A Revised Mechanism for the Inactivation of Bovine Liver Enoyl-CoA Hydratase by (Methylenecyclopropyl)formyl-CoA Based on Unexpected Results with the C114A Mutant†

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ABSTRACT: The compound (methylenecyclopropyl)formyl-CoA (MCPF-CoA) has been reported earlier as a potent active site-directed inactivator of bovine liver enoyl-CoA hydratase (ECH). It is believed that the mechanism of inactivation involves the attack of Cys114 at C-2′ of MCPF-CoA, resulting in ring cleavage and permanent covalent modification of the enzyme. Here, we describe studies with the C114A mutant of bovine liver ECH, which was constructed and purified to determine the role of this residue in the catalytic mechanism of the enzyme. The C114A mutant, which is catalytically competent, shows an unexpected susceptibility to inactivation by MCPF-CoA, indicating that Cys114 is not the primary nucleophile responsible for the inactivation of the enzyme. To determine if catalytic residues Glu115 and Glu135 play a role in the inactivation of the enzyme, the E115Q and E135Q mutants were also constructed and purified. It was determined that these mutants did not react with MCPF-CoA, indicating a possible role for both residues in the inactivation of the wild-type enzyme. Pepsin digestion and subsequent LC−MS/MS analysis of the inactivated wild-type enzyme and C114A mutant revealed that Glu115 was modified in each case, supporting the hypothesis that this residue is the true nucleophile that traps MCPF-CoA and indicating that the covalent modification of Cys114 reported earlier may be a postinactivation artifact. We propose a modified mechanism of inactivation involving Glu115 and Glu135, and suggest that MCPF-CoA may be a mechanism-based inhibitor for bovine liver ECH.

Enoyl-CoA hydratase (ECH)[1,2] catalyzes the reversible hydration of 2-*trans*-enoyl-CoA thioesters (**1**) to the corresponding (*S*)-3-hydroxyacyl-CoA thioesters (**2**) (*1*).

This physiologically important reaction constitutes the second step in the mitochondrial β-oxidation pathway of fatty acid metabolism. Most of the early biochemical studies on this enzyme were performed with the mitochondrial short-chain ECH, also known as crotonase (EC 4.2.1.17) (*2*−*6*). This enzyme, regardless of its source of origin, has been shown to be an extremely efficient catalyst that processes the C_4 substrate, crotonoyl-CoA, at a near-diffusion-controlled reaction rate ($k_{cat}/K_m = 5 \times 10^7$ M^{-1} s^{-1} for the bovine liver ECH). Studies on the stereochemical course of its catalysis have also established that the reversible dehydration−hydration reaction proceeds via a *syn* pathway. Such a *syn* addition−elimination reaction occurs at the *si* face of the conjugated thioester substrate, resulting in the formation of (*S*)-3-hydroxyacyl-CoA in the forward direction (*7*). However, an (*R*)-specific ECH was recently found from *Aeromonas caviae* (*8*). It should be noted that the genes of

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[1] Abbreviations: ECH, enoyl-CoA hydratase; DEAE, diethylaminoethyl; ESI-MS, electrospray ionization mass spectroscopy; LB, Luria-Bertani broth; MCPG, (methylenecyclopropyl)glycine; MCPA, (methylenecyclopropyl)acetic acid; MCPF, (methylenecyclopropyl)formic acid; PCR, polymerase chain reaction.

[2] The key residues of rat ECH, such as Gly141, Cys143, Glu144, and Glu164, are numbered according to the encoded sequence, including the N-terminal signal sequence, whereas the amino acid residue numbering for bovine liver ECH refers to the mature sequence of the protein, minus the signal sequence.

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many ECHs have been cloned and sequenced. Since the sequences of these enzymes are very homologous (≥80%; see Figure 3 in the Results and Discussion), their catalytic properties are expected to be highly conserved as well.

X-ray crystallography studies of rat liver mitochondrial ECH complexed with the tight-binding inhibitors acetoacetyl-CoA and octanoyl-CoA, determined at 2.5 and 2.4 Å, respectively, have shed light on the organization of the active site of ECH and the catalytic determinants presented by the enzyme (*9*, *10*). The mechanistic model for ECH that emerges from these studies involves the participation of Glu164 as a general acid that donates a proton to C_2 of the enoyl-CoA thioester, and Glu144 as the catalytic base for the activation of a water molecule in the active site. Apart from these two catalytic residues, the presence of a short hydrogen bond ($d = 2.7$ Å) between the substrate thioester carbonyl and the backbone NH group of Gly141 is also observed. These results are supported by extensive mechanistic and spectroscopic studies (*11*−*15*), which have demonstrated that the thioester substrate and its analogues undergo a reorganization of the enoyl π-electrons in the active site of the enzyme, presumably due to the hydrogen bond between the substrate carbonyl group and the backbone amide of Gly141, leading to increased electrophilicity at the β-carbon of the enoyl-CoA substrate. Such an active site-induced polarization of the conjugated π-bond could facilitate the addition of a water molecule across the α,β-unsaturated thioester. Results from kinetic isotope effect studies have suggested the existence of a single transition state during the C_2−H and C_3−OH bond formation steps, indicating that a concerted mechanism involving concomitant nucleophilic attack at the β-carbon and protonation at the α-carbon might be operational (*16*, *17*).

Recently, we have reported (methylenecyclopropyl)formyl-CoA (MCPF-CoA, **4**) as an irreversible inactivator for bovine liver short-chain ECH (*18*−*20*). This compound is a metabolite of (methylenecyclopropyl)glycine (MCPG, **3**) (*21*, *22*), a homologue of hypoglycin (**5**) which is the causative agent of Jamaican vomiting sickness (*23*−*25*). Hypoglycin is metabolized in vivo to (methylenecyclopropyl)acetyl-CoA (MCPA-CoA, **6**), which inactivates acyl-CoA dehydrogenases (*26*−*28*) by a mechanism involving radical-mediated ring scission (*29*−*31*). Similarly, MCPG is metabolized in vivo to MCPF-CoA which appears to be a specific inhibitor against ECHs (*21*), particularly bovine liver ECH (*19*). Since both stereoisomers (1*R* and 1*S*) of **4** inactivate the bovine liver enzyme in a time- and concentration-dependent fashion with nearly identical inhibition parameters, the observed inactivation of ECH is non-stereospecific (*20*). Using isotopically labeled MCPF-CoA analogues, the site of covalent modification was found to be Cys114 in the mature bovine liver ECH sequence (*20*). It was also established that during inactivation, the C-3 atom of MCPF-CoA is transformed to an alkene carbon. Thus, the mechanism of inactivation likely involves the attack of Cys114 at C-2′ of **4**, resulting in cleavage of the strained cyclopropyl ring and the covalent modification of the enzyme at Cys114 (*20*).

