Effect of Mutagenesis on the Stereochemistry of Enoyl-CoA Hydratase†

Yuguo Feng, Hilary A. Hofstein, Jacque Zwahlen, and Peter J. Tonge*

Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11794-3400

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ABSTRACT: Enoyl-CoA hydratase catalyzes the hydration of *trans*-2-crotonyl-CoA to 3(*S*)-HB-CoA, 3(*S*)-hydroxybutyryl-CoA with a stereospecificity (k_S/k_R) of 400000 to 1 [Wu, W. J., Feng, Y., He, X., Hofstein, H. S., Raleigh, D. P., and Tonge, P. J. (2000) *J. Am. Chem. Soc. 122*, 3987−3994]. Replacement of E164, one of the catalytic glutamates in the active site, with either aspartate or glutamine reduces the rate of formation of the 3(*S*) product enantiomer (k_S) without affecting the rate of formation of the 3(*R*) product (k_R). Consequently, k_S/k_R is 1000 and 0.33 for E164D and E164Q, respectively. In contrast, mutagenesis of E144, the second catalytic glutamate, reduces the rate of formation of both product enantiomers. Thus, only E144 is required for the formation of 3(*R*)-HB-CoA, 3(*R*)-hydroxybutyryl-CoA. Modeling studies together with analysis of α-proton exchange rates and experiments with crotonyl-oxyCoA, a substrate analogue in which the α-proton acidity has been reduced 10000-fold, support a mechanism of 3(*R*)-hydroxybutyryl-CoA formation that involves the E144-catalyzed stepwise addition of water to crotonyl-CoA which is bound in an *s-trans* conformation in the active site. Finally, we also demonstrate that hydrogen bonds in the oxyanion hole, provided by the backbone amide groups of G141 and A98, are important for the formation of both product enantiomers.

Enzymes have the remarkable ability to catalyze chemical transformations with an exceptional degree of stereospecificity. While substrates may have optimal conformations for a particular reaction, the interactions between the substrates and the amino acid residues in the active site dominate the energies and the reactivity of different substrate conformations (*1*, *2*). Consequently, knowledge of the reaction's stereochemistry can provide information on the topology of enzyme−substrate complexes, the distribution and use of catalytic groups, and the binding conformation of the substrate (*3*).

Enoyl-CoA[1] hydratase (crotonase) (EC 4.2.1.17), the second enzyme in the fatty acid *β*-oxidation pathway, catalyzes the stereospecific hydration of *trans*-2-enoyl-CoA thioesters to the corresponding 3(*S*)-hydroxyacyl-CoAs (*4*). The reaction proceeds with *syn* geometry, which is the common stereochemical preference for enzyme-catalyzed reactions where the proton abstracted is α to a carbonyl group of a thioester or ketone (*5*, *6*). In general, *syn* elimination/addition results from the requirement for only a single active site acid/base due to the relatively acidic nature of the thioester or ketone α-protons. This is in contrast to the abstraction of protons α to a carboxylate that requires two active site acid/bases and in which addition/elimination proceeds with *anti* stereochemistry (*7*). However, while enoyl-CoA hydratase is a *syn*-specific enzyme, X-ray crystallography and site-directed mutagenesis have demonstrated that the enzyme uses two active site glutamates (E144 and E164) to catalyze the *syn* addition/elimination reaction (*8*−*13*). In the X-ray structure of the unliganded enzyme (*9*, *10*) and the enzyme complexed with 4-dimethylaminocinnamoyl-CoA (*13*) and hexadienoyl-CoA (*34*), a water molecule is bound between the two glutamates, and we have suggested that these residues act in concert to catalyze the addition of water (*11*). Replacement of E164 with glutamine reduces k_{cat} 340000-fold while the E144 to glutamine substitution reduces k_{cat} 3000-fold. On the basis of structural and kinetic data it is thought that E164, a conserved residue in the crotonase superfamily, is the catalytic acid that protonates the substrate's C2 during hydration while E144 is the catalytic base that activates the H_2O molecule in the active site. In addition, the X-ray crystal structure reveals that the backbone N−H groups of G141 and A98 form an oxyanion hole that hydrogen bonds to the substrate carbonyl oxygen (*9*, *10*, *13*). The hydrogen bond provided by G141 is crucial for the enzyme activity, and elimination of this hydrogen bond by replacing the glycine with proline leads to a 10^6-fold decrease in k_{cat} (*14*).

The absolute stereospecificity of wild-type enoyl-CoA hydratase has previously been determined (*15*). The enzyme catalyzes the incorrect hydration of *trans*-2-crotonyl-CoA (Cr-CoA) to 3(*R*)-hydroxybutyryl-CoA [3(*R*)-HB-CoA] once every 4×10^5 turnovers. However, while the rate of 3(*R*)-HB-CoA formation is much slower than 3(*S*)-hydroxybutyryl-CoA [3(*S*)-HB-CoA] formation, the enzyme must still

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* To whom correspondence should be addressed. Telephone: (631) 632-7907. Fax: (631) 632-7960. E-mail: peter.tonge@sunysb.edu.

