Active-Site-Directed Inhibition of 3-Hydroxy-3-methylglutaryl Coenzyme A Synthase by 3-Chloropropionyl Coenzyme A†

Henry M. Miziorko* and Christine E. Behnke
Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226
Received October 10, 1984

ABSTRACT: 3-Chloropropionyl coenzyme A (3-chloropropionyl-CoA) irreversibly inhibits avian liver 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase). Enzyme inactivation follows pseudo-first-order kinetics and is retarded in the presence of substrates, suggesting that covalent labeling occurs at the active site. A typical rate saturation effect is observed when inactivation kinetics are measured as a function of 3-chloropropionyl-CoA concentration. These data indicate a K_i = 15 μM for the inhibitor and a limiting k_{inact} = 0.31 min^{-1}. [1-^{14}C]-3-Chloropropionyl-CoA binds covalently to enzyme with a stoichiometry (0.7 per site) similar to that measured for acetylation of enzyme by acetyl-CoA. While the acetylated enzyme formed upon incubation of HMG-CoA synthase with acetyl-CoA is labile to performic acid oxidation, the adduct formed upon 3-chloropropionyl-CoA inactivation is stable to such treatment. Therefore, such an adduct cannot solely involve a thio ester linkage. Exhaustive Pronase digestion of [^{14}C]-3-chloropropionyl-CoA-labeled enzyme produces a radioactive compound which cochromatographs with authentic carboxyethylcysteine using reverse-phase/ion-pairing high-pressure liquid chromatography and both silica and cellulose thin-layer chromatography systems. This suggests that enzyme inactivation is due to alkylation of an active-site cysteine residue.

Active-site-directed irreversible inhibitors (Shaw, 1970) can lend the crucial element of specificity to covalent modification of proteins and, consequently, offer a tremendous advantage over group-specific reagents in identifying active-site amino acid residues. The development of "suicide" reagents, i.e., relatively inert agents which are modified by target enzymes to generate highly reactive species bound at the active site (Bloch, 1969), represents an important extension of affinity labeling. Substantial structural and mechanistic information can result from successful affinity labeling studies, explaining the popularity of this approach.

A variety of active-site-directed and suicide reagents have been applied to the study of acyl coenzyme A (acyl-CoA)[1]-utilizing enzymes. The reagents are quite effective in modifying enzymes which catalyze formation of a carbanion at C2 of their acyl-CoA substrate. Both unsaturated and halogenated acyl-CoAs are effective irreversible inhibitors of thiolase (Holland et al., 1973). Acetylenic CoA derivatives have been profitably used to label acyl-CoA dehydrogenases (Frerman et al., 1980; Gomes et al., 1981; Fendrich & Abeles, 1982). 4-Bromo-2,3-dioxobutyl-CoA proved to be a potent irreversible inhibitor of citrate synthase, acetyl-CoA hydrolase, fatty acid synthase, and thiolase (Owens & Barden, 1978; Clements et al., 1982). In addition, the metabolite analogue fluoropropionyl-CoA has been useful in establishing that biotin-dependent carboxylation reactions do not proceed via a concerted process (Stubbe et al., 1980; Stubbe & Abeles, 1977).

HMG-CoA synthase catalyzes the three-step reaction sequence depicted below:

$$\text{acetyl-SCoA} + \text{HS-Enz} \rightleftharpoons \text{acetyl-S-Enz} + \text{CoASH} \quad (1)$$

$$\text{acetoacetyl-SCoA} + \text{acetyl-S-Enz} \rightleftharpoons \text{CoAS-HMG-S-Enz} \quad (2)$$

$$\text{CoAS-HMG-S-Enz} + H_2O \rightarrow \text{HMG-SCoA} + \text{HS-Enz} \quad (3)$$

Nucleophilic residues are involved in formation of the acetyl-S-enzyme intermediate (eq 1) and in the proton abstraction that produces the carbanionic species which attacks acetoacetyl-CoA (eq 2). A variety of precedents suggested that affinity labeling techniques might be profitably applied to identify a reactive basic residue at this enzyme's active site. Brief investigation of some of the reagents listed earlier suggested that, in the case of HMG-CoA synthase, they did not reliably function as classic affinity reagents. These observations prompted the synthesis of 3-chloropropionyl-CoA and the evaluation of this acyl-CoA analogue as an active-site-directed reagent. This report presents an account of these experiments and indicates the potentially broad utility of the reagent. A preliminary account of this work has appeared (Behnke & Miziorko, 1984).

Experimental Procedures

Materials

Homogeneous mitochondrial HMG-CoA synthase from chicken liver was prepared and assayed by the method of Reed et al. (1975). 3-Chloropropionyl chloride and 3-chloropropionic acid were purchased from Aldrich Chemical Co. The acyl chloride was redistilled before use; the free acid was recrystallized from petroleum ether before use. 3-Chloro[1-^{14}C]propionic acid was obtained from Pathfinder Laboratories, Inc. (St. Louis, MO). Oxalyl chloride and *S*-(carboxyethyl)-L-cysteine were purchased from Fluka AG. Mono- and bis(carboxyethyl)histidines (Chadha & Plapp, 1984) were a generous gift of Dr. Bryce Plapp. Mono- and bis(carboxyethyl)lysines were prepared as described by Cavins & Friedman (1967). CoASH (lithium salt) and dephospho-CoA were obtained from Pharmacia P-L Biochemicals. Dephospho-CoA

†This work was supported in part by a grant from the National Institutes of Health (AM 21491). H.M.M. is the recipient of a National Institutes of Health Research Career Development Award (AM 00645).

