Studies of the specificity and kinetics of rat liver spermidine/spermine N^1 -acetyltransferase

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(Received 17 March 1983/Accepted 16 May 1983)

The substrate specificity and kinetic mechanism of spermidine N^1 -acetyltransferase from rat liver was investigated using a highly purified (18 000-fold) preparation from the livers of rats in which the enzyme was induced by treatment with carbon tetrachloride (1.5 ml/kg body wt. 6 h before death). The enzyme catalysed the acetylation of spermidine, spermine, *sym*-norspermidine, *sym*-norspermine, *N*-(3-aminopropyl)-cadaverine, N^1 -acetylspermine, 3,3'-diamino-*N*-methyldipropylamine and 1,3-diaminopropane, but was inactive with putrescine, cadaverine, *sym*-homospermidine and N^1 -acetylspermidine. These results suggest that the enzyme is highly specific for the acetylation of a primary amino group that is separated by a three-carbon aliphatic chain from another nitrogen atom (i.e. the substrates are of the type $H_2N[CH_2]_3NHR$). The maximal rates of acetylation of 1,3-diaminopropane and 3,3'-diamino-*N*-methyldipropylamine were much lower than the maximal rates with spermidine or *sym*-norspermidine as substrates, suggesting a preference for a secondary amino group bearing the aminopropyl group that is acetylated. The best substrates for acetylation were *sym*-norspermidine and *sym*-norspermine, which had K_m values of about $10\mu M$ and V_{max} values of about $2\mu mol$ of product/min per mg of enzyme compared with a K_m of $130\mu M$ and V_{max} of $1.3\mu mol$ /min per mg for spermidine. N^1 -Acetylspermidine (the product of the reaction) and N^8 -acetylspermidine were weak inhibitors and were competitive with spermidine, having K_i values of about 6.6 mM and 0.4 mM respectively. N^1 -Acetylspermidine was a non-competitive inhibitor with respect to acetyl-CoA. CoA was also inhibitory to the reaction, showing non-competitive kinetics when either [acetyl-CoA] or [spermidine] was varied. These results suggest that the reaction occurs via an ordered Bi Bi mechanism in which spermidine binds first and N^1 -acetylspermidine is the final product to be released.

Acetylation of the polyamines spermidine and spermine has been shown to be the rate-limiting step in the loss of an aminopropyl group leading to putrescine and spermidine respectively (Bolkenius & Seiler, 1981; Seiler *et al.*, 1981*a,b*; Pegg *et al.*, 1981, 1982; Pösö & Pegg, 1982). N^1 -Acetylspermidine is rapidly degraded by polyamine oxidase, giving 3-acetamidopropanal and putrescine. A similar reaction of polyamine oxidase converts N^1 -acetylspermine into spermidine (Bolkenius & Seiler, 1981). The acetylation is catalysed by a highly inducible

enzyme that forms exclusively the N^1 -acetylated polyamine derivatives when incubated with the polyamines and acetyl-CoA (Matsui *et al.*, 1981; Della Ragione & Pegg, 1982). This inducible enzyme does not acetylate histones and appears to be present predominantly in the cytoplasmic compartment of the cell (Matsui & Pegg, 1980; Matsui *et al.*, 1981) but other enzymes that acetylate polyamines have been described. These include chromatin-associated enzymes, which also acetylate histones (Libby, 1978*a*, 1980; Blankenship & Walle, 1977, 1978; Cullis *et al.*, 1982), and enzymes present in microsomal fractions (Seiler & Al-Therib, 1974). The occurrence of multiple enzymes catalysing polyamine acetylation and the presence of active de-acetylases (Seiler & Al-Therib, 1974; Libby,

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1978b; Blankenship, 1978) necessitates the extensive purification of the acetylases in order to study their specificity and kinetics. Recently we have described the purification to homogeneity of the inducible spermidine N^1 -acetyltransferase from livers of rats treated with the hepatotoxin, carbon tetrachloride (Della Ragione & Pegg, 1982). In the present work the substrate specificity and kinetics of the reaction have been investigated. It is shown that the enzyme is highly specific for acetylation of the terminal amino group of structures of the form $R-NH[CH_2]_3NH_2$ and that the reaction mechanism appears to be an ordered Bi Bi mechanism in which spermidine is the first substrate to bind to the enzyme.

