Choline Acetyltransferase: Reversible Inhibition by Bromoacetyl Coenzyme A and Bromoacetylcholine†

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ABSTRACT: Choline acetyltransferase (EC 2.3.1.6) catalyzes the following reversible reaction: acetyl coenzyme A + choline ⇌ acetylcholine + coenzyme A. Bromoacetyl-CoA and bromoacetylcholine are potent inhibitors of the bovine brain enzyme. Although the enzyme is inactive immediately following Sephadex gel filtration to remove unreacted inhibitor, enzyme activity returns to normal in 1 day at 0°. When the enzyme is incubated with bromo[^{14}C]acetylcholine and the reaction mixture is passed through a Sephadex G-50 column, radiolabel is associated with the protein in the eluent. When bromoacetyl[^{14}C]choline is used, substantially less label is associated with the eluent protein. The bond between the bromoacetyl group and the enzyme shows the characteristics of a thio ester. Kinetic studies indicate that bromoacetyl-CoA is a competitive inhibitor with respect to acetyl-CoA, and bromoacetylcholine is competitive with respect to acetylcholine. These experiments are consistent with the notion that the bromoacetyl derivatives acylate the active site sulfhydryl group and thereby inhibit the enzyme.

Choline acetyltransferase (EC 2.3.1.6) catalyzes the reversible transfer of the acetyl group from acetyl-coenzyme A to choline. Previous studies are consistent with the hypothesis that an active-site sulfhydryl reacts with acetyl-coenzyme A to form an acetyl-thioenzyme intermediate and coenzyme A (Roskoski, 1973). Choline then reacts with the acetyl-enzyme to form acetylcholine and the regenerated enzyme. Consonant with the notion of an active-site sulfhydryl, thiol reagents inhibit choline acetyltransferase prepared from squid head ganglia (Reisberg, 1954), primate placenta (Schuberth, 1966), torpedo (Morris, 1967), and mammalian brain (Potter *et al.*, 1968; Chao and Wolfgram, 1973). However, the bovine brain enzyme is protected from *N*-ethylmaleimide inactivation at high substrate concentrations during turnover, but becomes susceptable to inactivation when the concentration of acetyl-coenzyme A (but not choline) is lowered (Roskoski, 1974). This suggests that the rate-limiting step during turnover is the reaction between choline with the acetyl-enzyme.

Chase and Tubbs (1969) reported that pigeon breast muscle carnitine acetyltransferase (EC 2.3.1.7) is inhibited by bromoacetyl-CoA (in the presence of carnitine) or by bromoacetylcarnitine (in the presence of CoA). They showed that a slowly dissociable *S*-carboxymethyl-CoA carnitine ester produced this inhibition. These workers subsequently showed that bromoacetylcarnitine irreversibly inhibited the enzyme by alkylation of a single histidine residue (Chase and Tubbs, 1970). Morris and Grewaal (1971) then reported that the human placental choline acetyltransferase is inhibited by bromoacetylcholine and analogous halogenated choline derivatives. The reversible nature of this inhibition led these workers to the conclusion that this inactivation does not involve alkylation of the enzyme.

Bromoacetyl-CoA and bromoacetylcholine have been found to inhibit the bovine brain choline acetyltransferase in the absence of added substrate. Following Sephadex gel filtration to remove the unreacted inhibitor, enzyme activity approaches control values within 1 day ($t_{1/2}$ = 6 hr). A bromoacetyl-enzyme derivative was isolated by Sephadex gel filtration using labeled bromo[^{14}C]acetylcholine and enzyme. The bromoacetyl-enzyme link shows the characteristics of a thio ester. The kinetic data show that bromoacetyl-CoA is a competitive inhibitor with respect to acetyl-CoA and bromoacetylcholine is competitive with respect to acetylcholine. These data are consistent with the hypothesis that the bromoacetyl derivatives react with the active site -SH forming a thio ester and thereby inhibit the enzyme. The reactivation of the enzyme is apparently due to spontaneous hydrolysis of this bond.

