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Review

Enoyl-CoA Hydratase: Reaction, Mechanism, and Inhibition

Gautam Agnihotri† and Hung-wen Liu*

Division of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry and Biochemistry, University of Texas, Austin, TX 78712, USA

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Abstract—Enoyl-CoA hydratase (ECH) catalyzes the second step in the physiologically important beta-oxidation pathway of fatty acid metabolism. This enzyme facilitates the *syn*-addition of a water molecule across the double bond of a *trans*-2-enoyl-CoA thioester, resulting in the formation of a β-hydroxyacyl-CoA thioester. The catalytic mechanism of this proficient enzyme has been studied in great depth through a combination of kinetic, spectroscopic, and structural techniques, and is proposed to occur via the formation of a single transition state. Sequence alignment and mutagenesis studies have implicated the key residues important for catalysis: Gly-141, Glu-144, and Glu-164 (rat liver ECH numbering). The two catalytic glutamic acid residues are believed to act in concert to activate a water molecule, while Gly-141 is proposed to be involved in substrate activation. Recently, two potent inhibitors of ECH have been reported in the literature, which result in the irreversible inactivation of the enzyme via covalent adduct formation. This review summarizes studies on the structure, mechanism, and inhibition of ECH.

Contents

*Corresponding author. Tel.: +1-512-232-7811; fax: +1-512-471-2746; e-mail: h.w.liu@mail.utexas.edu
†Current address: Assay Development, NeoGenesis, 840 Memorial Drive, Cambridge, MA 02139, USA.

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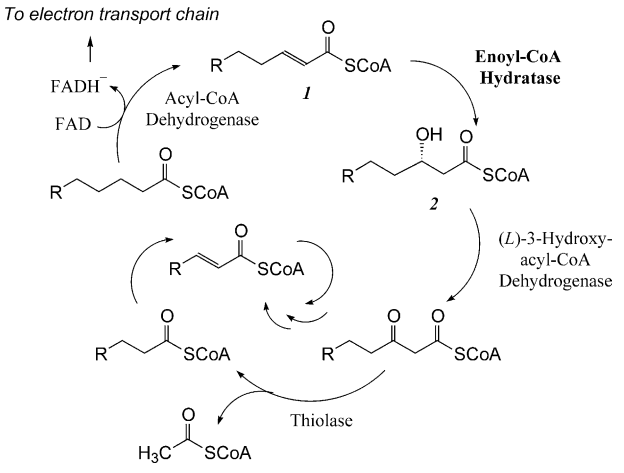
Introduction

The β-oxidation spiral of fatty acid degradation consists of four enzymatic transformations that reduce the acyl chain length by two carbons in each cycle, resulting in the formation of acetyl-CoA as the byproduct in each cycle.[1] When even chain fatty acids are used as the starting material, acetyl-CoA is formed as the ultimate product of the pathway itself. When odd chain fatty acids are used as the starting material, the final round of oxidation forms propionyl-CoA, which is converted to the more common metabolic intermediate succinyl-CoA through the action of propionyl-CoA carboxylase and methylmalonyl-CoA mutase.[2] The four enzymes that drive this cycle in mitochondria are acyl-CoA dehydrogenase (AD), enoyl-CoA hydratase (ECH), (L)-3-hydroxyacyl-CoA dehydrogenase (HAD), and thiolase (Scheme 1). Although the mitochondrial pathway is shown as being catalyzed by discrete enzymes, other cell organelles (e.g., peroxisomes) contain multifunctional proteins which can catalyze two and even three reactions in the pathway.[3,4] This review will focus on the second enzyme in the fatty acid oxidation pathway, ECH. As will be evident in the sections below, the catalytic mechanism of ECH has been extensively studied using a variety of methods. The intent of this review is to present a broad summary of what is currently known about this enzyme without going into the intricate details of the individual studies.



Scheme 1.

ECH catalyzes the reversible syn-hydration of *trans*-2-enoyl-CoA thioesters (**1**) to the corresponding (*S*)-3-hydroxyacyl-CoA thioesters (**2**).[5] However, the enzyme has also been shown to catalyze the hydration of *cis*-2-enoyl-CoA thioesters to the corresponding (*R*)-3-hydroxyacyl-CoA thioesters.[6] This physiologically important reaction constitutes the second step of the β-oxidation spiral of fatty acid degradation. Short-chain ECH is an extremely efficient catalyst, with a catalytic efficiency that approaches the diffusion-controlled limit. The equilibrium of the ECH-catalyzed reaction favors the formation of the product by a factor of 3.5 at 25 °C.[7] Although enzyme assays are commonly run at pH 7.0, maximum turnover occurs at pH 9.4.[7] The most active substrate for ECH is crotonoyl-CoA, with a k_{cat}/K_m value of 5×10^7 M^{-1} s^{-1}.[7] However, turnover number drops sharply with increasing chain length. For example, the rate of hydration of *trans*-2-hexadecenoyl-CoA, which represents the longest acyl chain studied, is only about 1% of that observed with crotonoyl-CoA.[7] In contrast, the values of K_m within the same series do not drop as sharply. Thus, while the K_m for crotonoyl-CoA is 20 μM, the corresponding value for *trans*-2-hexadecenoyl-CoA is 500 μM.[7]

Bovine liver ECH accepts a variety of substrates including 2- and 3-methyl-crotonoyl-CoAs, 2-, 3-, and 4-halo-crotonoyl-CoAs, acryloyl-CoA, 2-methyl-acryloyl-CoA, sorbyl-CoA, and 1-carboxycyclohexenoyl-CoA. In addition, 2-alkynoyl-CoAs are hydrated to the corresponding β-ketoacyl-CoAs, which result from the tautomerization of the enol-CoA product.[7] However, this tolerance for structural variation is confined to the acyl-chain portion of the substrate; ECH displays a stringent specificity for coenzyme A versus other pantetheine analogues. For example, the rate of hydration of crotonylpantetheine is only about 0.15% of that of crotonoyl-CoA.[7] Therefore, crotonylpantetheine and β-hydroxybutyrylpantetheine have often been used as analogues for kinetic studies of ECH in order to make the chemical step rate limiting (see below). Interestingly, crotonyl-oxyCoA, in which the thioester linkage between coenzyme A and crotonic acid has been replaced by an ester linkage, has been shown to act as a substrate for ECH, albeit with reduced efficiency.[8] In addition to catalyzing the hydratase reaction, ECH has also been shown to catalyze the positional isomerization of β,γ-unsaturated CoA thioesters to the corresponding