When the important physiological role played by this enzyme in the metabolism of fatty acids and branched chain amino acids is considered, the design of methods to control and/or regulate its activity may be therapeutically useful for alleviating disorders associated with these metabolic processes. Spurred by the promise of developing a selective inhibitor for this class of enzymes, we have sought to characterize the interaction of bovine liver ECH with MCPF-CoA in further detail. Since Cys114 appears to be directly involved in this process, we decided to construct the C114A mutant of bovine liver ECH and investigate its catalytic properties and sensitivity to MCPF-CoA. Here, we report that the C114A mutant is catalytically active and, interestingly, remains susceptible to inactivation by MCPF-CoA. Clearly, there are inconsistencies between the previously postulated inactivation mechanism and these recent findings. Therefore, we propose a modified mechanism of inactivation of this enzyme to reconcile our previous results with the data presented herein.

EXPERIMENTAL PROCEDURES

General. All protein purification operations were performed at 4 °C, unless otherwise specified. The protein concentration was determined by the Bradford method (*32*) using bovine serum albumin as the standard. However, for routine purposes, the enzyme concentration was estimated using an extinction coefficient of 16 000 M^{-1} cm^{-1} at 280 nm (*4*). The level of expression and the purity of the purified protein were assessed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) as described by Laemmli (*33*). Amino acid sequencing was carried out by the Mass Spectrometry Facility at the University of British Columbia. DNA sequencing was performed by the Advanced Genetic Analysis Center of the University of Minnesota (St. Paul, MN). DNA sequence analyses were performed using the Wisconsin Sequence Analysis Package of Genetics Computer Group (Madison, WI) and GeneWorks version 2.5 of IntelliGenetics, Inc. (Mountain View, CA). Putative ECH protein sequences were obtained from the San Diego Supercomputer Workbench (http://workbench.sdsu.edu). Sequence alignments were performed using the CLUSTALW 1.8 program available at http://searchlauncher.bcm.edu and are displayed using the formatting provided by the BOXSHADE server available at http://www.ch.embnet.org. Methods and protocols for recombinant DNA manipulations were according to the manufacturers' manuals or from general references (*34*).

Materials. The bovine liver enoyl-CoA hydratase gene was previously cloned into the plasmid pSD_6 and expressed in *Escherichia coli* strain JM105. The recombinant protein was purified as described previously (*20*). Agarose and bacterial cell growth media were purchased from GIBCO BRL (Gaithersburg, MD). Cloned *pfu* DNA polymerase was a product of Stratagene (La Jolla, CA). Geneclean II was from BIO101 Inc. (La Jolla, CA), and Bradford reagent was from Bio-Rad (Richmond, CA). Nucleotide triphosphates [dATP, dCTP, dGTP, and dTTP (10 mM solution)], Magic Mini Preps, Wizard Miniprep, and Wizard Megaprep DNA purification systems, and buffers used in PCR were obtained from Promega (Madison, WI). The vectors pET-24b(+) and pET-3b were obtained from Novagen (Madison, WI). The expression vectors pTrc99A, *E. coli* JM105, DEAE-Sepharose, and Phenyl-Sepharose CL-6 were purchased from Pharmacia (Uppsala, Sweden). Custom-designed oligonucleotide primers were made by Integrated DNA Technologies (Coralville, IA). Pepsin from porcine stomach mucosa, crotonoyl-CoA, and acetoacetyl-CoA were obtained from

Sigma (Milwaukee, WI). The molecular weight standards and other biochemicals and chemicals were purchased from Sigma or Aldrich (Milwaukee, WI), and were of the highest purity available. MCPF-CoA was kindly provided by D. Li (*18*, *19*).

Construction of the C114A Mutant of Bovine Liver ECH. The C114A point mutation in bovine liver ECH was introduced by the phosphorothioate method using the Sculptor in vitro mutagenesis kit of Amersham (Arlington Heights, IL). The primer 5′-GCCCTTGGTGGTGGC**GCT**GAACTTGCTATGATGTGTGAC-3′, where the bold codon represents the change from cysteine to alanine, was employed to introduce the mutation. The expected mutant plasmid was isolated and used to transform *E. coli* TG1 cells. The incorporation of the desired mutation was verified by DNA sequencing. To achieve optimum expression, the mutant gene was excised from pUC119 with restriction enzymes *Eco*RI and *Bam*HI and ligated into expression vector pTrc99A. The resulting recombinant plasmid was used to transform *E. coli* HB101 cells. The protocols for the mutagenesis reaction were carried out using standard molecular biology procedures and/or the manufacturer's instructions.

Construction of the E115Q and E135Q Mutants of Bovine Liver ECH. The E115Q and E135Q mutants of bovine liver ECH were prepared by a polymerase chain reaction-based method using the QuickChange Site-Directed Mutagenesis Kit protocol from Stratagene. The following primers were used to introduce the point mutations (the bold sequence denotes the codon change for the respective mutations): E115Q (forward), 5′-GGTGGTGGCTGT**CAG**CTTGCTATGATGTGT-3′; E115Q (reverse), 5′-ACACATCATAGCAAG**CTG**ACAGCCACCACC-3′; E135Q (forward), 5′-CAGTTTGGGCAGCCG**CAG**ATTCTAATAGGAACC-3′; and E135Q (reverse), 5′-GGTTCCTATTAGAAT**CTG**CGGCTGCCCAAACTG-3′. The PCRs were run using the wild-type bovine liver ECH gene ligated in a pTrc99A vector (50 ng) as the template, and the reaction was performed using the following temperature program: 5 min at 95 °C, 2 min at 55 °C, and 15 min at 68 °C (one cycle) and 30 s at 95 °C, 1 min at 55 °C, and 12 min at 68 °C (15 cycles total). After the thermal cycling was complete, the amplified gene was digested with *Dpn*I and purified by GeneClean II. The resulting DNA was used to transform competent *E. coli* XL1-blue cells. Five colonies from each transformation plate were used to inoculate LB medium supplemented with 100 μg/mL ampicillin, and the plasmid DNA from the cultures was isolated using the DNA Wizard Miniprep kit. The plasmids were checked by 0.7% agarose gel electrophoresis, and samples of the appropriate size were selected for sequencing to verify if the desired mutation was present.

Purification of Mutant Bovine Liver ECH Proteins. The bovine liver ECH C114A, E115Q, and E135Q mutants were purified according to the protocol developed previously for the wild-type enzyme (*20*). The concentration of the purified enzymes was determined by the Bradford assay (*32*), or calculated on the basis of an extinction coefficient of 16 000 M^{-1} cm^{-1} at 280 nm that was reported in the literature (*4*).

Enzyme Assays. The activity of the enzymes was measured by a continuous spectrophotometric assay based on the method of Steinman and Hill (*4*), except for the omission of ovalbumin from the reaction mixture and the use of 50 mM potassium phosphate, 3 mM EDTA buffer (pH 7.5). Briefly, the progress of the reaction was monitored at 263 nm (ϵ = 6700 M^{-1} cm^{-1}) which corresponds to the α,β-unsaturated enoyl moiety of the substrate. The assay cocktail consisted of 50 mM potassium phosphate, 3 mM EDTA (pH 7.5), and 150 μM crotonoyl-CoA in a total volume of 1 mL. The reaction was initiated by the addition of the enzyme, after which the contents were quickly mixed and placed in the spectrophotometer. The specific activity of the enzyme is defined in terms of the number of micromoles of substrate consumed per minute per milligram of the enzyme.

