The Crotonase Superfamily: Divergently Related Enzymes That Catalyze Different Reactions Involving Acyl Coenzyme A Thioesters

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ABSTRACT

Synergistic investigations of the reactions catalyzed by several members of an enzyme superfamily provide a more complete understanding of the relationships between structure and function than is possible from focused studies of a single enzyme alone. The crotonase (or enoyl-CoA hydratase) superfamily is such an example whereby members catalyze a wide range of metabolic reactions but share a common structural solution to a mechanistic problem. Some enzymes in the superfamily have been shown to display dehalogenase, hydratase, and isomerase activities. Others have been implicated in carbon−carbon bond formation and cleavage as well as the hydrolysis of thioesters. While seemingly unrelated mechanistically, the common theme in this superfamily is the need to stabilize an enolate anion intermediate derived from an acyl-CoA substrate. This apparently is accomplished by two structurally conserved peptidic NH groups that provide hydrogen bonds to the carbonyl moieties of the acyl-CoA substrates and form an "oxyanion hole".

Introduction

The study of enzyme superfamilies whose members catalyze different overall reactions allows a more complete understanding of the relationships between structure and function than is possible from studies of individual proteins.[1,2] Members of such mechanistically diverse superfamilies are thought to be related by divergent evolution from a common progenitor, therefore displaying low levels of sequence identity/similarity but adopting similar three-dimensional architectures. In each protein, a common structural strategy is employed to lower the free energies of chemically similar intermediates. Catalysis of the divergent chemistries is accomplished by both retaining those functional groups that catalyze the common partial reaction and incorporating new groups that direct the intermediate to new products. Indeed, as a specific example, the enolase superfamily has served as a paradigm for the study of catalytically diverse superfamilies.[3] The active sites of proteins in the enolase superfamily are located at the interfaces between two structural motifs: the catalytic groups are positioned in conserved regions at the ends of the β-strands forming $(\alpha/\beta)_8$-barrels, while the specificity determinants are found in flexible loops in the capping domains formed by the N- and C-terminal portions of the polypeptide chains. While the members of the enolase superfamily share similar three-dimensional architectures, they catalyze different overall reactions that share a common partial reaction: abstraction of an α-proton from a carboxylate anion substrate to form an enolic intermediate that is stabilized by interactions with an essential divalent metal ion. By virtue of this stabilization, the intermediates are kinetically competent. Depending upon the overall reaction, however, the enolic intermediates partition to products via 1,1-proton transfer (racemization or epimerization) or β-elimination (dehydration or deamination).

The crotonase superfamily described here displays mechanistic diversity at least equal to, or perhaps even greater than, that of the enolase superfamily. Indeed, the documented reactions catalyzed by members of this superfamily include dehalogenation, hydration/dehydration, decarboxylation, formation/cleavage of carbon−carbon bonds, and hydrolysis of thioesters. Additionally, members of the crotonase superfamily are involved in a wide range of metabolic pathways. The first structure to be solved for a member of this superfamily was 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* sp. strain CBS-3.[4] This investigation was followed by the elegant structural analyses of the rat enoyl-CoA hydratase (commonly referred to as crotonase, hence the name of the superfamily)[5,6] and the rat $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase.[7] Very recently the molecular motif of an additional member of the crotonase superfamily has been determined, namely methylmalonyl-CoA decarboxylase from *Escherichia coli*.[8] This X-ray analysis of methylmalonyl-CoA decarboxylase was especially enlightening in that several of the unexpected changes in three-dimensional structure could not have been predicted on the basis of amino acid sequence alignments alone.

Here we describe the overall three-dimensional structures and active site geometries of 4-chlorobenzoyl-CoA dehalogenase, crotonase, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase,

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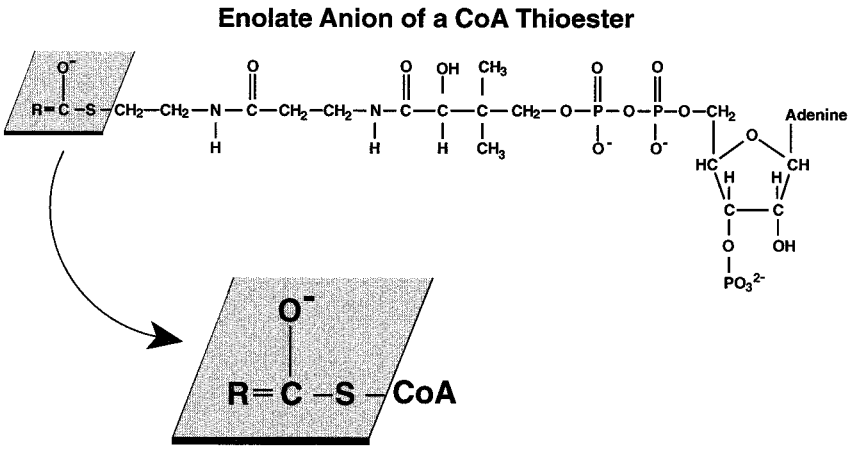

FIGURE 1. Enolate anion of a CoA thioester.

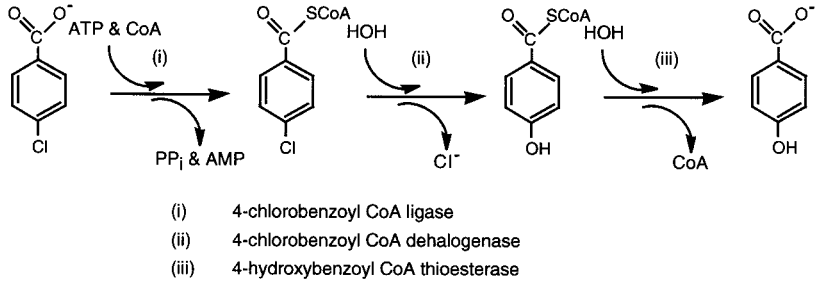

FIGURE 2. 4-Chlorobenzoic acid degrading pathway in *Pseudomonas* sp. strain CBS-3.

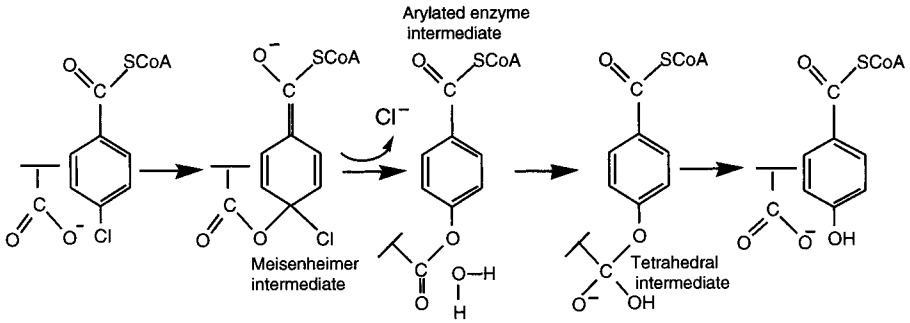

FIGURE 3. Catalytic mechanism for 4-chlorobenzoyl-CoA dehalogenase.

and methylmalonyl-CoA decarboxylase in the context of their known biochemical properties. The common theme behind all of the reactions catalyzed within this superfamily is the stabilization of an enolate anion intermediate of acyl-CoA substrates (Figure 1) by two structurally conserved peptidic NH groups that form an "oxyanion hole". The goal of this Account is to emphasize our current state of knowledge regarding both the similarities and the differences exhibited by members of the crotonase superfamily.

