Role of Glutamate 144 and Glutamate 164 in the Catalytic Mechanism of Enoyl-CoA Hydratase†

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ABSTRACT: The role of two glutamate residues (E164 and E144) in the active site of enoyl-CoA hydratase has been probed by site-directed mutagenesis. The catalytic activity of the E164Q and E144Q mutants has been determined using 3′-dephosphocrotonyl-CoA. Removal of the 3′-phosphate group reduces the affinity of the substrate for the enzyme, thereby facilitating the determination of K_m and simplifying the analysis of the enzymes' pH dependence. k_{cat} for the hydration of 3′-dephosphocrotonyl-CoA is reduced 7700-fold for the E144Q mutant and 630000-fold for the E164Q mutant, while K_m is unaffected. These results indicate that both glutamate residues play crucial roles in the hydration chemistry catalyzed by the enzyme. Previously, we reported that, in contrast to the wild-type enzyme, the E164Q mutant was unable to exchange the α-proton of butyryl-CoA with D_2O [D′Ordine, R. L., Bahnson, B. J., Tonge, P. J., and Anderson, V. E. (1994) *Biochemistry 33,* 14733−14742]. Here we demonstrate that E144Q is also unable to catalyze α-proton exchange even though E164, the glutamate that is positioned to abstract the α-proton, is intact in the active site. The catalytic function of each residue has been further investigated by exploring the ability of the wild-type and mutant enzymes to eliminate 2-mercaptobenzothiazole from 4-(2-benzothiazole)-4-thiabutanoyl-CoA (BTTB−CoA). As expected, reactivity toward BTTB−CoA is substantially reduced (690-fold) for the E164Q enzyme compared to wild-type. However, E144Q is also less active than wild-type (180-fold) even though elimination of 2-mercaptobenzothiazole (pK_a 6.8) should require no assistance from an acid catalyst. Clearly, the ability of E164 to function as an acid−base in the active site is affected by mutation of E144 and it is concluded that the two glutamates act in concert to effect catalysis.

Enoyl-CoA hydratase (EC 4.2.1.17) is the second enzyme in the fatty acid β-oxidation pathway and catalyzes the syn hydration of α,β-unsaturated fatty acid CoA thiolesters (*1*). On the basis of detailed kinetic isotope effect studies, it has been proposed that the mechanism is concerted (*2, 3*), with both C2−H and C3−O bonds being formed in the same transition state. In Scheme 1, an enzyme group (B−H) supplies the required proton at C2 concomitant with the unassisted attack of water at C3. The proposal for the use of an unactivated water molecule as the nucleophile is based on the lack of a solvent isotope effect on V/K for the dehydration of 3-hydroxybutyrylpantetheine. This could be accommodated by a single-base mechanism, which is consistent with a syn addition/elimination reaction based on the stereochemical course of the reaction (*4*).

Sequence alignment studies provided the first information on the identity of catalytic residues in the active site of enoyl-CoA hydratase. Site-directed mutagenesis demonstrated that E165 in enoyl-CoA isomerase, a homologous enzyme, was an important catalytic residue and sequence alignment data suggested that E164 was the equivalent residue in enoyl-CoA hydratase (*5*). Subsequently, we demonstrated that mutation of E164 to glutamine resulted in a large decrease in activity and resulted in the inability of the enzyme to catalyze α-proton exchange with solvent (*6*). Further kinetic analysis indicated that k_{cat} for the E164Q enzyme was reduced 100000-fold relative to wild-type enzyme (*7*). Furthermore, the equivalent residue in the fatty acid oxidation complex (E139) was also shown to be essential for hydratase activity (*8*). The importance of E164 as a catalytic acid−base has since been substantiated by the X-ray crystal structure of enoyl-CoA hydratase, which shows E164 in a position to catalyze protonation/deprotonation of the substrate's α-carbon (*9, 10*).

The X-ray crystal structure of enoyl-CoA hydratase (*9, 10*) together with homology-modeling studies based on the X-ray crystal structure of 4-chlorobenzoyl-CoA dehalogenase (*11*) provided evidence for the existence of a second acid−base residue, E144, in the active site of enoyl-CoA hydratase. Preliminary kinetic analysis of the E144Q enzyme suggested that this residue also played a role in catalysis (*11*). Structural analysis of acetoacetyl-CoA and octanoyl-CoA bound to the

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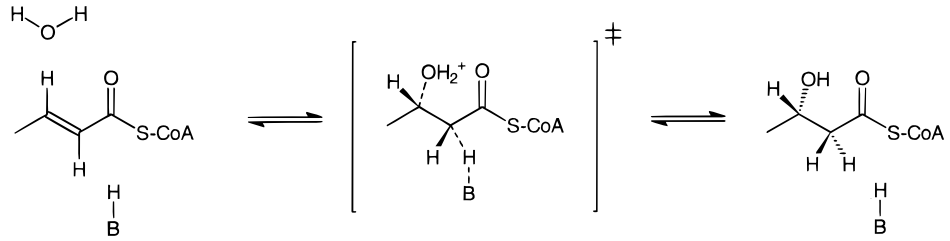
Scheme 1: Concerted Reaction



enzyme reveals that E144 is in a position to facilitate the addition of water to the C3 position of the substrate. The structural studies also reveal a water molecule bound between E144 and E164 in the unliganded enzyme which, following superposition of the ligand-bound structure, is positioned close to the substrate's C3 carbon.

To further investigate the role of E144 in the catalytic mechanism of enoyl-CoA hydratase, we have determined the pH dependence of k_{cat} and K_m for the hydration of 3′-dephosphocrotonyl-CoA (dpCr−CoA)[1] catalyzed by the wild-type, E144Q, and E164Q enzymes. Removal of the 3′ phosphate group from CoA raises K_m, thus facilitating accurate determination of the kinetic parameters, and removes an ionizable group in the substrate that could interfere with interpretation of the pH dependence data. In addition, the ability of E144Q to catalyze α-proton exchange has been analyzed together with the ability of E144Q and E164Q to catalyze the elimination of 2-mercaptobenzothiazole from 4-(2-benzothiazole)-4-thiabutanoyl-CoA (BTTB−CoA).[1] The results demonstrate that E144 and E164 act cooperatively to catalyze proton abstraction from the substrate.

EXPERIMENTAL PROCEDURES

Chemicals. Crotonic anhydride, butyric anhydride, 4-dimethylaminocinnamic acid, 1,1′-carbonyldiimidazole, and coenzyme A (lithium salt; CoA)[1] were purchased from Sigma Chemical Co. Deuterium oxide (99.9%) was purchased from Cambridge Isotope Labs, and calf intestinal phosphatase (CIP)[1] was from Stratagene. 4-(2-Benzothiazole)-4-thiabutanoic acid was a gift from Dr. Colin Thorpe, University of Delaware.