Determination of the Steady-State Kinetic Parameters. The steady-state kinetic parameters of wild-type bovine liver ECH and its C114A mutant were determined by measuring the initial velocities of the reaction when appropriate amounts of crotonoyl-CoA (2−300 μM) were mixed with the enzyme (7 nM). The data were fit to the Michaelis−Menten equation to determine the values of K_m and k_{cat} for the respective enzymes.

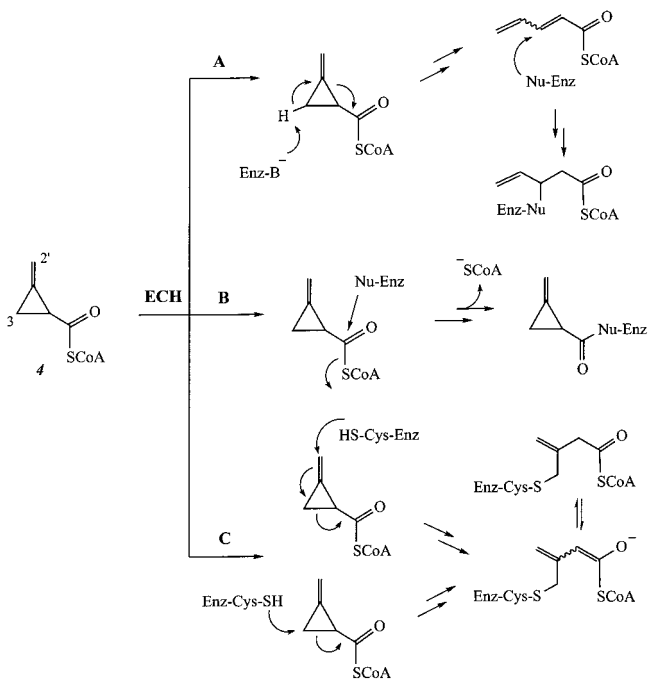
Kinetic Analysis of Inactivation. In a typical inactivation experiment, 200 μM inactivator (MCPF-CoA) was added to the bovine liver ECH C114A, E115Q, or E135Q mutant (9 μM) in 250 μL of 50 mM potassium phosphate buffer (pH 7.4) at room temperature. For the C114A mutant, aliquots (10 μL) of the reaction mixture were removed at various time intervals and mixed directly with 150 μM crotonoyl-CoA to determine the residual activity of the enzyme. For the "protection" experiments, the enzyme was incubated with 200 μM MCPF-CoA and 200 μM acetoacetyl-CoA in 500 μL of 50 mM potassium phosphate containing 3 mM EDTA (pH 7.5), and the residual activity was determined at various time points as described above.

Test for Reversibility of Inactivation. The bovine liver ECH C114A mutant (5 nmol) and MCPF-CoA (20 molar equiv) were incubated overnight at room temperature in 500 μL of 50 mM potassium phosphate containing 3 mM EDTA (pH 7.5). After complete inactivation of the enzyme had occurred, the enzyme was introduced into a dialysis bag and dialyzed against 1 L of 50 mM potassium phosphate (pH 7.5) for 2 days at 4 °C, with seven changes of buffer. Aliquots of the enzyme were withdrawn from the dialysis bag at various time points and checked for activity using the assay described above. A control experiment that included an equivalent amount of buffer instead of MCPF-CoA was performed in parallel.

Pepsin Digestion of Bovine Liver ECH. Proteolytic digestion of recombinant wild-type bovine liver ECH and its C114A variant was performed by mixing the inactivated enzyme (200 μg) with 20 μL of 2.1 M sodium phosphate (pH 1.7), 20 μL of 1 mg/mL pepsin from porcine stomach mucosa in 200 mM sodium phosphate (pH 2.0), and deionized water in a total volume of 200 μL. The digestion was conducted at room temperature for 1 h, after which the reaction mixture was frozen in liquid N_2, packed in dry ice, and shipped to the University of British Columbia for MS analysis. Control samples in which an appropriate volume of buffer was incubated with the enzyme in lieu of MCPF-CoA were also prepared and subjected to the identical treatment in parallel.

Electrospray Mass Spectrometry. Mass spectra were recorded on a PE-Sciex API 300 triple-quadrupole mass spectrometer (Sciex, Thornhill, ON) equipped with an ion-

Scheme 1



spray ion source. Peptides were separated by reverse phase HPLC on an Ultrafast Microprotein Analyzer (Michrom BioResources Inc., Pleasanton, CA) directly interfaced with the mass spectrometer through a postcolumn split tee with the split ratio of 9:1. In each of the MS experiments, the proteolytic digest was loaded onto a C_{18} column (Waters, 3.9 mm × 150 mm), which was then eluted with a gradient of 0 to 60% solvent B over the course of 60 min followed by 100% B over the course of 2 min at a flow rate of 700 μL/min (solvent A, 0.05% trifluoroacetic acid and 2% acetonitrile in water; solvent B, 0.045% trifluoroacetic acid and 80% acetonitrile in water). The eluant from the column was divided such that 10% was introduced into the mass spectrometer, and 90% was collected manually for further MS/MS experiments. Mass spectra were obtained in either the single-quadrupole scan mode (LC−MS), the tandem-MS product ion scan mode (MS/MS), or the MS/MS/MS mode.

In the single-quadrupole mode (LC−MS), the quadrupole mass analyzer was scanned over an *m*/*z* range of 300−2000 Da with a step size of 0.5 Da and a dwell time of 1 ms per step. The ion source voltage (ISV) was set at 5 kV, and the orifice energy (OR) was 50 V. In the tandem-MS product ion scan mode, the spectrum was obtained by selectively introducing the precursor ions (*m*/*z* 882^+, 850^+, 1191^+, 1223^+, or 1730^+) from the first quadrupole (Q1) into the collision cell (Q2) and observing the product ions in the third quadrupole (Q3). Thus, Q1 was locked on *m*/*z* 882 (1191, 1223, and 1730): Q3 scan range of 50−900 (870, 1750, or 1730), step size of 0.3, dwell time of 2 ms, ion source voltage (ISV) of 5 kV, orifice energy (OR) of 50, Q0 = −10, and IQ2 = −55(−55,−44,−44). In the MS/MS/MS product ion scan mode, the ion source voltage (ISV) and orifice energy (OR) were increased at 5.5 kV and 60 V to enrich the fragments of 1223^+ and 1191^+ and then Q1 was locked on *m*/*z* 1223 or 1191: Q3 scan from 50 to 1240 or 1210, Q0 = −10, and IQ2 = −73.