4-Chlorobenzoyl-CoA Dehalogenase from *Pseudomonas* sp. Strain CBS-3

During the latter part of the twentieth century, synthetic chlorinated organic compounds accumulated in the environment as a result of both commercial production and careless waste disposal. The chlorobenzoic acids, for example, now found in the environment often result from the microbial degradation of polychlorinated biphenyls, referred to as PCBs.[9] Some bacteria, such as *Pseudomonas* sp. strain CBS-3,[10] are able to utilize these various chlorinated compounds as their sole source of carbon via the 4-chlorobenzoate degrading pathway illustrated in Figure 2.[11,12] Of particular interest is the second enzyme in the pathway, namely 4-chlorobenzoyl-CoA dehalogenase, hereafter referred to simply as dehalogenase. This enzyme has attracted significant research attention due to its quite specialized mode of catalysis, as indicated in Figure 3.[13−20] According to all presently available biochemical data, the reaction mechanism of the dehalogenase proceeds via attack of the side-chain carboxylate group of Asp 145 on the benzoyl ring of the substrate at position C(4), leading to the formation of a Meisenheimer complex. Displacement of the halide results in an arylated enzyme intermediate, which subsequently is hydrolyzed by an activated water molecule.

An X-ray crystallographic analysis of the dehalogenase from *Pseudomonas* sp. strain CBS-3 was initiated in an

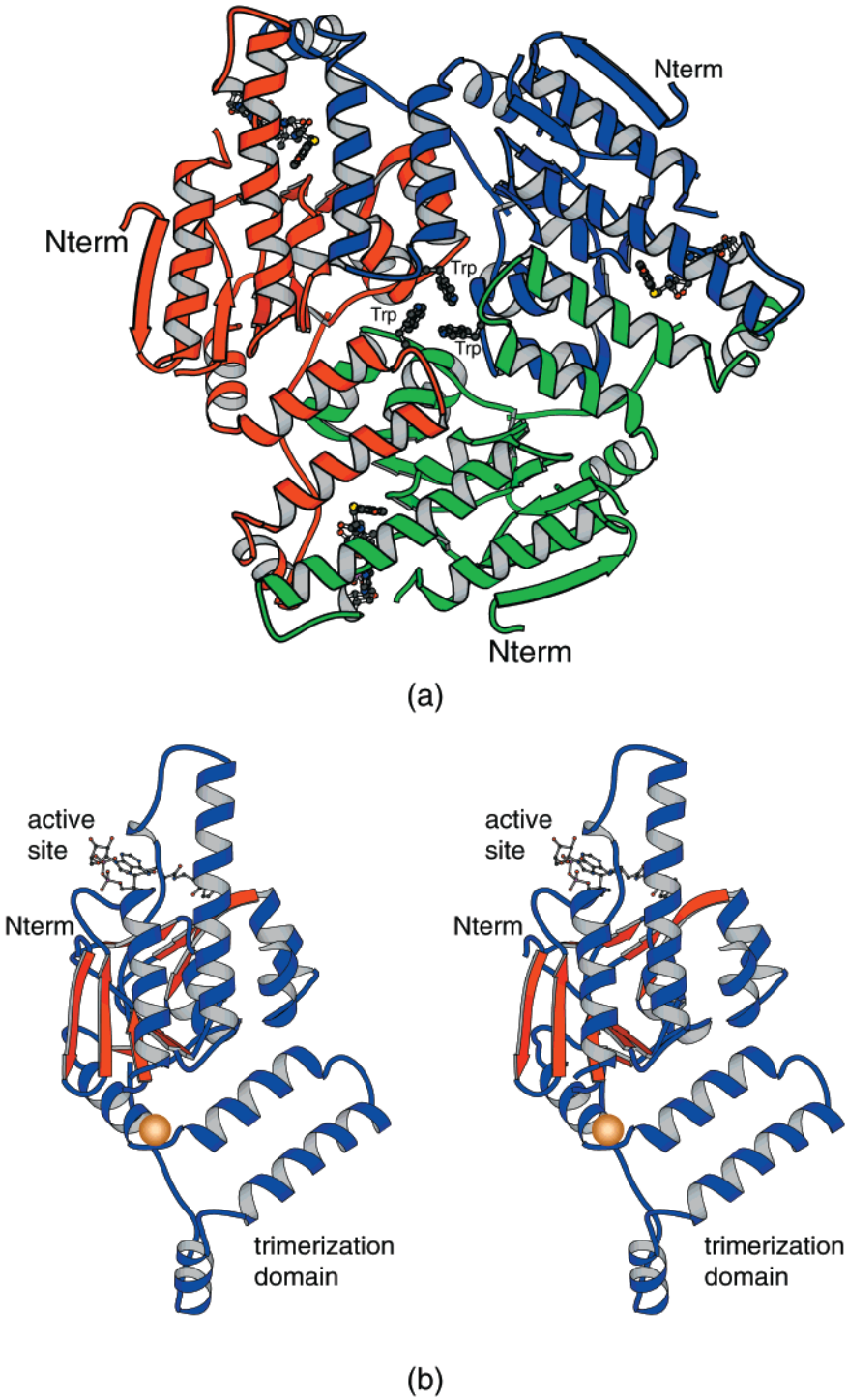

FIGURE 4. Ribbon representation of 4-chlorobenzoyl-CoA dehalogenase. The quaternary structure of the enzyme is trimeric as indicated in (a) with the three subunits color-coded in red, green, and blue. The positions of the active sites in each subunit are indicated by the 4-hydroxybenzoyl-CoA ligands drawn in ball-and-stick representations. Pairs of active sites are separated by approximately 42 Å. The crown of three tryptophan residues that play a role in maintaining the trimeric interface of the dehalogenase is displayed in a ball-and-stick representation. An individual subunit is shown in stereo in (b) with the α-helices and β-strands displayed in blue and red, respectively. As indicated by the orange sphere, a cation (most likely a calcium ion) is positioned between the two α-helices connecting the N-terminal and trimerization domains. X-ray coordinates for 4-chlorobenzoyl-CoA dehalogenase were determined in the Holden laboratory and can be obtained from the Protein Data Bank (1NZY).

effort to address several key structural features concerning its quite unique reaction mechanism. For example, how is the Meisenheimer complex stabilized? What amino acid residues serve to polarize the carbonyl moiety of the thioester, thereby making C(4) of the benzoyl ring more susceptible to nucleophilic attack by Asp 145? What amino acid residue serves to activate a water molecule for its subsequent attack of the arylated enzyme intermediate? What structural features allow for the polarization of the carbonyl moiety of the arylated intermediate to increase

