Articles

Evolution of Function in the Crotonase Superfamily: (3*S*)-Methylglutaconyl-CoA Hydratase from *Pseudomonas putida*†

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ABSTRACT: Members of the enoyl-CoA hydratase (crotonase) superfamily catalyze different overall reactions that utilize a common catalytic strategy delivered by a shared structural scaffold; the substrates are usually acyl esters of coenzyme A, and the intermediates are usually thioester enolate anions stabilized by a conserved oxyanion hole. In many bacterial genomes, orthologous members that contain homologues of acid/base catalyst Glu164 but not of Glu144 in rat mitochondrial crotonase are encoded by operons of which the functions have not been assigned. Focusing on the orthologues from *Pseudomonas aeruginosa* and *P. putida*, we have determined that these operons encode enzymes in leucine catabolism with the unknown enzyme assigned as (3*S*)-methylglutaconyl-CoA hydratase (MGCH), which catalyzes the *syn*-hydration of (*E*)-3-methylglutaconyl-CoA to (3*S*)-hydroxymethylglutaryl-CoA. The discovery that bacterial MGCHs catalyze hydration of enoyl-CoAs utilizing a single active-site residue contrasts with the paradigm crotonases as well as with the recently identified mammalian MGCHs that use homologues of both Glu144 and Glu164 in crotonase. Substrate analogues lacking a γ-carboxylate have been shown to be competitive inhibitors of the enzyme, and installation of a glutamate for the "missing" homologue of Glu144 fails to introduce hydratase activity with the substrate analogues. Thus, bacterial MGCHs may provide an example of opportunistic evolution in which a carboxylate group of the substrate functionally replaces one of the active site glutamate residues in the reactions catalyzed by crotonases and the eukaryotic MGCHs.

The study of mechanistically diverse enzyme superfamilies allows a greater understanding of the interplay between sequence, structure, and function than is possible from studying individual enzymes. Members of such superfamilies have homologous sequences and retain a common structural scaffold, consistent with divergent evolution from a common progenitor. Additionally, each member employs a common catalytic strategy to lower the free energy of chemically similar intermediates that would otherwise be too unstable to be kinetically competent. Divergent chemistries and functions are accomplished by retaining the functional groups needed to catalyze the common partial reaction while incorporating new groups that partition the intermediates to different products in the different active sites (*1*, *2*).

The growing number of sequences in the databases underscores the need to assign functions to newly discovered proteins. Even limited sequence identity (<20%) may allow an unknown protein to be placed in an enzyme superfamily for which there is a known catalytic strategy, thereby providing an important clue about the mechanism and identity of the reaction catalyzed by the unknown protein. When coupled with metabolic context, this information can be sufficient to assign the type of reaction catalyzed by the enzyme, for example, racemization or dehydration, and perhaps even allow the identity of the substrate to be specified (*3*).

As described in this article, we used this strategy to identify bacterial (3*S*)-methylglutaconyl-CoA hydratases (MGCHs), members of the enoyl-CoA hydratase (crotonase) superfamily. The crotonase superfamily encompasses enzymes that catalyze diverse reactions in a wide array of metabolic pathways. Reactions catalyzed by members of the superfamily include hydration/dehydration (rat mitochondrial crotonase, ECHM), dehalogenation (4-chlorobenzoyl-CoA dehalogenase, 4-CLD), and thioester hydrolysis (3-hydroxyisobutyryl-CoA hydrolase, HICH) among others (*1*, *4*). The partial reaction common to members of the superfamily is the formation of an oxyanion intermediate (*1*, *5*, *6*). In the case of ECHM and the majority of other members of the superfamily, the intermediate is a thioester enolate anion; in the 4-CLD-catalyzed reaction, it is an anionic Meisenheimer complex (*7*), and in the case of the HICH-catalyzed reaction, it is an anionic tetrahedral intermediate (*8*).

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[1] Abbreviations: 4-CLD, 4-chlorobenzoyl-CoA dehalogenase; ECHM, rat mitochondrial enoyl-CoA hydratase; GCT, glutaconyl-CoA transferase; HICH, 3-hydroxyisobutyryl-CoA hydrolase; HMG-CoA, (3*S*)-hydroxymethylglutaryl-CoA; MGCH, methylglutaconyl-CoA hydratase; MG-CoA, (*E*)-3-methylglutaconyl-CoA.

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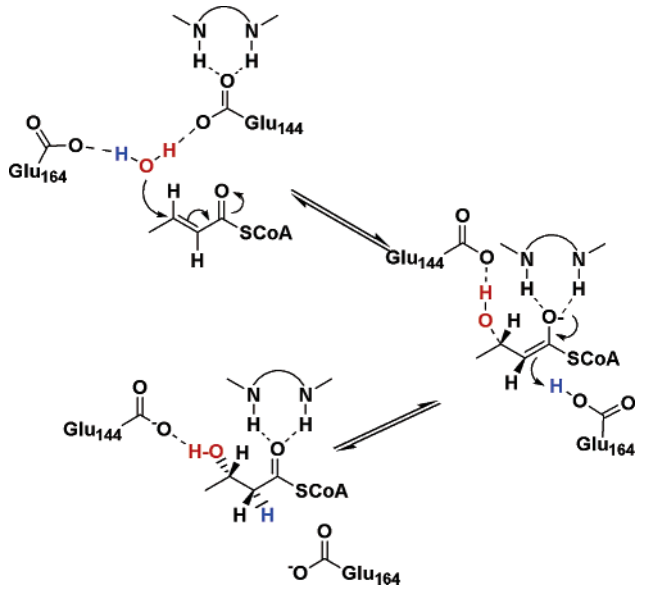
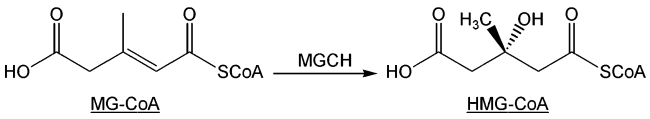


FIGURE 1: Mechanism of the reaction catalyzed by rat mitochondrial crotonase (enoyl-CoA hydratase).

The anionic intermediates are stabilized by a conserved oxyanion hole, as observed in the high-resolution structures for ECHM (*9*, *10*), 4-CLD (*11*), and several other superfamily members (*12*, *13*), with two peptidic NH groups forming the oxyanion hole. These hydrogen bond to the thioester oxygen, thereby providing enhanced stabilization of the anionic intermediate as the negative charge is localized on the carbonyl oxygen (*5*, *14*, *15*). The NH groups are located in separate consensus sequences found in all members of the superfamily, consistent with stabilization of an oxyanion intermediate being the driving force behind the evolution of function in the superfamily.

