm) of PCMB-Sepharose equilibrated with 50 mM-potassium phosphate buffer, pH 7.4, containing 0.5 M-KCl. The elution was performed stepwise with (a) 40 ml of the equilibration solution, (b) 80 ml of the phosphate buffer containing 2 M-KCl, (c) 90 ml of the buffer containing 2 M-KCl and 1 mM-2-mercaptoethanol, and (d) 90 ml of the buffer containing 2 M-KCl and 10 mM-2-mercaptoethanol. Fractions of volume 4 ml were collected. Most of the proteins were eluted at step (c), whereas the enzyme was eluted at step (d). The active fractions were pooled and then concentrated, by using an Amicon concentrator (Amicon Corp., Lexington, MA, U.S.A.). Dithiothreitol was added up to 1 mM, and the enzyme solution was dialysed overnight against 3 × 2 litres of 50 M-potassium phosphate buffer, pH 7.4, containing 0.1 M-KCl and 1 mM-2-mercaptoethanol. Dithiothreitol was added up to 5 mM, and the enzyme preparation was stored at 4°C. No significant decrease in enzyme activity was observed during storage for 1 month.

Assay of 5'-methylthioadenosine phosphorylase activity

The activity of 5'-methylthioadenosine phosphorylase was assayed by a modification of the method of Cacciapuoti *et al.* (1978), measuring 5'-[Me-¹⁴C]methylthioribose 1-phosphate formed from 5'-[Me-¹⁴C]methylthioadenosine. The standard reaction mixture contained 40 mM-potassium phosphate buffer, pH 7.4, 1 mM-dithiothreitol, 0.6 mM-5'-[Me-¹⁴C]methylthioadenosine (sp. radioactivity 0.041 Ci/mol) and the enzyme protein in a final volume of 0.2 ml. Enzyme was omitted from the reaction blank. After incubation for 30 min at 37°C the reaction was stopped by adding 0.05 ml of 1 M-trichloroacetic acid, and the precipitate was removed by centrifugation. A portion (0.2 ml) of the supernatant was applied to a Dowex 50 (H⁺ form) column (0.5 cm × 0.9 cm) placed in a plastic syringe plugged with a glass-fibre filter-paper disc (Whatman GF/A; Reeve Angel and Co.). The effluent was collected directly into a scintillation vial and the column was eluted with 0.8 ml of 0.2 M-trichloroacetic acid. The combined eluates were counted for radioactivity in 10 ml of ACS scintillation solution with an efficiency of 81%. 5'-Methylthioadenosine was quantitatively retained by the resin, whereas 5'-methylthioribose 1-phosphate was eluted directly through the column. The results are expressed as nmol of the product formed/min.

Protein determination

Protein was measured by the method of Lowry *et al.* (1951) either directly or after precipitation with

trichloroacetic acid, with crystalline bovine serum albumin as a standard.

Protein in the fractions of the chromatographic purifications was estimated by the u.v. absorption at 280 nm.

Treatment of data

All the kinetic data were measured in triplicates, with a standard deviation of $\pm 5\%$. The lines in the double-reciprocal plots and replots were drawn by the least-squares method of Wilkinson (1961), assuming equal variance for the velocities. The nomenclatures of Cleland (1970) and Dixon & Webb (1979) were used in this work. Eqn. (1) was used to fit the data for initial-velocity patterns and eqn. (2) for inhibition pattern by spermine:

$$v = \frac{V[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]} \quad (1)$$

$$v = \frac{V[A][B]}{K_{ia}K_b(1 + [I]/K_i) + K_b[A] + K_a[B](1 + [I]/K'_i) + [A][B]} \quad (2)$$

Results

Linearity of the spermine synthase reaction

The previously found product inhibition by 5'-methylthioadenosine made it difficult to study initial-velocity kinetics without removing 5'-methylthioadenosine formed in the reaction. Therefore spermine synthase activity was measured in this work by adding a large excess of 5'-methylthioadenosine phosphorylase to the reaction mixture to degrade the 5'-methylthioadenosine immediately it was formed. The phosphorylase was partially purified from human placenta. The final preparation was found to be free from spermidine synthase and spermine synthase activities and had a specific activity of 130 nmol of 5'-methylthioadenosine degraded/min per mg of protein (Table 1). The reaction velocity of spermine synthase in the presence of 5'-methylthioadenosine phosphorylase activity was linear with time, as shown in Fig. 1, and also at the lowest substrate concentrations used in the enzyme assays for the period of 5 min. The

consumption of decarboxylated *S*-adenosylmethionine was calculated to be always less than 15% of the initial amount.

