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Acetyl-Coenzyme A and Coenzyme A Analogues

THEIR EFFECTS ON RAT BRAIN CHOLINE ACETYLTRANSFERASE

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Choline acetyltransferase has the same affinity for acetyl-CoA, propionyl-CoA and butyryl-CoA ($K_m = 1.4\,\mu M$). Choline acetyltransferase may use the two latter compounds as substrate, but the longer the acyl chain the lower will be $V_{max.}$. CoA is an inhibitor ($K_i = 1.8\,\mu M$). The position of the 3′-phosphate is of primary importance. Desphospho-CoA is a weak inhibitor ($K_i = 500\,\mu M$). 5′-AMP is already an inhibitor ($K_i = 2500\,\mu M$). Phosphopantetheine is not an inhibitor. Dextran Blue is a potent inhibitor ($K_i = 0.05\,\mu M$). Choline acetyltransferase binds to hydrophobic affinity columns. Because of its affinity for nucleotides, affinity for Dextran Blue and hydrophobicity, it is proposed that it contains the 'nucleotide fold', which is a common structural domain present in several enzymes binding nucleotides.

The specificity of choline acetyltransferase (acetyl-CoA–choline *O*-acetyltransferase; E.C. 2.3.1.6) for its substrate choline has been discussed in numerous reports (Hemsworth & Smith, 1970*a*,*b*; Prince, 1971; Currier & Mautner, 1974; Barker & Mittag, 1975; Ilson & Collier, 1975; Collier *et al.*, 1976; for review see Rossier, 1977). Papers concerning the specificity of choline acetyltransferase for acetyl-CoA are not so numerous and the results were obtained before the introduction of radiolabelled substrates in the choline acetyltransferase assay system (Berman-Reisberg, 1957; Berry & Whittaker, 1959). The present study, using several radiolabelled analogues, was undertaken to delineate the specificity of choline acetyltransferase for acetyl-CoA and other longer acyl-CoA derivatives. The fluorescent analogue acetyl-1,N^6-etheno-CoA was also tested as a substrate for the enzyme.

Another part of the present study has centred on the properties of CoA analogues. It has already been well demonstrated that CoA, a product of the choline acetyltransferase reaction, is also a potent competitive inhibitor with respect to acetyl-CoA (Morris & Grewaal, 1971). It may be of interest that all choline acetyltransferase inhibitors synthesized recently (mainly styrylpyridine and halogenated acetylcholine analogues) are non-competitive or uncompetitive inhibitors (Morris & Grewaal, 1969; White & Cavallito, 1970). All the CoA analogues studied in the present paper were competitive inhibitors, including CoAS–SMe (CoA methyl disulphide), a compound described by Currier & Mautner (1976). A specific use of competitive inhibitors is affinity chromatography and the present work was primarily designed to screen good ligands for this purpose. Roskoski *et al.* (1975) have proposed purifying choline acetyltransferase by affinity chromatography by using Dextran Blue, a ligand without any structural relation with CoA. This paper shows that Dextran Blue is a competitive inhibitor of the enzyme with respect to acetyl-CoA. This result may indicate that the active site of the enzyme contains a 'nucleotide fold'.

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Experimental

Chemicals

CoA, desamino-CoA, 1-N^6-etheno-CoA, desphospho-CoA, butyryl-CoA, decanoyl-CoA, palmitoyl-CoA, 5′-AMP, 2′,5′-ADP, 3′,5′-ADP and hexane/agarose (6 μmol of hexane/ml of gel) were from P–L Biochemicals (Milwaukee, WI, U.S.A.). Dextran Blue was from Pharmacia (Uppsala, Sweden).

Desthio-CoA was prepared in the laboratory by the procedure of Chase *et al.* (1966).

4′-Phosphopantetheine, CoASeH (seleno-CoA), 3′-desphospho-CoASeH and iso-CoASeH were prepared in the laboratory by the procedure of Gunther & Mautner (1965).

CoAS–SMe was prepared by the procedure of Currier & Mautner (1976) as follows. CoA was dissolved in water and an equal amount in weight of methyl methanethiosulphonate was added. After

stirring at room temperature (20°C) for several hours, the mixture was passed through a Sephadex G-10 column that had been equilibrated with water. The CoA analogue was eluted first in one peak and was not contaminated by any unchanged CoA, as checked by the Ellman (1959) reaction.

