Interaction of Analogues of Coenzyme A with Choline Acetyltransferase†

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ABSTRACT: The finding that methyl methanethiolsulfonate appears to inhibit choline acetyltransferase from squid ganglia not by reacting with a thiol group of the enzyme but by reacting with the thiol group of coenzyme A to form a competitive inhibitor of acetyl coenzyme A led to the synthesis of the ethyl, propyl, and 3-carboxy-4-nitrophenyl disulfides of CoA. The methyl disulfide of 1,N^6-etheno-CoA, a fluorescent ligand, was also prepared. All the disulfides are powerful inhibitors of ChA, their K_i values being very similar. The K_m values for acetyl-, propionyl-, and butyryl-CoA were also found to be similar; however, modification of the acyl group alters the K_m values for choline. CoA, and dethia-CoA, showed similar abilities to be bound to ChA; however, the 3′-phospho groups of acetyl-CoA and CoA appear to be of importance in interacting with the enzyme. 8-Anilino-1-naphthalenesulfonate is a competitive inhibitor of acetyl-CoA binding.

Choline acetyltransferase (ChA,[1] EC 2.3.1.6) is responsible for the formation of the essential neurotransmitter acetylcholine. It catalyzes the alcoholysis of acetyl-CoA by choline. This reaction is favored only slightly thermodynamically, under physiological conditions (Pieklik and Guynn, 1975) and not at all kinetically. While thiol esters react much more rapidly with amines and carbanions than analogous esters, thiol esters react more slowly than esters with alcohols or hydroxide (Connors and Bender, 1961; Chu and Mautner, 1966).

Since the discovery of ChA (Nachmansohn and Machado, 1943), this enzyme has been studied extensively but little about its detailed mechanism of action is known. Recently, it was suggested 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 thiol ester group (Currier and Mautner, 1974). Confirmation for the involvement of an imidazole group, first postulated by White and Cavallito (1970), in the active site of ChA was obtained by Roskoski (1974c) and Malthe-Sørenssen (1976). It remained unclear, however, whether this reaction involves acetyl-CoA bound to the enzyme or whether, as suggested by Roskoski (1973, 1974a,b; Roskoski et al., 1975), this reaction involves an acetylthio-ChA intermediate formed by an essential thiol group of the enzyme reacting with acetyl-CoA.

Several workers have found that the catalytic action of ChA can be inhibited with thiol reagents (Berman-Reisberg, 1954, 1957; Schuberth, 1966; Morris, 1967, Potter et al., 1968; Mannervik and Sörbo, 1970; Roskoski, 1974a). However, attempts to protect ChA from inhibition by thiol reagents by the addition of substrates or products of the enzyme reaction led to conflicting results. Mannervik and Sörbo (1970) reported that choline, acetylcholine, and CoA did not protect ChA isolated from bovine caudate nuclei against the action of thiol reagents but noted some protection by acetyl-CoA. Later, Roskoski (1974a), using enzyme from the same source, claimed complete protection by acetyl-CoA, substantial protection by acetylcholine and, using human brain and placental ChA, complete protection by acetylthiolcholine (Roskoski et al., 1975).

Roskoski (1974a) also claimed that, using bovine brain ChA, it was possible, by incubating the enzyme with [^{14}C]acetyl-CoA or with [^{14}C]acetyl[^{3}H]choline, followed by passage through a Sephadex G-50 column, to obtain ^{14}C-labeled enzyme. In contrast to White and Cavallito (1970), who presented some evidence for formation of a ChA–acetyl-CoA complex, Roskoski believed that he was isolating a [^{14}C]acetylthio derivative of ChA which he claimed to be able to acetylate either choline or CoA. He also claimed (1974b) that reaction of ChA with bromoacetyl-CoA or with bromoacetylcholine to form bromoacetylthio-ChA derivatives provided evidence for the presence of an essential thiol group in the active site of the enzyme.

In view of the discrepancies between the work of Roskoski and the earlier experiments of Mannervik and Sörbo, our laboratory investigated this problem using ChA isolated from squid head ganglia. In agreement with the latter authors, we found that acetyl-CoA, but not acetylcholine or choline, provided protection of the enzyme against inhibition by thiol reagents. Furthermore, using [^{14}C]acetyl-CoA, we found (Currier and Mautner, 1974; Mautner et al., 1974) that incubation with very highly purified ChA (Husain and Mautner, 1973), followed by passage through a Sephadex column by the procedure of Roskoski (1973), cleanly separated ChA from [^{14}C]acetyl-CoA with no radioactivity associated with the enzyme. Recently, Malthe-Sørenssen (1976), using bovine brain enzyme, also proved unable to isolate the postulated acetylthio-ChA intermediate unless very impure enzyme was used.