[1] Abbreviations: CoA, coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; Cl_3CCOOH, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

0006-2960/85/0424-3174$01.50/0

kinase was generously provided by Dr. R. Naylor (Pharmacia P-L Biochemicals). Pronase protease from *Streptomyces griseus* was purchased from Calbiochem. [γ-^{32}P]ATP was obtained from New England Nuclear. All other chemicals were of the highest quality commonly available. Glass fiber filters were purchased from H. Reeve Angel. Silica gel 60 TLC plates came from EM Laboratories; MN cellulose TLC plates were from Analtech, Inc.

Methods

Synthesis of 3-Chloropropionyl Coenzyme A. 3-Chloropropionyl-CoA was prepared by the method of Simon & Shemin (1953). A 5-fold molar excess of redistilled 3-chloropropionyl chloride was added in small portions to a solution of CoASH (lithium salt) in 0.4 M $KHCO_3$, pH 7.0–7.5, at 0 °C. After the solution gave a negative response when tested with nitroprusside reagent, indicating the absence of free sulfhydryl, the pH of the solution was lowered to 3.5. The solution was then extracted with diethyl ether. The aqueous layer, containing the reaction product, was brought to pH 4.5–5.0. 3-Chloropropionyl-CoA was purified by column chromatography on Whatman DE52 cellulose, using a 20–200 mM LiCl linear gradient containing 3 mM HCl, followed by desalting on a Sephadex G10 column. The 3-chloropropionyl-CoA was stored at −20 °C in solution or as a lyophilized powder. The purity of the product was assessed by reverse-phase HPLC [LiChrospher RP-18; 100 mM tetrabutylammonium phosphate (pH 5.5)/methanol (45:55)].

Synthesis of [^{14}C]-3-Chloropropionyl-CoA. 3-Chloro[1-^{14}C]propionic acid was dissolved in a 25-fold molar excess of oxalyl chloride, and the resulting solution was incubated for 30 min at 50 °C to form the acyl chloride (Kass & Brock, 1969). Unreacted oxalyl chloride was volatilized by using a stream of dry N_2. The product was dissolved in diethyl ether, and the last traces of unreacted reagent were evaporated along with solvent under dry N_2. An aliquot of the radioactive product, as well as a sample of authentic 3-chloropropionyl chloride, was reacted with neutral hydroxylamine. The resulting hydroxamates migrated identically upon thin-layer chromatography (cellulose; water-saturated 1-butanol). [^{14}C]-3-Chloropropionyl-CoA was prepared by using the ^{14}C-labeled acyl chloride in the procedure described above. The DEAE-cellulose-purified product comigrated with unlabeled material upon reverse-phase HPLC.

Synthesis of 3-Chloropropionyl-[3′-^{32}P]CoA. A 4.0-mL reaction mixture containing 10 μmol of dephospho-CoA, 98 μmol of [γ-^{32}P]ATP (0.16 Ci/mol), and 60 milliunits of dephospho-CoA kinase in 40 mM Tris-HCl, pH 8.2, with 30 mM $MgCl_2$ and 1 mM DTT was incubated at 37 °C for 1 h. An additional 15 milliunits of enzyme was then added to the reaction mixture, and incubation was continued for a total of 4.8 h. The reaction was terminated by freezing the mixture. The product was purifed by column chromatography on DEAE-cellulose (1.5 × 25 cm) using a 10–200 mM LiCl linear gradient containing 3 mM HCl. Fractions were monitored for A_{260} and ^{32}P radioactivity. The [^{32}P]CoASH-containing fractions (verified by reverse-phase HPLC) were pooled and concentrated by using a rotary evaporator. The residue was dissolved in cold methanol and precipitated by addition of 4 volumes of cold acetone. The dried product was stored at −20 °C.

3-Chloropropionyl-[3′-^{32}P]CoA was prepared by reacting [3′-^{32}P]CoA with 3-chloropropionyl chloride, as described for the synthesis of unlabeled 3-chloropropionyl-CoA, except using 0.13 M Li_2CO_3 instead of the $KHCO_3$ buffer. The 3-chloropropionyl-[^{32}P]CoA was purified by repeated precipitation from methanol/acetone (1:5). The purified product was stored as an aqueous solution, pH 5, at −20 °C.