Methyl methanethiosulphonate was synthesized just before use by the procedure of Smith *et al.* (1975). CoAS–SEt (CoA ethyl disulphide) and CoAS–SPr (CoA propyl disulphide) were prepared by the same procedure by using respectively ethyl ethanethiosulphonate and propyl propanethiosulphonate.

Radiolabelled substrates

[1-^{14}C]Acetyl-CoA (specific radioactivity 54mCi/mmol), [1-^{14}C]propionyl-CoA (specific radioactivity 25.7mCi/mmol) and [1-^{14}C]butyryl-CoA (specific radioactivity 21.2mCi/mmol) were from New England Nuclear (Boston, MA, U.S.A.).

[1-^{14}C]Acetyletheno-CoA was prepared in the laboratory by the method of Dr. Michel Gaudry (personal communication). This method was adapted from the technique of Secrist *et al.* (1972) used to prepared various ethenoadenosine-containing coenzymes by reaction of the various coenzymes with chloroacetaldehyde in aqueous solution at mildly acidic pH. A mixture of 10mg of unlabelled acetyl-CoA with 10μCi (0.16mg) of [^{14}C]acetyl-CoA (The Radiochemical Centre, Amersham, U.K.) was dissolved in 1ml of water. The reaction was started by the addition of 1ml of freshly prepared chloroacetaldehyde obtained by two subsequent distillations under mild vacuum of a mixture containing an equal volume of chloroacetaldehyde dimethyl acetal (Aldrich, Milwaukee, WI, U.S.A.) and 50% (w/v) H_2SO_4. The acetyl-CoA/chloroacetaldehyde mixture was adjusted to pH4 with 5M-NaOH and was stirred at room temperature in the dark for 4 days. After extraction with diethyl ether of the unreacted chloroacetaldehyde, the solution was adjusted to pH6.5 and poured on the top of a DEAE-cellulose column (2.5cm × 50cm, DE 52 Whatman) equilibrated in 5mM-phosphate buffer, pH6.5. The column was eluted by a linear gradient of LiCl (0.0–0.3M, 700ml each). Fractions (12ml) were collected. A material absorbing at 232nm was present in tubes 91–118. A radioactive material was co-eluted with a fluorescent material whose u.v. spectrum is diagnostic of ethenoadenosine derivatives (Secrist *et al.*, 1972). Fractions 105–107 were freeze-dried and desalted on a Sephadex G-10 column. The material obtained was highly fluorescent with a maximal emission at 410nm when the excitation was at 310nm. In terms of the recovered radioactivity, the total procedure gave a yield of 16%.

Rat brain enzyme

Caudate nuclei of ten rats were dissected and homogenized directly after death in 10ml of 0.32M-sucrose. After a preliminary centrifugation (1000*g*, 10min), the supernatant was subjected to a second centrifugation (12500*g*, 30min), and the pellet (P_2) containing synaptosomes was washed once with 20ml of 0.32M-sucrose; after another centrifugation, the pellet was subjected to hypo-osmotic shock with 40ml of 1mM-1,10-phenanthroline hydrochloride and bovine serum albumin (1mg/ml) (pH was adjusted to pH6.3 with HCl). It was shown by Fonnum (1968) that, after such treatment, choline acetyltransferase was still bound to membranes, but could be released by increasing the ionic strength of the solution. To release choline acetyltransferase, and to avoid contamination by soluble cholinesterases and deacylases, which would interfere with the assay, the membrane fragments were submitted to centrifugation (17000*g*, 20min), the supernatant was discarded and then the membrane pellet was resuspended in 1ml of 5mM-Tris/HCl, pH7.2, containing 100mM-NaCl, 1mM-1,10-phenanthroline hydrochloride and bovine serum albumin (1mg/ml). The released enzyme appeared now in the 105000*g* (1h) supernatant and was further dialysed for 18h against 3 × 1 litres of the buffer used to resuspend the pellet. These preparations had a specific activity of between 0.05 and 0.1μmol/min per mg of endogeneous proteins.

Choline acetyltransferase assay

The revised technique of Fonnum (1975) was used. The assays were always done in the medium used to prepare the enzyme. The values were found similar when enzyme was assayed in the presence or absence of eserine; therefore cholinesterase inhibitors were not used. Choline hydrochloride final concentration was always 20mM. Radioactive substrates ([^{14}C]-acetyl-CoA, [^{14}C]propionyl-CoA and [^{14}C]butyryl-CoA) were not diluted with unlabelled materials, but used at final concentration ranging from 1μM to 100μM. To determine K_i values the following protocol was performed: incubation (5min, 37°C) was started by addition of 10μl of the enzyme solution, diluted 50-fold, to 10μl of an identical solution containing various concentration of inhibitors and labelled acetyl-CoA. The final concentrations of acetyl-CoA were 1.15, 2.3 and 4.6μM. K_i values were determined by Dixon plots; K_m values were determined by Lineweaver–Burk plots. The values found would be close to true K_m and K_i values as choline was used at a concentration 60 times above its K_m.