Preparation of Butyryl-CoA, Crotonyl-CoA, 3′-Dephosphocrotonyl-CoA, 4-Dimethylaminocinnamoyl-CoA, and 4-(2-Benzothiazole)-4-thiabutanoyl-CoA. Butyryl-CoA (Bu-CoA)[1] was synthesized by coupling butyric anhydride with CoA. Butyric anhydride was added dropwise to a solution of CoA in water, ethanol, and ethyl acetate (1:1:1) with stirring at RT. The reaction progress was monitored by following the concentration of free thiol in solution using 5,5′-dithio-bis-(2-nitrobenzoic acid) [DTNB[1] (*12*)]. When no free thiol was detected, the solution was concentrated in vacuo to remove the organic solvent and the Bu-CoA was purified by HPLC (Shimadzu) using an Alltech Econosil C-18 semipreparative column. Chromatography was performed using 0.2 M ammonium acetate/1.75% acetonitrile as buffer A and running a 0 to 25% gradient of 95% acetonitrile/5% water (buffer B) over 40 min at a flow rate of 4 mL/min. Elution was monitored at 260 and 290 nm using a Shimadzu SPD-10A UV−vis detector and fractions containing butyryl-CoA were pooled and lyophilized. The retention time for butyryl-CoA was 25.9 min. To remove all ammonium acetate, the lyophilized solid was redissolved in H_2O or D_2O and relyophilized twice. ^{1}H NMR (500 MHz, D_2O): δ 8.57 (s, 1H), 8.28 (s, 1H), 6.18 (d, 1H, ^3J = 7.2 Hz), 4.59 (s, 1H), 4.25 (s, 2H), 4.03 (s, 1H), 3.84 (q, 1H, ^3J = 4.8 Hz), 3.56 (q, 1H, ^3J = 4.8 Hz), 3.46 (t, 2H, ^3J = 6.6 Hz), 3.34 (t, 2H, ^3J = 6.6 Hz), 2.99 (t, 2H, ^3J = 7.2 Hz), 2.58 (t, 2H, ^3J = 7.2 Hz), 2.44 (t, 2H, ^3J = 6.6 Hz), 1.62 (m, 2H, ^3J = 7.2 Hz), 0.90 (t, 3H, ^3J = 7.2 Hz), 0.86 (s, 3H), 0.73 (s, 3H). MALDI MS ([M−H]$^-$) calcd for $[C_{25}H_{41}N_7O_{17}P_3S]^-$: 836.6. Found: 837.6.

Crotonyl-CoA (Cr−CoA)[1] was synthesized from crotonic anhydride using the method described for butyryl-CoA. The Cr−CoA had a retention time of 23.6 min upon HPLC. ^{1}H NMR (300 MHz, D_2O): δ 8.54 (s, 1H), 8.21 (s, 1H), 6.91 (d of q, 1H, J_{trans} = 15.6 Hz, ^3J = 6.9 Hz), 6.14 (d, 1H, J_{trans} = 15.3 Hz), 6.13 (d, 1H, ^3J = 5.4 Hz), 4.56 (s, 1H), 4.21 (s, 2H), 3.99 (s, 1H), 3.81 (q, 1H, ^3J = 4.5 Hz), 3.53 (q, 1H, ^3J = 4.5 Hz), 3.41 (t, 2H, ^3J = 6.6 Hz), 3.30 (t, 2H, ^3J = 6.0 Hz), 2.98 (t, 2H, ^3J = 6.3 Hz), 2.39 (t, 2H, ^3J = 6.3 Hz), 1.82 (d, 3H, ^3J = 7.2 Hz), 0.86 (s, 3H), 0.73 (s, 3H). ^{31}P NMR (121.4 MHz, D_2O; relative to phosphoric acid): δ 2.47 (s, 1P), −10.86 (m, 2P). MALDI MS ([M−H]$^-$) calcd for $[C_{25}H_{39}N_7O_{17}P_3S]^-$: 834.6. Found: 834.9.

3′-Dephosphocrotonyl-CoA (dpCr−CoA)[1] was prepared by dephosphorylating Cr−CoA using calf intestinal phosphatase (CIP)[1] as described (*13*). Four milliliters of 4.2 mM Cr−CoA was incubated with 10 units of CIP at 4 °C for 24 h. After incubation, the CIP was removed by centrifugation (5000 rpm) through a Centricon-10 (Amicon) ultrafilter, and the dpCr−CoA purified by HPLC (26.9 min retention time). ^{1}H NMR (300 MHz, D_2O): δ 8.50 (s, 1H), 8.24 (s, 1H), 6.91 (d of q, 1H, J_{trans} = 15.3 Hz, ^3J = 6.6 Hz), 6.15 (d, 1H, J_{trans} = 15.6 Hz), 6.10 (d, 1H, ^3J = 5.4 Hz), 4.71 (m, 1H), 4.51 (t, 2H, ^3J = 4.2 Hz), 4.37 (s, 1H), 4.21 (s, 2H), 4.00 (s, 1H), 3.84 (q, 1H, ^3J = 4.2 Hz), 3.56 (q, 1H, ^3J = 4.2 Hz), 3.41 (t, 2H, ^3J = 4.2 Hz), 3.31 (t, 2H, ^3J = 6.6 Hz), 2.99 (t, 2H, ^3J = 6.3 Hz), 2.40 (t, 2H, ^3J = 6.9 Hz), 1.82 (d, 3H, ^3J = 6.6 Hz), 0.89 (s, 3H), 0.76 (s, 3H). ^{31}P NMR (121.4 MHz, D_2O; relative to phosphoric acid): δ −10.86 (m, 2P). MALDI MS ([M−H]$^-$) calcd for $[C_{25}H_{40}N_7O_{14}P_2S]^-$: 756.6. Found: 756.1. Note: Dephosphorylation of the ribose alters the chemical shifts of the ribose 2′, 3′, and 4′ protons. Thus, the ribose 4′ proton changes from 4.56 ppm in Cr−CoA to 4.37 ppm in dpCr−CoA while the ribose 2′, 3′ protons are now resolved from the water peak and appear at 4.51 and 4.71 ppm, respectively.

[1] Abbreviations: BTTB−CoA, 4-(2-benzothiazole)-4-thiabutanoyl-CoA; Bu-CoA, butyryl-CoA; Cr−CoA, crotonyl-CoA.; dpCr−CoA, 3′-dephosphocrotonyl-CoA; CIP, calf intestinal phosphatase; CoA, coenzyme A (lithium salt); DAC−CoA, 4-dimethylaminocinnamoyl-CoA; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid).

4-Dimethylaminocinnamoyl-CoA (DAC−CoA)[1] was synthesized from 4-dimethylaminocinnamic acid using the procedure described previously for hexadienoyl-CoA (*14*). Briefly, the acid was activated by reaction with 1,1′-carbonyldiimidazole in anhydrous THF and subsequently coupled to CoA using a method similar to that described above for Bu-CoA. DAC−CoA was purified by reversed-phase HPLC on the Alltech Econosil C-18 semipreparative column using a solvent gradient of 0 to 20% B 0−5 min, 20% B 5−15 min, and 20 to 50% B 15−43 min. The DAC−CoA had a retention time of 29 min. ^{1}H NMR (300 MHz, D_2O): δ 8.44 (s, 1H), 8.09 (s, 1H), 7.42 (d, 1H, J_{trans} = 15.6 Hz), 7.37 (d, 2H, ^3J = 9 Hz), 6.74 (d, 2H, ^3J = 9 Hz), 6.54 (d, 1H, J_{trans} = 15.9 Hz), 6.03 (d, 1H, ^3J = 6.03 Hz), 4.57 (s, 1H), 4.25 (s, 2H), 4.05 (s, 1H), 3.87 (q, 1H, ^3J = 4.5 Hz), 3.59 (q, 1H, ^3J = 4.5 Hz), 3.45 (m, 4H), 3.12 (m, 2H), 2.98 (s, 6H), 2.47 (t, 2H, ^3J = 6.3 Hz), 0.93 (s, 3H), 0.79 (s, 3H). UV−vis (H_2O) ϵ_{400} 28 000 M^{-1} cm^{-1}. MALDI MS ([M−H]$^-$) calcd for $[C_{32}H_{46}N_8O_{17}P_3S]^-$: 939.7. Found: 940.7.