Table I: Acyl-CoA Modification of HMG-CoA Synthase[a]

acylating agent	radioact. in protein ppt: cpm	radioact. in protein ppt: mol/mol of enzyme sites
[1-^{14}C]acetyl-CoA (7420 cpm/nmol)	5370	0.70
[1-^{14}C]propionyl-CoA (12 000 cpm/nmol)	3112	0.25
[1-^{14}C]-3-chloropropionyl-CoA (5380 cpm/nmol)	4036	0.73
3-chloropropionyl-[3′-^{32}P]CoA (333 cpm/nmol)	326	0.47

[a] Homogeneous HMG-CoA synthase (1.03 nmol in the ^{14}C labeling experiments and 2.08 nmol in the ^{32}P labeling experiment) was incubated at 30 °C in 100 mM potassium phosphate buffer, pH 7.5, for 5 min in the presence of [1-^{14}C]acetyl-CoA (0.19 mM; 7420 cpm/nmol) or [1-^{14}C]propionyl-CoA (0.21 mM, 12 000 cpm/nmol) or for 25 min in the presence of 0.19 mM [1-^{14}C]-3-chloropropionyl-CoA (5380 cpm/nmol) or 3-chloropropionyl-[3′-^{32}P]CoA (333 cpm/nmol). Final volume of reaction mixtures was 0.1 mL. An aliquot of the 3-chloropropionyl-CoA-treated enzyme was assayed to verify virtually complete inactivation by the end of the incubation period. Reactions were terminated by addition of 10 volumes of ice-cold 10% trichloroacetic acid. Each sample of precipitated protein was centrifuged, resuspended in 1 mL of cold 10% Cl_3CCOOH, and pipetted onto a 2.5-cm glass fiber filter. The filters were washed 10 times with 5 mL of cold 10% Cl_3CCOOH, 6 times with 5 mL of cold 50 mM sodium pyrophosphate in 0.5 M HCl, and once with 10 mL of cold absolute ethanol. Radioactivity was determined by liquid scintillation counting.

Results

Formation of Covalent Adducts between HMG-CoA Synthase and Substrate Analogues. The acetyl-CoA binding site on HMG-CoA synthase will accomodate a variety of acyl-CoA molecules, as indicated by physical or kinetic competition experiments (Miziorko et al., 1979; Menahan et al., 1981). However, formation of a covalent acyl-enzyme adduct does not invariably result when an acetyl-CoA analogue binds to the enzyme. In this context, it is significant that propionyl-CoA, which does not appear to function as an alternate substrate, is able to acylate HMG-CoA synthase (Table I). A diminished level of enzyme acylation is supported by propionyl-CoA in comparison with that measured by using comparable levels of acetyl-CoA. This observation may merely reflect differences in equilibrium constants for the partial reaction (cf. eq 1) which involves acylation of enzyme by these metabolites. While the stoichiometry of loading is lower than that observed with the actual substrate, acetyl-CoA, propionylation of the active site occurs at a significant level, suggesting that a modified propionyl-CoA derivative might also serve to acylate the cysteinyl sulfhydryl that normally reacts with acetyl-CoA. Upon incubation of [1-^{14}C]-3-chloropropionyl-CoA with enzyme, there is formation of a covalent adduct (Table I) with a stoichiometry approaching that measured when enzyme is modified by using acetyl-CoA. This does not necessarily imply that the equilibrium constant for enzyme acylation is substantially different for the 3-chloropropionyl-CoA- vs. propionyl-CoA-supported reactions, since covalently modified enzyme measured in the [^{14}C]-3-chloropropionyl-CoA experiment reflects contributions from more than one species (vide infra). Halogenated substrate analogues have been frequently used to covalently label enzyme active sites (Hartman, 1977); these precedents prompted our synthesis and investigation of 3-chloropropionyl-CoA as an active-site-directed irreversible inhibitor of HMG-CoA synthase. It was anticipated that, while 3-chloropropionyl-CoA may acylate the enzyme, alkylation of the protein will also result (Figure 1). This alkylation may occur directly after formation

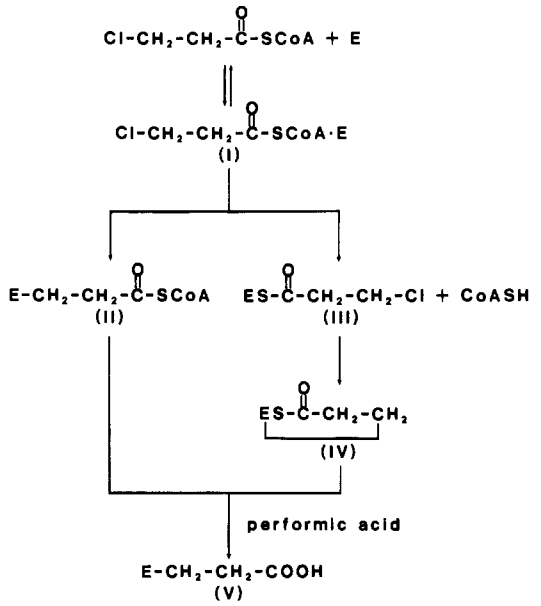

FIGURE 1: Possible mechanisms for 3-chloropropionyl-CoA inactivation of HMG-CoA synthase.

