3-Hydroxy-3-methylglutaryl Coenzyme A Lyase: Affinity Labeling of the *Pseudomonas mevalonii* Enzyme and Assignment of Cysteine-237 to the Active Site†

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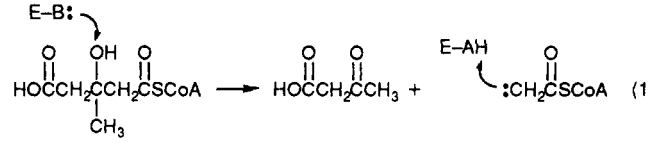
ABSTRACT: *Pseudomonas mevalonii* 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) lyase is irreversibly inactivated by the reactive substrate analog 2-butynoyl-CoA. Enzyme inactivation, which follows pseudo-first-order kinetics, is saturable with a K_I = 65 μM and a limiting k_{inact} of 0.073 min^{-1} at 23 °C, pH 7.2. Protection against inactivation is afforded by the competitive inhibitor 3-hydroxyglutaryl-CoA. Labeling of the bacterial enzyme with [1-^{14}C]-2-butynoyl-CoA demonstrates that inactivation coincides with covalent incorporation of inhibitor, with an observed stoichiometry of modification of 0.65 per site. Avian HMG-CoA lyase is also irreversibly inactivated by 2-butynoyl-CoA with a stoichiometry of modification of 0.9 per site. Incubation of 2-butynoyl-CoA with mercaptans such as dithiothreitol results in the formation of a UV absorbance peak at 310 nm. Enzyme inactivation is also accompanied by the development of a UV absorbance peak at 310 nm indicating that 2-butynoyl-CoA modifies a cysteine residue in HMG-CoA lyase. Tryptic digestion and reverse-phase HPLC of the affinity-labeled protein reveal a single radiolabeled peptide. Isolation and sequence analysis of this peptide and a smaller chymotryptic peptide indicate that the radiolabeled residue is contained within the sequence GGXPY. Mapping of this peptide within the cDNA-deduced sequence of *P. mevalonii* HMG-CoA lyase [Anderson, D. H., & Rodwell, V. W. (1989) *J. Bacteriol. 171,* 6468–6472] confirms that a cysteine at position 237 is the site of modification. These data represent the first identification of an active-site residue in HMG-CoA lyase.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[1] lyase (EC 4.1.3.4) catalyzes the cleavage of HMG-CoA into acetoacetate and acetyl-CoA in the final step of the ketogenic HMG-CoA cycle. In addition to its vital role in ketogenesis (Robinson & Williamson, 1980), the enzyme also functions in the catabolic pathway of the amino acid leucine. Inherited deficiency of this enzyme has been reported to cause severe metabolic acidosis following periods of fasting or infection (Gibson et al., 1988); this disease has proven fatal in a number of cases (Ozand et al., 1991). Despite the importance of HMG-CoA lyase, only recently has structural information become available for this enzyme. HMG-CoA lyase has been partially purified from a number of eukaryotic sources including pig heart (Bachawat et al., 1955) and bovine liver (Stegink & Coon, 1968), and homogeneous enzyme has been obtained from avian liver (Kramer & Miziorko, 1980). The enzyme has also been partially purified from the prokaryote *Pseudomonas mevalonii* (Scher & Rodwell, 1989). Anderson and Rodwell (1989) have reported the cDNA-deduced primary sequence of the *P. mevalonii* enzyme, and Mitchell et al. (1991) have recently reported the deduced primary sequences of both avian and human HMG-CoA lyases.

The lyase reaction is believed to be a retro-Claisen condensation with the abstraction of a proton from the 3-hydroxy group of HMG-CoA by a general base initiating cleavage of the C2–C3 bond, followed by quenching of the resulting carbanion of acetyl-CoA by a general acid:



The identity of the residues which participate in this reaction is unknown. In fact, no active-site structural information is currently available for this enzyme.