Crotonase, the paradigm member of the superfamily, catalyzes the *syn*-hydration of crotonyl-CoA to (3*S*)-hydroxybutyryl-CoA. The structure of ECHM implicates two glutamate residues as acid/base catalysts: Glu144 deprotonates water for attack on C3 of the substrate to form the thioester enolate anion intermediate, and Glu164 delivers a proton to C2 of the intermediate to form the product. The intermediate is stabilized by hydrogen bonding to the peptidic NH groups of Ala98 and Gly141, which form the oxyanion hole (Figure 1) (*16*−*18*).

The genomes of many bacteria, including *Pseudomonas aeruginosa* and *P. putida*, encode a member of the superfamily located in an operon that encodes part of the leucine catabolic pathway. These proteins, designated PA2013 in *P. aeruginosa* (GenBank 15597209) (*19*) and PP4066 in *P. putida* (GenBank 26990767), were each annotated as a "putative enoyl-CoA hydratase". We have determined that these members of the superfamily are (3*S*)-methylglutaconyl-CoA hydratases (MGCHs) that catalyze the hydration of the enoyl-CoA (*E*)-3-methylglutaconyl-CoA (MG-CoA) using a single glutamate residue as acid/base catalyst.



An alignment of the sequences of MGCHs with other members of the superfamily reveals that this glutamate (Glu138 in the MGCH from *P. putida*) is a homologue of Glu164 in the structurally and mechanistically characterized rat mitochondrial crotonase; a glycine in the MGCH replaces Glu144 in crotonase. This active site structure also contrasts with that recently established for eukaryotic MGCHs (also catalyzing hydration of an enoyl-CoA substrate) that contain homologues of both glutamate residues in crotonase (*20*, *21*). Thus, although the bacterial and eukaryotic MGCHs catalyze hydration of the same substrate to yield the same product, the details of their mechanisms are not conserved. Perhaps this represents a novel example of opportunism in the evolution of function in the crotonase superfamily with the γ-carboxylate group of the substrate in the reaction catalyzed by the bacterial MGCH replacing one of the active site glutamate residues in the reaction catalyzed by the eukaryotic enzyme.

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich and were of the highest quality grade commercially available unless otherwise stated. Restriction enzymes and PCR components were purchased from Invitrogen; T4 DNA ligase was purchased from Epicenter Technologies. Oligonucleotide primers were synthesized by Bio-Synthesis (Lewisville, TX). DNA sequencing was performed by the Genetic Engineering Facility at the University of Illinois Biotechnology Center.

All NMR spectra were recorded with a Varian narrow-bore 500 MHz spectrometer unless otherwise specified. A Bio-Pilot or AKTA FPLC system (Pharmacia Biotech) was used for purification of proteins. Spectrophotometric assays were performed with a Perkin-Elmer Lambda 14 spectrophotometer. A Beckman Gold HPLC System was used for the purification of CoA esters. Protein mass spectrometry (ESI and FAB) was performed at the University of Illinois School of Chemical Sciences Mass Spectrometry Laboratory.

Cloning, Expression, and Purification of MGCH. The genes encoding the MGCHs were PCR-amplified from *P. aeruginosa* and *P. putida* genomic DNA (obtained from American Type Culture Collection) using Pfx DNA polymerase. Oligonucleotide primers containing the first and last 39 bases of each gene were designed and modified with an *Nde*I 5′-end and a *Xho*I 3′-end restriction site. The amplified gene products were purified (Qiagen), restriction digested, and ligated into a modified version of the pET15B vector restricted with *Nde*I and *Xho*I; this vector encodes a 10-His tag instead of the usual 6-His tag at the N-terminus. The sequences were verified, and the proteins were expressed in *Escherichia coli* strain BL21(DE3) cells (Stratagene).

Transformed cells containing the plasmid were grown in 2 L of LB−Amp medium (100 μg/mL ampicillin) for 24 h at 37 °C and harvested by centrifugation (6600 × *g* at 4 °C) for 10 min. No IPTG was added to induce protein expression. The cell pellet was resuspended in 100 mL of binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9) and lysed by sonication. After centrifugation (27 000 × *g* at 4 °C for 30 min), the supernatant solution was applied to a chelating Sepharose Fast Flow column (Pharmacia Biotech, 50 mL, 1.6 cm × 40 cm) charged with Ni^{2+} and washed with 400 mL of binding buffer followed by 400 mL of wash buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9). The His-tagged protein was eluted with

400 mL of an equal mixture of binding buffer and strip buffer (100 mM EDTA, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9). The purified protein was concentrated and dialyzed against 10 mM Tris-HCl, pH 7.9. Purity was assessed by SDS−PAGE gel as well as ESI-MS. ESI-MS for MGCH from *P. aeruginosa*: expected mass of 31 179.5, observed mass of 31 187.9 ± 31.2. ESI-MS for MGCH from *P. putida*: expected mass of 32 247.5, observed mass of 32 251.4 ± 32.3.

The His-tag was removed by first dialyzing against 1× PBS buffer and then adding thrombin to a concentration of 1 unit/mg of protein. When thrombin cleavage was completed, the protein was applied to a ResourceQ anion-exchange column. The column was washed with 80 mL of 10 mM Tris-HCl, pH 7.9, and the protein was eluted with a 240 mL linear gradient of 0−0.5 M NaCl in 10 mM Tris-HCl, pH 7.9. Removal of the His-tag was confirmed by ESI-MS.

Cloning, Expression, and Purification of MvaB (HMG-CoA Lyase). The gene encoding HMG-CoA lyase (MvaB) from *P. aeruginosa* (PA2011, GenBank 15597207) was cloned into the *Nde*I and *Xho*I sites of the modified pET15b vector using the protocol described for MGCH. The His-tagged MvaB was expressed and purified on a chelating Sepharose Fast Flow column as described for MGCH except that 5 mM $MgCl_2$ was added to the buffers. After purification, the protein was dialyzed into 10 mM Tris-HCl, pH 7.9, containing 5 mM $MgCl_2$ and assayed.

Cloning, Expression, and Purification of MGCH Mutants. Mutants of MGCH were constructed using the overlap extension method. For each mutant, two complementary 39-base oligonucleotide primers were designed with the desired mutation located in the middle. One-half of the mutated gene was prepared by PCR amplification of the wild-type MGCH plasmid using the 3′-primer and the T7-promoter primer for the pET15b vector. The other half was created using the 5′-primer and T7-terminator primer. A final PCR reaction using the two halves of the gene and the T7-promoter and terminator primers gave the desired mutated product.

The mutated DNAs for the E138D/A/Q mutants were cloned into the *Nde*I and *Xho*I sites of the modified pET15b vector using the protocol described for MGCH. The His-tagged mutant proteins were expressed and purified in the same manner. After purification the protein was dialyzed into 10 mM Tris, pH 7.9, and assayed. The genes were sequenced to confirm the presence of the desired mutations.

