Studies of the acetyl-CoA-binding site of rat liver spermidine/spermine N^1 -acetyltransferase

Fulvio DELLA RAGIONE,* Bradley G. ERWIN and Anthony E. PEGG†

Department of Physiology and Specialized Cancer Research Center, The Milton S. Hershey Medical Center,
The Pennsylvania State University College of Medicine, P.O. Box 850, Hershey, PA, 17033, U.S.A.

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Rat liver spermidine/spermine N^1 -acetyltransferase was found to be strongly inhibited by the dyes Cibacron F3GA, Coomassie Brilliant Blue and Congo Red. Inhibition was competitive with respect to acetyl-CoA and K_i values of $0.7\ \mu\text{M}$ and $52\ \mu\text{M}$ were determined for Cibacron F3GA and Coomassie Brilliant Blue respectively. The enzyme was strongly retained by columns of Affi-Gel Blue, which contains Cibacron F3GA linked to agarose. It was not eluted from this adsorbent in the presence of 10 mM-spermidine/0.5 M-NaCl/50 mM-Tris/HCl, pH 7.5, but was released by 1 mM-CoA in 10 mM-spermidine/50 mM-Tris/HCl, pH 7.5. These results are consistent with the presence in the enzyme of a dinucleotide fold that binds acetyl CoA and has a high affinity for Cibacron F3GA. The spermidine/spermine N^1 -acetyltransferase was irreversibly inactivated by exposure to butane-2,3-dione in sodium borate, pH 7.8, or by exposure to phenylglyoxal or camphorquinone-10-sulphonic acid. All of these reagents are known to interact with arginine residues in proteins under the conditions in which they inactivated the acetyltransferase. Inactivation was prevented by the presence of acetyl-CoA or CoA, but to a lesser extent by 3'-dephospho-CoA and not at all by NAD or adenosine. This protection suggests that an arginine residue at the active site is involved in the binding of the acetyl-CoA substrate. Treatment of the assay mixture but not the spermidine N^1 -acetyltransferase with alkaline phosphatase prevented the reaction taking place. This suggests that the apparent loss of enzyme activity in response to alkaline phosphatase reported by Matsui, Otani, Kamei & Morisawa [(1982) *FEBS Lett.* **150**, 211–213] is due to dephosphorylation of the acetyl-CoA substrate and that the 3'-phosphate group is essential for activity.

A highly inducible and rapidly-turning-over acetyltransferase is the rate-limiting enzyme in the degradation of spermine to spermidine and of spermidine to putrescine (Matsui & Pegg, 1980; Matsui *et al.*, 1981; Seiler *et al.*, 1981; Pegg *et al.*, 1982). This degradation reverses the biosynthesis of spermine from spermidine and of spermidine from putrescine, which is catalysed by aminopropyltransferases using decarboxylated *S*-adenosylmethionine as the aminopropyl donor (Tabor & Tabor, 1976; Williams-Ashman & Pegg, 1981; Pegg & McCann, 1982). At present, the importance of the back reaction in the regulation of cellular polyamine

levels is not known and it is widely assumed that this content is regulated by the rate of the biosynthetic reactions. A more detailed understanding of the spermidine-spermine N^1 -acetyltransferase reaction and the development of specific inhibitors of its action may provide the means to investigate its role in controlling polyamine content. We have recently described the purification to homogeneity of this enzyme (Della Ragione & Pegg, 1982) and have investigated its substrate specificity and mechanism of action (Della Ragione & Pegg, 1983). In the present paper we have studied the interaction of the enzyme with several dyes that are competitive inhibitors with respect to acetyl-CoA and with dicarbonyl reagents that react with arginine residues in the protein. We have also examined the effects of treatment with alkaline phosphatase on the enzyme, since Matsui *et al.* (1982) have reported that a

* Present address: Department of Biochemistry, University of Naples 1st Medical School, Via Constantinopoli 16, 80138, Napoli, Italy.

† To whom correspondence and reprint requests should be addressed.

similar acetyltransferase from *E. coli* or bovine lymphocytes is inactivated by this enzyme.

