# Chemical Events in Chloropropionyl Coenzyme A Inactivation of Acyl Coenzyme A Utilizing Enzymes†

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ABSTRACT: Incubation of 3-chloropropionyl-CoA with 3-hydroxy-3-methylglutaryl-CoA synthase results in exchange of the C2 proton with solvent as inactivation of enzyme proceeds. This enzyme is also inhibited by *S*-acrylyl-*N*-acetylcysteamine; the limiting rate constant for inactivation by the acrylyl derivative (0.36 $min^{-1}$) slightly exceeds the value measured for chloropropionyl-CoA (0.31 $min^{-1}$). These observations support the intermediacy of acrylyl-CoA in the chloropropionyl-CoA-dependent inactivation of hydroxymethylglutaryl-CoA synthase. Inhibition of fatty acid synthase by chloropropionyl-CoA is primarily due to alkylation of a reactive cysteine, although secondary reaction with the enzyme's pantetheinyl sulfhydryl occurs. Modification of fatty acid synthase by *S*-acrylyl-*N*-acetylcysteamine occurs at a limiting rate (1.8 $min^{-1}$) that is comparable to that estimated for chloropropionyl-CoA-dependent inactivation. However, this enzyme lacks the ability to deprotonate C2 of an acyl group such as the chloropropionyl moiety. Since such a step would be required to generate an acrylyl group from chloropropionyl-*S*-enzyme, it is likely that a typical affinity labeling process accounts for inactivation of fatty acid synthase by chloropropionyl-CoA. HMG-CoA lyase is also inhibited by *S*-acrylyl-*N*-acetylcysteamine. In contrast to the ability of this reagent to serve as a mechanism-based inhibitor of hydroxymethylglutaryl-CoA synthase and an affinity label of fatty acid synthase, it acts as a group-specific reagent in modifying HMG-CoA lyase ($k_2$ = 86.7 $M^{-1}$ $min^{-1}$).

A variety of approaches have been used to design reactive analogues that label catalytic or regulatory sites of acyl-CoA-utilizing enzymes (Holland et al., 1973; Owens & Barden, 1978; Clements et al., 1982; Davis et al., 1987). Highly reactive analogues frequently modify the target protein at multiple sites, complicating the subsequent extraction and interpretation of structural information. With this complication in mind, we developed 3-chloropropionyl-CoA as an active-site probe. While a β-haloacyl derivative might be expected to show relatively low reactivity under the reaction conditions typical for protein modification, chloropropionyl-CoA efficiently inactivated 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)[1] synthase (Miziorko & Behnke, 1985a). The selectivity exhibited by this reagent facilitated isolation and sequence analysis of the modified site (Miziorko & Behnke, 1985b). Chloropropionyl-CoA also irreversibly modifies fatty acid synthase (Miziorko et al., 1986). A cysteine involved in the condensation reaction was implicated in this modification.

While the ability of chloropropionyl-CoA to stoichiometrically modify active sites of certain acyl-CoA-utilizing enzymes is clear, the detailed chemical steps in the inactivation processes have not been described. In particular, it would be interesting to determine whether modification of the target protein is the result of a typical affinity labeling process (Shaw, 1970) or whether the enzyme transforms the β-haloacyl group to a more reactive species, qualifying the process as a mechanism-based inactivation reaction. In the latter case, formation and direct participation of an acrylyl derivative might be envisioned. Additional work involving enzyme modification by thioester-containing derivatives of chloropropionic and acrylic acid has been performed in order to elucidate the mechanism(s) of inactivation; the results are communicated in this paper.

## Experimental Procedures

### *Materials*

*N*-Acetylcysteamine was purchased from Chemical Dynamics. High specific activity $^3H_2O$ was obtained from New England Nuclear. Organic acids and acyl chlorides were purchased from Aldrich and were recrystallized or redistilled before use.

Avian liver mitochondrial HMG-CoA synthase was prepared as previously described (Reed et al., 1975; Miziorko, 1984). Rat mammary fatty acid synthase was prepared according to the procedure of Ahmad et al. (1982). Avian liver HMG-CoA lyase was purified by using the following modification of the procedure of Kramer and Miziorko (1980). The DEAE-cellulose chromatography step was performed using a column equilibrated at pH 7.8; the enzyme binds under these conditions and is eluted with a potassium phosphate gradient (10–200 mM). After hydroxylapatite chromatography, the final affinity chromatography step involves substitution of a blue Sepharose matrix for agarose–hexane–CoA or agarose–hexane–3′,5′-ADP; elution with HMG-CoA (100 μM) is

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[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; PTH, phenylthiohydantoin; HPLC, high-pressure liquid chromatography.