4-(2-Benzothiazole)-4-thiabutanoyl-CoA (BTTB−CoA)[1] was synthesized from 4-(2-benzothiazole)-4-thiabutanoic acid using the mixed anhydride method as described (*15*), except that methylene chloride was replaced by anhydrous diethyl ether as solvent and triethylamine was used as the base. Following coupling to CoA, BTTB−CoA was purified by HPLC as described above using a solvent gradient of 15 to 40% B in 0−60 min. The BTTB−CoA had a retention time of 13 min on the Alltech Econosil C-18 semipreparative column. ^{1}H NMR (300 MHz, D_2O): δ 8.36 (s, 1H), 7.95 (s, 1H), 7.71 (d, 1H, ^3J = 7.5 Hz), 7.64 (d, 1H, ^3J = 8.1 Hz), 7.36 (t, 1H, ^3J = 7.2 Hz), 7.25 (t, 1H, ^3J = 7.2 Hz), 5.96 (d, 1H, ^3J = 4.5 Hz), 4.49 (s, 1H), 4.16 (s, 2H), 3.93 (s, 1H), 3.75 (q, 1H, ^3J = 4.5 Hz), 3.63 (q, 1H, ^3J = 4.5 Hz), 3.46 (t, 2H, ^3J = 6.6 Hz), 3.34 (t, 2H, ^3J = 6.0 Hz), 3.21 (t, 2H, ^3J = 5.7 Hz), 3.08 (t, 2H, ^3J = 6.3 Hz), 2.92 (t, 2H, ^3J = 6.3 Hz), 2.32 (t, 2H, ^3J = 6.6 Hz), 0.77 (s, 3H), 0.63 (s, 3H).

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 *E. coli* as described (*11*). The E164Q and E144Q mutants were constructed using the recombination polymerase chain reaction (RPCR) (*16*). The pET20b(+) plasmid carrying the enoyl-CoA hydratase cDNA was linearized through digestion with either *Pst*I (base 1144) or *Pvu*II (base 2692). The two complementary nonmutagenic primers were 5′-CGTTCCACTGAGCGTCAGACCCCG-3′ (NM1; 1584−1607) and 5′-CGGGGTCTGACGCTCAGTGGAACG-3′ (NM2; 1607−1584). The primers used to create the E144Q mutation were (base change underlined) 5′-GTGGGGGCTGTCAACTTGCCATG-3′ (EQ1_144; 3762−3784) and 5′-CATGGCAAGTTGACAGCCCCCAC-3′ (EQ2_144; 3784−3762), while the primers used to create the E164Q mutant were 5′-GGACAGCCACAAATCCTCCTG-3′ (EQ1_164; 3824−3844) and 5′-AGGAGGATTTGTGGCTGTCC-3′ (EQ2_164; 3843−3824). The linearized *Pst*I plasmid was used as a template for PCR (14 cycles) with primers, NM2 and EQ1_144 or EQ1_164 while the *Pvu*II digested plasmid was used as template with primers NM1 and EQ2_144 or EQ2_164. Following PCR, the two reactions were combined and used to transform XL1Blue cells (Stratagene). Mutant plasmid was purified using standard methods, sequenced using dideoxynucleotide methodology with [^{35}S]dATP (Sequenase 2, USB Biochemicals or CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit) and transformed into BL21-(DE3)pLysS cells (Novagen) for protein expression.

Cultures of BL21(DE3)pLysS cells carrying the wild-type or mutagenic plasmid were grown in LB-ampicillin 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-ampicillin containing 1 mM IPTG. After a further 3 h growth at 37 °C, the cells were harvested by centrifugation and used for protein purification. Cells from a 1 L culture were resuspended in 30 mL of 20 mM potassium phosphate (KH_2PO_4), 3 mM EDTA, pH 7.4 (buffer A), containing 1 mM DTT, and the cells cracked with a French Press. Cell debris was removed by centrifugation (20 min, 17 000 rpm), and the supernatant dialyzed twice against 1 L of the above buffer. The dialysate was centrifuged (17 000 rpm) and filtered through a 0.2 μm filter (Millex), and the enzyme was crystallized by addition of ethanol while cooling in a dry-ice−ethanol bath (*17*). The crystallized enzyme was dissolved in a minimal amount of buffer A, dialyzed against buffer A, and applied to a sepharose-CoA affinity column equilibrated with buffer A (*11*). The column was washed with buffer A, and the enzyme eluted with a linear gradient of 0 to 0.3 M KCl in buffer A. For experiments requiring enzyme in D_2O, the sepharose-CoA affinity column was washed with D_2O/buffer A (pD 7.4) and the enzyme eluted using 0.3 M KCl in D_2O/buffer A. The active-site concentration of the wild-type enzyme was determined by titration with DAC−CoA using ϵ_{496} 48 000 M^{-1} cm^{-1}. Enzyme eluted directly from the affinity column commonly had A_{280}/A_{496} ratios of 0.26 consistent with ϵ_{280} 12 300 M^{-1} cm^{-1} per active site of the protein or ϵ_{280} 73 800 M^{-1} cm^{-1} per hexamer.

Kinetic Assays. Initial steady-state kinetic assays were performed in 20 mM sodium phosphate and 3 mM EDTA, pH 7.4, at 25 °C. Substrate concentration was varied from 100 to 4 μM for Cr−CoA and from 400 to 5 μM for dpCr−CoA. Enzyme concentrations were 14.4 pM (wild-type), 22 nM (E144Q), and 6.8 μM (E164Q) for assays with Cr−CoA and 169 pM (wild-type), 290 nM (E144Q), and 6.8 μM (E164Q) for assays with dpCr−CoA.

Initial velocities for Cr−CoA and dpCr−CoA were measured by monitoring the decrease in absorbance at 280 nm using either a Perkin-Elmer λ5 UV−vis spectrometer or a CARY-100 spectrometer (Varian). An extinction coefficient of 3600 M^{-1} cm^{-1} at 280 nm was used for the hydration of the enoyl double bond. Initial velocities for the elimination of 2-mercaptobenzothiazole from BTTB−CoA were measured by monitoring the increase in absorbance at 310 nm. An extinction coefficient of 15 900 M^{-1} cm^{-1} at 310 nm was used.

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 3.09b (Erithacus Software Ltd.).

