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Identification of the Site of Acetyl-S-Enzyme Formation on Avian Liver Mitochondrial 3-Hydroxy-3-methylglutaryl-CoA Synthase†

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ABSTRACT: Avian liver mitochondrial hydroxymethylglutaryl-CoA synthase contains an active-site cysteine involved in forming the labile acetyl-S-enzyme intermediate. Identification of and assignment of function to this cysteine have been accomplished by use of an experimental strategy that relies upon generation and rapid purification of the *S*-acetylcysteine-containing active-site peptide under mildly acidic conditions that stabilize the thioester adduct. Automated Edman degradation techniques indicate the peptide's sequence to be Arg-Glu-Ser-Gly-Asn-Thr-Asp-Val-Glu-Gly-Ile-Asp-Thr-Thr-Asn-Ala-Cys-Tyr. The acetylated cysteine corresponds to position 129 in the sequence deduced from cDNA data for the hamster cytosolic enzyme [Gil, G., Goldstein, J. L., Slaughter, C. A., & Brown, M. S. (1986) *J. Biol. Chem. 261*, 3710–3716]. The acetyl-peptide sequence overlaps that reported for a tryptic peptide that contains a cysteine targeted by the affinity label 3-chloropropionyl-CoA [Miziorko, H. M., & Behnke, C. E. (1985) *J. Biol. Chem. 260*, 13513–13516]. Thus, availability of these structural data allows unambiguous assignment of the acetylation site on the protein as well as a refinement of the mechanism explaining the previously observed affinity labeling of the enzyme.

3-Hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA[1] synthase; EC 4.1.3.5) produces a key intermediate in ketogenesis and cholesterogenesis by a three-step reaction sequence:

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

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

$$\text{CoAS-HMG-S-Enz} + \text{H}_2\text{O} \rightarrow \text{HMG-SCoA} + \text{HS-Enz} \quad (3)$$

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A covalent acyl-enzyme intermediate was suggested by early work involving the yeast enzyme (Stewart & Rudney, 1966; Middleton & Tubbs, 1974). The enzyme isolated from avian liver mitochondria contains eight cysteines per 53-kDa subunit (Miziorko, 1985). Experiments on the liver enzyme demonstrated the involvement of one of these cysteines in acetyl-S-enzyme formation (reaction 1; Miziorko et al., 1975). Affinity-labeling experiments have recently led to the identification of a critical cysteine and elucidation of the structure of the peptide that harbors this residue (Miziorko & Behnke, 1985a,b). While the precise function of that cysteine could not be unambiguously assigned, its identification was facilitated by the fact that it was alkylated by the affinity label 3-chloropropionyl-CoA. The carboxyethylcysteine derivative that resulted was stable to the procedures commonly employed in protein chemistry. In order to determine whether the cysteine identified by the affinity-labeling approach is involved in acetyl-S-enzyme formation or participates in other key chemical steps in the reaction, a direct identification of the acetylation site on the enzyme has been undertaken. This paper outlines the strategy and methodology that was employed to isolate the peptide bearing the labile thioester-containing adduct and presents the structural information derived from Edman degradation of this active-site peptide.

A preliminary account of this work has appeared (Vollmer et al., 1988).

Experimental Procedures

Materials

Chicken liver mitochondrial HMG-CoA synthase was prepared and assayed by the method of Reed et al. (1975). Chymotrypsin was purchased from Sigma (St. Louis, MO). Mono-S FPLC and LiChrospher RP-18 HPLC columns were obtained from Pharmacia and E. Merck, respectively. Reagents for amino acid composition analyses were supplied by Beckman; peptide sequencing reagents were provided by Applied Biosystems.

Methods

Amino Acid Analysis. Peptides were performic acid oxidized according to the procedure of Hirs (1967). Hydrolysis was performed in constant boiling HCl (Pierce) containing 10 mM mercaptoethanol at 110 °C for 24 h. Hydrolysates were analyzed by using a Beckman Model AA-3 HPLC amino acid analysis system, which involves a cation-exchange separation followed by detection after postcolumn derivatization with *o*-phthalaldehyde (Benson & Hare, 1975). The system was equipped with a Spherogel amino acid column (4.6 × 250 mm) and a Kratos FS-970 fluorescence detector.

