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Stereospecificity of the Reaction Catalyzed by Enoyl-CoA Hydratase

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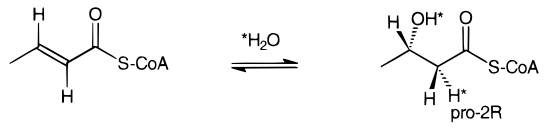
Abstract: Enoyl-CoA hydratase catalyzes the stereospecific hydration of α,β-unsaturated acyl-CoA thiolesters. Hydration of *trans*-2-crotonyl-CoA to 3(*S*)-hydroxybutyryl-CoA proceeds via the *syn* addition of water and thus the *pro*-2*R* proton of 3(*S*)-hydroxybutyryl-CoA is derived from solvent. Incubation of 3(*S*)-hydroxybutyryl-CoA with enzyme in D_2O results in the slow exchange of the *pro*-2*S* proton with solvent deuterium, in addition to the anticipated rapid exchange of the *pro*-2*R* proton. Further experiments have shown that the exchange of the *pro*-2*S* proton occurs in concert with the formation of the incorrect 3(*R*)-hydroxybutyryl-CoA enantiomer. The rate of 3(*R*)-hydroxybutyryl-CoA formation is 4×10^5-fold slower than the normal hydration reaction, but at least 1.6×10^6-fold faster than the non-enzyme-catalyzed reaction. This has allowed us to determine that the absolute stereospecificity for the enzyme-catalyzed reaction is 1 in 4×10^5. The initial formation of 3(*R*)-hydroxybutyryl-CoA is hypothesized to occur via the incorrect hydration of *trans*-2-crotonyl-CoA. Once formed, the 3(*R*)-hydroxybutyryl-CoA dehydrates to give *cis*-2-crotonyl-CoA. While the equilibrium constant for the hydration of *trans*-2-crotonyl-CoA to 3(*S*)-hydroxybutyryl-CoA is 7.5, the equilibrium constant for the hydration of *cis*-2-crotonyl-CoA to 3(*R*)-hydroxybutyryl-CoA is estimated to be ∼1000. To validate this reaction scheme, *cis*-2-crotonyl-CoA has been synthesized and characterized. These studies demonstrate that the enzyme is capable of catalyzing the epimerization of hydroxybutyryl-CoA.

Introduction

Enoyl-CoA hydratase (E.C. 4.2.1.17) catalyzes the stereospecific hydration of α,β-unsaturated acyl-CoA thiolesters.[1,2] Hydration of *trans*-2-crotonyl-CoA to 3(*S*)-hydroxybutyryl-CoA proceeds close to the diffusion-controlled limit for the encounter of enzyme and substrate, (k_{cat} 1790 s^{-1} and k_{cat}/K_m 3.6×10^8 $M^{-1}s^{-1}$).[3] The reaction occurs via the *syn* addition of water and thus the *pro*-2*R* proton of 3(*S*)-hydroxybutyryl-CoA is derived from solvent (Scheme 1).[2]

Scheme 1



trans-2-crotonyl-CoA 3(*S*)-hydroxybutyryl-CoA

Early studies on the mitochondrial enoyl-CoA hydratase revealed that the enzyme also catalyzes the hydration of *cis*-2-enoyl-CoAs to the corresponding 3(*R*)-hydroxyacyl-CoA products.[1] Since the subsequent enzyme in the fatty acid oxidation cycle is specific for the 3(*S*)-hydroxyacyl-CoA, enzymes occur that can catalyze the interconversion of 3(*R*)- and 3(*S*)-

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(1) Wakil, S. J. *Biochim. Biophys. Acta* **1956**, *19*, 497−504.
(2) Willadsen, P.; Eggerer, H. *Eur. J. Biochem.* **1975**, *54*, 247−252.
(3) Hofstein, H. S.; Feng, Y.; Anderson, V. E.; Tonge, P. J. *Biochemistry* **1999**, *38*, 9508−9516.

