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Purification and Characterization of Spermidine/Spermine N^1-Acetyltransferase from Rat Liver†

Fulvio Della Ragione and Anthony E. Pegg*

ABSTRACT: An enzyme catalyzing the acetylation of the polyamines, spermidine or spermine, has been purified 112 000-fold to homogeneity from livers of rats treated with carbon tetrachloride. Major purification steps involved affinity chromatography on *sym*-norspermidine-Sepharose, from which the enzyme was eluted by spermidine, and affinity chromatography on Cibacron blue agarose, from which the enzyme was released by coenzyme A. Similar final specific activities were obtained by using either method as a final purification step, providing strong evidence for homogeneity. The enzyme preparation gave a single band on polyacrylamide gel electrophoresis carried out under native or denaturing conditions. It had an apparent molecular weight of about 115 000 made up of two subunits of 60 000. The acetyltransferase acted on spermidine to form only N^1-acetylspermidine. Spermine was also a substrate, giving N^1-acetylspermine, and N^1-acetylspermine could be acetylated to form N^1,N^{12}-diacetylspermine. The V_{max} values for spermidine, spermine, and N^1-acetylspermine were 8, 1.8, and 1.2 μmol of product min^{-1} mg^{-1}, respectively, when assays were carried out in the presence of saturating concentrations of acetyl-CoA. The K_m for acetyl-CoA was 1.5 μM and the K_ms for spermidine, spermine, and N^1-acetylspermine were 130 μM, 35 μM, and 30 μM, respectively. These results suggest that both spermidine and spermine would be physiological substrates for this enzyme. Coenzyme A was quite strongly inhibitory, having a K_i of 40 μM, but an even more powerful inhibitor could be produced by reacting coenzyme A with methyl methanethiosulfate to form coenzyme A methyl disulfide. The purified enzyme had no deacetylase activity and the reaction was effectively irreversible. The name spermidine/spermine N^1-acetyltransferase is suggested for this enzyme, which may play an important role in the interconversion of polyamines.

The degradation of spermine into spermidine and of spermidine into putrescine, which reverses the normal biosynthetic pathway for polyamines (Jänne et al., 1978; Williams-Ashman & Canellakis, 1979), has now been shown to involve the sequential activity of two enzymes, an acetylase and polyamine oxidase (Pegg et al., 1981; Seiler et al., 1981a). Polyamine oxidase cleaves N^1-acetylspermine or N^1-acetylspermidine at the secondary nitrogen to yield 3-acetamidopropanal and spermidine or putrescine, respectively (Hölttä, 1977; Bolkenius & Seiler, 1981). An enzyme catalyzing the acetylation of these polyamines has been found to be induced by treatment with hepatotoxins, which are known to enhance the conversion of

† From the Department of Physiology and Specialized Center Research Center, The Milton S. Hershey Medical Center, Hershey, Pennsylvania 17033. *Received July 7, 1982.* This work was supported in part by Research Grant GM26290.

* Correspondence should be addressed to this author at the Department of Physiology.

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spermidine into putrescine (Pegg et al., 1981; Matsui et al., 1981). In the present paper we describe the purification of this enzyme to homogeneity using affinity chromatography on *sym*-norspermidine–Sepharose or on Cibacron blue agarose as the major step. More than 100 000-fold purification was needed to achieve a homogeneous preparation even with liver from rats maximally induced by treatment with carbon tetrachloride, 6 h before death. The specific activity of the purified enzyme is much greater than that of previously described polyamine acetylases (Seiler & Al-Therib, 1974; Libby, 1978, 1980; Blankenship & Walle, 1977). Its substrate specificity and physical properties, which are described here, clearly distinguish it from these enzymes and from histone acetylases.

Experimental Procedures

Materials. [1-^{14}C]Acetyl-CoA (49.8 mCi/mmol) was purchased from New England Nuclear, Boston, MA. N^1-Acetylspermidine and N^8-acetylspermidine were kindly given by Dr. M. M. Abdel-Monem, University of Minnesota, Minneapolis, MN, and Dr. Z. Canellakis, Departments of Pharmacology and Internal Medicine, Yale, University School of Medicine, Yale, CT. N^1-Acetylspermine was a generous gift from Dr. N. Seiler, Centre de Recherche Merrell International, 67084, Strasbourg Cedex, France. 3,3′-Diaminodipropylamine (*sym*-norspermidine) was obtained from Eastman Kodak Co., Rochester, NY. Coenzyme A methyl disulfide was synthesized according to Currier & Mautner (1977). 6-Aminohexanoic acid–Sepharose 4B was purchased from Pharmacia Fine Chemicals, Upsala, Sweden. Diaminodipropylamine–Sepharose CL-4B was obtained from Pierce Chemical Co., Rockford, IL. Ultrogel AcA 34 was purchased from LKB, Paramus, NJ. Affi-Gel blue (Cibacron blue agarose) was supplied by Bio-Rad Laboratories, Richmond, CA. All other biochemicals were obtained from Sigma Chemical Co., St. Louis, MO. Sprague-Dawley rats were used in all the experiments. Carbon tetrachloride was injected intraperitoneally at a dose of 1.5 mL/kg to rats weighing 200–350 g.