Experimental Section

Materials. [1-^{14}C]Acetate (sodium salt) (59 Ci/mol) was purchased from New England Nuclear Corp. Bromo[1-^{14}C]-acetate (55 Ci/mol) and [*methyl*-^{14}C]choline (60 Ci/mol) were products of Amersham/Searle Corp. Bromoacetyl bromide was obtained from Pfaltz and Bauer, Inc.

Bromoacetylcholine perchlorate was prepared by the procedure of Chiou and Sastry (1968) and bromoacetyl-CoA, Chase and Tubbs (1969).

To prepare the bromo[^{14}C]acetylcholine, 1 μmol of bromo-[^{14}C]acetate, 5 μmol of choline chloride, and 5 μmol of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (Sigma Chemical Co.) were incubated at ambient temperature for 24 hr in 60 μl of water. The reaction mixture was applied to a Dowex 50-X8 (H^+) column, washed, eluted, and concentrated as previously described (Roskoski, 1973). The final yield was 36%.

To prepare bromoacetylcholine, 7.5 μmol of bromoacetyl bromide was added to 5 μmol of labeled choline in 50 μl of pyridine at 0°. After 2 hr, the reaction mixture was applied to a Dowex 50-X8 (H^+) column as described above. The yield was greater than 90%.

Methods. The bovine brain choline acetyltransferase was prepared as previously described (Roskoski, 1973). Campagnari and Webster's procedure (1963) for preparing bovine heart acetate thiokinase (EC 6.2.1.1) was used to prepare the enzyme, to the first ammonium sulfate step, from bovine

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TABLE I: Bromoacetyl-CoA and Bromoacetylcholine Inhibition of Choline Acetyltransferase.[a]

Inhibitor	Concn (M)	Enzyme Activity (% Control)
Bromoacetyl-CoA	10^{-7}	9
	10^{-8}	79
Bromoacetylcholine	10^{-5}	10
	10^{-6}	32
	10^{-7}	86
	10^{-8}	100

[a] Enzyme activity was assayed by the formation of labeled acetylcholine from [^{14}C]acetyl-coenzyme A as previously described (Roskoski, 1973) in the presence of the specified inhibitor concentration. Enzyme was added to the incubation mixture to start the reaction. The control was 259 pmol for the 5-min incubation at 37°

brain. The specific activity of the choline acetyltransferase in this preparation was greater than that of the acetate thiokinase.

Assay of ATP Dependent Acetyl-CoA and Acetylcholine Biosynthesis. The incorporation of radioactive acetate into acetyl-CoA or acetylcholine or the incorporation of labeled bromoacetate into its corresponding derivatives is the basis for the assay. The final incubation mixture contained 100 μM labeled acetate, 100 μM coenzyme A, 25 mM morpholinopropanesulfonic acid (Sigma Chemical Co.), 1 mM ATP, 5 mM $MgCl_2$, 5 mM choline chloride, 0.1 mM eserine sulfate, 1% 1-butanol, and 100 μg of enzyme extract at pH 7.4 in 20 μl. Incubations were for 15 min at 37°. Choline was omitted when acetyl-CoA biosynthesis was measured. The reaction was stopped by the addition of 10 μl of 1 N formic acid. Labeled acetyl-CoA was resolved from precursor by PEI-cellulose thin-layer chromatography and labeled acetylcholine was resolved by paper electrophoresis as previously described (Roskoski, 1973). The corresponding bromoacetyl derivative standards comigrated with the acetyl derivatives in these systems.

The radiochemical enzyme assays and determinations, isolation of enzyme–substrate intermediates by Sephadex G-50 gel filtration, treatment of enzyme–substrate complexes by trichloroacetic acid precipitation, alkaline hydrolysis, hydroxylaminolysis, performic acid oxidation, and thin-layer chromatographic systems were previously described (Roskoski, 1973).