To prevent denaturation of spermine synthase by dilution, bovine serum albumin was added to the diluted enzyme solution. Some commercial samples of bovine serum albumin are known to contain amine oxidase as an impurity (see Al-Naji & Clarke, 1983), which might interfere with the enzyme assays. This possibility was ruled out in the present work by the following method. [1,4- ^{14}C]Spermidine and [1,4- ^{14}C]spermine (both at $10\ \mu\text{M}$ concentration) were incubated in the standard reaction mixture (minus decarboxylated *S*-adenosylmethionine) for 20 min in the presence of albumin (0.2 mg/ml). After incubation the recovery of the radioactive polyamines was complete, as demonstrated by paper electrophoresis (Raina *et al.*, 1976). It should also be pointed out that in method II (used in most

experiments of the present work) the formation of radioactive 5'-methylthioadenosine was measured, which is not affected by amine oxidase unless a considerable portion of the substrate (spermidine) is being oxidized. This was not the case, as described above.

Initial-velocity studies

Detailed kinetic studies were carried out by using various concentrations of one substrate in the presence of different fixed concentrations of the second substrate. Both pairs of the substrates gave intersecting or converging lines in Lineweaver-Burk double-reciprocal plots (Figs. 2 and 3). Intersection of nearly parallel reciprocal lines of plots of initial velocity versus decarboxylated *S*-adenosylmethionine concentration (Fig. 3) was confirmed with a Hanes plot of $[S]/v$ versus $[S]$ (Plowman, 1972), in which the lines did not have a common intercept on the vertical axis (plot not shown). When lower concentrations of the substrates were used

Table 1. Purification of 5'-methylthioadenosine phosphorylase from human placenta

The crude extract was prepared from 380 g of human placenta and processed in several batches as described in the Experimental section. 5'-Methylthioadenosine phosphorylase activity was determined under standard assay conditions.

Purification step	Total protein (mg)	Specific activity (units/mg)	Total activity (k units)	Yield (%)	Purification factor (-fold)
Crude extract	14960	1.30	19.4	100	1
55–75%-satn. $(\text{NH}_4)_2\text{SO}_4$	5400	3.17	17.1	87.9	2.4
Hydroxyapatite	665	9.73	6.4	33.3	7.5
Spermine-Sepharose	246	14.7	3.6	18.6	11
PCMB-Sepharose	9.8	131	1.3	6.6	101

Fig. 1. Effect of 5'-methylthioadenosine phosphorylase and albumin on spermine synthase activity

The reaction mixture (0.1 ml) contained 1 mM-spermidine, 40 μ M-decarboxylated adenosylmethionine and purified spermine synthase (0.04 unit and 0.1 μ g of protein). The additions were: O, none; ●, 30 μ g of albumin; □, 0.5 unit of 5'-methylthioadenosine phosphorylase; ■, 30 μ g of albumin + 0.5 unit of 5'-methylthioadenosine phosphorylase. Spermine synthase activity was determined by method I.

Fig. 2. Effect of spermidine concentration on the initial velocity of spermine synthase reaction at different fixed concentrations of decarboxylated adenosylmethionine

The reaction mixture contained 2 μ g of albumin, 0.4 unit of 5'-methylthioadenosine phosphorylase and 0.0015 unit of spermine synthase. The concentrations of decarboxylated adenosylmethionine were: O, 0.3 μ M; □, 0.4 μ M; Δ, 0.5 μ M; ▽, 0.8 μ M; ◇, 2.0 μ M. Spermine synthase activity was determined by method II. The initial velocity (v) was measured as pmol/min.

Fig. 3. Effect of decarboxylated adenosylmethionine concentration on the initial velocity of spermine synthase reaction at different fixed concentrations of spermidine

The reaction mixture and other conditions were the same as in the legend to Fig. 2. The concentrations of spermidine were: O, 25 μ M; □, 35 μ M; Δ, 50 μ M; ▽, 100 μ M; ◇, 250 μ M.

(decarboxylated *S*-adenosylmethionine 0.1–0.3 μ M and spermidine 10–35 μ M), the double-reciprocal plots clearly intersected (results not shown).

The apparent kinetic constants were determined from primary plots in accordance with the reciprocal of eqn. (1) in the Experimental section, and from secondary plots of intercepts and slopes of the primary plots versus reciprocal concentrations of the fixed substrate (Cleland, 1970; Plowman, 1972). The apparent K_m values were 0.1 μ M for decarboxylated *S*-adenosylmethionine and 60 μ M for spermidine respectively. The apparent dissociation constant K_{ia} was 0.06 μ M. The secondary plots were linear within the limits of experimental error (plots not shown).