Assay for Spermidine N^1-Acetyltransferase. The assay of spermidine N^1-acetyltransferase activity was performed essentially as previously described (Matsui et al., 1981). The standard reaction mixture contained in a total volume of 100 μL: 300 nmol of spermidine or the amount of polyamine mentioned, 10 μmol of Tris-HCl, pH 7.8, 0.8 nmol (40 nCi) of [1-^{14}C]acetyl-CoA, and the enzyme solution. After incubation at 30 °C for 10 min the reaction was terminated by addition of 20 μL of 1 M NH_2OH·HCl and heating in a boiling water bath for 3 min. Aliquots (50 μL) of the mixture were then applied to cellulose phosphate paper disks (Whatman P-81) that were washed 5 times with distilled water and 3 times with ethanol on a sintered glass filter. The disks were dried and then assayed for radioactivity in a Beckman LS-3133T liquid scintillation spectrometer after addition of 10 mL of Formula 949 counting fluid (New England Nuclear). Counting efficiency was about 65%. A unit of enzyme activity was defined as that amount which formed 1 nmol of N^1-acetylspermidine/min at 30 °C. Histone acetylase activity was measured by using the assay conditions described by Libby (1978) except that the incubation time was 10 min and calf thymus histones (type II AS from Sigma) were used as the substrate.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out at 4 °C by using 7.5% acrylamide separating gel (11 cm long) in 50 mM Tris-HCl, pH 7.5, and 3% acrylamide spacer gel (1 cm) in the same buffer. Electrophoresis was carried out in Tris–glycine buffer, pH 7.5. All buffers contained 1 mM spermidine. Samples containing about 10 μg of protein, 10% sucrose, and tracking dye (bromophenol blue) in a total volume of 200 μL were applied to the gels. After electrophoresis (2 mA/gel for 7 h) the gels were fixed in 40% methanol–7% acetic acid overnight and stained with 0.02% Coomassie brilliant blue in the same solution. The gels were destained at room temperature with 7% acetic acid and 6% methanol. In some cases the gels were not stained but were sliced into 2-mm sections, and each of these was incubated overnight with 150 μL of a solution containing 50 mM Tris-HCl, pH 7.8, 3 mM spermidine, and 150 μg of bovine albumin at 4 °C. Spermidine N^1-acetyltransferase activity was determined in each tube after the addition of 80 nCi of [1-^{14}C]acetyl-CoA as described above except that the incubation time was 30 min. Sodium dodecyl sulfate electrophoresis was carried out at room temperature by using 7.5% resolving gel (pH 8.9) and 3% stacking gel (pH 6.8) (Laemmli, 1970). Samples containing about 15 μg of protein were incubated for 5 min at 100 °C in 0.125 M Tris-HCl, pH 6.8, containing 1% $NaDodSO_4$, 1% 2-mercaptoethanol, 8 M urea, and 0.001% bromophenol blue. After being cooled, the samples were layered directly onto the stacking gel and electrophoresis carried out at 5 mA/gel for about 5 h. The gels were then processed as described above. Phosphorylase *b* (94 000), bovine serum albumin (68 000), ovalbumin (43 000), and carbonic anhydrase (30 000) were used as standards for molecular weight determination.

Identification of Products of Reaction. Paper chromatography was employed for identification of acetylated polyamines. The purified enzyme (about 3 units) was incubated with 3 μmol of polyamine (spermidine or spermine), 50 μmol of Tris-HCl, pH 7.8, 40 nmol of [1-^{14}C]acetyl-CoA (2 μCi), and 0.5 mg of bovine serum albumin in a final volume of 0.5 mL at 30 °C for 10 min. The reaction was terminated with 0.5 mL of 10% (w/v) trichloroacetic acid and then centrifuged. The supernatant was recovered and the pH adjusted to 12 with 100 μL of 10 M NaOH; the polyamines were then extracted with 8 mL of 1-butanol saturated with water. The organic phase was then added to a 1 mL of 1 N HCl and shaken for 5 min. After centrifugation the aqueous phase was recovered and evaporated under reduced pressure. The residue was coevaporated 3 times with a 5-mL fraction of water and resuspended in 200 μL of distilled water. Aliquots were subjected to paper chromatography on Whatman 3MM paper. The solvent system was 1-propanol–triethylamine–water (85:3:15) (Dublin & Rosenthal, 1960), and the R_f values for N^1-acetylspermidine, N^8-acetylspermidine, and N^1-acetylspermine were 0.33, 0.72, and 0.50, respectively.

Reversibility of the Reaction. The reversibility of the reaction catalyzed by spermidine N^1-acetyltransferase was checked in the following way. Purified enzyme (5 units) was incubated with 50 nmol of freshly prepared coenzyme A, [1-^{14}C]acetyl-labeled N^1-acetylspermidine (20 nmol, 1 μCi), 50 μmol of Tris-HCl, pH 7.8, and 0.3 mg of bovine serum albumin in a final volume of 0.3 mL for 60 min at 30 °C. The reaction was stopped by the addition of 1.7 mL of methanol and then centrifuged. Aliquots (100 μL) of supernatant were analyzed by paper chromatography with the solvent used for acetyl polyamines. The R_f of acetyl-CoA in this system was about 0.05. [1-^{14}C]Acetyl-labeled N^1-acetylspermidine was synthesized following the method described above for identification of reaction products except that the reaction mixture was scaled up 3 times. Unlabeled spermidine and labeled N^1-acetylspermidine were then separated by thin-layer chro-

matography as described by Blankenship & Walle (1978).