Affinity labeling has proven itself as a valuable tool for elucidating active-site structure with several advantages over modification of proteins with conventional group-specific reagents (Plapp, 1982). A number of halogenated and unsaturated acyl-CoA compounds have been used to affinity-label coenzyme A utilizing enzymes. Chloropropionyl-CoA has been used successfully to affinity-label HMG-CoA synthase (Miziorko & Behnke, 1985), the enzyme preceding HMG-CoA lyase in the HMG-CoA cycle. 3-Alkynoyl-CoA derivatives have been used to affinity-label pig heart thiolase (Holland et al., 1973), pig liver general acyl-CoA dehydrogenase (Frerman et al., 1980), and butyryl-CoA dehydrogenase (Fendrich & Abeles, 1982). 2-Octynoyl-CoA has also been used as a mechanism-based inhibitor of acyl-CoA dehydrogenase (Freund et al., 1985; Powell & Thorpe, 1988). With the goal of obtaining active-site structural information for HMG-CoA lyase, we have synthesized and tested 2-butynoyl-CoA for its ability to act as an active-site-directed inhibitor of this enzyme. We report here the affinity-labeling

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[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; 3-HG-CoA, 3-hydroxyglutaryl-CoA; PTH, phenylthiohydantoin; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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of the enzyme from *Pseudomonas mevalonii* and the identification of the site of 2-butynoyl-CoA modification.

MATERIALS AND METHODS

Materials

$Ba^{14}CO_3$, NAD^+, NADH, TPCK-trypsin, and immobilized chymotrypsin were purchased from Sigma (St. Louis, MO); propynyllithium was obtained from Organometallics Inc. (East Hampstead, NJ); Lichrospher and Vydac RP-18 HPLC columns were purchased from Merck and Vydac, respectively. Coenzyme A and Sephadex G-50 were obtained from Pharmacia.

Methods

Enzyme Purification and Assays. P. mevalonii HMG-CoA lyase was purified to homogeneity from *Escherichia coli* BL21 (DE3) containing the pT7-2600 plasmid (Anderson & Rodwell, 1989) by methods to be reported elsewhere (C. Narasimhan, unpublished results). Avian HMG-CoA lyase was purified by the method of Kramer and Miziorko (1980) with the following modification. Following hydroxylapatite chromatography, the enzyme was loaded onto a blue Sepharose column equilibrated in 20 mM sodium phosphate, pH 7.0, containing 0.5 mM DTT and eluted with the same buffer containing 100 μM HMG-CoA. Activity was measured by the citrate synthase coupled assay reported by Kramer and Miziorko (1980) using a Cary 219 double-beam spectrophotometer. Protein concentrations were determined using the Bio-Rad microprotein assay (Bradford, 1976).

Synthesis of [1-^{14}C]-2-Butynoyl-CoA. The method of Powell and Thorpe (1988) in which a lithium acetylide is carboxylated using $^{14}CO_2$ was used to prepare a radiolabeled form of 2-butynoic acid. Propynyllithium (50 mg) was added to one flask (a) of a two-flask system in a dry anaerobic chamber. Twenty-two milligrams of barium [^{14}C]carbonate (24.4 mCi/mmol) and 44 mg of unlabeled barium carbonate were added to the second flask (b). The system was then sealed, removed from the anaerobic chamber, and partially evacuated. Dry tetrahydrofuran (5.0 mL) was added with stirring to flask a at −70 °C, and 2.0 mL of concentrated sulfuric acid was added to flask b. After 90 min, the reaction was quenched by the slow addition of 5.0 mL of H_2O to flask a. The product was acidified and extracted into ether. The extracts were dried over anhydrous sodium sulfate and evaporated to dryness. The ^{1}H NMR spectrum of the product gave the expected pattern [10.8 ppm (singlet); 2.0 ppm (singlet)] and agreed with that of the commercially available unlabeled 2-butynoic acid.

[1-^{14}C]-2-Butynoyl-CoA was synthesized from the radiolabeled acid using the mixed-anhydride procedure of Bernert and Sprecher (1977) as modified by Freund et al. (1985) in their synthesis of 2-octynoyl-CoA. HPLC analysis of the product was performed on a Lichrospher RP-18 column equilibrated in methanol/100 mM sodium phosphate (25:75), pH 3.0, at a flow rate of 1.0 mL/min. A_{260} and ^{14}C radioactivity profiles, monitored in tandem using an Applied Biosystems Model 757 spectrophotometric detector and a Radiomatic Fl/B flow-through radioactivity detector, revealed a single product eluting at 7.7 min. This indicated that the product was free of unreacted acid, CoA, or CoA dimer. Concentrations of the product as determined by A_{260} (ϵ = 20.9 M^{-1} cm^{-1}) agreed with that determined from the specific activity of the acid (16 400 dpm/nmol).

