Enoyl-Coenzyme A Hydratase-Catalyzed Exchange of the α-Protons of Coenzyme A Thiol Esters: A Model for an Enolized Intermediate in the Enzyme-Catalyzed Elimination?†

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ABSTRACT: 3-Quinuclidinone catalyzes the exchange of the α-protons of butyryl-coenzyme A (CoA) with a second-order rate constant of 2.4×10^{-6} M^{-1} s^{-1}. In contrast, enoyl-CoA hydratase catalyzes the stereospecific exchange of the *pro*-2*S* proton of butyryl-CoA with a maximum second-order rate constant of ca. 8×10^{2} M^{-1} s^{-1}. This isotope exchange reaction is completely stereospecific within the limits of experimental detection (over 600-fold). The enzyme-catalyzed exchange is dependent on pD, decreasing above a pK_a of 8.8 and below a pK_a of 8.1, but independent of the buffer concentration. The stereospecificity of the exchange was unexpected because the *pro*-2*R* hydrogen is abstracted during the enzyme-catalyzed dehydration of 3(*S*)-hydroxybutyryl-CoA. In spite of the ability to exchange the *pro*-2*S* hydrogen, the stereospecificity of the dehydration reaction was determined to be better than 1 in 10^5 as no incorporation of ^2H into the α-position of crotonyl-CoA or into the *pro*-2*S* position of 3(*S*)-hydroxybutyryl-CoA was detected during prolonged equilibrations with enoyl-CoA hydratase. Both the exchange of the α-proton and the dehydration activity of the enzyme are diminished by over 100-fold in a site-directed mutation of rat liver enoyl-CoA hydratase, where glutamate-164 is changed to glutamine, strongly suggesting that the same active site base is responsible for proton abstraction in both the dehydration and solvent exchange reactions. The enoyl-CoA hydratase-catalyzed exchange of the α-protons becomes nonstereospecific when the acidity of the α-protons is enhanced. While α-proton abstraction can be observed when no elimination reaction is possible, there is no evidence for proton abstraction without elimination in the crotonase equilibrations with 3(*S*)-hydroxybutyryl-CoA, 3-hydroxypropionyl-CoA, or 3-chloropropionyl-CoA. The differences in the isotope exchange and dehydration reactions emphasize the importance of the 3-hydroxyl group in promoting elimination and are consistent with a concerted elimination mechanism.

Coenzyme A (CoA)[1] serves as the acyl carrier for a wide variety of enzyme-catalyzed reactions. The enhanced acidity of the thiol ester α-proton, relative to that of the free fatty acid, is utilized in many acyl-CoA dependent reactions. Abstraction of an α-proton generates an enolized intermediate. The prototype of this reaction, for which the crystal structure is well defined, is citrate synthase. The nucleophilic α-carbon of the enolized acetyl-CoA adds to the carbonyl of oxalacetate to form citryl-CoA, as shown in Scheme 1. A second mechanistic pathway from the enolized intermediate is reketonization of the thiol ester with concomitant elimination of a leaving group from the β-carbon of the thiol ester. Enoyl-CoA hydratase (crotonase, EC 4.2.1.17) and acyl-CoA dehydrogenase are examples of this class of reaction.

Gerlt and Gassman considered the general problem of proton abstraction from carbon acids such as the α-carbon of CoA thiol esters. They concluded that both the condensation reactions and the elimination reactions would require the presence of an enolized intermediate stabilized by a "short, strong hydrogen-bond" from the enzyme to the thiol ester carbonyl (Gerlt & Gassman, 1992, 1993). The similarities of these two reaction classes proceeding through the common enolized CoA thiol ester are depicted in Scheme 1.

The acidity of the α-protons of thiol esters was quantified by Amyes and Richard (1992) when they demonstrated the general base-catalyzed α-proton exchange of model thiol esters in aqueous solution. We have extended these studies to determine whether the structure of CoA plays a role in enhancing the solution acidity of the α-protons of CoA thiol esters and to determine whether crotonase, which normally catalyzes the elimination of H_2O, is capable of functioning as a catalyst to abstract the α-protons of CoA thiol esters lacking a leaving group at C3. During this study, we were

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[1] Abbreviations: Ac-CoA, acetyl-CoA; Bu-CoA, butyryl-CoA; CoA, coenzyme A; CP-CoA, 3-chloropropionyl-CoA; crotonase, enoyl-CoA hydratase; Cr-CoA, crotonyl-CoA; DAC-CoA, 4-(*N*,*N*-dimethylamino)-cinnamoyl-CoA; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; G6PDH, glucose-6-phosphate dehydrogenase; HB-CoA, 3(*S*)-hydroxybutyryl-CoA; 3-HP-CoA, 3-hydroxypropionyl-CoA; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; 3-quin, 3-quinuclidinone.

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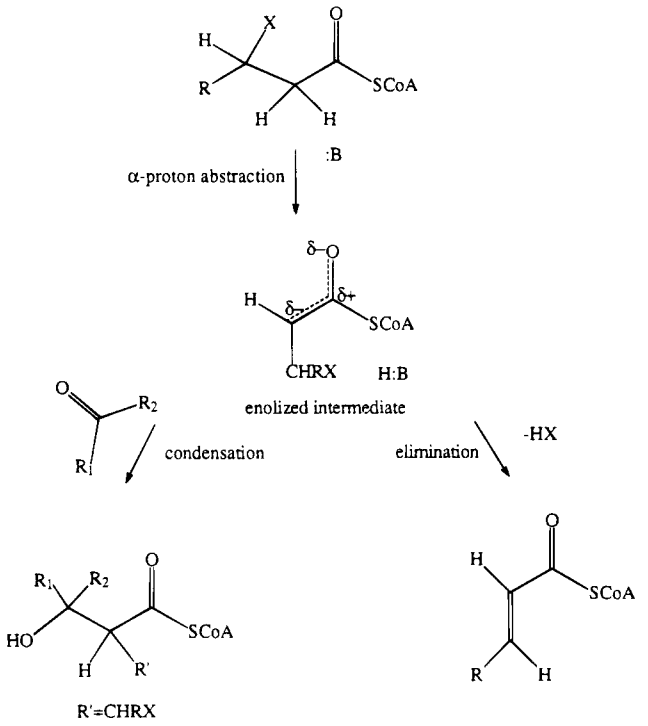
Scheme 1



surprised by the observation that the 3(*S*)-hydroxyl group was required for the abstraction of the *pro*-2*R* proton from 3(*S*)-hydroxybutyryl-CoA and in the absence of a leaving group that the *pro*-2*S* proton was exchanged with solvent. These results emphasize the importance of the interactions of crotonase with the 3-hydroxyl group during the elimination reaction and indicate the important role the 3-hydroxyl group plays in controlling both the stereochemistry and the rate of the elimination reaction. These observations are easily accounted for by a concerted elimination as proposed earlier (Bahnson & Anderson, 1991).

EXPERIMENTAL PROCEDURES

Chemicals. Deuterium oxide (99.9%) was from Cambridge Isotope Labs. Potassium hydroxide, potassium bicarbonate, monobasic potassium phosphate, and PrepSep C18 mini columns were from Fisher. CoA as the lithium salt, diketene, glucose 6-phosphate, malonyl-CoA, 2-methylmalonyl-CoA, Dowex 1-X2 (Cl^-) resin, and NADH were from Sigma. Acetic anhydride, butyric anhydride, 3-chloropropionic acid chloride, octanoyl chloride, *trans*-2-hexenoic acid, *trans*-3-hexenoic acid, thiophenol, 2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-phosphetane 2,4-disulfide (Lawesson's reagent), dicyclohexylcarbodiimide, 2-ethylbutyric acid, β-propiolactone, and 3-quinuclidinone (3-quin) were from Aldrich. All other chemicals were of reagent grade or better and were used without further purification.