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hydroxyacyl-CoAs in order to facilitate the metabolism of fatty acids containing *cis* double bonds. Pathways for the metabolism of fatty acids containing *cis* double bonds exist in peroxisomes and include a dienoyl-CoA reductase and a hydroxyacyl-CoA epimerase.[4−8] The epimerase activity is linked to an enoyl-CoA hydratase that catalyzes the interconversion of *trans*-2-enoyl-CoAs and 3(*R*)-hydroxyacyl-CoAs.[8−10] Although each of these enzymes is stereospecific, the absolute stereospecificity has not previously been reported. Here we demonstrate that mitochondrial enoyl-CoA hydratase can catalyze the formation of 3(*R*)-hydroxybutyryl-CoA from *trans*-2-crotonyl-CoA.

Using NMR spectroscopy we have observed that the enzyme catalyzes incorporation of solvent deuterium into the *pro*-2*S* position of 3(*S*)-hydroxybutyryl-CoA, in addition to the expected exchange of the *pro*-2*R* proton. Exchange of the *pro*-2*S* proton has been shown to occur in concert with the formation of 3(*R*)-hydroxybutyryl-CoA. This has allowed us to determine the absolute stereospecificity for the enzyme-catalyzed reaction. During the time course of the NMR experiment, the total amount of crotonyl-CoA decreases, indicating a shift in the equilibrium to the hydroxylated species. This observation is rationalized by the knowledge that, once formed, the 3(*R*)-hydroxybutyryl-CoA will dehydrate to give *cis*-2-crotonyl-CoA. While the equilibrium constant for the hydration of *trans*-2-crotonyl-CoA to 3(*S*)-hydroxybutyryl-CoA is 7.5, the equilibrium constant for the hydration of *cis*-2-crotonyl-CoA to 3(*R*)-hydroxybutyryl-CoA is estimated to be ∼1000 (vide infra), explaining the overall decrease in crotonyl-CoA during epimerization. This model has been supported by studying the enzyme-catalyzed hydration of *cis*-2-crotonyl-CoA.

Experimental Procedures

Chemicals. Coenzyme A (CoA) lithium salt, 3(*S*)-hydroxybutyryl-CoA dehydrogenase, L-lactate dehydrogenase, pyruvate, thrombin, and NAD^+ were purchased from Sigma Chemical Co. Deuterium oxide (99.9%) was purchased from Cambridge Isotope Labs. His-bind resin was purchased from Novagen.

Preparation of *trans*-2-Crotonyl-CoA, 3(S)-Hydroxybutyryl-CoA and 4-Dimethylaminocinnamoyl-CoA. *trans*-2-Crotonyl-CoA was synthesized from crotonic acid and coenzyme A using the mixed anhydride method described previously.[3] 3(*S*)-Hydroxybutyryl-CoA was synthesized enzymatically from *trans*-2-crotonyl-CoA. In a typical reaction, 3 mM *trans*-2-crotonyl-CoA was incubated with 1 μM enoyl-CoA hydratase for 5 min. This enzyme concentration and incubation time is sufficient to completely hydrate the *trans*-2-crotonyl-CoA without resulting in any formation of 3(*R*)-hydroxybutyryl-CoA. Following removal of the enzyme using a Centricon, 3(*S*)-hydroxybutyryl-CoA was purified by HPLC using conditions identical to those used for *trans*-2-crotonyl-CoA. 3(*S*)-Hydroxybutyryl-CoA eluted with a retention time of 17.6 min compared to 23.6 min for *trans*-2-crotonyl-CoA. 4-Dimethylaminocinnamoyl-CoA was synthesized from 4-dimethylaminocinnamic acid and CoA following activation of the acid using 1,1′-carbonyl diimidazole.[3]

Preparation of *cis*-Crotonyl-CoA. *cis*-Crotonic acid (*iso*crotonic acid) was prepared following the procedure of Rappe as follows.[11] 2-Butanone (7.2 g, 0.1 mol) was mixed with 10 mL of 48% hydrobromic acid in an ice−water bath. Bromine was added (10 mL, 0.2 mol) dropwise, followed by the addition of 30 mL of water. The organic layer was separated and distilled under reduced pressure. The fraction boiling between 84 and 85 °C (10 mmHg) was collected. The yield of 1,3-dibromobutan-2-one was 9.31 g (41%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.8 (d, CH_3), 4.1 (d, 1 H, CHH′Br), 4.3 (d, 1 H, CHH′Br), 4.8 (q, 1 H, CHBr).