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performed to recover homogeneous enzyme.

*Methods*

*Synthesis of Unlabeled and Radiolabeled Acyl-CoA Analogues.* Preparation of 3-chloro[2-$^3$H]propionyl-CoA required synthesis of the appropriate tritiated acid precursor. This was performed by reaction of $^3$HCl with acrylic acid according to the procedure of Moureu et al. (1921). Oxalyl chloride was used to activate the acid to the acyl chloride, and this intermediate was used to thioesterify CoASH (Miziorko & Behnke, 1985a). *S*-Acrylyl-*N*-acetylcysteamine was prepared by the procedure of Stubbe et al. (1980). The lead salt of *N*-acetylcysteamine (Kass & Brock, 1969) was reacted with acrylyl chloride in benzene. The product was purified by chromatography on silicic acid ($CH_2Cl_2$/MeOH, 99:1) and its identity confirmed by proton NMR.

*Amino Acid and Protein Sequence Analyses.* Samples of fatty acid synthase that had been modified with chloro-[$^{14}$C]propionyl-CoA were hydrolyzed in constant-boiling HCl containing 10 mM mercaptoethanol (24 h, 115 °C) and taken to dryness prior to analysis on a Beckman 6800 amino acid analyzer. The instrument was modified to permit collection of the effluent for radioactivity determination by liquid scintillation counting. Tryptic digestion of radiolabeled protein was performed after exhaustive carboxymethylation. A radiolabeled, pantetheine-containing peptide was isolated by DEAE chromatography (1.5 × 20 cm column; elution performed by using a 1.5-L $NH_4HCO_3$ gradient, 10–600 mM) and reverse-phase HPLC (EM LiChrospher RP-18 column, 4 × 250 mm; elution performed by using a gradient of $CH_3CN$, 0–70%, in aqueous 0.1% trifluoroacetic acid). Amino acid composition was determined as described above. Sequence analysis was performed at the Protein and Nucleic Acid Shared Research Facility of the Medical College of Wisconsin using an Applied Biosystems Model 477A pulsed liquid-phase sequencer (Hewick et al., 1981).

## Results

*Correlation between Inactivation of HMG-CoA Synthase by 3-Chloro[2-$^3$H]propionyl-CoA and Tritium Exchange.* On chemical grounds, the β-haloacyl group of chloropropionyl-CoA is not expected to be highly reactive in modification of nucleophilic active-site amino acids. One possible explanation for this reagent's efficacy in inactivating HMG-CoA synthase is that chloropropionyl-CoA functions as a mechanism-based inhibitor rather than as a typical affinity label. If this hypothesis is correct, enzyme would deprotonate C2 of the acyl group, forming highly reactive acrylyl-CoA. The feasibility of this mechanism is supported by the well-established ability of HMG-CoA synthase to catalyze proton exchange from the carbon adjacent to the thioester carbonyl in the absence of the second substrate and without completion of an entire reaction cycle (Miziorko et al., 1975). Enzyme inactivation would be attributable to reaction of this acrylyl moiety with the active-site cysteine that has previously been established as the modification site (Miziorko & Behnke, 1985b). In order to test whether mechanism-based inactivation occurs, chloro[2-$^3$H]propionyl-CoA was prepared and incubated with HMG-CoA synthase. Residual enzyme activity was measured at various times after inactivation was started by assaying aliquots of the reaction mix. Additionally, determination of the level of tritium in aqueous solvent was performed by subjecting additional aliquots to bulb-to-bulb distillation in order to separate water from reaction components. As would be expected for mechanism-based inactivation, tritium levels in aqueous solvent increased concomitantly with enzyme inactivation (Figure 1). A partition ratio (exchange/inactivation ~6/1) may be estimated from these data. In control experiments, no substantial tritium exchange was observed when enzyme was omitted from the incubations.

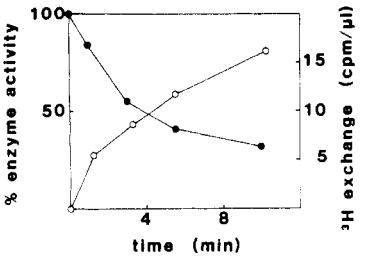


FIGURE 1: Inhibition of HMG-CoA synthase by chloro[2-$^3$H]-propionyl-CoA: coordinate loss of enzyme activity and formation of $^3H_2O$. HMG-CoA synthase (214 μg) is incubated at 30 °C in a reaction mixture (1.2 mL) containing 100 mM potassium phosphate, pH 7.5, and 3-chloro[2-$^3$H]propionyl-CoA (42 μM; 2500 cpm/nmol). At the times indicated, aliquots are withdrawn and used for enzyme activity measurements as well as determinations of $^3H_2O$ production, measured after separation from radioactive substrate and other nonvolatile components by bulb-to-bulb sublimation.