Synthesis of Coenzyme A Thiol Esters. Butyryl-CoA (Bu-CoA) and acetyl-CoA (Ac-CoA) were synthesized by standard methods using either the acid chloride or anhydride (Stadtman, 1957). 3-Chloropropionyl-CoA (CP-CoA) was synthesized using the method of Miziorko and Behnke (1985). 4-(*N*,*N*-Dimethylamino)cinnamoyl-CoA (DAC-CoA) and 2-ethylbutyryl-CoA were synthesized from their respective acids by the acylimidazole method of Kawaguchi *et al.* (1981). Dithiobutyryl-CoA was synthesized following the procedure of Wlassics and Anderson (1989). Both *trans*-2- and *trans*-3-hexenoyl-CoA's were synthesized by the method of Bahnson (1991). The reaction mixtures were monitored for free thiol by an assay with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (1.0 mM in pH 8.5 phosphate buffer) (Ellman, 1959). When no free thiol was observed, the reaction mixtures were titrated to pH 4.5 with 1 M H_3PO_4 or acetic acid and then extracted with 5 equal vol of ethyl acetate to remove any excess free acid. The CoA thiol esters were then purified by high-performance liquid chromatography (HPLC).

3(*S*)-Hydroxybutyryl-CoA (HB-CoA) was synthesized as described by Bahnson and Anderson (1989). Briefly, 40 mg of CoASH (52 μmol) were reacted with an excess of freshly distilled diketene (150 μmol) in 100 mM phosphate (pH 8.0). When no free thiol was observed, the reaction mixture was titrated to pH 4.5 with 1 M H_3PO_4 and extracted with 5 equal vol of ethyl acetate, and the pH of the aqueous phase was adjusted to pH 7.0 with 1 M KOH. The product acetoacetyl-CoA was then reduced by ca. 1.5 mM (2.0 μmol) NADH in the presence of 3-hydroxyacyl-CoA dehydrogenase, which resulted in a slow but steady reduction of the absorbance at 380 nm. Glucose-6-phosphate dehydrogenase (G6PDH) and an excess of glucose 6-phosphate (60 μmol) were used to recycle the NADH. The addition of G6PDH caused an increase in the absorbance at 380 nm to a value less than that of the initial absorbance, indicating a steady state NADH/NAD^+ ratio. The reaction was determined to be complete when this intermediate absorbance increased steadily to the original absorbance for NADH at 380 nm. The HB-CoA was titrated to pH 4.5 with 1 M H_3PO_4, extracted with 5 equal vol of ethyl acetate, and then purified by HPLC.

3-Hydroxypropionyl-CoA was synthesized from CoA and β-propiolactone. To a solution of CoA in 200 mM potassium phosphate at pH 7.6 was added 3−5 equiv of the lactone. As before, the reaction was monitored by DTNB assay. Upon completion, the sample was titrated to pH 4.5, extracted with 5 equal vol of ethyl acetate, and purified by HPLC at both pH 4.5 and 6.5. The second HPLC at pH 6.5 was required to separate a contaminant, presumably CoAS-3-propionate.

Cr-CoA and 2(*R*,*S*)-methylmalonyl-CoA used in these experiments were purchased from Sigma. The Cr-CoA was found to have <5−10% of the cis isomer by ^1H nuclear magnetic resonance (NMR) and was used without further purification.

High-Performance Liquid Chromatography. HPLC purifications of the thiol ester substrates were performed on an Alltech Econosil octadecylsilyl reverse phase column (10 × 250 mm) eluted with methanol, 1−10 mM phosphate (pH 4.5) (or 6.5), and water at 3.0 mL/min. CoA thiol esters were detected by ultraviolet absorbance of the adenine at 260 nm.

NMR Spectroscopy. All ^1H NMR spectra were acquired, in D_2O, on a Bruker AM 400 MHz NMR spectrometer. The probe temperature was maintained at 24−26 °C. Chemical shifts are reported with respect to external (trimethysilyl)-propanesulfonic acid. The internal chemical shift standard was the triplet at 2.43 ppm, which was assigned to the two protons (t, 2H) at 6″ on the pantetheine backbone of CoA. Proton resonances from the coenzyme were used as internal integration standards: the triplet at 2.43 ppm was due to protons (t, 2H) at 6″, the singlets were due to the diastereotopic pantetheine methyl groups at 0.89 and 0.75 ppm (s, 3H), the doublet at 6.18 ppm was due to the anomeric

ribose proton (d, 1H), or the triplet at 2.96 ppm (t, 2H) was due to the protons at 9″ (Sarma & Lee, 1975).

Cr-CoA: δ 8.54 (s, 1H), 8.25 (s, 1H), 6.93 (d of q, 1H, J = 8.6, 6.8 Hz, Cβ), 6.18 (m, 1H, Cα), 6.16 (d, 1H, J = 6.5 Hz), 4.59 (s, 1H), 4.24 (s, 2H), 4.02 (s, 1H), 3.84 (q, 1H), 3.56 (q, 1H), 3.45 (t, 2H), 3.34 (t, 2H), 3.02 (t, 2H), 2.43 (t, 2H), 1.86 (d of d, 3H, J = 1.7, 5.3 Hz, Cγ), 0.89 (s, 3H), 0.75 (s, 3H).

HB-CoA: δ 8.66 (s, 1H), 8.41 (s, 1H), 6.21 (d, 1H), 4.59 (s, 1H), 4.24 (m, 3H, Cβ, C5′), 4.02 (s, 1H), 3.85 (q, 1H), 3.57 (q, 1H), 3.45 (t, 2H), 3.34 (t, 2H), 3.34 (t, 2H), 3.02 (t, 2H), 2.76 (d, 2H, Cα), 2.43 (t, 2H), 1.19 (d, 3H, Cγ), 0.92 (s, 3H), 0.79 (s, 3H).

3-HP-CoA: δ 8.56 (s, 1H), 8.27 (s, 1H), 6.17 (d, 1H), 4.59 (s, 1H), 4.24 (s, 2H), 4.01 (s, 1H), 3.85 (t & q, 3H, Cβ), 3.54 (q, 1H), 3.45 (t, 2H), 3.34 (t, 2H), 3.01 (t, 2H), 2.85 Cα (t, 2H), 2.43 (t, 2H), 0.87 (s, 3H), 0.74 (s, 3H).

Bu-CoA: δ 8.56 (s, 1H), 8.27 (s, 1H), 6.17 (d, 1H), 4.59 (s, 1H), 4.24 (s, 2H), 4.02 (s, 1H) 3.83 (q, 1H), 3.55 (q, 1H), 3.45 (t, 2H), 3.32 (t, 2H), 2.98 (t, 2H), 2.98 (t, 2H), 2.56 (t, 2H, Cα), 2.43 (t, 2H), 1.61 (m, 2H, Cβ), 0.88 (t, 3H, Cγ), 0.87 (s, 3H), 0.74 (s, 3H).

Ac-CoA: δ 8.56 (s, 1H), 8.27 (s, 1H), 6.18 (d, 1H), 4.60 (s, 1H), 4.24 (s, 2H), 4.02 (s, 1H), 3.83 (q, 1H), 3.55 (q, 1H), 3.45 (t, 2H), 3.32 (t, 2H), 2.98 (t, 2H), 2.43 (t, 2H), 2.35 (s, 3H, Cα), 0.89 (s, 3H), 0.76 (s, 3H).

CP-CoA: δ 8.58 (s, 1H), 8.30 (s, 1H), 6.18 (d, 2H), 4.60 (s, 1H), 4.24 (s, 2H), 4.03 (s, 1H), 3.83 (m, 3H, Cβ), 3.57 (q, 1H), 3.45 (t, 2H), 3.34 (t, 2H), 3.09 (t, 2H, Cα), 3.04 (t, 2H), 2.43 (t, 2H), 0.90 (s, 1H), 0.77 (s, 1H).