Preparation, Isolation, and Sequence Analysis of [^{14}C]-2-Butynoyl-CoA-Modified Peptides. Homogeneous *P. mevalonii* HMG-CoA lyase (21 nmol; 70 units/mg) was incubated with 450 μM [^{14}C]-2-butynoyl-CoA (16 400 dpm/nmol) at 23 °C in 100 mM potassium phosphate (pH 7.2)/20% glycerol (v/v) until <10% of the initial activity remained. The reaction was quenched by passing the sample over a 2.0-mL Sephadex G-50 centrifugal desalting column equilibrated in 100 mM potassium phosphate, pH 7.8 (Penefsky, 1977). In order to fully carboxymethylate enzyme sulfhydryls, the protein was denatured in 6 M urea and incubated with 1 mM DTT at 30 °C for 30 min. Iodoacetate was then added to a final concentration of 3 mM and the incubation continued at 30 °C for 30 min in the dark. The reaction was quenched by the addition of β-mercaptoethanol to a final concentration of 10 mM. Urea and unbound reagents were removed by exhaustive dialysis against 0.1 M ammonium bicarbonate. Digestion of the dialysate was performed by adding 2% (w/w) TPCK-treated trypsin and incubating at 37 °C with gentle stirring. After 2 h, a second aliquot of trypsin (2% w/w) was added, and the digestion was continued for an additional 2 h. Since HPLC analysis (see below) indicated incomplete digestion, the sample was further digested with trypsin (2% w/w) at 37 °C for 4 h in 1 M guanidine hydrochloride. The digest was frozen on dry ice and stored at −20 °C. Prior to preparative HPLC, the sample was lyophilized to remove bicarbonate and dissolved in H_2O to give a final guanidine concentration of 4 M.

Analysis of peptides was performed on a Vydac RP-18 column (4.6 × 250 mm) using a linear gradient (5–65% acetonitrile/0.1% trifluoroacetic acid) over 110 min at a flow rate of 0.7 mL/min. Absorbance at 215 nm and ^{14}C radioactivity were recorded in tandem using an Applied Biosystems Model 757 spectrophotometer and a Radiomatic Flo-one/B radioactivity detector.

For preparative isolation of the ^{14}C-modified tryptic peptide, 4 nmol of the trypsin digest was chromatographed as described above. The absorbance at 215 nm was monitored and the effluent collected in 0.5-min fractions. Ten-microliter aliquots were removed for radioactivity determination by liquid scintillation counting. The leading edge of the radioactivity peak (fraction 98; 150 pmol) was analyzed by Edman degradation using an automated protein sequencer (Applied Biosystems Model 477A) equipped with an on-line PTH analyzer (Model 120A).

For chymotryptic digestion, HPLC fractions containing the radiolabeled tryptic peptide were pooled and concentrated with a SpeedVac concentrator. Guanidine hydrochloride and ammonium bicarbonate were added to a final concentration of 0.4 M and 100 mM, respectively. Chymotrypsin digestion was performed by adding 250 μg of agarose-immobilized chymotrypsin (160 units/g of solid) and incubating at 37 °C for 4 h with gentle stirring. Following digestion, the sample was spun in a microfuge to remove the insoluble chymotrypsin, and the agarose pellet was washed 2 times with 50 μL of H_2O. The combined supernatants were concentrated with a SpeedVac and stored at −20 °C.

Preparative chromatography of the chymotrypsin digest was performed as described above with 730 pmol of radiolabeled peptide. Twenty-microliter aliquots were removed for radioactivity determination. Approximately 150 pmol of the radioactive peak which eluted at 24 min was sequenced by Edman degradation. Two-thirds of the sample from each cycle was used for detection of the PTH derivative. The remaining sample which was not injected onto the column