Determination of Kinetic Parameters. The K_m for acetyl-CoA was determined by using assay conditions identical with those described above except that 10 nCi was used in each assay and the final concentration of acetyl-CoA varied from 2 to 20 μM. The K_m values for spermidine, spermine, and N^1-acetylspermine were determined as described above except that the final concentration of polyamine varied from 0.01 to 3 mM. Data were analyzed by plotting the reciprocal of initial velocities against the reciprocal of substrate concentration. The linear regression method was used to calculate the value of the kinetic parameters.

Determination of Protein. Protein concentration was determined by the method of Bradford (1976) using bovine liver aldolase as a standard protein.

Preparation of sym-Norspermidine Sepharose. sym-Norspermidine was linked to CH-Sepharose 4B as follows. Ten grams of 6-aminohexanoic acid–Sepharose 4B was swollen overnight in 1 L of 0.5 M NaCl and washed with four changes of 1 L of 0.5 M NaCl and then with 4 L of water to remove the salt. The washed gel was mixed with 18 mmol of *sym*-norspermidine (using a solution adjusted to pH 5.5 with 2 M HCl) and the total volume made to 100 mL with water. The mixture was stirred slowly while 2 g of 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide dissolved in water was added dropwise. The pH was maintained at pH 5.5, and the mixture is shaken gently at room temperature for 24 h. The gel was washed with 8 L of 0.5 M NaCl and stored in 50 mL Tris-HCl, pH 7.5, containing 1 M NaCl and 0.02% sodium azide at 4 °C until use.

Determination of Molecular Weight. The estimation of the molecular size of the purified spermidine N^1-acetyltransferase was made with a calibrated column of Ultrogel AcA 34 (1.6 × 70 cm) by using a flow rate of 1 mL/h in 50 mM Tris-HCl, pH 7.5, and 10 mM spermidine. The column was calibrated by using standard proteins of known molecular weight (bovine liver aldolase, 158 000; hemoglobin, 64 500; chymotrypsinogen A, 25 000; cytochrome *c*, 12 500).

Results

Purification of Spermidine N^1-Acetyltransferase. The activity of the enzyme was increased at least 50-fold by treatment with 1.5 mL/kg doses of carbon tetrachloride 6 h prior to sacrifice. Livers from 50 rats were then removed and homogenized in 2 volumes of 0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, 25 mM KCl, and 5 mM $MgCl_2$. The homogenate was centrifuged at 25000*g* for 30 min and the supernatant removed and centrifuged at 105000*g* for 1 h. This and all following steps were carried out at 4 °C. Proteins precipitating between 20 and 50% saturation with ammonium sulfate were then collected, dissolved in the smallest possible volume of 50 mM Tris-HCl, pH 7.5, and dialyzed against 100 volumes of the same buffer for 4 h.

The dialyzed sample, clarified by centrifugation at 25000*g* for 20 min, was applied to a column (5 cm × 45 cm) of DEAE-cellulose (Whatman DE-52) previously equilibrated with 50 mM Tris-HCl, pH 7.5. The sample was applied at a flow rate of 60 mL/h. The column was then washed with 300 mL of equilibration buffer and eluted with a linear gradient (1.4 L of total volume) of from 0.1 to 0.6 M NaCl in this buffer at a flow rate of 80 mL/h. Spermidine N^1-acetyltransferase activity eluted between 0.21 and 0.3 M NaCl and was concentrated by ultrafiltration and diluted to a salt concentration of 0.1 M NaCl with 50 mM Tris-HCl, pH 7.5.

A column of *sym*-norspermidine–Sepharose (1.5 × 10 cm) was prepared and equilibrated with 50 mM Tris-HCl, pH 7.5.

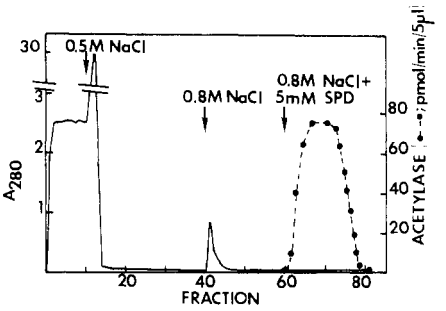

FIGURE 1: Purification of spermidine N^1-acetyltransferase by chromatography on *sym*-norspermidine–Sepharose. The enzyme was applied to the column in 50 mM Tris-HCl, pH 7.5, and the column was washed successively with 0.5 M NaCl, 0.8 M NaCl, and 0.8 M NaCl plus 5 mM spermidine in this buffer as indicated. Fractions of 20 mL were collected until fraction 60 when the volume per fraction was reduced to 8 mL. Every fraction was assayed for the absorbance at 280 nm (—) and the acetylase activity (●). No acetylase activity was detected prior to fraction 60.