Results

Specificity of acetyl-CoA as substrate

Acetyl-CoA K_m values were shown to increase with the ionic strength (Spantidakis *et al.*, 1976; Rossier, 1977). Also, purification procedures change the

kinetic parameters of choline acetyltransferase (Rossier, 1977). Therefore, during this study, a crude enzyme was tested at only one fixed ionic strength (approx. 126mM).

Table 1 shows that propionyl-CoA and butyryl-CoA may be used as substrate by choline acetyltransferase. Acetyl-CoA, propionyl-CoA and butyryl-CoA have exactly the same affinity for the enzyme. Nevertheless, the turnover number of the enzyme decreased with substitution of acetyl-CoA for propionyl-CoA or butyryl-CoA, and the longer was the chain length the slower was the turnover number. Other long-chain fatty acid analogues were tested for their inhibitory properties with respect to acetyl-CoA. Dixon plots showed that butyryl-CoA, decanoyl-CoA and palmitoyl-CoA were competitive inhibitors and had around the same K_i values. This similarity in K_i values could indicate that all acyl-CoA molecules may be substrates for the enzyme. Thus acylcholine of various chain lengths will be synthesized by the enzymic reaction, but the longer the chain length of the acyl moiety the slower will be the velocity of the reaction.

Table 1. *$V_{max.}$, K_m and K_i values for acetyl-CoA and other acyl-CoA analogues*

Compound	K_m (μM)	K_i (μM)	$V_{max.}$ (% of value obtained with acetyl-CoA)
Acetyl-CoA	1.4	—	100
Propionyl-CoA	1.5	—	65
Butyryl-CoA	1.4	4.5	12
Acetyl-1,N^6-etheno-CoA	2.5	—	33
Decanoyl-CoA	—	4.0	—
Palmitoyl-CoA	—	3.0	—

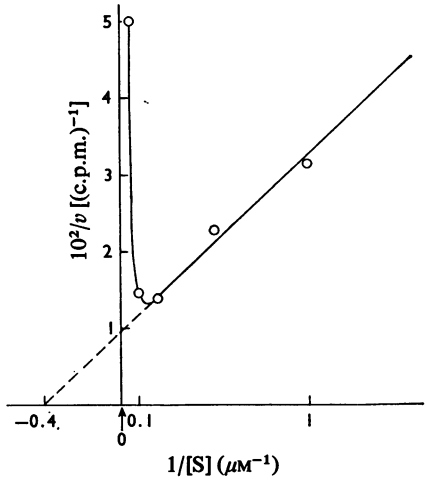

Fig. 1. *Lineweaver–Burk plot for acetyletheno-CoA*

Acetyletheno-CoA as substrate

Fig. 1 shows that the compound prepared as labelled acetyl-1,N^6-etheno-CoA is a substrate of the reaction. The K_m is around 2.5μM, but this preparation of radiolabelled acetyletheno-CoA gave substrate inhibition. In the absence of any further criteria of purity as those presented in the Experimental section, it may be proposed that the substrate inhibition was the result of some contaminating compounds coeluted with acetyletheno-CoA.

Modification of the amino group of the adenosine moiety of CoA

Suppression of the primary amino group of CoA yielding desamino-CoA decreases the inhibition promoted by plain CoA. Incorporation of the amino group in a non-saturated aromatic ring (etheno-CoA) decreased the inhibition to a similar extent. The K_i values are: CoA, 1.8μM; desamino-CoA, 9.0μM; 1,N^6-etheno-CoA, 10.5μM. Desamino-CoA and etheno-CoA are both competitive inhibitors with respect to acetyl-CoA. From the comparison of the K_i values, it may be concluded that the primary amino group, although important, is not essential. Therefore chemical modifications of this group may be done without much affecting the enzyme affinity for CoA.