[1] Abbreviations: CoA, coenzyme A (lithium salt); dpCr-CoA, 3′-dephospho-*trans*-2-crotonyl-CoA; Cr-CoA, *trans*-2-crotonyl-CoA; Cr-oxyCoA, *trans*-2-crotonyl-oxyCoA; HD-CoA, *trans*,*trans*-2,4-hexadienoyl-CoA; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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catalyze 3(*R*)-HB-CoA formation since the rate is $>10^6$-fold faster than the uncatalyzed reaction. To probe the mechanism of 3(*R*)-HB-CoA formation, the stereochemistry of the reaction catalyzed by several enoyl-CoA hydratase mutants has been studied. The E164Q and E164D mutants, in which the ability to generate 3(*S*)-HB-CoA is substantially reduced, have almost the same efficiency as the wild-type enzyme for catalyzing the formation of 3(*R*)-HB-CoA. The stereospecificity of these two mutants therefore decreases as the rate of 3(*S*)-HB-CoA formation decreases, and for E164Q k_S/k_R is 0.33. However, the E144Q mutant does not catalyze the formation of any appreciable 3(*R*)-HB-CoA. Thus, while both glutamates are crucial for the normal hydration reaction, only E144 is involved in the reaction that generates the 3(*R*) product enantiomer. Since wild-type enoyl-CoA hydratase catalyzes the exchange of the *pro*-2*S* hydroxybutyryl proton with solvent deuterium at a rate similar to the formation of 3(*R*)-HB-CoA, we propose that 3(*R*)-HB-CoA is formed by the E144-catalyzed addition of water to the incorrect face of the substrate's C3═C2 double bond. Modeling studies suggest that this can occur if the substrate is bound in an *s-trans* conformation in the active site. Model building also suggests that E144 is unlikely to be positioned to protonate C2, and consequently, we hypothesize that E144 catalyzes the stepwise addition of water to the substrate with formation of the enol(ate) intermediate being rate limiting. This hypothesis is consistent with the observation that crotonyl-oxyCoA, a substrate analogue that has a 460-fold lower k_{cat} compared to crotonyl-CoA due to the reduced acidity of the α-protons (*16*), does not form any appreciable 3(*R*)-HB-oxyCoA over a period of 5 days with wild-type enzyme. The experiments with crotonyl-oxyCoA also demonstrate that product epimerization does not occur via the reversible abstraction and addition of the hydroxybutyryl C3 proton. Finally, experiments with G141P and A98P, which have respectively 1.6×10^6- and 3400-fold decrease in k_{cat} for substrate hydration, do not catalyze the formation of 3(*R*)-hydroxybutyryl-CoA, indicating that the two hydrogen bonds provided by G141 and A98 to the substrate carbonyl oxygen are very important for the formation of both enantiomers.

EXPERIMENTAL PROCEDURES

Chemicals. Crotonic anhydride, 1,1′-carbonyldiimidazole and coenzyme A (lithium salt) were purchased from Sigma Chemical Co. Deuterium oxide (99.9%) was purchased from Cambridge Isotope Laboratories. Calf intestinal phosphatase (CIP) was purchased from Stratagene.

Preparation of Ligands. 2-*trans*-Crotonyl-CoA (Cr-CoA) was synthesized from crotonic anhydride as previously described (*11*), and 3(*S*)-hydroxybutyryl-CoA [3(*S*)-HB-CoA] was synthesized from Cr-CoA using enoyl-CoA hydratase (*11*). 2-*trans*-Crotonyloxypantetheine was synthesized from crotonic acid and pantoic acid and then converted into 2-*trans*-crotonyl-oxyCoA (Cr-oxyCoA) using enzymes from the CoA biosynthesis pathway (*16*).

Preparation and Purification of Wild-Type and Mutant Enoyl-CoA Hydratases. Recombinant wild-type rat mitochondrial enoyl-CoA hydratase was expressed and purified from cultures of *Escherichia coli* using an N-terminal $(His)_6$ tag as described (*15*). The E164Q, E144Q, and G141P mutants were available from a previous study (*11*, *14*) while the E164D and A98P mutants as well as E144Q/E164Q double mutant were constructed using QuikChange mutagenesis (Stratagene). Mutant plasmids were purified using standard methods, sequenced using the ABI PRISM dye terminator cycle sequencing kit with Ampli Taq DNA Polymerase FS (Perkin-Elmer, Foster City, CA), and transformed into BL21-(DE3) pLysS cells (Novagen) for protein expression. Cultures of BL21(DE3) pLysS cells carrying the wild-type or mutant plasmids were grown in 500 mL of media at 37 °C to an OD_{600} of 0.8. The cells were harvested by centrifugation and resuspended in an equal volume of fresh LB media containing 1 mM isopropyl thiogalactoside (IPTG). The induction was carried out overnight at 37 °C for wild-type, E164D, E164Q, E144Q, and A98P enoyl-CoA hydratases, while an overnight induction at 25 °C was used for the G141P and E144Q/E164Q mutants. The cells were harvested by centrifugation, and the cell pellets were stored at −20 °C prior to protein purification. After thawing, the cells were resuspended in 30 mL of His-bind buffer and lysed using a French press, and the cell debris was removed by ultracentrifugation at 33000 rpm for 1 h. Following metal-affinity chromatography using His-bind resin (Novagen), the His tag was removed using thrombin, and the cleaved His tag and thrombin were removed using a CoA affinity column (*11*).

Active Site Titration with 4-Dimethylaminocinnamoyl-CoA (DAC-CoA). Binding of the active site titrant 4-dimethylaminocinnamoyl-CoA (DAC-CoA) to wild-type enoyl-CoA hydratase results in a 96 nm red shift in λ_{max} for the dimethylamino chromophore from 400 to 496 nm (*11*). The concentration of wild-type enzyme and the E144D, E164D, E144Q, E164Q, E164Q/E144Q mutants was determined by titration with DAC-CoA: ϵ_{max} 48000 M^{-1} cm^{-1} for bound DAC-CoA. Binding of DAC-CoA to the G141P, A98P, and E164D/E144Q mutants did not result in a change in λ_{max} for DAC-CoA, and consequently, the concentrations of these enzymes were determined using the protein absorbance at 280 nm and ϵ_{280} 12300 M^{-1} cm^{-1}.

Kinetic Assays. Steady-state kinetic assays were performed at 25 °C in 20 mM sodium phosphate buffer, pH 7.4, containing 3 mM EDTA. Initial velocities for the hydration of Cr-CoA were measured by monitoring the absorbance change at 280 nm using a Cary-100 spectrometer (Varian). Kinetic parameters, k_{cat} and K_M, were determined by nonlinear least-squares fits of the initial velocity data to the Michaelis−Menten equation using Grafit and ϵ_{280} 3600 M^{-1} cm^{-1}.

