S-METHANESULFONYL-CoA: A THIOL-SPECIFIC REAGENT FOR AFFINITY LABELING OF SHORT CHAIN ACYL-CoA SITES

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1. Introduction

The objective of our research is to develop new reagents that are active-site-directed irreversible inhibitors of enzymes that accept an acyl-CoA as a substrate. We have described photoaffinity labeling studies with a photolabile analog of acyl-CoA [1,2] as well as studies with a classical affinity label of the α-bromoketone type [3,4]. Here, we report the synthesis of *S*-methanesulfonyl-CoA (MES-CoA) and describe affinity labeling studies with succinate thiokinase and irreversible inhibition studies with 3 other acyl-CoA enzymes. MES-CoA contains the thiolsulfonate functional group, which is known to undergo a nucleophilic displacement reaction with thiols to form a mixed disulfide [5,6]. The very high specificity of the thiolsulfonate group for thiols suggests that MES-CoA can be used to detect the presence of a reactive thiol in an acyl-CoA binding site.

2. Materials and methods

Succinate thiokinase (EC 6.2.1.4), pig heart, was purchased from Boehringer Mannheim. Other enzymes and reagents were obtained, or synthesized, as in [1–4].

For the synthesis of MES-CoA, 0.1 ml freshly prepared 1.0 M $KHCO_3$ is mixed with 0.2 ml water, then with 1.0 ml acetone. To the $KHCO_3$–acetone solution, 0.25 ml methanesulfonyl chloride (3.2 mmol) is added with swirling. This solution is combined with 0.5 ml water containing 2.5 mg CoA (~3 μmol) and thoroughly mixed. Within 20 s of combining the reagents, 5 ml diethyl ether are added to the system with mixing. The aqueous phase is extracted and immediately chromatographed on a 1.5 × 20 cm column of Sephadex G-15 equilibrated with 1 mM HCl. The column separates MES-CoA from oxidized CoA and small M_r species. On the basis of A_{260} measurements the yields range from 40–60%.

The purity of the analog is assessed by TLC on cellulose plates, as in [3]. The R_F value for MES-CoA is 0.47, which can be compared to an R_F of 0.15 for oxidized CoA, 0.47 for CoA, and 0.54 for acetyl-CoA. MES-CoA and CoA have the same R_F in this system; however, CoA gives a yellow spot with a spray reagent consisting of 3 mM 5,5′-dithiobis (2-nitrobenzoic acid), 0.05 M Tris–HCl (pH 8.0) but MES-CoA does not.

The activity of succinate thiokinase was measured as in [7]; freshly prepared solutions of GTP were used. Fatty acid synthetase, citrate synthase and β-hydroxyacyl-CoA dehydrogenase were assayed as in [3,4].

Our protocol for affinity labeling experiments has been described [3,4]. The kinetics of irreversible inhibition were evaluated according to [8], and where appropriate, data were fitted to a straight line equation by the method of least squares.

3. Results and discussion

The chemical reactivity of MES-CoA was confirmed by incubating the analog in 20 mM Na-phosphate (pH 7.3) for 30 min at room temperature in the presence of equimolar CoA or a 5-fold molar excess of pyridine, histidine, or imidazole. MES-CoA was also incubated in 50 mM Tris–acetate (pH 7.5) for 30 min. Analysis of the incubated samples by TLC showed that MES-CoA was converted to CoA-disulfide in the presence of added CoA, as expected. MES-

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CoA was not affected by the other reagents tested. However, in buffers of pH 8, or higher, MES-CoA is not stable as it converts to CoA-disulfide within 1–2 h.

In [9] succinate thiokinase contained a reactive cysteinyl thiol group within its active site. Thus, since succinyl thiokinase both accepts an acyl-CoA as a substrate and contains an active site thiol group, we selected this enzyme to test whether MES-CoA can function as an active-site-directed irreversible inhibitor. Incubation of succinate thiokinase with low levels of MES-CoA causes a rapid and irreversible inhibition of the enzyme (fig.1). The rate of inactivation followed pseudo-first-order kinetics at each MES-CoA concentration tested. A double reciprocal plot of k^{-1}_{app} *vs* $[\text{MES-CoA}]^{-1}$ gave 47 μM for K_i and 0.37 min^{-1} for k_3 (inset, fig.1). The K_i determined for MES-CoA compares very favorably to the K_m reported for succinyl-CoA (20–60 μM [7]), suggesting that MES-CoA binds at the acyl-CoA site. Chemically modified succinate thiokinase (fully inhibited) was incubated for 1 day without a detectable increase in activity, which demonstrates the irreversible nature of the inhibition.

Further evidence that MES-CoA is an active-site-directed reagent was obtained in experiments in which acetyl-CoA was added as a reversibly binding ligand to protect the acyl-CoA site. The presence of acetyl-CoA slows the rate of inactivation, as predicted by the model for such a system [8]; an app. k_b 0.09 mM was obtained for acetyl-CoA from this study. In a separate study we showed that acetyl-CoA is a competitive inhibitor *vs* CoA (a substrate), using the method of steady state kinetics; the value determined for K_i was 0.1 mM.