Table II: Stability of the Adduct Formed upon 3-Chloropropionyl-CoA Inactivation of HMG-CoA Synthase[a]

acylating agent	^{14}C radioact. (cpm) in protein ppt	
	−performic acid	+performic acid
3-chloropropionyl-CoA	4036	3068 (76%)
acetyl-CoA	5370	113 (2%)

[a] HMG-CoA synthase (1.03 nmol) was reacted with [1-^{14}C]-3-chloropropionyl-CoA or [1-^{14}C]acetyl-CoA as described in the legend of Table I. Paired samples were denatured with 10% trichloroacetic acid. Precipitated protein was loaded onto glass fiber filters and washed first with cold 10% trichloroacetic acid, then with 50 mM sodium pyrophosphate in 0.5 M HCl, and finally with absolute ethanol. One of the paired filters was counted immediately for ^{14}C radioactivity; the other was incubated overnight over performic acid and washed again with cold ethanol prior to counting for ^{14}C radioactivity.

of the initial noncovalent enzyme–3-chloropropionyl-CoA complex (I), resulting in the species indicated by II, or may be delayed until after acylation of enzyme to form a 3-chloropropionyl-S-enzyme species (III). An indication that alkylation occurs at both stages is provided by mixing enzyme with 3-chloropropionyl-[3′-^{32}P]CoA prior to denaturation with trichloroacetic acid. Since 0.47 nmol of ^{32}P radioactivity remains covalently bound per nanomole of denaturated enzyme (Table I), substantial alkylation of enzyme must occur prior to formation of 3-chloropropionyl-S-enzyme, which would labilize the ^{32}P label. The higher stoichiometry of ^{14}C labeling reflects the formation of two additional species, neither of which contains a CoA moiety. Acylation of enzyme occurs to form an intermediate (III) which can react further to alkylate the enzyme (IV). Protein modified in either fashion is stable to trichloroacetic acid precipitation. To determine how much acylated enzyme fails to react further to produce an alkylated species, the sample is subjected to performic acid oxidation (Table II). Such treatment labilizes modified enzyme which contains only thio ester linked groups by destroying the thio ester linkage with formation of a cysteic acid residue and regeneration of the free acid form of the acylating agent (Miziorko et al., 1975). Less than 25% of the radioactivity bound to [^{14}C]-3-chloropropionyl-CoA-inactivated enzyme is liberated under these conditions. Thus, a substantial fraction of covalently modified enzyme contains a performic acid stable enzyme–inhibitor adduct *in addition to* any thio ester bridge. The nature of this new adduct will be discussed below.

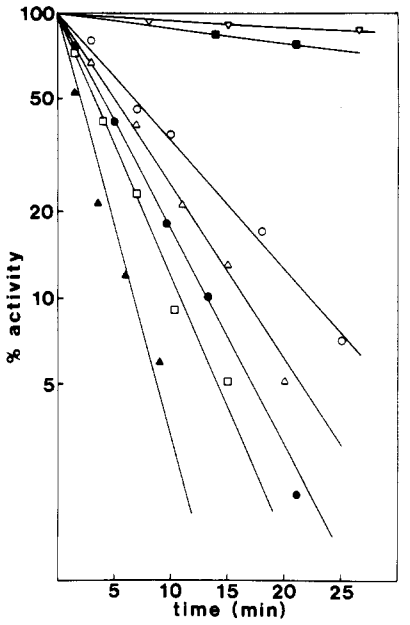

FIGURE 2: Kinetics of 3-chloropropionyl-CoA inhibition of HMG-CoA synthase. Reaction mixtures (300 μL) were prepared containing potassium phosphate buffer, pH 7.0 (50 mM), HMG-CoA synthase (18 μg), 8 (○), 12 (△), 24 (●), 48 (□), and 118 μM (▲) 3-chloropropionyl-CoA, 100 μM acetoacetyl-CoA plus 48 μM 3-chloropropionyl-CoA (■), or 100 μM acetyl-CoA plus 48 μM 3-chloropropionyl-CoA (▽). Incubations were initiated by addition of 3-chloropropionyl-CoA and were performed at 30 °C. Aliquots were withdrawn at the times indicated and assayed for enzyme activity by standard spectrophotometric procedures (Reed et al., 1975) using mixtures containing 50 μM acetoacetyl-CoA.

Kinetics of HMG-CoA Synthase Inactivation by 3-Chloropropionyl-CoA. Upon incubation with 3-chloropropionyl-CoA, HMG-CoA synthase displays a time-dependent decrease in activity which is compatible with first-order kinetics (Figure 2). Exhaustive dialysis of inactivated enzyme, under conditions which preserve full activity in control experiments performed with native enzyme, does not restore enzyme activity. The rate of inactivation is a function of inhibitor concentration. A rate constant for inactivation (k_{inact}) can be calculated from the half-life of processes which follow first-order kinetics (Laidler, 1958); a replot of $1/k_{inact}$ values vs. the reciprocal of the corresponding inhibitor concentrations yields a straight line which has a finite intercept on the vertical axis, indicating that the inactivation process is characterized by saturation kinetics (Meloche, 1967). Such behavior is predicted if irreversible inhibition occurs subsequent to formation of a Michaelis–Menten-type complex between 3-chloropropionyl-CoA and enzyme.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E\text{–}I \qquad (4)$$

The numerical value of the vertical intercept of the replot is a measure of the reciprocal of the limiting rate constant (k_2 = 0.31 min^{-1}). The horizontal intercept provides an estimate of the reciprocal of the binding constant ($K_I = k_1/k_{-1}$) for 3-chloropropionyl-CoA in the initial noncovalent complex with enzyme. The K_I = 15 μM indicates tight binding of 3-chloropropionyl-CoA at the acetyl-CoA site comparable to that observed upon substrate inhibition by acetoacetyl-CoA (K_i = 10 μM; Menahan et al., 1981) or upon product inhibition by HMG-CoA (K_i = 12 μM; Miziorko, 1984).