Experimental

Materials

[1- ^{14}C]Acetyl-CoA (48.8 Ci/mol) was purchased from New England Nuclear, Boston, MA, U.S.A. N^1 -Acetylspermidine and N^8 -acetylspermidine were gifts from Dr. H. M. Abdel-Monem, Department of Medicinal Chemistry, University of Minnesota, MN, U.S.A., and from Dr. Z. Canellakis, Departments of Pharmacology and Internal Medicine, Yale University School of Medicine, Yale, CT, U.S.A. Monoacetylspermine was kindly given by Dr. N. Seiler, Centre de Recherche Merrell International, Strasbourg, France. N -(3-Aminopropyl)cadaverine (1,9-diamino-4-azanonane) and *sym*-homospermidine (1,9-diamino-5-azanonane) were gifts from Dr. S. S. Cohen, Department of Pharmacology, State University of New York at Stony Brook, Long Island, NY, U.S.A. *sym*-Norspermidine (3,3'-diaminodipropylamine) and *sym*-norspermine [NN' -bis-(3-aminopropyl)propane-1,3-diamine] were purchased from Eastman Kodak Co., Rochester, NY, U.S.A. All other biochemical reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Purification and assay of spermidine N^1 -acetyltransferase

This enzyme was purified from the livers of rats treated with 1.5 ml/kg body wt. doses of carbon tetrachloride 6 h before death as previously described (Della Ragione & Pegg, 1982) through the affinity chromatography step on *sym*-norspermidine-Sepharose. This resulted in an 18000-fold purification and gave an enzyme preparation with a specific activity of 1300 units per mg, where 1 unit represents the production of 1 nmol of N^1 -acetylspermidine per min at 30°C in the standard assay medium. This preparation is about 20% pure and is free from contaminating acetyltransferases and deacetylases. The enzyme is very unstable in the absence of spermidine and was stored at -20°C in 10 mM-spermidine/50 mM-Tris/HCl, pH 7.5 (Della

Ragione & Pegg, 1982). The polyamine was removed by addition of 1 mg of bovine serum albumin/ml and dialysis against two changes of 50 mM-Tris/HCl, pH 7.4 (200 vol.) for 8 h. Activity was assayed in a reaction mixture of 0.1 ml volume containing 8 μ M-[1- ^{14}C]acetyl-CoA (40 nCi), 100 mM-Tris/HCl, pH 7.8, and 3 mM-spermidine. Where indicated the spermidine was replaced by other amines or the concentration of substrates was varied as shown. Assays were carried out for 5 min at 30°C and the reaction was terminated by the addition of 0.2 ml of 1 M-hydroxylamine hydrochloride followed by heating in a boiling-water bath for 3 min. Portions (0.05 ml) were applied to cellulose phosphate discs (Whatman P81), which were washed to remove [1- ^{14}C]acetyl-CoA and assayed for radioactivity as previously described (Della Ragione & Pegg, 1982). In all cases where the possible reaction product was not known to be retained on cellulose phosphate under the washing conditions used, the assays for acetylated products were conducted by the extraction procedure of Blankenship & Walle (1977). About 0.01 unit (0.008 μ g) of enzyme was used in the assays. It was necessary to have the bovine serum albumin present to stabilize the enzyme in such dilute solutions. This gave a linear rate of reaction. All results were checked by the addition of other proteins as stabilizing agents (see Della Ragione & Pegg, 1982) to ensure that albumin did not influence the reaction by binding substrates or inhibitors. The bovine serum albumin used in our experiments did not exhibit any polyamine oxidase activity and similar kinetics were observed when aldolase was used as a stabilizing protein. This rules out the possibility that oxidation of the polyamines tested as substrates influences the results. Protein was determined by the method of Bradford (1976) using reagents from Bio-Rad Laboratories, Richmond, CA, U.S.A., and rabbit muscle aldolase as standard. Lineweaver-Burk plots were drawn after assays using amounts of enzyme sufficient to convert 1–10% of substrate into product. The points plotted in each Figure are the actual data points, whereas the lines are those derived by weighted linear regression analysis. A Texas Instruments digital computer model TI programmable 51 III with a linear regression program was used to calculate all the kinetic data.

