EVIDENCE FOR A THIOL REAGENT INHIBITING CHOLINE ACETYLTRANSFERASE BY REACTING WITH THE THIOL GROUP OF COENZYME A FORMING A POTENT INHIBITOR

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Summary: Evidence is presented for the thiol reagent methyl methanethiolsulfonate inhibiting choline acetyltransferase (EC 2.3.1.6), not by reaction with an enzymic thiol group, but by reaction with the thiol group of CoA. The resulting CoA methyl disulfide is a potent inhibitor of this enzyme. Its action is reversed competitively by acetyl CoA.

Introduction

Choline acetyltransferase, the enzyme responsible for the transfer of the acetyl group of acetyl CoA to choline, has since its discovery by Nachmansohn and Machado (1) been studied extensively. Recently, it was suggested (2) that general-base catalysis by an imidazole residue enhances the nucleophilicity of the hydroxy group of enzyme bound choline enabling it to react with a thiolester group. However, it remained unclear whether this reaction involved the thiolester group of enzyme-bound acetyl CoA or an acetylthio-enzyme intermediate.

It has been known for a long time that choline acetyltransferase is inhibited by thiol reagents (3,4,5), sensitivity varying widely in enzymes isolated from different species. As evidence for the involvement of an enzymatic thiol group in the transfer of the acetyl group of acetyl CoA to choline, with deacylation of the acetylthio-enzyme being the rate limiting step, Roskoski (6), using bovine brain enzyme has cited the following observations: the enzyme can be protected against thiol reagents by acetyl CoA, acetylcholine and acetylthiocholine, and an

acetylthio-enzyme can be isolated which is capable of acetylating either CoA or choline. Mannervik and Sörbo (7), using the same enzyme could not protect against inhibition by DTNB with acetylcholine but were able to protect to some degree with acetyl CoA.

Working with choline acetyltransferase isolated from the head ganglia of squid (8), an enzyme known to be unusually sensitive to inhibition by thiol reagents (3), we found acetyl CoA, but not choline or acetylcholine to provide some protection against inhibition by DTNB (unpublished data). With the squid enzyme an acetylthio-enzyme intermediate could not be isolated under the conditions of Roskoski (2). Since it appeared possible that a thiol group near but not at the active site might be reacting, we investigated the inhibitory action of a thiol reagent methyl methanethiolsulfonate (MMTS) (9). This reagent by reacting with an enzyme thiol would attach a methylthio rather than a larger group to the postulated SH residue. This would minimize steric interference with the substrates and products of this enzyme. MMTS proved to be a very powerful inhibitor but, because preincubation with acetyl CoA and acetylcholine did not protect against inhibition, we considered the possibility of another mode of inhibition by this reagent: reaction of MMTS with the thiol group of enzyme-formed CoA yielding an inhibitor.

Materials and Methods

Choline acetyltransferase was isolated from squid ganglia and purified (8) to an activity of 2.55 μmoles/min./mg. protein with a protein concentration of 8.0 mg./ml. The enzyme was assayed by the procedure of Fonnum (10) and diluted 2,000 fold into 0.1M sodium phosphate (pH 7.4), 1mM EDTA, and 2 mg/ml lysozyme. K_m and K_i values were calculated from Lineweaver-Burk plots. The time study, Figs. 1 and 2, involved an aliquot method, extracting 20 μl samples at one minute intervals. Incubation temperature was 15°C and final concentrations were: AcCoA 155 μM, choline 10 mM, and NaCl 150 mM.

Reagents

Methyl methanethiolsulfonate (MMTS) was synthesized by the procedure of Smith et al. (9).

TABLE I

Binding of Substrates and Inhibitors to Squid Choline Acetyltransferase

	K_m	K_i
Acetyl CoA	47×10^{-6}M	
Choline	1880×10^{-6}M	
CoA		$7{,}500 \times 10^{-6}$M
MMTS		8×10^{-6}M
Dethia CoA		$7{,}800 \times 10^{-6}$M
CoA methyl disulfide		2×10^{-6}M