Specificity of inhibition is implied by the high affinity with which 3-chloropropionyl-CoA binds to form the initial non-

Table III: Thin-Layer Chromatographic Characterization of the Modified Amino Acid Recovered from Pronase Digests of 3-Chloropropionyl-CoA-Inactivated HMG-CoA Synthase[a]

chromatographic system	R_f of ^{14}C radioact. and (carboxyethyl)-cysteine	^{14}C radioact. recovered (%)
silica; 1-propanol/H_2O (70:30)	0.48	80
cellulose; 1-butanol/acetic acid/H_2O (4:1:1)	0.40	67

[a] Aliquots of the redissolved residue from the Pronase digest of [^{14}C]-3-chloropropionyl-CoA-inactivated HMG-CoA synthase were applied with authentic (carboxyethyl)cysteine to cellulose or silica gel thin-layer sheets. Chromatograms were developed in the solvent systems indicated. The (carboxyethyl)cysteine was detected by spraying the dried chromatograms with 0.25% ninhydrin in acetone. ^{14}C was determined by scraping the cellulose or silica gel from 0.5-cm zones into liquid scintillation vials and counting in liquid scintillator. In each case, the peak of ^{14}C activity coincided with cochromatographed (carboxyethyl)cysteine. R_f values of other amino acid standards (silica; propanol/H_2O systems) were as follows: N^{ϵ}-(carboxyethyl)lysine, 0.04; N^{ϵ},N^{ϵ}-bis(carboxyethyl)lysine, 0.09; N^1- and N^3-(carboxyethyl)-histidine, 0.24; N^1,N^3-(dicarboxyethyl)histidine, 0.30.

covalent complex. An additional argument for specific inhibition is based on the observation that substantial protection against 3-chloropropionyl-CoA inhibition is afforded when either acetyl-CoA or acetoacetyl-CoA is present in the incubation mix (Figure 2). HMG-CoA synthase displays the classical ping-pong Bi-Bi pattern for binding of substrates and release of products. Acetyl-CoA is the first substrate to bind, and its ability to compete with 3-chloropropionyl-CoA for a common binding site and retard the inactivation process is expected if the irreversible modification is active site directed. Acetoacetyl-CoA binds productively as a second substrate only after enzyme has been acetylated by acetyl-CoA. However, in accordance with the ping-pong scheme for substrate binding, it can also bind unproductively to the free enzyme, occupying the acetyl-CoA site and functioning as a substrate inhibitor. In this capacity, it also competes with 3-chloropropionyl-CoA for a common specific binding site (i.e., the acetyl-CoA site), reducing the probability of forming the initial enzyme–3-chloropropionyl-CoA complex and, consequently, decreasing the rate of inactivation due to production of a covalently modified enzyme.

Reaction of 3-Chloropropionyl-CoA with an Active-Site Cysteine. Acetyl-, propionyl-, and 3-chloropropionyl-CoA form covalent adducts with HMG-CoA synthase (Table I). Thio ester linked acyl groups are cleaved from enzyme by performic acid oxidation (Miziorko et al., 1975; Miziorko & Lane, 1977) under conditions which labilize less than 25% of the acyl groups bound in 3-chloropropionyl-CoA-modified enzyme (Table II). These observations suggest that a reactive active-site nucleophilic group reacts with the bound chloropropionyl group (or some derivative produced from this species) to form a performic acid stable linkage. To identify this active-site nucleophile, the following strategy was employed. [^{14}C]-3-Chloropropionyl-CoA-inactivated enzyme was precipitated with 10% trichloroacetic acid, loaded onto a glass fiber filter, washed exhaustively with 10% trichloroacetic acid and 50 mM sodium pyrophosphate in 0.5 M HCl, and rinsed with cold ethanol. The labeled protein was digested by incubating the glass fiber filter in a buffered solution (25 mM NH_4HCO_3, pH 7.8) of Pronase, as previously described (Miziorko & Lane, 1977). Digestion was performed at room temperature for 4 days. The digestion was terminated by freeze-drying, which also removed volatile buffer salts. This product was dissolved in water, and aliquots of the resulting