To a molar solution of potassium bicarbonate (50 mL) was added 1,3-dibromobutan-2-one (4.5 g, 0.02 mol) over a 5-minute period. The mixture was stirred for 2.5 h followed by extraction (2 × 15 mL) with ether. The water layer was acidified with dilute hydrochloric acid and again extracted with 6 × 20 mL portions of ether. The organic layer was dried over $MgSO_4$ overnight followed by concentration in vacuo (bath temperature around 0 °C) to give 1.1 g (65%) *cis*-crotonic acid. ^{1}H NMR showed the sample to be >98% *cis*. This compound was refrigerated until further use. ^{1}H NMR (300 MHz $CDCl_3$) δ 2.1 (d, d, 3H), 5.8 (d, q, 1 H), 6.4−6.6 (m, 1 H).

cis-Crotonyl-CoA was synthesized from *cis*-crotonic acid using the mixed anhydride method. Briefly, *cis*-crotonic acid (30 mg, 0.35 mmol) was dissolved in anhydrous ether (4 mL) with triethylamine (44 mg, 0.43 mmol), followed by the addition of ethyl chloroformate (37 mg, 0.35 mmol). The solution was stirred in an ice−water bath for 4 h. The mixed anhydride was then filtered and added dropwise to a solution of CoA in Na_2CO_3 (50 mM, pH 8), ethanol, and ethyl acetate (1:1:1) with stirring at 0 °C. The reaction progress was monitored by following the concentration of free thiol in solution using 5,5′-dithiol-bis(2-nitrobenzoic acid) (DTNB). When no free thiol was detected the solution was concentrated in vacuo to remove the organic solvent and purified by HPLC (Shimadzu) using a Vydac C_{18} 250 × 4.60 mm preparative column. Chromatography was performed using ammonium acetate (100 mM)/1.75% acetonitrile as buffer A and running a 0 to 30% gradient of 95% acetonitrile/5% H_2O (buffer B) over 40 min at a flow rate of 8 mL/min. Elution was monitored at 260 and 290 nm using a Shimadzu SPD-10A UV−vis detector, and fractions containing *cis*-2-crotonyl-CoA were pooled and lyophilized. The desired product was obtained in 25% yield as a white powder. ^{1}H NMR (500 MHz, D_2O) δ 8.56 (s, 1 H), 8.28 (s, 1 H), 6.27−6.22 (dt, 1H, J = 11.5, 7.0 Hz), 6.14 (d, 1 H, J = 11 Hz), 4.85 (t, 1 H), 4.61 (s, 1 H), 4.26 (s, 2 H), 4.04 (s, 1 H), 3.85 (q, 1 H), 3.58 (q, 1 H), 3.47 (t, 2 H), 3.03 (t, 2 H), 2.45 (t, 2 H), 2.04 (d, 3 H, J = 7.0 Hz), 0.91 (s, 3 H), 0.78 (s, 3 H). MALDI-MS calculated for $[C_{25}H_{39}N_7O_{17}P_3S]^-$ $[M - H]^-$: 834.5; found: 833.1.

Subsequent HPLC analysis revealed that the *cis*-2-crotonyl-CoA was contaminated by ∼25% of the *trans* isomer. However, prolonged incubation in solution at room temperature resulted in no further increase in the amount of *trans* and consequently we hypothesized that isomerization had occurred during the coupling reaction. The kinetics of the hydration of *cis*-2-crotonyl-CoA were performed at pH 7.4 in 20 mM phosphate buffer by monitoring the decrease in absorbance at 280 nm as described for the *trans* isomer.[3] Spectrophotometric analysis of the contaminated *cis*-2-crotonyl-CoA following addition of 0.28 nM enzyme revealed a fast decrease in absorbance at 280 nm followed by a slower phase. After the reaction was followed to completion, the ratio of the absorbance change in the fast and slow phases was 1:4, in keeping with the observed ratio of *trans* to *cis* isomers in the substrate. Subsequent modification of the HPLC method enabled us to separate the *cis* and *trans* isomers. Chromatography was performed using 50 mM KH_2PO_4 as buffer A and running a 0 to 50% gradient of methanol (buffer B) over 50 min at a flow rate of 8 mL/min. Under these conditions the retention time for *cis*- and *trans*-2-crotonyl-CoA was 38.4 and 38.0 min, respectively. Kinetic analysis of the repurified *cis*-2-crotonyl-CoA revealed monophasic rates of reaction comparable to the slow phase observed previously. The repurified *cis*-2-crotonyl-CoA was used for determination of k_{cat} and K_m using $\Delta\epsilon_{280}$ 4300 M^{-1} cm^{-1}.