Peptide Sequencing. Peptide sequencing was performed by using an Applied Biosystems Model 477A pulsed liquid-phase automated sequencer. Samples were loaded onto precycled Biobrene-impregnated glass fiber disks and subjected to repetitive Edman degradation (Hewick et al., 1981), using the "normal" program for reaction and conversion cycles, which involves double coupling. Phenylthiohydantoin (PTH) amino acids were identified by subjecting 50 μL of the 132 μL recovered from each conversion cycle to reverse-phase HPLC using an on-line Model 120A PTH amino acid analyzer. Radioactivity released in each Edman cycle was determined by liquid scintillation counting measurements performed on fractions remaining after HPLC analyses.

Results

The [^{14}C]acetyl-S-enzyme form of HMG-CoA synthase was prepared according to well-established methodology (Miziorko et al., 1975). Enzyme (3.6 mg) was incubated at 30 °C in 100 mM potassium phosphate, pH 7.5, prior to addition of [^{14}C]acetyl-CoA (200 μM final concentration; 10 600 dpm/nmol). After 5 min at 30 °C, the enzyme was quickly frozen on dry ice and then thawed for further workup. This freeze–thaw procedure appears to diminish the rate at which enzyme catalyzes the abortive hydrolysis of the thioester linkage to the acetyl group. Increases of up to 25% in acylation stoichiometry have been measured when this step is included in the experimental procedure. The thawed sample was maintained at 4 °C during the separation of [^{14}C]acetyl-S-enzyme from CoASH and unreacted [^{14}C]acetyl-CoA by chromatography on Sephadex G-50 (0.9 × 25 cm, equilibrated with 10 mM potassium acetate, pH 5.0). The pH as well as temperature was lowered during this isolation in order to retard chemical hydrolysis of the thioester linkage in the acetyl-S-enzyme. The center fractions of the acetyl-S-enzyme peak (65% of enzyme applied to Sephadex G-50) were reserved, and the stoichiometry of modification was measured to be 0.94 acetyl/enzyme protomer. The volume of the recovered sample was reduced to 0.5 mL by centrifugal ultrafiltration using an Amicon CF-25 Centriflow filter.

Chymotryptic digestion of the sample was performed by using an initial 0.3:1 molar ratio of chymotrypsin to HMG-CoA synthase; high levels of chymotrypsin were used due to the need for performing the digestion at suboptimal pH (5.0). Digestion was allowed to proceed at 0 °C for 2.5 h prior to addition of another aliquot of chymotrypsin. After a total of 5 h at 0 °C, a third aliquot of chymotrypsin was added, and the temperature was raised to 30 °C for 1.5 h to bring the digestion to an end point. Progress of the digestion was followed by subjecting aliquots of the mixture to HPLC peptide mapping (Fullmer & Wasserman, 1979). Some precipitation of enzyme occurred as temperature was elevated during the final period of digestion. This precipitate, which presumably represents largely undigested sample, was removed by filtration; the filtrate represented 75% of the ^{14}C radioactivity from the isolated acetyl-S-enzyme and displayed a single radiolabeled component upon HPLC analysis.

Initial purification of the radiolabeled peptide was performed at 4 °C by using a cation-exchange FPLC Mono-S column equilibrated with 20 mM potassium formate, pH 3.8. The peptide binds only weakly under these conditions and elutes as a broad peak prior to application of a salt gradient (Figure 1). Retention is, however, adequate for the radiolabeled peptide to be well resolved from the peak of unbound material; thus, a substantial purification is accomplished. Approximately 55% of the radioactivity applied to the column was recovered in the peak of weakly bound material. No other substantial radioactive peaks were detectable; the remaining radiolabel may be diluted and lost upon slow hydrolysis of the thioester linkage during the purification procedure or may be distributed at low levels among a series of peptides that represent incomplete digestion products.

Final purification of the radiolabeled peptide was accomplished by reducing the volume of ^{14}C-containing FPLC fractions in vacuo and subjecting the resulting sample to reverse-phase HPLC. Elution of a LiChrospher RP-18 column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid was accomplished by application of a 0–60% gradient of

[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography; PTH, phenylthiohydantoin; PITC, phenyl isothiocyanate.