Covalent modification of the enzyme proceeds by attack of an enzyme–thiol on the thiol portion of the thiolsulfonate bond [5,6]. In this reaction, methanesulfonate is displaced and a mixed disulfide of CoA and enzyme is formed. Consequently, the 'irreversible' inhibition produced by MES-CoA should be reversed simply by adding a reducing agent such as dithiothreitol. When an excess of dithiothreitol was added to an incubating solution of modified succinate thiokinase, enzyme activity was recovered (fig.2). Control experiments showed that unmodified succinate thiokinase neither gains nor loses activity when incubated with dithiothreitol for 150 min. In some applications of affinity labels (e.g., in site stoichiometry studies) it is necessary to quench the reagent without affecting the chemically modified enzyme. We found that CoA can quench the excess MES-CoA in an incubation sample without producing a detect-

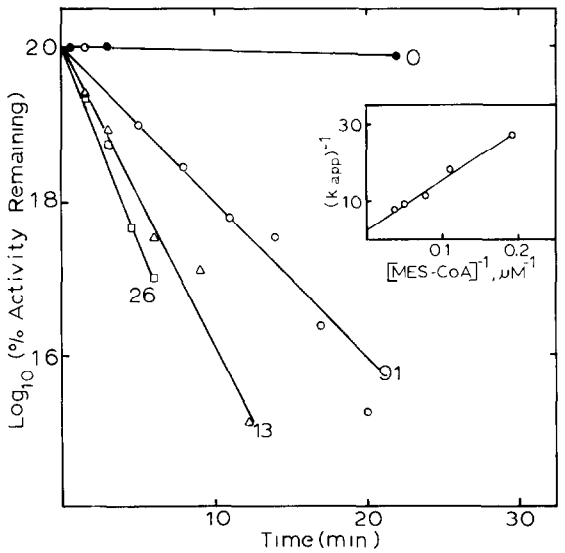

Fig.1. Irreversible inhibition of succinate thiokinase. Enzyme (25 μg) was incubated in 1.0 ml 70 mM Tris–acetate (pH 7.0) at 0°C. Concentrations of MES-CoA (μM) are listed. In the interest of clarity, only 4 lines are shown. (Inset) A double reciprocal plot of the slope (K_{app}) of each line *vs* the corresponding MES-CoA concentration is shown.

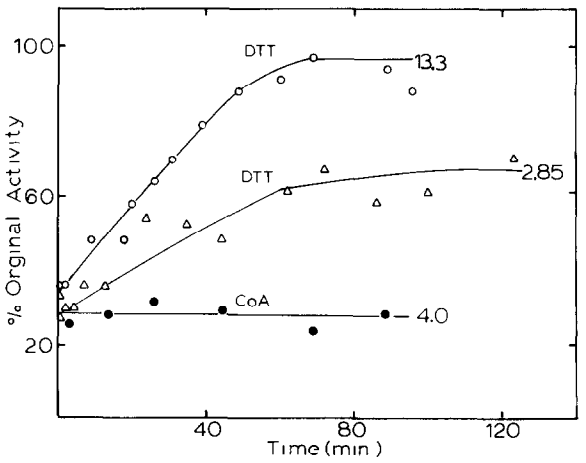

Fig.2. Effect of dithiothreitol and CoA on chemically modified succinate thiokinase. Enzyme (25 μg) and MES-CoA (13 μM) were incubated for 5 min at room temperature in 0.2 ml 0.25 M Tris–acetate (pH 8.0). The system was then brought to 1.0 ml 0.1 M Tris–acetate (pH 8.0) by adding buffer containing, as shown, dithiothreitol or CoA (both mM).

able recovery of enzyme activity (fig.2) [experiment suggested by Dr J. S. Nishimura]. Apparently, the bulk of the enzyme-bound CoA prevents a second CoA from approaching the mixed disulfide bond.

Electrophoretic analysis of the commercial enzyme preparation on polyacrylamide gels [10] indicated that only ~10% of the protein was succinate thiokinase (M_r ~ 76 000). Analysis of an enzyme sample labeled with MES-[(G)^{3}H]CoA by SDS–polyacrylamide gel electrophoresis and subsequent autoradiography [10] (except that we did not use mercaptoethanol in the gel or buffers) showed that the β-subunit (M_r ~ 42 500) was highly labeled whereas the α-subunit (M_r ~ 34 500 was not. This result is consistent with the work in [9]. In addition, an unidentified protein band of M_r ~ 48 000 was highly labeled; this protein presumably contains a CoA (or nucleotide) binding site. The autoradiogram, when compared to a gel with stained protein bands, also showed that dominant (contaminant) protein bands are lightly labeled by the radioactive reagent. Thus, while MES-CoA is highly selective for the acyl-CoA site of succinate thiokinase, it also acts as a non-specific thiol-modification reagent to a limited degree.