Preparation of Enoyl-CoA Hydratase. Recombinant wild-type rat mitochondrial enoyl-CoA hydratase was expressed and purified from cultures of *Escherichia coli* as described.[3,12] This method involves

(4) Yang, S. Y.; Cuebas, D.; Schulz, H. *J. Biol. Chem.* **1986**, *261*, 12238−43.
(5) Smeland, T. E.; Li, J. X.; Chu, C. H.; Cuebas, D.; Schulz, H. *Biochem. Biophys. Res. Comm.* **1989**, *160*, 988−92.
(6) Hiltunen, J. K.; Palosaari, P. M.; Kunau, W. H. *J. Biol. Chem.* **1989**, *264*, 13536−40.
(7) Smeland, T. E.; Cuebas, D.; Schulz, H. *J. Biol. Chem.* **1991**, *266*, 23904−8.
(8) Malila, L. H.; Siivari, K. M.; Mäkelä, M. J.; Jalonen, J. E.; Latipää, P. M.; Kunau, W. H.; Hiltunen, J. K. *J. Biol. Chem.* **1993**, *268*, 21578−85.
(9) Yang, S. Y.; Elzinga, M. *J. Biol. Chem.* **1993**, *268*, 6588−6592.
(10) Qin, Y. M.; Haapalainen, A. M.; Conry, D.; Cuebas, D. A.; Hiltunen, J. K.; Novikov, D. K. *Biochem. J.* **1997**, *328 (Pt 2)*, 377−82.
(11) Rappe, C. *Org. Synth.* **1973**, *53*, 123−126.
(12) Wu, W. J.; Anderson, V. E.; Raleigh, D. P.; Tonge, P. J. *Biochemistry* **1997**, *36*, 2211−2220.

Table 1. Kinetic and Equilibrium Constants for the Hydration of *trans*- and *cis*-2-Crotonyl-CoA

k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	K (HBCoA/CrtCoA)
	trans-2-crotonyl-CoA		
1790 ± 120	5 ± 1	360 ± 96	7.5[b]
	cis-2-crotonyl-CoA		
152 ± 4	5 ± 1	30 ± 7	1000[c]

[a] Kinetic parameters determined in 20 mM phosphate buffer at 25 °C. Data for *trans*-2-crotonyl-CoA were taken from Hofstein et al.[3] k_{cat} and K_m for *cis*-2-crotonyl-CoA were determined using 0.29 nM enzyme and varying the substrate concentration from 1 to 50 μM. [b] The equilibrium constant for the hydration of *trans*-2-crotonyl-CoA (K_1 in Scheme 2) was determined in D_2O phosphate buffer at 25 °C following the addition of 1 μM enoyl-CoA hydratase to 3 mM *trans*-2-crotonyl-CoA. *K* was calculated from the ratio of the C4 methyl resonances for 3(*S*)-hydroxybutyryl-CoA (1.21 ppm) and *trans*-2-crotonyl-CoA (1.86 ppm). [c] The equilibrium constant for the hydration of *cis*-2-crotonyl-CoA (K_3 in Scheme 2) was estimated on the basis of the relative stability of *cis*- and *trans*-(*S*)-ethyl thiocrotonate calculated using ab initio methods.[19]

ethanol recrystallization and a CoA affinity column.[3] However, since bacterial cells contain an endogenous epimerase, we needed to confirm that the activity we were monitoring was not due to a contaminant in our enzyme preparation. Consequently, we subcloned the enoyl-CoA hydratase gene into a pET15(b) vector downstream of a $(His)_6$ amino acid tag. After metal-affinity chromatography using His-bind resin (Novagen), the His-tag was removed using thrombin, and the cleaved His-tag and thrombin were removed using the CoA affinity column.