The pH dependence of k_{cat} and k_{cat}/K_m was measured for dpCr−CoA with wild-type, E144Q and E164Q enzymes at 25 °C. All buffers were 20 mM and contained 100 mM NaCl. Buffers used were sodium acetate (pH 4.5), MES (sodium salt; pH 5.0−6.0), sodium phosphate (monobasic; pH 6.5−7.0), Hepes (sodium salt; pH 8.0), Tris (chloride salt; pH

9.0), and borate (sodium salt; pH 9.6). Enzyme concentrations were 440 pM (wild-type) at pH 4.5, 271 pM (wild-type), 195 nM (E144Q) at pH 5.0, 176 pM (wild-type) at pH 5.5, 108 pM (wild-type), 195 nM (E144Q) at pH 6.0, 271 pM (wild-type), 195 nM (E144Q) at pH 6.5, 54 pM (wild-type), 273 nM (E144Q) at pH 7.0, 264 pM (wild-type), 312 nM (E144Q) at pH 8.0, 352 pM (wild-type), 390 nM (E144Q) at pH 9.0, and 542 pM (wild-type), 390 nM (E144Q) at pH 9.6. E164Q was 6.8 μM at pH 5−7 and 4.7 μM at pH 7.5−9.6.

The following equations were used to fit the variation of k_{cat} and k_{cat}/K_m with pH where y is the parameter to be fitted and C is the pH independent plateau value (*18*).

single ionization, activity increasing as pH increases:

$$\log y = \log[C/(1 + [H^+]/K)] \quad (1)$$

single ionization, activity decreasing as pH increases:

$$\log y = \log[C/(1 + K/[H^+])] \quad (2)$$

double ionization, bell-shaped pH dependence:

$$\log y = \log[C/(1 + [H^+]/K_1 + K_2/[H^+])] \quad (3)$$

NMR Spectroscopy. The ^{1}H NMR spectra were recorded at the SUNY at Stony Brook NMR Center on a Bruker AMX-600 spectrometer, a Varian Inova 500 MHz spectrometer and a Varian Inova 300 MHz spectrometer. All experiments were performed at 25 °C using (trimethylsilyl)-propanesulfonic acid as a standard.

Measurement of α*-Proton Exchange.* To monitor the exchange of the Bu-CoA *pro*-2*S* α-proton with solvent, the integrated intensity of the Bu-CoA C2 resonance at 2.58 ppm was monitored as a function of time using the pantetheine 6" methylene resonance at 2.44 ppm as an internal standard. Each exchange reaction contained 3 mM butyryl-CoA in 0.76 mL of 100 mM phosphate in D_2O, pD 7.0. Following acquisition of a zero time spectrum, the exchange reactions were initiated by the addition of 1.5 μM wild-type or E144Q mutant enzyme buffered in 50 mM phosphate D_2O, pD 7.0. Exchange rates were calculated as described previously (*6*) by fitting the decrease in the C2 Bu-CoA proton resonance to a first-order decay (eq 4):

$$\text{no. of protons} = (A_i - A_f)e^{-k_{obs}t} + A_f \quad (4)$$

where the number of protons was determined as a ratio to the internal integration standard, A_i and A_f were the initial and final number of α-protons and k_{obs} is the observed first-order rate constant. The exchange rate, k_{exc}, was calculated from eq 5, which takes into account the total amount of Bu-CoA bound at any time.

$$k_{exc} = k_{obs}[\text{Bu-CoA}]_T/[\text{Bu-CoA}]_B \quad (5)$$

where $[\text{Bu-CoA}]_T$ is the total Bu-CoA concentration (3 mM) and $[\text{Bu-CoA}]_B$ is the concentration of bound Bu-CoA (0.86 μM) calculated using K_d 0.5 mM for the binding of Bu-CoA to enoyl-CoA hydratase. The K_d value is assumed to be the same as that reported previously for the bovine enzyme (*6*).

RESULTS AND DISCUSSION

Kinetic Analysis of Wild-Type and Mutant Enzymes Using 3′-Dephosphocrotonyl-CoA. Kinetic parameters for Cr−CoA and dpCr−CoA are given in Table 1.

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

enzyme	substrate[b]	k_{cat} (s^{-1})	K_m (μM)	K_{cat}/K_m ($\mu M^{-1}\,s^{-1}$)
wild-type	Cr−CoA	1790 ± 120	5 ± 1	360 ± 96
wild-type[c]	Cr−CoA	3500	70	50
wild-type[d]	Cr−CoA	2145	40	54
E164Q[e]	Cr−CoA	0.0053 ± 0.0003	41 ± 6	0.000 12 ± 0.000 02
E164Q[c]	Cr−CoA	0.023	15	0.0015
E144Q	Cr−CoA	1.9 ± 0.2	9.7 ± 3.9	0.20 ± 0.10
wild-type	dpCr−CoA	1380 ± 50	89 ± 9	15.5 ± 2.1
E164Q	dpCr−CoA	0.0022 ± 0.0001	103 ± 5	(2.1 ± 0.2) × 10^{-5}
E144Q	dpCr−CoA	0.18 ± 0.01	97 ± 15	0.0020 ± 0.0004
wild-type	BTTB−CoA	0.18 ± 0.01	35 ± 2	0.005 ± 0.001
E164Q	BTTB−CoA	0.000 26 ± 0.000 02	ND[f]	ND[f]
E144Q	BTTB−CoA	0.0011 ± 0.0001	ND[f]	ND[f]

[a] Kinetic parameters were determined at pH 7.4 in 20 mM sodium phosphate buffer containing 3 mM EDTA at 25 °C. [b] Substrates used were crotonyl-CoA (Cr−CoA[1]), 3′-dephosphocrotonyl-CoA (dpCr−CoA[1]) and 4-(2-benzothiazole)-4-thiabutanoyl-CoA (BTTB−CoA[1]). [c] Data from ref *7*, pH 7.4, 10 mM Na_2HPO_4, 2.9 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl. [d] Data from ref *19*, pH 7.4, 0.3 M Tris-HCl, 5 mM EDTA. [e] This work, pH 7.4 in 20 mM sodium phosphate buffer containing 3 mM EDTA and 0.5 M NaCl at 25 °C. [f] ND, not determined.

The k_{cat} determined for Cr−CoA with wild-type enoyl-CoA hydratase was 1790 s^{-1}, similar to values of 3500 and 2145 s^{-1} previously reported for the rat liver enzyme [Muller-Newen et al. (*7*) and Furuta et al. (*19*), respectively]. However, under the conditions used in the present study, K_m was significantly smaller than the previously reported values of 70 and 40 μM, [Muller-Newen et al. (*7*) and Furuta et al. (*19*), respectively]. Indeed, the K_m value of 5 μM reported in Table 1 represents an upper limit, as the lowest substrate concentration used in the kinetic measurements was 4 μM. Using a K_m of 5 μM gives a lower limit for k_{cat}/K_m of 3.6×10^8 $M^{-1}\,s^{-1}$, which is close to the diffusion-controlled limit for encounter of enzyme and substrate. Consequently, it is likely that the K_m for Cr−CoA under our conditions is not substantially lower than 5 μM.