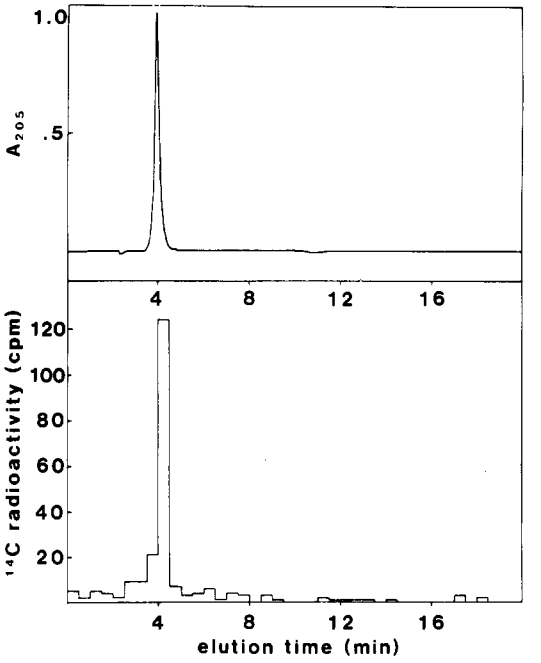

FIGURE 3: Elution profile of (carboxyethyl)cysteine (top) and ^{14}C-labeled material from a Pronase digest of [^{14}C]-3-chloropropionyl-CoA-inactivated HMG-CoA synthase (bottom). High-pressure liquid chromatography using a Lichrospher RP-18 column (4.6 × 250 mm; 10-μm particle size) was performed isocratically (10 mM tetrabutylammonium phosphate, pH 5.5) at a flow rate of 1.0 mL/min. The absorbance of the effluent was monitored at 205 nm. Fractions of the effluent were collected, and ^{14}C radioactivity was measured by standard liquid scintillation techniques.

solution, along with appropriate standards, were subjected to HPLC (Figure 3) and thin-layer chromatography (Table III) in order to identify the amino acid residue that reacts with the 3-chloropropionyl moiety (or a reactive derivative thereof) accounting for enzyme inactivation. In each of the chromatographic systems employed, the ^{14}C radioactivity is primarily accounted for by a single component (recovery: HPLC, 66%; TLC, 67–80%) which coincides with authentic (carboxyethyl)cysteine. Thus, all data generated in this series of experiments are consistent with the proposal that enzyme inactivation results from attack of the sulfhydryl group of an active-site cysteine on a reactive acyl group derived from 3-chloropropionyl-CoA.

Discussion

The ability of HMG-CoA synthase to bind a variety of acyl-CoA derivatives suggested that several previously reported active-site-directed reagents might serve as affinity labels for this enzyme. Contrary to such expectations, 3-butynoyl-CoA, which is an effective inhibitor of thiolase (Holland et al., 1973) as well as long- and short-chain acyl-CoA dehydrogenases (Frerman et al., 1980; Gomes et al., 1981), inactivates HMG-CoA synthase rather poorly (H. Miziorko, unpublished results). Only a few potential irreversible inhibitors of HMG-CoA synthase have been screened, and therefore, it has not been possible to draw any correlation between the ability of an inhibitor to reversibly acylate the enzyme and the efficacy of subsequent inactivation. As indicated in Figure 1, enzyme may be covalently modified (II) directly after formation of an enzyme–3-chloropropionyl-CoA complex (I) or may be acylated (III), with release of CoASH, prior to inactivation (IV). Since the stoichiometry of labeling using [1-^{14}C]-3-chloropropionyl-CoA is 50% higher than that measured using 3-chloropropionyl-[3′-^{32}P]CoA (Table I), it seems clear that acylation does occur. When the ^{14}C-labeled sample is per-

formic acid oxidized to remove contributions from label that is solely thio ester linked, the stoichiometry of incorporation is slightly in excess of that measured in the ^{32}P labeling experiment. This indicates that conversion of III to IV proceeds less smoothly than that of I to II. Thus, in the case of HMG-CoA synthase, most of the 3-chloropropionyl-CoA-inactivated enzyme is formed without prior acylation by the inhibitor. In contrast, when similar experiments were performed with fatty acid synthetase, good stoichiometries of incorporation were measured in the ^{14}C labeling experiment, and no substantial incorporation was observed in the parallel experiment using 3-chloropropionyl-[3′-^{32}P]CoA. (H. Miziorko, C. Behnke, P. A. Ahmad, and F. Ahmad, unpublished results).