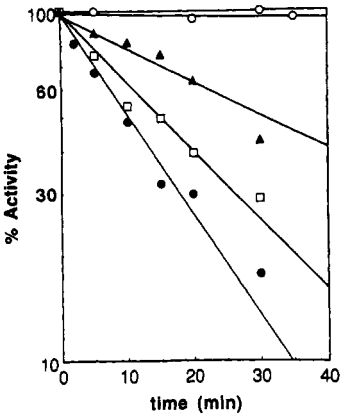


FIGURE 1: Irreversible inactivation of *P. mevaloni* HMG-CoA lyase by 2-butynoyl-CoA. Enzyme (2.2 μg; 64 units/mg) was incubated with 0 (○), 32 (▲), 130 (□), and 500 (●) μM 2-butynoyl-CoA at 23 °C in 100 mM potassium phosphate, pH 7.8, and 20% (v/v) glycerol. At the indicated times, aliquots were removed and assayed using the spectrophotometric assay described by Kramer and Miziorko (1980).

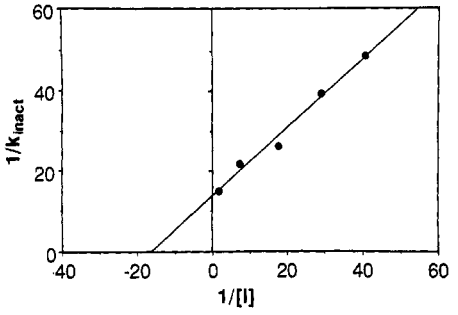


FIGURE 2: Saturability of 2-butynoyl-CoA inactivation and determination of kinetic constants K_I and k_3. Incubations were carried out at 23 °C in 100 mM potassium phosphate buffer, pH 7.8, and 20% (v/v) glycerol. Values of k_{inact} were obtained from plots of percent activity vs time. Intercepts indicate a K_I = 65 μM and a limiting k_{inact} = 0.073 min^{-1}.

was collected into fractions for determination of radioactivity by liquid scintillation counting.

RESULTS

Inactivation Kinetics. The rationale in designing an affinity label is to create a compound which resembles the natural substrate of the enzyme while possessing a reactive functionality that can covalently modify an active-site residue. The reactivity of an acetylenic group in conjunction with the binding affinity afforded by coenzyme A made 2-butynoyl-CoA an attractive candidate for affinity labeling of HMG-CoA lyase. When this analog was tested on HMG-CoA lyase, it was indeed found to be effective in causing time-dependent irreversible inactivation (Figure 1). As expected for an active-site-directed inhibitor (Plapp, 1982), pseudo-first-order kinetics are observed. Saturability is clearly demonstrated in the plot of $1/[I]$ vs $1/k_{inact}$ (Figure 2) by the observation of a finite intercept on the vertical axis. From this plot, the kinetic constants K_I and k_3 can be determined. The k_3 or limiting k_{inact} for 2-butynoyl-CoA inactivation is 0.073 min^{-1} at pH 7.2 and 23 °C. The K_I, a measure of the binding affinity of the inhibitor for the enzyme, is expected to be substantially lower for an affinity reagent than for a comparable group-specific reagent. The K_I of 2-butynoyl-CoA for the bacterial enzyme (65 μM) indeed indicates tight binding. In comparison, the K_m for the substrate, S-HMG-CoA, is 20 μM (C. Narasimhan, unpublished results). Although much of the binding affinity of 2-butynoyl-CoA is attributable to the CoA moiety, more subtle interactions between the inhibitor and binding determinants within the active site may exist. The K_I for 2-butynoyl-CoA with the avian HMG-CoA lyase is 320 μM even though the K_m for S-HMG-CoA is only 8 μM (Kramer & Miziorko, 1980).

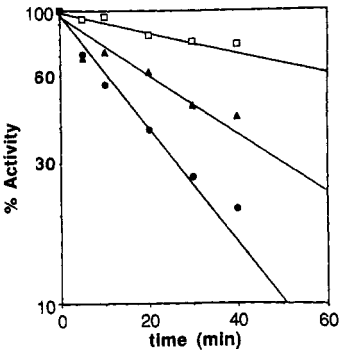


FIGURE 3: Protection of HMG-CoA lyase from 2-butynoyl-CoA inactivation by 3-hydroxyglutaryl-CoA. Bacterial HMG-CoA lyase was incubated with 90 μM 2-butynoyl-CoA in the presence of 0 (●), 0.52 (▲), and 3.15 (□) mM 3-hydroxyglutaryl-CoA. Reactions were carried out at 23 °C in 100 mM potassium phosphate, pH 7.8, and 20% (v/v) glycerol. At the indicated times, aliquots were removed and assayed for activity as described by Kramer and Miziorko (1980).