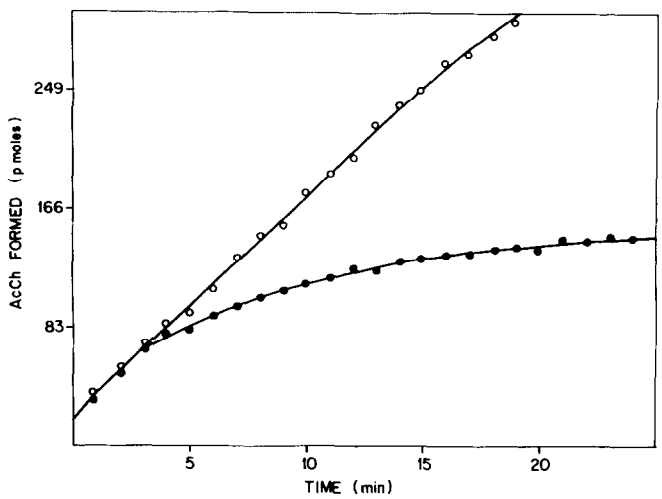

Figure 1 Inactivation of choline acetyltransferase o - o Control, • - • 2.5 x 10^{-6}M MMTS

CoA methyl disulfide was prepared by the reaction of 25 mg. (31.2 µmoles) of CoA dissolved in 0.6 ml of water with 25 mg. (198 µmoles) of MMTS. After stirring the mixture at room temperature for several hours, the solution was passed through a Sephadex column which had been equilibrated with water. Product was monitored by absorbance at 260 nm, active fractions were pooled and evaporated to dryness in vacuo at 35°C. A yield of 15.5 mg (61%) of white, microcrystalline product was obtained. Synthesis of CoA methyl disulfide using ^{14}C-methyl methanethiolsulfonate (.518 µCi/µmole) yielded CoA-^{14}C-methyl disulfide with the same specific radioactivity. Anal.: $C_{22}H_{36}Li_2P_3N_7O_{16}S_2.4H_2O$ found: C, 29.71; H, 4.23; N, 10.71 theory: C, 29.44; H, 4.94; N, 10.92

Dethia-CoA was prepared by the procedure of Chase and Tubbs (11).

Results

As can be seen in Table I, MMTS and, even more so, CoA methyl disulfide are very potent inhibitors of choline acetyltransferase. Both are reversed competitively by acetyl CoA. Dethia CoA is a much poorer inhibitor, its K_i being similar to that of CoA. The observation that the inhibitory action of MMTS shows a lag period (Fig. 1) corresponding to an amount of enzyme-produced CoA equal to that of MMTS suggests that the MMTS reacts with CoA to form the inhibitor CoA methyl disulfide. Synthesized CoA methyl disulfide added at t=0 shows no lag period and addition of an equi-molar amount of CoA to MMTS at t=0 also eliminates the lag period (Fig. 2).

Since, in any system as complicated as an enzymatic reaction singular effects are rare we tested the effect of preincubation of the enzyme and MMTS prior to addition of substrates and found the inhibitory potency of MMTS is somewhat increased. It seems likely that MMTS has inhibitory effects in addition to its ability to react with CoA.

Discussion

These findings suggest that MMTS reacts with CoA to form the inhibitory disulfide. Competitive reversal by acetyl CoA of inhibition by the latter compound (Fig. 3) eliminates the possibility that binding of CoA methyl disulfide might lead to formation of enzyme methyl disulfide by trans-methylthiolation to an enzymic thiol group.

In summary, studies of the inhibitory action of CoA methyl disulfide, the most potent active site directed inhibitor of choline acetyltransferase hitherto reported, make it very unlikely that an enzymatic thiol group is essential for the transacetylation reaction. However, more information dealing with the reactivity and inhibitory activity of unsymmetrical CoA disulfides with thiol groups is necessary before eliminating a possible