DAC-CoA: δ 8.40 (s, 1H), 8.03 (s, 1H), 7.37 (d, 1H, J_{trans} = 15.7 Hz, Cβ), 7.31 (d, 2H, J = 8.9 Hz), 6.67 (d, 2H, J = 8.9 Hz), 6.48 (d, 1H, J_{trans} = 15.7 Hz, Cα), 6.00 (d, 1H), 4.53 (s, 1H), 4.21 (s, 2H), 4.01 (s, 1H), 3.83 (q, 1H), 3.54 (q, 1H), 3.43 (m, 4H), 3.08 (m, 2H), 2.92 (s, 6H), 2.43 (t, 2H), 0.88 (s, 3H), 0.74 (s, 3H).

2-Methylmalonyl-CoA: δ 8.66 (s, 1H), 8.41 (s, 1H), 6.21 (d, 1H), 4.59 (s, 1H), 4.02 (s, 1H), 3.85 (q, 1H), 3.65 (q, 1H, Cα), 3.57 (q, 1H), 3.45 (t, 2H), 3.34 (t, 2H), 3.02 (t, 2H), 2.43 (t, 2H), (q, 1H), 1.35 (d, 3H, α-methyl), 0.92 (s, 3H), 0.79 (s, 3H).

trans-3-Hexenoyl-CoA: δ 8.54 (s, 1H), 8.25 (s, 1H), 6.16 (d, 1H, 6.5 Hz), 5.75 (m, 1H, Cγ), 5.45 (m, 1H, Cβ), 4.59 (s, 1H), 4.24 (s, 2H), 4.02 (s, 1H), 3.84 (q, 1H), 3.56 (q, 1H), 3.45 (t, 2H), 3.34 (t, 2H), 3.25 (d, 2H, Cα), 3.00 (t, 2H), 2.43 (t, 2H), 2.00 (m, 2H, Cδ), 0.90 (t, 3H, Cϵ) 0.89 (s, 3H), 0.75 (s, 3H).

Dithiobutyryl-CoA: δ 8.56 (s, 1H), 8.27 (s, 1H), 6.17 (d, 1H), 4.59 (s, 1H), 4.24 (s, 2H), 4.02 (s, 1H), 3.83 (q, 1H), 3.55 (q, 1H), 3.40 (m, 2H), 3.32 (t, 2H), 2.97 (t, 2H, Cα), 2.56 (t, 2H), 2.43 (t, 2H), 1.75 (m, 2H, Cβ), 0.88 (s, 3H), 0.87 (t, 3H, Cγ), 0.74 (s, 3H).

2-Ethylbutyryl-CoA: δ 8.56 (s, 1H), 8.28 (s, 1H), 6.18 (d, 1H), 4.58 (s, 1H), 4.24 (s, 2H), 4.02 (s, 1H), 3.83 (q, 1H), 3.54 (q, 1H), 3.46 (m, 2H), 3.24 (t, 2H), 3.02 (t, 2H), 2.50 (m, 1H, Cα), 2.43 (t, 2H), 1.52 (m, 4H, Cβ), 0.89 (s, 3H), 0.84 (t, 6H, Cγ), 0.76 (s, 3H).

Enzymes. Crotonase was isolated using a procedure based on that of Steinman and Hill (1975) as modified by Bahnson (1991). The enzyme used in these experiments was recrystallized with ethanol as a precipitant and gave a single band by Coomassie Blue-visualized sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The concentration of the crystalline crotonase was determined using an extinction coefficient of 27 800 M^{-1} cm^{-1} at 494 nm for the crotonase/DAC-CoA complex. 3-Hydroxyacyl-CoA dehydrogenase (type V) from bovine liver and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (type xxiii) were from Sigma. Medium chain acyl-CoA dehydrogenase from porcine kidney was a generous gift from Dr. Colin Thorpe. The rat liver enoyl-CoA hydratase $Glu^{164}\rightarrow Gln$ mutant was generated from a plasmid containing the rat liver mitochondrial enoyl-CoA hydratase sequence (Minami-Ishii *et al.*, 1989) generously provided by Dr. T. Osumi. The enzyme was isolated using a CoA–Sepharose affinity column and recrystallized with ethanol as the precipitant. A full characterization of the overepxression system and mutant will be described elsewhere.

Exchange Reactions with 3-Quinuclidinone. A solution of 500 mM 3-quin in D_2O was titrated to pD 9.0 with 3 M KOD. A sample containing the desired amount of CoA thiol ester was dissolved in 0.5 mL of 1.0 M KCl and concentrated to dryness. This sample was resuspended in 0.5 mL of the 500 mM 3-quin solution to initiate the reaction. Each of the samples contained 1.0–1.5 mM CoA thiol ester and 500 mM 3-quin at pD 9.0 ([I] = 1.0 (KCl)). The pD was measured before and after the reaction to ensure that no changes had taken place. The reactions were quenched at various time intervals by the addition of 1 M H_3PO_4 to pH 4.0. The 3-quin was separated from the CoA's by using 0.5 mg of Dowex 1-X2 anion exchange resin, which selectively retains the CoA trianions. This minicolumn was washed with 40–50 mL of 10 mM phosphate (pH 4.5) to remove any trace of 3-quin and then with 50–60 mL of 1.4 M KCl (pH 2.0) to elute the CoA thiol esters. The CoA thiol esters were then adsorbed on a reverse phase C18 Prep-Sep column and washed with 40–50 mL of 10 mM phosphate (pH 4.5) to desalt the sample. CoA's were recovered by elution with 15–30 mL of 20% methanol in 10 mM phosphate (pH 4.5) and concentrated. Fractions from both columns were monitored by UV absorbance at 260 and 284 nm. NMR analysis demonstrated that this method afforded total separation of the 3-quin from the CoA substrates. Deuterium incorporation was observed by NMR and quantified by ratio to the nearest CoA integration standard. Controls at the same pD's and containing all components except the 3-quin were run. Controls in which Cr-CoA was used as the substrate were performed under the reaction conditions described earlier.

Exchange Reactions with Bovine Liver and $Glu^{164}\rightarrow Gln$ Rat Liver Crotonase. The exchange reactions of the CoA thiol ester α-protons with solvent catalyzed by crotonase were initiated by the addition of the desired amount of enzyme (1–10 μM) in phosphate-buffered D_2O (50 mM, pD 7.0). Stock solutions of the CoA substrates were concentrated to dryness under vacuum and resuspended in D_2O at least twice. An appropriate amount of the CoA substrate, usually 1.0–3.0 mM in 0.5 mL of 100 mM phosphate in D_2O, was titrated to the desired pD with 1 or 3 M KOD and lyophilized once more. The solution was resuspended in D_2O, filtered or centrifuged to remove pariculates, and added to a dry 5 mm NMR tube. A zero-time spectrum was acquired prior to the addition of enzyme. Controls containing all components except the enzyme were performed. The crotonase was shown to retain full activity for at least 7 days. The exchange reactions were effectively inhibited by the

Table 1: α-Proton Exchange with 3-Quinuclidinone at pD 9.0

CoA substrate	k_{obs} (s^{-1})	k_{exc2} (M^{-1} s^{-1})[a]	k_{hd} (M^{-1} s^{-1})[b]	V/K (M^{-1} s^{-1}) (with crotonase)
Ac-CoA	$(1.8 \times 10^{-6}) \pm (0.22 \times 10^{-6})$	$(4.5 \times 10^{-6}) \pm (0.55 \times 10^{-6})$	7.0×10^{-7}	
Bu-CoA	$(9.5 \times 10^{-7}) \pm (0.67 \times 10^{-7})$	$(2.4 \times 10^{-6}) \pm (0.17 \times 10^{-6})$	2.5×10^{-7}	$\approx 8 \times 10^{2}$
HB-CoA	$(2.5 \times 10^{-5}) \pm (0.30 \times 10^{-5})$	$(6.3 \times 10^{-5}) \pm (0.75 \times 10^{-5})$	5.4×10^{-6}	$\approx 5 \times 10^{7}$

[a] Ethyl thioacetate k_{exc2} (M^{-1} s^{-1}) ca. 1×10^{-6} (Amyes & Richard, 1992). [b] The rate constant for hydrolysis was determined by monitoring the decrease in the NMR resonances for the CoA thiol ester.

addition of a stoichiometric amount of DAC−CoA. Samples in which the Glu[164]→Gln crotonase was used were treated exactly the same as those with the wild-type crotonase. The mutant was found to be as stable as the wild type, as judged by the binding of DAC-CoA.