*Inactivation of HMG-CoA Synthase by S-Acrylyl-N-acetylcysteamine.* If chloropropionyl-CoA functions as a mechanism-based inhibitor in inactivating HMG-CoA synthase, then acrylyl-CoA forms at the enzyme's active site, and this species accounts for alkylation of a critical cysteine residue. Direct demonstration of the participation of acrylyl-CoA is hindered by the lack of any reasonable synthetic route for preparation of the compound (Stubbe et al., 1980; Patel & Walt, 1988). This obstacle can be bypassed by forming the acrylyl thioester with *N*-acetylcysteamine rather than CoA; this strategy relies on the observation that *N*-acetylcysteamine, which represents the terminal portion of the CoA molecule, can substitute for the nucleotide in partial reactions catalyzed by HMG-CoA synthase (Miziorko et al., 1975). When tested as an irreversible inhibitor of the enzyme, *S*-acrylyl-*N*-acetylcysteamine was observed to cause time-dependent inactivation. First-order kinetics are followed (Figure 2), and the substrate acetyl-CoA affords protection against inactivation, as expected if the analogue is functioning as an affinity label (Meloche, 1967; Shaw, 1970). Since the cysteamine moiety does not contain all of the binding determinants found in CoA, *S*-acyl-*N*-acetylcysteamine analogues typically bind to CoA-utilizing enzymes with affinity that is much weaker than the affinity of the corresponding acyl-CoA's (Jaenicke & Lynen, 1960). For example, *S*-(acetoacetyl)-*N*-acetylcysteamine substitutes for acetoacetyl-CoA as a second substrate in the HMG-CoA synthase catalyzed condensation reaction and supports $V_{max}$ comparable to that measured using acetoacetyl-CoA. The apparent $K_m$ for this alternate substrate (10 mM) indicates binding that is weaker by several orders of magnitude than that observed ($K_m \leq 1$ μM) for acetoacetyl-CoA (Menahan et al., 1981; Page & Tubbs, 1978). Thus, it is not surprising that a replot of the inactivation data for *S*-acrylyl-*N*-acetylcysteamine (Figure 3) indicates a $K_i$ (1.9 mM; Table I) that is much higher than the value observed for chloropropionyl-CoA (15 μM).

Importantly, the limiting rate constant for inactivation by these two inhibitors can be extracted by extrapolation of the observed inactivation rates to infinite inhibitor concentration (Figure 3). The rate constant for the cysteamine-containing analogue (0.36 min$^{-1}$; Table I) slightly exceeds the value measured for chloropropionyl-CoA (0.31 min$^{-1}$). Thus, the acrylyl-containing inhibitor is kinetically competent to be an intermediate in the chloropropionyl-CoA-dependent inacti-

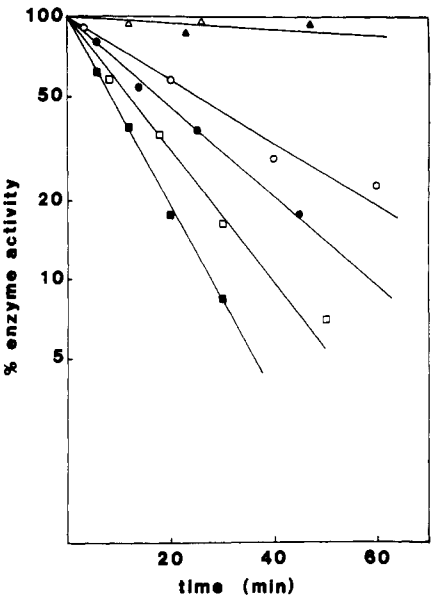


FIGURE 2: Kinetics of *S*-acrylyl-*N*-acetylcysteamine inhibition of HMG-CoA synthase. Reaction mixtures (100 μL) were prepared containing potassium phosphate buffer, pH 7.0 (50 mM), HMG-CoA synthase (41 μg), and 0 mM (▲), 0.1 mM (○), 0.2 mM (●), 0.3 mM (□), and 0.5 mM (■) *S*-acrylyl-*N*-acetylcysteamine or 0.1 mM acetyl-CoA plus 0.5 mM *S*-acrylyl-*N*-acetylcysteamine (△). Incubations were initiated by addition of *S*-acrylyl-*N*-acetylcysteamine 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).