Results

General Characteristics of Enzyme Inhibition. Bromoacetyl-CoA and bromoacetylcholine proved to be potent inhibitors of choline acetyltransferase (Table I). These compounds inhibit the enzyme more than 90% at a concentration two orders of magnitude less than the apparent K_m's of their substrate analogs [acetyl-CoA, $K_m = 10^{-5}$ M; acetylcholine, $K_m = 10^{-3}$ M (Glover and Potter, 1971)]. The addition of bromoacetyl-CoA (10^{-7} M) or bromoacetylcholine (10^{-5} M) to the enzyme at 0°, followed immediately (15 sec) by Sephadex gel filtration (45 min) to remove the low molecular weight compounds, inhibits activity about 80%. In contrast to the carnitine acetyltransferase (Chase and Tubbs, 1969), the alternate substrate is not required for this inactivation. The decreased enzyme activity immediately following Sephadex gel filtration at first suggested that these compounds irreversibly inhibit the enzyme. However, when the Sephadex eluent is assayed the following day, the enzyme activity returns to normal (Table II). There is a steady increase in activity with a half-time of about 6 hr. These experiments argue that the mechanism of bromoacetyl-CoA and bromoacetylcholine inhibition does not involve the alkylation of an active-site SH, or imidazole group, which would be expected to be irreversible.

TABLE II: Reversibility of the Bromoacetyl-CoA and Bromoacetylcholine Inhibition.[a]

	Enzyme Activity (nmol/5 min)	
	0 hr	24 hr
Control	9.3	9.1
Bromoacetyl-CoA	1.6	9.5
Bromoacetylcholine	2.1	9.4
Bromoacetyl-CoA and *N*-ethylmaleimide[b]	1.8	8.7
Bromoacetylcholine and *N*-ethylmaleimide[b]	2.0	9.2
N-Ethylmaleimide	2.1	1.8

[a] The enzyme extract (250 μg) was incubated with bromoacetyl-CoA (10^{-7} M) or bromoacetylcholine (10^{-5} M) for 5 min at 37° in 100 μl of buffer B (50 mM potassium phosphate–100 mM KCl–0.1 mM EDTA (pH 7.4)). After chilling on ice, the mixture was passed through a Sephadex G-50 (fine) column as previously described (Roskoski, 1973) and 1-ml fractions were collected. Then 10-μl aliquots of the enzyme-containing fractions were assayed for choline acetyltransferase. The fractions were stored on ice for 22–26 hr, then assayed again. The results are expressed as total enzyme activity recovered in the eluent. [b] The enzyme was incubated with the bromoacetyl-CoA and bromoacetylcholine for 2 min at 37°. Then 1 μl of *N*-ethylmaleimide was added to give a final concentration of 10^{-4} M and the incubation was continued an additional 5 min prior to Sephadex gel filtration.

Isolation of the Complex Formed by the Enzyme and Inhibitor by Sephadex Gel Filtration. When bromo[^{14}C]acetylcholine is incubated with the partially purified choline acetyltransferase, and the reaction mixture is chilled and passed through a Sephadex G-50 column, radioactivity is associated with the enzyme protein in the eluent (Figure 1). An equivalent amount of radioactivity is associated with the eluent protein using [^{14}C]acetyl-CoA as previously described (Roskoski, 1973, 1974). On the other hand, when bromoacetyl[^{14}C]choline is incubated with the enzyme extract, less than 10% as much labeled choline is associated with the protein eluent (Figure 1). The enzyme activity in the eluent is inhibited about 85% by each of these radioactive compounds (data not shown). The enzyme extract forms a complex rather specifically with the bromoacetyl group, and not with the choline group. This is consistent with the isolation of a bromoacetyl-enzyme complex, but not with a bromoacetylcholine–enzyme complex.

Properties of the Bromoacetyl-Enzyme Link. A thio ester link between the bromoacetyl group and enzyme, analogous to the postulated acetyl-thioenzyme intermediate (Roskoski, 1973), is a plausible alternative to an irreversible alkylation of an enzymic imidazole or thiol. The bromo[^{14}C]acetyl-enzyme complex was isolated by Sephadex gel filtration and