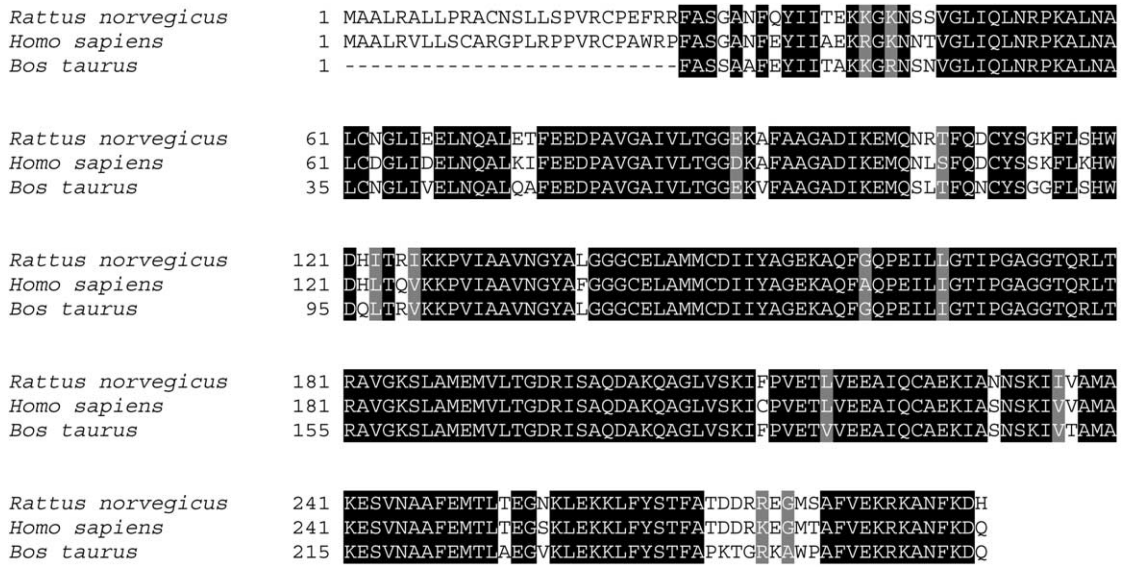


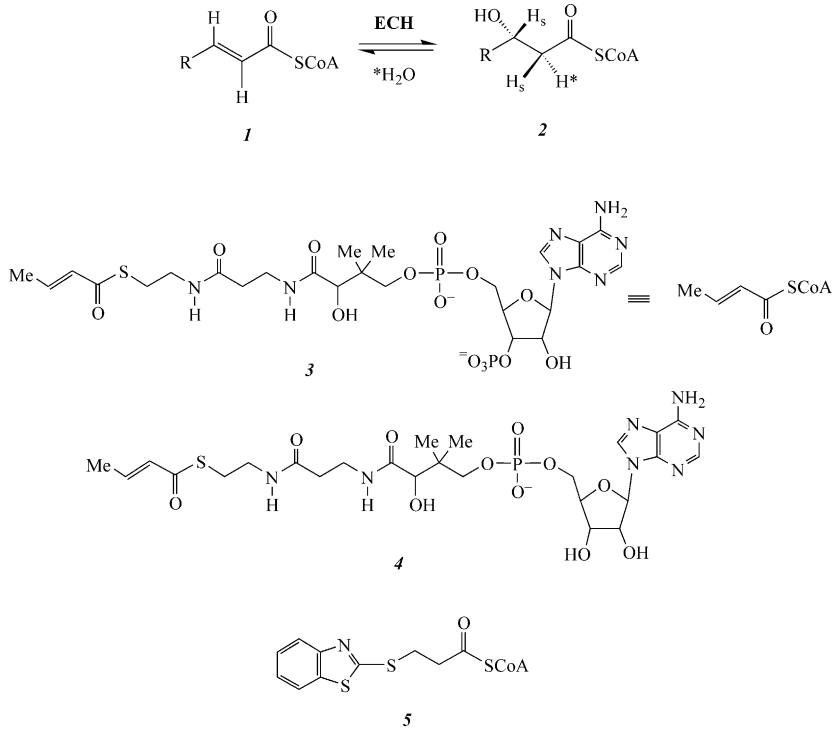
Figure 1. Sequence alignment of representative mammalian ECHs from rat liver, bovine liver, and human sources. The 29-residue N-terminal leader sequence for bovine liver ECH has not been published.

α,β unsaturated CoA thioesters,[9] as well as the stereospecific exchange of the *pro*-2*S* hydrogen of butyryl-CoA with the solvent.[10]

Sources and Homology

The most detailed studies on ECH have been performed on the enzyme from mammalian sources. ECH was initially purified and characterized from bovine liver,[7] although the enzyme has also been obtained from other sources such as pig heart,[11] pig kidney,[12] and rat liver.[13] Recently, bovine liver ECH has also been purified after heterologous overexpression in *Escherichia coli*, and the properties of the recombinant enzyme have been shown to be similar to that of the native enzyme.[14]

ECH genes from various sources show a fairly high degree of sequence homology to each other, especially among genes from related phylogenetic groups. For instance, the mammalian ECHs are highly conserved (Fig. 1), and show sequence identities in the range of 85% and higher. On the basis of sequence alignments, several conserved residues such as Glu-144 and Glu-164 (rat liver ECH numbering) were identified and shown to be important for catalysis (Fig. 2). It should be noted that ECHs also display significant sequence identity with other hydratases and isomerases such as Δ^2,Δ^3-



enoyl-CoA isomerase, 4-chlorobenzoyl-CoA dehalogenase etc. This has resulted in the classification of these enzymes into a common hydratase/isomerase superfamily.[15,16]

Physical and Structural Properties

Sedimentation equilibrium analysis revealed that ECH is a homohexamer in its native state with a subunit molecular mass of approximately 29 kDa.[17] This value is in good agreement with the calculated molecular mass of 28,287 Da for the rat liver ECH which is based on the deduced amino acid sequence as derived from the cDNA sequences for these enzymes. As in the case of acyl-CoA dehydrogenases, which are also involved in fatty acid β-oxidation, ECHs are synthesized in the cytosol with an N-terminal signal sequence that is removed proteolytically.[18] The signal sequence is a necessary recognition element to direct the enzyme to its proper location within the cell.

Studies on the catalytic mechanism of ECH received a significant impetus with the determination of the three dimensional structures of the rat liver enzyme with and without bound acetoacetyl-CoA and octanoyl-CoA, with resolutions of 2.5 and 2.4 Å, respectively (Fig. 3).[19,20] These studies, reported by Rik Wierenga and coworkers, represented the first structural information among hydratases. Recently, Petsko and coworkers have also published the crystal structure of rat liver ECH with bound 4-(*N*,*N*-dimethylamino)cinnamoyl-CoA with a resolution of 2.3 Å.[21] Interestingly, 4-chlorobenzoyl-CoA dehalogenase and other coenzyme A binding proteins have been found to have similar overall folds and coenzyme A binding motifs, indicating that this motif may be a conserved structural adaptation among these proteins. Mutation of 4-chlorobenzoyl-CoA dehalogenase to introduce key catalytic residues of ECH in the proper orientation conferred the previously absent hydratase activity to the enzyme, illustrating the overall structural similarities within the hydratase/isomerase superfamily of enzymes.[22]