To accurately compare the wild-type and mutant enzymes, Cr−CoA was dephosphorylated in an attempt to reduce the affinity of the substrate for the enzyme. Analysis of the X-ray crystal structure determined by Wierenga and co-workers (*9*, *10*) indicates that the CoA 3′-phosphate group interacts with K101 and K282, and consequently, we hypothesized that dephosphorylating CoA would reduce the affinity of the substrate for the enzyme. It can be seen from Table 1 that 3′-dephosphocrotonyl-CoA (dpCr−CoA) has a k_{cat} of 1380 s^{-1}, similar to the k_{cat} for Cr−CoA but a K_m of 89 μM giving a k_{cat}/K_m of 1.55×10^7 $M^{-1}\,s^{-1}$. Removal of the 3′-phosphate group thus increases K_m by at least 18-fold and facilitates the accurate determination of the kinetic parameters. In addition, the use of dpCr−CoA also has the potential advantage that an ionizable group in the substrate is removed, thus facilitating analysis of the enzyme's pH dependence.

It has previously been shown that E164 is involved in the chemical steps of the addition reaction (*6*, *7*). Mutation of E164 to glutamine reduced k_{cat} by 150000-fold (*7*) and

abolished the ability of the enzyme to catalyze the exchange of the α-proton of butyryl-CoA with solvent (*6*). Supporting the role of E164 in catalysis, the X-ray structure of the enzyme (*9, 10*) shows that E164 is perfectly positioned to catalyze protonation/deprotonation of the substrate's α-carbon. Using sequence-homology modeling, we have postulated that E144, a second glutamate in the active site, is in a position to participate in the chemical steps of catalysis (*11*). This hypothesis was substantiated by the X-ray crystallographic studies undertaken by Wierenga and co-workers that showed E144 oriented such that it could participate as a general base in the addition of water to the substrate's C3 carbon (*9, 10*). To probe the role of E144 in catalysis, we have undertaken a kinetic analysis of the E144Q enzyme, and to compare the result of altering E144 with the effect of altering E164, we have also determined kinetic parameters for E164Q.

The kinetic parameters for Cr−CoA and dpCr−CoA with E144Q and E164Q are given in Table 1. For E144Q, k_{cat} and K_m were 0.18 s^{-1} and 97 μM with dpCr−CoA as substrate giving a k_{cat}/K_m of 2.0×10^3 M^{-1} s^{-1}. Replacement of E144 with glutamine consequently results in a 7700-fold decrease in k_{cat} without changing K_m appreciably. A somewhat smaller decrease in k_{cat} was observed using Cr−CoA as substrate. The effect on k_{cat} is even larger for the E164Q mutant where k_{cat} and K_m were 0.0022 s^{-1} and 103 μM with dpCr−CoA as substrate giving a k_{cat}/K_m of 21 M^{-1} s^{-1}. Thus, replacement of E164 with glutamine results in a 630000-fold reduction in k_{cat} compared to wild-type enzyme. Using Cr−CoA as substrate gave qualitatively similar results for k_{cat} in accord with previously published kinetic parameters for this mutant (*7*).

Clearly both E144 and E164 are critical residues in the reaction catalyzed by enoyl-CoA hydratase. To further probe the role of E144 in catalysis, we analyzed the ability of the E144Q mutant to catalyze the exchange of the α-protons of butyryl-CoA.

Exchange Reaction with Butyryl-CoA. Previously, we reported that wild-type bovine enoyl-CoA hydratase catalyzed the stereospecific exchange of the *pro*-2*S* proton of butyryl-CoA with solvent and that the ability to catalyze α-proton exchange was abolished by mutating E164 to glutamine. This observation is consistent with the X-ray crystal structure which shows E164 positioned within hydrogen-bonding distance of the substrate's C2 carbon (*9, 10*). However, in the E144Q mutant, E164 is intact and so we investigated the ability of this mutant to catalyze α-proton exchange.

The original exchange experiments were performed using the bovine liver enzyme (*6*). To compare data for the mutant enzymes in this study, we determined the rate of α-proton exchange using the recombinant wild-type enzyme. The exchange reaction was monitored by following the decrease in intensity of the C2−H NMR peak (Figure 1).

Using 1.5 μM wild-type enzyme and 3 mM butyryl-CoA, the C2−H resonance decreased from an integrated intensity of 2 protons to 1 proton over the course of 13 h, giving k_{obs} 6.8×10^{-5} s^{-1}. Assuming $K_d = 0.5$ mM for butyryl-CoA, this gave $k_{exc} = 0.24$ s^{-1}. This compares favorably with $k_{exc} = 0.2$ s^{-1} determined for the bovine liver enzyme (*6*).

In contrast to the wild-type enzyme, no α-proton exchange was observed using E144Q over a period of 4 days (5760 min) (Figure 1). This result is identical to that obtained using the E164Q mutant. Consequently, replacement of *either* glutamate with glutamine abolishes the ability of the enzyme to catalyze α-proton exchange. To further investigate the role of E144 and E164 in the active site, the ability of both wild-type and mutant enzymes to catalyze the elimination of 2-mercaptobenzothiazole from BTTB−CoA was investigated.

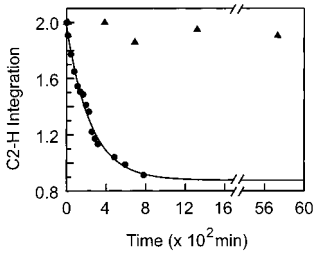


FIGURE 1: Comparison of the rate of α-proton exchange with D_2O catalyzed by wild-type (●) and E144Q (▲) enoyl-CoA hydratase. Experiments utilized 3 mM butyryl-CoA in 20 mM phosphate buffer pD 7.4 with 1.5 μM enzyme. Each time point is the sum of 64 scans and the integrated intensity of the butyryl-CoA C2−H resonance has been plotted on the *y*-axis. The data for wild-type enzyme have been fit to eq 4 with k_{obs} 6.8×10^{-5} s^{-1}.

Elimination of 2-Mercaptobenzothiazole from BTTB−CoA. Addition of wild-type enoyl-CoA hydratase to BTTB−CoA resulted in an increase in absorbance at 310 nm. Comparison of the spectra of the product formed with that reported for the reaction of acyl-CoA dehydrogenase with BTTB−CoA strongly suggests that enoyl-CoA hydratase catalyzes the elimination of 2-mercaptobenzothiazole from BTTB−CoA. This reaction initially forms acryloyl-CoA which is presumably immediately hydrated by the enzyme to 3-hydroxypropionyl-CoA. Steady-state kinetics revealed a k_{cat} of 0.18 s^{-1} and a K_m of 35 μM for wild-type enoyl-CoA hydratase. This contrasts with a k_{cat} of 0.12 s^{-1} and a K_m of 6 μM for acyl-CoA dehydrogenase (*20*). Thus, enoyl-CoA hydratase and acyl-CoA dehydrogenase have similar abilities to eliminate 2-mercaptobenzothiazole from BTTB−CoA. This reaction presumably proceeds via abstraction of the BTTB−CoA α-proton followed by elimination of 2-mercaptobenzothiazole. As 2-mercaptobenzothiazole has a pK_a of 6.8, then this substrate should not require an acid catalyst to assist in the elimination of the product. Consequently the activity of E144Q and E164Q toward BTTB−CoA was also tested.