the bond was identified as a thio ester using the methodology previously given (Roskoski, 1973). Summarizing, the bond between the bromo[^{14}C]acetyl group and protein is not cleaved by 10% trichloroacetic acid (90°, 20 min) nor by treatment with 6 M guanidinium chloride. On the other hand, treatment with 3 M hydroxylamine (pH 5.7) quantitatively liberates the radiolabel. Moreover, the radioactivity liberated by dilute alkali (pH 10) comigrated with bromoacetate on silica gel thin-layer chromatograms using procedures previously given (Roskoski, 1973). Bromoacetate, which has an R_f of 0.3 compared with acetate (R_f 0.6), was identified. These results are consistant with the hypothesis that the bromoacetyl group is linked to enzyme protein as thio ester. To substantiate this idea, the bromo[^{14}C]acetyl-enzyme was subjected to performic acid oxidation. Thio esters are cleaved by this procedure, but oxygen esters are not (Harris *et al.*, 1963). The protein bound radioactivity was quantitatively cleaved by performic acid oxidation and it comigrated with standard bromoacetate on the silica gel thin layers.

Bromoacetyl-CoA and Bromoacetylcholine Protection against N-Ethylmaleimide Inactivation. Previous studies have shown that choline acetyltransferase is inactivated by thiol reagents such as *N*-ethylmaleimide and that the acetyl donor substrates, acetyl-CoA and acetylcholine, protect against this inactivation (Roskoski, 1974). If bromoacetyl-CoA and bromoacetylcholine also form an analogous thio ester intermediate, then they ought to protect against thiol reagent inactivation. The enzyme was first incubated with the bromoacetyl donor substrates and then with *N*-ethylmaleimide. Sephadex gel filtration was used to remove these low molecular weight reagents. The eluent protein was assayed immediately and again after 1 day. The results in Table II show that the enzyme activity is decreased by bromoacetyl-CoA, bromoacetylcholine, and *N*-ethylmaleimide immediately following gel filtration. After 1 day, enzyme activity returned to the control level in the enzyme samples incubated with the bromoacetyl donor substrates, but not in the untreated sample. This provides evidence that the two bromoacetyl donors acylate the same group and, furthermore, this is the group with which the acetyl-CoA and acetylcholine react.

Kinetic Analysis of Bromoacetyl-CoA and Bromoacetylcholine Inhibition of Choline Acetyltransferase. Double reciprocal plots of the substrate concentration and initial velocity were made in the presence of increasing concentrations of inhibitor. For the forward reaction (acetyl-CoA and choline), bromoacetyl-CoA was competitive with respect to acetyl-CoA and noncompetitive with respect to choline (Figure 2). The secondary plots were linear with bromoacetyl-CoA concentrations from 5×10^{-9} to 10^{-7} M. Bromoacetylcholine was a linear noncompetitive inhibitor with respect to both substrates in the forward direction (data not shown). For the reverse reaction (acetylcholine and CoA), bromoacetylcholine was a competitive inhibitor with respect to acetylcholine and a noncompetitive inhibitor with respect to coenzyme A. The secondary plots were linear with bromoacetylcholine concentrations from 5×10^{-7} to 10^{-5} M. These kinetic data substantiate the impression that the observed inhibition is rather specific and involves residue(s) in the active site.

Attempts to Biosynthesize Bromoacetyl-CoA and Bromoacetylcholine in Vitro. The present studies demonstrate that bromoacetyl-CoA and bromoacetylcholine are potent inhibitors of choline acetyltransferase. These compounds would be promptly inactivated by specific and nonspecific esterases when injected into animals. Specific choline acetyltransferase inhibition *in vivo* would require the generation of bromoacetyl-CoA as well as the requisite substrate specificity for succeeding biochemical reactions (Tucek, 1970). Therefore, attempts were made to synthesize bromoacetyl-CoA and bromoacetylcholine using enzyme extracts prepared from bovine brain. The extracts were active in the ATP dependent biosynthesis of [^{14}C]acetyl-CoA and [^{14}C]acetylcholine (Table III). The incorporation of labeled acetate into these products was decreased by the addition of unlabeled acetate, but not by the addition of bromoacetate or fluoroacetate. This suggests that bromoacetate is not an effective substrate for acetate thiokinase. Furthermore, it was not possible to demonstrate the biosynthesis of bromo[^{14}C]acetyl-CoA using labeled bromoacetate as precursor in this system. The methods would detect about 1% of the activity compared with acetate substrate. These data argue that bromoacetate would not be an effective precursor of bromoacetyl-CoA *in vivo* and therefore predict no affect on the choline acetyltransferase reaction. However, Brady (1955) was not able to show that fluoroacetate is a substrate for acetate thiokinase *in vitro*, although it is metabolized to fluorocitrate *in vivo* (Peters, 1957).