3-Hydroxyglutaryl-CoA Protection. Since HMG-CoA lyase only uses a single substrate and has a relatively high turnover number, it was not practical to investigate the ability of HMG-CoA to protect against 2-butynoyl-CoA inactivation. However, 3-hydroxyglutaryl-CoA (3-HG-CoA) has been shown to be a competitive inhibitor of the avian enzyme with a K_I = 50 μM for the cleavage reaction and 95 μM for the tritium exchange reaction (Kramer & Miziorko, 1983). The effect of 3-HG-CoA on the rate of inactivation of the bacterial lyase by 90 μM 2-butynoyl-CoA is shown in Figure 3. The K_I = 130 μM for 3-hydroxyglutaryl-CoA estimated from the protection data (Wold, 1977) using the bacterial enzyme is in reasonable agreement with that obtained from inhibition experiments with avian enzyme.

Stoichiometry of Labeling. The selectivity of 2-butynoyl-CoA modification was evaluated by determining the stoichiometry of labeling using radiolabeled 2-butynoyl-CoA. As shown in Figure 4A, the loss in bacterial lyase activity upon incubation with [1-^{14}C]-2-butynoyl-CoA coincides with incorporation of radiolabel with a stoichiometry approaching 0.65 per site. Since binding of the affinity reagent is dependent upon an intact active site, a stoichiometry which is less than unity may reflect the presence in the preparation of some denatured protein. Alternatively, overestimation of lyase protein by the Bradford assay may account for the discrepancy. The identical experiment performed with homogeneous avian HMG-CoA lyase also reveals that incorporation of [1-^{14}C]-2-butynoyl-CoA coincides with loss in enzymatic activity; measured stoichiometry approaches 0.9 per site (Figure 4B). Therefore, it appears that the inactivation of HMG-CoA lyase by 2-butynoyl-CoA involves the modification of a single residue.

UV Spectroscopy of the Inhibitor–Enzyme Adduct. The above data clearly demonstrate that 2-butynoyl-CoA acts as a true affinity label in irreversibly inactivating HMG-CoA lyase. However, the ultimate goal of affinity labeling is the identification of the active-site residue which is covalently modified by the affinity reagent. The observation that dithiothreitol (DTT) protected against 2-butynoyl-CoA inactivation of the lyase (P. W. Hruz, unpublished observation) suggested

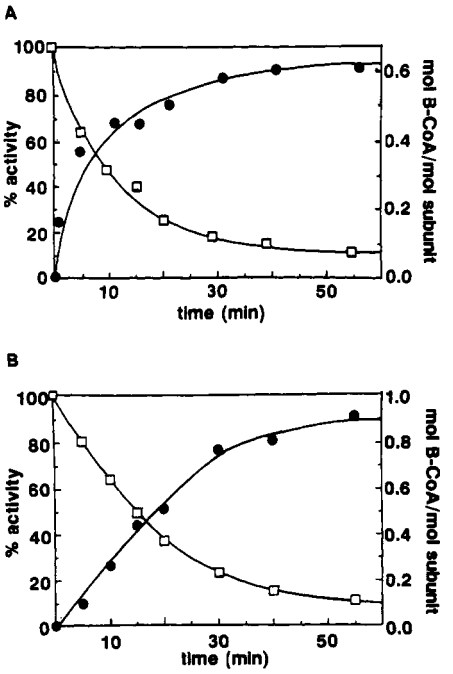


FIGURE 4: Correlation of incorporation of [1-^{14}C]-2-butynoyl-CoA with the extent of enzyme inactivation. (A) *P. mevalonii* HMG-CoA lyase (33 μg; 70 units/mg) was incubated with 360 μM [1-^{14}C]-2-butynoyl-CoA (16 400 dpm/nmol) at 23 °C in 100 mM potassium phosphate buffer, pH 7.8, and 20% (v/v) glycerol. (B) Avian HMG-CoA lyase (53 μg; 360 units/mg) was incubated with 260 μM [1-^{14}C]-2-butynoyl-CoA at 30 °C in 100 mM potassium phosphate, pH 7.5. At the indicated times (□), aliquots were removed and assayed for enzyme activity as described by Kramer and Miziorko (1980). Bound ^{14}C (●) was assayed by applying aliquots of the incubation mixtures to nitrocellulose membranes. Membranes were washed once with 5 mL of cold 10% trichloroacetic acid and 12 times with 10 mL of 100 mM potassium phosphate, pH 7.2. Radioactivity was determined by liquid scintillation counting.