Both E144Q and E164Q catalyzed the elimination of 2-mercaptobenzothiazole from BTTB−CoA more slowly than wild-type enzyme. The low activity of the mutants toward BTTB−CoA precluded obtaining an accurate estimate of K_m, however, estimates of k_{cat} were obtained using 250 μM BTTB−CoA with 20 μM E164Q and 2.6 μM E144Q. Initial velocities obtained using 150 and 250 μM BTTB−CoA were almost identical, suggesting that at these substrate concentrations the enzymes were saturated. Assuming that $[S] \gg K_m$ enabled us to estimate a k_{cat} of 2.6×10^{-4} s^{-1} for E164Q and 1.1×10^{-3} s^{-1} for E144Q. The decrease in k_{cat} is thus 690-fold for E164Q and 180-fold for E144Q. The larger decrease in rate for E164Q compared to E144Q is qualitatively similar to the decreases in rate observed with 3′-dephosphocrotonyl-CoA as substrate. However, if E144 is acting solely as a general acid in the elimination reaction, we would have expected that mutation of E144 to glutamine would have had no impact on the ability

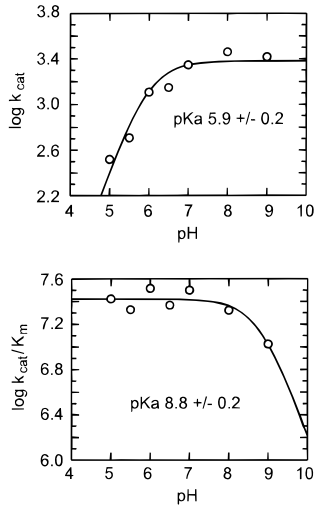


FIGURE 2: Variation of k_{cat} and k_{cat}/K_m with pH for the hydration of 3′-dephosphocrotonyl-CoA by wild-type enoyl-CoA hydratase. The pH dependence of k_{cat} has been fit to eq 1 giving a pK_a of 5.9 ± 0.2 and a limiting k_{cat} value of 2400 ± 300 s^{-1} (C) at high pH. The pH dependence of k_{cat}/K_m has been fit to eq 2 giving a pK_a of 8.8 ± 0.2 and a limiting k_{cat}/K_m value of (2.6 ± 0.2) × 10^7 M^{-1} s^{-1} (C) at low pH.

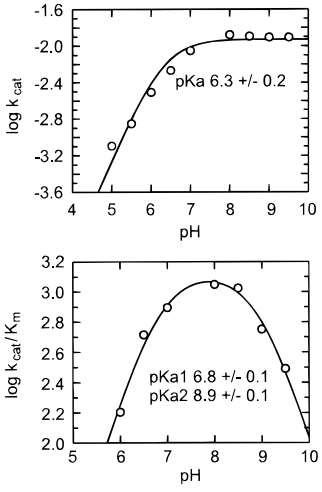


FIGURE 3: Variation of k_{cat} and k_{cat}/K_m with pH for the hydration of 3′-dephosphocrotonyl-CoA by E164Q enoyl-CoA hydratase. The pH dependence of k_{cat} has been fit to eq 1 giving a pK_a of 6.3 ± 0.2 and limiting a k_{cat} value of 0.018 ± 0.001 s^{-1} (C) at high pH. The pH dependence of k_{cat}/K_m has been fit to eq 3 giving pK_a1 6.8 ± 0.1, pK_a2 8.9 ± 0.1 and a limiting k_{cat}/K_m value of 1370 ± 150 s^{-1} M^{-1} s^{-1} (C).

to eliminate 2-mercaptobenzothiazole from BTTB−CoA. These data are further evidence that the two glutamate residues communicate with each other and that the ability of E164 to act as a general acid−base catalyst requires a second intact glutamate in the active site.

As described above, it is likely that E164 is the base that catalyzes α-proton exchange. While it is possible that the lack of α-proton exchange observed with the E144Q enzyme could result from the inability of solvent-hydrogen exchange to occur in this mutant, the 180-fold reduction in the rate of BTTB elimination catalyzed by E144Q indicates that the ability of E164 to abstract the substrates' α-proton has been compromised by the replacement of E144 with glutamine. This effect is presumably exerted either by altering the pK_as of E164 and/or the α-proton of butyryl-CoA or by altering the relative positions of E164 and the α-proton. As yet no

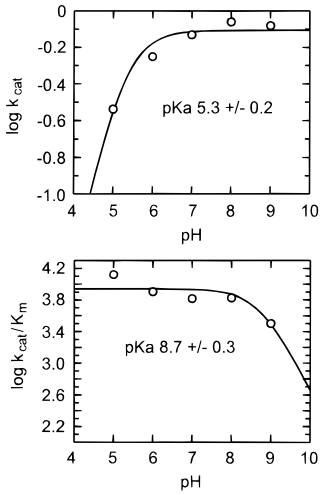


FIGURE 4: Variation of k_{cat} and k_{cat}/K_m with pH for the hydration of 3′-dephosphocrotonyl-CoA by E144Q enoyl-CoA hydratase. The pH dependence of k_{cat} has been fit to eq 1 giving a pK_a of 5.3 ± 0.2 and a limiting k_{cat} value of 0.78 ± 0.05 s^{-1} (C) at high pH. The pH dependence of k_{cat}/K_m has been fit to eq 2 giving a pK_a of 8.7 ± 0.3 and a limiting k_{cat}/K_m value of 8700 ± 1300 M^{-1} s^{-1} (C) at low pH.

Scheme 2: Ionization of the Active-Site Glutamate(s)

Wild-type

ECH-[Glu164•HOH•Glu144]$^{-1}$ $\underset{k_{-1}}{\overset{k_1S}{\rightleftharpoons}}$ ECH-[Glu164•HOH•Glu144]$^{-1}$• S $\xrightarrow{k_{cat}}$

pK_a< 5.0 ⇅ pK_a= 5.9 ⇅

ECH-[Glu164•HOH•Glu144] $\underset{k_{-1}}{\overset{k_1S}{\rightleftharpoons}}$ ECH-[Glu164•HOH•Glu144]• S

E164Q

ECH-[Gln164•HOH•Glu144]$^{-1}$ $\underset{k_{-1}}{\overset{k_1S}{\rightleftharpoons}}$ ECH-[Gln164•HOH•Glu144]$^{-1}$• S $\xrightarrow{k_{cat}}$

pK_a= 6.8 ⇅ pK_a= 6.3 ⇅

ECH-[Gln164•HOH•Glu144] $\underset{k_{-1}}{\overset{k_1S}{\rightleftharpoons}}$ ECH-[Gln164•HOH•Glu144]• S

E144Q

ECH-[Glu164•HOH•Gln144]$^{-1}$ $\underset{k_{-1}}{\overset{k_1S}{\rightleftharpoons}}$ ECH-[Glu164•HOH•Gln144]$^{-1}$• S $\xrightarrow{k_{cat}}$

pK_a< 5.0 ⇅ pK_a< 5.3 ⇅

ECH-[Glu164•HOH•Gln144] $\underset{k_{-1}}{\overset{k_1S}{\rightleftharpoons}}$ ECH-[Glu164•HOH•Gln144]• S

X-ray crystal structure is available which would provide information on the position of the catalytic residues in the mutant enzymes.