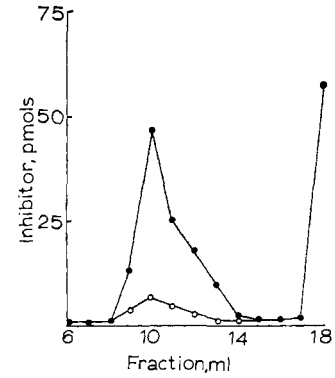

FIGURE 1: Formation of bromo[^{14}C]acetyl-enzyme from bromo-[^{14}C]acetylcholine and choline acetyltransferase. The reaction mixture, which contained 0.5 mg of protein, was incubated with 10^{-4} M bromo[^{14}C]acetylcholine or bromoacetyl[^{14}C]choline for 5 min at 37° in buffer B. After chilling on ice, Sephadex gel filtration and elution were carried out as previously documented (Roskoski, 1973): (●) bromo[^{14}C]acetylcholine precursor; (○) bromoacetyl[^{14}C]choline precursor.

Discussion

The bromoacetylcholine inhibition of the bovine brain choline acetyltransferase parallels the halogenated acetyl-

TABLE III: ATP Dependent Acetyl-CoA and Acetylcholine Bioformation.[a]

Labeled Precursor	Product (pmoles)	
	Acetyl-CoA	Acetyl-choline
[^{14}C]Acetate, 100 μM control	105	124
−ATP	0	0
+bromo acetate, 1 mM	112	118
+fluoro acetate, 1 mM	102	116
+unlabeled acetate, 1 mM	14	18
Bromo[^{14}C]acetate, 100 μM control	0.1	0.2

[a] Bovine brain enzyme extract containing acetate thiokinase and choline acetyltransferase was prepared and assayed by the methodology outlined in the Experimental Section.

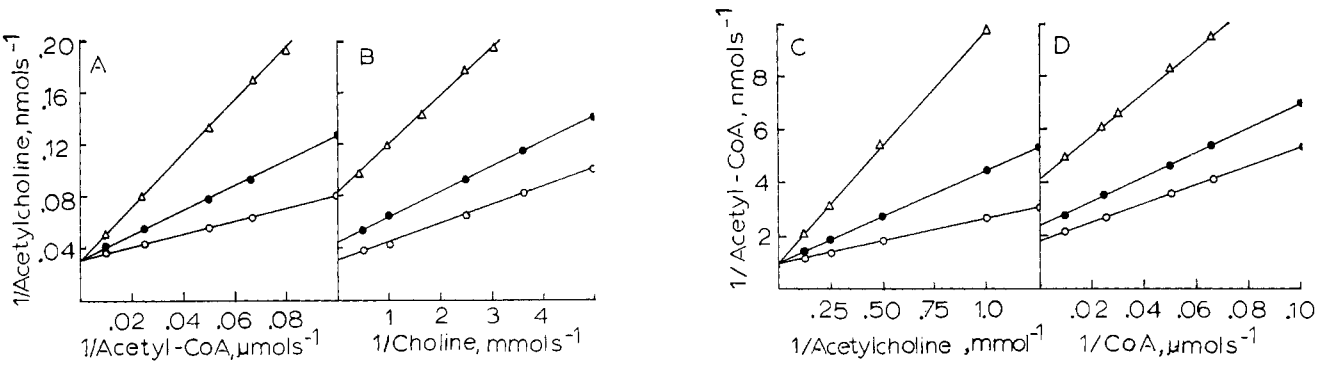

FIGURE 2: Double reciprocal plots of bromoacetyl-CoA and bromoacetylcholine inhibition of choline acetyltransferase. (A and B) Initial rates of [^{14}C]acetylcholine synthesis were carried out radiochemically using 2.5 μg of enzyme per 5-min assay as previously described (Roskoski, 1973). (C and D) Initial rates of [^{14}C]acetyl-CoA synthesis using 15 μg of protein per 15 min assay were also carried out as previously described (Roskoski, 1973) using PEI-cellulose to resolve the labeled product from the precursor control A and B: (○) control; bromoacetyl-CoA (●) 5×10^{-9} M; (△) 10^{-8} M. C and D: (○) control; bromoacetylcholine (●) 2.5×10^{-5} M; (△) 5×10^{-5} M.