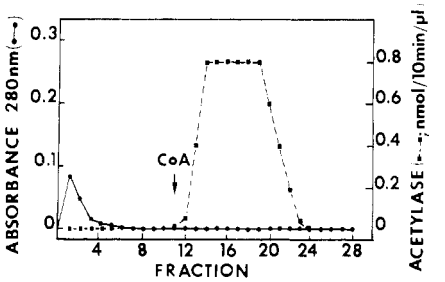

FIGURE 2: Purification of spermidine N^1-acetyltransferase by chromatography on Cibacron blue agarose. The enzyme was applied to the column in 50 mM Tris-HCl, pH 7.5, and 1 mM spermidine, and the column washed with this buffer until the absorbance at 280 nm fell to zero. At the time indicated the column was then washed with this buffer containing 1 mM coenzyme A. Fractions of 3 mL were collected and the acetylase activity (■) and absorbance at 280 nm (●) measured after the removal of coenzyme A by ultrafiltration.

The enzyme solution was applied to this column at a flow rate of 40 mL/h. The column was then washed with 0.5 M NaCl in 50 mM Tris-HCl, pH 7.5, until the absorbance of the eluate at 280 nm was about 0.05. The eluting solution was then changed to 0.8 M NaCl in 50 mM Tris-HCl, pH 7.5, and washing continued until the absorbance of the eluate was less than 0.01 at 280 nm. Spermidine N^1-acetyltransferase was eluted with 5 mM spermidine in 0.8 M NaCl and 50 mM Tris-HCl, pH 7.5 (Figure 1). Fractions containing activity were concentrated to 1 mL by ultrafiltration and freed from NaCl by repeated dilution to 5 mL with 50 mM Tris-HCl, pH 7.5, containing 1 mM spermidine. This step produced more than 450-fold purification of the material eluted from the DEAE-cellulose column.

The material eluted from the norspermidine–Sepharose column was further purified in two ways. One method was to apply the sample to a column of Cibacron blue agarose (0.8 × 4 cm) which had been equilibrated with 50 mM Tris-HCl, pH 7.5, and 1 mM spermidine. The column was loaded and eluted at a flow rate of 18 mL/h, and fractions of 3 mL were collected. The column was then washed with 30 mL of equilibration buffer until the absorbance at 280 nm of the eluate was reduced to zero for at least two fractions. The enzyme activity was then eluted with freshly prepared solution of 1 mM coenzyme A in the equilibration buffer. The activity was assayed in each fraction after removal of coenzyme A by successive concentration and dilution with 50 mM Tris-HCl, pH 7.5, and 10 mM spermidine. The activity was usually present starting with the second fraction. As shown in Figure 2 about 40 mL of the eluting buffer was necessary to recover

Table I: Purification of Spermidine N^1-Acetyltransferase from Carbon Tetrachloride Induced Rat Liver[a]

purifcn step	total protein (mg)	total units	sp act. (units/mg)	purifcn (x-fold)	yield (%)
(1) 105000*g* supernatant	28 864	2078	0.072	1	100
(2) ammonium sulfate	12 332	1726	0.14	2	83
(3) DEAE-cellulose	532.1	1500	2.82	39	72
(4) chromatography on *sym*-norspermidine–Sepharose	0.66	860	1303	18 037	41
(5a) chromatography on Cibacron blue agarose	0.048	389	8102	112 527	19
(5b) second chromatography on *sym*-norspermidine–Sepharose	0.052	419	8069	112 069	20

[a] Results are shown for purification from 50 rats. Steps 5a and 5b are alternative procedures, and the final yield of enzyme from each is given based on using all the material from 50 rats obtained at step 4. One unit represents 1 nmol of product/min at 30 °C.

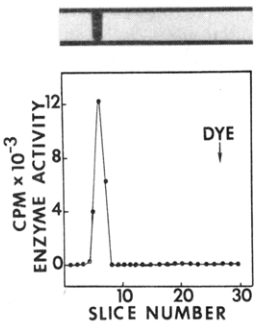

FIGURE 3: Separation of spermidine N^1-acetyltransferase by polyacrylamide gel electrophoresis. About 8 μg of purified enzyme was subjected to electrophoresis on 7.5% gels. The gel was sliced into 2-mm slices and the enzyme activity determined in each slice as described under Experimental Procedures. Activity is expressed as cpm and N^1-acetylspermidine formed per slice per 30 min (●). The arrow indicates the position of the tracking dye.

all of the activity. The active fractions were then pooled and concentrated to a total volume of 0.4 mL.

An alternative step to further purify the enzyme from the *sym*-norspermidine–Sepharose column was developed utilizing the same affinity absorbent. A column of *sym*-norspermidine–Sepharose (1 × 2 cm) was prepared and equilibrated with 50 mM Tris-HCl, pH 7.5. The enzyme from step 4 was applied at a flow rate of 20 mL/h, and the washing and elution were carried out as reported above except that the absorbance at 280 nm at the end of washing with 0.8 M NaCl was <0.005. The enzyme was then eluted with 0.8 M NaCl in 50 mM Tris-HCl, pH 7.5, and 5 mM spermidine. The fractions containing enzyme activity were freed from the salt by concentration and dilution with 50 mM Tris, pH 7.5, and 10 mM spermidine to a final volume of 0.4 mL.