Table I: Comparison of Chloropropionyl-*S*-CoA and *S*-Acrylyl-*N*-acetylcysteamine as Inhibitors of Acyl-CoA-Utilizing Enzymes

| | chloropropionyl-CoA | *S*-acrylyl-*N*-acetylcysteamine |
|---|---|---|
| HMG-CoA synthase | | |
| $K_i$ (mM) | 0.015[a] | 1.9 |
| $k_{inact}$ ($min^{-1}$) | 0.31[a] | 0.36 |
| fatty acid synthase | | |
| $K_i$ (mM) | ≤0.020[b] | 5.0 |
| $k_{inact}$ ($min^{-1}$) | ≤2[b] | 1.8 |
| HMG-CoA lyase | | |
| $k_2$ ($M^{-1}$ $min^{-1}$) | | 86.7 |

[a] Values reported by Miziorko and Behnke (1985a). [b] Values reported by Miziorko et al. (1986).

vation of HMG-CoA synthase.

*Inactivation of HMG-CoA Lyase by S-Acrylyl-N-acetylcysteamine.* In view of the high chemical reactivity of an acrylyl thioester, it was, a priori, unclear how well *S*-acrylyl-*N*-acetylcysteamine would function in active-site-directed modification as opposed to group-specific reaction. While the saturable inactivation kinetics and protection by substrate argued that modification of HMG-CoA synthase by this inhibitor is active site directed, this argument is strongly confirmed by the contrasting results obtained when HMG-CoA lyase is incubated with this analogue. As in the case of HMG-CoA synthase, lyase is inhibited in a time-dependent fashion, with pseudo-first-order kinetics being exhibited. In contrast to the data for HMG-CoA synthase, the rate of lyase inactivation is *not* saturable (Figure 4). Thus, the acrylyl derivative functions as an efficient group-specific inhibitor of HMG-CoA lyase ($k_2$ = 86.7 $M^{-1}$ $min^{-1}$; Table I). While the modification site(s) has (have) not yet been identified, it has been established that the enzyme has a sensitive sulfhydryl group (Kramer & Miziorko, 1980), suggesting that an active-site cysteine may be targeted for alkylation.

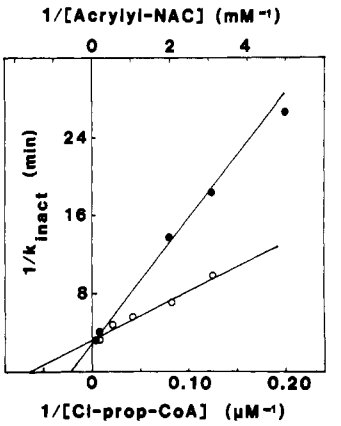


FIGURE 3: Double-reciprocal plot of $k_{inact}$ of HMG-CoA synthase as a function of inhibitor concentration. Rate constants for inactivation by *S*-acrylyl-*N*-acetylcysteamine (●) were derived from the data shown in Figure 2. Data for inactivation by 3-chloropropionyl-CoA (○) are shown for comparison and were obtained under comparable experimental conditions (Miziorko & Behnke, 1985a).

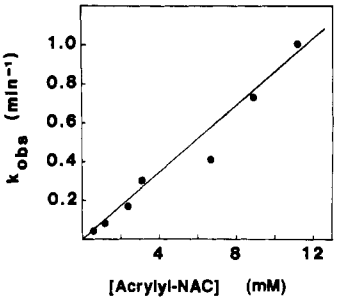


FIGURE 4: Kinetics of *S*-acrylyl-*N*-acetylcysteamine inhibition of HMG-CoA lyase. Reaction mixtures (100 μL) were prepared containing potassium phosphate, pH 7.0 (100 mM), bovine serum albumin (100 μg), dithiothreitol (0.2 mM), magnesium chloride (10 mM), HMG-CoA lyase (1.4 μg), and *S*-acrylyl-*N*-acetylcysteamine as indicated. HMG-CoA lyase in phosphate buffer with bovine serum albumin, dithiothreitol, and magnesium chloride was preincubated at 30 °C for 5 min. Incubations were then initiated by addition of *S*-acrylyl-*N*-acetylcysteamine and were performed at 30 °C. Aliquots were withdrawn at the times indicated and assayed for enzyme activity by standard spectrophotometric procedures (Kramer & Miziorko, 1980). A plot of $k_{observed}$ for HMG-CoA lyase inhibition as a function of *S*-acrylyl-*N*-acetylcysteamine concentration is shown. The slope of the line shown fit to the data indicates a bimolecular rate constant, $k_2$ = 86.7 $M^{-1}$ $min^{-1}$.