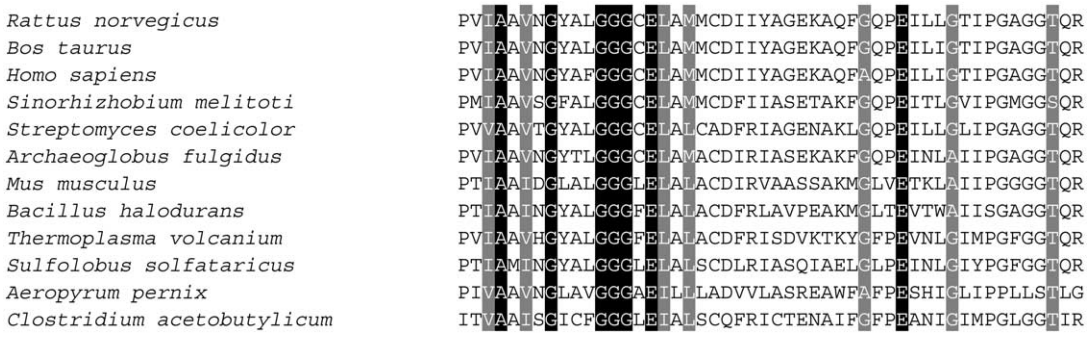


Figure 2. Partial sequence alignment of proteins assigned as ECHs from bacterial and mammalian sources indicating the conserved residues present near the active site.

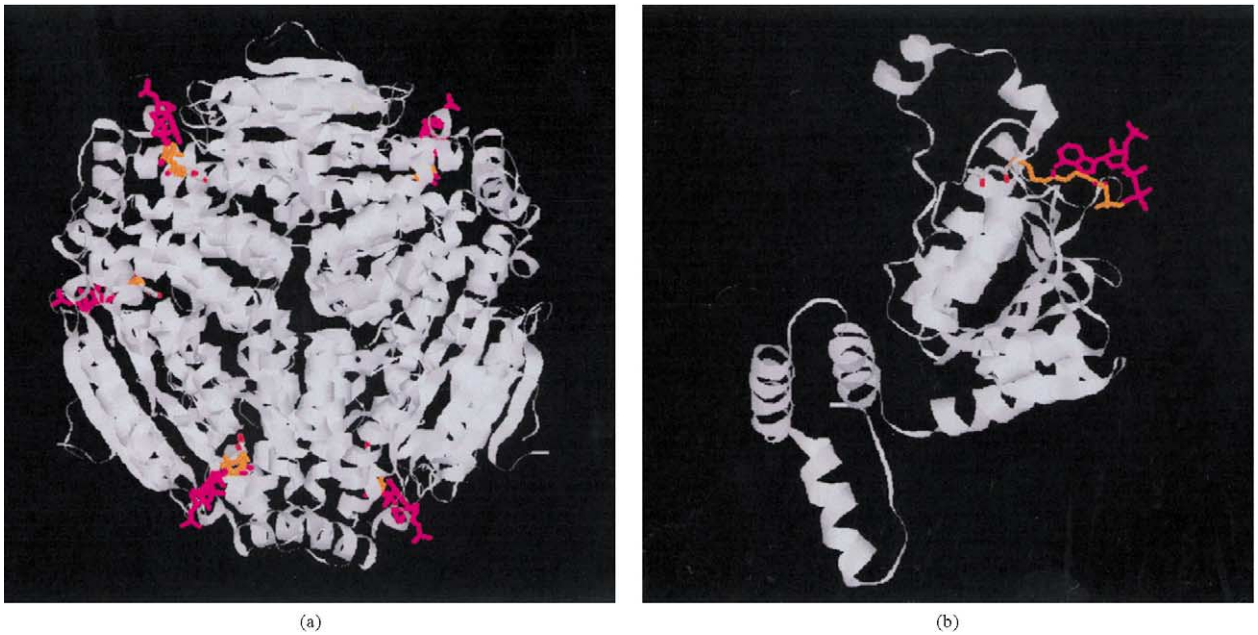


Figure 3. (a) Global quarternary structure of the ECH hexamer; (b) structure of ECH monomer with bound acetoacetyl-CoA. These figures were obtained using the co-ordinates deposited in the Protein Data Bank under the entry 1DUB.

The X-ray crystal structure of the enzyme revealed that the ECH monomers form a tightly associated trimeric structure.[19] Two such trimers are then held loosely as a 'dimer of trimers' to form the hexameric complex (Fig. 3a). The X-ray structure further revealed that the hexamer contains six distinct active sites which were fully contained within the individual monomeric units with the exception of contact from one amino acid residue (Lys 282) from a neighboring subunit. The coenzyme A binding pocket is formed by four turns of a ββα spiral (Fig. 3b). The coenzyme A part of the molecule serves as the primary binding determinant, which is not surprising given the rich functionality presented by this moiety, especially when compared to the enoyl-part of the molecule. In the active site of the enzyme, the pantetheine portion of the inhibitor is highly bent, adopting an almost U-shape around the pyrophosphate group. The pyrophosphate group itself is highly exposed to the external medium, and also forms three salt–bridge interactions with positively charged lysine residues. There is one intramolecular hydrogen bond between the adenine ring and the pantetheine group. The adenine ring also makes three hydrogen bonds to the protein. Comparison of the active sites structure when the ligand is bound to that of the unliganded active site revealed minimal structural differences, indicating that the active site is rigid and that the binding of the inhibitor (or the substrate) would not be expected to induce any major structural perturbations in the protein.

Stereochemical Issues

There is no evidence for the participation of metal ions or cofactors in the catalytic mechanism of ECH. Stereochemical investigations have revealed that the enzymatic hydration/dehydration reaction proceeds via a *syn*-pathway.[23] Thus, the proton and the hydroxyl groups are added to/removed from the same side of the *trans*-double bond. It has been determined that ECH introduces a bias towards the *syn*-pathway of greater than 8.8 kcal/mol.[10] Tonge and coworkers have recently demonstrated that incubation of 3(*S*)-hydroxybutyryl-CoA with ECH in D_2O results in the slow exchange of the *pro*-2*S* proton with solvent deuterium, in addition to the rapid exchange of the *pro*-2*R* proton.[6] Further studies revealed that the enzyme was able to form 3(*R*)-hydroxybutyryl-CoA at a rate that was 4×10^5-fold slower than the normal hydration reaction, but that was 1.6×10^6-fold faster than the uncatalyzed reaction. Thus, the enzyme is capable of epimerization of hydroxybutyryl-CoA. The stereospecificity of ECH was calculated to be 1 in 4×10^5.[6]

Interestingly, *syn*- stereochemical outcomes are usually observed for enzyme catalyzed hydration/dehydration reactions where a hydrogen on a carbon that is adjacent to a carbonyl group of a ketone or a thioester is involved.[24,25] However, non-enzymatic proton addition/abstraction reactions generally proceed via the *anti*-stereochemical pathway, probably due to stereoelectronic and steric reasons since *syn*-reactions involve a high energy eclipsed conformation in the transition state.[26] The fact that ECH and several other enzymes catalyze reactions via apparently unfavorable pathways could indicate that evolution has forced these enzymes to use their catalytic machinery, perhaps sub-optimally, in their current roles.[27]