Experimental procedures

Materials

Camphorquinone-10-sulphonic acid was obtained from Pierce Chemical Company, Rockford, IL, U.S.A. Affi-Gel Blue (Cibacron F3GA linked to agarose) was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. [$1\text{-}^{14}\text{C}$]Acetyl-CoA (sp. radioactivity 49.8 Ci/mol) was purchased from New England Nuclear, Boston, MA, U.S.A. All other biochemicals, including alkaline phosphatase (from calf intestine; type I; 1.4 units/mg), Cibacron F3GA, Coomassie Brilliant Blue R, Congo Red, butane-2,3-dione and phenylglyoxal, were products of Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation and assay of spermidine N^1 -acetyltransferase

This was carried out exactly as described by Della Ragione & Pegg (1983), except that in assays employing dyes as inhibitors, albumin was omitted from the assays. In the absence of albumin, the reaction time was reduced to 2 min in order to ensure a linear reaction rate as a function of time. The standard assay conditions contained 8 μM -acetyl-CoA and 3 mM-spermidine in a total volume of 0.1 ml.

Modification of spermidine N^1 -acetyltransferase by dicarbonyl reagents

The enzyme was dialysed against the desired buffer and 15 μl portions (about 0.1 unit) of the solution were equilibrated at 25°C for 3 min before the addition of 35 μl of reagent mixture containing inhibitors or protecting agents. The reaction was then allowed to proceed for up to 20 min at 25°C with 3 μl portions withdrawn at various intervals (starting immediately after mixing for the zero-time sample) to measure the progress of the reaction. These portions were added to 97 μl of the standard assay medium and activity was measured. A sample of enzyme was incubated in 50 μl of buffer alone and 3 μl portions were withdrawn at intervals to monitor any loss of activity during the incubation at 25°C. The kinetics of inactivation were estimated by plots of the logarithm of residual activity against time, which followed pseudo-first-order kinetics. In experiments where the protection from inactivation by various nucleotides was studied, the enzyme in 50 mM- NaHCO_3 , pH 7.8, was incubated with phenylglyoxal and the putative protecting agent in a total volume of 0.02 ml at 25°C for 20 min. The reaction was then halted by the addition of 0.18 ml of an ice-cold solution of 1 mg of bovine serum albumin/ml in the same buffer. The mixture was freed from

the protecting agent by dilution to 5 ml with 50 mM- NaHCO_3 , pH 7.8, and concentrated to 0.5 ml using an ultrafiltration cell. This procedure was repeated three times and 0.05 ml portions of the final 0.5 ml used for assay of residual spermidine N^1 -acetyltransferase activity. This method was used because it was more rapid and effective than prolonged dialysis (in which some enzyme activity was lost due to instability in dilute solution). All experiments included control tubes in which enzyme alone and enzyme plus phenylglyoxal alone were processed under the same conditions.

Effect of alkaline phosphatase on spermidine N^1 -acetyltransferase

These experiments were carried out with crude preparations of the enzyme from livers of rats treated with carbon tetrachloride (Matsui *et al.*, 1981). Approx. 0.15 mg of liver protein was used per assay. The extract was pre-incubated with alkaline phosphatase in a volume of 65 μl for 10 min at 30°C before addition of 35 μl containing the substrates and assay of activity in a further 10 min incubation. In parallel experiments the substrates were incubated with alkaline phosphatase in a volume of 77 μl for 10 min at 30°C before addition of the liver protein in a further 23 μl and assay of activity in a further 10 min incubation. Finally, in a third series of experiments the alkaline phosphatase was added to the complete assay medium with substrates and liver protein and the formation of N^1 -acetylspermidine measured in the standard 10 min assay at 30°C. In some cases, 10 mM-sodium pyrophosphate was added as indicated to inhibit the alkaline phosphatase. The presence of 10 mM-sodium pyrophosphate did not affect the acetylation when added to the normal assay mix.

Results

A number of enzymes utilizing acetyl-CoA have been shown to have a 'dinucleotide fold' that has a high affinity for dyes such as Cibacron F3GA (Stellwagen, 1977). These include choline acetyltransferase (Roskoski *et al.*, 1975; Currier & Mautner, 1977). Therefore, the interaction between spermidine N^1 -acetyltransferase and Affi-Gel Blue was studied. The enzyme in 50 mM-Tris/HCl (pH 7.5)/10 mM-spermidine was completely retained by a column of this adsorbent (Fig. 1). The acetyltransferase was not eluted from this column by the addition of 0.1 M- or 0.5 M-NaCl to the buffer and only appeared in the eluate when the NaCl concentration was raised to 2 M (Fig. 1a). In contrast, the enzyme was eluted by 1 mM-CoA, the product of the reaction (Fig. 1b). These results indicate that the binding to Affi-Gel Blue is likely to involve the active