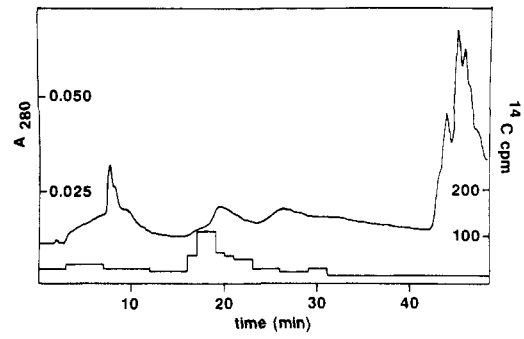

FIGURE 1: FPLC purification of the acetyl-S-peptide following chymotryptic digestion. A Mono-S cation-exchange column, equilibrated with 20 mM potassium formate, pH 3.8, was loaded with sample and eluted with equilibration buffer at a flow rate of 1 mL/min. After 30 min, a linear gradient (0–0.5 M KCl in 20 mM potassium formate, pH 3.8) was developed over 50 min. Effluent was continuously monitored for A_{280} and 1-mL fractions were collected. ^{14}C radioactivity was determined by using 3-μL aliquots for liquid scintillation counting. The acetyl-S-peptide elutes as a broad peak resolved from the material that is unretained by the column.

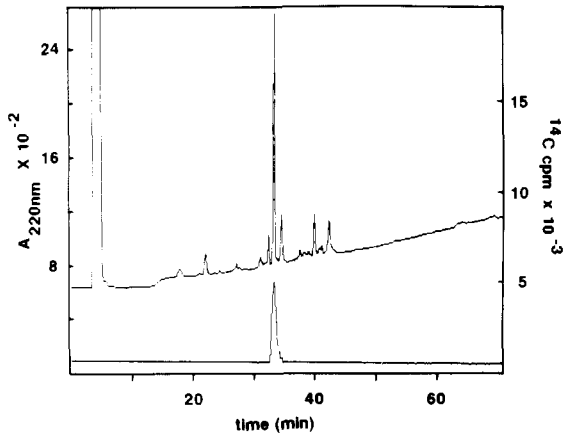

FIGURE 2: Reverse-phase HPLC purification of the acetyl-S-peptide. The FPLC-purified chymotryptic peptide was applied to a LiChrospher RP-18 column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid and eluted over 60 min by using a linear gradient (0–60% acetonitrile in 0.1% trifluoroacetic acid) at a flow rate of 1 mL/min. Effluent was continuously monitored for A_{220} and ^{14}C radioactivity by using in-line absorbance and radioactivity detectors. The effluent containing the radioactive, UV-absorbing material was reduced in volume and used for Edman degradation.

CH_3CN containing 0.1% trifluoroacetic acid over 60 min at a flow rate of 1.0 mL/min. Effluent was monitored for 220-nm absorbance and ^{14}C radioactivity. Detection of radioactivity was accomplished by using an in-line detector with a flow cell containing a CaF_2 scintillator. Only one peak of radioactivity was recovered (Figure 2), accounting for 60% of the injected radiolabel. This labeled material coincides with the major UV-absorbing peptide peak that was eluted by the gradient. The UV profile further indicates the efficacy of the FPLC purification step, since relatively few peptide components appear in the mixture subjected to the reverse-phase HPLC procedure. The effluent containing the radioactive peptide essentially free of unlabeled peptide contaminants was taken to dryness in vacuo and used for composition and sequence analyses.

Aliquots of the *S*-[^{14}C]acetylcysteine-containing peptide were performic acid oxidized (Hirs, 1967); this process destroys the thioester linkage and produces cysteic acid as well as volatile [^{14}C]acetic acid. After the performic acid oxidized sample was taken to dryness in vacuo, the expected loss of ^{14}C radioactivity was verified, and the peptide was acid hydrolyzed.

Table I: Amino Acid Composition of the Acetyl Peptide[a]

amino acid	nmol of amino acid/nmol of peptide
Cys[b]	1.0 ± 0.10 (1)
Asx	3.7 ± 0.10 (4)
Thr	2.7 ± 0.10 (3)
Ser	1.1 ± 0.00 (1)
Glx	2.5 ± 0.00 (2)
Gly	1.8 ± 0.00 (2)
Ala	1.1 ± 0.10 (1)
Val	1.1 ± 0.00 (1)
Met[c]	0.0 (0)
Ile	1.1 ± 0.00 (1)
Leu	0.6 ± 0.03 (0)
Tyr	0.1 ± 0.04 (1)
Phe	0.2 ± 0.02 (0)
His	0.0 (0)
Lys	0.0 (0)
Arg	0.8 ± 0.04 (1)
Pro	not determined (0)
Trp	not determined (0)

[a] The peptide (0.49 nmol) recovered after the HPLC purification procedure was performic acid oxidized (Hirs, 1967) for 4 h, lyophilized, and then hydrolyzed in vacuo for 24 h at 110 °C in 6 N HCl containing 10 mM mercaptoethanol. The sample was frozen in 100–200-pmol aliquots, which were lyophilized and stored at −20 °C until analyses were performed. Data for the mean and deviation for three determinations are shown; values in parentheses represent the number of residues found by Edman degradation. [b] Cys was determined as cysteic acid. [c] Met determination includes oxidized species (sulfoxide and sulfone).