For the NMR experiments, enzyme was exchanged into D_2O buffer (20 mM Na_2PO_4, pD 7.4) prior to elution from the CoA-sepharose affinity column using the D_2O buffer containing 0.3 M KCl. Enzyme concentration was determined by titration with 4-dimethylaminocinnamoyl-CoA.

NMR Spectroscopy. The ^1H NMR spectra were recorded at the SUNY Stony Brook NMR Center on a Bruker AMX-600 spectrometer and a Varian Inova 500 MHz spectrometer. All experiments in this study were performed at 25 °C, unless otherwise specified. Sixty-four scans were accumulated for each spectrum. NMR data were analyzed using Felix software (Biosym, MSI).

Analysis of NMR Data as a Function of Time. Alterations in NMR peak intensities during the incubation of hydroxybutyryl-CoA with enzyme in D_2O buffer were analyzed using eq 1 where A_t is the intensity at time t, A_i is the initial peak intensity, A_f is the final peak intensity, and k_{obs} is the observed first-order rate constant.[13]

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

The exchange rate, k_{exc}, was calculated from eq 2 which takes into account the total amount of acyl-CoA bound at any time.

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

where $[\text{acyl-CoA}]_T$ is the total acyl-CoA concentration and $[\text{acyl-CoA}]_B$ is the concentration of bound acyl-CoA. Since the concentration of acyl-CoA (2−3 mM) was significantly larger than the enzyme concentration (14−80 μM) and also the K_m for both hydroxybutyryl-CoA (10 μM, unpublished data) and crotonyl-CoA (5 μM, Table 1), the concentration of bound acyl-CoA ($[\text{acyl-CoA}]_B$) was equal to the enzyme concentration used.

Formation of 3(R)-Hydroxybutyryl-CoA. The stereochemistry of hydroxybutyryl-CoA was analyzed using the 3(*S*)-hydroxyacyl-CoA dehydrogenase/lactate dehydrogenase coupled assay. In a typical reaction mixture (total volume 1 mL), 0.067 mM of hydroxybutyryl-CoA was incubated with 3.6 units of 3(*S*)-hydroxyacyl-CoA dehydrogenase, 0.03 mM NAD^+, 42 units of l-lactate dehydrogenase, and 1.2 mM pyruvate. After 30 min incubation, the enzymes were removed by Centricon (Centricon-10, Amicon). The reaction mixture was analyzed by HPLC (Shimadzu) using an Alltech Econosil C-18 analytical column.[3] Chromatography was performed using 20 mM ammonium acetate/1.75% acetonitrile as buffer A and running a 0−25% gradient of 95% acetonitrile/5% water (buffer B) over 54 min at a flow rate of 1 mL/min. Elution was monitored at 260 and 290 nm using a Shimadzu SPD-10A UV−vis detector. The retention time for acetoacetyl-CoA was 13.6 and 14.4 min for hydroxybutyryl-CoA. Alternatively, chromatography was performed using the same column with 50 mM KH_2PO_4 as buffer A and by running a gradient of 0 to 100% methanol (buffer B) over 40 min at 1 mL/min. Under these conditions the acetoacetyl-CoA had a retention time of 19.8 min, and hydroxybutyryl-CoA a retention time of 20.5 min.

The formation of 3(*R*)-hydroxybutyryl-CoA was monitored using the above method following incubation of 300 μM 3(*S*)-hydroxybutyryl-CoA with 14 μM enoyl-CoA hydratase in 20 mM Na_2PO_4, 3 mM EDTA, and 300 mM NaCl pH 7.4 buffer at room temperature for 72 h. Samples were collected after 2, 4, 6, 20, 48, and 72 h for coupled assayed reaction analysis. In each case the enoyl-CoA hydratase was removed using a Centricon (5 min spin) prior to running the coupled assay. To confirm that epimerization was catalyzed by the enzyme's active site, incubations were also performed in the presence of 300 μM 4-dimethylaminocinnamoyl-CoA, a competitive inhibitor of the enzyme.