Fig. 1. Binding of spermidine N^1 -acetyltransferase to Affi-Gel Blue

A column of Affi-Gel Blue (0.8 cm $\times$ 5 cm) was equilibrated with 50 mM-Tris/HCl (pH 7.5)/1 mM-spermidine and 0.5 ml of a solution of partially purified spermidine N^1 -acetyltransferase (1.5 mg of protein; about 4 units) was applied. The column was washed at a flow rate of 18 ml/h and 6 ml fractions were collected. In the experiments shown in (b), the column was eluted with 0.1 M-, 0.5 M- and 2 M-NaCl in the equilibration buffer as shown. In the experiment shown in (a), the column was eluted with 1 mM-CoA in the equilibration buffer. The A_{280} and enzymic activity in each fraction was measured as shown. The CoA was removed before assay by repeated dilution and concentration in 50 mM-Tris/HCl (pH 7.5)/10 mM-spermidine using an ultra-filtration cell.

Fig. 2. Effect of various dyes on acetylation of spermidine

The standard assay medium contained 3 mM-spermidine, 8 μ M-[1- 14 C]acetyl-CoA, 8 ng of enzyme and the inhibitors shown. The incubation time was 2 min. Results are shown as the percentage of the N^1 -acetylspermidine formed in the absence of inhibitor. Results are shown by inhibition by Cibacron F3GA (●), Congo Red (▲) and Brilliant Blue (■). The inset shows the effect of Cibacron F3GA at low concentration.

site of the enzyme rather than less specific ionic or hydrophobic interactions.

This observation was extended by testing the effects of Coomassie Brilliant Blue, Congo Red and Cibacron F3GA on the activity of spermidine N^1 -acetyltransferase (Fig. 2). All of these dyes were powerful inhibitors of the reaction in the standard assay mixture (which contains 8 μ M-acetyl-CoA), giving more than 70% inhibition at 50 μ M concentrations. Cibacron F3GA was the most potent, giving 50% inhibition at 0.5 μ M (Fig. 2). The inhibition by all of these dyes at up to 200 μ M concentrations was completely reversed by the addition of 100 μ g of bovine serum albumin, which has a high affinity for these dyes (results not shown). More detailed kinetic analysis of the inhibition by these dyes indicated that both Cibacron F3GA (K_i 0.7 μ M) and Coomassie Brilliant Blue (K_i 52 μ M) were competitive inhibitors with respect to acetyl-CoA (Fig. 3).

Fig. 3. Double-reciprocal plots showing inhibition by Cibacron F3GA (a) and Brilliant Blue (b) of spermidine N^1 -acetyltransferase

The assay medium contained 3 mM-spermidine, 8 ng of enzyme, [1- 14 C]acetyl-CoA and the amount of inhibitor shown. It was incubated for 2 min at 30°C and the reciprocal of the velocity (v) was measured as the amount of product formed (nmol of [1- 14 C]acetyl-CoA incorporated/mg per min) plotted against the reciprocal of the concentration of acetyl-CoA. Results are shown for 0 (▲), 0.5 μ M- (▼), 1 μ M- (■) and 2 μ M-Cibacron F3GA (●) in (a) and for 0 (▲), 100 μ M- (■) and 200 μ M-Brilliant Blue (●) in (b).

There is evidence based on spectral changes that Cibacron F3GA interacts directly with polyamines, particularly spermine (Subramanian, 1982). All of the assays showing powerful inhibition of acetylation by this dye were carried out in the presence of saturating (mM) concentrations of the polyamine substrate, and it is unlikely that the inhibition is mediated via alterations in the free polyamine concentration. It is, however, quite possible that the inhibitory agent is actually a polyamine: Cibacron F3GA complex.

A number of reagents that interact specifically with arginine residues in proteins under certain conditions are known (Riordan, 1979; Pande *et al.*, 1980) and the effects of some of these on spermidine *N*¹-acetyltransferase were examined. When incubated with the enzyme in 50 mM-Tris/HCl, pH 7.8, phenylglyoxal and camphorquinone-10-sulphonic acid led to a loss of activity, whereas butane-2,3-dione had little effect (Fig. 4). It is well known that the interaction of butane-2,3-dione with arginine in proteins is enhanced in the presence of borate (Riordan, 1979; Chang & Huang, 1981). As shown in Fig. 4, butane-2,3-dione did inactivate the spermidine *N*¹-acetyltransferase when incubated with the enzyme in 50 mM-sodium borate, pH 7.8, instead of the Tris/HCl buffer. Similarly, interaction of phenylglyoxal with arginine residues is facilitated in the presence of HCO_3^- (Riordan, 1979) and this reagent inactivated the enzyme more rapidly in the presence of 50 mM- NaHCO_3 , pH 7.8 (Fig. 4).