To gain further information on the ionization state of the two glutamates, we undertook a study of the pH dependence of the enzyme activity. Previously, we have been unable to detect a pH dependence using Cr−CoA as substrate which was presumed to be due to a large external commitment to catalysis for this substrate (data not shown). Consistent with this observation is that there is no detectable primary ^{D}(V/K) for the dehydration of 3(*S*)-hydroxybutyryl-CoA. We hypothesized that deletion of the CoA 3′-phosphate group might decrease the external commitments, enabling ionizations affecting the chemical steps of the reaction to be observed.

pH Dependence for dpCr−CoA. k_{cat} and K_m were measured for the wild-type enzyme between pH 5 and 9 using dpCr−CoA as substrate. Below pH 5 and above pH 9, the enzyme was unstable, preventing accurate determination of the kinetic

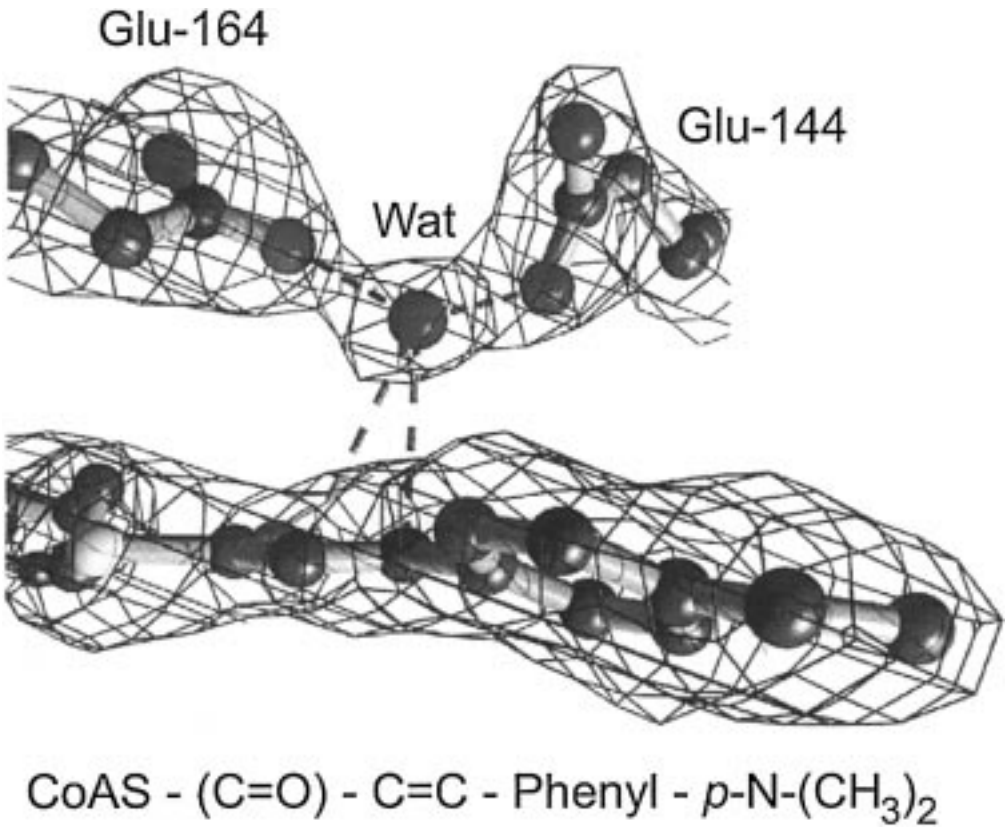


FIGURE 5: Structure of 4-dimethylaminocinnamoyl-CoA (DAC−CoA) bound to wild-type enoyl-CoA hydratase. A water molecule (Wat) is hydrogen bonded between the two catalytic glutamate residues, E144 and E164. The oxygen of the water molecule is 2.48 Å from the closer oxygen of E144, 2.63 Å from the closer oxygen of E164 and 3.50 and 3.14 Å from C2 and C3 of DAC−CoA, respectively.

parameters. As the pH was increased from 5 to 9, k_{cat} increased to a maximum value of 2 400 s^{-1}. The pH dependence of k_{cat} was fit to a single ionization (eq 1) with pK_a 5.9 ± 0.2 (Figure 2).

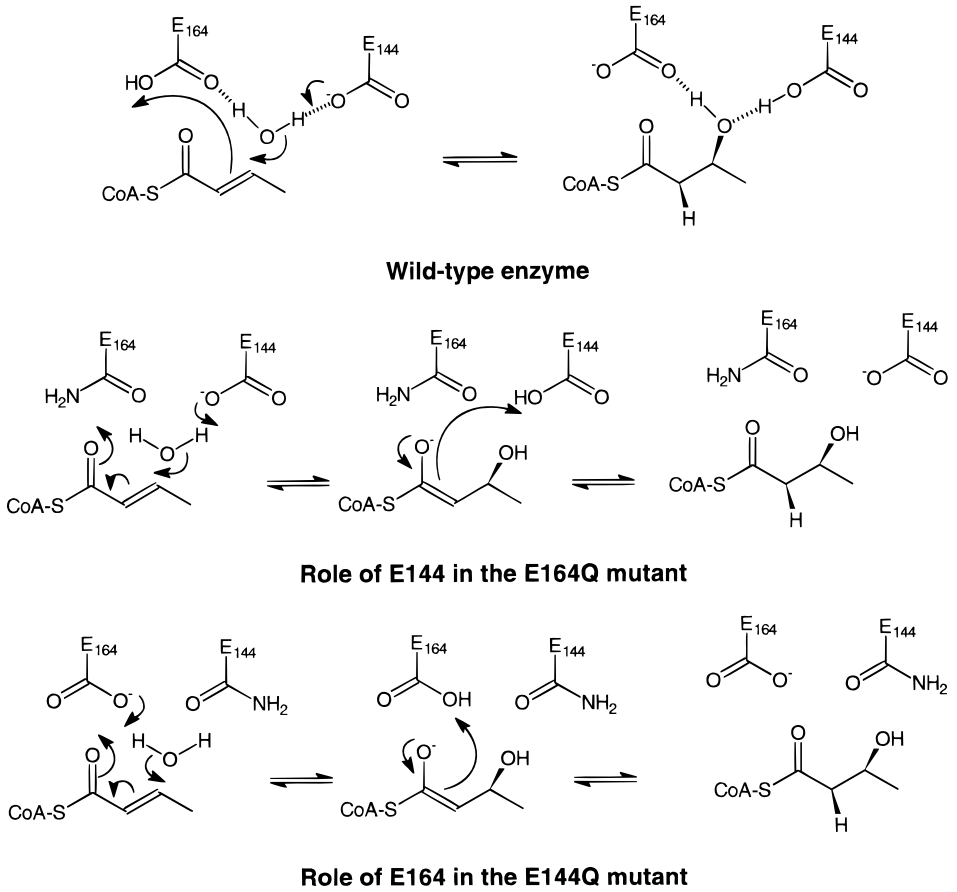
In contrast, k_{cat}/K_m was pH independent between pH 5 and 8. Above pH 8, k_{cat}/K_m started to decrease, due mainly to the large increase in K_m that occurs at basic pH. Figure 2 shows the variation in k_{cat}/K_m with pH. The data have been fitted to a single ionization with pK_a 8.8 ± 0.2 (eq 2); however, this value is close to the most basic pH used in the study. Consequently, the true value for the k_{cat}/K_m pK_a is likely to be greater than 8.8.