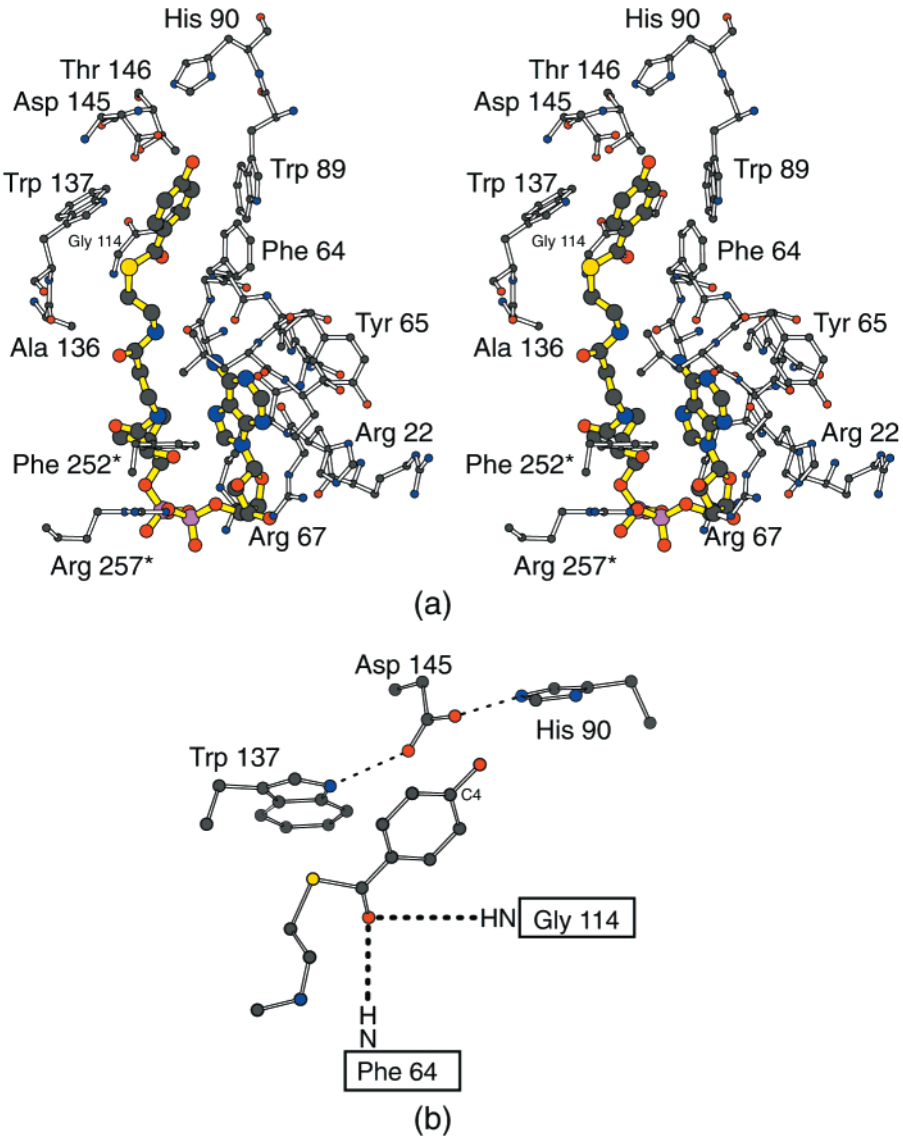

FIGURE 5. Active site for 4-chlorobenzoyl-CoA dehalogenase. Those amino acid residues that lie within approximately 3.8 Å of the 4-hydroxybenzoyl-CoA ligand are shown in stereo in (a). The ligand is highlighted in yellow bonds. Amino acid residues marked with an asterisk belong to a neighboring subunit in the trimer. The dispositions of the "key" players in the dehalogenase reaction mechanism are depicted in (b). Dashed lines indicate hydrogen-bonding interactions. Gly 114 is located at the N-terminus of an α-helix.

its susceptibility to nucleophilic attack by the activated water molecule?

The first model for the dehalogenase from *Pseudomonas* sp. strain CBS was determined to 1.8 Å resolution. From this study it was obvious that the quaternary structure of the enzyme was trimeric, with each subunit containing 269 amino acid residues.[4,11] Shown in Figure 4a is a ribbon representation of the complete trimer.[4] As can be seen, the enzyme has the shape of a propeller with overall dimensions of ~45 Å × 86 Å × 79 Å. The subunit−subunit interactions within the trimer are extensive such that the surface area buried upon trimer formation is ~4600 Å^2, as calculated according to the method of Lee and Richards[21] with a probe sphere of 1.4 Å. The structure of the dehalogenase was solved in the presence of its product, 4-hydroxybenzoyl-CoA, and the positions of these ligands are indicated by the ball-and-stick representations in Figure 4a. Strikingly, the three dehalogenase active sites of the trimer are not solely contained within individual subunits but rather are positioned at the subunit−subunit interfaces. Three tryptophans, Trp 221 from each subunit, form a crown at the top of the trimer, as indicated in Figure 4a. These tryptophans participate in both stacking and hydrogen-bonding interactions.

A ribbon representation of a single dehalogenase subunit is shown in Figure 4b. As can be seen, the subunit folds into two separate and quite distinct domains: the N-terminal motif formed by Met 1−Ala 205 and Val 263−Val 269, and the C-terminal region delineated by Pro 206−Gln 262. The N-terminal domain is dominated by 10 strands of β-sheet that form two layers of mixed sheet lying nearly at right angles to one another. The first layer of β-sheet contains six strands with the N-terminal strand running antiparallel and the remaining lying parallel. Likewise, the smaller β-sheet is a mixture of three parallel β-strands and one antiparallel β-strand. These two layers

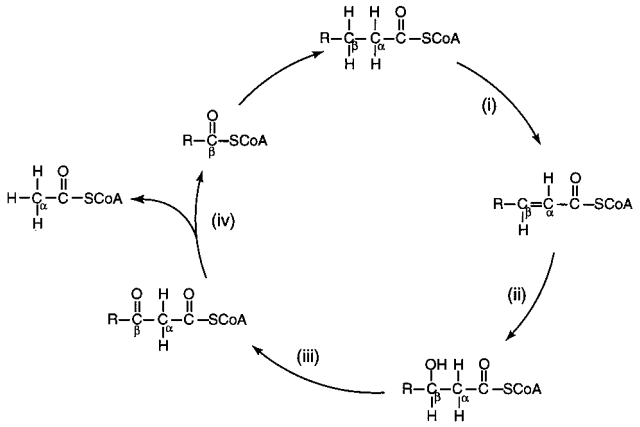

FIGURE 6. β-Oxidation cycle for long-chain saturated fatty acids.

of β-sheet are surrounded by a total of eight α-helices. Much simpler in terms of tertiary structure, the C-terminal domain of the dehalogenase subunit is composed of three amphiphilic α-helices. In that the C-terminal domain is involved extensively in subunit−subunit contacts, it has also been referred to as the trimerization domain. As indicated by the orange sphere in Figure 4b, the connecting α-helices between the catalytic and trimerization domains, defined by Phe 191−Ala 204 and Thr 207−Ala 219, are stabilized by the presence of a cation, most likely a calcium ion.

A close-up view of the dehalogenase active site with bound 4-hydroxybenzoyl-CoA is given in Figure 5a. While the 4-hydroxybenzoyl and adenosine portions of the ligand are buried, the pantothenate unit and the pyrophosphate group are more solvent exposed. Three residues, Phe 64, Trp 89, and Trp 137, form a decidedly aromatic and hydrophobic ring around the 4-hydroxybenzoyl moiety of the ligand. The thioester carbonyl of the product molecule lies within hydrogen-bonding distance to the peptidic NH groups of Phe 64 and Gly 114. Additionally, Gly 114 is located at the N-terminal end of an α-helix formed by Gly 114 to Ala 121. It has been speculated that these hydrogen bonds and the positive end of the helix dipole moment serve both to polarize the carbonyl group of the thioester (thereby activating the benzoyl ring C(4) toward nucleophilic attack by Asp 145) and to stabilize the resulting Meisenheimer complex.[4]

A summary of the "key" players in the reaction mechanism of the dehalogenase is depicted in Figure 5b. Accordingly, the carboxylate group of Asp 145 serves as the nucleophile, which initiates the reaction mechanism by attacking the benzoyl ring C(4), thereby leading to the formation of the Meisenheimer complex. This intermediate is most likely stabilized by both hydrogen bonds donated by the backbone peptidic NH groups of Phe 64 and Gly 114 and the positive end of a helix dipole moment. On the basis of the X-ray model for the dehalogenase, most likely His 90 serves as the base responsible for activating a water molecule that attacks the arylated enzyme intermediate. Additionally, it is believed that $N^{\epsilon 1}$ of Trp 137 serves to position the carbonyl carbon of the arylated intermediate for nucleophilic attack by the water molecule and to stabilize the resulting oxyanion.