Data Analysis. The decrease in the integration of the α-proton NMR resonance relative to the internal integration standard as a function of time was fit to a first-order decay:

$$\text{no. of protons} = (A_i - A_f)e^{-k_{obs}t} + A_f \quad (1)$$

where the number of protons was determined as a ratio to the internal integration standard. A_i and A_f, the initial and final number of α-protons, respectively, were fixed integers as dictated by each experiment, and k_{obs} is the observed first-order rate constant. The exchange rate was calculated from eq 2, where k_{obs} is the first-order rate constant obtained from the fit to eq 1.

$$k_{exc} = \frac{k_{obs}[\text{Bu-CoA}]}{[\text{crotonase·thiol ester}]} \quad (2)$$

For the 3-quin-catalyzed reaction, the second-order rate constant was calculated using

$$k_{exc2} = \frac{k_{obs}}{[\text{3-quin}]} \quad (3)$$

Where k_{obs} is the observed pseudo-first-order rate constant obtained from the plots.

The variation in k_{obs} with pD for the crotonase-catalyzed exchange of a single Bu-CoA α-proton was fit to a bell-shaped curve defined by two pK_a's, as shown by

$$k_{obs} = \frac{k_{max}}{1.0 + 10^{(pK_{a1} - pH)} + 10^{(pH - pK_{a2})}} \quad (4)$$

where k_{max} is the exchange rate constant for the enzyme in the correct protonation state. All equations were fit and the data analyzed using GraFit software (Leatherbarrow, 1990).

The stereospecificity of the dehydration reaction was determined by the dynamic equilibrium method of Anderson and LaReau (Anderson & LaReau, 1988; LaReau & Anderson, 1989), where the incorporation of ^2H into C2 of Cr-CoA was monitored instead of that of ^3H into lactate. The probability, P, of the crotonase-catalyzed elimination/addition proceeding through an anti transition state can be calculated from

$$f = (1 - P)^n \quad (5)$$

where f is the fraction of ^1H remaining at C2 of Cr-CoA and n is the average number of reactions each Cr-CoA molecule has undergone. Analysis of the splitting pattern of the C3 vinylic ^1H by NMR provides a direct measure of f, since the incorporation of ^2H into C2 results in more complex splitting patterns of the ^1H resonance at C3 (Bahnson, 1991). The average number of reactions, n, is given by

$$n = \{[\text{crotonase}]k_{cat}t\}/[\text{CoA thiol ester}] \quad (6)$$

where t represents time.

RESULTS

3-Quinuclidinone-Catalyzed α-Proton Exchange. 3-quin is a nonnucleophilic base that catalyzes the exchange of the α-protons of thiol esters while minimizing hydrolysis (Amyes & Richard, 1992). The rates of exchange of the α-protons and hydrolysis of the various thiol ester substrates catalyzed by 3-quin are presented in Table 1. In control reactions under identical conditions, excluding 3-quin, neither exchange nor hydrolysis occurred at a measurable rate during the time observed, indicating that both reactions were catalyzed by 3-quin. The observations that both α-protons exchange and that no biphasic behavior was observed indicate that the α-proton exchange catalyzed by 3-quin is not stereospecific.

3-quin did not catalyze the elimination of water from HB-CoA. No evidence of the elimination product, Cr-CoA, was observed, nor was any other form of unsaturated CoA species present in the ^1H NMR spectra of any of the reaction mixtures. 3-quin did not catalyze an addition reaction to Cr-CoA in control reactions performed with Cr-CoA incubated under the same conditions as the exchange reactions. After 24 h, the vinylic resonances of Cr-CoA remained unchanged, indicating that 3-quin did not form a Michael adduct or catalyze the hydration of Cr-CoA to form HB-CoA. Consequently, application of the principle of microscopic reversibility confirms the observation that 3-quin does not catalyze the dehydration of HB-CoA to Cr-CoA, in spite of the fact that it catalyzes the enolization.

Crotonase-Catalyzed Exchange of the pro-2S Proton of Saturated Acyl-CoA's. Crotonase catalyzes the stereospecific exchange of only a single α-proton of HB-CoA, Bu-CoA, and octanoyl-CoA, as shown in Table 2 and Figures 1−3. For the experiments with Bu-CoA and octanoyl-CoA, the *pro*-2*S* proton was proven to be the stereospecifically abstracted hydrogen when the remaining proton was completely removed by acyl-CoA dehydrogenase, which exchanges only the *pro*-2*R* hydrogen (Biellman & Hirth, 1970), and is shown in Figure 1. The resonance at 2.56 ppm is due to the α-protons, which show a small shift to higher field and a broadening effect when a single ^2H is incorporated. The crotonase-catalyzed exchange was shown to be highly stereospecific as Bu-CoA incubated with 1−10 μM crotonase for 7 days at various pD values still retains the

Table 2: Specificity of Crotonase-Catalyzed α-Proton Exchange

CoA thiol ester	stereospecificity	relative rates[a]
HB-CoA	*pro*-2*R*	3800
CP-CoA	ND[b]	≫7.5
3-HP-CoA	*pro*-2*R*[c]	≫7.5
Bu-CoA	*pro*-2*S*	1.0
octanoyl-CoA	*pro*-2*S*	1
dithiobutyryl-CoA	nonspecific	0.7
2-methylmalonyl-CoA	nonspecific	7.5
3-hexenoyl-CoA	nonspecific	>7.5

[a] Rates are relative to Bu-CoA exchange (for absolute rate see Table 1). [b] Not determined by the experimental protocol. [c] By analogy with HB-CoA.

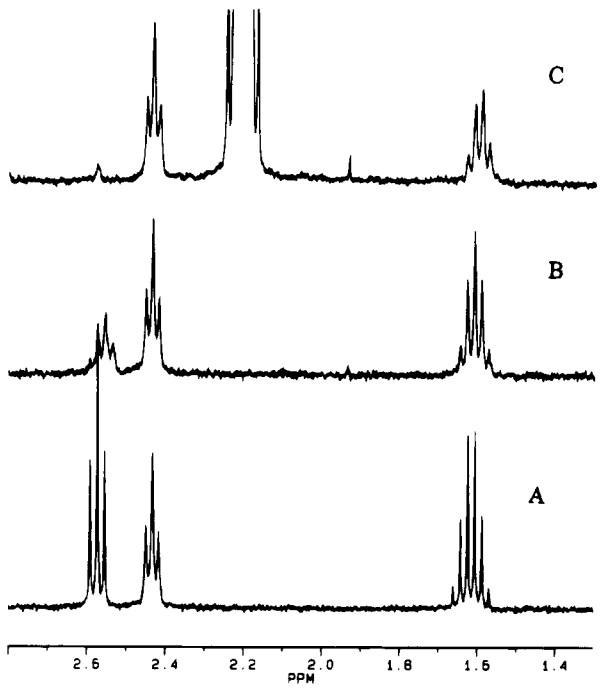


FIGURE 1: Crotonase-catalyzed α-proton exchange of Bu-CoA: (A) Bu-CoA, 1.1 mM in phosphate buffer (pD 7.0); (B) sample A after a 1040 min incubation with 1 μM crotonase. Besides the integration, the 1:4:6:4:1 quintet for the C3 protons at 1.62 ppm is the predicted overlaid doublet of quartets if a single stereospecific exchange has occurred. (C) Sample B following a 200 min incubation with 4 μM acyl-CoA dehydrogenase. The resonance for the C3 protons is simplified to a quartet. The large resonance at 2.2 ppm was introduced with the acyl-CoA dehydrogenase.

pro-2*R* proton. This limits the k_{obs} for exchange of the *pro*-2*R* proton of Bu-CoA to $<3 \times 10^{-7}$ s^{-1}, which is over 600 times slower than the exchange of the *pro*-2*S* hydrogen. Identical results were obtained with octanoyl-CoA and were independent of whether the crotonase or the acyl-CoA dehydrogenase was added first.