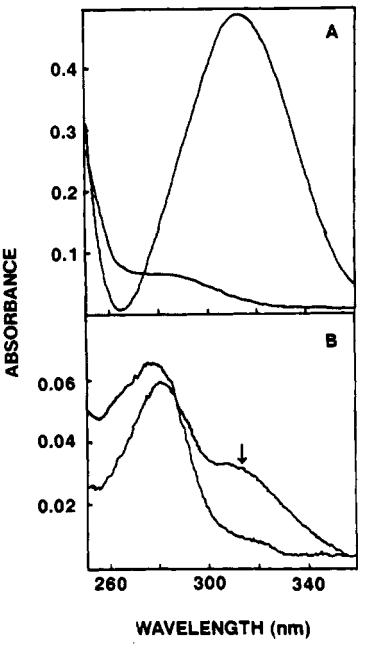


FIGURE 5: Spectrophotometric demonstration of the reaction of 2-butynoyl-CoA with dithiothreitol (A) and HMG-CoA lyase (B). (Panel A) 1 mM dithiothreitol in 100 mM potassium phosphate, pH 7.8, and 20% (v/v) glycerol (lower trace) and the same concentration of DTT following incubation with 40 μM 2-butynoyl-CoA at 30 °C for 10 min (upper trace). (Panel B) 3.3 μM bacterial HMG-CoA lyase in potassium phosphate, pH 7.8, and 20% (v/v) glycerol (lower trace) and enzyme following incubation at 30 °C with 150 μM 2-butynoyl-CoA (upper trace). The arrow indicates the 310-nm peak of the enzyme–inhibitor adduct. The contribution of 2-butynoyl-CoA to the absorbance profiles has been corrected for by addition of identical concentrations of the inhibitor to the reference cuvette.

that the affinity reagent readily reacts with mercaptans at slightly alkaline pH. Robinson et al. (1963) had previously shown that 2-butynoyl-CoA reacts with 2-mercaptoethanol or reduced glutathione to produce an adduct with an absorbance maximum at 308 nm. In a similar manner, incubation of 2-butynoyl-CoA with DTT results in the formation of an adduct with an absorbance maximum at 310 nm (Figure 5A). Incubation of 2-butynoyl-CoA with other nucleophiles and incubation of 2-butynoic acid with DTT fail to produce such a peak. As shown in Figure 5B, labeling of *P. mevalonii* HMG-CoA lyase with 2-butynoyl-CoA results in the formation of a similar 310-nm peak, suggesting the formation of an adduct between 2-butynoyl-CoA and an enzyme sulfhydryl. Using the calculated extinction coefficient of the DTT–2-butynoyl-CoA adduct (12 000 M^{-1}), the 310-nm absorbance of the 2-butynoyl-CoA labeled lyase suggests a stoichiometry of labeling of approximately 0.6 per site, in agreement with the stoichiometry calculated using the radiolabeled inhibitor.

Peptide Mapping and Sequencing. Confirmation of a cysteine as the target of 2-butynoyl-CoA modification as well as mapping of the active site within the primary sequence of the enzyme was accomplished by isolation and sequencing of peptides containing the active-site residue. Although the difficulties in obtaining sufficient quantities of purified avian HMG-CoA lyase precluded the isolation of an affinity-labeled peptide from this source, the overexpression and purification of recombinant *P. mevalonii* HMG-CoA lyase (C. Narasimhan, unpublished results) provided sufficient material to allow isolation and sequencing of the 2-butynoyl-CoA-modified peptide. An HPLC profile of tryptic peptides from [1-^{14}C]-2-butynoyl-CoA-modified bacterial HMG-CoA lyase reveals a single radiolabeled peptide (Figure 6). Although the UV absorbance profile failed to indicate heterogeneity in this sample, Edman degradation of approximately 150 pmol of the radioactive peak identified the presence of two tryptic peptides beginning at residues 137 and 226 of the cDNA-deduced *P. mevalonii* sequence (Anderson & Rodwell, 1989). The empirically determined partial sequences of these peptides are shown in Table I (peptides 1 and 2).