Importance of the 3′-phosphate

Suppression of the 3′-phosphate on the ribose ring greatly altered the inhibition (Table 2). The K_i value of desphospho-CoA was two orders of magnitude greater than the K_i value of CoA. Displacement of the phosphate from the 3′- to the 2′-position gives iso-CoA. As iso-CoA was not available, experiments were performed with the selenium analogues of CoA. IsoSeH-CoA was around 4 times less potent than CoASeH. The importance of the 3′-position of the

Table 2. *Importance of the phosphate in the 3′-position shown by the K_i values of various analogues*

Compound	K_i (μM)
CoA	1.8
Desphospho-CoA	500
CoASeH	7
Iso-CoASeH	25
Desphospho-CoASeH	700
3′,5′-ADP	415
2′,5′-ADP	2200
5′-AMP	2500

phosphate group was also substantiated by comparison of 5′-AMP, 2′,5′-ADP and 3′,5′-ADP. 5′-AMP was already a competitive inhibitor with respect to acetyl-CoA, but a poor one. Incorporation of a second phosphate group in the 2′-position (2′,5′-ADP) did not increase the affinity, but incorporation of a second phosphate group in the 3′-position increased markedly (6 times) the affinity.

Modification of the SH group

Already it was shown (Table 3) that substitution of the sulphur atom by selenium did not affect the inhibition too much. Seleno compounds may be used only in the oxidized form (CoA–Se–Se–CoA). The K_i value for oxidized CoA was determined to be close to the value obtained for reduced CoA (see Table 3). Desthio-CoA is known to be a competitive inhibitor with respect to acetyl-CoA (Morris & Grewaal, 1971). Similar results were obtained here and K_i values of CoA and desthio-CoA were close.

I have also tested on the rat brain enzyme the mixed CoA disulphides prepared in the laboratory (Currier & Mautner, 1976). These compounds, CoAS–SMe and its analogues CoAS–SEt and CoAS–SPr, were better inhibitors than CoA itself. Their K_i values (0.1–0.34 μM) are also lower than the K_m value found for acetyl-CoA (1.4 μM).

Blue Dextran: a competitive inhibitor with respect to acetyl-CoA

It was observed that most of the nucleotide-metabolizing enzymes contained a common structural domain, 'the nucleotide fold'. Amino acid sequencing has shown that the primary structure of this common structural domain has not changed during evolution and may have evolved from a common ancestor. One of the characteristics of this 'nucleotide fold' is its high hydrophobicity (Rossman *et al.*, 1974).

Blue Dextran is commonly used to evaluate the void volume of gel-filtration columns. This molecule is composed of one chromophore, Reactive Blue, covalently bound to dextran, a polysaccharide. It was observed that all the enzymes containing the so-called 'nucleotide fold' were inhibited by Blue Dextran (Thompson *et al.*, 1975). Here I have observed that Blue Dextran is a potent choline acetyltransferase competitive inhibitor with respect to acetyl-CoA. The K_i value (0.05 μM) is more than one order of magnitude lower than the K_m of acetyl-CoA. This may indicate that choline acetyltransferase contains this common structural domain, the 'nucleotide fold'. This part of the molecule will be involved in the binding of the nucleotide-containing coenzymes: acetyl-CoA or CoA.

The importance of the nucleotide moiety in the binding of acetyl-CoA by choline acetyltransferase agrees with the observation that 5′-AMP was an inhibitor, and that, on the other hand, 4′-phosphopantheteine did not inhibit, even slightly, at a concentration up to 1 mM. Therefore the panthothenic acid part of CoA seems not to be very important in the mechanisms involved in the binding of CoA by choline acetyltransferase.

Hydrophobic nature of choline acetyltransferase

It is proposed that choline acetyltransferase contains the nucleotide fold. One of the characteristics of the nucleotide fold is its high hydrophobicity. The hydrophobic nature of the enzyme has already been suggested by the observation that high glycerol concentrations protect the enzyme against denaturation (Rossier, 1976). Fonnum (1972) had also suggested that the binding of the enzyme to hydroxyapatite columns was related to the hydrophobic nature of the enzyme. To demonstrate its hydrophobic nature the following experiment was performed. An agarose/hexane column (1 cm × 0.4 cm), prewashed with 10 ml of 5 mM-Tris/HCl, pH 7.2, supplemented with 100 mM-NaCl, was loaded with 100 μl of the crude enzyme described under 'Rat brain enzyme'. Bovine serum albumin, present at 1 mg/ml in the enzyme solution for stabilization, did not bind to the column, although choline acetyltransferase did bind and was not eluted by washing, even if the washing solution contained 20 μM-acetyl-CoA.