A summary of the purification procedure is given in Table I. Approximately 50 μg of purified enzyme was obtained from 50 rats in about 20% yield. Both methods for the final purification step gave similar yields and a similar specific activity. Since these steps rely on entirely different properties of the enzyme the similarity in final specific activity provides strong evidence for the homogeneity of the final preparation. Passage of the material eluted from the Cibacron blue agarose column through the second *sym*-norspermidine–Sepharose column did not increase the specific activity. The enzyme purified by either method gave a single band on polyacrylamide gel electrophoresis under both denaturing (results not shown) and nondenaturing conditions (Figure 3). In the latter case the activity corresponded to the protein band when assays were carried out on material eluted from the gel slices.

The rationale for the use of *sym*-norspermidine–Sepharose as an affinity absorbant is based on the very high affinity (K_m = 8 μM) of the enzyme for this amine as a substrate. Since this matrix is strongly positively charged it is conveivable that part of the binding of the enzyme to it is due to less specific ionic interactions but it is unlikely that these play a major role for the following reasons. First, the enzyme could be eluted by spermidine or by *sym*-norspermidine at a concentration of 40 mM in the presence of 0.2 M NaCl but could not be eluted by 1 M NaCl in the absence of the polyamines. Second, diaminodipropylamine–Sepharose CL-4B (Pierce Chemicals), a commercially available product in which *sym*-norspermidine is linked directly to the Sepharose without a spacer arm, did not bind the enzyme (nor did 6-aminohexanoic acid–Sepharose 4B itself).

The rationale for the use of Affi-Gel blue for purification of the enzyme was based on the finding that Cibacron F3GA, the chromophore of blue dextran inhibited enzyme activity by 70% at 1 μM in the presence of 8 μM acetyl-CoA. The enzyme was retained on the Affi-Gel blue column even when this was washed with 0.5 M NaCl in 50 mM Tris-HCl, pH 7.5, but was readily released by coenzyme A. The material from the DEAE-cellulose column could be applied directly to the Affi-Gel blue column, and after the material was washed, the enzyme then eluted with coenzyme A. This gave a purity of about 50% (results not shown).

Stability of the Enzyme. Crude preparations from steps 1 and 2 could be stored at −20 °C for about 1 week with less than 20% loss of activity, but the material from step 3 (DEAE-cellulose chromatography) was very unstable, losing 40–45% of activity within 24 h at 4 °C or at −20 °C. Therefore, the enzyme was taken through the further purification steps as rapidly as possible. The purified enzyme could be stored at −20 °C in 50 mM Tris-HCl, pH 7.5, and 10 mM spermidine with a loss of activity of about 30% per week. The enzyme is very sensitive to heat denaturation. When incubated in 10 mM spermidine and 50 mM Tris-HCl, pH 7.5, at temperatures of 45–60 °C, activity was lost rapidly with straightforward first-order kinetics. The $t_{1/2}$ at 45 °C was about 2.2 min, at 52 °C it was about 0.5 min, and at 60 °C 90% of the activity was lost in less than 0.5 min.

Molecular Size and Subunit Composition. Estimations of molecular size of the enzyme were carried out by gel filtration on Ultrogel AcA 34. If only a small or moderate molecular asymmetry is assumed, an approximate molecular weight of 115 000 was obtained. The single band of enzyme protein seen on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate corresponds to a molecular weight of 60 000, suggesting that the native enzyme is a dimer.

Properties of Enzyme. The homogeneous enzyme preparation catalyzed the acetylation of spermidine and spermine but was totally inactive with putrescine or histones as a substrate. When spermidine was the substrate, the product of the reaction was exclusively (<98%) N^1-acetylspermidine. Only N^1-acetylspermine was detected as the product when spermine was used as a substrate; under the reaction conditions

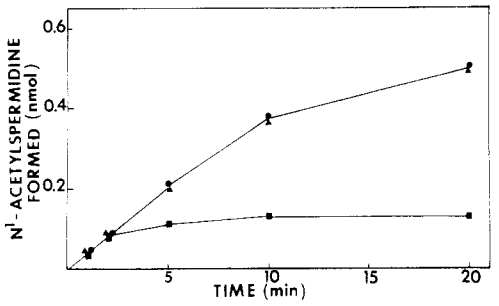

FIGURE 4: Effect of protein addition on production of N^1-acetylspermidine. The enzyme (8 ng) was incubated in the standard assay medium with no addition (■), with 0.5 mg/mL bovine serum albumin (▲), and with 0.5 mg/mL bovine liver aldolase (●) and the amount of N^1-acetylspermidine formed given as shown.