Fig. 4. Effect of various dicarbonyl reagents on acetylation of spermidine

The rat liver spermidine *N*¹-acetyltransferase was incubated alone (○), with 25 mM-phenylglyoxal (◆), 25 mM-camphorquinone-10-sulphonic acid (●) or 25 mM-butane-1,2-dione (■) in 50 mM-Tris/HCl, pH 7.8, with 10 mM-butane-2,3-dione in the presence of 50 mM-sodium borate, pH 7.8 (▲) or with 20 mM-phenylglyoxal in 50 mM- NaHCO_3 , pH 7.8 (▼). The activity remaining after the time indicated is shown as a percentage of that at the start of the incubation.

More detailed examination of the rate of inhibition of the acetylase by phenylglyoxal was carried out using a range of concentrations from 1 mM to 20 mM in 50 mM- NaHCO_3 , pH 7.8 (Fig. 5). A pseudo-first-order reaction was observed with a $t_{1/2}$ dependent on the concentration. Plots of the pseudo-first-order rate constant against the inhibitor concentration indicated an approximate second-order rate constant of about $1.7 \text{ M}^{-1} \cdot \text{min}^{-1}$. The inactivation was irreversible and activity could not be removed by extensive dialysis for 24 h with four changes of 50 mM-Tris/HCl (pH 7.8)/1 mM spermidine. The loss of activity could be prevented by the inclusion of acetyl-CoA or CoA in the buffer at the same time as the phenylglyoxal (Table 1). Acetyl-CoA was more

Fig. 5. Rate of inactivation of spermidine *N*¹-acetyltransferase by phenylglyoxal

Rat liver spermidine *N*¹-acetyltransferase was incubated with 1 mM- (▼), 5 mM- (●), 10 mM- (▲) or 20 mM-phenylglyoxal (■) in 50 mM- NaHCO_3 , pH 7.8, and the activity remaining after the time indicated plotted as the logarithm of the percentage activity remaining.

Table 1. *Effect of nucleosides on inhibition of spermidine N¹-acetyltransferase by phenylglyoxal*

The enzyme in 50mM-NaHCO₃, pH 7.8, was incubated at 25°C for 15 min in the presence of the concentration of phenylglyoxal shown and the nucleotide indicated. The remaining enzyme activity was then determined and expressed as a percentage of that found when no inhibitor was added during the 15 min incubation.

[Phenylglyoxal] (mM)	Addition	Activity remaining (% of control)
4	None	54
4	2.8 mM-Acetyl-CoA	104
4	2.8 mM-CoA	64
1	None	71
1	5 mM-CoA	101
1	5 mM-3'-Dephospho-CoA	89
1	10 mM-Adenosine	70
1	10 mM-NAD	73

potent (i.e., protected at a lower concentration) than CoA, which was in turn more active than 3'-dephospho-CoA. Adenosine and NAD did not protect the enzyme.

Recently, it has been suggested that spermidine N¹-acetyltransferase from bovine lymphocytes or bacteria may be a phosphoprotein because activity was lost on pre-incubation with alkaline phosphatase (Matsui *et al.*, 1982). As shown in Table 2 (Expt. 1), exposure to alkaline phosphatase did reduce the acetylation of spermidine by the rat liver enzyme but this effect could also be demonstrated by pre-incubation of the assay medium with the phosphatase. In fact, the assay medium was more sensitive to alkaline phosphatase than the enzyme. Pre-incubation of the former with 0.03 unit of alkaline phosphatase reduced activity by 45%, but pre-incubation of the enzyme with this amount of the phosphatase had no effect. Furthermore, when the enzyme was pre-incubated with alkaline phosphatase and pyrophosphate added along with the assay medium to prevent any effect on the substrate, there was no inhibition (Table 2, Expt. 2). Therefore, it appears likely that the inhibitory effect of the alkaline phosphatase is due to the removal of the 3'-phosphate from the acetyl-CoA substrate rather than to an effect on the enzyme itself.

Discussion

The powerful inhibition of spermidine N¹-acetyltransferase activity by dyes such as Cibacron F3GA and Congo Red and the strong affinity of the enzyme for Affi-Gel Blue suggest that the enzyme contains a 'dinucleotide fold' region, which is well

Table 2. *Effect of alkaline phosphatase on spermidine N¹-acetyltransferase activity*

The experiments were carried out as described in the Experimental procedures section by pre-incubating the enzyme or assay mix with alkaline phosphatase as shown. Such pre-incubation of the enzyme or assay mix in the absence of phosphatase had no effect on the activity. Results are expressed as the percentage of the activity found when no pre-incubation or addition of phosphatase was carried out.