To confirm that 3(*R*)-hydroxybutyryl-CoA was indeed being formed by enoyl-CoA hydratase, the compound that did not react with 3(*S*)-hydroxyacyl-CoA dehydrogenase was purified for further analysis. Incubations of 3(*S*)-hydroxybutyryl-CoA with enoyl-CoA hydratase were performed in both H_2O and D_2O until the coupled assay revealed no further increase in acetoacetyl-CoA. The peak corresponding to hydroxybutyryl-CoA and presumed to be the 3(*R*) isomer was then isolated by preparative HPLC, lyophilized and characterized by NMR spectroscopy.

Results and Discussion

α-Proton Exchange in 3(S)-Hydroxybutyryl-CoA. Part of the ^1H NMR spectrum of 3.0 mM 3(*S*)-hydroxybutyryl-CoA in D_2O buffer is shown in Figure 1a.

This region of the spectrum shows a resonance at 2.43 ppm due to the CoA 6″-methylene group and resonances at 2.77 and 1.21 ppm due to the hydroxybutyryl C2−H methylene and C4−H methyl protons, respectively. Following addition of 80 μM enzyme (Figure 1a, 8 min) there is an immediate decrease in the hydroxybutyryl C2−H resonance at 2.77 ppm due to the rapid interconversion of 3(*S*)-hydroxybutyryl-CoA and *trans*-2-crotonyl-CoA that results in the exchange of the hydroxybutyryl *pro*-2*R* proton with solvent deuterium (Scheme 1). The decrease in the C2−H intensity is accompanied by a small upfield shift in frequency to 2.75 ppm attributed to an isotope effect on δ_H resulting from replacement of one methylene proton with deuterium (Figure 1b). The upfield C2−H resonance is characterized by a coupling constant (^3J) of 4 Hz, in contrast to $^3J = 6.5$ Hz for the C2−H resonance when two protons are present. In addition, the intensity of the C2−H resonance also decreases due to establishment of an equilibrium between crotonyl-CoA and hydroxybutyryl-CoA. The formation of crotonyl-CoA is demonstrated by the appearance of a peak at 1.86 ppm (Figure 1a, C4(Cr−CoA); Figure 1c) assigned to the crotonyl C4 methyl group. The integrated intensity of the hydroxybutyryl-CoA C4 methyl resonance at 1.21 ppm (Figure 1a, C4(HB−CoA)) decreases 12% upon addition of enzyme, in agreement with the previously determined equilibrium constant of 7.5 between 3(*S*)-hydroxybutyryl-CoA and *trans*-2-crotonyl-CoA (Table 1).

At lower concentrations of enzyme (e.g., 2.1 μM) there is no detectable change in the NMR spectrum over a 2 day period, following the initial rapid decrease in the C2−H intensity (data not shown). However, in the presence of 80 μM enzyme a time-

(13) D'Ordine, R. L.; Bahnson, B. J.; Tonge, P. J.; Anderson, V. E. *Biochemistry* **1994**, *33*, 14733−14742.