Mechanistic Options and KIE Studies

Enzyme catalyzed dehydration (elimination) reactions such as those catalyzed by ECH may occur in a stepwise fashion (E1cb), through the intermediacy of an unstable carbanionic species. It has been suggested that in certain cases, a stepwise mechanism without the formation of a discrete carbanion intermediate but with electrophilic catalysis may also be possible.[28] Similarly, concerted elimination reactions (E2) either with or without electrophilic catalysis might also occur. Whether a concerted or stepwise reaction occurs is related to the stability of the carbanion of the E1cb path. Thus, it has been proposed that there is a distinct transition from E2 to an E1cb mechanism as the stability of the carbanion is enhanced.[29] The initial hypothesis regarding the catalytic mechanism of ECH was that it proceeds through a stepwise mechanism. This proposal was supported by the strong affinity (micromolar) of acetoacetyl-CoA to the enzyme.[30] This molecule was shown to exist predominantly as the enol(ate) tautomer, indicating that the enzyme could stabilize negatively charged intermediates. However, the most important evidence regarding the catalytic mechanism of ECH issue was obtained through an elegant series of experiments by Vernon Anderson and coworkers.

The question of whether a concerted or a stepwise mechanism is operational may be addressed by kinetic isotope effect (KIE) experiments. For such experiments to yield valid results, the identity of the rate-limiting step is important. In case of ECH, which is one of the most proficient catalysts known, the rate of the enzyme catalyzed reaction approaches the diffusion controlled limit. Therefore, alternative substrates such as 3-hydroxybutyrylpantetheine and crotonylpantetheine, which lack the adenosine part of the coenzyme A moiety, were used for the KIE experiments to ensure that the chemical steps were completely rate limiting. For the ECH catalyzed dehydration of 3-hydroxybutyrylpantetheine, the primary $^{D}(V/K)$ was found to be 1.60 and the primary $^{18O}(V/K)$ was found to be 1.053.[31] When significant primary isotope effects on V/K are observed for a reaction involving two bond cleavage events, it may indicate that either both bonds may be broken in the same transition state or that two distinct transition states of similar energies, both of which may be partially rate-limiting, may exist. In such a case, double isotope effects can be used to probe whether two different isotopic substitutions affect the same or different chemical steps.[32,33] Thus, for instance, the measurement of secondary (V/K) for the hydration reaction in H_2O and D_2O should provide the distinction between a concerted and a stepwise reaction. It was determined that the ratio of $^{\alpha\text{-}D}(V/K)_{H_2O}$/ $^{\alpha\text{-}D}(V/K)_{D_2O}$ was very close to unity, which requires that there be a single kinetically

significant transition state.[28] This deduction was supported by other isotope effect studies such as Solvent Discrimination Isotope Effect (SDIE) and isotope exchange in D_2O,[18] all of which pointed to the absence of a discrete carbanion in the reaction mechanism of ECH. Thus, it was concluded that the hydration/dehydration reaction was concerted.[28]

Spectroscopic Studies

The negligible solvent isotope effect observed in the KIE studies with ECH indicated that an unactivated water molecule acts as a nucleophile during the hydration reaction. Thus, some activation of the substrate may be required to promote catalysis, since water is not an effective nucleophile. Spectroscopic studies using a series of α,β-unsaturated CoA thioesters that formed non-covalent complexes at the active site of ECH revealed that the enzyme indeed induces a polarization effect to activate the substrate and make it a better electrophile for reaction with unactivated water.[34–36]

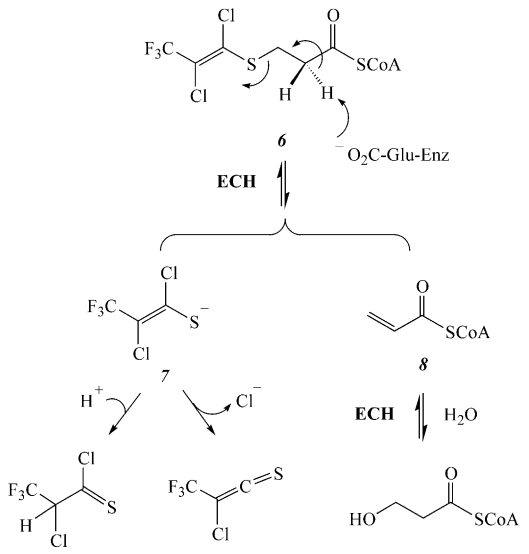
When a series of substituted cinnamoyl-CoA thioesters were incubated with ECH, the UV spectra of these compounds displayed significant red shifts.[34] The red shift was found to increase with the ability of a *para* substituent to donate electrons by resonance. Similarly, the affinity of the substituted cinnamoyl-CoA thioester was enhanced by electron donating substituents, with the slope of the log of the ratio of the inhibition constants versus σ_p^+ being near unity.[34] Affinity was also increased by either *para* or *meta* electron-withdrawing substituents, suggesting that the enzyme stabilizes a partial positive charge at C-3. NMR experiments revealed that the shielding of [3-^{13}C, 3-^{2}H]cinnamoyl-CoA was decreased by $+3.2$ ppm, consistent with an increased positive charge at C-3 upon binding to the enzyme.[34] Raman spectroscopic analysis of cinnamoyl-CoA bound at the active site of ECH similarly showed that significant electronic changes occurred in the ground state of the π electrons in the bound substrate. Overall, the results from the comprehensive spectral analysis showed that a major rearrangement of electrons occurred at the acryloyl portion of the cinnamoyl group upon binding, while only minor changes occurred in the distribution of electrons in the phenyl ring.[34] This indicated that the active site of the enzyme induced a polarization in order to activate the substrate by providing an electrophile near the thioester carbonyl oxygen, either in the form of a postive charge, or in the form of a strong hydrogen bond. The overall result of this polarization was to decrease the electron density at C-3 and induce a partial positive charge at this position, which would promote the attack of water and subsequent protonation at C-2.