Amino acid analysis of the hydrolysate (Table I) was accomplished by using standard cation-exchange methodology and postcolumn *o*-phthalaldehyde derivatization to permit sensitive fluorimetric detection. Another aliquot of the isolated radiolabeled peptide was subjected to automated Edman degradation (Hewick et al., 1981). The results[2] establish the peptide's primary structure: Arg-Glu-Ser-Gly-Asn-Thr-Asp-Val-Glu-Gly-Ile-Asp-Thr-Thr-Asn-Ala-Cys-Tyr. Cysteine was assigned at cycle 17 on the basis of the appearance of ^{14}C radioactivity due to the PTH derivative of [^{14}C]-acetyl-S-cysteine.[3] There is good agreement between the peptide composition predicted from the sequencing data and that calculated from the amino acid analysis results. The direct determination underestimates the tyrosine content, probably due to oxidation or formation of halogenated derivatives during performic acid oxidation or acid hydrolysis (Sanger & Tuppy, 1951; Sanger & Thompson, 1963). Additionally, amino acid analyses detected small amounts of a leucine contaminant that

[2] A 310-pmol sample was used for analysis. Average repetitive yield was 96.1%; average initial yield was 20.2%. Aliquots corresponding to 62% of the material recovered from each Edman cycle were used for radioactivity determinations, which showed ^{14}C released in cycle 17 and, at a slightly lower level, in cycle 18. PTH-derivative yields (pmol) were as follows: Cycle 1, 15.5; 2, 38.6; 3, 42.2; 4, 64.4; 5, 47.2; 6, 41.1;, 7, 30.5; 8, 45.5; 9, 23.4; 10, 44.6; 11, 55.3; 12, 26.0; 13, 28.7; 14, 33.4; 15, 39.4; 16, 40.8; 17, identification based on appearance of radioactivity due to PTH *S*-[^{14}C]acetylcysteine; 18, 4.7.

[3] It has previously been established that the acetyl group is linked to the protein via a thioester involving a cysteine residue; these data indicate *which* of the enzyme's eight cysteines is the site of modification. Sensitivity of the adduct between the acetyl group and enzyme to performic acid vapor (Miziorko et al., 1975; Middleton & Tubbs, 1974), which selectively destroys thioesters while leaving oxygen esters intact (Lynen, 1967), suggested the chemical nature of the linkage. Identification of a cysteine as the source of the sulfhydryl involved in forming the thioester was also accomplished. After [^{14}C]acetyl enzyme was subjected to exhaustive proteolysis, the cysteinyl amino group becomes available and intramolecular S → N acyl transfer occurs (Harris et al., 1963; Pratt & Bruice, 1971). The ^{14}C radioactivity becomes stable to performic acid oxidation, and the resulting radioactive species was isolated and identified as *N*-acetylcysteic acid (Miziorko et al., 1975).