While the postulated acetylthio-ChA intermediate became increasingly elusive, it remained to establish why thiol reagents inhibit this enzyme and whether a thiol group in the active site was involved. In investigating this problem, it seemed important to consider the possibility that a thiol reagent, by attachment to a sulfur distal from the active site, might still be able to cause noncompetitive inhibition of substrate attachment

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[1] Abbreviations used: CoA, coenzyme A; AcCoA, acetyl-coenzyme A; Ans, 8-anilino-1-naphthalenesulfonate; MMTS, methyl methanethiolsulfonate; EETS, ethyl ethanethiolsulfonate; PPTS, propyl propanethiolsulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ChA, choline acetyltransferase; DEAE, diethylaminoethyl; NMR, nuclear magnetic resonance; UV, ultraviolet.

through steric hindrance. For this reason, it seemed desirable to use small uncharged thiol reagents, attachment of which to a mercapto group would minimize interaction with other portions of the enzyme.

Alkyl alkanethiolsulfonates are convenient reagents for this purpose. Smith et al. (1975) reported them to be easy to synthesize, to label, and to remove and showed that, in rabbit muscle creatine kinase, inhibitory action of these reagents increased with the size of the alkylthio group being introduced, leading them to the conclusion that the thiol group being blocked was not essential to the functions of the active site. These reagents react with thiols as follows:

$$RSSO_2R' + R''SH \rightarrow R'SO_2H + R''SSR$$

Methyl methanethiolsulfonate (MMTS) was found to be an excellent inhibitor of ChA isolated from squid ganglia (Currier and Mautner, 1976). Inhibition was seen after a short lag period and was reversed competitively by acetyl-CoA. These findings suggested that MMTS might inhibit ChA not be reacting with an active-site thiol group of the enzyme but by reacting with the thiol group of CoA. Since CoA is one of the products of the enzyme reaction, a lag period in the formation of the inhibitor, CoA methyl disulfide would be expected. To test this hypothesis, we synthesized the mixed methyl disulfide of CoA. This compound inhibited ChA without a lag period with a K_i of 2×10^{-6} M. Inhibition was reversed competitively by acetyl-CoA (Currier and Mautner, 1976).

In the present work we are reporting the synthesis and the interactions with ChA of a series of mixed disulfides of CoA, 3′-dephospho-CoA, and of the fluorescent analogue 1,N^6-etheno-CoA and are investigating the specificity of CoA binding.

Materials and Methods

Reagents. [1-^{14}C]Acetyl-CoA, [1-^{14}C]propionyl-CoA, [1-^{14}C]butyryl-CoA, [1-^{14}C]acetic anhydride, and [^{14}C]-methyl iodide were purchased from New England Nuclear Corp. CoA, 1,N^6-etheno-CoA, and unlabeled butyryl-CoA were purchased from P-L Biochemicals Inc.; 3′-dephospho-CoA was from Sigma.

Acetyl 3′-dephospho-CoA was prepared from 3′-dephospho-CoA and [^{14}C]acetic anhydride by a procedure similar to that used for the preparation of acetyl-CoA by Mannervik and Sörbo (1970). The crude material was purified using a DEAE-Sephadex column as described by Stewart et al. (1968) and obtained in a yield of 64% (specific radioactivity, 0.3 μCi/μmol).

MMTS, ethyl ethanethiolsulfonate (EETS), and propyl propanethiolsulfonate (PPTS) were prepared by a procedure similar to that of Smith et al. (1975) starting with the disulfides.