Mechanism of Catalysis

ECH catalyzes the hydration of α,β-unsaturated enoyl-CoA thioesters to the corresponding β-hydroxybutyryl-CoA thioesters without the assistance of any cofactors or metal ions. Sequence alignment studies and comparisons with other homologous enzymes provided the first hints about the identity of the catalytic residues in the active site of the enzyme. Site directed mutagenesis revealed that Glu-165 was an important catalytic residue in enoyl-CoA isomerase.[37] Using sequence alignments, the corresponding residue in ECH was found to be Glu-164 (rat liver ECH numbering). In early experiments, mutation of Glu-164 to Gln-164 resulted in a large decrease in the enzyme activity and rendered the mutant enzyme unable to catalyze α-proton exchange of saturated CoA thioesters with the solvent.[10] It was found that k_{cat} for the E164Q enzyme was reduced 10^5-fold relative to wild type ECH.[15] Further studies on the kinetics of the E164Q mutant were performed using 3′-dephosphocrotonyl-CoA (**4**), which had reduced affinity for the enzyme as compared to crotonyl-CoA (**3**). Studies using this substrate analogue revealed that while the value of K_m was unaffected, the value of k_{cat} was reduced by 630,000-fold.[38] In addition, the ability of the E164Q mutant to eliminate 2-mercaptobenzothiazole from 4-(2-benzothiazole)-4-thiabutanoyl-CoA (**5**, BTTB-CoA) was reduced by 690-fold.[38] The importance of Glu-164 was further substantiated by the three-dimensional structure of ECH, which showed this residue to be in a position to catalyze the protonation/deprotonation of the α-carbon of the substrate.

The X-ray crystal structure of ECH together with homology modeling studies based on the X-ray crystal structure of 4-chlorobenzoyl-CoA dehalogenase provided evidence for the existence of a second acidic residue, Glu-144 (rat liver ECH numbering), that could play a role in the catalytic mechanism of ECH.[19–21] Mutation of Glu-144 to Gln-144 did not affect the value of K_m for 3′-dephosphocrotonyl-CoA (**4**), although the value of k_{cat} was reduced by 7700-fold.[38] This indicated that Glu-144 also played a crucial role in the catalytic mechanism of ECH. Since Glu-164 was intact in the E144Q mutant, it would be expected that the E144Q mutant should be competent in performing the functions ascribed to Glu-164, such as the ability to catalyze the exchange of the α-protons of butyryl-CoA with the solvent and the ability to catalyze the elimination of 2-mercaptobenzothiazole from BTTB-CoA (**5**).[38] However, it was revealed that the E144Q mutant was unable to catalyze the Glu-164 mediated α-proton exchange. Similarly, the reactivity of the E144Q mutant was reduced by 180-fold as compared to the wild type enzyme. These results provided an interesting picture of the catalytic mechanism of ECH, wherein the two glutamate residues, Glu-144 and Glu-164, form part of a catalytic diad and act in concert to facilitate the hydration reaction.[38]

X-ray crystallography studies further revealed the presence of a strong hydrogen bond between the backbone amide nitrogen of Gly-141 (rat liver ECH numbering) and the thioester carbonyl of the bound acetoacetyl-CoA.[19–21] This strong hydrogen bond could be responsible for providing the electrophilic pull necessary to polarize the enone π-system in order to reduce the electron density at C-3, as predicted by the spectroscopic



Scheme 2.

analysis described in an earlier section. Interestingly, replacement of Gly-141 with a proline in rat liver ECH resulted in an approximately 10^6-fold decrease in k_{cat} while K_m was relatively unchanged.[39] Furthermore, this substitution completely abolished the ability of the enzyme to polarize the ground state of hexadienoyl-CoA, indicating a direct link between polarization and reactivitiy. On the basis of these data the hydrogen bond from Gly-141 could potentially contribute 34 kJ/mol stabilization to the transition state formed during the catalytic mechanism of ECH.[39]

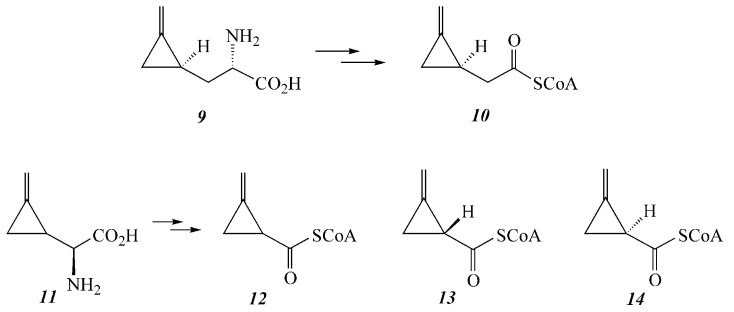
Overall, the thorough mechanistic studies on ECH described in the preceding sections represent an elegant convergence of the techniques of modern enzymology. Based on results from kinetic, isotope effect, spectroscopic, crystallographic, and mutagenesis studies of ECH, the current picture regarding its catalytic mechanism is as follows: a concerted attack by water at C-3 and protonation at C-2 promoted by Glu-144/Glu-164, and the overall process facilitated by polarization of the enone π-system through a strong hydrogen bond between the backbone amide of Gly-141 and the thioester carbonyl group of the substrate.

Inhibition Studies of ECH

Studies with inhibitors have often yielded valuable insights into the mechanistic workings of many enzymes. Any inhibitors of enzymes involved in fatty acid metabolism have added significance due to the crucial physiological role of this pathway in the living cell and to the fact that several disorders of human metabolism have been shown to have their molecular basis in the malfunction of these enzymes. Recently, Thorpe and coworkers described 5,6-dichloro-7,7,7-trifluoro-4-thia-5-heptenoyl-CoA (**6**, DCTFTH-CoA) as an inactivator of rat liver enoyl-CoA hydratase.[40] This compound is an analogue of 4-thia-acyl-CoA thioesters that can undergo a β-elimination reaction to form highly unstable thiolate fragments, which subsequently form electrophilic thioketene or thionoacyl halide species. As depicted in Scheme 2, DCTFTH-CoA (**6**) can be directly activated by ECH with the release of 1,2-dichloro-3,3,3-trifluoro-1-propenethiolate (**7**) and acryloyl-CoA (**8**).[40] Thus, incubation of 0.4 μM rat liver ECH with 10 μM DCTFTH-CoA led to the inactivation of the enzyme with only 0.5% activity remaining after 90 min at pH 9.0. Interestingly, the stoichiometry of inactivation with DCTFTH-CoA was found to be strongly dependent on protein concentration, indicating some form of intersubunit communication, possibly prompted by covalent modification.[40] The inactivation of ECH could be prevented by the addition of CoA thioesters, nucleophiles such as DTT (1 mM), *N*-acetylcysteamine, and GSH, as well as by a variety of other bulky nucleophiles and proteins. Further studies revealed that DCTFTH-CoA-modified ECH could still bind CoA thioester ligands. Interestingly, the modified enzyme retained β-elimination activity towards BTTB-CoA (**5**) and continued to release cytotoxic metabolites upon treatment with additional DCTFTH-CoA, even though the hydratase activity of the enzyme had been reduced to $<2\%$ of native activity.[40] MALDI-TOF analysis of the inactivated enzyme indicated a modest gain in mass of 89 ± 5 Da after inactivation. Peptide mapping subsequent to tryptic digestion of DCTHTF-CoA treated ECH identified the formation of a mixed disulfide between Cys-62 and Cys-111. These results clearly point to an interesting mechanism of inactivation of rat liver ECH, and further studies are currently ongoing to unravel the molecular basis of this phenomenon.