The new reagent was also tested for its effect on other enzymes that accept a short chain acyl-CoA as a substrate. Citrate synthase (pig heart) is not inhibited irreversibly by MES-CoA (26 μM) after incubation for 1 h as described [3], with or without 0.1 mM oxalacetate; however, simple competitive inhibition *vs* acetyl-CoA was observed (K_i 50 μM). β-Hydroxyacyl-CoA dehydrogenase is a homodimer with only 1 cysteinyl thiol/subunit [11]; the cysteine is not believed to be part of the active site [12]. This dehydrogenase is slowly inhibited by simple thiol reagents like iodoacetamide and *N*-ethylmaleimide [12], and also by MES-CoA (fig.3). This result also suggests that MES-CoA can act as a non-specific thiol-modification reagent. The non-linear kinetics suggest that the 2 thiols in the homodimer are modified at different rates. NAD^+ (250 μM) or acetoacetyl-CoA (500 μM) did not pretect the enzyme against MES-CoA. However, NAD^+ and acetoacetyl-CoA together provide a modest protection (fig.3); the ternary complex apparently undergoes a conformational change that alters the reactivity of the thiol groups.

A catalytic mechanism proposed for mammalian fatty acid synthetase (FAS) assigns a functional role to both an enzyme–pantetheinyl thiol and a cysteinyl thiol [13]. MES-CoA (≤12 μM) causes an extremely

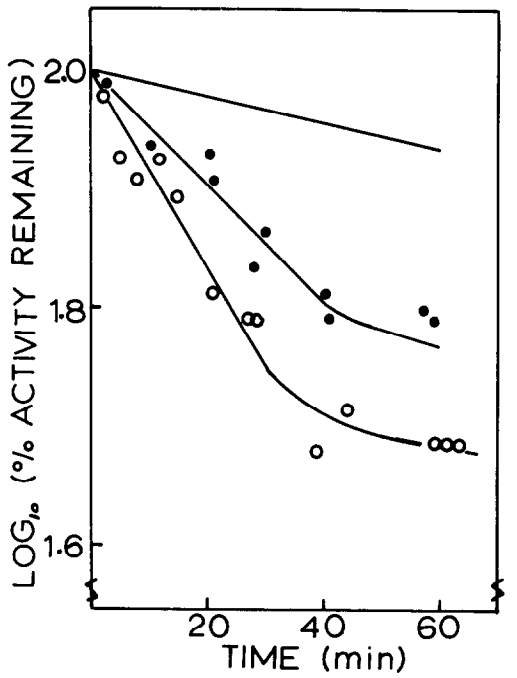

Fig. 3. Effect of MES-CoA on β-hydroxyacyl-CoA dehydrogenase. In each experiment enzyme (10 μg) was incubated at room temperature in 20 mM Na-phosphate (pH 7.3) at 0.5 ml initial vol.: (○) effect of 225 μM MES-CoA on enzyme activity; (●) effect of 225 μM MES-CoA on activity in presence of both 250 μM NAD and 500 μM acetoacetyl-CoA. The top line is the control in which enzyme was incubated in buffer only.

rapid and irreversible inhibition when added to FAS incubated as in [4]. The kinetics were quite similar to those observed with FAS and *S*-(4-bromo-2,3-dioxobutyl)-CoA [4]. The reagent appears to react with different classes of thiol groups since the rates of inactivation did not follow pseudo-first-order kinetics. The addition of 10 mM dithiothreitol to FAS that had been totally inactivated with MES-CoA led to the recovery of ~95% of the initial enzyme activity within 30 min; in contrast, the addition of 5 mM CoA did not lead to a recovery of activity. FAS is a very complex enzyme and an extensive investigation will be required before the identity of the thiols modified by MES-CoA can be ascertained.

We conclude that MES-CoA is a useful affinity label for investigating acyl-CoA binding sites. The reagent is easy to prepare and frozen solutions can be stored for several weeks without breakdown. The high selectivity of the thiolsulfonate group for thiols

means that MES-CoA will only label binding sites that contain a suitably located thiol. The fact that the chemical modification can be reversed at the discretion of the investigator adds to the attractiveness of the reagent. Less attractive features of MES-CoA are the following:

(i) The high reactivity of MES-CoA with thiols makes it necessary to remove agents such as dithiothreitol from the enzyme prior to incubation with the reagent. Some enzymes show a significant loss of activity when incubated in the absence of added thiols;

(ii) Our studies show that MES-CoA can act as a simple thiol reagent and modify (albeit slowly) a cysteinyl thiol not located in an acyl-CoA binding site. Thus, when using MES-CoA as an affinity label one must demonstrate that the reagent is active-site-directed (cf. [8,14]).

The synthesis of methoxycarbonyl-CoA disulfide, a reagent which may also be selective for thiol groups, has been described in [15].

Acknowledgements

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