[^{14}C]Methyl Methanethiolsulfonate (MMTS). A modification of the procedure of Smith et al. (1975) was used. A solution of NaHS dried over P_2O_5 (11.2 g, 0.20 mol) in absolute ethanol (150 mL) was filtered. To this solution was added dropwise 11.4 g (0.10 mol) of methanesulfonyl chloride at a temperature of 25 °C. After stirring for an additional 2 h at 25 °C, the reaction flask was heated to 65–70 °C. The NaCl ppt was filtered and the ethanol removed in vacuo at 45 °C. Sodium methanethiolsulfonate was recrystallized from ethanol yielding 10.5 g (78%) of white crystals. Sodium methanethiolsulfonate (268 mg, 2.0 mmol) was suspended in 3 mL of absolute ethanol and cooled to −20 °C. [^{14}C]Methyl iodide (14.2 mg, 0.10 mmol, specific activity, 2 mCi/mmol) was cooled to −20 °C and cold ethanol (2 mL) was added to dissolve the methyl iodide. The solution was then carefully transferred to the reaction mixture which was allowed to warm to room temperature. After stirring for an additional 2 h, 285 mg (2 mmol) of unlabeled methyl iodide was added and stirring continued for 2 h. Ethanol was then removed in vacuo at 40 °C and the residue resuspended in methylene chloride. After filtration, the filtrate was concentrated to 2 mL and applied to a silica gel column (2 × 30 cm) and eluted with methylene chloride. Fractions of 4 mL were collected and assayed for radioactivity. Radioactive fractions were pooled and the methylene chloride was removed in vacuo. After drying, a clear oil was obtained in a yield of 0.196 g (77%). Identity with unlabeled MMTS was proved with NMR spectroscopy.

CoA Methyl Disulfide. The synthesis of Currier and Mautner (1976) was used.

CoA Ethyl Disulfide. EETS (40 mg, 0.26 mmol) was dissolved in 0.3 mL of ethanol before mixing with CoA (50 mg, 61 μmol) and 0.3 mL of water. After reacting for 2 h at room temperature, the mixture was evaporated in vacuo at 40 °C, the residue dissolved in 2 mL of methanol, and the product precipitated with 20 volumes of acetone. The solution was centrifuged, the pellet dissolved in 1 mL of water, and the solution chromatographed on Sephadex G-10. Elution with water yielded 45 mg (84%) of UV-absorbing product (λ_{max}, 260 nm). Anal. Calcd for $C_{23}H_{38}Li_2N_7O_{16}P_3S_2 \cdot 2H_2O$: C, 31.55; H, 4.83; N, 11.20. Found: C, 31.14; H, 4.62; N, 10.89.

CoA Propyl Disulfide. A procedure analogous to that described above using PPTS (110 mg, 0.62 mmol) in 0.4 mL of ethanol and CoA (50 mg; 61 μmol) in 0.3 mL of water yielded 37.5 mg (69%) of product. Anal. Calcd for $C_{24}H_{40}Li_2N_7O_{16}P_3S_2 \cdot 2H_2O$: C, 32.40; H, 4.99; N, 11.02. Found: C, 32.99; H, 4.59; N, 10.93.

CoA 3-Carboxy-4-nitrophenyl Disulfide. The reaction of CoA (40 mg, 48 μmol), DTNB (25 mg, 62 μmol), and 10 mg of $NaHCO_3$ in 0.6 mL of water for 2 h at room temperature, after chromatography on Sephadex G-10, yielded 42.1 mg (83%) of slightly yellow microcrystalline material. Anal. Calcd for $C_{28}H_{37}Li_2N_8O_{20}P_3S_2 \cdot 2H_2O$: C, 31.99; H, 4.57; N, 10.66. Found: C, 32.09; H, 4.19; N, 10.81.

1,N^6-Etheno-CoA Methyl Disulfide. Using a modification of the procedure of Secrist et al. (1972), CoA methyl disulfide (50 mg, 58 μmol) was dissolved in 6 mL of 1.0–1.6 M freshly prepared chloroacetaldehyde and the pH adjusted to 4.3 with 0.1 N LiOH. The solution was protected from light and stirred at 32 °C until the ratios 265/275 appeared constant in the UV absorption spectrum (24 h). The reaction mixture was extracted 10 × 6 mL with benzene and 4 × 6 mL with Et_2O, evaporated in vacuo at 40 °C to 1.5 mL, and chromatographed on a Sephadex G-10 column. The 1-mL fractions were monitored at 275 nm and tested for chloride with $AgNO_3$. When CoA [^{14}C]methyl disulfide was used as starting material, radioactivity was followed. Appropriate fractions were pooled from the single product peak and evaporated in vacuo at 40 °C. The resulting microcrystalline material was coevaporated with ethanol and dried to yield 43 mg (77%) of product: UV λ_{max} (10^{-4} N HCl) 227 nm (ϵ 36 935), 266 nm (ϵ 10 000), 274 nm (ϵ 9286); fluorescence λ_{ex} (10^{-4} N HCl) 265 nm, 229 nm; λ_{em} 409 nm. Anal. Calcd for $C_{24}H_{36}Li_3N_7O_{16}P_3S_2 \cdot 6H_2O$: C, 29.89; H, 4.91; N, 10.17. Found: C, 29.60; H, 4.33; N, 10.10.