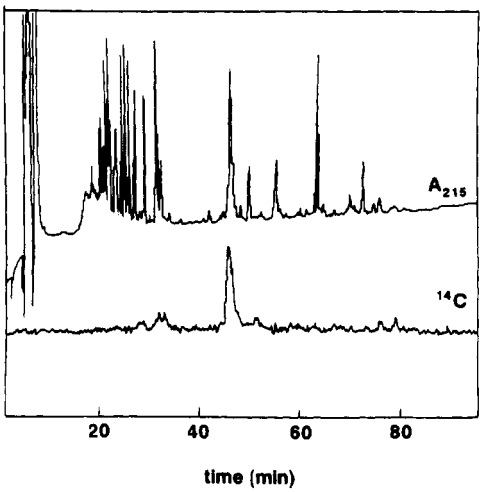


FIGURE 6: Tryptic map of [1-^{14}C]-2-butynoyl-CoA-modified HMG-CoA lyase. A tryptic digest of [1-^{14}C]-2-butynoyl-CoA-modified HMG-CoA lyase (1 nmol) was loaded onto a Vydac RP-18 column (4.6 × 250 mm) and eluted with a 5–65% gradient of CH_3CN in 0.1% trifluoroacetic acid. Radioactivity (^{14}C) and UV absorbance at 215 nm (A_{215}) were monitored in tandem, and the ^{14}C profile has been adjusted to account for the lag time between detectors.

Table I: Sequence Comparison of HMG-CoA Lyase Active-Site Peptides

peptide source	amino acid sequence
(1) tryptic[a]	Gly-Tyr-Val-Ser-Cys[b]-Val-Leu-Gly-Cys[b]-Pro-Phe-Ser-Gly-Ala-Val-Ala
(2) tryptic[a]	Thr-Phe-Asp-Ser-Ser-Val-Ala-Gly-Leu-Gly-Gly-Xaa-Pro-Tyr-Ser-Pro
(3) chymotryptic	Gly-Gly-Xaa-Pro-Tyr
(4) bacterial[c]	Thr-Phe-Asp-Ser-Ser-Val-Ala-Gly-Leu-Gly-Gly-Cys-Pro-Tyr-Ser-Pro
(5) avian[d]	Val-Val-Asp-Ala-Ser-Val-Ala-Gly-Leu-Gly-Gly-Cys-Pro-Tyr-Ala-Gln
(6) human[d]	Val-Val-Asp-Ser-Ser-Val-Ala-Gly-Leu-Gly-Gly-Cys-Pro-Tyr-Ala-Gln

[a] PTH-amino acid derivatives from peptide 1 were observed at levels significantly higher than those from peptide 2, allowing identification of two peptides. Average repetitive yields for peptides 1 and 2 were 79.9% and 84.1%, respectively. Assignments were confirmed by comparison with the cDNA-deduced sequence of the bacterial enzyme. Unambiguous assignments were not possible beyond cycle 16. [b] PTH-carboxymethyl-Cys elutes between PTH-Ser and PTH-Gln. [c] cDNA-deduced sequence (Anderson & Rodwell, 1989). [d] cDNA-deduced sequence (Mitchell et al., 1991).

The presence of two peptides along with the failure to obtain full-length sequences prevented definitive identification of the modified residue from this analysis. However, subsequent digestion of the radioactive tryptic peptide with chymotrypsin allowed the isolation of a smaller radiolabeled peptide fragment. Automated Edman degradation of this peptide (Table II) identified the sequence Gly-Gly-X-Pro-Tyr which maps (Table I; peptide 3) within one of the tryptic peptides isolated by HPLC on the basis of radioactivity. The residue from cycle 3 (designated X) corresponds to Cys-237 of the deduced cDNA sequence of *P. mevalonii* HMG-CoA lyase (Table I, peptide 4). The assignment of this residue as the target of 2-butynoyl-CoA modification is indicated by the observation of radioactivity released in cycle 3. This assignment is further supported by the failure to observe PTH-(carboxymethyl)cysteine, which elutes between PTH-Ser and PTH-Gln, at this cycle. Modification of the denatured and reduced protein with iodoacetate following 2-butynoyl-CoA labeling would have carboxymethylated this residue if it had not been previously blocked by the affinity label.