NMR Spectroscopy. The ^1H NMR spectra were recorded at the SUNY Stony Brook NMR Center using Varian 600 and 500 MHz spectrometers. All experiments in this study were performed at 25 °C, unless otherwise specified. Sixty-four scans were accumulated for each spectrum, and the NMR data were analyzed using Felix software (Biosym, MSI). Alternations in NMR peak intensities during the incubation of hydroxybutyryl-CoA with enzyme in D_2O buffer were analyzed using eq 1 where A_t is the intensity at

$$A_t = (A_i - A_f) \exp(-k_{obs}t) + A_f \quad (1)$$

time t, A_i is the initial peak intensity, A_f is the final peak intensity, and k_{obs} is the observed first-order rate constant (*17*). The exchange rate, k_{exc}, was calculated from eq 2 which takes into account the total amount of acyl-CoA bound at

$$k_{exc} = k_{obs}[\text{acyl-CoA}]_T/[\text{acyl-CoA}]_B \quad (2)$$

any time. In eq 2, $[\text{acyl-CoA}]_T$ is the total acyl-CoA concentration and $[\text{acyl-CoA}]_B$ is the concentration of bound acyl-CoA. Since the concentration of acyl-CoA (2−3 mM) was significantly larger than the enzyme concentration (14−80 μM) and also than the K_M for both hydroxybutyryl-CoA (10 μM) and crotonyl CoA (5 μM), the concentration of bound acyl-CoA was equal to the enzyme concentration used.

Formation of 3(R)-Hydroxybutyryl-CoA. The stereochemistry for the formation of hydroxybutyryl-CoA was analyzed using the 3(*S*)-HB-CoA dehydrogenase/lactate dehydrogenase coupled assay (*15*). In a typical reaction mixture (total volume of 1 mL), 50 μM HB-CoA was incubated at room temperature with 3.6 units of 3(*S*)-HB-CoA dehydrogenase, 0.03 mM NAD^+, 42 units of lactate dehydrogenase, and 1.2 mM pyruvate in 20 mM sodium phosphate buffer, pH 7.4. After incubation for 1 h, the enzymes were removed by Centricon, and the reaction mixture was analyzed by reversed-phase HPLC using a Vydac C-18 analytical column. Chromatography was performed using 50 mM potassium phosphate/H_2O as buffer A and running a 0−100% gradient of methanol (buffer B) over 40 min at a flow rate of 1 mL/min while monitoring at 260 and 290 nm. The retention time for acetoacetyl-CoA (AcAc-CoA) was 19.8 min and 20.5 min for HB-CoA.

The formation of 3(*R*)-HB-CoA was monitored using the above method following incubation of 3 mM 3(*S*)-HB-CoA or *trans*-Cr-CoA with 14 μM enoyl-CoA hydratase in 20 mM phosphate buffer, pH 7.4 at room temperature, for 72 h. Samples were collected after 2, 4, 6, 20, 48, and 72 h for coupled assay reaction analysis. When the coupled assay revealed there was no further increase in AcAc-CoA, all HB-CoA in the reaction mixture was applied to the coupled assay reaction and then purified using HPLC. The peak corresponding to HB-CoA and presumed to be the 3(*R*) isomer was then isolated by semipreparative reversed-phase HPLC, lyophilized, and characterized by NMR spectroscopy.

RESULTS

Kinetic Parameters for Mutant Enoyl-CoA Hydratases. Kinetic parameters of the mutant enoyl-CoA hydratases were determined using Cr-CoA (Table 1). The data for E164Q has previously been reported (*11*). E144D, E164D, E144Q, and E164Q show respectively 60-, 1200-, 3000-, and 340000-fold decreases in k_{cat} with little change in K_M. Finally, G141P has a 1.6×10^6-fold decrease in k_{cat} with no change in K_M while k_{cat} for the A98P mutant is decreased 3400-fold compared to wild type and K_M is increased 13-fold.

Stereochemistry of the Reaction Catalyzed by E164Q, E164D, and E144Q. The ability of E164Q, E164D, and E144Q to catalyze the formation of 3(*R*)-HB-CoA was studied using the coupled assay. For E164D and E144Q, 3 mM 3(*S*)-HB-CoA was incubated with 14 μM enzyme in 20 mM phosphate buffer, pH 7.4 at room temperature, for 2 days. Samples were taken from the reaction mixture at various times during the incubation, and the coupled assay was used to determine the ratio of 3(*S*)-HB-CoA to 3(*R*)-HB-CoA that was present. The coupled assay involved the stereospecific oxidation of 3(*S*)-HB-CoA to acetoacetyl-CoA, followed by HPLC separation of acetoacetyl-CoA from any remaining hydroxybutyryl-CoA [3(*R*)-HB-CoA] (*15*). The percentage of 3(*R*)-HB-CoA calculated from the ratio of the two HPLC peaks was plotted against time. Curve fitting and data analysis yielded a first-order rate constant for the formation of 3(*R*)-HB-CoA (k_R) of 0.0015 s^{-1} for E164D (Figure 1, Table 2), which is similar to the value of k_R previously determined for wild-type enoyl-CoA hydratase (0.0044 s^{-1}) (*15*). In contrast, experiments with E144Q indicated that this mutant was unable to catalyze the formation of 3(*R*)-HB-CoA even when the incubation was extended to 4 days. Assuming we could detect the formation of 5% 3(*R*)-HB-CoA, k_R for E144Q must be less than 2.1×10^{-5} s^{-1} (Table 2).

While E144Q and E164D, as well as wild-type enoyl-CoA hydratase, catalyze the rapid interconversion of substrate and the 3(*S*) product relative to the rate of 3(*R*)-HB-CoA formation, E164Q catalyzes the formation of both product enantiomers at similar rates. Consequently, to simplify direct measurement of k_R and k_S, the stereochemical studies with E164Q were initiated using Cr-CoA (3 mM) rather than 3(*S*)-HB-CoA. Samples were taken at various intervals and analyzed using the coupled assay. These data demonstrated that k_R for E164Q is actually larger than k_S, since the ratio of 3(*R*)-HB-CoA to (*S*)-HB-CoA in the initial samples was around 3:1 (Figure 2). This ratio remained constant over the first 2 h of the reaction while the total amount of hydroxybutyryl-CoA increased due to slow hydration of the substrate. The 3:1 ratio of 3(*R*) to 3(*S*) product enables us to estimate that k_S/k_R for E164Q was around 0.33 (Table 2). Since k_{cat} for E164Q (0.0053 s^{-1}) determined previously should be the sum of k_S and k_R, we estimate k_S and k_R to be 0.0013 $s^{-1}$and 0.004 s^{-1}, respectively (Table 2). The value of k_R for E164Q is identical to that for the wild-type enzyme, and thus, replacement of E164 with aspartate or glutamine does not affect the rate of 3(*R*)-HB-CoA formation.