Table 3. *K_i values after the modification of the SH group of CoA*

Compound	Structure	K_i (μM)
CoA	CoA–SH	1.8
Oxidized CoA	CoA–S–S–CoA	1.0
Seleno-CoA	CoA–Se–Se–CoA	7.0
Desthio-CoA	CoA–H	2.0
CoA methyl disulphide	CoA–S–S–CH_3	0.1
CoA ethyl disulphide	CoA–S–S–CH_2–CH_3	0.34
CoA propyl disulphide	CoA–S–S–CH_2–CH_2–CH_3	0.3
Dextran Blue	—	0.05

Discussion

Affinity chromatography

The values found here for acetyl-CoA K_m and CoA K_i were one order of magnitude lower than the values previously reported (Morris & Grewaal, 1971). Spantidakis *et al.* (1976) have also found acetyl-CoA K_m values ranging between 20 and 50 μM, but with a purified rat brain enzyme. Here, with a crude enzyme, the acetyl-CoA K_m value was found to be 1.4 μM. It appears that purification procedures decrease the affinity of choline acetyltransferase for acetyl-CoA.

Therefore, when affinity chromatography is used, it is worth while to use such techniques during the earlier stages of the purification procedure.

Already a preliminary communication has described the successful use of an affinity chromatography column with bound CoA (Ryan & McClure, 1976). It is worth while to note that Blue Dextran is an excellent inhibitor ($K_i = 0.05\,\mu M$) and may also be used to purify choline acetyltransferase. Roskoski *et al.* (1975) have described the use of Blue Dextran linked to Sepharose to purify the enzyme isolated from human placenta; unfortunately, the results obtained were very poor. On the other hand, L. Hersh (personal communication) has presented convincing data showing that the simple use of Blue Dextran affinity chromatography gave in one step a purification factor around 100, also starting from human placenta.

Blue Dextran and CoA columns have also been used to purify choline acetyltransferase from squid optic lobes. So far, the results obtained show that squid enzyme does not bind to Blue Dextran or CoA columns. This lack of binding may be due to the low affinity of the squid enzyme for CoA and Blue Dextran. In general, the kinetic parameters of the enzyme from squid optic lobes differ markedly from those of the vertebrate enzymes. In vertebrates, the ratio between CoA K_i value and acetyl-CoA K_m value is about 1. With squid optic lobes enzyme, this ratio is close to 200 (acetyl-CoA K_m, $48\,\mu M$; CoA K_i, $7500\,\mu M$) (Currier & Mautner, 1976).

It has been shown that squid optic-lobe enzyme is less susceptible than vertebrate enzyme to styrylpyridine inhibition (Husain & Mautner, 1973). I have also found no immunological cross-reactivity between choline acetyltransferase from rat brain and squid optic lobes. Antiserum produced against the enzyme from rat brain did not inactivate the enzyme from squid brain optic lobes. Similarly, antiserum produced against enzyme from squid optic lobes did not inactivate enzyme from rat brain (J. Rossier & H. G. Mautner, unpublished work). These kinetic and immunological data show the dissimilarities between choline acetyltransferase from squid optic lobes and rat brain.

Active site

The active site of choline acetyltransferase contains an imidazole ring. This ring would be involved in the transacetylation reaction (Roskoski, 1974; Currier & Mautner, 1974; Malthe-Sørenssen, 1976). From data presented here, it seems possible to propose that acetyl-CoA will bind through its adenosine moiety in a hydrophobic nucleotide-binding site close to the imidazole ring. The importance of the 3′-phosphate on the ribose ring, the low specificity of the enzyme in that it may use longer fatty acid analogues of acetyl-CoA, and the absence of any inhibition promoted by 4′-phosphopantetheine may indicate that the binding of acetyl-CoA to the active site occurs mainly through the adenosine moiety of the molecule. The importance of the nucleotide part in the binding to the active site is in agreement with the hypothesis presented here that choline acetyltransferase from rat brain is related to the family of enzymes containing a common structural domain, the nucleotide fold. Rossman *et al.* (1974) have shown, by amino acid sequence analysis of several nucleotide-metabolizing enzymes, that the nucleotide fold was highly hydrophobic. Therefore, if choline acetyltransferase has evolved from the same family of proteins, part of the active site would be hydrophobic. The observation by Mannervik & Sörbo (1970) that, among thiol reagents, the hydrophobic ones more strongly inhibited enzyme activity and that acetyl-CoA protected against this inhibition may also indicate that the part of active site involved in acetyl-CoA binding is hydrophobic.

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