The identities of the residues contributing to the observed pH dependence are unknown. For the wild-type enzyme, we cannot rule out the possibility that a physical step is partially or completely rate limiting even using 3′-dephosphocrotonyl-CoA as substrate. In the reaction catalyzed by the enzyme, it is probable that one glutamate has to be ionized while the other is protonated. Activity is then expected to be bell-shaped with a rising acidic limb and basic descending limb. It is possible that the pK_a observed in the pH dependence of k_{cat} can be ascribed to the first ionization of the glutamate pair. The basic pK_a observed in the k_{cat}/K_m profile can be assigned to a residue involved in substrate/product binding. The fact that no acidic limb is observed in the pH dependence of k_{cat}/K_m suggests that the pK_a of 5.9 observed for k_{cat} is decreased in the free enzyme. If the acid pK_a is attributable to ionization of one of the active-site glutamates, then binding of the substrate must raise the pK_a. This change in pK_a could be due to the desolvation of the active site.

In contrast to the wild-type enzyme, it is assumed that bond cleavage reactions are rate limiting for the E144Q and E164Q enzymes. The pH dependence of k_{cat} for each enzyme should therefore reflect the ionization of residues involved in modulating the chemistry of the hydration reaction, rather than physical steps such as conformational changes induced by substrate binding and/or product dissociation. k_{cat} for the E164Q enzyme increases as the pH is raised and this pH dependence is shown in Figure 3. The data can be adequately described by a single ionization with pK_a 6.3 ± 0.2 using eq 1. We assign this pK_a to E144 in the enzyme−substrate complex. Figure 3 also shows the pH dependence of k_{cat}/K_m for this enzyme which has been fitted to eq 3. The plot is bell-shaped with pK_as of 6.8 ± 0.1 and 8.9 ± 0.1. The acidic pK_a is assigned to E144 while the basic pK_a is assigned to a residue involved in substrate binding. Clearly, the enzyme requires E144 in its anionic form for activity in the E164Q mutant. It should be noted that the activity of this enzyme is very low compared to the wild-type enzyme, so that even when the E144 residue is ionized, the E164Q enzyme is still 130000-fold less active than wild-type.

Figure 4 shows the pH dependence of k_{cat} and k_{cat}/K_m for the E144Q mutant. Over the pH range 5−9, k_{cat} increases by less than a factor of 3. The data have been fit using a single ionization having pK_a 5.3 ± 0.2 (eq 1), although this is close to the lowest pH used to measure activity. If E164 does ionize over this pH range, then the ionization state of E164 does not significantly affect activity. Alternatively, the pK_a of E164 lies outside this range. The most likely possibility, consistent with data for the wild-type and E164Q enzymes, is that E164 in the E144Q mutant must be in its

Scheme 3



anionic form for the reaction to proceed. Consequently, the pK_a of E164 is likely <5.3 in the enzyme−substrate complex of the E144Q enzyme. k_{cat}/K_m also exhibits only a relatively small variation between pH 5 and 9. The data have been fitted to a single basic ionization that results in decreased activity with pK_a 8.7 ± 0.3 (eq 2). This is supported by the previous observations that K_m for the wild-type and E164Q mutants increases at basic pH. Importantly, k_{cat}/K_m does not decrease at low pH, consistent with the hypothesis that the pK_a of E164 in the free E144Q enzyme is <5.0.

The main conclusion that is drawn from the pH dependence data is that the active form of the enzyme is one in which one of the glutamates (wild-type) or the one remaining glutamate (E144Q and E164Q) must be ionized for the reaction to occur. The pH dependence data for the wild-type, E164Q, and E144Q enzymes are summarized in Scheme 2 which shows the active form of the enzyme as carrying a −1 charge based on the ionization of the catalytic glutamate(s).

Role of E164 and E144: X-ray Crystal Structure of DAC−CoA Bound to Enoyl-CoA Hydratase. The X-ray structure of DAC−CoA bound to wild-type enoyl-CoA hydratase shows a water molecule hydrogen bonded between E144 and E164 (Figure 5; B. Bahnson and G. Petsko, personal communication).

This is presumably the catalytic water that adds to C3 of crotonyl-CoA in the hydration reaction. Clearly, removal of either glutamate will affect the location of the water molecule in the active site, which must still bind for hydration to occur.

Replacement of either glutamate with glutamine results in a large decrease in the rate of hydration, k_{cat} is 7700-fold lower for E144Q and 630000-fold lower for E164Q at pH 7.4. Clearly both glutamates are important catalytic residues. In addition, neither E164Q nor E144Q are able to catalyze the exchange of the α-proton of butyryl-CoA. Since E164 is the residue that abstracts the α-proton, the ability of E164 to function as an acid−base must be severely impaired in the E144Q mutant. This is supported by the reduction in activity of both E164Q and E144Q toward BTTB−CoA.

In the concerted reaction catalyzed by the wild-type enzyme, E144 abstracts a proton from the bound water molecule as E164 protonates the substrate. A reaction scheme depicting the roles of E144 and E164 in this process is shown in Scheme 3. However, this mechanism must now be rationalized with the lack of a solvent isotope effect on V/K for the dehydration of 3-hydroxybutyrylpantetheine. The presence of a single water molecule connecting E164 and E144 in the unliganded active site of enoyl-CoA hydratase (*10*) alters the interpretation of any solvent isotope effects. In the ground state, the fractionation factors of the two protons involved in the hydrogen bonds between the nucleophilic water molecule and these glutamate residues may well be significantly less than unity. To the extent that this is true, the fractionation factors of the two protons in the transition state may also be less than unity, implying that a minimal solvent deuterium isotope effect on dehydration does not rule out activation of the nucleophilic water by general base catalysis.

In either E144Q or E164Q, a water molecule must bind in the active site. In addition, analysis of the X-ray crystal structure reveals that no other residues are present in the active site that could function as acid−base catalysts.

Therefore, the single remaining glutamate in each mutant must fulfill both roles—increasing the nucleophilicity of the water molecule and protonating the substrate at C2. Reaction schemes depicting the role of the remaining glutamate in the E164Q and E144Q enzymes are shown in Scheme 3.

k_{cat} for E164Q shows a well defined pH dependence, presumably reflecting ionization of E144. However, this mutant is significantly less active than E144Q at all pHs, reflecting the importance of E164 in protonating the substrate. In contrast, k_{cat} for the E144Q mutant changes only slightly between pH 5 and 9. A reasonable hypothesis is that E164 in the E144Q enzyme is ionized over this pH range. Thus, although E164 is relatively acidic, it must first deprotonate the water molecule to generate hydroxide before it can protonate the substrate. In line with this hypothesis, we would predict that the reaction is no longer concerted for the mutant enzymes. We would then propose that the slow step in both E164Q and E144Q is substrate protonation/deprotonation and that catalysis proceeds via an enolate intermediate. This is depicted in Scheme 3 for the E164Q and E144Q enzymes.

ACKNOWLEDGMENT

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