Stereochemistry of G141P and A98P. To examine the importance of the oxyanion hole in catalyzing the formation of 3(*R*)-HB-CoA, the stereochemistry of the G141P and A98P mutants was studied. After 72 h of incubating 3 mM Cr-CoA with 14 μM G141P in 20 mM phosphate buffer, pH 7.4 (H_2O), coupled assays demonstrated that there was less than 5% 3(*R*)-HB-CoA present in the reaction mixture. Thus k_R must be less than 4.3×10^{-5} s^{-1} for G141P (Table 2). In addition, NMR spectroscopy was used to monitor the α-proton exchange reaction catalyzed by G141P (Figure 3). Addition of 80 μM G141P to 3 mM Cr-CoA in D_2O buffer resulted in the immediate appearance of resonances at 2.75 and 1.21 ppm, assigned to the C2−H and C4−H HB-CoA protons, and a large decrease in intensity of the C4−H Cr-CoA resonance at 1.86 ppm. Following these initial changes in the NMR spectrum, resulting from the hydration of Cr-CoA, the ratio of the peak areas for the C2−H and C4−H HB-CoA resonances remained at 1:3 over the next 48 h (Figure 3). The lack of exchange of the C2−H HB-CoA proton is consistent with the observation that G141P is unable to catalyze the formation of 3(*R*)-HB-CoA over the course of the experiment (*15*). Assuming we could detect a 2% change in C2−H peak integration, k_{exc} for G141P must be less than 4.3×10^{-6} s^{-1} (Table 2). In addition, the coupling constant of the C2−H HB-CoA resonance was 4.0 Hz, consistent with the assignment of this peak to the *2*(*S*) proton of 3(*S*)-HB-CoA (*15*). Consequently, the formation

Table 1: Kinetic Parameters for Wild-Type and Mutant Enoyl-CoA Hydratases[a]

enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1} s^{-1}$)	decrease in k_{cat} relative to wild type
wild type	1790 ± 120	15 ± 2	119 ± 24	1
E144D[b]	26 ± 1	118 ± 10	0.22 ± 0.03	50
E164D	1.50 ± 0.01	32 ± 5	0.047 ± 0.008	1200
E144Q	0.60 ± 0.01	25 ± 2	0.024 ± 0.002	3000
E164Q[c]	0.0053 ± 0.0003	41 ± 6	$(1.3 \pm 0.3) \times 10^{-4}$	340000
A98P	0.53 ± 0.02	195 ± 10	0.0027 ± 0.0002	3400
G141P[d]	0.0011 ± 0.0001	3 ± 1	$(3.7 \pm 1.5) \times 10^{-4}$	1630000

[a] Kinetic parameters were determined at pH 7.4 in 20 mM sodium phosphate buffer containing 3 mM EDTA at 25 °C. The substrate used was crotonyl-CoA (Cr-CoA). [b] Data with 3′-dephosphocrotonyl-CoA. The decrease in k_{cat} relative to k_{cat} was determined for wild-type enzyme with the same substrate (1380 s^{-1}) (*11*). [c] Data from Hofstein et al. (*11*). [d] Data from Bell et al. (*14*).

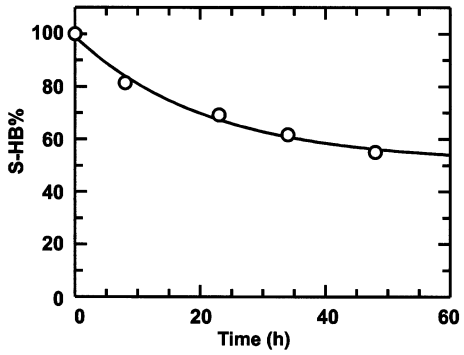


FIGURE 1: Formation of 3(*R*)-hydroxybutyryl-CoA catalyzed by E164D. The amount of 3(*S*)-hydroxybutyryl-CoA present (percent) as a function of time was determined at room temperature in a reaction mixture containing 3 mM 3(*S*)-hydroxybutyryl-CoA and 14 μM E164D (20 mM phosphate, 0.3 M NaCl, 3 mM EDTA, pH 7.4). Samples were collected at various times and analyzed using HPLC following reaction with 3(*S*)-hydroxyacyl-CoA dehydrogenase in a coupled assay.

of 3(*S*)-HB-CoA catalyzed by G141P also occurs with the stereospecific protonation of the C2 center, consistent with the *syn* addition of water (D_2O) to the Cr-CoA double bond.

Since k_{exc} is a rate constant and independent of substrate and enzyme concentration, using a lower substrate concentration or a higher enzyme concentration will increase k_{obs} (eq 2), thereby facilitating experimental determination of k_{exc}. Consequently, the substrate concentration was reduced from 3 to 0.3 mM for experiments with the A98P mutant. However, following incubation of 0.3 mM Cr-CoA with 14 μM A98P in 20 mM phosphate buffer (pH 7.4, H_2O) for 72 h, which is equivalent to a 720 h incubation of 3 mM Cr-CoA with 14 μM enzyme, less than 5% of 3(*R*)-HB-CoA was formed on the basis of the coupled assay. In addition, similar to G141P, NMR spectroscopy revealed that A98P only catalyzed the exchange of the *pro*-2*R* proton with solvent deuterium over 72 h (data not shown). Consequently, k_R for A98P must be less than 4.3×10^{-6} s^{-1} while k_{exc} must be less than 2.9×10^{-6} s^{-1} (Table 2). Therefore, like G141P, the A98P mutant has a severely compromised ability for catalyzing the formation of 3(*R*)-HB-CoA.