*Inactivation of Fatty Acid Synthase by S-Acrylyl-N-acetylcysteamine.* Low levels of chloropropionyl-CoA efficiently inhibit fatty acid synthase (Miziorko et al., 1986). Available evidence indicates that, in contrast to the situation observed with HMG-CoA synthase, the chloropropionyl group is transferred to the protein and CoASH liberated. However, the chemical nature of the acyl group that accounts for alkylation of the enzyme has not been established. On the basis of the rationale outlined above for HMG-CoA synthase, it was envisioned that *S*-acrylyl-*N*-acetylcysteamine should function as an affinity label for fatty acid synthase if a mechanism-based process accounts for the sensitivity of this enzyme to chloropropionyl-CoA. When the cysteamine derivative was tested, time-dependent inactivation was observed; loss of activity followed first-order kinetics, and protection was afforded by substrates. As expected for site-directed modification, saturable binding of the inhibitor is observed. A $K_i$ = 5.0 mM (Table I) is calculated from this replot of the data, indicating somewhat weaker binding than observed with HMG-CoA synthase. The limiting rate constant for inactivation of fatty acid synthase (1.8 $min^{-1}$) indicates a more rapid rate of in-

activation than that measured with HMG-CoA synthase. However, as will be discussed later, kinetic considerations may be insufficient to qualify an acrylyl-containing species as an intermediate in the chloropropionyl-CoA-dependent inactivation process.

*Protein Chemistry Studies on Chloropropionyl-CoA-Modified Fatty Acid Synthase.* The inactivation of fatty acid synthase by chloropropionyl-CoA has been explained by invoking carboxyethylation of a cysteinyl sulfhydryl in the condensing enzyme domain (Miziorko et al., 1986). The assignment was based on the observation that, after hydrolysis of enzyme that had been inactivated using radiolabeled inhibitor, the radiolabeled amino acid adduct comigrated with authentic (carboxyethyl)cysteine in two different thin-layer chromatographic systems. Subsequent evaluation of acid-hydrolyzed samples of fatty acid synthase that had been modified with chloro[$^{14}$C]propionyl-CoA, by more sensitive and higher resolution amino acid analysis techniques, indicates that our previous conclusion is substantially correct. However, 25–30% of the radioactivity recovered from the amino acid analyzer is attributable to (carboxyethyl)cysteamine, indicating that secondary labeling of the acyl carrier peptide occurs. Following exhaustive carboxymethylation of denatured, radiolabeled protein and tryptic digestion, it is possible to isolate the peptide bearing the minor radiolabeled component. Amino acid analysis indicates the presence of $\beta$-alanine as well as radiolabeled (carboxyethyl)cysteamine in hydrolysates of the isolated material, suggesting that this fragment is derived from the pantetheine-containing acyl carrier peptide. This assignment has been directly confirmed by sequence analysis,[2] which indicates the structure Asp-Leu-Ala-Gly-Ile-Asn-Leu-Asp-Ser-Ser-Leu-Ala-Asp-Leu-Gly-Gln,(Leu)-Asp-Ser-Leu-Met-Gly-Val-Glu-Val,(Gln)-Arg. The serine at position 18 represents the attachment site for the panthetheine cofactor which, in this case, is carboxyethylated. The sequence is in good agreement (>95% homology) with a stretch of the primary sequence deduced for the rat protein from cDNA analysis (Witkowski et al., 1987). The region flanking the pantetheine-bearing serine also shows good homology with the recently published chicken acyl carrier peptide sequence (Chang & Hammes, 1988).