The G118E mutant was cloned into the pET17 vector due to its insolubility when expressed with an N-terminal 10-His tag. The cell lysate was loaded onto a DEAE column, which was washed with 800 mL of 20 mM Tris-HCl, pH 8.0, and eluted with a 1800 mL linear gradient of 0−0.5 M NaCl. Fractions that contained the protein were pooled, concentrated, and dialyzed against 20 mM Tris-HCl, pH 8.0. The dialyzed protein was further purified with a ResourceQ column eluted with a linear gradient of 0−0.5 M NaCl in 240 mL of wash buffer. The fractions were run on an SDS−PAGE gel, and those that were >90% in purity were pooled and used for assays. The protein was dialyzed as described for the other mutants, and the gene was sequenced.

HPLC Assay for MGCH. Samples containing 5.4 mM racemic HMG-CoA, 19 mM Tris-HCl, pH 7.9, and 5.7 μM MGCH from either organism were incubated at room-temperature overnight. Control samples contained no enzyme. The formation of 3-MG-CoA was monitored at 254 nm using a Vydac semipreparative C18 reverse-phase HPLC column (10 mm × 25 cm, 10 μm particle size) eluted at a flow rate of 2 mL/min. The solvent system was comprised of acetonitrile (Buffer A) and 100 mM ammonium acetate, pH 6.5 (Buffer B). An initial wash of 1% acetonitrile and 99% ammonium acetate was performed for 2 min followed by a linear gradient from 1% to 40% acetonitrile over 55 min. Under these conditions, MG-CoA eluted at 18.3 min, and HMG-CoA eluted at 17.0 min. MG-CoA was identified by ESI-MS (expected 893.2, observed $[M - H]^+$ 892.4).

To determine the stereochemistry of the reaction, 0.5 mM of either (*R*)- or (*S*)-HMG-CoA was incubated with 0.5 μM MGCH in 5 mM Tris-HCl, pH 7.9. Control reactions contained no enzyme. The reactions were allowed to incubate at room temperature for 90 min before the enzyme was removed by ultrafiltration. The reaction mixtures were analyzed by HPLC according to the protocol previously described. Products were collected and identified by ESI-MS.

Spectrophotometric Assay of MGCH. A continuous spectrophotometric assay was used to derive kinetic constants for the MGCH-catalyzed reaction. The rate of disappearance of the conjugated MG-CoA substrate was monitored at 280 nm. Assays (0.9 mL at 25 °C) contained 20 mM Tris-HCl, pH 7.9, one of three substrates, and enzyme. Extinction coefficients of 11.3 (*22*), 5.7, and 3.6 cm^{-1} mM^{-1} (*17*) were used for 3-methylglutaconyl-CoA (MG-CoA), 3-methylcrotonyl-CoA and crotonyl-CoA, respectively. When the enzymatic activity was low, the enzyme was incubated with a high concentration of substrate for an extended period of time and the change in absorbance was used to approximate a value for k_{cat}.

The ability of 3-methylcrotonyl-CoA and crotonyl-CoA to inhibit MGCH activity was performed using the assay conditions described above. The rate of hydration of MG-CoA was determined using five different concentrations of methylcrotonyl-CoA (0−150 μM) or crotonyl-CoA (0−200 μM). The values of the K_i for each inhibitor were calculated using the program Enzkin (Cleland).

Synthesis of Racemic HMG-CoA. 3-Hydroxymethylglutaric acid (162.1 mg, 1 mmol) was dissolved in 20 mL of dry THF at 0 °C. Triethylamine (138.6 μL, 1 mmol) and ethylchloroformate (95.6 μL, 1 mmol) were added. The mixture was stirred under nitrogen for 1 h; triethylammonium chloride was removed by filtration. The filtrate was added dropwise over 1 h to a solution of coenzyme A (30 mg, 35 μmol) in 3 mL of water under nitrogen at 0 °C. The pH was maintained at 7 by addition of 0.1 M NaOH. The reaction was stirred for 2 h before 3 M HCl was added to lower the pH to 1. The solution was extracted three times with ethyl acetate, and the aqueous layer was lyophilized (*23*).

The HMG-CoA product was purified using a Vydac semipreparative C18 HPLC column (10 mm × 25 cm, 10 μm particle size) using the conditions described previously. The retention time of 3-HMG-CoA under these conditions was 17.0 min. Final concentrations were typically ∼10 mM (30% yield). ESI-MS: expected 911.2, observed $[M - H]^+$ 910.3. ^1H NMR (D_2O): δ 0.59 (s, 3H), 0.74 (s, 3H), 1.17 (s, 3H), 2.28 (t, $J = 6.2$ Hz, 2H), 2.32 (q, $J = 14.5$ Hz, 2H), 2.76 (q, $J = 14.8$ Hz, 2H), 2.83 (t, $J = 6.2$ Hz, 2H), 3.18 (t,

$J = 6.2$ Hz, 2H), 3.30 (t, $J = 6.2$ Hz, 2H), 3.40 (dd, $J = 4.9$ Hz, 1H), 3.68 (dd, $J = 4.9$ Hz, 1H), 3.84 (s, 1H), 4.07 (broad s, 2H), 4.40 (s, 1H), 4.67 (s, 2H), 6.02 (d, $J = 6.6$ Hz, 1H), 8.13 (s, 1H), 8.39 (s, 1H).

Synthesis of (E)-3-Methylglutaconic Acid. Sodium hydroxide (4.84 g, 120 mmol) was added to a solution of ethylisodehydroacetate (6 g, 30 mmol) in water (53 mL). The mixture was heated for 3 h at 70 °C, cooled on ice, and treated with 12 N HCl (10.5 mL). Sodium chloride was added to saturation. The mixture was extracted with ether (3 × 25 mL); the extracts were dried with $MgSO_4$ and evaporated to afford an off-white solid (2.98 g, 69%). FAB-MS: expected 144.1, observed $[M - H]^+$ 143.1. ^{1}H NMR (DMSO): for (*E*)-isomer, δ 2.17 (d, $J = 1$ Hz, 3H), 3.20 (s, 2H), 5.70 (s, 1H); for (*Z*)-isomer, δ 1.89 (d, $J = 1$ Hz, 3H), 3.62 (s, 2H), 5.75 (s, 1H). The ratio of the integrals of the resonances at δ 2.17 and 1.89 revealed a 2:1 ratio of the (*E*)- and (*Z*)-isomers (*24*).

The isomers were separated by suspending a mixture of the acids (1.5 g, 10.4 mmol) in dry benzene (10 mL) and stirring with acetic anhydride (490 μL, 5.2 mmol) at 45−50 °C for 12 h. The remaining solid was collected, washed with benzene, and dried to yield pure (*E*)-3-methylglutaconic acid (650 mg) (*25*). ^{1}H NMR (DMSO): δ 2.17 (d, $J = 1$ Hz, 3H), 3.20 (s, 2H), 5.70 (s, 1H).