Stereochemistry of the Reaction with trans-2-Crotonyl-oxyCoA. trans-2-Crotonyl-oxyCoA (Cr-oxyCoA) is a substrate analogue of Cr-CoA in which the thioester sulfur atom has been substituted by oxygen. k_{cat} for the reaction of Cr-oxyCoA with wild-type enoyl-CoA hydratase (3.9 ± 0.1 s^{-1}) is ca. 460-fold slower than for Cr-CoA (k_{cat} 1790 ± 120 s^{-1}), presumably due to the decreased acidity of the oxyester α-protons (*16*). To determine if α-proton acidity also influenced the formation of the incorrect substrate enantiomer, we attempted to measure the rate of formation of 3(*R*)-hydroxybutyryl-oxyCoA [3(*R*)-HB-oxyCoA]. Consequently, 3 mM Cr-oxyCoA was incubated with 10 μM wild-type enoyl-CoA hydratase in D_2O buffer (20 mM phosphate, pD 7.4). However, even after 2 weeks no appreciable formation of the 3(*R*) enantiomer could be detected using the coupled assay. If the limit of our ability to detect formation of the 3(*R*) enantiomer is 5%, then k_R for Cr-oxyCoA must be less than 1.2×10^{-5} s^{-1} (Table 2), a decrease of at least 56-fold compared to the formation of 3(*R*)-HB-CoA by wild-type enoyl-CoA hydratase in D_2O (k_R 6.8×10^{-4} s^{-1}) (*15*). Similar results were obtained from analysis of α-proton exchange rates using NMR spectroscopy (Figure 4). Thus, addition of 80 μM enzyme to 3 mM Cr-oxyCoA in D_2O phosphate buffer (20 mM, pD 7.4) resulted in the immediate appearance of the hydroxybutyryl C2−H resonance at 2.55 ppm with an integration of 1 proton, as well as the hydroxybutyryl C4−H resonance at 1.22 ppm. The coupling constant of the C2−H resonance was 4.5 Hz, indicating the peak was due to the 2(*S*) proton. However, no change was observed in the intensity of the 2(*S*) proton over 2 weeks. Since we estimate that we could detect a 2% change in C2−H peak integration, k_{exc} for the oxyCoA substrate must be less than 5.0×10^{-6} s^{-1} (Table 2), a decrease of at least 140-fold compared to the equivalent reaction with Cr-CoA.

DISCUSSION

The hydration reaction catalyzed by enoyl-CoA hydratase is highly stereospecific. Our previous studies showed that the wild-type enzyme catalyzes the incorrect hydration of Cr-CoA to 3(*R*)-HB-CoA once every 4×10^5 turnovers. Although k_R is much slower than k_S, it is still $>10^6$-fold faster than the uncatalyzed reaction. To explore the strategy used by the enzyme to achieve its stereospecificity, we have analyzed the impact of site-directed mutagenesis on the relative ability of the enzyme to form the 3(*S*) and 3(*R*) product enantiomers.

Initial proposals for the role of E164 and E144 in catalysis involved E164 acting as a catalytic acid to protonate C2 while E144 acted as a catalytic base to activate the bound H_2O molecule (*11*). Bahnson and co-workers have recently revised this model and suggested that E164 acts as both the acid and base while E144 is involved in positioning the bound water molecule (*13*). While the present studies cannot distinguish between these two possibilities, the mutagenesis data reinforce the critical role for both residues in the

Table 2: Rate of 3(*R*) Product Enantiomer Formation (k_R) and α-Proton Exchange (k_{exc}) Catalyzed by Wild-Type and Mutant Enoyl-CoA Hydratases

enzyme	k_S (s^{-1})[a]	k_R (s^{-1})[b]	k_S/k_R	k_{exc} (s^{-1})[c]
wild type	1790 ± 120	$(4.4 \pm 0.1) \times 10^{-3}$	4.1×10^5	6.8×10^{-4}
E164D	1.50 ± 0.01	$(1.5 \pm 0.2) \times 10^{-3}$	1000	ND[d]
E144Q	0.60 ± 0.01	$<2.1 \times 10^{-5}$ [e]	$>2.9 \times 10^4$	$<2.0 \times 10^{-6}$ [f]
E164Q	0.0012 ± 0.0001	$(4.1 \pm 0.1) \times 10^{-3}$	0.33	ND[d]
A98P	0.53 ± 0.02	$<4.3 \times 10^{-6}$ [e]	$>1.2 \times 10^5$	$<2.9 \times 10^{-6}$ [f]
G141P	0.0011 ± 0.0001	$<4.3 \times 10^{-5}$ [e]	>25	$<4.3 \times 10^{-6}$ [f]
wild type (Cr-oxyCoA)[g]	3.9 ± 0.1	$<1.2 \times 10^{-5}$ [h] (D_2O)	$>3.3 \times 10^5$	$<5.0 \times 10^{-6}$ [f]

[a] k_S is k_{cat} determined for each enzyme using Cr-CoA. [b] k_R is the rate constant for the formation of 3(*R*)-hydroxybutyryl-CoA determined using the coupled assay. [c] k_{exc} is the rate constant for exchange of the *pro*-2*S* proton with solvent deuterium determined by NMR spectroscopy. [d] Not determined. [e] Upper limit for k_R based on the sensitivity of the coupled assay to detect 3(*R*)-hydroxybutyryl-CoA (estimated at 5%). [f] Upper limit for k_{exc} based on the sensitivity of NMR spectroscopy to detect exchange of the C2−H (estimated at 2%). [g] Experiments using *trans*-2-crotonyl-oxyCoA (Cr-oxyCoA). k_S (k_{cat}) is taken from Dai et al. (*16*). [h] Attempts to detect formation of 3(*R*)-hydroxybutyryl-oxyCoA (k_R determination) involved coupled assay analysis of the same sample in D_2O that was used for the NMR experiment (k_{exc} determination).