Addition to reaction	Spermidine N ¹ -acetyltrans- ferase activity (% of control)
Expt. 1	
Normal assay	100
Pre-incubated assay mix with 0.3 unit of alkaline phosphatase	0
Pre-incubated assay mix with 0.03 unit of alkaline phosphatase	55
Pre-incubated enzyme with 0.3 unit of alkaline phosphatase	25
Pre-incubated enzyme with 0.03 unit of alkaline phosphatase	114
Added 0.3 unit of alkaline phosphatase directly to assay	20
Added 0.03 unit of alkaline phosphatase directly to assay	107
Expt. 2	
Normal assay	100
Added 0.3 unit of alkaline phosphatase directly to assay	12
Added 0.15 unit of alkaline phosphatase directly to assay	25
Added 0.3 unit of alkaline phosphatase plus 10 mM-sodium pyrophosphate to assay	55
Added 0.15 unit of alkaline phosphatase plus 10 mM-sodium pyrophosphate to assay	91
Preincubated enzyme with 0.3 unit of alkaline phosphatase; then added 10 mM-sodium pyrophosphate to assay	49
Preincubated enzyme with 0.15 unit of alkaline phosphatase; then added 10 mM-sodium pyrophosphate to assay	98

documented for many other enzymes, including those having specificities for FAD, NAD/NADP, ATP and acyl-CoA (Stellwagen, 1977; Thompson *et al.*, 1975). The competitive nature with respect to acetyl-CoA of this inhibition and the release of the spermidine N¹-acetyltransferase from Affi-Gel Blue in the presence of CoA but not with 0.5 M-NaCl also support the idea that this region is present as part of the active site of the enzyme (Thompson *et al.*, 1975). In these respects the spermidine N¹-acetyl-

transferase shows considerable similarity to choline acetyltransferase, which is inhibited by aromatic dyes and binds to Sepharose linked to these dyes (Roskoski *et al.*, 1975; Mautner *et al.*, 1981). However, it should be noted that the original concept that Cibacron F3GA is a specific probe for the dinucleotide fold (Thompson *et al.*, 1975) has not been confirmed in all cases. There are a significant number of exceptions in which binding does not occur with proteins containing this region or in which binding to a dye affinity column and release by a physiological ligand occurs even when the dinucleotide fold does not exist (Ashton & Polya, 1978; Beissner *et al.*, 1979; Reisler & Liu, 1981; Clonis *et al.*, 1981).

X-ray crystallographic studies have shown that the binding of Cibacron Blue F3GA to alcohol dehydrogenase involves an interaction between the dye and an arginine residue in this fold (Biellmann *et al.*, 1979). Arginine residues serve a general role as anionic binding sites in enzymes (Riordan, 1979) and choline acetyltransferase is known to be inhibited by arginine-specific reagents (Mautner *et al.*, 1981). The present finding that spermidine *N*¹-acetyltransferase is inactivated by camphor-quinone-10-sulphonic acid, phenylglyoxal and by butane-2,3-dione in the presence of borate provides strong evidence for an essential arginine residue in this enzyme since these reagents form specific adducts with arginine under the conditions used (Riordan, 1979; Pande *et al.*, 1980; Chang & Huang, 1981; Fortin *et al.*, 1981; Wong & Wong, 1981). Since the inactivation was prevented by acetyl-CoA or CoA, it is probable that this arginine residue is located at the active centre of the 'dinucleotide fold'. The weaker protection by 3'-dephospho-CoA is in accordance with this concept because this derivative is a less potent inhibitor of the enzyme than CoA (Della Ragione & Pegg, 1983) and implies that the 3'-phosphate group may be involved in the binding. The importance of the 3'-phosphate group of acetyl-CoA in the reaction is in agreement with other work on the specificity of the spermidine *N*¹-acetyltransferase (Della Ragione & Pegg, 1982, 1983) and provides an explanation for the apparent inactivation of the enzyme in response to alkaline phosphatase that was observed by Mautner *et al.* (1982). These workers suggested that the apparent rapid rate of turnover of the enzyme (Matsui & Pegg, 1980) may be due to dephosphorylation converting the enzyme into an inactive form but our results with the rat liver enzyme do not support this interpretation.

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