Spermine synthase was not inhibited by decarboxylated *S*-adenosylmethionine under standard assay conditions even at a concentration of 0.4 mM (results not shown).

Product-inhibition studies

Inhibition by spermine was mixed with respect to spermidine (Fig. 4) but competitive with respect to decarboxylated *S*-adenosylmethionine (Fig. 5) at fixed non-saturated concentration of the other substrate. At saturated concentrations of the fixed-concentration substrate no inhibition was observed with spermidine, but it was still competitive with decarboxylated *S*-adenosylmethionine (results not shown). The results fit to eqn. (2) in the Experimental section. The effect of 5'-methylthio-

Fig. 4. Product inhibition of spermine synthase by spermine with spermidine as the varied-concentration substrate

The reaction mixture contained $0.5 \mu\text{M}$ -decarboxylated adenosylmethionine, $2 \mu\text{g}$ of albumin, 0.4 unit of $5'$ -methylthioadenosine phosphorylase and 0.0015 unit of spermine synthase. The concentrations of spermine were: $\bigcirc$, none; $\square$, 0.05 mM ; $\triangle$, 0.10 mM ; ∇ , 0.25 mM . Spermine synthase activity was determined by method II, and v was measured as pmol/min .

Fig. 5. Product inhibition of spermine synthase by spermine with decarboxylated adenosylmethionine as the varied-concentration substrate

The reaction mixture contained $100 \mu\text{M}$ -spermidine. Spermine concentrations and other conditions were the same as indicated in the legend to Fig. 4.

adenosine on the spermine synthase activity was studied in the absence of $5'$ -methylthioadenosine phosphorylase, by using non-saturated and saturated concentrations of the fixed-concentration substrate and varying the concentration of the other substrate in the region of its Michaelis constant. Inhibition by $5'$ -methylthioadenosine was mixed with respect to both spermidine (Fig. 6) and decarboxylated S -adenosylmethionine (Fig. 7) at non-saturated and saturated concentrations (as high as 50 mM -spermidine) of the fixed-concentration substrate.

Fig. 6. Product inhibition of spermine synthase by $5'$ -methylthioadenosine with spermidine as the varied-concentration substrate

The reaction mixture contained $10 \mu\text{M}$ -decarboxylated adenosylmethionine (sp. radioactivity 2.85 Ci/mol) and 0.03 unit of spermine synthase. The concentrations of $5'$ -methylthioadenosine were: $\bigcirc$, none; $\square$, $1 \mu\text{M}$; $\triangle$, $2 \mu\text{M}$; ∇ , $5 \mu\text{M}$. Spermine synthase activity was determined by method II, and v was measured as pmol/min .

Fig. 7. Product inhibition of spermine synthase by $5'$ -methylthioadenosine with decarboxylated adenosylmethionine as the varied-concentration substrate

The reaction mixture contained $100 \mu\text{M}$ -spermidine, $2 \mu\text{g}$ of albumin and 0.0015 unit of spermine synthase. The concentrations of $5'$ -methylthioadenosine were: $\bigcirc$, none; $\square$, $0.25 \mu\text{M}$; $\triangle$, $0.5 \mu\text{M}$; ∇ , $1.0 \mu\text{M}$. Spermine synthase activity was determined by method II, and v was measured as pmol/min .

The apparent K_i values were calculated from secondary plots, i.e. slopes and intercepts of the primary plots plotted versus the inhibitor concentration. The secondary plots were linear within the limits of experimental error (plots not shown). The inhibitor constants for spermine as calculated from eqn. (2) were $10 \mu\text{M}$ (K_i) and $40 \mu\text{M}$ (K_i'), affecting K_{ia} and K_a respectively. The inhibition

patterns by 5'-methylthioadenosine were all mixed type, suggesting complex effects on all kinetic terms in eqn. (1). The apparent K_i values for 5'-methylthioadenosine, determined as the horizontal intersection points at the horizontal axis of the secondary plots, were: K_i (slope) $10\ \mu\text{M}$ and K_i (intercept) $1\ \mu\text{M}$ with spermidine as the varied-concentration substrate; K_i (slope) $0.2\ \mu\text{M}$ and K_i (intercept) $2\ \mu\text{M}$ with decarboxylated *S*-adenosylmethionine as the varied-concentration substrate.