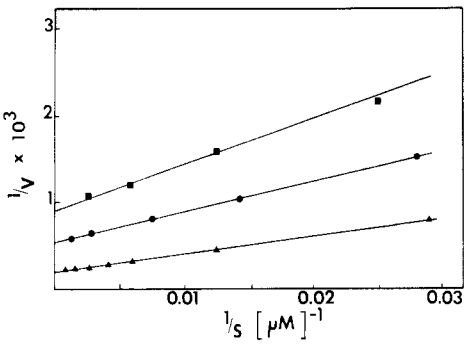

FIGURE 5: Determination of K_m for spermidine, spermine, and N^1-acetylspermine. The enzyme (8 ng) was incubated for 5 min in the standard assay medium containing 8 μM acetyl coenzyme A, 50 μg of albumin, various concentrations of spermidine (▲), spermine (●), and N^1-acetylspermine (■) as shown and the amount of acetylated product determined. Results are shown as double-reciprocal plot of the rate of reaction (as nmol mg^{-1} min^{-1} of [1-^{14}C]acetyl-CoA incorporated) against the substrate concentration.

used, therefore, in which less than 10% of the substrate was consumed, no diacetylspermine was formed. However, monoacetylspermine was a substrate for the reaction when added in the absence of spermine. The reaction was not reversible and the purified enzyme had no deacetylase activity. No loss of labeled N^1-acetylspermidine or formation of acetate or acetyl-CoA occurred when the enzyme was incubated with 67 μM N^1-[^{14}C]acetylspermidine for 60 min with or without 0.17 mM coenzyme A (results not shown).

When the purified enzyme was used alone (8 ng/assay), the reaction rate was linear for only 2 min (Figure 4) whereas the enzyme from earlier steps in the purification gave a linear rate of reaction for at least 10 min at 30 °C. This loss of linearity was due to instability of the protein in very dilute concentrations and could be prevented by addition of other proteins such as bovine serum albumin or liver aldolase (Figure 4). The kinetic experiments were, therefore, carried out by using an incubation time of 5 min in the presence of 0.5 mg/mL bovine serum albumin which ensured a linear rate of reaction. When spermidine was used as acceptor, the K_m for acetyl-CoA was 1.5 μM; the K_ms for spermidine, spermine, and monoacetylspermine were 130 μM, 35 μM, and 30 μM, respectively (Figure 5). Coenzyme A was inhibitory, having a K_i value of about 40 μM. The rate of acetylation under saturating substrate conditions was considerably greater for spermidine (V_{max} of 8 μmol min^{-1} mg^{-1}) than for spermine (1.8 μmol min^{-1} mg^{-1}) or N^1-acetylspermine (1.2 μmol min^{-1} mg^{-1}).

The enzyme was inhibited by exposure to 0.5 mM *p*-(chloromercuri)benzoate which might indicate the involvement of a thiol group in its mechanism of action. Therefore, the effect of methyl methanethiosulfonate was tested since this

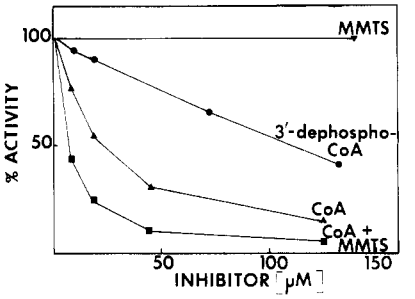

FIGURE 6: Effect of various inhibitors on acetylation of spermidine. The standard assay medium contained 3 mM spermidine, 8 μM [1-^{14}C]acetyl-CoA, 8 ng of enzyme, 50 μg of albumin, and the potential inhibitors shown. Results are shown as the percentage of the N^1-acetylspermidine formed in the absence of inhibitor. Results are shown for inhibition by methyl methanethiosulfonate [MMTS (▼)], 3′-dephosphocoenzyme A [3′-dephospho-CoA (●)], coenzyme A [CoA (▲)], and coenzyme A methyl disulfide [CoA + MMTS (■)].

compound adds only a methylthio group rather than a larger group and reaction with it minimizes the steric modification of the enzyme structure (Smith et al., 1975). It was found that no inhibition was produced over exposure to this compound in a range of 10–500 μM. These results suggest that the loss of activity in response to *p*-(chloromercuri)benzoate is due to changes in the protein structure brought about by this reagent rather than by the inactivation of an essential thiol. More interestingly, high concentrations (0.5–10 mM) of methyl methanethiosulfonate added directly to the assay mixture did produce a small (ca. 10%) inhibition of the reaction rate without any concentration dependence. This result resembles earlier findings with choline acetyltransferase (Currier & Mautner, 1976) and could be explained by the formation of a strong inhibitor when methyl methanethiosulfonate reacts with coenzyme A, the product of the reaction. As shown in Figure 6 we were able to confirm this hypothesis by comparing the inhibition of the reaction by coenzyme A alone and the coenzyme A methyl disulfide. The latter was a very strong inhibitor of the reaction considerably more potent than coenzyme A itself which was in turn more strongly inhibitory than 3′-dephosphocoenzyme A.

Discussion

The specific activity of the final preparation of the acetyltransferase described here is very much greater than that previously reported for enzymes acetylating polyamines (Libby, 1978, 1980; Matsui et al., 1981) but is comparable to that of other acetylases which have been purified extensively such as choline acetyltransferase (Husain & Mautner, 1973), aromatic amine acetyltransferase (Drummond et al., 1980), and histone acetylase A (Belikoff et al., 1980). Our earlier work in which the inducible spermidine N^1-acetyltransferase was purified only 70-fold (Matsui et al., 1981) suggested that it was a completely different enzyme from the previously described histone/polyamine acetyltransferases and the present results confirm this. The enzyme purified by us does not acetylate putrescine or form the N^8-acetylspermidine isomer and is completely inactive with histones as a substrate. It is localized in the cytoplasm as indicated by its presence in the supernatant fraction after centrifugation of homogenates. The nuclear enzymes detected by Libby (1978, 1980) and by Blankenship & Walle (1977, 1978) acetylate histone, putrescine, and polyamines, forming predominantly N^8-acetylspermidine when acting on spermidine as a substrate. These enzymes have been purified 5000-fold from calf liver and 500-fold from rat liver (Libby, 1978, 1980). In both cases, two distinct enzymes both having histone and polyamine acetylase activity were obtained