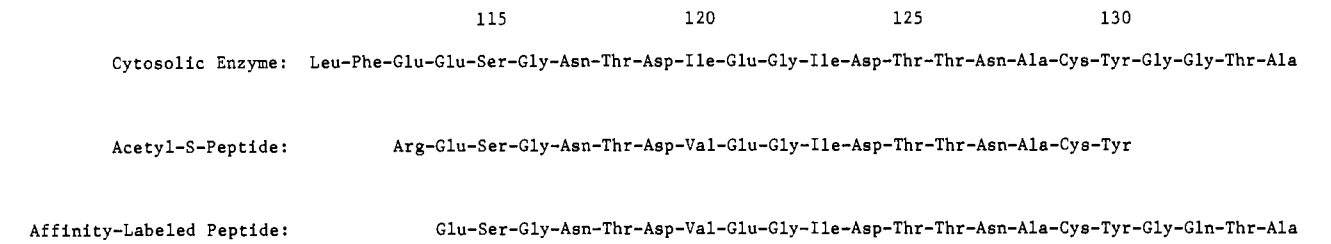

FIGURE 3: Comparison of available sequence data for the HMG-CoA synthase active site. A portion of the hamster cytosolic enzyme sequence (as deduced from cDNA data; Gil et al., 1986) is shown for comparison with sequences obtained by direct Edman analysis of peptides from the avian mitochondrial enzyme. The cysteine in the sequence labeled "acetyl-S-peptide" is the residue involved in forming the acetyl-S-enzyme intermediate. The cysteine in the sequence labeled "affinity-labeled peptide" is alkylated by 3-chloropropionyl-CoA (Miziorko et al., 1985a,b). Residue numbering corresponds to the cytosolic enzyme sequence.

does not complicate the Edman degradation analysis. While the repetitive yield encountered during sequence analysis was good (96%), the initial yield was lower than usual. Similar observations have occasionally been made in sequencing other radiolabeled peptides and may be explained by poor retention of particular samples on the Biobrene saturated disk that is loaded into the microsequencer's reaction cartridge.

The level of radioactivity recovered in cycle 17 corresponds to 26% of that expected from the initial load, after corrections are made for initial yield (20%) and losses expected after 17 cycles with a repetitive yield of 96%. If the radiolabel recovered in the material (cycle 18) that lags after the initial appearance of PTH-*S*-acetylcysteine were to be included in this calculation, it would almost double the recovery estimate. The chemical nature of the acyl-enzyme intermediate has been well established, and the recovery of some radioactivity in cycle 18 is attributable to lag in the Edman chemistry and is not due to the presence of *O*-acetyltyrosine in the peptide. Given the lability of thioesters at alkaline pH (Fedor & Bruice, 1965), achievement of this level of radiolabel recovery is notable. Presumably, the *S*-acetylcysteine linkage is sufficiently stable so that only modest chemical hydrolysis of the thioester occurs during the relatively short time periods and under the alkaline conditions employed for PITC coupling in the Applied Biosystems 477A sequencer. Demonstration of the ability to detect this class of derivative at the small sample levels normally used for microsequencing may facilitate future experimental design for detection of labile adducts in peptides.

Discussion

It is not uncommon to identify an interesting protein domain that contains a chemically labile amino acid adduct. Such a derivative might result from formation of a covalent enzyme–substrate reaction intermediate or could be generated upon modification of a protein by certain group-specific reagents or affinity labels. In order to elucidate the sequence of the peptide that harbors the modified residue, a choice must be made between *direct* isolation and sequencing of the target peptide or *indirect* methodology, which involves destruction of the original labile adduct and selective replacement with a more convenient linkage prior to peptide purification and analysis. While we have successfully utilized indirect approaches (Krieger et al., 1987), the direct approach is appealing due to its methodological simplicity. This paper outlines our application of this direct approach in isolating and analyzing the acetylated peptide of HMG-CoA synthase. The freeze–thaw step that attenuates the endogenous hydrolase activity (Miziorko et al., 1975) is important for maximizing yield of acetyl-S-enzyme. Equally critical is the use of low pH for digestion and peptide isolation procedures. It was difficult to predict how well the *S*-acetylcysteine adduct would survive multiple cycles of Edman chemistry, especially the PITC coupling step, which is accomplished under alkaline conditions that also support thioester hydrolysis (Fedor & Bruice, 1965). At this step in the analysis, it was not envisioned that loss of the radiolabel and recovery of cysteine as a species other than PTH cysteine would obscure the final sequence assignment. It was, however, gratifying to find that, due to the short time periods during which the peptide is subjected to alkaline pH, the *S*-acetylcysteine adduct is largely preserved.

The validity of the sequence deduced for the acetyl peptide is clear upon comparison with available protein (Miziorko & Behnke, 1985b) and cDNA (Gil et al., 1986) sequence data. We have previously reported the stoichiometric alkylation of mitochondrial HMG-CoA synthase by the affinity label 3-chloropropionyl-CoA, as well as a 21-residue sequence of the N-terminal portion of a tryptic peptide that bears this modification site. Residues 1–17 of that peptide (Figure 3) overlap the sequence of the chymotryptic peptide described in this paper. The assignment of the acetyl-peptide residue corresponding to position 113 as arginine is in accord with the observation that the tryptic peptide containing the affinity-labeled cysteine has an N-terminus corresponding to position 114 (Figure 3). Despite the fact that cytosolic and mitochondrial HMG-CoA synthases are not immunologically closely related (Reed et al., 1975), there is a high level of homology (>90%) between the acetyl peptide and residues 113–130 of the hamster cytosolic enzyme (Gil et al., 1986). Conservation of this sequence in two proteins that are, overall, probably not highly homologous suggests the importance of this peptide (vide infra).