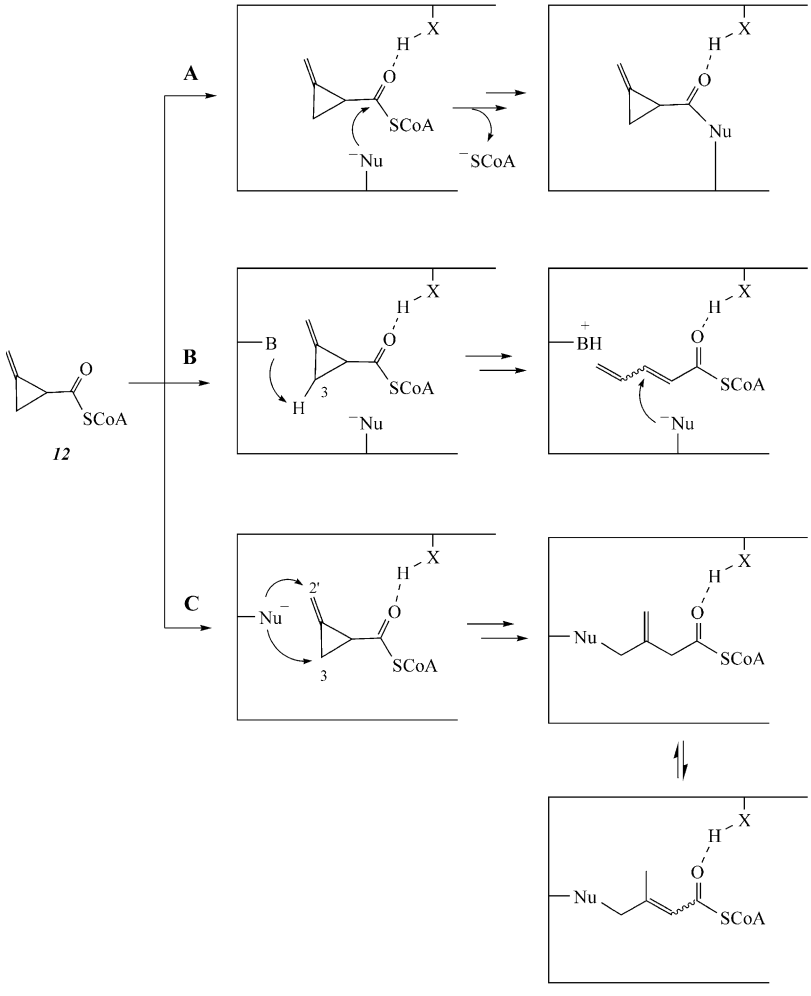
Cyclopropyl natural products with an exomethylene functionality have been shown to have intriguing biological effects on the enzymes of the fatty acid oxidation cycle. For example, hypoglycin (**9**), which is the causative agent of Jamaican Vomitting Sickness,[41–43] was shown to be metabolized in vivo to (methylenecyclopropyl)acetyl-CoA (**10**, MCPA-CoA), and was shown to inhibit several acyl-CoA dehydrogenases such as MCAD and SCAD.[44–49] Detailed studies by our group on the inhibition of MCAD by MCPA-CoA provided valuable insights into the catalytic mechanism of this enzyme. It was shown that MCPA-CoA acted as a mechanism-based inhibitor, undergoing ring scission in the active site of the enzyme and resulting in the covalent modification of FAD.[47–49]

The lower homologue of hypoglycin A, (methylenecyclopropyl)glycine (**11**), was also shown to have hypoglycemic activity in fasting rats. It was postulated that this compound undergoes a metabolic fate analogous to that of hypoglycin A, and was converted in vivo to (methylenecyclopropyl)formyl-CoA (**12**, MCPF-CoA). Early reports on MCPF-CoA indicated that it was an inhibitor of thiolases and ECHs, although its activity against ECHs depended on the source of the enzyme.[50] Spurred by our previous experience with MCPA-CoA, we decided to undertake a detailed investigation into the inhibition of ECHs by MCPF-CoA.



MCPF-CoA As An Inhibitor Of Bovine Liver ECH

MCPF-CoA (**12**) was found to show moderate inhibition against thiolase from pig kidney. It was also found that MCPF-CoA showed different inhibition profiles against ECHs from different organisms. However, contrary to what was reported earlier, MCPF-CoA was found to be a better inhibitor of bovine liver ECH as compared to pig kidney ECH.[51,52] Detailed studies on the inhibition of the bovine liver enzyme revealed that the enzyme lost its activity in a time-dependent manner with a half life of approximately 38 min. The loss of activity was found to be irreversible in nature since the activity could not be restored upon prolonged dialysis. Furthermore, protection experiments using acetoacetyl-CoA revealed that the loss of activity was related to events that occurred in the active site of the enzyme. Detailed kinetic analysis of the inactivation provided values for k_{inact} (13.1×10^{-3} min^{-1}) and K_I (43.7 μM), indicating that although the rate of inactivation was slow, MCPF-CoA bound to the active site of ECH with approximately the same affinity as that of the natural substrates.[52] Although initial studies were performed with racemic MCPF-CoA, the (*R*)- and (*S*)- enantiomers of MCPF-CoA (**13** and **14**) were separately synthesized and used to probe the stereochemical



Scheme 3.

preference of the enzyme.[14] Interestingly, it was found that both enantiomers were equally potent in their inhibition. Thus, the values of k_{inact}, K_I, and partition ratio were found to be 3.36×10^{-3} min^{-1}, 49.2 μM, and 10, for (*R*)-MCPF-CoA (**13**), and 2.65×10^{-3} min^{-1}, 57.1 μM, and 10 for (*S*)-MCPF-CoA (**14**), respectively.

Results with rat liver ECH provided an intriguing contrast to those with bovine liver ECH. The inhibition of the rat liver enzyme occurred at a slower rate, with a half life of 4.28 h.[52] More surprisingly, however, the interaction of the rat liver ECH with MCPF-CoA (**12**) was reversible in nature, since the activity of the enzyme could be recovered after gel permeation chromatography. Kinetic analysis of the inhibition revealed that MCPF-CoA bound to the rat liver enzyme competitively with a K_i of 30 μM which is comparable to the affinity of the substrate for the enzyme.[52] Given the high degree of sequence homology (~90% sequence identity) between ECHs from rat liver and bovine liver, especially in the active site region which is virtually identical, the divergent inhibition profiles of MCPF-CoA with these enzymes are unexpected, although it is certainly possible that subtle changes in the architecture of the active site dictate the nature of the respective interactions. However, it was clear that MCPF-CoA was a potent irreversible inhibitor of bovine liver ECH, and further studies on this aspect were performed to address the mechanism of inactivation.