Enoyl-CoA Hydratase (Crotonase) from Rat Liver Mitochondria

Enoyl-CoA hydratase, also referred to as crotonase, plays a key role in fatty acid metabolism by catalyzing the

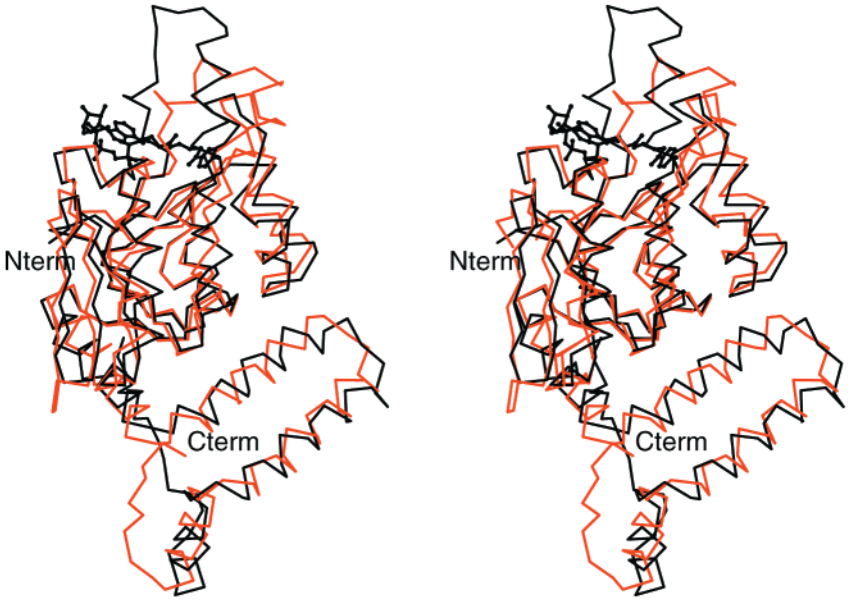

FIGURE 7. Superposition of the α-carbon traces for the dehalogenase (in black) and the crotonase (in red). For the sake of clarity, only the 4-hydroxybenzoyl-CoA ligand bound to the dehalogenase is shown in a black stick representation. X-ray coordinates for the crotonase were obtained from the Protein Data Bank (1DUB).

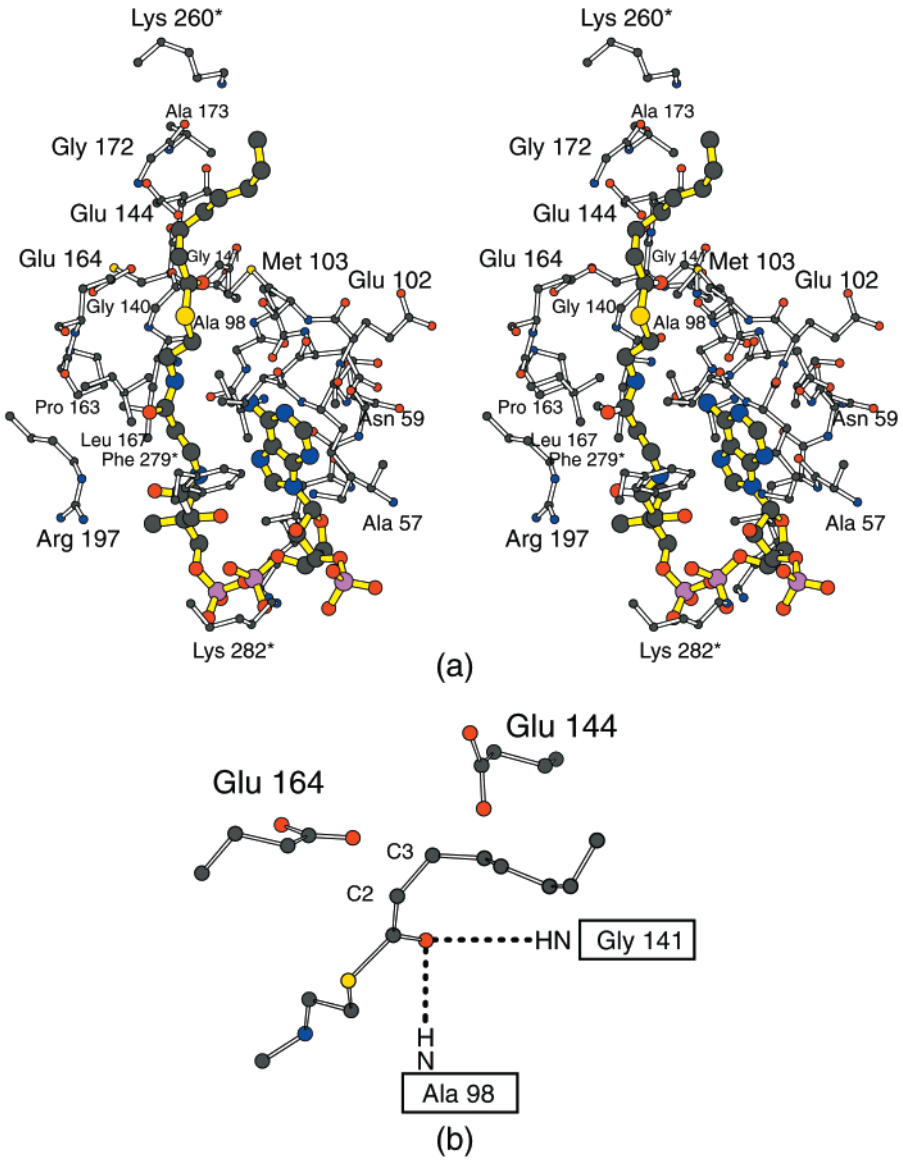

FIGURE 8. Active site for the crotonase. Those amino acid residues that lie within approximately 3.8 Å of octanoyl-CoA are shown in stereo in (a). As in Figure 5, those amino acid residues marked by an asterisk are contributed by another subunit within the hexamer. Octanoyl-CoA is highlighted in yellow bonds. The dispositions of the "key" players in the crotonase reaction mechanism are indicated in (b). X-ray coordinates for the crotonase were obtained from the Protein Data Bank (2DUB). Hydrogen bonds are indicated by the dashed lines.

reversible addition of water to α,β-unsaturated enoyl-CoA thioesters.[22] A schematic of the steps involved in the β-oxidation of long-chain fatty acids in mitochondria is presented in Figure 6. Four enzymes, acyl-CoA dehydrogenase, crotonase, L-β-hydroxyacyl-CoA dehydrogenase, and β-ketoacyl-CoA thiolase, are required to reduce the long-chain fatty acid by two carbon units upon each turn of the cycle. As indicated, the specific biochemical role of crotonase is to convert 2-*trans*-enoyl-CoA into (*S*)-β-hydroxyacyl-CoA.[23] The enzyme is extremely efficient, with the value of k_{cat}/K_m for the C_4 unsaturated fatty acid substrate essentially diffusion-controlled.[24]

From the structural work conducted in the laboratory of Dr. Rik K. Wierenga, the three-dimensional structures of rat liver mitochondrial crotonase complexed with either acetoacetyl-CoA or octanoyl-CoA are now known to 2.5 and 2.4 Å resolution, respectively.[5,6] The quaternary structure of crotonase has been described as a dimer of trimers with each subunit containing 261 amino acid residues.[5,25] In the initial description of the structure, Dr. Wierenga aptly described the N-terminal domain of the subunit as a right-handed spiral of four turns with each turn composed of two β-strands and an α-helix. A superposition of the crotonase subunit with bound acetoacetyl-CoA onto the dehalogenase subunit complexed with 4-hydroxybenzoyl-CoA is displayed in Figure 7, and, as can be seen, the similarity between these two enzymes is especially striking. The α-carbon traces for these two proteins superimpose with a root-mean-square deviation of 1.4 Å for 188 structurally equivalent atoms.