Dethia-CoA. The procedure of Chase and Tubbs (1966) was used. ChA was partially purified from squid head ganglia to an activity of 2.55 μmol min^{-1} mg^{-1} protein as described by Husain and Mautner (1973). The enzyme was assayed ac-

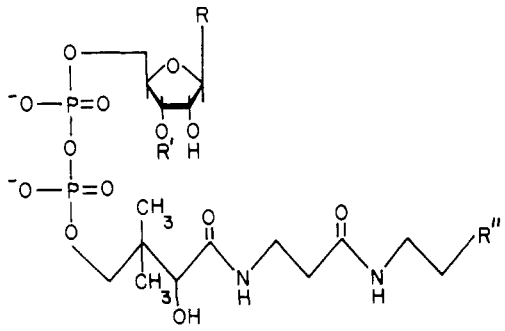
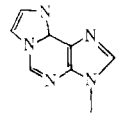


FIGURE 1: For compounds and substituent groups, see:

Compound	R	R′	R″
CoA	Adenine	$-PO_3H^-$	$-SH$
Dethia-CoA	Adenine	$-PO_3H^-$	$-H$
Acetyl-CoA	Adenine	$-PO_3H^-$	$-SCCH_3$ (O)
CoA methyl disulfide	Adenine	$-PO_3H^-$	$-SSCH_3$
3′-Dephospho-CoA	Adenine	$-H$	$-SH$
1-N^6-Etheno-CoA methyl disulfide		$-PO_3H^-$	$-SSCH_3$

cording to the procedure of Fonnum (1975) in which [^{14}C]acetylcholine formed is extracted as a tetraphenylboron complex. K_m and K_i values were calculated from Lineweaver-Burk plots.

Carnitine acetyltransferase from pigeon breast muscle was purchased from Sigma as a crystalline suspension in 3.2 M $(NH_4)_2SO_4$ (pH 6.0). Assays for initial rates were obtained by the procedure described by Rossier et al. (1973), except that all solutions were buffered with 100 mM tris(hydroxymethyl)aminomethane (pH 7.8); K_m values for acetyl-CoA (66 μM) and carnitine (286 mM) were similar to the values published by Chase and Tubbs (1966). CoA methyl disulfide inhibited carnitine acetyltransferase competitively with a K_i of 95 μM.

Nuclear magnetic resonance spectroscopy was carried out using a Hitachi Perkins-Elmer Model R-20B spectrometer. Fluorescence emission and excitation spectra were obtained using an American Instrument Co. SPF 100 CS spectrophotometer. All chemical analysis were obtained by H. Agahigian, Baron Consulting Co., Orange, Conn.

Results

The abilities of the substrates and products of the ChA reaction to be bound to the enzyme isolated from squid ganglia are summarized in Table I as are the binding constants of the methyl-, ethyl-, propyl-, and 3-carboxy-4-nitrophenyl disulfides of CoA and the methyl disulfide of 1,N^6-etheno-CoA. Table II compares acetyl-CoA, propionyl-CoA, butyryl-CoA, and acetyl-3′-dephospho-CoA as substrates of the ChA reaction and shows the effect of the acyl-CoA derivatives on the binding of choline.

As part of an investigation of the specificity of the binding of CoA to ChA, we investigated the binding of analogues of CoA lacking the sulfur or the 3′-phospho group (Figure 1). The binding constants of these compounds are compared in Table I.

Because of recent reports that Reactive Blue 2, the chromophore of Blue Dextran, is structurally related to the adenosine diphosphoryl portion of coenzymes (Thompson et al., 1975), there has been interest in the use of Blue Dextran-Sepharose columns for the purification of enzymes containing the "dinucleotide fold". Such columns have been used for the purification of ChA from brain and placenta (Roskoski et al., 1975) and from *Drosophila* heads (B. H. Weber, personal communication). More recently, 8-anilino-1-naphthalenesulfonate (Ans) was shown to compete with the binding of acetyl-CoA in the pyruvate-dehydrogenase complex (Shepherd and Hammes, 1976). In spite of the complete lack of relevance of the structure of Ans to the "dinucleotide fold", we found Ans to be a good, competitive antagonist of acetyl-CoA in the squid ChA preparation (Figure 2).