For the sake of clarity, Figure 1 does not explicitly indicate the possible intermediacy of a reactive derivative of 3-chloropropionyl-CoA in the modification process. An active-site nucleophile may react directly with the thioesterified 3-chloropropionyl group, accounting for inactivation. Alternatively, proton abstraction from the α-carbon and subsequent chloride elimination may result in transient formation of an acrylyl moiety which then irreversibly reacts with an active-site nucleophile. In either case, hydrolysis of thio ester linkages which are relatively labile at physiological pH accounts for recovery of the modified enzyme as a carboxyethylated derivative. While participation of an acrylyl moiety in the inactivation process has not been demonstrated, there is some indirect evidence in support of its formation. Development of methodology for cleanly preparing acrylyl-CoA has been hindered by the inherent reactivity of an acrylyl group with other nucleophiles (Stubbe et al., 1980). The heterogeneous samples which can be prepared contain a potent irreversible inhibitor of HMG-CoA synthase, but, due to the presence of multiple CoA-containing components, no assignment of the reactive species can be made with certainty. Perhaps a more compelling argument involves the proven ability of HMG-CoA synthase to catalyze the chemical steps needed for formation of an acrylyl derivative. HMG-CoA synthase deprotonates the α-carbon of the acetyl-S-enzyme intermediate prior to the condensation reaction. In fact, even in the absence of the second substrate, acetoacetyl-CoA, the enzyme slowly catalyzes proton exchange between [^{3}H]acetyl-CoA and water (Miziorko et al., 1975). Such observations suggest that formation of an acrylyl derivative from a 3-chloropropionyl derivative is a realistic possibility. Direct measurement of chloride elimination cannot be made with the sensitivity that allowed Stubbe & Abeles (1977) to use fluoride formation to conveniently monitor acrylyl-CoA production from fluoropropionyl-CoA. Thus, the distinction between whether 3-chloropropionyl-CoA functions as a suicide reagent, generating the reagent which ultimately accounts for enzyme inactivation, or whether it is strictly an affinity reagent must await the results of future investigation.

Regardless of the identity of the actual inactivating agent, there is no doubt that irreversible inhibition occurs by the classic active-site-directed process (Meloche, 1967). In contrast to inactivation of yeast HMG-CoA synthase by bromoacetyl-CoA (Middleton & Tubbs, 1972), the avian liver enzyme is inactivated by 3-chloropropionyl-CoA in a process which follows first-order kinetics. In addition, as expected for a process which involves a prebound inhibitor, the inactivation displays saturation kinetics. Good protection is afforded by acetyl-CoA or acetoacetyl-CoA, metabolites that compete with 3-chloropropionyl-CoA for the acetyl-CoA site. Finally, incorporation of reagent approaches the stoichiometry observed for acetylation of enzyme by the substrate acetyl-CoA.

The recovery of [^{14}C](carboxyethyl)cysteine from digests of [^{14}C]-3-chloropropionyl-CoA-inactivated enzyme underscores the importance of active-site cysteinyl –SH residues. There are a total of eight cysteines per 53 000-dalton HMG-CoA synthase subunit, as determined by titration of SDS-denatured enzyme with Ellman's reagent or by amino acid analysis (Miziorko, 1984). One of these residues is involved in forming a thio ester with the acyl group of reaction intermediates or with certain analogues that bind at the acetyl-CoA site (Miziorko et al., 1975; Miziorko & Lane, 1977). There is no reason to expect, a priori, that this cysteinyl –SH is the target of the 3-chloropropionyl-CoA modification. In fact, the observation that some irreversible modification of protein occurs after acylation of this active-site sulfhydryl suggests that another amino acid residue is the target of the reagent. Assigning a role to such a residue is not entirely straightforward. A nucleophilic group participates as a base catalyst by abstracting a proton from the acetyl-S-enzyme prior to condensation of the resulting carbanion with acetoacetyl-CoA. In addition to several uncharged basic groups, any anionic group, including a deprotonated cysteinyl sulfhydryl, could function in this capacity. The pK of a typical cysteinyl sulfhydryl is somewhat higher than that of several other potential bases, and, thus, unless an unusual environment is involved, such a residue seems to be a less likely candidate. In the active-site-directed inactivation of ketodeoxyphosphogluconate aldolase by bromopyruvate (Meloche, 1973), both a carboxyl group and a cysteinyl sulfhydryl were identified as targets, but the former residue was ultimately implicated in proton abstraction. In other similar proton abstractions (Fendrich & Abeles, 1982; O'Connell & Rose, 1973), there is also evidence that carboxylate anions function as the base. However, active-site cysteinyl sulfhydryls with pK's less than 5 have been detected in fatty acid synthetase (Oesterhelt et al., 1977) and papain (Lewis et al., 1976) so that a role for a deprotonated cysteinyl sulfhydryl as the nucleophile should not be ruled out. Middleton & Tubbs (1972) reported that yeast HMG-CoA synthase is extremely susceptible to inhibition by Cd^{2+} and arsenical compounds. On the basis of these data, it was postulated that enzyme activity depends on two vicinal thiol groups. In titration of the native enzyme with dithiobis(nitrobenzoic acid), we observe that, during the course of modification of the second of eight possible cysteines, precipitation of the enzyme begins (H. Miziorko, unpublished results). Thus, several pieces of data argue for the presence of two cysteines at the enzyme's active site.

Unlike many reagents which have been synthesized for use as affinity labels, 3-chloropropionyl-CoA is stable upon storage as a lyophilized powder or as a frozen solution at −20 °C. This contrasts sharply with the instability of several brominated inactivators (Clements et al., 1981; Barden et al., 1981). In addition, 3-chloropropionyl-CoA appears to be a very selective reagent, on the basis of the stoichiometry observed in the HMG-CoA synthase modification. Use of brominated affinity reagents to inactivate other lipogenic enzymes has resulted in the labeling of multiple sites (Clements et al., 1982). As pointed out by Hartman (1977), bromine is a much better leaving group than chlorine, and consequently, brominated reagents are usually quite reactive; there is usually a trade-off involving lowered selectivity in exchange for the high reactivity of such reagents. Thus, in view of the stability and specificity of 3-chloropropionyl-CoA, it seems possible that this reagent may find wide use in affinity labeling of acyl-CoA-utilizing enzymes.