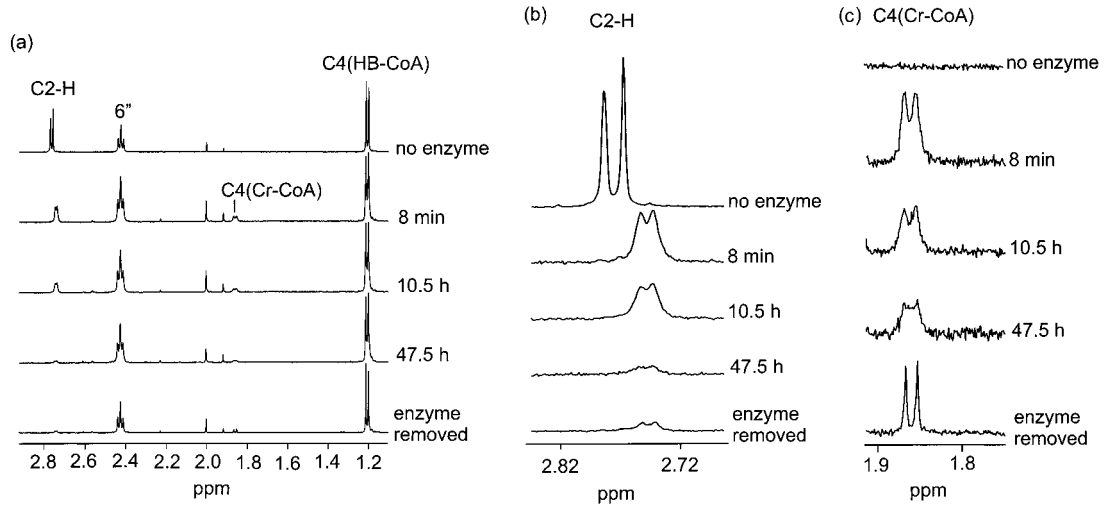


Figure 1. Selective ^{1}H NMR spectra of 3(*S*)-hydroxybutyryl-CoA over the region 2.92 to 1.10 ppm. The NMR samples all contained 300 mM KCl, 20 mM NaH_2PO_4 in D_2O at pD 7.4. All of the experiments were performed at 25 °C. Sixty-four scans were accumulated for each spectrum. The peak labeled C2−H corresponds to the hydroxybutyryl α-proton(s), the peak labeled 6″ corresponds to the CoA 6″ CH_2, the peak labeled C4(HB−CoA) corresponds to the hydroxybutyryl methyl group, and the peak labeled C4 (Cr−CoA) corresponds to the C4 methyl group of crotonyl-CoA. The singlets around 2 and 1.9 ppm are contaminants. (a) 3.0 mM 3(*S*)-hydroxybutyryl-CoA incubated with 80.0 μM enoyl-CoA hydratase with the incubation time shown at the right-hand side of each spectrum. The top spectrum was obtained prior to addition of enzyme, and the bottom spectrum was obtained following removal of the enzyme by Centricon filtration. (b) Enlargement of the region from 2.85 to 2.68 ppm. (c) Enlargement of the region from 1.75 to 2.08 ppm. The enlargement factor is 4-fold larger than that used for (b).

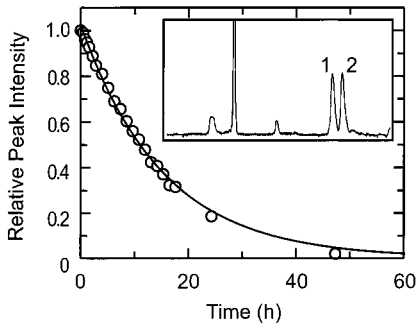


Figure 2. Plot of peak intensity of the *pro*-2*S* proton as a function of time for the sample in Figure 1. All peak intensities were normalized to that of the first data point. The data were fit to eq 1 giving k_{obs} 0.065 h^{-1}. Insert: HPLC analysis of the NMR sample after 47.5 h incubation following reaction with the 3(*S*)-hydroxyacyl-CoA dehydrogenase/lactate dehydrogenase coupled assay. Peak 1 (50%) corresponds to acetoacetyl-CoA, and peak 2 (50%) corresponds to the unreacted 3(*R*)-hydroxybutyryl-CoA. In a control experiment, under the same reaction conditions, 3(*S*)-hydroxybutyryl-CoA was completely converted into acetoacetyl-CoA.

dependent decrease in the C2−H resonance can be observed. Figure 1a shows NMR spectra of 3(*S*)-hydroxybutyryl-CoA in the presence of 80 μM enzyme at 8 min and at 10.5 and 47.5 h. The data demonstrate that the C2−H resonance decreases as a function of time until, after 48 h, it has almost vanished (Figure 1a and 1b). Clearly, the hydroxybutyryl *pro*-2*S* proton is also exchanging with solvent deuterium. Figure 2 shows a plot of the C2−H integration as a function of time, following the initial rapid decrease in intensity. The change in the intensity of the C2−H resonance can be reasonably accounted for by a first-order exponential decay with k_{obs} 0.065 ± 0.01 h^{-1}. This yields a value of (6.8 ± 1.1) × 10^{-4} s^{-1} for the exchange rate (k_{exc}) of the *pro*-2*S* proton (Table 2).