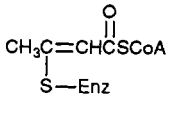
Table II: Amino Acid Sequence of [^{14}C]-2-Butynoyl-CoA-Labeled Chymotryptic Peptide

cycle	residue	pmol[a]	cycle	residue	pmol[a]
1	Gly	117	4	Pro	27
2	Gly	80	5	Tyr	11
3	[^{14}C]X	–[b]			

[a] Initial yield was 81%; average repetitive yield was 68%. [b] ^{14}C radioactivity was recovered in this cycle. Comparison with the deduced amino acid sequence data indicates Cys-237 at this position. Modification of this residue by 2-butynoyl-CoA is supported by the failure to detect PTH-carboxymethyl-Cys in this cycle.

DISCUSSION

In contrast to 2-octynoyl-CoA's role as a mechanism-based inhibitor of medium-chain acyl-CoA dehydrogenase (Powell & Thorpe, 1986), 2-butynoyl-CoA appears to act as an affinity reagent in inactivating HMG-CoA lyase. The inherent reactivity of this compound is clearly demonstrated by its ability to react with free thiols such as β-mercaptoethanol and dithiothreitol. This is not unexpected since the acetylenic group at the 2 position is conjugated to the thioester bond and does not require prior activation to act as a Michael acceptor. Tight binding of the CoA derivative to the enzyme's active site apparently allows selective labeling despite this reactivity. The extended conjugation of the proposed 2-butynoyl-CoA–enzyme adduct shown in the following structure could account for the observed 310-nm absorbance in the modified protein:



Thorpe's observation of a 312-nm absorbance maximum for 4-thia-2-octenoyl-CoA (Lau et al., 1989) also supports this proposed structure. The residue which has been assigned as the site of modification of the active-site-directed inhibitor 2-butynoyl-CoA is found to reside within a highly conserved region of the protein. Comparison of the three cDNA-deduced primary sequences presently available for HMG-CoA lyase shows an extensive region of homology with greater than 68% identity in the region surrounding Cys-237 (Table I). This cysteine is invariant in these three enzymes. A search of the Swiss protein database failed to detect other proteins with any significant homology to the active-site region.

The presence of a cysteine residue at the active site of HMG-CoA lyase had been suggested by the extreme sensitivity of the enzyme to inactivation by sulfhydryl-directed group-specific reagents such as iodoacetate. The pH vs activity profiles of the avian (Kramer & Miziorko, 1980) and bacterial (Scher & Rodwell, 1989) enzymes have shown that peak activity occurs at a pH of 8.5–9.0 which may reflect the requirement for a deprotonated base for catalysis. Cysteine, with a pK_a near 8.5, would be an excellent candidate for such a residue. The possibility for significant shifts in amino acid pK values as a result of the microenvironment within the active site requires one to interpret such data with caution. Nevertheless, the identification of a conserved active-site cysteine residue in HMG-CoA lyase is consistent with the above speculation and suggests that further investigation of this cysteine as a catalytic residue is warranted.

The utilization of cysteine as an active-site base is not without precedent. A cysteine residue in isopentenyl-diphosphate: dimethylallyl-diphosphate isomerase has been identified as an active-site nucleophile from affinity-labeling studies (Street & Poulter, 1990). The utilization of two equivalent cysteines as the bases responsible for the isomerization of proline in proline racemase, as implicated on the basis of chemical modification studies (Rudnick & Abeles, 1975), has been established from fractionation factors of the enzyme's catalytic groups (Belasco et al., 1986). Most recently, cysteine has been found to serve as the catalytic base for biosynthetic thiolase from *Zooglea ramigera* (Palmer et al., 1991). Thus, while the precise role of Cys–237 in catalysis remains to be established, the affinity labeling of this residue in HMG-CoA lyase from *P. mevalonii* has allowed the first assignment of an active-site residue for this enzyme.

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