Experiments varying the buffer (phosphate) concentration and pH were performed with Bu-CoA to carefully determine the rate of exchange, as shown in Figure 2, and replotted in Figure 3 to extract k_{obs}. The exchange rate constant was independent of buffer concentration (data not shown). The preclusion of buffer involvement contends that either water or an enzyme functional group must accept the abstracted proton. Controls indicated that no exchange was observed in the absence of crotonase. The rate of exchange of the Bu-CoA α-proton by crotonase was pD dependent. The data fit a bell-shaped curve with pK_a values of 8.1 and 8.8, as shown in Figure 4. The exchange reaction, consequently,

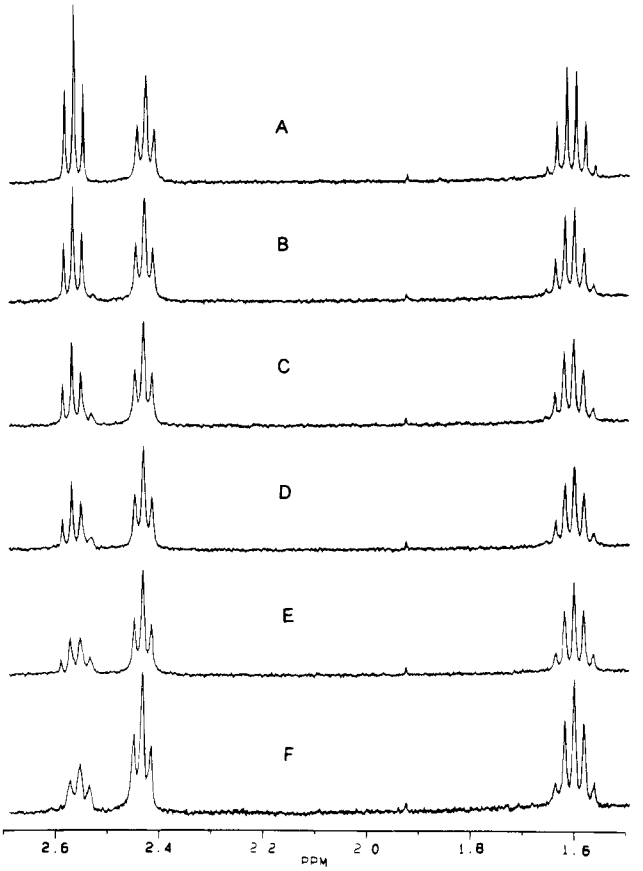


FIGURE 2: Selected ^{1}H NMR spectra of Bu-CoA over the region 2.7−1.5 ppm, demonstrating the time course of α-proton exchange. The spectra were processed with 0.3 Hz line broadening and are the result of 72 scans each. The α-proton resonance at 2.56 ppm decreased in intensity and shifted upfield due to the exchange of the *pro*-2*S* ^{1}H of Bu-CoA for ^{2}H. Also note the collapse of the sextet at 1.61 ppm, due to the β-protons on Bu-CoA, to a broadened quintet. The *pro*-2*S* proton of Bu-CoA is stereospecifically exchanged by crotonase (see Figure 1). Time and integration values for the α-proton resonance at 2.56 ppm are as follows: (A) 0 min, 2.003 protons; (B) 240 min, 1.735 protons; (C) 510 min, 1.473 protons; (D) 690 min, 1.340 protons; (E) 780 min, 1.260 protons; (F) 1480 min, 1.005 protons.

requires two general acid−base catalysts: one protonated and one unprotonated, with an average pK_a of 8.45.

In an effort to probe the binding site accessibility, 2-ethylbutyryl-CoA was employed as a substrate for the crotonase-catalyzed α-proton exchange. No exchange was observed. This observation indicates that there are constraints on the mobility of the substrate in the active site and the access of the base to the α-protons of the thiol esters. Controls showed the crotonase used in these experiments to be fully active throughout.

To verify that the exchange occurred at the crotonase active site, DAC-CoA, a competitive inhibitor of crotonase with a K_i of ca. 1 μM (D'Ordine *et al.*, 1994), was used to inhibit the α-proton exchange of Bu-CoA. When 50 μM DAC-CoA was included in a standard exchange sample containing 100 mM potassium phosphate (pD 8.1), 1.4 mM Bu-CoA, and 1.6 μM crotonase, <25% exchange of the Bu-CoA *pro*-2*S* proton was observed over 15 h, while in a parallel control reaction exchange was complete.

Glu^{164}→Gln Mutant Does Not Catalyze α-Proton Exchange. The site specific Glu^{164}→Gln mutation has a dramatically reduced catalytic activity and failed to catalyze

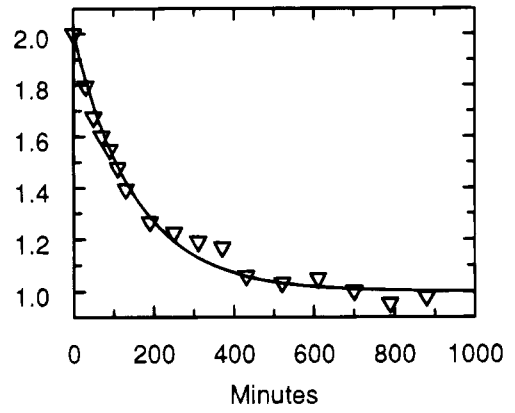


FIGURE 3: Rate of proton exchange with D_2O by crotonase. Plot (eq 1) of the loss of the *pro*-2*S* proton of Bu-CoA versus time in minutes. Each time point is the result of an individual spectrum comprising 72 scans. The integration of each time point was performed over a uniform chemial shift range. Integration values for the plotted points are the result of duplicate integrations versus the internal integration standard at 2.43 ppm, given a set value of 2.00 protons. The sample contained 100 mM potassium phosphate (pD 8.7), 1.4 mM Bu-CoA, and 1.0 μM crotonase in a volume of 500 μL. The observed rate was $(6.4 \times 10^{-3}) \pm (3.0 \times 10^{-4})$ min^{-1}.

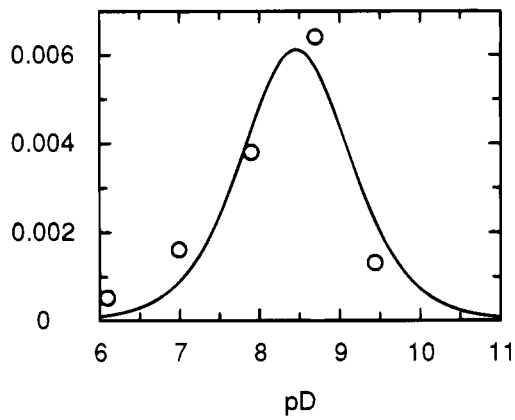


FIGURE 4: Effect of pD variation on the observed rate of exchange, k_{obs}, of the *pro*-2*S* proton of Bu-CoA with D_2O by crotonase. The graph shows the results of five separate exchange samples with varying pD's, all in 100 mM phosphate with 1.4 mM Bu-CoA and 1.0 μM crotonase. The solid line represents the fit to eq 2 with $pK_{a1} = 8.1$ an $pK_{a2} = 8.8$.

any detectable exchange of the α-protons of Bu-CoA (data not shown). The mutant is capable of binding DAC-CoA with high affinity and induces a slightly greater red shift (6 nm) in the UV absorbance than wild-type enzyme. When DAC-CoA binds to native enzyme, a red shift of 90 nm is observed; with Glu^{164}→Gln crotonase the red shift is 96 nm. The pH variation of λ_{max} is also intriguing as the wild-type enzyme from both bovine and rat liver is pH dependent, with the λ_{max} decreasing to 488 nm above a pK_a of 8.45. The mutant λ_{max} is pH independent, as shown in Figure 5.