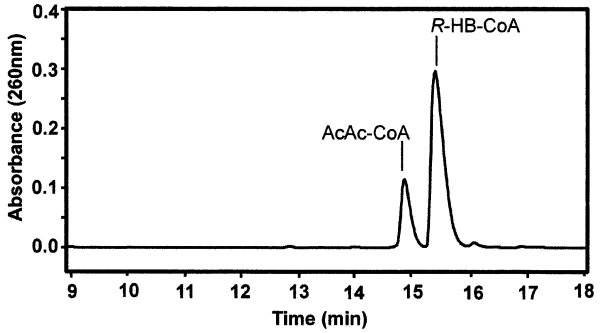


FIGURE 2: HPLC analysis of the E164Q-catalyzed formation of 3(*R*)-hydroxybutyryl-CoA. The reaction mixture contained 3 mM *trans*-2-crotonyl-CoA and 14 μM E164Q in 20 mM phosphate, 0.3 M NaCl, and 3 mM EDTA, pH 7.4. Following incubation at room temperature for 30 min, an aliquot was withdrawn, and the amount of 3(*R*)-hydroxybutyryl-CoA was determined by HPLC following reaction with 3(*S*)-hydroxyacyl-CoA dehydrogenase using the coupled assay. The peak labeled AcAc is acetoacetyl-CoA generated by oxidation of 3(*S*)-HB-CoA while the peak labeled *R*-HB-CoA is hydroxybutyryl-CoA that did not react with the dehydrogenase and is consequently assigned to 3(*R*)-HB-CoA.

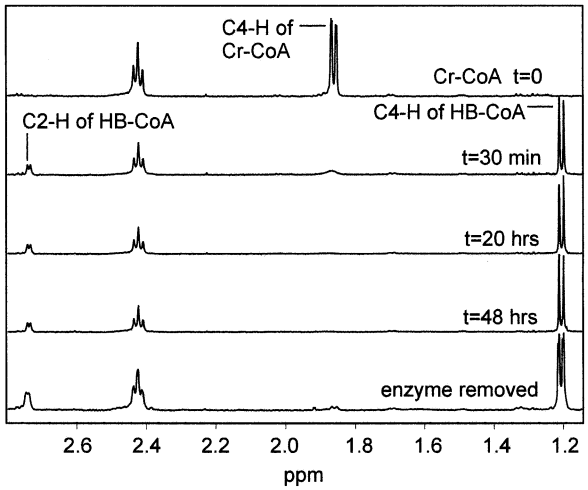


FIGURE 3: Selected ^1H NMR spectra of *trans*-2-crotonyl-CoA (3.0 mM) incubated with G141P (80.0 μM) in D_2O buffer, pD 7.4 (20 mM NaH_2PO_4, 300 mM KCl), at 25 °C. Incubation times are shown on the right-hand side of each spectrum, and only the region from 2.82 to 1.26 ppm is shown. Sixty-four scans were accumulated for each spectrum. The peak labeled C2−H corresponds to the hydroxybutyryl α-proton(s), the peak labeled C4 (HB-CoA) corresponds to the hydroxybutyryl methyl group, and the peak labeled C4 (Cr-CoA) corresponds to the C4 methyl group of crotonyl-CoA. The top spectrum was obtained prior to addition of enzyme, and the bottom spectrum was obtained following removal of the enzyme by Centricon filtration.

formation of 3(*S*)-HB-CoA. Both E164D and E144D, in which the functional groups are still present but have moved about 1.5 Å away from C2 and C3, respectively, show substantial reductions in the rate of the normal hydration reaction. This indicates that the precise positioning and orientation of the catalytic residues in the active site of the wild-type enzyme are critical for efficient substrate hydration. In addition, E164D, E164Q, and wild-type enoyl-CoA hydratase all have similar values of k_R, and thus the stereospecificity of the enzyme-catalyzed reaction is reduced substantially in these mutants. The ratios of k_S/k_R for E164D and E164Q are 1000 and 0.33, respectively. Consequently, the reaction catalyzed by E164Q is no longer stereospecific. The fact that mutation of E164 does not affect the enzyme's ability to form the 3(*R*) enantiomer leads to the conclusion that only E144 is involved in this reaction. Interestingly, no 3(*R*)-HB-oxyCoA was formed by wild-type enoyl-CoA hydratase over a 2 week period using Cr-oxyCoA, a substrate analogue in which the α-protons are ca. 10000-fold less acidic. This suggests that protonation/deprotonation at C2 is rate limiting for formation of the 3(*R*) product and that E144 in the E164Q mutant must be equally efficient at catalyzing proton transfer at C2. Studies with wild-type and the aspartate mutants clearly indicate that both glutamates have to be optimally positioned for generation of the correct 3(*S*) product enantiomer. For formation of 3(*R*)-HB-CoA, the substrate cannot be oriented in the active site to make optimal use of both glutamates simultaneously.

G141 is conserved throughout the crotonase superfamily members and resides at the N-terminus of a short α-helix (*18*, *19*). The G141 peptidic NH group together with other NH groups, such as A98 in enoyl-CoA hydratase, comprises the oxyanion hole for stabilizing negative charge accumulation on the thioester carbonyl group. The hydrogen bond between G141 and the substrate is eliminated in the G141P mutant. Although this is a nonconservative mutation, the similarity of K_M values for G141P and wild-type enzymes suggests that the active site is largely intact in G141P, a hypothesis substantiated by preliminary X-ray crystallographic studies (unpublished data). The extremely low k_{cat} of G141P indicates the hydrogen bond provided by the amide group from G141 is crucial for catalysis, and Raman studies of G141P have shown that it is not able to polarize the substrate analogue hexadienoyl-CoA, supporting a role for