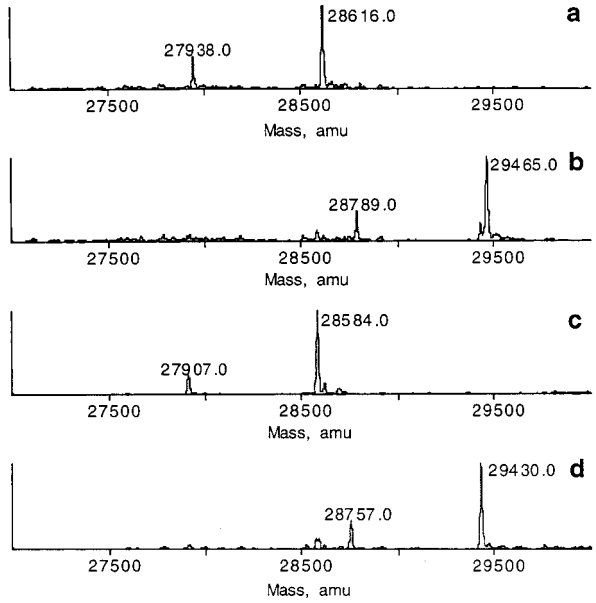


FIGURE 1: (a) ESI-MS of wild-type bovine liver ECH. The predicted molecular mass of the enzyme is 28 616 Da. (b) ESI-MS of wild-type bovine liver ECH inactivated with MCPF-CoA, which has a molecular mass of 848 Da. (c) ESI-MS of the bovine liver ECH C114A mutant. The predicted molecular mass of the enzyme is 28 586 Da. (d) ESI-MS of the bovine liver ECH C114A mutant inactivated with MCPF-CoA. The smaller peaks at 27 938, 28 789, 27 907, and 28 757 Da are due to an ATG start codon nine base pairs downstream in the plasmid pSD_6, which was used for the overexpression of the wild-type enzyme as well as a template for the mutagenesis reactions.

RESULTS AND DISCUSSION

Earlier reports have described MCPF-CoA (**4**) as a potent irreversible inhibitor of bovine liver ECH (*18*−*20*). On the basis of the structure of this compound and the chemical logic of its functional groups, a few possible mechanisms of inactivation were initially proposed to account for the inactivation of bovine liver ECH, shown in Scheme 1 (*19*, *20*). Of the three putative mechanisms, the mechanism involving an initial deprotonation at C-3 (Scheme 1, mechanism A) was ruled out on the basis of the lack of tritium wash-out from [3-^{3}H]MCPF-CoA (*18*, *19*). Similarly, the transesterification mechanism involving the formation of an acyl−enzyme adduct and the liberation of coenzyme A (mechanism B) was ruled out on the basis of the resistance of the enzyme−inhibitor adduct toward alkaline hydrolysis and on the basis of the association of the adenine chromophore with the inactivated enzyme despite prolonged dialysis (*18*, *19*). This was also corroborated by mass spectrometric data on the inactivated enzyme. Thus, ESI-MS revealed that while the native enzyme had a molecular mass of 28 616 Da, the inactivated enzyme had a molecular mass of 29 465 Da (see Figure 1a,b). The increase in the mass of the inactivated enzyme by 849 Da over that of the native enzyme indicates the formation of a covalent adduct between 1 equiv of MCPF-CoA and bovine liver ECH. In addition, these results further confirmed that the inactivation does not occur via the transesterification mechanism. Subsequently, use of the isotopically labeled MCPF-CoA analogues supported mechanism C, involving the attack of Cys114 at the exomethylene C-2′ atom (*20*).

Construction and Purification of the C114A Mutant of Bovine Liver ECH. As mentioned above, experiments using [3-^{3}H]MCPF-CoA and [3-^{13}C]MCPF-CoA had led to the identification of Cys114 as the nucleophile being trapped in the inactivation of bovine liver ECH by this inhibitor (*20*). It should be noted that Cys114 is expected to be in or near the active site of the enzyme since Glu115, which is conserved in all ECHs, has been shown to be catalytically essential in the rat liver enzyme (*35*). Interestingly, structural studies on the rat liver ECH had revealed that Cys143 (the Cys114 equivalent in rat liver ECH) is part of a hydrogen bond network that may be important for substrate binding (*9*, *10*). In view of the apparent direct involvement of Cys114 in reacting with MCPF-CoA and the perceived importance of this residue for bovine liver ECH, we decided to explore whether Cys114 plays a role in the catalytic mechanism of this enzyme. Accordingly, the C114A mutant was constructed and the mutant protein was purified as per the methods developed for the wild-type recombinant bovine liver ECH. ESI-MS analysis of the mutant protein showed a molecular mass of 28 584 Da (see Figure 1c), which matches well with the calculated molecular mass of 28 586 Da, confirming the presence of the Cys-to-Ala mutation in this protein.

Kinetic Parameters of the C114A Mutant. The steady-state kinetic parameters of the C114A mutant were determined by measuring the initial rates of the C114A-catalyzed reaction at different substrate concentrations. As shown in Figure 2, the C114A mutant displayed classical saturation kinetics under conditions where the concentration of the substrate was high. By fitting the Michaelis−Menten equation to a plot of rate versus substrate concentration, we determined the value of K_m to be 17.4 ± 3.8 μM and that of k_{cat} to be 1.16×10^3 s^{-1} (Figure 2A). These values are very similar to those determined for the wild-type enzyme ($K_m = 15.6 \pm 1.3$ μM, $k_{cat} = 2.06 \times 10^3$ s^{-1}; Figure 2B) as well as those reported for the rat liver enzyme (*35*). Therefore, it is evident that replacement of the side chain methanethiol group of Cys114 with a methyl side chain does not have a major impact on either the binding of the substrate to the active site of bovine liver ECH or the rate at which the enzyme catalyzes the hydration of the substrate. The apparent lack of a catalytic role for Cys114 in bovine liver ECH is also supported by a comparison of the sequences of genes with putative enoyl-CoA hydratase activity from various species. A partial sequence alignment shown in Figure 3 reveals several conserved residues, including Ala103, Gly107, Gly113, and Glu115 (bovine liver ECH numbering) near Cys114. However, less than half of the sequences have a Cys residue at this locus. In addition, those amino acids found at this position in different sequences do not fall into a discrete pattern in terms of their side chain functionalities. Thus, one can conclude that residue 114 does not play a significant role in the catalytic mechanism of ECHs in general.