Molecular Basis Of Inactivation Of Bovine Liver ECH

Based on the inherent chemical logic of the functional groups in MCPF-CoA (**12**), three mechanisms were proposed to account for the inactivation of bovine liver ECH (Scheme 3).[52,14] Of these, the first mechanism is a transesterification mechanism involving the attack of a nucleophilic residue on the thioester carbonyl, formation of a presumed tetrahedral adduct and its subsequent collapse resulting in the liberation of coenzyme A. The second mechanism involves a deprotonation at C-3 with subsequent ring-opening and attack by an nucleophilic residue. The third mechanism involves a direct attack of a nucleophile on the strained methylenecyclopropyl unit itself, resulting in ring fragmentation and the covalent modification of the enzyme. Such a mechanism could proceed via two routes, both of which would result in a similar enzyme–inhibitor adduct although the fates of the individual carbons of MCPF-CoA would be different. The attack of the nucleophile could occur either at C-3, in which case the double bond between C-2 and C-2′ would be retained, or the attack of the nucleophile could occur at the C-2′ position of the exomethylene double bond, in which case the double bond would move between C-2 and C-3, resulting in the conversion of C-3 from an sp^3 carbon to an sp^2 (alkene) carbon.

The transesterification mechanism for inactivation of bovine liver ECH seemed unlikely for a variety of reasons. For example, there was no rationale for this mechanism to operate with MCPF-CoA when it does not occur with other thioester based substrates and/or inhibitors. In addition, prolonged dialysis of the inactivated enzyme did not result in a loss of the adenine chromophore of coenzyme A, which would be expected if free coenzyme A were to be formed in the active site as predicted by this mechanism.[52] More convincing evidence against this mechanism was obtained by ESI-MS experiments. Thus, while the mass of the native enzyme was found to be 28,616 Da, the mass of the enzyme-inhibitor adduct was found to be 29,465 Da, which was close to the predicted mass of one molecule of ECH reacting with one molecule of MCPF-CoA (molecular mass 849 Da).[53]

Studies using synthetic [3-^{3}H]MCPF-CoA also indicated a 1:1 stoichiometry of inactivation of MCPF-CoA. Furthermore, studies with this analogue failed to show washing out of the radioisotope when incubated with ECH.[52] If an initial deprotonation occurred at C-3, the radioisotope would be taken up by the base, from whence it could be expected to exchange with the solvent. However, the lack of tritium washout from MCPF-CoA argued against the proposal involving a deprotonation at C-3 as part of the inactivation mechanism.[52]

In order to determine if the remaining alternative, namely nucleophile-assisted ring opening was operational, [3-^{13}C]MCPF-CoA was synthesized and incubated with bovine liver ECH.[14] The inactivated enzyme was subjected to gHMQC experiments, which revealed that the signal at 14.4 ppm representing the C-3 carbon had disappeared, and had been replaced by a cross-peak at 105 ppm in the ^{13}C dimension and 4.8 and 5.0 ppm in the ^{1}H dimension. This strongly suggested that the sp^3 carbon at C-3 had been probably transformed into a alkene (sp^2) carbon, indicating that the putative mechanism of inactivation was a nucleophilic attack at C-2′, leading to ring fragmentation and covalent modification of the enzyme.[14]

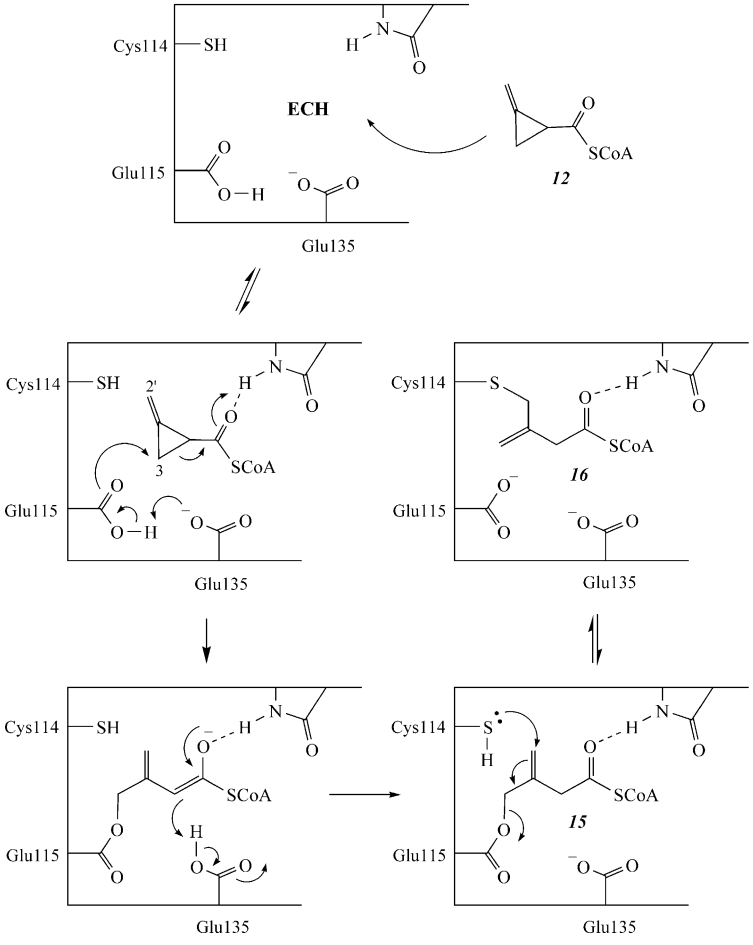
Identity Of The Reactive Nucleophile

Peptide-mapping is a powerful tool in unraveling the nature and identities of residues participating in covalent adduct formation. In order to determine which residue of bovine liver ECH was responsible for nucleophilic attack, [3-^{3}H]MCPF-CoA was incubated with bovine liver ECH. The inactivated enzyme was processed with trypsin and two radiolabeled tryptic fragments were isolated by reverse-phase HPLC.[14] The N-terminal sequence of both peptides were identical (Y-A-L-G-G-G-**X**-E-L), indicating that they were probably the result of incomplete digestion. The radioisotope was predominantly associated with residue 'X', whose identity could not be determined, presumably due to its covalent modification with MCPF-CoA. A sequence comparison of ECHs from difference sources indicated that the peptide sequence was part of the active site, and included Glu-115 (bovine liver ECH numbering, equivalent to Glu-144 in rat liver ECH), which was adjacent to the modified residue. Since the sequence of

bovine liver ECH was not known, the identity of residue 'X' was tentatively assigned as Cys based on the sequences of other homologous ECHs.[14] In an effort to conclusively identify the reactive nucleophile, a bovine liver cDNA library was constructed and used to isolate the coding sequence for the enzyme. Cloning and sequencing of the cDNA insert indeed confirmed that residue 'X' was Cys-114 (bovine liver ECH numbering; the corresponding sequence number for rat liver ECH, including the signal sequence, is Cys-143).[14] Therefore, with the deduction of the mechanism of inactivation using isotopically labeled MCPF-CoA analogues and the tentative identification of the reactive nucleophile, the mechanistic picture regarding the inactivation of bovine liver ECH seemed complete.