Nonstereospecific Exchange When the CoA Thiol Ester α-Protons Had Enhanced Acidity. When the crotonase-catalyzed exchange of the α-protons of 3-hexenoyl-CoA, dithiobutyryl-CoA, and (*R*/*S*)-methylmalonyl-CoA was examined, no stereospecificity was observed. In all cases, the integrated intensity of the ^{1}H NMR resonance of the α-protons decreased to 0 with a single-exponential decay, and the rates of exchange are given in Table 2. Controls under the same conditions, excluding crotonase, were run, and the background rate was subtracted.

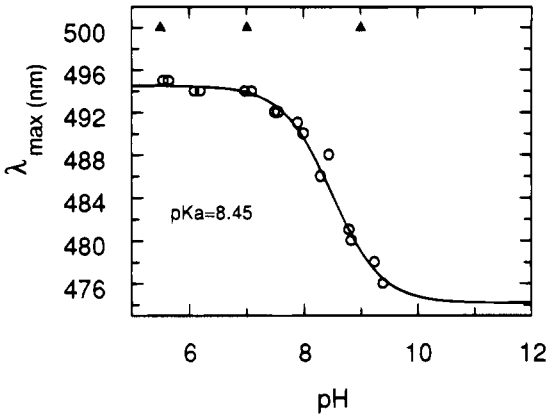


FIGURE 5: Plot of DAC-CoA binding to native crotonase and the Glu^{164}→Gln mutant at various pH's. The pH range for which the binding experiments were carried out is 5.5−9.4. The buffers used were 50 mM acetate, phosphate, Tris-HCl, or TAPS at the appropriate pH. Open circles are the results for the native crotonase. The pK_a obtained for the wild-type crotonase was 8.45. The filled triangles are for the Glu^{164}→Gln mutant.

With 3-hexenoyl-CoA an alternative reaction pathway for the enolized intermediate exists. Abstraction of the α-proton of 3-hexenoyl-CoA generates a dienolized intermediate that can reprotonate at C2 or C4. The thermodynamically favored reprotonation at C4 would result in isomerization to the conjugated 2-hexenoyl-CoA. The 2-hexenoyl-CoA would be subsequently hydrated to 3-hydroxyhexanoyl-CoA. This isomerization occurs to a minor extent over 24 h (Bahnson, 1991), in spite of the report that crotonase catalyzes the isomerization (Stern, 1961).

Stereospecificity of the Crotonase-Catalyzed Dehydration of HB-CoA. In the case of HB-CoA, when an equilibrium mixture of HB-CoA and Cr-CoA was incubated with crotonase over 13 days at pD 7.4, no change in the integrated area of the HB-CoA *pro*-2*S* proton relative to the CoA internal standard was observed. The ^{1}H NMR spectrum of the equilibrium mixture obtained at the end of the incubation is shown in Figure 6. The lack of α-proton exchange is observable both in the integration of the doublet for the remaining α-hydrogen of HB-CoA and in the splitting pattern for the β-hydrogen of Cr-CoA. If the *pro*-2*S* proton of HB-CoA exchanged, the dehydration reaction would produce [2-^{2}H]-Cr-CoA. The ^{2}H substitution would remove the large trans $^3J_{HCCH}$ coupling observed for the β-hydrogen (doublet of quartets). The superimposable splitting pattern observed for the β-hydrogen consequently is strong evidence that minimal, if any, exchange has occurred and that the fraction of ^{1}H remaining, f in eq 5, is >0.9. Thus, the probability of an anti elimination or addition occurring with the *pro*-2*S* proton is less than 4×10^{-7}, and the energy of activation difference for the syn and anti eliminations must be greater than 8.8 kcal/mol.

Stereospecificity of Dehydrochlorination. CP-CoA presents the possibility of enzyme-catalyzed β-elimination of chloride, as well as exchange of the α-protons. If CP-CoA is dehydrochlorinated, the expected product, acryloyl-CoA, should be hydrated to form 3-hydroxypropionyl-CoA. This expected result is demonstrated in Figure 7. The resonances of the C2 and C3 protons of CP-CoA have disappeared, and resonances corresponding to 3-[2-^{2}H]-HP-CoA (a doublet at 3.86 ppm and a ^{2}H-broadened triplet at 2.83 ppm) have appeared. The appearance of the clean doublet for the C3

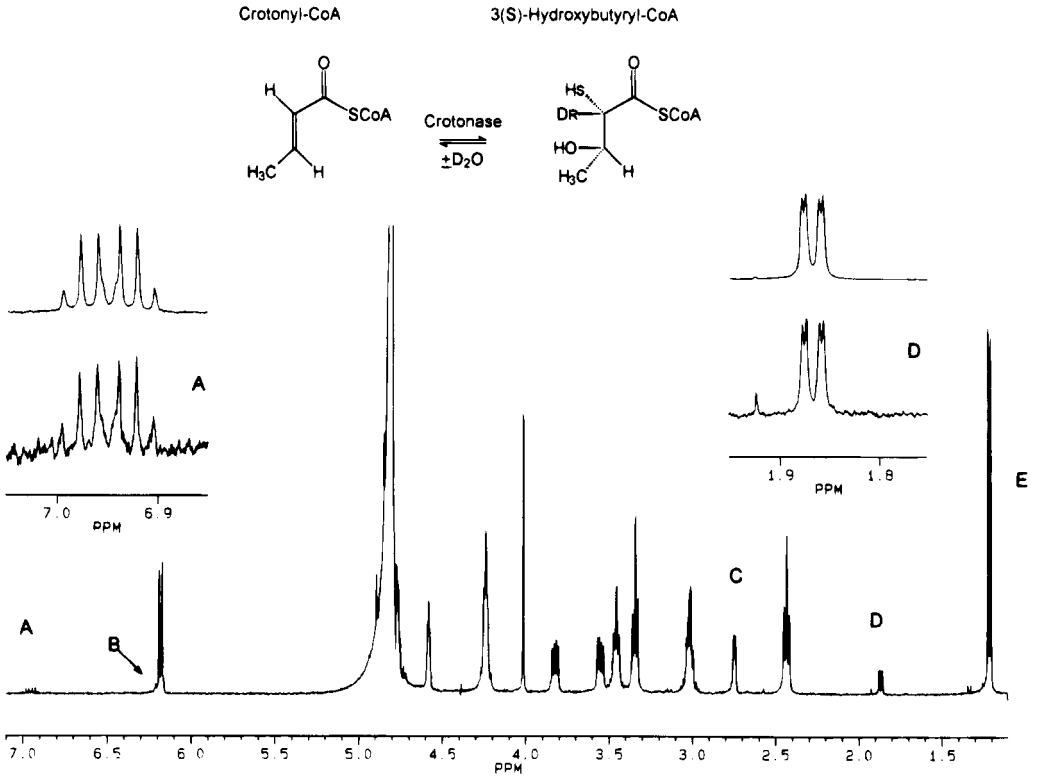


FIGURE 6: 400 MHz ^{1}H NMR spectra of an equilibrium mixture of HB-CoA and Cr-CoA in the presence of crotonase. Sample initially contained 10 mM HB-CoA, 2.5 μM crotonase, 100 mM phosphate (pD 7.4), and 100 μM EDTA. Equilibrium was reached in <5 min. The spectrum shown was obtained after 13 days and is identical to that taken 5.0 min after the addition of crotonase. The spectrum was processed using 0.3 Hz line broadening. The region from 7.1 to 1.0 ppm is shown. (A) C3 proton of Cr-CoA; (B) C2 proton of Cr-CoA; (C) C2 *pro-S* ^{1}H of HB-CoA; (D) C4 methyl resonance of Cr-CoA (the smaller splitting, J_{HCCCH}, is evidence for ^{1}H remaining at C2); (E) C4 methyl resonance of HB-CoA. The relative integration of the *pro-2S* proton of HB-CoA to the integration standard at 2.43 ppm remained constant over the duration of the experiment. The C2 proton of Cr-CoA (B) is only partially visible, with half of the doublet obscured by the resonance from the anomeric carbon of CoA; however, convincing evidence of its retention is clearly indicated by couplings to C3 and C4 protons. See the insets of signals A and D. The top expansion in each set indicates the coupling in Cr-CoA prior to reaction with crotonase. The bottom set shows the equilibrium Cr-CoA resonances of the same sample. The stability of these proton couplings is inconsistent with any significant ^{2}H exchange for the *pro-2S* ^{1}H of HB-CoA.