In contrast with our experiences in isolating and analyzing a variety of active-site peptides, including the acyl carrier peptide mentioned above, work on the peptide containing the active cysteine from the condensing enzyme domain has not been conclusive. The radiolabeled peptide is recovered in low yield upon chromatography using conventional ion-exchange or HPLC methodology. Instead of the usual sharp peaks that characterize HPLC peptide maps, we observed a radioactivity profile for the [$^{14}$C](carboxyethyl)cysteine-containing peptide which is broad, coinciding with a UV profile that exhibits a broad feature upon which are superimposed sharp peaks due to cochromatographing contaminant peptides. Sequence analysis of the partially purified sample releases several PTH derivatives at each cycle, as expected from the HPLC results. Radiolabel due to PTH-[$^{14}$C](carboxyethyl)cysteine is not detected in the effluent containing the PTH derivatives (30 Edman cycles were performed). Instead, radioactivity was largely accounted for when the Polybrene-coated sample filter was recovered from the reaction cartridge and counted using liquid scintillation techniques. Two explanations could account for these observations. The peptide could be artifactually N-blocked during sample work-up after trypsin digestion; the standard procedures for sample handling and chromatography were, however, not substantially different from those successfully utilized in our lab or in other labs pursuing peptide isolation and analysis. Alternately, the cysteine from the condensing enzyme domain maps within the tryptic peptide generated from the N-terminus of the rat fatty acid synthase. It has been well established that the N-terminus of this enzyme is blocked (Stoops et al., 1975), and it has been suggested that the condensing enzyme domain is located close to the N-terminus of the polypeptide (Wakil et al., 1983). While this latter possibility appears attractive, it seems difficult to reconcile with the report (Chang & Hammes, 1988) that a tryptic peptide that harbors a cysteine assigned to the condensing enzyme domain is isolable and sequenceable.

## Discussion

Recent structural work on HMG-CoA synthase (Vollmer et al., 1988) allowed the assignment of function to the cysteine modified by chloropropionyl-CoA. The study demonstrated that the sequence of the chymotryptic peptide which harbors the cysteine involved in formation of the acetyl-*S*-enzyme reaction intermediate is identical with the sequence of a portion of the tryptic peptide containing the cysteine alkylated by chloropropionyl-CoA. The results contained in this report allow us to further refine the description of the chloropropionyl-CoA-mediated inactivation process. Tritium exchange from chloro[2-$^{3}$H]propionyl-CoA into the aqueous medium occurs as enzyme inactivation proceeds. This observation indicates that the enzyme is capable of catalyzing the first step in formation of the putative acrylyl-SCoA species. Additionally, it has been shown that an acrylyl thioester is kinetically competent to function as an intermediate in the inactivation process. These data strongly argue for the assignment to chloropropionyl-CoA of a role as a mechanism-based ("suicide") inhibitor.

In the case of fatty acid synthase, it is less likely that chloropropionyl-CoA functions as a mechanism-based inhibitor. An unequivocal distinction between that type of inactivation and the simpler affinity labeling process is complicated by the fact that direct comparison of $K_i$'s and $k_{inact}$'s that characterize the chloropropionyl-CoA- and *S*-acrylyl-*N*-acetylcysteamine-mediated inactivations is not straightforward. Chloropropionyl-CoA is a very potent inhibitor of fatty acid synthase; this constraint required inhibition studies to be performed at concentrations which precluded graphical analysis of the type shown in Figure 3. Data analysis employed the method of Owens and Barden (1978), which predicts the observed linear relationship between log percent activity and [I] if [I] $\ll K_i$. The analysis suggested that $K_i/k \simeq 10$ $\mu$M/min$^{-1}$. Thus, if a $K_i = 20$ $\mu$M were assigned, then a value of approximately 2 min$^{-1}$ is calculated as the rate constant. If a value of this magnitude were correct, then comparison with the $k_{inact}$ for acrylylcysteamine (1.8 min$^{-1}$; Table I) would suggest that the acrylyl derivative might function as an intermediate. In fact, $K_i$ is likely to be lower since the rate of fatty acid synthase inactivation by chloropropionyl-CoA was close to saturation at 20 $\mu$M (Miziorko et al., 1986). Additionally, $K_i$'s in the 20 $\mu$M range are large enough so that the usual graphical methods of analysis can be employed (e.g., a $K_i = 15$ $\mu$M was determined for HMG-CoA synthase by replots of the type shown in Figure 3). These observations

[2] Cycle number, PTH-amino acid assignment, and yield (picomoles), respectively, are the following: 1, Asp, 220; 2, Leu, 142; 3, Ala, 355; 4, Gly, 227; 5, Ile, 160; 6, Asn, 183; 7, Leu, 166; 8, Asp, 162; 9, Ser, 91.3; 10, Ser, 183; 11, Leu, 153; 12, Ala, 73.4; 13, Asp, 85.5; 14, Leu, 46.2; 15, Gly, 60.2; 16, Gln (Leu), 40.1 (31.1); 17, Asp, 56.2; 18, Ser, 7.7; 19, Leu, 31.2; 20, Met, 37.0; 21, Gly, 40.1; 22, Val, 30.1; 23, Glu, 31.0; 24, Val (Gln), 20.1 (15.0); 25, Arg, 26.4.

suggest that the $K_i$ value for chloropropionyl-CoA is substantially numerically smaller than 20 $\mu$M; the estimate for the limiting value for $k_{inact}$ would also be proportionally lower. Therefore, if fatty acid synthase were to form an acrylyl-containing intermediate from chloropropionyl-CoA, it could react rapidly enough to account for the observed inactivation.