The proposals regarding the mechanism of inactivation of bovine liver ECH were, however, thrown into doubt following the construction and purification of the C114A mutant of bovine liver ECH.[53] The mutant was initially constructed to determine if Cys-114 played a role in the catalytic mechanism of bovine liver ECH. However, the kinetic properties of the C114A mutant were found to be similar to that of the wild type enzyme. Sequence analysis of ECHs from different sources indicated that Cys114, although present in a large number of sequences, is not absolutely conserved (Fig. 2). Early thiol-modification experiments had also indicated that while a cysteine residue was present close to the active site, it was probably not critical for catalysis. The kinetic properties of the C114A mutant provided experimental validation that Cys-114 did not participate in the reaction mechanism of bovine liver ECH.[53] More surprisingly, however, the C114A showed the same susceptibility towards inactivation by MCPF-CoA (**12**) as that of the wild type enzyme. The inhibition of the C114A mutant was irreversible, with a half-life of 40.8 min, and was active-site directed as indicated by protection experiments using acetoacetyl-CoA. Further, ESI-MS analysis showed that the molecular mass of the labeled Cys114A mutant was 29430 Da, close to the predicted mass (28,584 + 849 Da).[53] These results were unexpected and contradicted the earlier conclusion that Cys-114 reacted with MCPF-CoA. It appeared that the reaction with Cys-114 was an artifact and indicated that the participation of other nucleophilic residue(s) of ECH needed to be investigated.

The nucleophilic residues in closest proximity to the active site of bovine liver ECH were expected to be the two catalytic glutamates (Glu-115 and Glu-135) themselves. Construction and purification of the E115Q and E135Q mutants revealed that they showed trace levels of activity (10^4–10^5-fold lower than wild type ECH),



Scheme 4.

consistent with published results from studies on rat liver ECH.[38,53] However, based on ESI-MS analysis, these mutants were unable to form a covalent complex with MCPF-CoA. This indicated that Glu-115 and/or Glu-135 played a critical role in the reaction of bovine liver ECH with MCPF-CoA. The inactivation of wild type ECH and the C114A mutant was re-examined by using MS/MS as a peptide-mapping tool. Pepsin-derived fragments were separated by LC–MS and the labeled peptides were located using the neutral-loss technique. Once the labeled peptides had been identified, the location of the label was determined using MS/MS sequencing. It was revealed that Glu-115 was labeled in both wild type as well as the C114A mutant of bovine liver ECH.[53]

The hypothesis regarding the inactivation of bovine liver ECH by MCPF-CoA (**12**) that emerged required the presence of both Glu-115 and Glu-135 for the reaction to occur (Scheme 4). The role of Glu-115 was to carry out a nucleophilic attack at C-3, presumably promoted by the Gly-112 induced polarization of the enone π-system. The role of Glu-135 may be to modulate the reactivity of Glu-115 and/or donate a proton to C-2. While Cys-114 did not play a direct role in the inactivation, it was possible that the basic conditions used during trypsin digestion may have promoted the attack of this residue on the covalent Glu-115-MCPF-CoA adduct (**15**), resulting in the formation of an adduct **16** with Cys-114 and the liberation of Glu-115.[53] Thus, the reaction with Cys-114 was explained as a 'post-inactivation' artifact. It was concluded that since the reaction of MCPF-CoA with bovine liver ECH showed the characteristics of normal catalysis, requiring the presence and collaboration of Glu-115 and Glu-135, MCPF-CoA displayed characteristics of a mechanism-based inhibitor of bovine liver ECH.

Conclusion

ECH represents a unique and interesting case study since virtually every existing technique in the enzymologist's toolbox has been used in its investigation. The convergence of a wide variety of techniques and strategies has provided a thorough picture of the innermost workings of this proficient enzyme. The importance of these studies lies in the fact that ECH is part of a physiologically important pathway, disorders in which are related to several pathological conditions in humans. In a broader sense, the vast body of evidence accumulated on ECH should be valuable in the study of related enzymes from other pathways. For example, the prokaryotic fatty acid biosynthetic pathway, which is also a target of significant therapeutic interest,[54,55] can be imagined as the β-oxidation spiral acting in reverse, since it utilizes four discrete enzymes each of which performs functions that are analogous to their counterparts in the degradative pathway. It would be to our benefit to take advantage of Nature's elegant symmetry by applying the lessons learnt from ECH towards investigations on the relatively less known β-hydroxyacyl-ACP dehydratase (FabZ). Most notable is that interesting stories about ECH and other members of the hydratase/isomerase family are bound to arise, as the search for agents to alleviate human disease and suffering associated with these enzymes continues.

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Gautam Agnihotri was born in New Delhi, India, in 1970. After finishing high school, he spent two years at the National Defence Academy, Khadakvasla. Thereafter, he graduated with his Bachelor of Science degree from Rani Durgavati University, Jabalpur, in 1992. After obtaining his Master of Science degree in Organic Chemistry from the Indian Institute of Technology (Bombay) in 1994, he joined the graduate program at the Department of Chemistry, University of Minnesota, Minneapolis. A desire to work at the interface of chemistry and biology prompted him to join the research group of Professor Hung-wen Liu, under whose guidance he obtained his Ph.D. degree in Biological Chemistry in 2000. After graduating, he worked as a Postdoctoral Scientist at GlaxoSmithKline, Collegeville, PA untill April 2002. He is currently working as a Senior Scientist, Assay Development at NeoGenesis, Inc. in Cambridge, MA. His research focus includes the application of enzymology and biochemistry to support pharmaceutical lead discovery and screening. His hobbies include reading and traveling.

Hung-wen (Ben) Liu was born in Taipei, Taiwan, in 1952. He graduated with a Bachelor of Science degree in Chemistry from Tunghai University, Taichung, in 1974. After two years of military service, he began his graduate study at Columbia University where he carried out research under Professor Koji Nakanishi. His work on the additivity relation in exciton-split circular dichroism curves and its application to the structural studies of oligosaccharides earned him a PhD in 1981. He then joined the laboratory of Professor Christopher Walsh at Massachusetts Institute of Technology as a postdoctoral associate where he was introduced to the field of mechanistic enzymology. In 1984, he joined the faculty of Chemistry at the University of Minnesota, Minneapolis, where he was promoted to the rank of Full Professor in 1994, and to Distinguished McKnight University Professor in 1999. In 2000, he moved to the University of Texas, Austin, where he is now the George H. Hitchings Regents Chair in Drug Design and Professor of Medicinal Chemistry, Chemistry, and Biochemistry. His research lies at the crossroads of organic and biological chemistry, with particular emphasis on enzymatic reaction mechanisms, natural product biosynthesis, metabolic pathway engineering, inhibitor design and synthesis, and protein function regulation. The goal of his work is to establish a more thorough understanding of the mechanisms of various biological processes and to rationally design methods to control or mimic their actions. In his spare time, he enjoys spending time with his family, reading, traveling, photography, music, and playing with model train sets.