Results

Substrate specificity of rat liver spermidine N^1 -acetyltransferase

Table 1 summarizes the investigation of substrates for the acetylase. The compounds tested include putrescine, spermidine, spermine, and their monoacetylated derivatives that occur naturally in mammalian cells, and a variety of other compounds that,

Table 1. *Substrate specificity of spermidine N¹-acetyltransferase*

Results are shown as the apparent K_m (in the presence of $8\text{ }\mu\text{M}$ -acetyl-CoA) and the maximal rate of reaction (with a 3 mM concentration of the amine shown). The reaction rates are expressed as percentages of that found with spermidine as substrate ($1.27\text{ }\mu\text{mol/min per mg}$). K_m values were not determined for some compounds and this is indicated as N.D.

Compound	Structure	Apparent K_m (μM)	Maximal rate of reaction (%)
Spermidine	$\text{H}_2\text{N}[\text{CH}_2]_3\text{NH}[\text{CH}_2]_4\text{NH}_2$	130	100
<i>N</i> ¹ -Acetylspermidine	$\text{CH}_3\text{CO} \cdot \text{NH}[\text{CH}_2]_3\text{NH}[\text{CH}_2]_4\text{NH}_2$	Inactive	<1
Spermine	$\text{H}_2\text{N}[\text{CH}_2]_3\text{NH}[\text{CH}_2]_4\text{NH}[\text{CH}_2]_3\text{NH}_2$	34	24
<i>N</i> ¹ -Acetylspermine	$\text{H}_2\text{N}[\text{CH}_2]_3\text{NH}[\text{CH}_2]_4\text{NH}[\text{CH}_2]_3\text{NHCOCH}_3$	51	16
<i>sym</i> -Norspermidine	$\text{H}_2\text{N}[\text{CH}_2]_3\text{NH}[\text{CH}_2]_3\text{NH}_2$	9	140
<i>sym</i> -Norspermine	$\text{H}_2\text{N}[\text{CH}_2]_3\text{NH}[\text{CH}_2]_3\text{NH}[\text{CH}_2]_3\text{NH}_2$	10	160
	CH_3		
3,3-Diamino- <i>N</i> -methyldipropylamine	$\text{H}_2\text{N}[\text{CH}_2]_3\text{N}[\text{CH}_2]_3\text{NH}_2$	N.D.	31
<i>sym</i> -Homospermidine	$\text{H}_2\text{N}[\text{CH}_2]_4\text{NH}[\text{CH}_2]_4\text{NH}_2$	Inactive	<1
<i>N</i> -(3-Aminopropyl)cadaverine	$\text{H}_2\text{N}[\text{CH}_2]_3\text{NH}[\text{CH}_2]_5\text{NH}_2$	N.D.	30
1,3-Diaminopropane	$\text{H}_2\text{N}[\text{CH}_2]_2\text{NH}_2$	460	7.6
1,4-Diaminobutane (putrescine)	$\text{H}_2\text{N}[\text{CH}_2]_4\text{NH}_2$	Inactive	<1
1,5-Diaminopentane (cadaverine)	$\text{H}_2\text{N}[\text{CH}_2]_5\text{NH}_2$	Inactive	<1
<i>N</i> ⁸ -Acetylspermidine	$\text{H}_2\text{N}[\text{CH}_2]_3\text{NH}[\text{CH}_2]_4\text{NHCOCH}_3$	Inactive	<1
<i>N</i> ¹ -Acetylputrescine	$\text{H}_2\text{N}[\text{CH}_2]_4\text{NHCOCH}_3$	Inactive	<1