ACKNOWLEDGMENTS

We are grateful to Dr. Frank Frerman for many useful suggestions during the course of this work, to Dr. JoAnne Stubbe for generously providing us with a sample of fluoropropionyl-CoA, and to Dr. Bryce V. Plapp for supplying the carboxyethylated histidine standards.

Registry No. HMG-CoA synthase, 9027-44-5; 3-chloropropionyl-CoA, 96212-36-1; [^{14}C]-3-chloropropionyl-CoA, 96212-37-2; 3-chloropropionyl-[3′-^{32}P]CoA, 96212-38-3; CoASH, 85-61-0; [3′-^{32}P]CoA, 35364-62-6; 3-chloropropionyl chloride, 625-36-5; 3-chloro[1-^{14}C]propionic acid, 83565-55-3; oxalyl chloride, 79-37-8; cysteine, 52-90-4.

REFERENCES

Barden, R. E., Owens, M. S., & Clements, P. R. (1981) *Methods Enzymol. 72*, 580–583.

Behnke, C. E., & Miziorko, H. M. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 1783.

Block, K. (1969) *Acc. Chem. Res. 2*, 193–202.

Bradbury, A. F., & Smyth, D. G. (1973) *Biochem. J. 131*, 637–642.

Cavins, J. F., & Friedman, M. (1967) *Biochemistry 6*, 3766–3770.

Chadha, V. K., & Plapp, B. V. (1984) *Biochemistry 23*, 216–221.

Clements, P. R., Wallace, J. C., & Keech, D. B. (1981) *Methods Enzymol. 72*, 578–579.

Clements, P. R., Barden, R. E., Ahmad, P. M., Chisner, M. B., & Ahmad, F. (1982) *Biochem. J. 207*, 291–296.

Fendrich, G., & Abeles, R. H. (1982) *Biochemistry 21*, 6685–6695.

Frerman, F. E., Miziorko, H. M., & Beckman, J. D. (1980) *J. Biol. Chem. 255*, 11192–11198.

Gomes, B., Fendrich, G., & Abeles, R. H. (1981) *Biochemistry 20*, 1481–1490.

Hartman, F. C. (1977) *Methods Enzymol. 46*, 130–153.

Holland, P. C., Clark, M. G., & Bloxham, D. P. (1973) *Biochemistry 12*, 3309–3315.

Kass, L. R., & Brock, D. J. H. (1969) *Methods Enzymol. 14*, 696–698.

Laidler, K. R. (1958) *The Chemical Kinetics of Enzyme Action*, p 23, Oxford University Press, London.

Lewis, S. D., Johnson, F. A., & Shafer, J. A. (1976) *Biochemistry 15*, 5009–5017.

Meloche, H. P. (1967) *Biochemistry 6*, 2273–2280.

Meloche, H. P. (1973) *J. Biol. Chem. 248*, 6945–6951.

Menahan, L. A., Hron, W. T., Hinckelman, D. G., & Miziorko, H. M. (1981) *Eur. J. Biochem. 119*, 287–294.

Middleton, B., & Tubbs, P. K. (1972) *Biochem. J. 126*, 27–34.

Miziorko, H. M. (1984) *Methods Enzymol. 110*, 19–26.

Miziorko, H. M., & Lane, M. D. (1977) *J. Biol. Chem. 252*, 1414–1420.

Miziorko, H. M., Clinkenbeard, K. C., Reed, W. D., & Lane, M. D. (1975) *J. Biol. Chem. 250*, 5768–5773.

Miziorko, H. M., Lane, M. D., & Weidman, S. W. (1979) *Biochemistry 18*, 399–403.

O'Connell, E., Rose, I. A. (1973) *J. Biol. Chem. 248*, 2225–2231.

Oesterhelt, D., Bauer, H., Kresze, G. B., Steber, L., & Lynen, F. (1977) *Eur. J. Biochem. 79*, 173–180.

Owens, M. S., & Barden, R. E. (1978) *Arch. Biochem. Biophys. 187*, 299–306.

Reed, W. D., Clinkenbeard, K. C., & Lane, M. D. (1975) *J. Biol. Chem. 250*, 3117–3123.

Shaw, E. (1970) *Enzymes, 3rd. Ed. 1*, 91.

Simon, E. J., & Shemin, D. (1953) *J. Am. Chem. Soc. 75*, 2520.

Stubbe, J. A., & Abeles, R. H. (1977) *J. Biol. Chem. 252*, 8338–8340.

Stubbe, J. A., Fish, S., & Abeles, R. H. (1980) *J. Biol. Chem. 255*, 236–242.

Weil, L., & Seibles, T. S. (1961) *Arch. Biochem. Biophys. 95*, 470–473.