A close-up view of the crotonase active site, with bound octanoyl-CoA, is displayed in Figure 8a. Similar to that observed in the dehalogenase structure, the octanoyl-CoA adopts a curved conformation with the pantothenate and pyrophosphate groups exposed to the solvent. The active site is primarily contained within one subunit, but some

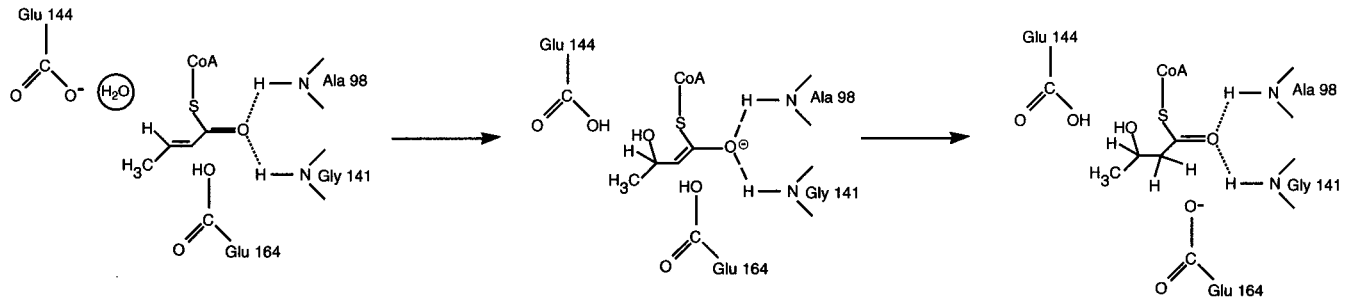

FIGURE 9. Possible reaction mechanism for crotonase based on its three-dimensional X-ray structure. The reaction is written in the direction of hydration.

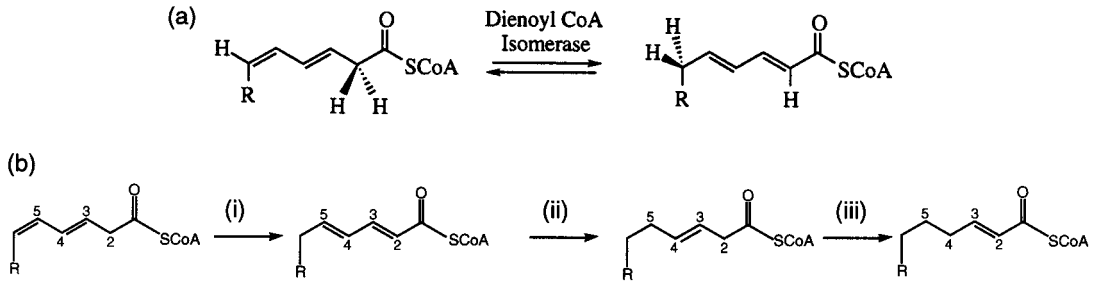

FIGURE 10. Reaction catalyzed by $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase. The specific reaction and stereochemistry is given in (a), while the metabolic pathway in which the isomerase participates is depicted in (b).

amino acid residues that line the binding pocket, such as Lys 260, Phe 279, and Lys 282, are contributed by a neighboring subunit in the hexamer. Again, as in the dehalogenase, the carbonyl group of octanoyl-CoA lies within hydrogen-bonding distance to the structurally conserved backbone amide groups of Ala 98 and Gly 141. Additionally, Gly 141 is positioned at the N-terminal end of an α-helix.

On the basis of the three-dimensional structure of crotonase, a catalytic mechanism has been proposed with the "key" players being Glu 144 and Glu 164, as indicated in Figure 8b.[5] Accordingly, in the physiologically relevant hydration reaction, Glu 144 activates a water molecule for nucleophilic attack at C(3), and Glu 164 protonates C(2) of the unsaturated fatty acid. This mechanism is summarized in Figure 9. Although the dehalogenase and crotonase catalytic mechanisms are seemingly quite distinct, the common underlying theme between them is the need to stabilize an enolate anion intermediate (or possibly a transition state with substantial anionic character).

$\Delta^{3,5},\Delta^{2,4}$-Dienoyl-CoA Isomerase from Rat

$\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase catalyzes a 1,5-proton transfer as outlined in Figure 10a. The enzyme plays a key role in the metabolism of certain fatty acids, namely the *cis*-unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms. As indicated in Figure 10b, three enzymes are required for this auxiliary fatty acid metabolic pathway: $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase, 2,4-dienoyl-CoA reductase, and Δ^{3},Δ^{2}-enoyl-CoA isomerase.[26] The end product, 2-enoyl-CoA, enters the β-oxidation pathway for subsequent degradation.

The structure of the unliganded form of rat $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase was recently solved to 1.5 Å resolution.[7] Each subunit of the hexameric protein contains 327 amino acid residues. A superposition of the isomerase onto the dehalogenase is displayed in Figure 11a. These two proteins superimpose with a root-mean-square deviation of 1.4 Å for 199 structurally equivalent α-carbon atoms. While the structure of isomerase was solved in the absence of bound ligands, it was possible to model into the active site 3-*trans*,5-*cis*-octadienoyl-CoA on the basis of its similarity to both 4-chlorobenzoyl-CoA dehalogenase and crotonase.[7] This modeling study indicated that the NH peptidic groups of the structurally conserved Ile 117 and Gly 173 are most likely involved in hydrogen-bonding interactions with the thioester oxygen of the 3-*trans*,5-*cis*-octadienoyl-CoA substrate and that Glu 196 and Asp 204 play key roles in the catalytic mechanism. Again, as is a hallmark for this family of enzymes, Gly 173 is located at the N-terminus of an α-helix, suggesting that the helix dipole moment may play a role in stabilizing the enolate anion intermediate during the reaction mechanism. Note that Glu 196 is the structural equivalent to Glu