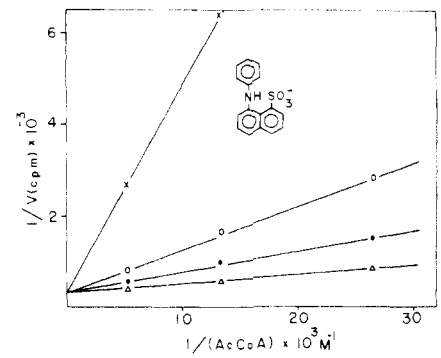


FIGURE 2: Double-reciprocal plot of Ans inhibition of ChA under the conditions described in Figure 1; Ans (△) 0, (●) 4.23 × 10^{-4} M, (○) 8.45 × 10^{-4} M, (X) 2.11 × 10^{-3} M.

TABLE I: Inhibitors of Choline Acetyltransferase.

Compound	K_i (μM)
CoA-methyl disulfide	2.3
CoA-ethyl disulfide	1.8
CoA-propyl disulfide	5.0
CoA-3-carboxy-4-nitrophenyl disulfide	4.0
1,N^6-Etheno-CoA-methyl disulfide	4.0
CoA	75.0
Dethia-CoA	78.0
1,N^6-Etheno-CoA	400.0
3′-Dephospho-CoA	7 000
8-Anilino-1-naphthalenesulfonate	200
Acetylcholine	37 000

Discussion

It can be seen in Table I that attachment of a methylthio group to the sulfur of coenzyme A results in an increase in the tightness of binding of the coenzyme to ChA isolated from squid head ganglia. From the differences in K_i (75 μM for CoA and 2.3 μM for CoA methyl disulfide), it is possible to calculate a difference in the free energy of binding to ChA of 2.06 kcal. Since acetyl-CoA and CoA methyl disulfide differ only in the presence of a sulfide rather than a carbonyl group, the difference of 1.78 kcal in the binding of these compounds seems surprising. However, anomalously tight binding brought about by the introduction of alkylthio groups has been noted before (Baker, 1967). If the differences in the binding constants between CoA and its derivatives involve hydrophobic interactions, the size of the enzyme site involved in such interactions must be rather small since the K_i's for the binding to ChA by the methyl, ethyl, and propyl disulfides of CoA are very similar. This would indicate that only the α-methylene portion of the alkyl chain is involved in binding. The possibility that substitution of the sulfur of CoA might introduce a conformational

TABLE II: Substrate Activity of Acyl Derivatives of CoA.

Compound	K_m (μM)	K_i (μM)	V_{max} (μmol min^{-1} mL^{-1})	Choline K_m (μM)
Acetyl-CoA	47		1.6	1900
Propionyl-CoA	31		1.5	2660
Butyryl-CoA	20	55	0.094	6670
Acetyl-3′-dephospho-CoA	820		0.53	753

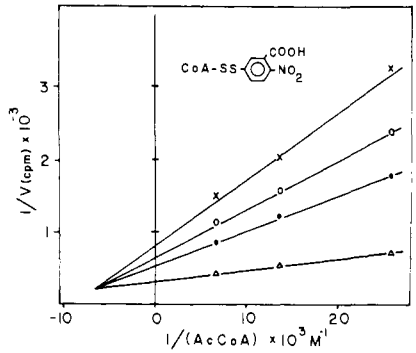


FIGURE 3: Double-reciprocal plot of CoA 3-carboxy-4-nitrophenyl disulfide inhibition of ChA. Initial rates were measured by the assay of Fonnum (1975) under the following conditions: choline 10 mM; NaCl, 150 mM; t = 5 min; incubation temperature, 32 °C; CoA-3-carboxyl-4-nitrophenyl disulfide (△) 0, (●) 1.17×10^{-5} M, (○) 1.63×10^{-5} M, (X) 2.33×10^{-5} M.

change in the coenzyme which might alter the abilities of other portions of this molecule to interact with its apoenzyme (Mautner, 1970) cannot be excluded. The methyl, ethyl, and propyl disulfides were competitive antagonists of acetyl-CoA binding while the 3-carboxy-4-nitrophenyl disulfide of CoA was a noncompetitive inhibitor (Figure 3).