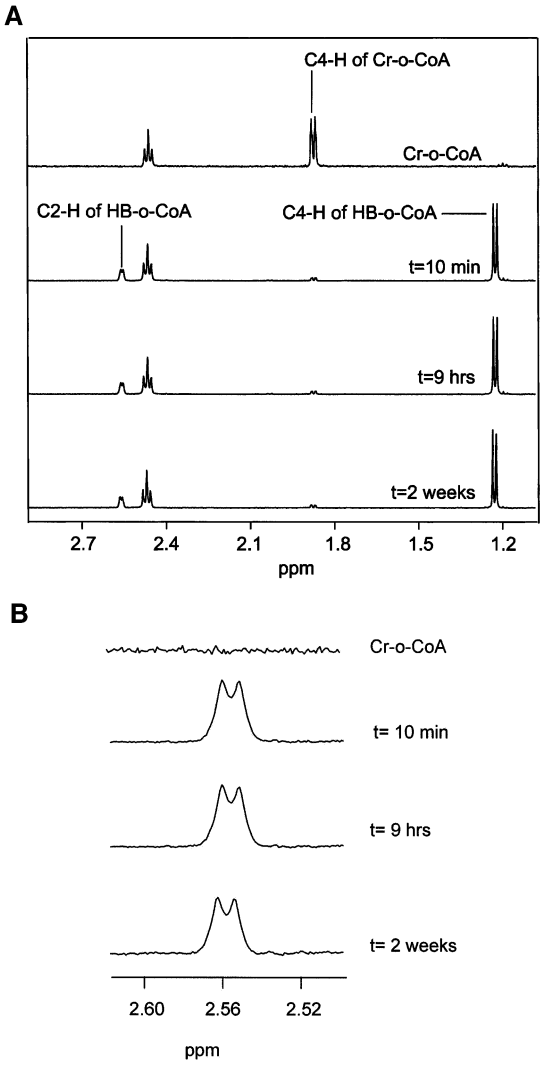


FIGURE 4: Selective ^{1}H NMR spectra of *trans*-2-crotonyl-oxyCoA (3.0 mM) incubated with wild-type enoyl-CoA hydratase (80 μM) in D_2O buffer, pD 7.4 (20 mM NaH_2PO_4, 300 mM KCl) at 25 °C. Only the region from 2.90 to 1.08 ppm is shown. Sixty-four scans were accumulated for each spectrum. The peak labeled C2−H corresponds to the hydroxybutyryl α-proton(s), the peak labeled C4 (HB-o-CoA) corresponds to the hydroxybutyryl methyl group, and the peak labeled C4 (Cr-o-CoA) corresponds to the C4 methyl group of crotonyl-oxyCoA. (A) Incubation times are shown on the right-hand side of each spectrum, and the top spectrum was obtained prior to addition of enzyme. (B) Enlargement of the region from 2.62 to 2.50 ppm.

the oxyanion hole in substrate activation (*14*). The present studies demonstrate that A98 also plays an important role in substrate hydration and k_{cat} for A98P is reduced 3000-fold compared to wild-type enzyme. Importantly, neither G141P nor A98P make any appreciable 3(*R*)-HB-CoA during extended incubations with Cr-CoA. Thus, the oxyanion hole is also critical for the incorrect hydration of the substrate.

Mechanism of 3(R)-HB-CoA Formation. There are two possible mechanisms for the formation of 3(*R*)-HB-CoA (Scheme 1). In the first mechanism, the enzyme catalyzes the epimerization of HB-CoA by reversible deprotonation/reprotonation at C3 (*20*), while in the second mechanism the water molecule is added to the incorrect face of the substrate's double bond. Additionally, there are two possible ways in which the incorrect addition of water can occur. The water molecule can be positioned on the opposite side of the active site (mechanism 2a) or rotation about the substrate's C1−C2 single bond can present the opposite face of the substrate's double bond to the water molecule (mechanism 2b).

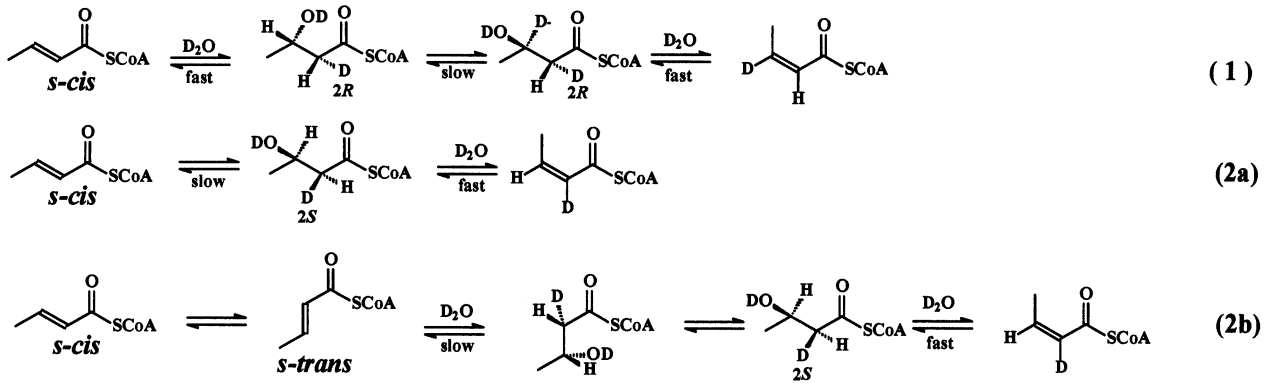
Mechanism 1 must be considered unlikely at the outset since it involves deprotonation/reprotonation at an unactivated stereo center in which the pK_a of the proton is presumably very high (pK_a > 30) (*20*). Three experimental observations are consistent with this expectation. First, reversible removal of the C3 proton would result in deuteration at C3 if the proton that was removed exchanged with solvent deuterium before being replaced. However, the integration of the C3−H proton showed no change during the NMR experiments in D_2O. Second, even if the abstracted C3 proton cannot exchange with solvent, mechanism 1 does not explain how solvent exchange at C2 can occur. Last, it might be expected that the rate of epimerization at C3 would be unaffected by the chemical nature of the bond linking the fatty acid to CoA. However, incubation of crotonyl-oxyCoA with wild-type enzyme over a period of 5 days resulted in no detectable formation of 3(*R*)-HB-oxyCoA.