Successful sequencing of the complete acetyl peptide was expedited by the relatively low molecular weight of this fragment. Tryptic cleavage of [^{14}C]chloropropionyl-CoA-modified enzyme produced a relatively large radiolabeled fragment. While the entire peptide was not sequenced, it is likely, on the basis of homology with the cytosolic enzyme, that it accounts for approximately 40 residues. The cytosolic enzyme contains chymotrypsin cleavage sites at Phe-112 and Tyr-130. This observation, together with preliminary work that suggested that the sites of acetylation and chloropropionyl-CoA modification might be identical (S. Vollmer, unpublished data), prompted our use of chymotrypsin. For the reasons discussed above, it is clear that the mitochondrial enzyme contains Arg at the position corresponding to Glu-113 of the cytosolic enzyme. Chymotrypsin specificity argues for a hydrophobic residue upstream from Arg. Whether this residue in the mitochondrial enzyme is, in fact, a phenylalanine remains unclear at present.

It is interesting that residues 128–132 (Ala-Cys-Tyr-Gly-Gln), which flank the HMG-CoA synthase acetylation site, show a high degree of homology with residues 125–129 (Ala-Cys-Tyr-Gly-Glu) of another acetyl-CoA-utilizing en-

zyme, chloramphenicol acetyltransferase (Shaw et al., 1979). While reactive thiols have been suggested to be near the active site of that enzyme, cysteines at positions other than 126 have been implicated. Moreover, Shaw (1983) points out that kinetic evidence that would support participation of an acetyl-enzyme intermediate is lacking. Nonetheless, acyl-enzyme intermediates can be elusive species, and lack of evidence rarely proves a point. If additional homology develops as acetylation sites on other proteins are identified, reevaluation of the function of Cys-126 in chloramphenicol acetyltransferase might be warranted.

There are 8 and 11 cysteines per subunit of the mitochondrial (Miziorko, 1985) and cytosolic (Gil et al., 1986) HMG-CoA synthases, respectively. Our sequence determination allows unambiguous assignment of an important function to one of these sulfhydryls, Cys-129 (residue numbering corresponds to the cytosolic enzyme sequence). While it is now clear that this residue is involved in acetyl-S-enzyme formation, there was previously some ambiguity concerning whether it functioned in this capacity (cf. reaction 1) or as an active-site nucleophile that catalyzes carbanion formation at C_2 of acetyl-S-enzyme prior to condensation with acetoacetyl-CoA (reaction 2). The observation (Miziorko & Behnke, 1985a) that modification stoichiometry measured with chloropropionyl-[3′-^{32}P]CoA (0.5/site) was lower than that measured with [1-^{14}C]chloropropionyl-CoA (0.7/site) was originally interpreted as suggesting that the analogue could acylate enzyme (with resultant loss of [^{32}P]CoA) as well as directly alkylate enzyme. Thus, the stoichiometry of ^{14}C incorporation could be viewed as the sum of acylation and/or alkylation events. The discrepancy between stoichiometry of ^{32}P and ^{14}C incorporation can also be explained by partial hydrolysis of the CoA thioester linkage after alkylation of enzyme by chloropropionyl-CoA has occurred; the enzyme has been established to possess an abortive acyl-CoA hydrolase activity (Miziorko et al., 1975; Middleton & Tubbs, 1974). In retrospect, this latter interpretation deserves serious consideration. Not all acyl-CoA derivatives that bind tightly or stoichiometrically to HMG-CoA synthase react further to form covalent acyl-S-enzyme adducts (Miziorko et al., 1979). In contrast with its ability to acylate other targets prior to alkylation (e.g., fatty acid synthase; Miziorko et al., 1986), it is certainly possible that chloropropionyl-CoA binds to HMG-CoA synthase and does not acylate enzyme but merely forms a reversible Michaelis-type complex prior to alkylation of Cys-129 and inactivation of enzyme. This mechanism is consistent with all available experimental data.

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