Enzymatic Synthesis of (E)-3-Methylglutaconyl-CoA (MG-CoA). Plasmid pMM2 containing the genes encoding glutaconyl-CoA transferase (GCT) from *Acidaminococcus fermentans* was obtained from Dr. Wolfgang Buckel at the Philipps-University of Marburg, Germany. GCT was expressed in *E. coli* BL21(DE3) cells grown in 2 L of LB−Amp at 37 °C to an OD_{600} of 0.6 and induced with 1 mM IPTG. The cells were grown for an additional 24 h and harvested (6600 × *g* at 4 °C for 10 min). The cells were suspended in 20 mM potassium phosphate, pH 7.4, and centrifuged (27 000 × *g* at 4 °C for 30 min) to remove cell debris. The cell extract was subjected to ammonium sulfate fractionation (50−80%, 31.4−56.1 mg/100 mL), and the precipitated protein was collected by centrifugation. The protein was redissolved in a minimal amount of 20 mM potassium phosphate, pH 7.4, and saturated ammonium sulfate was added dropwise until a slight turbidity occurred. The solution was incubated at 4 °C for 3 days, and crystals that formed were collected by centrifugation. SDS−PAGE analysis of both the crystals and the supernatant showed the presence of partially purified transferase (*26*, *27*).

MG-CoA was synthesized from methylglutaconate and acetyl-CoA. Partially purified GCT (0.3 μM) was added to a solution containing 0.67 mM acetyl-CoA, 30 mM (*E*)-3-methylglutaconate, and 50 mM potassium phosphate, pH 7.0. The reaction (30 mL) was performed at room temperature, and progress was monitored by the increase in absorbance at 280 nm. Equilibrium was reached in 45 min. The pH was adjusted to 4.0 with 0.5 M H_2SO_4, the precipitate was removed by filtration, and 120 mL of H_2O was added.

The solution was applied to a DEAE-cellulose column (1.6 cm × 40 cm) preequilibrated with 3 mM HCl. After a wash of 600 mL of 3 mM HCl, MG-CoA was eluted with a 700 mL linear gradient of 0−0.25 M LiCl. Fractions (10 mL) containing the CoA products as assessed by absorbance at 280 nm were combined and concentrated to a volume of ~2 mL. The product was desalted with a PD-10 column (Amersham) following the supplier's instructions. Fractions (1 mL) containing the CoA product were combined and lyophilized to yield MG-CoA (*28*).

The purity of the MG-CoA was verified using a semipreparative reverse-phase HPLC column using the conditions described previously. A peak at 18.3 min was confirmed to be MG-CoA by ESI-MS (expected 893.2, observed $[M - H]^+$ 892.2). ^{1}H NMR (D_2O): δ 0.62 (s, 3H), 0.79 (s, 3H), 1.92 (d, $J = 1.3$ Hz, 3H), 2.28 (t, $J = 6.2$ Hz, 2H), 2.89 (t, $J = 6.2$ Hz, 2H), 3.14 (s, 2H), 3.21 (t, $J = 6.2$ Hz, 2H), 3.30 (t, $J = 6.2$ Hz, 2H), 3.40 (dd, $J = 4.9$ Hz, 1H), 3.68 (dd, $J = 4.9$ Hz, 1H), 3.84 (s, 1H), 4.07 (broad s, 2H), 4.40 (s, 1H), 4.67 (s, 2H), 6.01 (q, $J = 1.1$ Hz, 1H), 6.09 (d, $J = 6.6$ Hz, 1H), 8.23 (s, 1H), 8.51 (s, 1H).

Synthesis of (*R*,*S*)-*Mevalonic Acid.* (*R*,*S*)-Mevalonic acid lactone (250 mg, 1.69 mmol) and 99.6 mg (1.77 mmol) of potassium hydroxide were stirred in 3.46 mL of water for ~30 min at 37 °C. The pH was lowered to 7.5 with 0.1 M HCl. The solution was lyophilized, and the resulting (*R*,*S*)-mevalonic acid was stored at −20 °C (*29*). ^{1}H NMR (D_2O): δ 1.15 (s, 3H), 1.70 (t, 2H), 2.35 (q, 2H), 3.62 (t, 2H).

Enzymatic Synthesis of (*S*)-*HMG-CoA.* The plasmid containing the gene encoding HMG-CoA reductase (MvaA) from *P. mevalonii* was a gift from Professor Victor Rodwell (Purdue University). BL21 cells transformed with this plasmid were grown at 37 °C for 24 h in 2 × 2 L of LB−Amp and collected (6600 × *g* at 4 °C for 10 min). The cells were resuspended in 10 mM Tris-Cl, pH 7.9, disrupted by sonication, and centrifuged (27 000 × *g* at 4 °C for 30 min) to remove cell debris.

The extract was subjected to ammonium sulfate fractionation (40%, 24.2 mg/100 mL), stirred on ice for 20 min, and centrifuged for 30 min (27 000 × *g*). The protein was redissolved in a minimal amount of 10 mM Tris-Cl, pH 7.9, and dialyzed overnight against 20 mM Tris-Cl, pH 7.9, containing 100 mM NaCl. The protein that precipitated was collected by centrifugation and redissolved in a minimal amount of 20 mM Tris-Cl, pH 7.9. SDS−PAGE analysis showed the presence of partially purified reductase.

Assays for MvaA (1 mL, 25 °C) contained 60 mM Tris-HCl, pH 7.9, 60 mM (*R*,*S*)-mevalonate, 10 mM NAD^+, and 1 mM CoA. The reaction was initiated by adding 11.6 units (7.9 μM) of HMG-CoA reductase, and the reduction of NAD^+ was monitored at 340 nm.

(*S*)-HMG-CoA was prepared from a reaction (34 mL) containing 60 mM Tris-HCl, pH 7.9, 60 mM (*R*,*S*)-mevalonate, 5 mM NAD^+, and 1 mM CoA. Pyruvate (60 mM) and lactate dehydrogenase (300 units) were added to regenerate NAD^+. MvaA (200 units) was added, and the solution was stirred at room temperature for 2 h. The reaction was monitored at 340 nm (*30*). After equilibrium was reached, the pH was adjusted to 4 with 0.5 M H_2SO_4. The precipitate was removed by filtration, and 120 mL of H_2O was added. The solution containing (*S*)-HMG-CoA was applied to a DEAE-cellulose column (1.6 cm × 40 cm) and purified as described for MG-CoA. Fractions containing the product were collected, evaporated, desalted, and lyophilized as described previously. (*S*)-HMG-CoA was further purified by HPLC on a semipreparative reverse-phase column using the conditions described previously. A peak was observed at 17.0 min, and ESI-MS and NMR spectra matched that of racemic HMG-CoA.