The similarity of k_R and k_{exc} suggests that incorrect substrate hydration is coupled to α-proton exchange. This can most readily be envisaged by the *syn* addition of water to the opposite face of Cr-CoA substrate which will result in the formation of 3(*R*)-HB-CoA in which the *pro*-2*S* proton is derived from solvent (mechanism 2). In mechanism 2a, a water molecule must approach the substrate's double bond from the opposite side of the active site, and modeling studies suggest that there is sufficient space in the active site to permit a water molecule to be located in the correct orientation. However, experiments with E144Q indicate that at least one glutamate is involved in 3(*R*)-HB-CoA formation. Consequently, the water molecule must be able to interact with this glutamate, which would not be possible if it were located on the far side of the active site.

In an alternative mechanism (2b), the required water molecule is located in similar positions for formation of both 3(*S*) and 3(*R*) products, and the two product enantiomers result from the addition of water to the *s-cis* and *s-trans* substrate conformers, respectively. In the *s-cis* conformer, the enoyl double bond is optimally positioned with respect to both glutamates in contrast to the *s-trans* conformer. As a result, mutation of either glutamate, even with the conservative change to an aspartate, has a dramatic effect on the hydration of the *s-cis* conformer. In contrast, only E144 is involved in hydration of the *s-trans* conformer, and so mutation of E164 does not affect the rate of 3(*R*)-HB-CoA formation. However, the carbonyl oxygen in both *s-cis* and *s-trans* conformations must still be hydrogen bonded to G141 and A98, consistent with the importance of the oxyanion hole in the formation of both product enantiomers. In support of mechanism 2a, recent Raman studies suggest that Cr-CoA is likely bound to the enzyme in two conformations, *s-cis* and *s-trans*, about the C1−C2 single bond (*34*).

Implications for the Stereochemistry of Fatty Acid Metabolism. The complete degradation of fatty acids containing *cis* double bonds requires the action of enzymes in addition to those that comprise the main β-oxidation pathway, since the action of enoyl-CoA hydratase on *cis*-2-enoyl-CoA results in the formation of 3(*R*)-hydroxyacyl-CoAs which cannot be oxidized by 3(*S*)-hydroxyacyl-CoA dehydrogenase. The

Scheme 1: Proposed Mechanisms for Formation of 3(*R*)-Hydroxybutyryl-CoA



two main mechanisms for overcoming this problem involve either the epimerization of 3(*R*)-hydroxyacyl-CoA or the reduction and isomerization of *cis*-4-*trans*-2-dienoyl-CoA to *trans*-2-enoyl-CoA (*21*−*24*). The latter pathway is active in mammalian mitochondria while the former is present in peroxisomes, and both pathways are operative in bacteria and plants (*23*, *25*, *26*). The interconversion of 3(*R*)- and 3(*S*)-hydroxyacyl-CoAs has been postulated to occur either via the direct epimerization of hydroxyacyl-CoA or by a dehydration/rehydration pathway that involves an *R*-specific enoyl-CoA hydratase (*27*−*29*). While the latter enzyme is clearly distinct from the normal *S*-specific enoyl-CoA hydratase, Schulz, Yang, and co-workers have demonstrated that the enoyl-CoA hydratase active site in the multienzyme bacterial fatty acid complex can catalyze the epimerization of 3-hydroxyacyl-CoA (*30*−*32*). The latter result strongly suggests that a single active site can catalyze the dehydration of 3(*R*)-hydroxyacyl-CoA to *trans*-2-enoyl-CoA.

Our present results indicate that the *S*-specific enoyl-CoA hydratase can catalyze the formation of both 3(*S*)- and 3(*R*)-HB-CoA from CR-CoA but by different mechanisms. Formation of 3(*R*)-HB-CoA relies on a single acid/base and occurs 400000-fold slower than the formation of the 3(*S*) product in which two catalytic glutamates are involved. While the interconversion of Cr-CoA and 3(*R*)-HB-CoA catalyzed by this enoyl-CoA hydratase is likely to be too slow to be metabolically significant, our results are germane to the mechanistic hurdles faced when a single active site catalyzes the hydration of *trans*-2-enoyl-CoA to both the 3(*S*)- and 3(*R*)-hydroxyacyl-CoAs. On the basis of the present studies we postulate that enzymes such as the bacterial fatty acid oxidation complex that can catalyze both reactions (*33*) do so by hydration of the *s-cis* and *s-trans* conformers of enoyl-CoA by a water molecule that is similarly positioned for both conformers. This requires only one set of catalytic acid/base residue(s) rather than the duplication that would result from addition of water to either face of a single bound enoyl-CoA conformer in which two water molecules would have to be positioned on opposite sides of the active site. Consistent with this hypothesis, mutagenesis of E139, the putative catalytic base in the bacterial fatty acid oxidation complex and the homologue of E164 in the mammalian enoyl-CoA hydratase, prevents the dehydration of both 3(*S*)- and 3(*R*)-hydroxyacyl-CoAs (*33*). Further similarity between the bacterial and mammalian enzymes is found in the observation that replacement of G116 in the bacterial enzyme with a phenylalanine also eliminates the hydratase/epimerase activity of the fatty acid oxidation complex. G116 is the homologue of G141 in the mammalian enzyme and is presumably involved in stabilizing the transition states for the dehydration of both hydroxyacyl-CoA enantiomers.

CONCLUSION

Enoyl-CoA hydratase catalyzes the hydration of Cr-CoA to 3(*S*)-HB-CoA with a stereospecificity of 1 in 4×10^5. Formation of the correct product enantiomer requires an intact oxyanion hole and optimal positioning of the substrate with respect to two catalytic glutamates (E144 and E164) in the active site. Formation of the incorrect product enantiomer is also catalyzed by the enzyme, since the uncatalyzed hydration reaction is still at least 10^6-fold slower than the rate of 3(*R*)-HB-CoA formation. However, while generation of the 3(*R*) product enantiomer also requires the oxyanion hole, only one of the two active site glutamates (E144) is involved. These observations are rationalized by the hypothesis that the substrate is bound in two conformers to the enzyme, only one of which is optimally positioned with respect to the enzyme's catalytic machinery.

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