It should be noted that differences between the abilities of CoA and acetyl-CoA to be bound to ChA depend on the species from which this enzyme has been isolated. CoA is bound loosely as compared with acetyl-CoA in enzyme obtained from horseshoe crabs, snails, or cockroaches (Emson et al., 1974). On the other hand, in brain enzyme, the K_i's for the binding of CoA and acetyl-CoA are very similar (Ryan, 1976). This situation is complicated by the observation that the binding of choline as well as that of acetyl-CoA is affected by salt concentration (Schuberth, 1966; Prince, 1971; Spantidakis et al., 1976). In rat brain ChA, CoA methyl disulfide also proved to be a very powerful, competitive antagonist of the binding of acetyl-CoA.

The tight binding of disulfide derivatives of CoA to ChA provides a convenient tool for studying the mechanism of action of this enzyme. Radioactive labeling of the alkyl group can be carried out conveniently, while the fluorescence of the 1,N^6-etheno derivative provides a convenient method for following the binding of the purine portion of the coenzyme or of its derivatives.

As can be seen in Table I, the K_i values for the binding of CoA and of its dethia analogue (Chase et al., 1966) proved to be quite similar suggesting that, in the squid ChA system, the importance of the sulfur of CoA in binding to the enzyme is slight. Nor does the 6-amino group of CoA appear to play an important role in binding to the active site of ChA. Table I shows the binding of CoA methyl disulfide and 1,N^6-etheno-CoA methyl disulfide to be very similar, while the binding constants for CoA and its etheno derivative differ by a factor of 5.3. On the other hand, the 3′-phospho group plays a role in the binding of acetyl CoA (the K_i's for acetyl-CoA and for 3′-acetyldephospho-CoA being 47 and 820 μM, respectively) as well as in the binding of CoA.

The observation that a molecule with as little similarity to the structure of CoA as Ans is a competitive antagonist of the binding of acetyl-CoA in squid ChA strengthens the belief that relatively nonspecific binding is involved in the enzyme-coenzyme interaction.

The similarity in the binding constants of the methyl, ethyl, and propyl disulfides of CoA suggested an examination of the relative substrate activities of acetyl, propionyl, and butyryl CoA. These data are summarized in Table II. As had been reported earlier (Berman et al., 1953; Berman-Reisberg, 1957), ChA is capable of catalyzing the formation of either acetylcholine or propionylcholine from acetyl- or propionyl-CoA, while butyryl-CoA is a poor substrate for the transacylation reaction. As can be seen, the maximal velocities for the ChA catalyzed formation of acetyl- and propionylcholine are identical, while the rate of butyrylcholine formation is negligible. However, the K_m values for acetyl-, propionyl-, and butyryl-CoA are very similar with binding becoming slightly tighter as the chain length of the acyl group is extended. Of interest is the observation that the binding of the acyl-CoA substrates affects the binding of choline, the other substrate. In the presence of butyryl-CoA which is a poor substrate of the ChA reaction and which is a competitive antagonist of acetyl-CoA binding, the binding of choline to the enzyme is interfered with. On the other hand, acetyl-3′-dephospho-CoA facilitates the binding of choline.

The relative lack of specificity in the binding of CoA and acyl or other derivatives of CoA to ChA stands in striking contrast to the great structural specificity (Currier and Mautner, 1974) and stereospecificity (Hemsworth and Smith, 1970) seen in the binding of analogues of choline. Most available evidence suggests that acetyl-CoA is the leading substrate in the transacylation reaction (White and Cavallito, 1970; Morris et al., 1971; Rama Sastry and Henderson, 1972). It seems likely that the acetyl-coenzyme plays a role in facilitating the binding of choline and, possibly, in facilitating the interaction of choline and the imidazole group of histidine (Currier and Mautner, 1974), within the active site of the enzyme.

It seems likely that the utility of disulfides of CoA as competitive antagonists of acetyl-CoA binding will not be restricted to the ChA system. For instance, we have found CoA methyl disulfide to be a competitive inhibitor of carnitine acetyltransferase, another enzyme catalyzing the alcoholysis of acetyl-CoA.

Acknowledgments

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