Incubation of the C114A Mutant of Bovine Liver ECH with MCPF-CoA. The incubation of the C114A mutant of bovine liver ECH (9 μM) with MCPF-CoA (200 μM) was performed as described in Experimental Procedures. To our great surprise, the C114A mutant was susceptible to inactivation by MCPF-CoA. As shown in Figure 4, the activity of the C114A mutant decreased in a time-dependent manner,

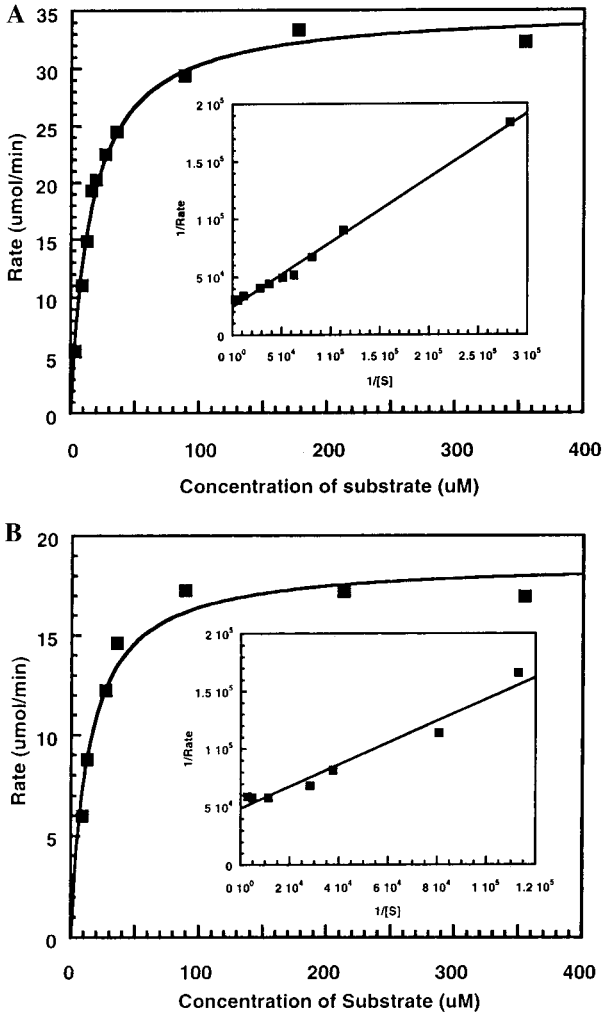


FIGURE 2: (A) Determination of steady-state kinetic parameters for wild-type bovine liver ECH. Rates of individual reactions were obtained by incubating varying amounts of crotonoyl-CoA (2−300 μM) with 7 nM bovine liver ECH as described in Experimental Procedures. The initial rates were plotted against the substrate concentrations to obtain the data shown above. The inset shows data from panel A plotted in a Lineweaver−Burk format. (B) Steady-state kinetic parameters for the bovine liver ECH C114A mutant.

whereas the activity of a control sample remained unchanged. By fitting a simple exponential equation to a plot of percent activity versus time, we calculated the rate constant for loss of activity (k_{obs}) to be 17.0×10^{-3} min^{-1}, and the half-life of the inactivation was calculated to be 40.8 min. The magnitudes of the k_{obs} and $t_{1/2}$ values determined for the C114A mutant are similar to those reported earlier for the inactivation of the wild-type enzyme (*19*). Since our earlier labeling experiments had shown Cys114 to be the nucleophile that reacts with MCPF-CoA (*20*), substitution of the cysteine residue at this position with alanine should abolish this reaction, and should bestow immunity to the enzyme against inactivation by MCPF-CoA. However, the fact that the C114A mutant is also susceptible to inactivation by MCPF-CoA is clearly contrary to this assumption. Intrigued by these results, we decided to pursue studies on this aspect in greater detail.

Inhibition of the C114A Mutant Is Irreversible and Occurs at the Active Site. The C114A mutant, after being inactivated by MCPF-CoA, could not regain its activity upon prolonged

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Rattus norvegicus          KKPV AA NGYAL GGCE A MCDIIYAG--EKAQFG
Bos taurus                 KKPV AA NGYAL GGCE A MCDIIYAG--EKAQFG
Homo sapiens               KKPV AA NGYPF GGCE A MCDIIYAG--EKAQFA
Caenorhabditis elegans     KKPV AA NGFAL GGNE A MCDIIYAG--EKARFG
Mycobacterium leprae       RTPM AA AGYAL GGCE A MCDLLIAA--DTAKFG
Mycobacterium tuberculosis TKPL AA EGYAL GGTE A AADLIVAA--RDSAFG
Mesorhizobium loti         TKPI AA NGYAL GGLE A LCDIVIAS--QAAQFA
Escherichia coli           NKPL AA NGYAL GGCE A LCDVVVAG--ENARFG
Archaeglobus fulgidus      EIPV AA NGYTL GGCE A ACDIRIAS--EKAKFG
Rhodococcus fascians       IKPL AA EGWAL GGCE A SADLIVAS--REAKFG
Bacillus halodurans        PQPT AA NGYAL GGFE A ACDFRLAV--PEAKMG
Leishmania major           PIAT AA EGKAL GGME A SLDMRVAG--DGATVG
Sulfolobus solfataricus    KKLI AS NGHCM GGLE A ACDLRFGANDENIKFG
Deinococcus radiodurans    EKPT AA HGTAL GGLE A GCTYRVAV--KDAQLG
Thermoplasma volcanium     PHPI AA NGLAA GGAE L LTLDYVVSV--KDAWFW
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FIGURE 3: Partial sequence alignment showing active site regions of putative enoyl-CoA hydratases from several bacterial and mammalian sources. Conserved residues, including Glu115, are shaded black, while the cysteine residue implicated in the inactivation of bovine liver ECH by MCPF-CoA is marked with an asterisk.

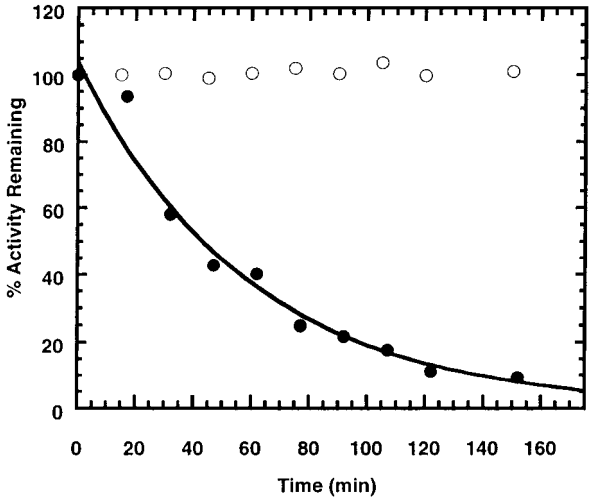


FIGURE 4: Inactivation of the bovine liver ECH C114A mutant by MCPF-CoA [(○) control and (●) with MCPF-CoA]. The mutant enzyme was incubated with a ~22-fold excess of MCPF-CoA, while control reaction mixtures contained an equivalent volume of buffer. At the indicated time points, aliquots were withdrawn from the inactivation and control reaction mixtures, and assayed for residual activity as described. The line represents an exponential fit to the data obtained from the inactivation curve. Values of k_{obs} and $t_{1/2}$ are reported in the text.