but the specific activities achieved were only 1.4 nmol min^{-1} mg^{-1} for the calf liver enzyme and 1.9 nmol min^{-1} mg^{-1} for the rat liver enzyme. These values are more than 3000 times lower than the specific activity of our preparation of spermidine/spermine N^1-acetyltransferase and 1500 times lower than that of histone acetylase A (Belikoff et al., 1980). It appears unlikely that there would be such a large difference if the purified nuclear polyamine *N*-acetyltransferase preparations were actually homogeneous, and it is, therefore, still an open question whether the same protein is responsible for acetylation of polyamines and histones in such nuclear extracts. Whether the chromatin-bound polyamine *N*-acetyltransferase actually plays any physiological role in the acetylation of intracellular polyamines also remains to be determined. N^8-Acetylspermidine has not been detected in cellular extracts but is present in urine (Abdel-Monem & Ohno, 1977; Seiler et al., 1981a,b), and monoacetylputrescine is present in only small amounts which could result from the action of a microsomal enzyme (Seiler & Al-Therib, 1974) which has not yet been fully characterized.

The evidence provided in this paper offers convincing proof that the spermidine/spermine N^1-acetyltransferase isolated is homogeneous. In addition to the analysis on polyacrylamide gel electrophoresis, the attainment of similar specific activities by different preparative methods suggests that a single protein has been isolated. This protein catalyzes the acetylation of both spermidine and spermine which are presumably its normal physiological substrates. The K_m values for both substrates are sufficiently low that it is likely that the enzyme is saturated and can operate at a maximal rate. This rate is considerably greater for spermidine but both polyamines are likely to be acetylated in vivo. The presence of N^1-acetylspermidine has been detected in rat liver with peak levels at the time at which the induced acetyltransferase activity is highest (Seiler et al., 1980, 1981a; Pegg et al., 1981; Abdel-Monem & Merdink, 1981). N^1-Acetylspermine was not found in these experiments but this substance is an excellent substrate with a very low K_m (0.6 μM) for polyamine oxidase (Bolkenius & Seiler, 1981) and polyamine oxidase activity is very high in the rat (Seiler et al., 1981a,b; Pösö & Pegg, 1982). N^1-Acetylspermine was detected in mouse liver after treatment with carbon tetrachloride (Seiler et al., 1981a; Pösö & Pegg, 1982) which also induces the acetyltransferase in this species (Pösö & Pegg, 1982). However, the mouse liver contains much lower levels of polyamine oxidase than the rat, allowing a larger buildup of the acetylated derivatives (Seiler et al., 1981a; Pösö & Pegg, 1982). The relative proportions of spermine, spermidine, and N^1-acetylspermine in the liver are so largely in favor of the free polyamines that it is unlikely that any N^1,N^{12}-diacetylspermine would be formed in vivo even though the enzyme can carry out this reaction in vitro.

The spermidine/spermine N^1-acetyltransferase is a rather unstable enzyme even in the presence of spermidine which greatly enhances its stability. It is very heat sensitive and is, therefore, best assayed at 30 °C. No evidence for multiple forms of this enzyme was achieved in the present work. There is only a very small amount of this enzyme present in the liver even after maximal induction (about 9 ng/mg of soluble protein). This corresponds to only about 60 000 molecules per hepatocyte (assuming that the enzyme is confined to these cells) and is comparable to the situation with ornithine decarboxylase which is present at about 1.4 ng/mg of soluble protein after maximal induction (Pritchard et al., 1981) which corresponds to about 12 000 molecules per cell. Both enzymes are increased 50–100-fold by maximal induction (Pegg et al., 1981) so the normal liver cell has only a few hundred molecules of ornithine decarboxylase and spermidine/spermine N^1-acetyltransferase which are respectively the limiting factors in the production of putrescine from ornithine and from spermidine.

The mechanism of induction of spermidine/spermine N^1-acetyltransferase which increases at least 50-fold within 6 h of exposure to carbon tetrachloride (Pegg et al., 1981) and had a very rapid rate of biodegradation (Matsui & Pegg, 1981) is of obvious interest. The results of the present investigation may provide techniques with which these can be approached. The preparation of sufficient enzyme of high purity for the production of antibodies should be possible by using the affinity chromatography techniques described here which give more than 100 000-fold purification in 20% yield. The role of this enzyme in polyamine metabolism and in the development of liver damage by hepatotoxins which induce it (Pegg et al., 1981) may be approachable if specific and potent inhibitors can be devised; the finding that the enzyme is very strongly inhibited by coenzyme A methyl disulfide suggests that compounds of this type may be used. Further studies of inhibitors of the reaction utilizing the purified enzyme are in progress.