The chemistry of the fatty acid synthase reaction strongly argues for assignment of chloropropionyl-CoA as an affinity label rather than as a mechanism-based inhibitor. It has been established that chloropropionyl-CoA acylates this enzyme prior to alkylating the target residue (Miziorko et al., 1986). Transfer of the reactive acyl group to the pantetheinyl sulfhydryl of the acyl carrier peptide would account for this observation. There is no precedent for enzyme-catalyzed deprotonation of the $\alpha$ carbon of the acyl group thioesterified to pantetheine. When rat mammary fatty acid synthase was incubated with chloro[2-$^3$H]propionyl-CoA, we did not observe the progressive appearance of tritium in the aqueous medium as inactivation proceeded, in contrast to the results obtained in a comparable experiment with HMG-CoA synthase. Moreover, we did not observe "burst kinetics" or stoichiometric exchange in measuring tritium exchange into the medium. In this context, it should be mentioned that in experiments with yeast fatty acid synthase (Arnstadt et al., 1975), loading acyl carrier peptide with a dideuteromalonyl group resulted in no primary isotope effect on the overall reaction, which is limited by condensation of that malonyl group with the nascent fatty acid chain. When the bacterial condensing enzyme is reacted with dideuteromalonyl acyl carrier peptide, no isotope effect on condensation is observed. Additionally, when the reaction is carried out in deuterated solvent, no isotope effect is observed, and when tritiated solvent is used, no labeling of product occurs (Arnstadt et al., 1975). Regardless of enzyme source, it appears that the carbanion which attacks the acyl group attached to the condensing domain's reactive cysteine does not form prior to decarboxylation of the malonyl-*S*-pantetheine adduct. Thus, it would be unproductive for a protein-derived base to deprotonate the $\alpha$ carbon of the acyl-*S*-pantetheine adduct. We conclude that the driving force for alkylation of fatty acid synthase by 3-chloropropionyl-CoA, an analogue that is much lower in reactivity than an $\alpha$-halo ketone or $\alpha$-haloacyl derivative, is primarily attributable to the extreme reactivity that has been documented (Oesterhelt et al., 1977) for the cysteinyl sulfhydryl at the condensing enzyme's active site. The secondary labeling of the pantetheinyl sulfhydryl by this reagent could occur either after transfer of the reactive acyl group from CoA to a serine hydroxyl or after transfer of the thioesterified acyl group from pantetheine to the cysteine at the condensing site. This secondary modification, while unexpected, is similar to the observation made by McCarthy and Hardie (1982) when they used the more reactive analogue chloroacetyl-CoA to modify the pantetheinyl sulfhydryl of fatty acid synthase.

Despite the ability of *S*-acrylyl-*N*-acetylcysteamine to function as an affinity label of HMG-CoA synthase and fatty acid synthase, it behaves as a group-specific reagent in alkylating HMG-CoA lyase. While the modification site on this protein remains to be established, ii has been demonstrated that the enzyme requires reduced sulfhydryls if catalytic activity is to be maintained (Kramer & Miziorko, 1980). Given the reactivity of sulfhydryls with acrylic acid derivatives (Weil & Seibles, 1961), it would not be surprising if a cysteine was the alkylated residue. However, precedent exists for alkylation of amino groups (Cavins & Friedman, 1967). Thus, further study on HMG-CoA lyase is required before any serious hypothesis concerning active-site amino acids can be advanced.

## Acknowledgments

We thank Dr. Keith Brew (University of Miami) for performing a preliminary structural analysis on the fatty acid synthase. Dr. Liane Mende-Mueller (Medical College of Wisconsin) performed the composition and sequence analyses of peptides derived from chloro[$^{14}$C]propionyl-CoA-inactivated fatty acid synthase.