although produced by some living organisms, are not normally present in mammalian tissues. As previously reported (Della Ragione & Pegg, 1982) the greatest rate of reaction with the naturally occurring polyamines was seen with spermidine, but spermine has a lower K_m and a maximal rate only four times less than spermidine. Both of these polyamines are present in mammalian cells at concentrations well above their K_m values and are likely to be substrates *in vivo*. Putrescine, *N*¹-acetylputrescine, *N*¹-acetylspermidine and *N*⁸-acetylspermidine were not substrates for the acetylase. Although *N*¹-acetylspermine was a moderately good substrate with a K_m of about $50\text{ }\mu\text{M}$, it is unlikely to be acetylated *in vivo* because its concentration in mammalian cells is very low (Seiler *et al.*, 1981a; Pösö & Pegg, 1982). Therefore, formation of *N*¹*N*¹²-diacetylspermine *in vivo* appears questionable and this product has not yet been detected *in vivo*.

The best substrates for the spermidine *N*¹-acetyltransferase were *sym*-norspermidine and *sym*-norspermine, which had apparent K_m values an order of magnitude less than spermidine and gave maximal rates of reaction about 1.5 times greater. The remarkable specificity of the enzyme for the addition of an acetyl group to a primary amino group linked to a second nitrogen atom by an aliphatic chain of three carbon atoms is illustrated by the comparison of *sym*-norspermidine with *sym*-homospermidine, which was inactive as a substrate. However, an unsymmetrical isomer of homospermidine, namely *N*-(3-aminopropyl)cadaverine, which has the required site for acetylation, was a substrate as was 3,3-diamino-*N*-methyldipropylamine. The only

diamine tested that was a substrate was 1,3-diaminopropane. This is also consistent with the absolute requirement for an aminopropyl group attached to a nitrogen atom for reaction. It is also apparent that the reaction occurs best when this nitrogen atom is a secondary amino group, since the rates of reaction with 1,3-diaminopropane and 3,3-diamino-*N*-methyldipropylamine were much lower than with *sym*-norspermidine.

Neither of the *N*¹- and *N*⁸-acetyl isomers of monoacetylspermidine were substrates for the acetylase. In order to test these substances as potential substrates, it was necessary to use the extraction of products into butanol as the assay technique. This assay gave a much higher blank value ($500\text{--}600\text{ c.p.m.}$) compared with $80\text{--}120\text{ c.p.m.}$ in the paper disc method, but even in assays with additional amounts of enzyme no increment over the blank was observed. The lack of activity of *N*¹-acetylspermidine is not surprising since the aminopropyl group is already acetylated. However, *N*⁸-acetylspermidine has the required aminopropyl moiety and was the only compound tested with this structure that was inactive. Possibly the enzyme requires a free amino group at both ends of the substrate.

Inhibition of the acetylation of spermidine by the acetyltransferase

All of the compounds found to be inactive as substrates for the acetylase were tested as potential inhibitors of the reaction by addition at a concentration of 2 mM to assays containing 0.3 mM-spermidine. Only the acetylated spermidine derivatives were active as inhibitors (Table 2). As

Table 2. *Effect of potential inhibitors on acetylation of spermidine*

The substances shown were added to the standard assay mixture containing $8\mu\text{M}$ -acetyl-CoA and $300\mu\text{M}$ -spermidine. Results are shown as percentages of the formation of N^1 -acetylspermidine when no inhibitor was added.

Compound	Concentration	N^1 -Acetyl-spermidine formed (%)
None	—	100
Putrescine	2 mM	100
Cadaverine	2 mM	100
N^1 -Acetylspermidine	2 mM	81
N^8 -Acetylspermidine	2 mM	69
CoA	$36\mu\text{M}$	49
3'-Dephosphocoenzyme A	$36\mu\text{M}$	70
CoA methyl disulphide	$36\mu\text{M}$	21
3'-Dephospho-CoA methyl disulphide	$36\mu\text{M}$	79

Fig. 1. *Inhibition of spermidine N^1 -acetyltransferase activity by N^8 -acetylspermidine*