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S-Methylated Nucleoside Phosphorothioates as Probes of Enzyme Metal·Nucleotide Binding Sites†

Bernard A. Connolly and Fritz Eckstein*

ABSTRACT: The S-methylated derivatives of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαSCH$_3$) have been prepared by the reaction of both diastereomers of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) with methyl iodide. At physiological pH ATPαSCH$_3$ was unstable, decomposing predominantly to adenosine 5′-*O*-(*S*-methyl thiophosphate) (AMPSCH$_3$) and pyrophosphate. A minor degradation pathway also yielded ATP and methyl mercaptan. Greatly enhanced stability was observed at lower pH. The S_P diastereomer of ATPαSCH$_3$ was a substrate for hexokinase and acetate kinase, and both diastereomers were active with fructose-6-phosphate kinase. The products of these reactions were the appropriate sugar or acyl phosphate, AMPSCH$_3$, and inorganic phosphate, the latter two species arising from the breakdown of the transient intermediate 5′-*O*-(*S*-methyl 1-thiodiphosphate) (ADPαSCH$_3$). No measurable substrate activities were observed with creatine and phosphoglycerate kinase. These results are interpreted as meaning that creatine and phosphoglycerate kinase require Mg^{2+} coordination to the α-phosphate group during the enzyme-catalyzed reaction whereas the other three enzymes do not. Attempts to prepare adenosine 5′-*O*-(*S*-methyl 2-thiotriphosphate) (ATPβSCH$_3$) and ADPαSCH$_3$ by similar methods were unsuccessful with adenosine 5′-*O*-(*S*-methyl 2-thiodiphosphate) (ADPβS) and AMPSCH$_3$ being respectively isolated as the major products.

The determination of the structure of the metal·nucleotide complex active in a particular enzyme-catalyzed reaction has attracted a great deal of attention [see review, Eckstein (1980)]. One approach to this problem uses the diastereomers of ATPαS[1] and ATPβS in combination with hard and soft metal ions. This method relies on the preferential coordination of Mg^{2+} to oxygen and Cd^{2+} to sulfur in the phosphorothioate analogues (Jaffe & Cohn, 1978a, 1979). These authors found that only Mg·ATPβS B and Cd·ATPβS A showed appreciable activity with hexokinase. This reversal of diastereomeric selectivity arises because Mg·ATPβS B (Mg^{2+} to oxygen binding) and Cd·ATPβS A (Cd^{2+} to sulfur binding) have identical metal·nucleotide chelate structures and provides excellent evidence for Mg^{2+} coordination to the β-phosphate group of ATP during the hexokinase-catalyzed reaction. A lack of reversal in diastereomeric stereoselectivity (as was in fact observed with the ATPαS isomers) is most simply interpreted by a lack of metal coordination to the phosphate group under study although such an interpretation is by no means conclusive. For instance, within the constraints imposed at the active site of enzymes Mg^{2+} could be forced to bind to sulfur or Cd^{2+} to oxygen, leading to a lack of reversal of isomeric specificity even though metal ion binding to the phosphate group occurs. Many enzymes have now been studied by this technique, and although all combinations of reversal or lack of it have been observed, the most common pattern seems to be a reversal of stereoselectivity with the ATPβS diastereomers coupled with no reversal at the α phosphorus. This has been observed with all the nucleotidyl transferases so far tested [DNA polymerase (Burgers & Eckstein, 1979), RNA polymerase (Armstrong et al., 1979), phenylalanyl-tRNA synthetase (Connolly et al., 1980), methionyl-tRNA synthetase (Smith & Cohn, 1982)] and about half of the phosphoryl transferases thus studied [hexokinase (Jaffe & Cohn, 1979), myosin (Connolly & Eckstein, 1981), acetate kinase (Romaniuk & Eckstein, 1981)]. These results are usually interpreted as meaning that the β,γ-bidentate metal·nucleotide chelate is the active one in these enzyme-catalyzed reactions. This conclusion is based on the simple interpretation that lack of reversal at the α-phosphate group means no metal ion co-

† From the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, West Germany. *Received June 29, 1982.*

[1] Abbreviations: ATPαS A and B, the S_P and R_P diastereomers of adenosine 5′-*O*-(1-thiotriphosphate); ATPαSCH$_3$ A and B, the S_P and R_P diastereomers of adenosine 5′-*O*-(*S*-methyl 1-thiotriphosphate); ADPαS A and B, the S_P and R_P diastereomers of adenosine 5′-*O*-(1-thiodiphosphate); ADPαSCH$_3$ A and B, the S_P and R_P diastereomers of adenosine 5′-*O*-(*S*-methyl 1-thiodiphosphate); ATPβSCH$_3$, adenosine 5′-*O*-(*S*-methyl 2-thiotriphosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ADPβSCH$_3$, adenosine 5′-*O*-(*S*-methyl 2-thiodiphosphate); AMPS, adenosine 5′-*O*-(thiophosphate); AMPSCH$_3$, adenosine 5′-*O*-(*S*-methyl thiophosphate); N^1-CH$_3$AMPS, N^1-methyladenosine 5′-*O*-(thiophosphate); N^1-CH$_3$AMPSCH$_3$, N^1-methyladenosine 5′-*O*-(*S*-methyl thiophosphate); TEAB, triethylammonium bicarbonate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; NAD, nicotinamide adenine dinucleotide; EPR, electron paramagnetic resonance.

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