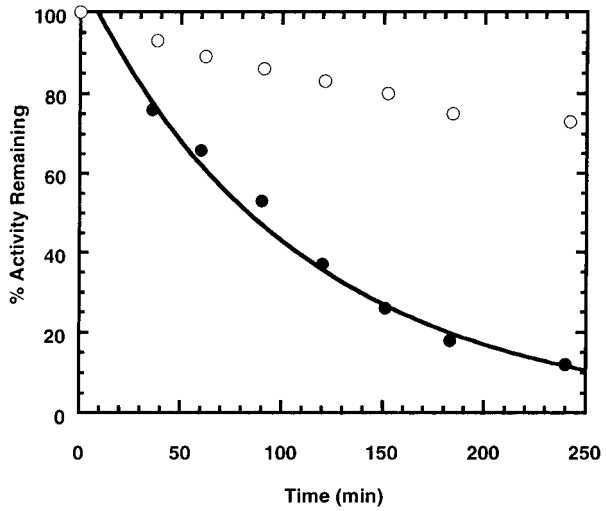


FIGURE 5: Inactivation of bovine liver ECH by MCPF-CoA is active site-directed. The bovine liver ECH C114A mutant was incubated with equimolar amounts of MCPF-CoA and AcAc-CoA as described. A control reaction mixture contained an equivalent volume of buffer instead of AcAc-CoA. At the indicated time points, aliquots from the protection reaction mixture (○) as well as the control reaction mixture (●) were withdrawn and assayed for activity as described. The line represents an exponential fit through the control data.

dialysis at 4 °C, a result similar to that observed with the wild-type enzyme. The inhibition of the C114A mutant is therefore irreversible, and is likely the result of formation of a covalent adduct between MCPF-CoA and the mutant enzyme. Covalent modification of the C114A mutant was also supported by mass spectroscopy data. As shown in Figure 1d, the ESI-MS spectrum of the inactivated enzyme displayed a peak corresponding to a mass of 29 430 Da, which correlates well with the molecular mass of the mutant enzyme (28 584 Da, Figure 1c) plus that of one molecule of MCPF-CoA. The covalent modification is active site-directed since the rate of inactivation of the C114A mutant (9 μM) by MCPF-CoA (200 μM) was significantly reduced when an equimolar amount of a known competitive inhibitor for ECH, acetoacetyl-CoA (AcAc-CoA), was added to the incubation (Figure 5). Taken together, all evidence indicated a high degree of resemblance between the inactivation (by MCPF-CoA) behavior of wild-type enzyme and that of its C114A mutant. It is conceivable that inactivation of the wild-type and the C114A enzyme is initiated through a common mechanism involving irreversible trapping of an active site nucleophile. It is also possible that the labeling of Cys114 in wild-type ECH may be a secondary event that took place after the initial inactivation had occurred. In other words, the labeling of Cys114 may not be a direct consequence of the reaction between the enzyme and MCPF-CoA, but may be a "postinactivation" phenomenon that may be an artifact of tryptic digestion and/or subsequent sample processing.

Construction and Purification of the E115Q and E135Q Mutants of Bovine Liver ECH. Amid speculation regarding the true identity of the nucleophile that reacts with MCPF-CoA leading to the inactivation of the wild-type enzyme, the needle of suspicion pointed to either one or both of the catalytic glutamic acid residues (E115 and E135). These active site residues are expected to be located in the proximity of the cyclopropyl ring of the bound MCPF-CoA and possess

the capacity to act in a nucleophilic fashion. Therefore, we decided to make mutants of bovine liver ECH that lack these side chains and to test the ability of the resulting proteins to react with MCPF-CoA. Accordingly, the E115Q and E135Q mutants were constructed and purified as described.

The E115Q and E135Q Mutants of Bovine Liver ECH Do Not React with MCPF-CoA. Kinetic analysis of the E115Q and E135Q mutants revealed that they displayed trace levels of activity which were $\sim 10^4-10^5$-fold lower than that of the wild-type enzyme. This correlates well with the corresponding data reported for the rat liver E144Q and E164Q mutants (*35*). Due to the low activities of these mutants, their ability to react with MCPF-CoA could not be tested on the basis of the conventional activity assay. However, when these proteins were incubated with excess MCPF-CoA and examined by ESI-MS, it was found that, unlike the wild-type enzyme and the C114A mutant, the E115Q and E135Q mutants did not form a covalent adduct with the inhibitor. Samples from the control incubations and incubations containing an excess of MCPF-CoA both display peaks near 28 613 Da (data not shown), which matches the calculated molecular masses of the E115Q and E135Q mutants. These results indicate that both Glu115 and Glu135 play a key role in the inactivation of the wild-type enzyme. To address whether one of these residues is the actual nucleophile that traps MCPF-CoA, we decided to use MS/MS experiments to characterize the reaction of wild-type bovine liver ECH and its C114A mutant with MCPF-CoA and to determine the identity of the reactive nucleophile in each case.

Glu115 Reacts with MCPF-CoA in Wild-Type Bovine Liver ECH. As described in our earlier report, Cys114 was identified as the residue labeled by [3-^{3}H]MCPF-CoA on the basis of peptide mapping that was performed using trypsin as the proteolytic enzyme. However, there is a possibility that labile linkages such as ester bonds may not survive the neutral and/or basic conditions used for trypsin digestion. Thus, pepsin which hydrolyzes peptide bonds under acidic conditions (pH $\sim$2) was used. As detailed in Experimental Procedures, pepsin-mediated digestion of the inactivated wild-type bovine liver ECH resulted in a number of fragments that were separated by reverse phase HPLC using ESI-MS as a detector. When scanned in the normal LC−MS mode, the total ion chromatogram (TIC) exhibited a large number of peaks. However, the peptide that was labeled with MCPF-CoA could be readily detected by comparative peptide mapping, as follows. When the spectrometer was scanned in the normal LC−MS mode, the total ion chromatograms (TIC) of the control and inhibited ECH digests displayed similar spectra (spectra a and c of Figure 6, respectively), consisting of a large number of peaks, with the exception of one extra peak at 31.7 min in the inhibited ECH digest. The mass spectrum of this peak (Figure 6d) showed a peptide with a mass of 1730 Da which was not present in the control ECH digest. In addition, the control ECH digest (Figure 6b) contained a peptide with a mass of 882.5 Da which elutes at 29.9 min, while in the inhibited ECH digest, the intensity of the peak with a mass of 882 Da was significantly reduced. The difference in mass of these peptides is 848 Da and is exactly that expected for the attached label. A search in the ECH sequence for possible peptides with a mass of 882 Da that could be formed gave three candidates, including peptide 118−126 (YALGGGCEL).