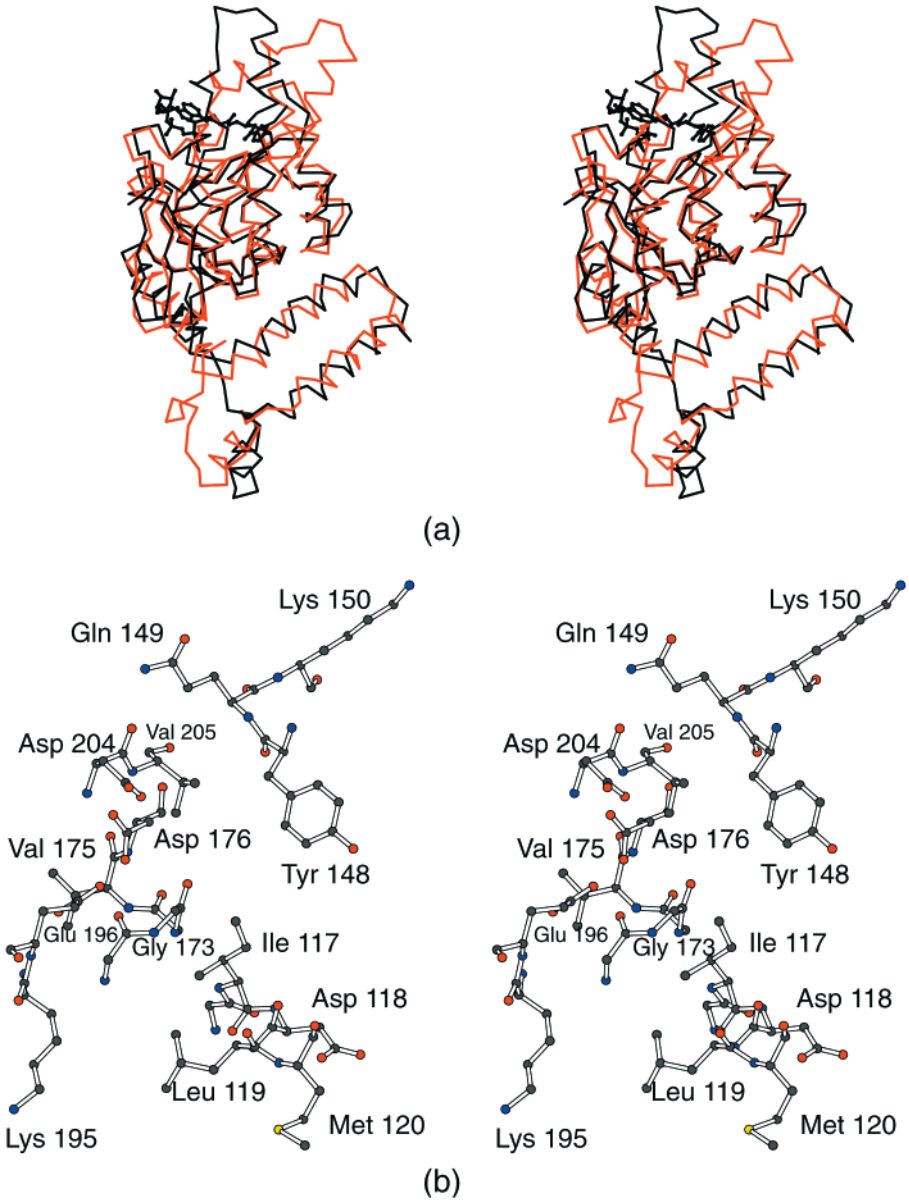

FIGURE 11. Superposition of the α-carbon traces for the dehalogenase (in black) and the $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase (in red) (a). A close-up view of the active site for $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase is shown in stereo in (b). X-ray coordinates were obtained from the Protein Data Bank (1DCI). The enzyme structure was solved in the absence of bound ligands.

164 in the crotonase active site (Figure 8b), which is consistent with proton transfers to/from C(2). Additionally, Asp 204 is the structural equivalent to Asp 145 in the dehalogenase (Figure 5b) and most likely is involved in proton transfer to/from C(6). Strikingly, C(6) of dienoyl-CoA and C(4) of 4-hydroxybenzoyl-CoA are approximately equidistant from the oxyanion hole. Finally, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase contains Asp 176, which is the structural homologue of Glu 144 in crotonase (Figure 8b). The distribution of these residues in the active site of $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase is shown in Figure 11b.

On the basis of the X-ray crystallographic model, a reaction mechanism was proposed for $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase. Accordingly, Glu 196 abstracts a proton from C(2) of the substrate. This proton abstraction leads to a rearrangement of the double bonds, thereby causing Asp 204 to donate its proton to C(6) of the substrate. The role of Asp 176 may be to increase, via a hydrogen bond, the pK_a of Glu 196 to allow it to function as the ultimate catalytic base.[27]

Methylmalonyl-CoA Decarboxylase from *E. coli*

Methylmalonyl-CoA decarboxylase from *E. coli* is a novel, biotin-independent decarboxylase that converts methylmalonyl-CoA to propionyl-CoA.[27] The gene for this enzyme is encoded in an operon that also includes methylmalonyl-CoA mutase and a propionyl-CoA:succinate-CoA transferase, thereby providing a previously unknown pathway for the decarboxylation of succinate in *E. coli*.[27] Prior to an X-ray analysis, it was shown, on the basis of amino acid sequence alignments, that the decarboxylase belonged to the crotonase superfamily, although the configuration of the functional groups within the active

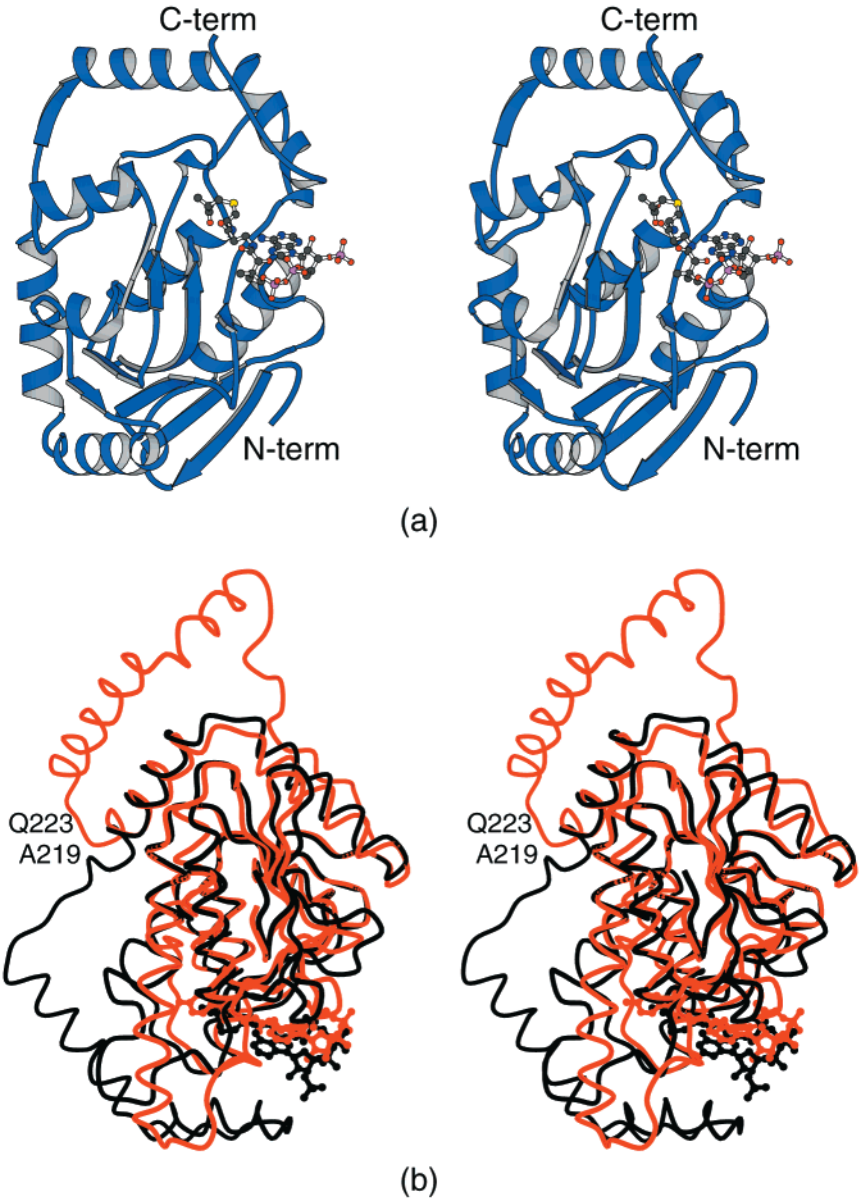

FIGURE 12. Ribbon representation of methylmalonyl-CoA decarboxylase, displayed in stereo (a). The 2(*S*)-carboxypropyl-CoA inhibitor is depicted in a ball-and-stick representation. A superposition of the α-carbon traces for the dehalogenase (in black) and methylmalonyl-CoA decarboxylase (in red) is shown in stereo in (b). X-ray coordinates for methylmalonyl-CoA decarboxylase were determined in the Holden laboratory and can be obtained from the Protein Data Bank (1EF8, 1EF9).

site appeared to be unique.[8] An X-ray crystallographic investigation to 1.85 Å resolution of the apo-enzyme revealed the quaternary structure of the enzyme to be hexameric, like those of the crotonase and the isomerase. A ribbon representation of one subunit of the decarboxylase is displayed in Figure 12a. In sharp contrast to other members of the crotonase superfamily, the decarboxylase subunit is roughly spherical with molecular dimensions of 52 Å × 52 Å × 55 Å. Overall, there are 10 β-strands contained within the subunit, the first nine of which form two layers of mixed β-sheet.