choline inhibition of the placental enzyme reported by Morris and Grewaal (1971). In each case less than 10^{-4} M bromoacetylcholine inhibits the enzyme more than 90%. In addition, the rate of inactivation is equally rapid, and the enzyme kinetic analysis are similar. Inhibition of the brain enzyme and placental enzyme is reversed by gel filtration or dialysis, respectively. However, the rate of reactivation is more rapid for the placental enzyme (Morris and Grewaal, 1971). These similarities suggest that the placental enzyme is bromoacetylated and inactivated in a manner similar to that proposed here for the brain enzyme. The 3-bromoacetonyltrimethylammonium halide inhibition of the placental enzyme was not reversed by dialysis (Morris and Grewaal, 1971), and suggests in this case an inactivation by alkylation.

Yeast 3-hydroxy-3-methylglutaryl-CoA synthetase (EC 4.1.3.5) is also rapidly inactivated by low concentrations of bromoacetyl-CoA (Middleton and Tubbs, 1972). Acetyl-CoA protects against this inactivation. The enzyme exhibits parallel line kinetics which indicates that the enzyme reaction proceeds by a two-stop chemical mechanism (Middleton, 1972). Moreover, the enzyme is inhibited by thiol reagents and is substantially protected from inhibition by acetyl-CoA. On the basis of their experiments, Middleton and Tubbs (1972) propose that the reaction proceeds through an acetyl-enzyme intermediate. The similarity of these results and those reported here and previously (Roskoski, 1973, 1974) for the bovine brain choline acetyltransferase suggests that their enzyme reaction may involve a thio ester intermediate and that the bromoacetyl-CoA inhibition may involve acylation of this postulated thiol group.

On the other hand, the bromoacetyl-CoA and bromoacetylcholine inhibition of the choline acetyltransferase differs from the corresponding inhibition of the carnitine acetyltransferase. In the latter case, bromoacetyl-CoA and carnitine (or bromoacetylcarnitine and CoA) inhibit reversibly by forming a very slowly dissociating ($t_{1/2}$ = 14.8 days) enzyme bound *S*-carboxymethyl coenzyme A carnitine ester (Chase and Tubbs, 1969). The alternate substrate is not required for the choline acetyltransferase inhibition reported here. Bromoacetylcarnitine also irreversibly inhibits the carnitine acetyltransferase by the alkylation of an enzymic histidine residue in the active site (Chase and Tubbs, 1970).

The reaction of bromoacetyl-CoA and bromoacetylcholine with the choline acetyltransferase is rather specific. The large bromo group does not prevent the bromoacetyl group from entering the active site. The observation that propionyl-CoA is a good enzyme substrate (Potter, 1971) also indicates that the catalytic site can accommodate these larger acyl derivatives.

In the absence of choline or acceptor substrate, enzyme activation is apparently due to the spontaneous hydrolysis of the thio ester link. The reversible nature of the inhibition argues against significant alkylation of residues in the active site. One possible target is the postulated active site sulfhydryl which mediates the acetyl transfer. Instead of alkylation, the enzymic SH forms a thio ester with these inactivating compounds. In a somewhat similar manner, bromoacetylcholine, which is a substrate and not an inhibitor of bovine erythrocyte acetylcholinesterase (EC 3.1.1.7) (Chiou and Sastry, 1968), forms an oxygen ester link with an enzymic serine hydroxyl. The acetyl group, and presumably the bromoacetyl analog, is transfered to water to complete the hydrolytic reaction (Froede and Wilson, 1971).

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