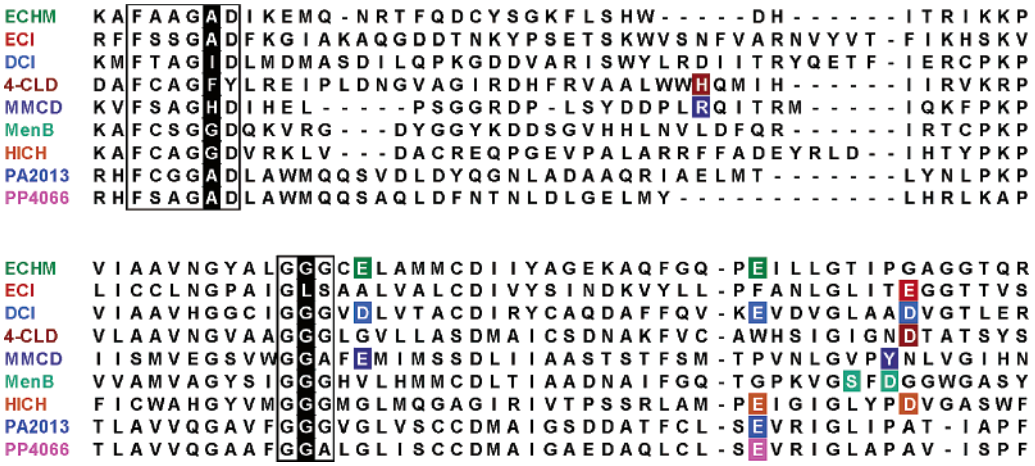


FIGURE 2: Partial sequence alignment of PA2013 and PP4406 with eight members of the crotonase superfamily: ECHM, rat mitochondrial crotonase; ECI, 3,2-*trans*-enoyl-CoA isomerase; DCI, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase; 4-CLD, 4-chlorobenzoyl-CoA dehalogenase; MMCD, methylmalonyl-CoA decarboxylase; MenB, 1,4-dihydroxynaphthoyl-CoA synthase; and HICH, 3-hydroxyisobutyryl-CoA hydrolase. Residues outlined in black are the oxyanion hole motifs; the residues highlighted in black contribute amide hydrogens to the oxyanion hole. Colored residues are known or suspected catalytic residues.

Enzymatic Synthesis of (*R*)*-HMG-CoA.* Assays (1 mL, 25 °C) for HMG-CoA lyase (MvaB) contained 100 mM Tris-HCl, pH 7.9, 7.7 mM $MgCl_2$, 0.5 mM NAD^+, 3.1 mM L-malate, 1.9 μM HMG-CoA lyase, 4 mM DTT, 1.4 units malate dehydrogenase, and 0.3 units of citrate synthase. The reaction was initiated by adding 0.4 mM (*R*,*S*)-HMG-CoA, and the reduction of NAD^+ was monitored at 340 nm.

(*R*)-HMG-CoA was prepared from a reaction (56.9 mL) containing 100 mM Tris-HCl, pH 7.9, 7.7 mM $MgCl_2$, 0.5 mM NAD^+, 0.2 μM HMG-CoA lyase, and 0.4 mM (*R*,*S*)-HMG-CoA. The solution was stirred at room temperature for 3 h, and the course of the reaction was followed at 340 nm (*30*).

After equilibrium was reached, the pH was adjusted to 4 with 0.5 M H_2SO_4. The (*R*)-HMG-CoA was purified in a manner similar to that described for the purification of (*S*)-HMG-CoA. (*R*)-HMG-CoA was separated from acetyl-CoA on a semipreparative reverse-phase HPLC column using the conditions described previously. A peak was observed at 17.0 min, and ESI-MS and NMR spectra both matched those of racemic HMG-CoA.

RESULTS AND DISCUSSION

Genomic Context of PA2013 and PP4066. A BLAST search of the proteins encoded by bacterial genomes using ECHM as the query sequence identified a group of apparently orthologous proteins encoded by a number of bacterial genomes, including those of *P. aeruginosa* and *P. putida*. The sequence of PA2013 from *P. aeruginosa* is 27.9% identical and 43.9% similar to the ECHM query sequence; the sequence of PP4066 from *P. putida* is 26.6% identical and 46.1% similar to the query. The two sequences are 67.2% identical to one another and both are annotated as "probable enoyl-CoA hydratases/isomerases" (*19*); based on this level of sequence identity, we assumed that PA2013 and PP4066 are orthologues.

A partial alignment of the sequences of these proteins with those of other members of the crotonase superfamily, including ECHM, rat dienoyl-CoA isomerase, and 4-CLD, is displayed in Figure 2. This alignment highlights the consensus sequences for the oxyanion hole as well as residues

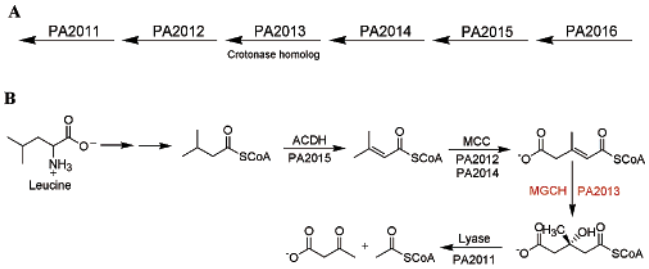


FIGURE 3: (A) Operon containing the gene encoding MGCH from *P. aeruginosa* and (B) assignment of genes in the pathway for leucine catabolism in *P. aeruginosa*. ACDH, acyl-CoA dehydrogenase; MCC, 3-methylcrotonyl carboxylase; MGCH, 3-methylglutaconyl-CoA hydratase.

that directly participate in the reactions catalyzed by these enzymes, including Glu144 and Glu164 in the active site of ECHM. PA2013 and PP4066 contain a homologue of Glu164 but not of Glu144 in crotonase.

PA2013 is the third gene in a six-gene operon (Figure 3A). PA2015 is 76% similar to an isovaleryl-CoA dehydrogenase from rat that participates in the catabolism of branched-chain amino acids. PA2012 and PA2014 are homologous to the two subunits of the biotin-dependent acetyl-CoA carboxylase; in the leucine catabolic pathway, β-methylcrotonyl-CoA, the product of a branched chain acyl-CoA dehydrogenase, is carboxylated by a biotin-dependent carboxylase to yield methylglutaconyl-CoA. PA2011 is 64% similar to hydroxymethylglutaryl-CoA lyase (HMG-CoA lyase) from *P. mevalonii*; HMG-CoA lyase catalyzes the final step in leucine catabolism in which hydroxymethylglutaryl-CoA is cleaved into acetyl-CoA and acetoacetate. Transcription of the operon is likely regulated by PA2016. PP4066 from *P. putida* is encoded by a five-gene operon that lacks the gene encoding the lyase.

Given these clues, we hypothesized that these operons encode enzymes in the established pathway for leucine catabolism (Figure 3B) that includes isovaleryl-CoA dehydrogenase, β-methylcrotonyl-CoA decarboxylase, and HMG-CoA lyase. Thus, we hypothesized that PA2013 and PP4066 are β-methylglutaconyl-CoA hydratases that convert MG-CoA to HMG-CoA (*22*), a function that conforms to the

mechanistic paradigm of the crotonase superfamily and involves a thioester enolate anion intermediate stabilized by the oxyanion hole.