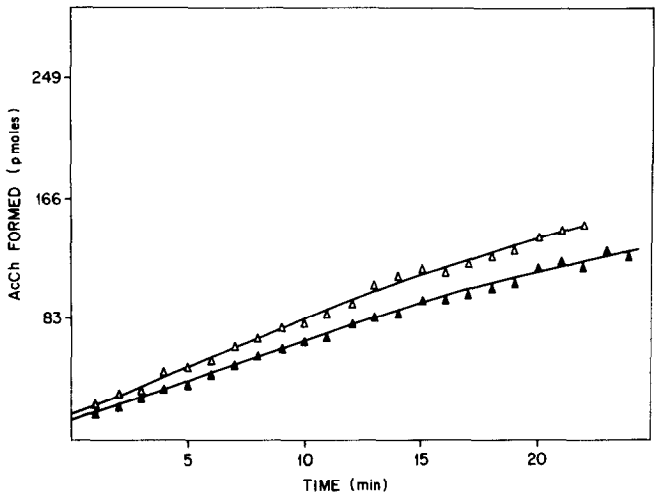

Figure 2 Inactivation of choline acetyltransferase ▲ - ▲ 6.4×10^{-6}M synthetic CoA methyl disulfide at t=0, △ - △ 2.5×10^{-6}M CoA and 2.5×10^{-6}M MMTS at t=0.

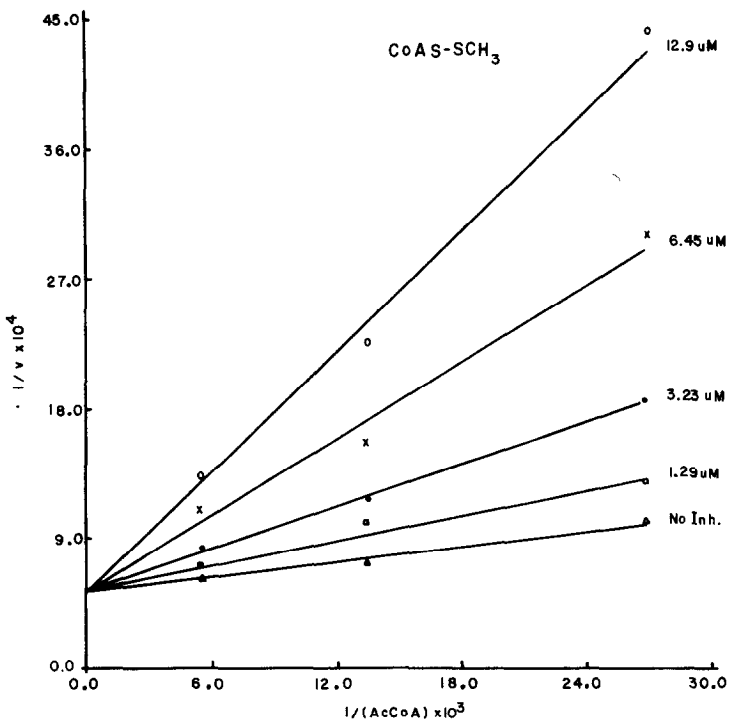

Figure 3 Lineweaver-Burk plot of inhibition by CoA methyl disulfide.

role for a thiol group in this enzyme and before concluding that all thiol reagents inhibit indirectly by reacting with CoA. Work is progressing with the synthesis and testing of other CoA disulfide derivatives.

The very striking difference in the abilities of CoA, acetyl CoA, dethia CoA and CoA methyl disulfide to be bound to the enzyme raises the question whether the differences are due to hydrophobic bonding interactions of the groups attached to the sulfur of CoA or to differences in CoA conformation brought about by such substituents.

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Bibliography

1 Nachmansohn, D. and Machado, A.L. (1943) J. Neurophysiol., 6, 397.
2 Currier, S. F. and Mautner, H. G. (1974) Proc. Nat. Acad. Sci. USA, 71, 3355.
3 Berman-Reisberg, R. (1954) Biochim. Biophys. Acta, 14, 442.
4 Berman-Reisberg, R. (1957) Yale J. Biol. Med., 29, 403.
5 Potter, L. T., Glover, V.A.S. and Saelens, J.K. (1968) J. Biol. Chem., 243, 3864.
6 Roskoski, R. (1974) J. Biol. Chem., 249, 2156.
7 Mannervik, B. and Sörbo, B. (1970) Biochem. Pharmacol., 19, 2509.
8 Husain, S. S. and Mautner, H. G. (1973) Proc. Nat. Acad. Sci. USA 70, 3749.
9 Smith, D. J., Maggio, E. T. and Kenyon, G. L (1975) Biochem., 14, 766.
10 Fonnum, F. (1975) J. Neurochem., 24, 407.
11 Chase, J. F. A., Middleton, B. and Tubbs, R. K. (1966) Biochem. Biophys. Res. Comm., 23, 208.