protons indicates that a single proton from C2 has been lost during the dehydrochlorination/rehydration sequence. This indicates that no nonstereospecific exchange of the α-protons of CP-CoA has occurred. No conclusion about the stereochemical course of the dehydrochlorination can be drawn since either a syn or an anti elimination would generate the same product, namely, acryloyl-CoA. When authentic 3-HP-CoA was incubated with crotonase under conditions identical to the CP-CoA incubation (Figure 7), only a single α-proton was exchanged, and the ^{1}H resonance for the C3 protons was simplified from a triplet at δ 3.85 ppm to a clean doublet, indicating that a single ^{1}H remained at C2. This again requires that the dehydration of 3-HP-CoA be stereospecific, although we have not rigorously quantified the number of reaction cycles. No ^{1}H resonances corresponding to the vinylic protons of acryloyl-CoA could be detected. This is anticipated since the C−C double bond in acryloyl-CoA is less stable than that present in Cr-CoA.

DISCUSSION

3-Quinuclidinone-Catalyzed α-Proton Exchange. The abstraction of the α-proton of CoA thiol esters is central to the role of thiol esters in C−C bond forming reactions and in β-elimination reactions. As such, the recent observation that nonnucleophilic amine bases can catalyze this proton abstraction in aqueous solution becomes a simple model for the enzyme-catalyzed reaction (Amyes & Richard, 1992). Amyes and Richard demonstrated that, in aqueous solution, 3-quin-generated stable enolates of acetone and ethyl thiol acetate that had lifetimes of 10^{-9}−10^{-10} s. On the basis of this observation, the authors suggested that an enolate could exist for a significant amount of time, on a mechanistic scale, in the presence of a basic amino acid residue at the active site of an enzyme.

The existence of an enzyme-bound CoA thiol ester enolate is of specific interest to the crotonase reaction since two of us proposed that this was a concerted reaction without a discrete enolized intermediate (Bahnson & Anderson, 1991). We sought to utilize 3-quin to generate enolates of selected CoA thiol esters to determine whether the CoA moiety confers special acidity on one or both of the diastereotopic α-protons of the thiol ester. Results in Table 2 show that the 3-quin can abstract the α-proton from CoA thiol esters with a second-order rate constant for acetyl-CoA that is not significantly greater than the second-order rate constant for *S*-ethyl thioacetate (Amyes & Richard, 1992). Consequently, the CoA structure does not, by itself, impart a greater acidity to the α-protons than that observed for the simpler model compound. The exchange rate constant for HB-CoA is 25-fold greater than that for Bu-CoA. This increase is readily attributable to the inductive effect of the hydroxyl substitution (More O'Ferral, 1982). Using the Taft σ* values, this increase corresponds to a ϱ* of 2.4, which is between the

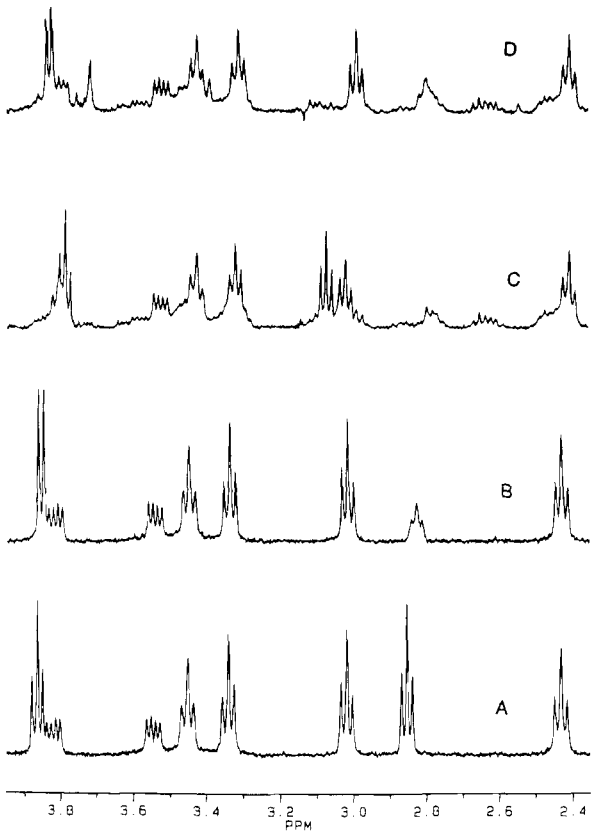


FIGURE 7: 400 MHz ^{1}H NMR spectrum of the equilibration of 3-HP-CoA and CP-CoA with crotonase. (A) Standard 3-HP-CoA (1.7 mM) in phosphate (pH 7.0 ± 0.1). (B) Same sample after crotonase-catalyzed (1.5 μM) stereospecific exchange of a single α-proton. The clean doublet at 3.87 ppm requires that the exchange be stereospecific. (C) 1.4 mM CP-CoA in phosphate (pH 7.0 ± 0.1). (D) Same CP-CoA sample incubated with crotonase (3.0 μM). The conversion to 3-HP-CoA is identified by the complete loss of the resonances for the C-2 and C-3 protons of CP-CoA and the appearance of the doublet at 3.86 ppm for 3-HP-CoA, indicating that a single proton is present at C2. The peak at 2.82 ppm is broadened due to the geminal ^{2}H. All spectra were processed with 0.3 Hz line broadening.

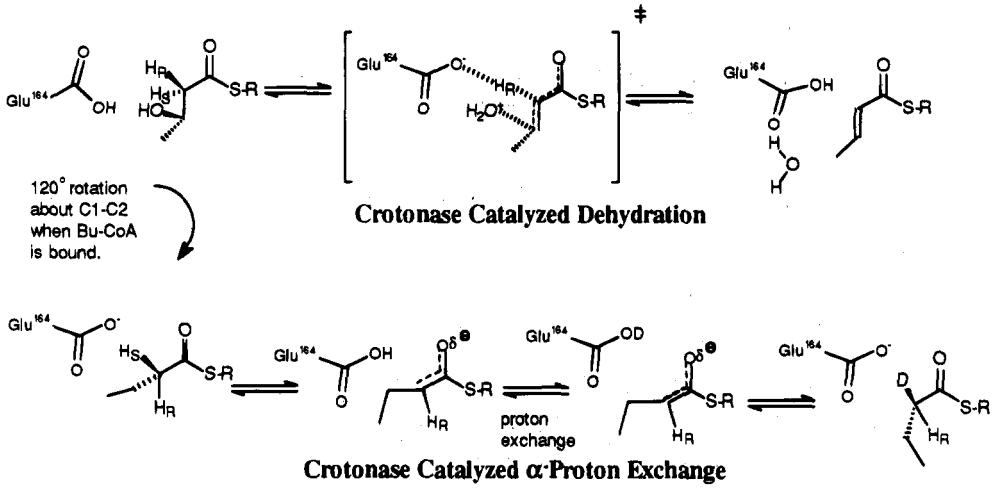
ϱ^* values of 2.2 and 3.2 for substituted fluoren-9-ylmethyl carboxylate esters (Thibblin, 1988; More O'Ferral, 1982) and propiophenones (Thomas & Stirling, 1977), respectively. The acidity of these compounds bracket that of the thiol ester as well, as predicted by the correlation of Stirling (Marshall *et al.*, 1977).