A superposition of the decarboxylase model onto the dehalogenase structure is depicted in Figure 12b. On the basis of amino acid sequence alignments, it was expected that these two proteins would have similar overall topological arrangements. Indeed, the root-mean-square deviation between α-carbons for these two proteins is approximately 1.4 Å for 178 structurally equivalent atoms, up to Val 215 of the decarboxylase. After this residue, however, the two polypeptide chains diverge significantly, primarily as a result of a change in the dihedral angles for Ala 219 in the decarboxylase structure and for the equivalent residue, Gln 223, in the dehalogenase. In both enzymes, the C-terminal residues adopt α-helical conformations, but the dispositions of these α-helices with respect to the N-terminal domains of the individual molecules are completely different. This type of molecular rearrangement was not obvious from primary structural alignments and yet has profound consequences with respect to the active site pockets. Indeed, the decarboxylase is the first member of the crotonase superfamily to be identified whereby the active site is contained solely

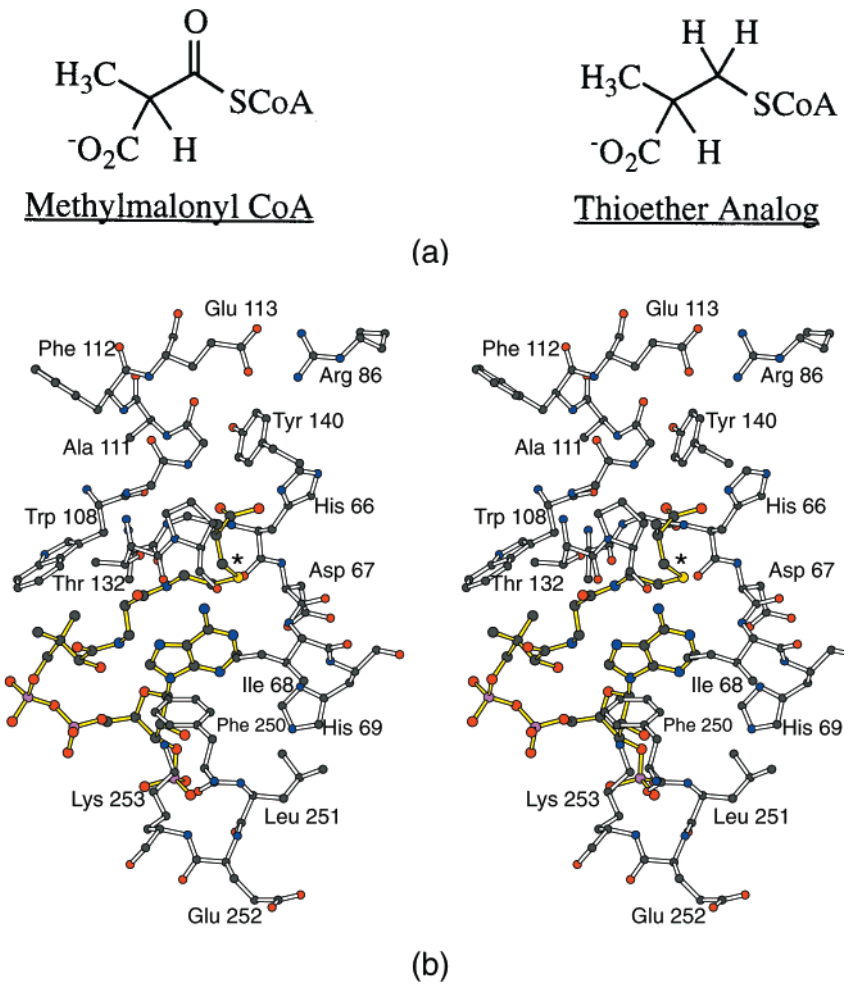

FIGURE 13. Inhibitor employed in the X-ray analysis of methylmalonyl-CoA decarboxylase (a) along with the natural substrate for the enzyme. A close-up view of the active site for the enzyme is shown in (b). Those residues that lie within approximately 3.8 Å of the inhibitor are displayed. Unlike that observed for the other members of the crotonase superfamily, in methylmalonyl-CoA decarboxylase the active site is formed by only one subunit. The inhibitor is highlighted in yellow bonds. The position of the methylene bridging group characterizing the inhibitor is indicated by the asterisk.

within one subunit of the hexamer (or trimer as in the case of the dehalogenase).

Following the initial structural analysis of the apo-form of the decarboxylase, crystals of the enzyme were grown in the presence of 2(*S*)-carboxypropyl-CoA, an inert thioether analogue of methymalonyl-CoA shown in Figure 13a. Note that in this inhibitor, the carbonyl moiety of the substrate has been substituted with a methylene group. A close-up view of the binding region for this thioether analogue is given in Figure 13b. As can be seen by comparing Figure 13b with Figures 5a and 8a, the thioether ligand binds to the decarboxylase in a somewhat different manner. Clearly, substitution of a carbonyl functionality with a bridging methylene group resulted in the binding of this ligand in a manner that does not mimic natural substrate positioning.

On the basis of the observed binding mode of 4-hydroxybenzoyl-CoA to the dehalogenase, however, a model of methylmalonyl-CoA was built into the decarboxylase active site as presented in Figure 14a. According to this model building study, the NH peptidic groups of the structually conserved His 66 and Gly 110 form hydrogen bonds with the carbonyl oxygen of the substrate, thereby producing the required polarization of this bond. As in all members of the crotonase superfamily examined thus far, Gly 110 is located at the N-terminal region of a conserved α-helix, implicating the possible importance of a helix dipole moment in the catalytic mechanisms of these enzymes. Additionally, O^{η} of Tyr 140 lies within hydrogen-bonding distance to the carboxylate group of the substrate and most likely plays a key role in orienting this moiety orthogonal to the plane of the thioester carbonyl group. The "conserved" Glu 113 in the decarboxylase, which corresponds to Glu 144 in the crotonase model, is most likely not involved in catalysis but rather forms a salt-bridge with the guanidinium group of Arg 86.

The protein region surrounding the ligand is decidedly hydrophobic, and this nonpolar environment would serve both to destabilize the negatively charged carboxylate group and to enhance the decarboxylation process. The formation of the resulting thioester enolate anion intermediate would be stabilized by hydrogen-bonding interactions with the backbone amide groups of His 66 and Gly 110. A putative catalytic mechanism for the decar-