The assays were carried out in the presence of $8\mu\text{M}$ -acetyl-CoA and 0.1 mM - (●) or 0.3 mM -spermidine (■). The reciprocal of the initial velocity of reaction (v) is plotted as a function of the concentration of N^8 -acetylspermidine. Units of v are nmol of product/mg per min.

shown in Fig. 1, N^8 -acetylspermidine was a competitive inhibitor with respect to spermidine, having an apparent K_i of 0.4 mM . Table 2 also shows that the acetylation of spermidine was inhibited by CoA and a number of CoA derivatives. 3'-Dephospho-CoA was less inhibitory than CoA itself, whereas the disulphide derivative formed by the reaction of CoA with methyl methanethiosulphate was a more potent inhibitor.

Kinetic measurements and product inhibition studies

Double-reciprocal plots of the initial velocity data obtained with variable concentrations of spermidine

Fig. 2. *Effect of spermidine concentration on spermidine N^1 -acetyltransferase activity*

Assays were carried out in the presence of $2.4\mu\text{M}$ - (▼), $4.8\mu\text{M}$ - (●), $7.2\mu\text{M}$ - (■) and $9.6\mu\text{M}$ -acetyl-CoA (▲) and the reciprocal of the initial velocity of reaction (v) plotted against the reciprocal of the spermidine concentration as shown.

Fig. 3. *Inhibition of spermidine N^1 -acetyltransferase by CoA*

Assays were carried out in the presence of 0.4 mM -spermidine in the presence of 0 (▲), $9\mu\text{M}$ - (■), $18\mu\text{M}$ - (▼) and $27\mu\text{M}$ -CoA (●) and the reciprocal of the initial velocity of reaction (v) plotted against the reciprocal of the acetyl-CoA concentration as shown.

(ranging from $80\mu\text{M}$ to $1580\mu\text{M}$) at various fixed concentrations of acetyl-CoA (from $2.4\mu\text{M}$ to $9.6\mu\text{M}$) yielded a series of lines that intersected to the left of the vertical axis (Fig. 2). The same result was obtained when double-reciprocal plots of the initial velocities were made with acetyl-CoA as a variable substrate and spermidine as fixed substrate (results not shown).

Inhibition of acetylation of spermidine by fixed concentrations of CoA, with concentrations of acetyl-CoA varying from 2.4 to $9.6\mu\text{M}$ and at a non-saturating concentration of spermidine ($400\mu\text{M}$) produced changes in both slopes and intercepts of

double reciprocal plots (Fig. 3). A similar pattern (not shown), indicative of a non-competitive type of inhibition, was obtained when CoA was assayed as inhibitor at various concentrations of spermidine and fixed concentrations of acetyl-CoA. When the other product of reaction, *N*¹-acetylspermidine, was used as an inhibitor of the rate of reaction at various concentrations of spermidine and fixed concentrations of acetyl-CoA the double-reciprocal plots revealed changes in the slopes, but not in the intercepts, consistent with a competitive type of inhibition (Fig. 4). Finally, with varying concentrations of acetyl-CoA and fixed concentrations of spermidine, *N*¹-acetylspermidine appeared to be a non-competitive inhibitor (results not shown). These observations and the inhibition constants *K*_{is} and *K*_{ii} (Rudolph, 1979) obtained from these plots are summarized in Table 3. These observations are consistent with the mechanism indicated

Fig. 4. Inhibition of spermidine *N*¹-acetyltransferase by *N*¹-acetylspermidine
Assays were carried out in the presence of 4.8 μM-acetyl-CoA in the presence of 0 (▲), 1.9 mM- (■), 3.8 mM- (▼) and 5.7 mM-*N*¹-acetylspermidine (●) and the reciprocal of the initial velocity of reaction (*v*) plotted against the reciprocal of the spermidine concentration as shown.

in Fig. 5, which is an ordered Bi Bi mechanism with spermidine binding first and *N*¹-acetylspermidine as the last product released.