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# Any of Several Lysines Can React with 5′-Isothiocyanatofluorescein To Inactivate Sodium and Potassium Ion Activated Adenosinetriphosphatase†

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ABSTRACT: Determinations of reaction stoichiometry demonstrate that the covalent incorporation of one molecule of 5′-isothiocyanatofluorescein can inactivate one molecule of sodium and potassium ion activated adenosinetriphosphatase in agreement with earlier determination of this stoichiometry. Several different modified peptides are produced, however, when the modified enzyme is digested with trypsin. One of these peptides has been identified as HLLVMK(thioureidylfluorescein)GAPER by use of a specific immunoadsorbent. The modified lysine is lysine 501 in the amino acid sequence of the $\alpha$ polypeptide of ($Na^+$ + $K^+$)-ATPase. This peptide has been previously isolated from such digests [Farley, R. A., Tran, C. M., Carilli, C. T., Hawke, D., & Shively, J. E. (1984) *J. Biol. Chem. 259*, 9532-9535]. The other specifically modified peptides have been purified and identified by amino acid sequencing. Their sequences identify lysine 480 and lysine 766 from the $\alpha$ polypeptide as amino acids modified by 5′-isothiocyanatofluorescein in reactions sensitive to the addition of ATP and responsible for inactivation of the enzyme.

Sodium and potassium ion activated adenosinetriphosphatase [($Na^+$ + $K^+$)-ATPase][1] (Skou, 1957) is the enzyme responsible for the coupled, active transport of sodium and potassium across the plasma membranes of all animal cells (Kyte, 1981). The enzyme is composed of one $\alpha$ subunit and one $\beta$ subunit. Recent advances in recombinant DNA technology have facilitated the determination of the primary structure of the two polypeptides. The $\alpha$ subunit, composed from a polypeptide with a length of 1016 amino acids (Shull et al., 1985; Ovchinnikov et al., 1986), is responsible for catalysis (Kyte, 1981). The $\beta$ subunit is a glycoprotein (Kyte, 1972) composed of a polypeptide with a length of 302 amino acids (Shull et al., 1986; Ovchinnikov et al., 1986). The physiological role of this $\beta$ subunit in cation transport, however, still remains unknown.

One of the major problems to be solved in the understanding of active transport is the molecular mechanism by which the hydrolysis of a chemical bond of high energy is coupled to the unidirectional movement of a small molecule. Its solution requires information about the structure of the protein and the relative locations of the sites for hydrolysis and transport as well as an understanding of subunit interactions and conformational changes accompanying transport. Little is known about the structural features of the active site of ($Na^+$ + $K^+$)-ATPase, which is a participant in the coupling between hydrolysis and ion transport. One approach to this problem has been to use reagents specific for particular functional groups to investigate the active site of this protein. Treatment of purified ($Na^+$ + $K^+$)-ATPase with such reagents can demonstrate the presence of essential functional groups and may enable us to elucidate the relationships between structure and function.

The active site of ($Na^+$ + $K^+$)-ATPase has been investigated with a variety of chemical reagents. It has been shown that an aspartate is the amino acid within this active site that accepts the phosphate of ATP during the first step of its hydrolysis, and the tripeptide containing this essential residue has been shown to be SD($PO_3^{2-}$)K (Post et al., 1973; Bastide et al., 1973). This identifies it as aspartate 369 of the $\alpha$ polypeptide (Shull et al., 1985). Nucleotide affinity reagents, which might react covalently with amino acid residues within the active site, have been used in an effort to identify other sequences surrounding the active site (Munson, 1981; Patzelt-Wenczler et al., 1975; Haley et al., 1974; Cooper et al., 1983; Ohta et al., 1986). A cysteine residue (Schoot et al., 1978), an arginine residue (De Pont et al., 1977), and a tyrosine residue (Cantley et al., 1978) have been suggested to play an important role in the active site. Dzhandzhugazyan et al. (1988) have suggested that aspartate 710 and aspartate 714 both are functionally important residues because they are modified by adenosine 5′-[*N*-[4-[*N*-(2-chloroethyl)-*N*-methylamino]benzyl]-$\gamma$-amidotriphosphate] in native ($Na^+$ + $K^+$)-ATPase. All of this information suggests that the active site is formed from amino acids distant from each other in the

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[1] Abbreviations: TUF, 5′-thioureidylfluorescein; ($Na^+$ + $K^+$)-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate; HPLC, high-pressure liquid chromatography; PTH-amino acid, phenylthiohydantoin of the amino acid; ($H^+$ + $K^+$)-ATPase, proton and potassium ion activated ATPase; $Ca^{2+}$-ATPase, calcium ion activated ATPase; $H^+$-ATPase, proton-activated ATPase; $K^+$-ATPase, potassium ion activated ATPase.

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