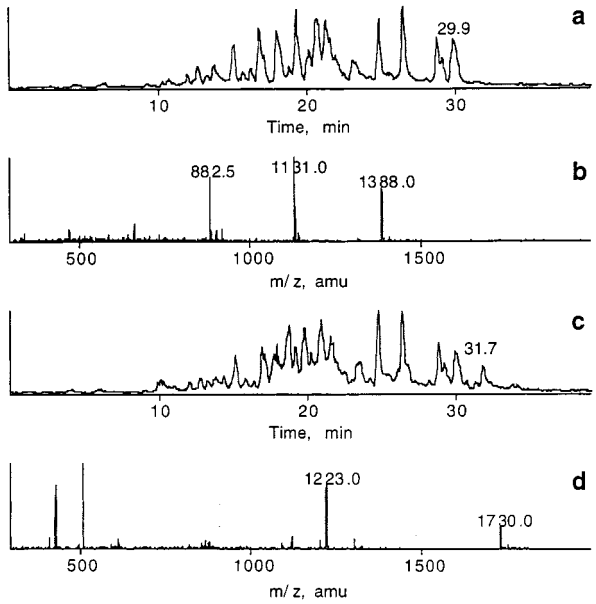
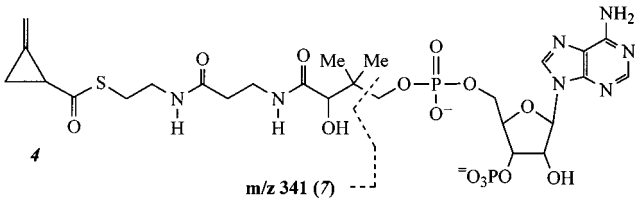


FIGURE 6: LC−MS analyses of peptic digests of labeled and unlabeled ECH. (a) LC−MS chromatogram of the unlabeled digest, (b) mass spectrum of the peak eluting at 29.9 min in the unlabeled digest, (c) LC−MS chromatogram of the labeled digest, and (d) mass spectrum of the peak eluting at 31.7 min in the labeled digest.

MS/MS spectra of the 882 Da peptide confirmed this sequence (not shown).

When the 1730 Da peptide (corresponding to 882 Da plus 848 Da, the latter being the molecular mass of the inhibitor) was examined in the MS/MS mode, a complicated spectrum dominated by the fragmentation of MCPF-CoA was obtained. It was later determined that under the high-orifice voltage conditions that were employed, MCPF-CoA degrades into a 341 Da fragment (**4** → **7**) that is still attached to the peptide. Thus, under MS/MS conditions, the mass of the modified peptide was 1223 Da (882 Da + 341 Da) and not 1730 Da because of the fragmentation of the bound inhibitor molecule under these conditions. Therefore, MS/MS sequencing of the 1223 Da fragment was attempted. The MS/MS spectrum of this fragment revealed a series of y ions and two b ions that contain the label with a mass of 341 Da, as shown in Figure 7. The presence of 602 Da (261 Da + 341 Da) and 1092 Da (751 Da + 341 Da) fragments confirms that the inhibitor was located on E115.



Glu115 Reacts with MCPF-CoA in the C114A Mutant of Bovine Liver ECH. Following an approach analogous to that described above, fragments resulting from pepsin-mediated digestion of the inactivated C114A mutant were separated by reverse phase HPLC using ESI-MS as a detector. A peptide with a molecular mass of 850 Da (^{108}Y-A-L-G-G-G-^{114}A-E-L) was found to be associated with the inhibitor by comparative peptide mass mapping, as described above. Under the conditions that were employed, MCPF-CoA

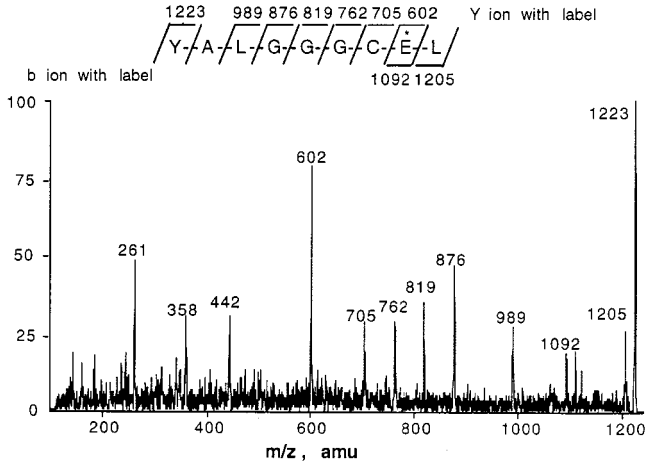


FIGURE 7: MS/MS analysis of the 1223 Da peptide showing covalent modification of E115 in the inactivation of wild-type bovine liver ECH by MCPF-CoA. The inactivated enzyme was subjected to pepsin digestion and LC−MS/MS as described.

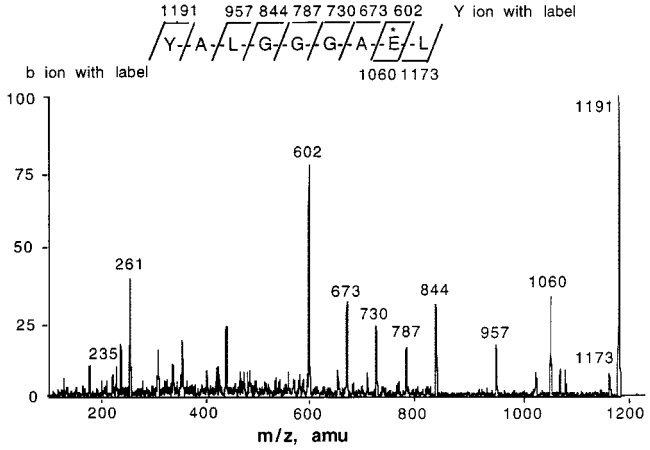


FIGURE 8: MS/MS analysis of the 1191 Da peptide showing covalent modification of E115 in the inactivation of the bovine liver ECH C114A mutant by MCPF-CoA. The inactivated enzyme was subjected to pepsin digestion and LC−MS/MS as described.

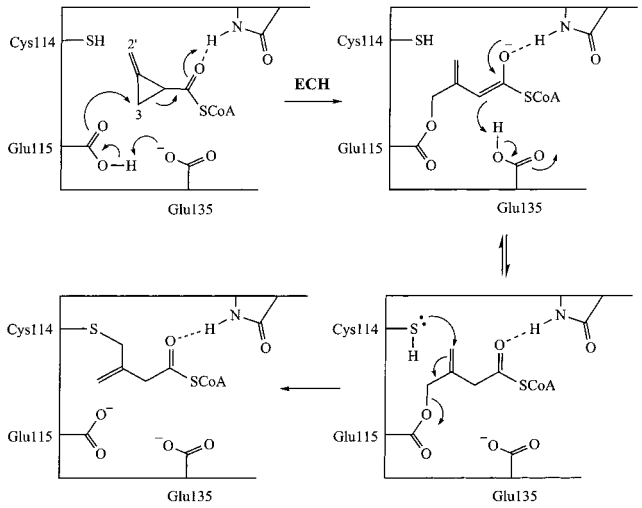
degrades into a 341 Da fragment that was still attached to the peptide. Therefore, MS/MS sequencing of the peptide with a molecular mass of 1191 Da was attempted (where 1191 Da equals the the mass of the 850 Da peptide plus the mass of the 341 Da inhibitor fragment). The MS/MS spectrum of the peptide with a mass of 1191 Da established that the inhibitor was located on Glu115 (Figure 8).