Mechanism of Elimination. While crotonase clearly catalyzes the enolization of CoA thiol esters (Table 2), the relevance to the mechanism of elimination is not established. The over 1000-fold difference in rates for exchange of the Bu-CoA α-proton and the dehydration reaction suggests that the role of the 3-hydroxyl substituent is significant. Since proton exchange requires exchange of the abstracted proton with solvent while the elimination reaction does not, it is possible that the smaller rate constant for exchange than for elimination is due to slow solvent exchange of the abstracted proton. However, the solvent exchange rate probably does not limit the exchange with the saturated acyl-CoA's since faster exchange is observed with CoA thiol esters whose α-protons are more acidic. The stereochemistry of the exchange reaction also highlights the difference in the two mechanisms since the *pro*-2*S* proton is exchanged with the saturated acyl-CoA's while the *pro*-2*R* hydrogen of HB-CoA is abstracted during the elimination reaction (Willadsen & Eggerer, 1975). *A priori*, three possible explanations of the differing stereospecificities could be considered: (i) Bu-CoA and HB-CoA bind in the same conformation, but different active site bases promote the proton abstraction. This arrangement of active site groups would be analogous to mandelate racemase, where there is an enzyme general base functional group on either face of an enolized intermediate (Landro *et al.*, 1991). (ii) HB-CoA and Bu-CoA bind in the same active site in the same manner, but the *pro*-2*S* proton of Bu-CoA is removed by solvent (or the base form of the buffer) and the *pro*-2*R* proton of HB-CoA is removed by an active site base. (iii) The stereospecific exchange of the α-protons of HB-CoA and Bu-CoA are catalyzed by the same active site base, and Bu-CoA and HB-CoA bind to the active site in different conformations, as suggested by Scheme 2.

The identification of a glutamate as the active site base required for catalysis in a homologous enzyme, enoyl-CoA isomerase (Müller-Newen & Stoffel, 1993), suggested a way of distinguishing mechanisms i and ii from iii. In addition to homology, Glu^{164} in crotonase is suggested to be the active site base by three observations. The Glu^{164}→Gln mutant has a decrease in catalytic activity of over 1000-fold, the mutant has lost the ability to exchange the α-proton of Bu-CoA while retaining the ability to polarize the CoA thiol ester, and the λ_{max} of bound DAC-CoA is 500 nm. Although it may have been postulated that Glu^{164} functions as an electrophilic catalyst by H-bonding to the carbonyl, the ability of the mutant to bind and induce the dramatic red shift in DAC-CoA indicates that the CoA binding site and the interactions that polarize the α,β-unsaturated thiol ester have been retained (D'Ordine *et al.*, 1994). These UV spectral results are analogous to those observed in ketosteroid isomerase, where mutation of the aspartate general base to asparagine resulted in at least a 1000-fold decrease in activity while not affecting the UV spectra of the α,β-unconjugated product (Kuliopoulos *et al.*, 1989).

It appears that Glu^{164} is the active site base that abstracts the α-proton in both the elimination and exchange reactions, as suggested in Scheme 2. Therefore, the explanation of the observed difference in stereospecificity must arise from which proton is presented to the basic form of the catalyst. We suggest that this could be accomplished by a relative rotation about the C1−C2 bond when HB-CoA and Bu-CoA are bound at the active site. Favorable interactions of the 3-hydroxyl group with the enzyme must provide the necessary energy to promote the difference in bound rotamer population. The experimental results with 2-ethylbutyryl-CoA rule out such a simple rotamer population preference as the *only* explanation, since it should be a substrate for the α-proton exchange reaction if there are two different sites available for the ethyl substituent at C2. This exchange was not detected, indicating that the exchange reaction preference between the two substrates is most likely due to an interaction of the enzyme with the hydroxyl group, which confers a specific orientation on the HB-CoA in the active site, as opposed to the Bu-CoA where there are no polar interactions with the alkyl moiety of the CoA thiol ester. Since 2-methylcrotonyl-CoA (Stern, 1961), 1-cyclohexenylcarboxyl-CoA (Steinman & Hill, 1973), and 2-propyl-3-hydroxypentanoyl-CoA (Li *et al.*, 1991) are substrates, crotonase can tolerate an alkyl substituent at the α-carbon.

Scheme 2



Crotonase Catalyzed Dehydration

Crotonase Catalyzed α-Proton Exchange

It is clear from model studies that the acidity of the α-protons of the thiol esters can be enhanced by the structure of the other substituents on the α-carbon, with increased acidity arising from both inductive and resonance effects. The enhanced acidity could lead to nonstereospecific exchange through at least two different mechanisms: a conformation with the *pro*-2*R* proton facing Glu[164] that is nonproductive with the saturated CoA's could now generate the intermediate. Alternatively, the increased acidity could make a weaker base on the opposite face catalytically functional, so that the active site will function like mandelate racemase (Landro *et al.*, 1991).

The evidence given in this paper suggests that Glu[164] is protonated during the normal catalytic cycle for interconverting HB-CoA and Cr-CoA. The UV−vis titration of the DAC-CoA−crotonase complex suggests that in the wild-type complex there is a functional group with a pK_a of 8.5. The pH variation of the exchange (Figure 4) indicates that one general acid−base functional group has to be deprotonated for the exchange to occur. This titratable group is not observed in the Glu[164]→Gln mutant. The simplest interpretation is that this pK_a is for Glu[164]. The decrease in the λ_{max} upon ionization of Glu[164] may be rationalized if the placement of a negative charge next to C2 of the acryloyl moiety decreases the polarization of the previously demonstrated polarization of the C2−C3 double bond (D'Ordine *et al.*, 1994). The pH variation of the exchange reaction further supports this assignment. The data only define the average of the two pK_a's from the bell curve (Cleland, 1977), and it is consistent with the conclusion that Glu[164], with a pK_a of 8.5, has to be ionized for exchange to occur, while a second electrophilic catalyst must be protonated. In contrast, the pH dependence of *V*/*K* for Cr-CoA does not identify a functional group that has to be deprotonated until below pH 4 (data not shown). We propose that Glu[164] has to be deprotonated for the α-proton exchange reaction to occur, but protonated for the addition−elimination reaction (see Scheme 2).

Dehydrochlorination of CP-CoA. The crotonase-catalyzed dehydrochlorination of CP-CoA shown in Figure 7 is analogous to the dehydrohalogenation reactions catalyzed by enolase (Stubbe & Abeles, 1980) and fumarase (Blanchard & Cleland, 1980). As with HB-CoA, the presence of an activated leaving group has made the reaction stereospecific. The pH variation of the dehydrochlorination reaction may well provide insight into the protonation state of the Glu[164] during the reaction, since it is not anticipated that the 3-chloro leaving group would require protonation for activation. These studies are being pursued.

Conclusion. Gerlt and Gassman have suggested that the mechanism of β-elimination reactions will always be stepwise (Gerlt *et al.*, 1991). They reason that stabilizing an enolized intermediate by "short, strong H-bonds" will necessarily lead to elimination from the intermediate (Gerlt & Gassman, 1992; 1993). The data presented in this paper indicate the importance of the 3-hydroxyl group in enhancing the enzyme-catalyzed rate of α-proton abstraction. In crotonase, we have demonstrated polarization of the carbonyl and agree that electrophilic polarization of the carbonyl is essential for catalysis (D'Ordine *et al.*, 1994). However, the results of the exchange reaction with 3-quin and HB-CoA in aqueous solution indicate that the formation of an enolized intermediate is not always sufficient to effect the catalysis of dehydration reactions. From the differences in rate, pH variation, and stereochemistry, it is clear in the crotonase-catalyzed dehydration that the leaving group must be activated to promote the elimination reaction. This is consistent with the existence of a significant primary ^{18}O isotope effect on the crotonase-catalyzed dehydration (Bahnson & Anderson, 1989) and for other dehydratases, such as fumarase where the proton abstraction comes to equilibrium prior to C−O bond cleavage (Blanchard & Cleland, 1980) and in enolase where the transition state for C−O bond cleavage also is partially rate-limiting (Anderson, 1981; Anderson *et al.*, 1994). If the activation of the leaving group is sufficiently great that the enolized intermediate proposed by Gerlt *et al.* (1991) does not have a significant lifetime, the elimination reaction will be concerted (Jencks, 1980), as we have previously proposed.

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