Discussion

The enzyme studied in the present experiments is clearly distinct from the histone acetylases, which also acetylate putrescine and polyamines (Libby, 1978*a*, 1980; Belikoff *et al.*, 1980; Wiktorowicz *et al.*, 1981; Wiktorowicz & Bonner, 1982; Cullis *et al.*, 1982). The spermidine *N*¹-acetyltransferase does not acetylate putrescine (Table 1) or histones (Della Ragione & Pegg, 1982). Also, the product when spermidine was used as a substrate was exclusively *N*¹-acetylspermidine (Della Ragione & Pegg, 1982), whereas the histone acetylases form predominantly *N*⁸-acetylspermidine when acting on spermidine (Blankenship & Walle, 1978; Matsui *et al.*, 1981). The selective formation of *N*¹-acetylspermidine is entirely consistent with the observed substrate specificity since the acetyl group is added to the aminopropyl moiety of spermidine and the aminobutyl end of the molecule is not attacked. Production of exclusively the *N*¹-isomer is also consistent with the postulated role of this enzyme in the interconversion of putrescine and polyamines. Polyamine oxidase cleaves the *N*¹-acetylated polyamines at the secondary nitrogen atom to yield 3-acetamidopropanal, whereas *N*⁸-acetylspermidine is not a substrate for polyamine oxidase and is deacetylated (Blankenship, 1978; Bolkenius & Seiler, 1981; Seiler *et al.*, 1981*b*).

Inhibitors of polyamine biosynthesis have been of great value in the investigation of the function of polyamines and may have considerable pharmacological importance [for review, see Pegg & McCann (1982) and Heby (1981)]. At present, there are no known specific inhibitors likely to be useful for interference with the interconversion of poly-

Table 3. Summary of product inhibition studies
The results of the studies described in Figs. 3 and 4 and in the text are shown.

Inhibitor	Variable substrate	Fixed substrate	<i>K</i> _{is}	<i>K</i> _{ii}	Type of inhibition
CoA	Acetyl-CoA	Spermidine	25 μM	3 μM	Non-competitive
CoA	Spermidine	Acetyl-CoA	13 μM	12 μM	Non-competitive
<i>N</i> ¹ -Acetylspermidine	Spermidine	Acetyl-CoA	6.7 mM	—	Competitive
<i>N</i> ¹ -Acetylspermidine	Acetyl-CoA	Spermidine	6.6 mM	14.6 mM	Non-competitive

Fig. 5. Proposed mechanism for spermidine *N*¹-acetyltransferase

amines via the acetylase/oxidase pathway and knowledge of the mechanism of the acetylase reaction should help in the rational design of such inhibitors. The strong inhibition by CoA, the product of the reaction, may be helpful in this respect. The 3'-phosphate group must aid in the binding of CoA since the 3'-dephospho-derivative was less inhibitory. More significantly, addition of a thiomethyl group via the formation of CoA methyl disulphide enhances the inhibition and *sym*-nor-spermidine is an excellent substrate with a very low K_m . These findings suggest that a stable multi-substrate analogue such as *N*-[2-(*S*-CoA)acetyl]nor-spermidine amide might prove to be a very potent inhibitor. The corresponding spermidine derivative, *N*-[2-(*S*-CoA)acetyl]spermidine amide has recently been found to inhibit histone acetylases with a K_i of less than 10 nM (Cullis *et al.*, 1982).

The present finding that spermidine *N*¹-acetyltransferase acts via an ordered Bi Bi reaction contrasts with the suggested, rapid-equilibrium ordered Bi Bi mechanism for rat liver histone acetyltransferase (Wiktorowicz *et al.*, 1981) and the rapid-equilibrium random Bi Bi mechanism of mitochondrial acetyl-CoA:L-glutamate *N*-acetyltransferase (Bachman *et al.*, 1982). It is apparent that despite the similarities in these reactions, the mechanism cannot be predicted by comparisons but must be determined experimentally.

This work was supported in part by grants GM-26290 and 1 P30 CA-18450 from the National Institutes of Health, Department of Health, Education and Welfare. We thank Mrs. Bonnie Merlino for the typing of this paper.

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