Functions of PA2013 and PP4066. The genes encoding PA2013 and PP4066 were expressed and purified to homogeneity using an N-terminal His-tag. Both SDS−PAGE and ESI-MS of PA2013 revealed a contaminating species with a molecular weight of about 29 000 kD, ~2 kD less than that of the native protein. This species, likely a proteolytically truncated form, could not be removed by ion-exchange chromatography. The purified PP4066 did not contain such a contaminant.

HPLC first was used to determine whether PA2013 and PP4066 catalyze the reversible hydration of MG-CoA. Because racemic HMG-CoA is easily synthesized and the MGCH-catalyzed hydration is expected to be reversible, racemic HMG-CoA was incubated separately with PA2013 and PP4066, and formation of MG-CoA was monitored. With both proteins, a new peak with a retention time of ~18.3 min was observed. Analysis by ESI-MS gave a mass of 892.2, which is the $[M - H]^+$ species expected for MG-CoA. ^{1}H NMR analysis also confirmed the identity of the product: the disappearance of the AB quartet at 2.70 ppm corresponding to the α-protons of HMG-CoA coincided with the appearance of a quartet at 5.93 ppm ($J = 1.1$ Hz) due to the vinyl proton of MG-CoA (data not shown). Thus, both PA2013 and PP4066 catalyze the reversible dehydration of HMG-CoA to yield MG-CoA. Due to the contaminant associated with PA2013, our further characterization of the MGCH-catalyzed reaction focused on PP4066, the MGCH from *P. putida*.

Kinetic Parameters and Substrate Specificity for MGCH. A chemical synthesis of MG-CoA, the substrate for MGCH, was not possible because CoA and other nucleophiles add to α,β-unsaturated acids such as methylglutaconate (*31*). Instead, we synthesized MG-CoA using a glutaconyl-CoA transferase (GCT). Buckel and co-workers reported the synthesis of glutaconyl-CoA from glutaconate and acetyl-CoA using GCT from *A. fermentans* (*26*). We modified this strategy to allow the synthesis of MG-CoA from methylglutaconate and acetyl-CoA. Only the (*E*)-isomer of MG-CoA could be prepared, because GCT utilizes only the (*E*)-isomer of glutaconate. However, Messner and co-workers reported that (*E*)-MG-CoA is the substrate for MGCH isolated from sheep liver (*22*), and we assume that the configuration of the MG-CoA substrate is the same in bacteria and eukaryotes given the significant sequence identities relating bacterial and eukaryotic isovaleryl-CoA dehydrogenases.

A continuous spectrophotometric assay was used to determine the values of the kinetic constants with MG-CoA as well as the structurally truncated analogues 3-methylcrotonyl-CoA and crotonyl-CoA (Table 1); no activity was detected with either modified substrate. We evaluated the importance of the γ-carboxylate in binding of these analogues. Double reciprocal plots of the inhibition by 3-methylcrotonyl-CoA (Figure 4) and crotonyl-CoA (Figure 5) reveal that both are competitive inhibitors; the values of K_i's, 31.7 μM for 3-methylcrotonyl-CoA and 94.9 μM for crotonyl-CoA (Table 1), are comparable to the K_m for the MG-CoA substrate, 29.1 μM, confirming that these analogues bind in the active site. Thus, we conclude that the γ-carboxylate group of MG-CoA is essential for the activity although it is not essential for binding. The role of the γ-carboxylate group as a catalytic functional group is a possibility although this cannot be evaluated unequivocally in the absence of a structure (vide infra).

Table 1: Kinetic Constants for Reactions Catalyzed by MGCH

substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	K_i (μM)
crotonyl-CoA	$\leq 1.4 \times 10^{-4}$	*a*	*a*	94.9 ± 20.3
methylcrotonyl-CoA	$\leq 4.9 \times 10^{-3}$	*a*	*a*	31.7 ± 7.2
methylglutaconyl-CoA	2.1 ± 0.1	29.1 ± 5.6	7.2×10^4	

[a] Not determined.

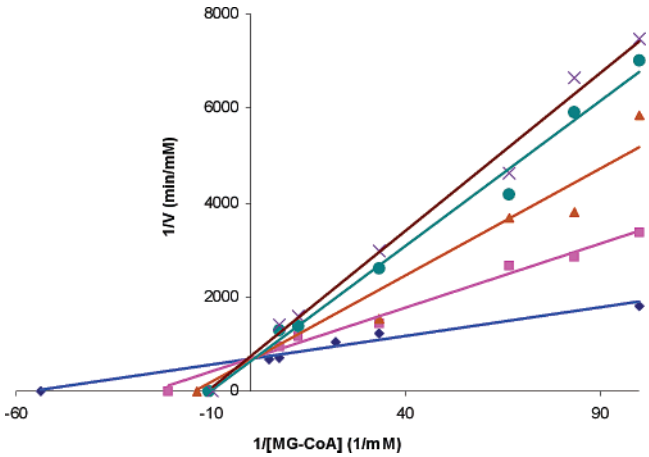


FIGURE 4: Inhibition of MGCH by methylcrotonyl-CoA. Rates were measured in the presence of 0 (◆), 50 (■), 94 (▲), 130 (●), and 150 μM MG-CoA (×).

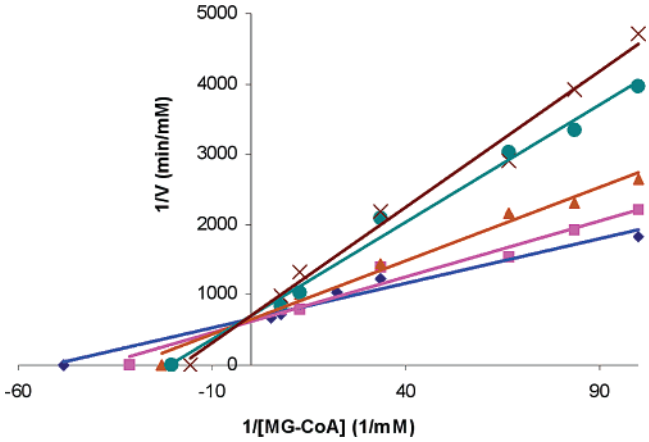


FIGURE 5: Inhibition of MGCH by crotonyl-CoA. Rates were measured in the presence of 0 (◆), 50 (■), 100 (▲), 130 (●), and 200 μM MG-CoA (×).