Mechanistic Implications of the Inactivation of Bovine Liver ECH by MCPF-CoA. As mentioned earlier, two peptides were found to be radiolabeled by [3-^{3}H]MCPF-CoA in the experiments with the wild-type enzyme (*20*). Sequence analysis of the N-termini of the two peptides gave an identical sequence, corresponding to residues 109−116 of bovine liver ECH with the inhibitor covalently linked to Cys114. However, peptide mapping using MS/MS as described above revealed that the residue being modified by MCPF-CoA was Glu115 instead of Cys114, a result clearly contradictory to that obtained using [3-^{3}H]MCPF-CoA. It should be pointed out that the inactivated proteins in both experiments were digested by protease, and the peptide fragments were separated followed by Edman degradation or MS/MS analysis. In the case of incubation with [3-^{3}H]-MCPF-CoA, the inactivated protein was treated with trypsin at pH 8.1 prior to N-terminal sequencing analysis, whereas pepsin, which operates at a much lower pH (1.8−2), was used to hydrolyze the modified protein for MS/MS analysis. Several steps in the sample processing sequence could potentially cause unwanted secondary reactions resulting in artifacts. In particular, as mentioned above, the digestion step performed with trypsin at a basic pH may be problematic. The enhanced nucleophilicity of the methanethiol side chain of Cys114 at high pH and the proximity of this residue to Glu115 could facilitate the attack of Cys114 on the adduct, transferring the inhibitor that was initially harbored on Glu115. While the ester linkage between Glu115 and MCPF-CoA in the inactivated enzyme might be labile at high pH, the enzyme−inhibitor adduct had been shown to be resistant to basic hydrolysis. This phenomenon may again be ascribed to the migration of the entrapped inhibitor from Glu115 to Cys114 during alkaline treatment. The stability of the thiol ether linkage in the enzyme−inhibitor adduct and the proximity of the modified residue to the active site must have rendered the resulting enzyme inactive and also resistant to alkaline hydrolysis. Therefore, a scenario in which MCPF-CoA reacts first with Glu115 to inactivate the enzyme, which is followed by the capture of the entrapped inhibitor by Cys114 in a postinactivation event, is an appealing hypothesis that could reconcile the two apparently contradictory sets of observations.

The above explanation is also consistent with the results obtained with the C114A mutant. Since replacement of the cysteine residue at position 114 of bovine liver ECH with an alanine did not show any deleterious effect on the catalytic efficiency of this enzyme, binding of MCPF-CoA to the C114A mutant should proceed unhindered. Since Glu115, which remains intact in the C114A mutant, is the nucleophile that actually reacts with MCPF-CoA, it is now clear why the C114A mutant is still susceptible to the inactivation by MCPF-CoA. Our current hypothesis is also supported by the demonstrated inability of the E115Q mutant to react with MCPF-CoA, which reinforces the validity of the results from the MS/MS experiments and strongly suggests that Glu115 is the initial nucleophile that traps MCPF-CoA.

Interestingly, replacement of Glu135 with glutamine leads to a mutant enzyme that is also immune to MCPF-CoA. If the inactivation by MCPF-CoA is merely the result of a simple nucleophilic addition of Glu115 to the inhibitor, the absence of Glu135 should not hamper this reaction. Therefore, a more elaborate mechanism in which Glu135 plays a definite role should be considered. As mentioned earlier, the current mechanistic model for the catalysis of ECH, as exemplified by the rat enzyme, involves the participation of Glu164 (Glu135 equivalent in rat ECH) as a general acid that donates a proton to C_2 of the substrate CoA thioester (*9*, *10*). Mutagenesis studies on the rat enzyme had already indicated the critical role played by this residue in the catalytic mechanism of ECH (*35*). It is worth mentioning that Glu164 is responsible for the stereospecific exchange of the *pro*-2*S* hydrogen with solvent when butyryl-CoA is incubated with rat liver ECH (*12*). However, the exchange reaction was abolished not only in the E164Q mutant (*12*) but also in the E144Q mutant of rat liver ECH (*35*). Thus, it was concluded that Glu144 and Glu164 act in concert, and that the presence of both is essential for the catalytic

Scheme 2



efficiency of rat liver ECH. Our findings that the E135Q mutant of the bovine liver enzyme has only trace levels of catalytic activity and is immune to the inactivation by MCPF-CoA are consistent with results from the rat liver enzyme. Consequently, a similar role can be assigned to Glu135 in the catalytic mechanism of bovine liver ECH.

A mechanistic proposal that accommodates all of the current evidence regarding the inactivation of bovine liver ECH by MCPF-CoA is presented in Scheme 2. It is proposed that Glu115 is responsible for the initial attack at C-3 of MCPF-CoA. This can be thought of as being a "Michael addition", wherein the nucleophilic carboxylate of Glu115 reacts with an electrophilic center at C-3 that is activated due to the polarization induced by binding. Such a polarization might also weaken the bond between C-1 and C-3 of MCPF-CoA, promoting the scission of the strained three-membered ring. The role of Glu135 may be to modulate the reactivity of Glu115 and/or donate a proton to C-1, which is akin to its normal role in the catalytic mechanism of ECH. Since the reaction with MCPF-CoA cannot proceed in the absence of Glu135, it appears that either the reactivity of Glu115 is greatly reduced in the E135Q mutant, or that no surrogate proton source may be available, or both. Therefore, this hypothesis envisions a role for both Glu115 and Glu135 in the inactivation: Glu115 clearly acts as the nucleophile, and Glu135 may act as an activator or as a proton source. In the presence of both Glu115 and Glu135, the cyclopropane ring is cleaved and a covalent enzyme−inhibitor adduct is formed. The presence or absence of Cys114 should not affect the formation of the adduct, which is the basis for the susceptibility of the enzyme to inactivation by MCPF-CoA in the C114A mutant. However, when Cys114 is present in the vicinity, it is possible that the greater nucleophilicity of the side chain methanethiol of this residue results in an S_N'-type attack at C-2′ that results in the liberation of Glu115 and the formation of another covalent enzyme−inhibitor adduct, this one with Cys114. It is most likely that this is the adduct that was detected by us in our earlier experiments. Clearly, this is an interesting problem, and further studies are needed to resolve whether this hypothesis is grounded in reality and to determine the chemical nature of the initial Glu115−MCPF-CoA adduct.

In summary, these results highlight the importance of verification of peptide mapping results using an alternate technique, a fact that has also been stressed in a recent report describing 5,6-dichloro-7,7,7-trifluoro-4-thia-5-heptenoyl-CoA as an irreversible inhibitor for rat liver ECH (*36*). Our studies suggest that Glu115 and Glu135 collaborate in some manner to react with the active site-bound MCPF-CoA, and that the absence of either one abolishes this reaction. As the reaction of bovine liver ECH with MCPF-CoA requires the collaboration of Glu115 and Glu135, with Glu135 serving as an activator and/or proton donor, this would classify MCPF-CoA as a mechanism-based inhibitor, since the catalytic mechanism of the enzyme also requires the action of these residues. MCPF-CoA can therefore serve as a lead for the design of specific agents targeted against enzymes of the fatty acid oxidation pathway, a fact which assumes significance given the many disorders of fatty acid metabolism that abound in the human population.

ACKNOWLEDGMENT

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