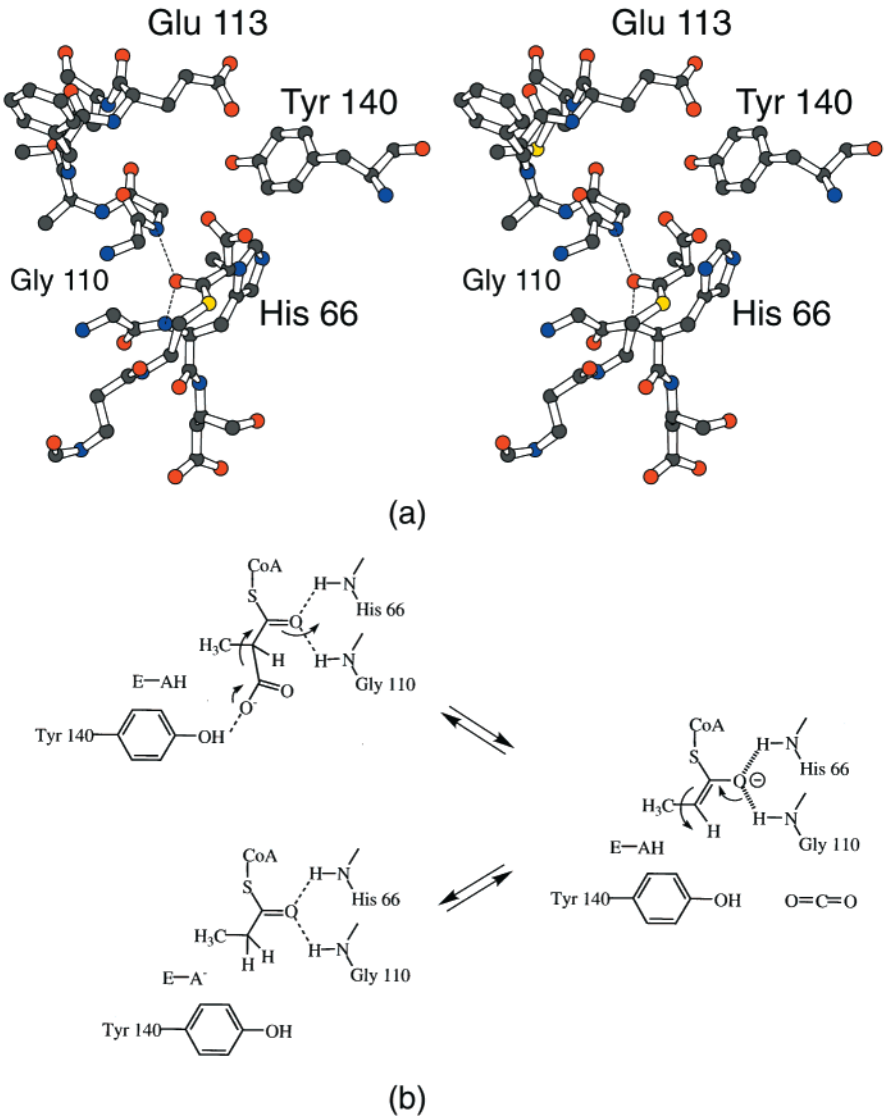

FIGURE 14. Possible binding mode for methylmalonyl-CoA within the active site of the decarboxylase (a). The reactive end of the substrate is shown and highlighted in yellow bonds. Potential hydrogen bonds are indicated by the dashed lines. On the basis of the X-ray structure, a catalytic mechanism for the enzyme has been proposed as indicated in (b). Figures 4, 5, 7, 8, 11, 12, 13b, and 14a were prepared with the software package MOLSCRIPT.[36]

boxylase has been set forth as outlined in Figure 14b. From the present structure, it is not clear which residue serves as the general acid to deliver a solvent-derived proton to the α-carbon to generate the propionyl-CoA product.

Perhaps one of the most important "take home" lessons from the structural analysis of the decarboxylase was the fact that the unexpected changes in the structure of the enzyme, relative to other members of the crotonase superfamily, could not have been predicted on the basis of amino acid sequence alignments alone. This result clearly undermines the growing assumption in the field of structural genomics that knowledge of the three-dimensional structure of one protein of a superfamily completely defines the structures of all other members.

Conclusions

Until recently, homologous enzymes were thought to catalyze identical reactions with, perhaps, different specificities, e.g., the chymotrypsin fold of serine proteases.[28] However, several superfamilies have been identified whose members catalyze different reactions.[1,2] In each, a common structural strategy is used to lower the free energies of similar intermediates. Catalysis of divergent chemistries is accomplished by (1) retaining those functional groups that catalyze the common partial reaction and (2) incorporating new groups that direct the intermediates to new products. While the crotonase superfamily is mechanistically diverse, in each reaction negative charge is localized on the oxygen of a thioester carbonyl, and stabilization of this negative charge occurs via hydrogen-bonding interactions with two peptide NH groups in a conserved oxyanion hole. The presence of the two NH groups of the oxyanion hole can be recognized in sequence alignments by two consensus sequences (highlighted in green in Figure 15), where the fifth amino acid in the first consensus sequence and the second amino acid in the second consensus sequence contribute the NH bonds. This conserved structural feature allows the hypothesis that the

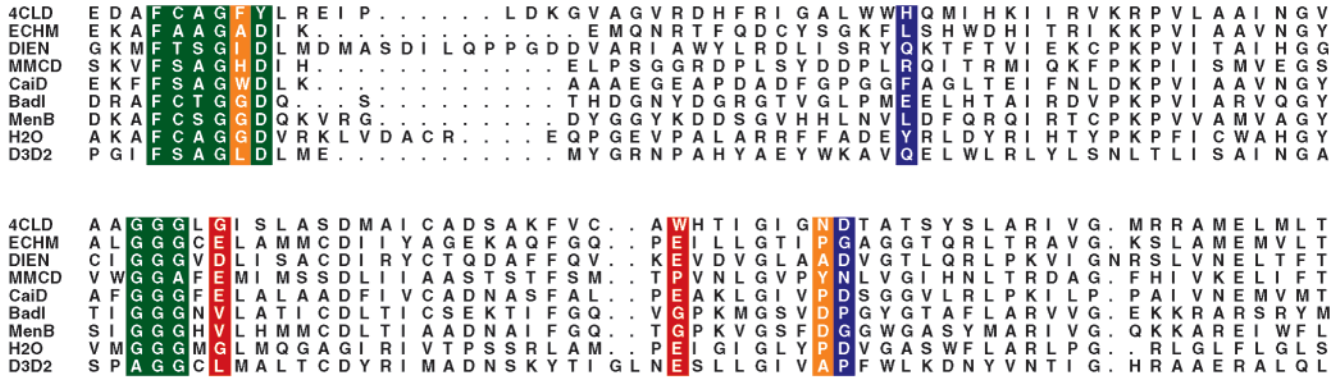

FIGURE 15. Partial sequence alignment of nine members of the crotonase superfamily: 4CLD, dehalogenase, ECHM, rat mitochondrial crotonase; DIEN, dienoyl-CoA isomerase; MMCD, methylmalonyl-CoA decarboxylase, CaiD, carnitinyl-CoA epimerase; BadI, 2-ketocyclohexanecarboxyl-CoA hydrolase; MenB, dihydroxynaphthoyl-CoA synthase; H2O, β-hydroxyisobutyryl-CoA hydrolase; and D3D2, 3,2-*trans*-enoyl-CoA isomerase.

evolution of this superfamily has been dominated by the need to stabilize enolate anion intermediates that otherwise would be too unstable to be kinetically competent.

Both three-dimensional structural and amino acid sequence comparisons have demonstrated that no active site catalytic groups are strictly conserved within this superfamily. To illustrate this conclusion, the partial sequence alignment in Figure 15 includes the structurally characterized dehalogenase (4CLD), rat mitochondrial crotonase (ECHM), dienoyl-CoA isomerase (DIEN), and methylmalonyl-CoA decarboxylase (MMCD) as well as several other members of the superfamily. The positions of the active site residues in crotonase are highlighted in red, those in dehalogenase are highlighted in blue, and those in the decarboxylase are highlighted in orange. In some members, such as the rat mitochondrial crotonase, a glutamate residue is thought to be involved in proton removal from or delivery to C(2) of the thioester substrate. In those reactions that involve the addition of water to an enoyl-CoA, a conserved glutamate residue sometimes activates the attacking water molecule for addition to the C(3) such as Glu 144 in the crotonase. Interestingly, in the reactions catalyzed by $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase and 4-chlorobenzoyl-CoA dehalogenase, a conserved Asp residue serves either as an acid/base catalyst (Asp 204 in the isomerase) or as a nucleophile (Asp 145 in the dehalogenase). For most members of this superfamily, however, the correlation between structure and function is still too ill-defined to allow prediction of either the mechanism or the identities of the acid/base catalysts. The fact that the same secondary structural elements within the N-terminal domain deliver both the groups necessary for catalysis and for substrate specificity adds to the ambiguities. On the basis of amino acid sequence alignments, other members belonging to the crotonase superfamily have been identified and include carnitinyl-CoA epimerase,[29] 2-ketocyclohexanecarboxyl-CoA hydrolase,[30] dihydroxynaphthoyl-CoA synthase,[31,32] β-hydroxyisobutyryl-CoA hydrolase,[33,34] and 3,2-*trans*-enoyl-CoA isomerase,[35] among others. In addition to the reactions described in this Account for the dehalogenase, crotonase, isomerase, and decarboxylase, enzymes in the crotonase superfamily have been shown to catalyze carbon−carbon bond formations and cleavages and hydrolysis of thioesters, for example. Additional X-ray structural and mechanistic studies will be required to more fully characterize the manner in which members of this superfamily carry out quite different chemical reactions by implementing a common strategy of stabilizing oxyanion intermediates. This work is in progress within our research groups and in other laboratories.

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