Product of MGCH-Catalyzed Reaction. The configuation of C3 in the HMG-CoA product was established by separately incubating the diastereomers of HMG-CoA with MGCH. (*S*)-HMG-CoA was prepared from the enzymatic conversion of (*R*)-mevalonate by HMG-CoA reductase; (*R*)-HMG-CoA was prepared by incubating a racemic mixture of HMG-CoA with HMG-CoA lyase that converts (*S*)-HMG-CoA to acetyl-CoA and acetoacetate and leaves the (*R*)-diastereomer intact. [Because PA2011 from *P. aeruginosa* was annotated as a probable HMG-CoA lyase, we cloned

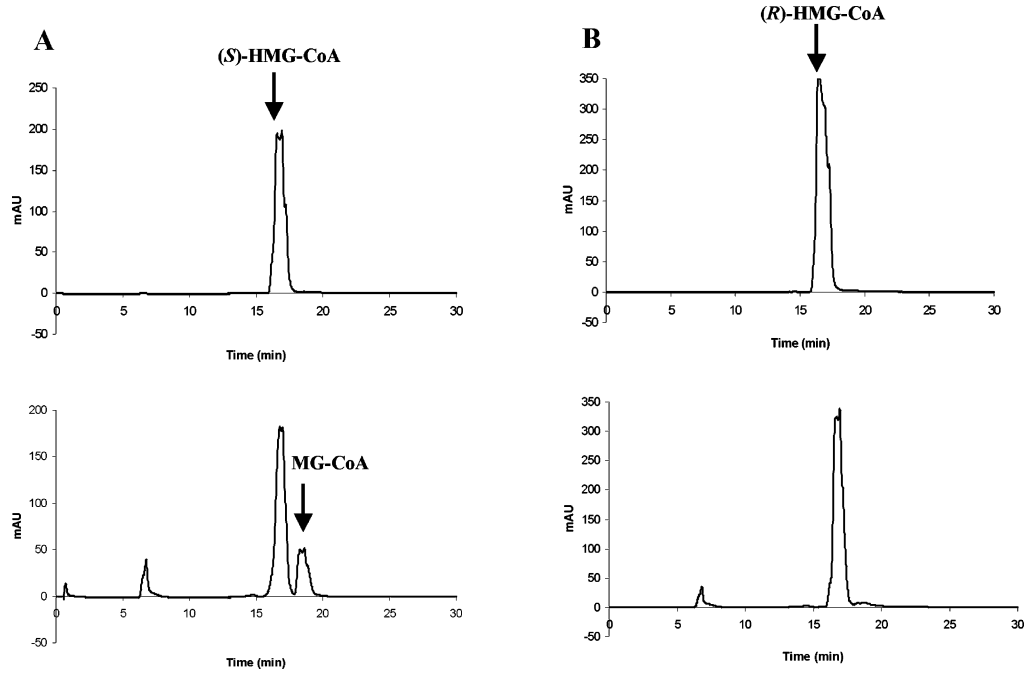


FIGURE 6: HPLC assay of (A) (*S*)-HMG-CoA and (B) (*R*)-HMG-CoA in the absence (top) and in the presence (bottom) of MGCH.

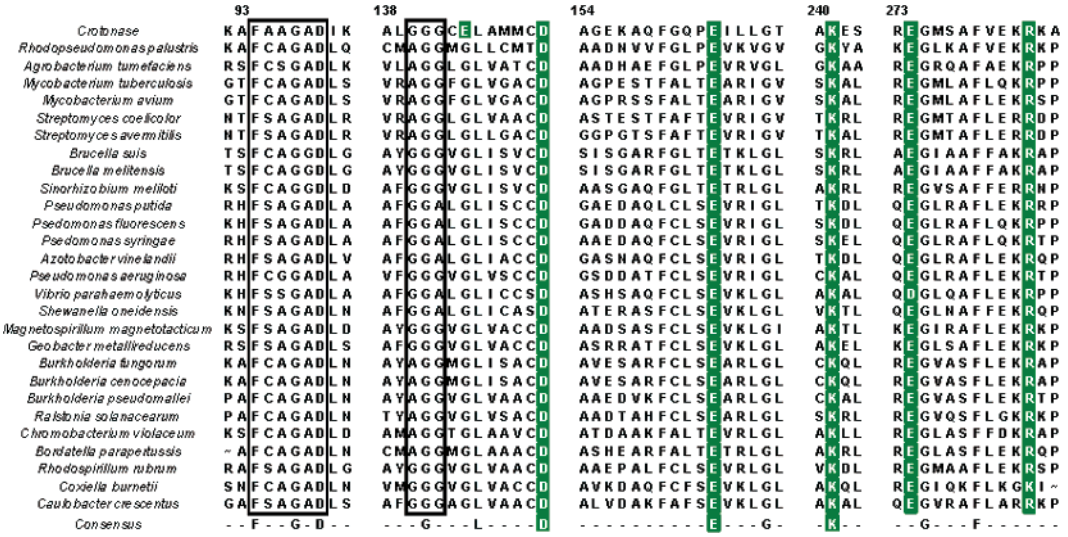


FIGURE 7: Partial sequence alignment of ECHM with 27 orthologs of MGCH. Fully conserved acid/base residues are highlighted in green. The alignment is numbered according to the crotonase sequence.

the gene, verified that it encoded HMG-CoA lyase, and used it to prepare (*R*)-HMG-CoA from racemic HMG-CoA.]

HPLC analyses of incubations of the separate diastereomers of HMG-CoA with MGCH revealed that only the (*S*)-diastereomer is dehydrated to form MG-CoA (Figure 6). Studies of a eukaryotic MGCH established the same substrate specificity (*20*), despite the recent discovery that the eukaryotic MGCHs contain homologues of both active site glutamate residues in crotonase.

We did not investigate the stereochemical course of protonation of C2 in the MGCH-catalyzed reaction. However, on the basis of the presence of Glu138, a homologue of the general acidic glutamate residues found in crotonases and the eukaryotic MGCHs, we are confident that protonation will occur on the *re*-face of the thioester enolate anion intermediate in the active site of the bacterial MGCH, resulting in a *syn*-hydration reaction.

Mechanism of the MGCH-Catalyzed Reaction. In the absence of a crystal structure for MGCH, we aligned the sequences of the 27 identifiable bacterial MGCHs in the databases to identify those conserved residues that could provide acid/base functional groups (Figure 7). MGCH contains a homologue (Glu138) of Glu164 in crotonase, the general acid catalyst that delivers a proton to the α-carbon of the thioester enolate anion intermediate. Based on the structure of ECHM, no other conserved residues are expected to be located in the active site, suggesting that the reaction catalyzed by MGCH utilizes a single protein-supplied acid/base catalyst.

Mutants of Glu138 were constructed. As summarized in Table 2, the E138D and E138Q substitutions decrease k_{cat}/K_M by 90- and 3500-fold, respectively. No activity could be detected for the E138A substitution. We conclude that Glu-138 is essential for the MGCH-catalyzed hydration reaction.

Table 2: Kinetic Constants for Mutants of MGCH Using MG-CoA as Substrate

	k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M (M^{-1} s^{-1})
wild-type	2.1 ± 0.1	$(2.9 \pm 0.56) \times 10^{-5}$	7.2×10^4
E138D	0.64 ± 0.044	$(8.0 \pm 1.6) \times 10^{-5}$	8.0×10^3
E138Q	$0.000\,86 \pm 0.000\,01$	$(4.3 \pm 1.8) \times 10^{-5}$	2.0×10^1
E138A	$\leq 1.3 \times 10^{-6}$	*a*	*a*

[a] Not determined.