Discussion

Purified spermine synthase activity is powerfully inhibited by the nucleoside product, 5'-methylthioadenosine (Pajula & Raina, 1979). The compound is, however, rapidly degraded *in vivo* to methylthioribose 1-phosphate and adenine by 5'-methylthioadenosine phosphorylase (Pegg & Williams-Ashman, 1969a), which has a high affinity for the nucleoside (Zappia *et al.*, 1978; Ferro *et al.*, 1979; Seidenfeld *et al.*, 1980; White *et al.*, 1982). Thus the presence of 5'-methylthioadenosine phosphorylase in the reaction mixture of the purified spermine synthase mimicked physiological conditions in the mammalian cell (for references on auxiliary enzymes see Rudolph *et al.*, 1979).

Spermine synthase has an extremely high affinity for decarboxylated *S*-adenosylmethionine. The K_m value obtained in the present investigation was somewhat lower than that previously reported for the purified brain enzyme (Pajula & Raina, 1979; Pajula *et al.*, 1979). Decarboxylated *S*-adenosylmethionine has been found to act as a substrate inhibitor of spermidine synthase isolated from rat prostate (Coward *et al.*, 1977; Hibasami *et al.*, 1980; Samejima & Yamano, 1982) and bovine brain (Raina *et al.*, 1983a), in contrast with spermine synthase from bovine brain, which was not inhibited at concentrations over 100-fold higher than those found in tissues (Pegg & Coward, 1981). The K_m value for spermidine was of the same order as previously reported (Hannonen *et al.*, 1972a; Pajula *et al.*, 1979; Pegg *et al.*, 1981b).

The initial-velocity patterns for spermidine and decarboxylated *S*-adenosylmethionine suggest a reaction mechanism with ternary complexes for the brain spermine synthase (Cleland, 1970; Plowman, 1972). On the other hand, Zappia *et al.* (1980) have proposed a Ping Pong mechanism for the bacterial spermidine synthase, with an aminopropylated enzyme as an intermediate. Studies with transition-state analogues by Tang *et al.* (1980, 1981), with spermidine synthase from rat prostate, and stereochemical investigations with the enzyme from bacteria (Golding & Nassereddin, 1982), also support the reaction mechanism with ternary complexes for the aminopropyl-transfer reaction. The

reaction catalysed by *E. coli* spermidine synthase has been reported to exhibit a classical S_N2 -substitution, i.e. inversion of configuration at the reacting methylene group (Golding & Nassereddin, 1982). In agreement with the stereochemical investigations, spermine synthase could also operate with a single displacement mechanism.

The product-inhibition patterns of the purified spermine synthase do not fit precisely with any of compulsory-order or random-order reaction mechanisms (Cleland, 1970; Plowman, 1972; Rudolph, 1979). The inhibition pattern by spermine is consistent with a compulsory-order Bi Bi mechanism with ternary complexes and also with a Theorell–Chance mechanism involving isomerization of ternary complexes. An uncompetitive inhibition by 5'-methylthioadenosine was, however, not observed at fixed saturated concentrations of the substrates, as predicted in those mechanisms. The mixed-inhibition types of inhibition by 5'-methylthioadenosine, predominantly competitive with respect to decarboxylated *S*-adenosylmethionine and predominantly uncompetitive with respect to spermidine, suggest that the nucleoside product affects all the kinetic constants and the maximum velocity in eqn. (1). With the present enzyme assay methods an equation for inhibition by 5'-methylthioadenosine could not be constructed.

The inhibition by 5'-methylthioadenosine was previously determined to be competitive with respect to decarboxylated *S*-adenosylmethionine (Pajula & Raina, 1979). The enzyme assay method (method I) used in the previous work is, however, not sensitive enough to show intersection of the lines of the reciprocal plot slightly to the left of the vertical axis at non-saturated substrate concentrations, as seen in Fig. 7.

Actually, the spermine synthase reaction should be considered as a Bi Ter type, having proton (H^+) as the third product (Tabor, 1962). All the kinetic studies on spermine synthase were performed at pH 7.4, assuming that the proton concentration was constant. The present kinetic results give no direct information about the step in which the proton is released from the enzyme–substrate complex.

From the data discussed above, the only reaction mechanism that seems to be certainly excluded is a compulsory-order mechanism without ternary complexes, i.e. a Ping Pong mechanism. Although the product-inhibition patterns would suggest a compulsory-order rather than a random-order mechanism, it is very difficult to get more accurate information about the reaction mechanism of spermine synthase with the present enzyme-assay techniques.

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