Table 3: Kinetic Constants for G118E Mutant Using Truncated Substrates

substrate	k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M ($M^{-1}s^{-1}$)
crotonyl-CoA	$\leq 2.2 \times 10^{-5}$	*a*	*a*
methylcrotonyl-CoA	$\leq 3.1 \times 10^{-5}$	*a*	*a*
methylglutaconyl-CoA	≤ 0.084	*a*	*a*

[a] Not determined.

The γ-carboxylate group of the MG-CoA substrate is necessarily spatially proximal to the β-carbon that is the site for nucleophilic attack by water in the hydration reaction. In ECHM, the general basic catalyst is Glu144; the sequences of all of the identifiable MGCHs contain a glycine at this position (Gly118 in the PP4066). As summarized in Table 1, neither 3-methylcrotonyl-CoA nor crotonyl-CoA, which lack the γ-carboxylate group in MG-CoA are substrates for MGCH although these bind in the active site. We prepared the G118E substitution of PP4066 to assess whether this mutation might allow 3-methylcrotonyl-CoA and crotonyl-CoA to be substrates. As summarized in Table 3, G118E was unable to catalyze the hydration of either of these substrate analogues lacking the γ-carboxylate group; this substitution compromised but did not eliminate the hydration of MG-CoA.

That (1) 3-methylcrotonyl-CoA and crotonyl-CoA, which lack the γ-carboxylate group in MG-CoA, are not substrates but competitively inhibit the enzyme and (2) adding a second protein-supplied acid/base functional group in the form of the G118E mutant does not catalyze their hydration is consistent with the hypothesis that the γ-carboxylate group of MG-CoA activates water for nucleophilic attack on the β-carbon of MG-CoA to form the thioester enolate anion intermediate stabilized by the conserved oxyanion hole. The structure of the bound MG-CoA likely would constrain the orientation of the γ-carboxylate relative to the nucleophilic water so that one of the less basic antiorbitals would participate in proton abstraction (*32*). The conserved Glu138 should be properly positioned to deliver a proton to C2 of the stabilized intermediate to complete the *syn*-hydration (Figure 8A).

This proposed mechanism for hydration of MG-CoA has the novel feature that the substrate provides an essential acid/base catalyst. An analogous catalytic strategy has been suggested for the structurally characterized dehydroquinate synthase in which a phosphate group may catalyze its own *syn*-elimination (*33*).

Alternatively, Glu138 may function as a base in the activation of the nucleophilic water with its resulting conjugate acid completing the reaction by delivering a solvent-derived proton to C2 (Figure 8B). The 1,3-proton-transfer reaction catalyzed by $\Delta^{3,2}$-enoyl-CoA isomerase is also catalyzed by a single glutamate that is a homologue of Glu164 in crotonase and Glu138 in the MGCH from *P. putida* (*34*).

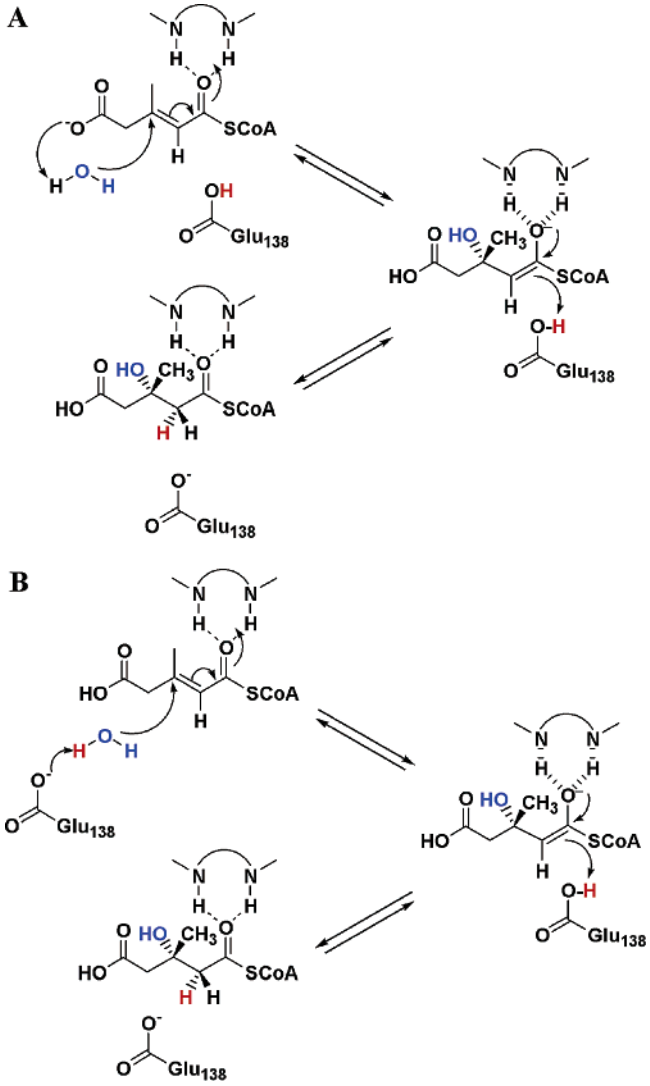


FIGURE 8: Proposed mechanism for MGCH using the (A) γ-carboxylate as a second acid/base catalyst and (B) Glu138 as a single acid/base catalyst.

A high-resolution structure for a bacterial MGCH may allow these mechanisms to be distinguished. Indeed, if our suggestion is correct that a substrate-derived functional group is utilized by the bacterial MGCHs, the mechanistic differences between the bacterial and eukaryotic MGCHs are an intriguing example of opportunistic recruitment and exploitation of the structure of the substrate in the evolution of function in the crotonase superfamily.

CONCLUSIONS

Using genomic context and the catalytic paradigm of the crotonase superfamily, we have assigned function to unknown proteins in *P. putida* and *P. aeruginosa* as MGCHs that catalyze the hydration of MG-CoA to (*S*)-HMG-CoA. The available evidence suggests that a single glutamate (Glu138 in the MGCH from *P. putida*) is the only protein-derived catalytic group in the *syn*-hydration reactions. This

contrasts with the *syn*-hydration of enoyl-CoAs catalyzed by crotonase and the eukaryotic MGCHs that involve two conserved glutamate residues. The discovery that substrate analogues lacking the γ-carboxylate group are competitive inhibitors lends persuasive support to the hypothesis that the substrate functional group is the second acid/base catalyst in the reaction catalyzed by the bacterial MGCHs. If correct, the mechanism of the MGCH-catalyzed reaction provides a novel example of opportunism in the evolution of function in the crotonase superfamily.

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