As expected, exchange of the hydroxybutyryl-CoA *pro*-2*S* proton results in loss of the crotonyl C2−H resonance at 6.19 ppm (data not shown). However, *pro*-2*S* exchange is also accompanied by an overall decrease in intensity of the C3−H and C4−H crotonyl resonances at 6.95 ppm (data not shown) and 1.86 ppm, respectively (Figure 1c). Fluctuations in the background made it difficult to quantitate the decrease in the crotonyl C3−H resonance; however, this decrease is not due to exchange of the C3−H proton with solvent deuterium, as the intensity of a peak at 4.24 ppm that includes the hydroxybutyryl C3−H and the CoA H5″ did not change appreciably during the experiment (data not shown) and as the coupling constant of the hydroxybutyryl C4−H methyl resonance remained constant (Figure 1a). The crotonyl methyl resonance at 1.86 ppm broadened and decreased in intensity during the experiment. The broad nature of this resonance made it difficult to accurately quantitate the decrease; however, it was hypothesized that the peak broadening was due to an exchange process between free and enzyme-bound crotonyl-CoA. Consequently, enzyme was removed by Centricon filtration and the NMR spectrum reacquired. Removal of the enzyme results in a dramatic narrowing of the crotonyl methyl resonance (Figure 1c), enabling the overall decrease in intensity to be estimated as 50%. In keeping with the observed decrease in the crotonyl-CoA concentration, the intensity of the hydroxybutyryl C4 methyl resonance increases ∼6% during exchange of the *pro*-2*S* proton. Comparison of the integrated intensity of the hydroxybutyryl-CoA and crotonyl-CoA C4 methyl resonances indicated that the hydroxybutyryl-CoA:crotonyl-CoA ratio had increased from a value of 7.5 following the initial addition of enzyme to a final value of 16. Since no other peaks grew in appreciably during the experiment, we conclude that the crotonyl-CoA-to-hydroxybutyryl-CoA equilibrium increased significantly during the experiment, accounting for the decrease in the concentration of crotonyl-CoA.

α-Proton Exchange Occurs in Concert with 3(*R*)-Hydroxybutyryl-CoA Formation. A plausible explanation for the alteration in the crotonyl-CoA/hydroxybutyryl-CoA equilibrium constant is that the exchange of the hydroxybutyryl-CoA *pro*-2*S* proton occurs via incorrect hydration of *trans*-2-crotonyl-CoA to give 3(*R*)-hydroxybutyryl-CoA and that the 3(*R*)-hydroxybutyryl-CoA is subsequently dehydrated to give *cis*-2-crotonyl-CoA. The alteration in the crotonyl-CoA/hydroxybutyryl-CoA equilibrium could then be rationalized by proposing that

Table 2. Time-Dependent Changes Observed in the ^{1}H NMR Spectra of Hydroxybutyryl-CoAs Catalyzed by Enoyl-CoA Hydratase in D_2O Buffer[a]

resonance	chemical shift (ppm)	^3J (Hz)	rate of change in D_2O (k_{exc} s^{-1})	rate of racemization (k_{RHB} s^{-1})	
				H_2O	D_2O
3(*S*)-Hydroxybutyryl-CoA					
C2−H[b]	2.77	6.5	ND[c]	d	d
C2−HD[e]	2.75	4	$(6.8 \pm 1.1) \times 10^{-4}$[f]	$(4.4 \pm 0.6) \times 10^{-3}$[g]	$(7 \pm 1) \times 10^{-4}$[g]
3(*R*)-Hydroxybutyryl-CoA					
C2−H[b]	2.77	6.5	ND[c]	d	d
C2−HD[e]	2.75	8	$(7.2 \pm 0.2) \times 10^{-4}$[f]	ND[c]	ND[c]
C4−H (HB−CoA)[h]	1.21	6.5[i]	$(8.5 \pm 0.9) \times 10^{-4}$[j]	ND[c]	ND[c]

[a] NMR experiments were performed in 20 mM phosphate buffer pD 7.4 at 25 °C. [b] C2−H refers to the hydroxybutyryl C2 methylene group. [c] Not determined. [d] In the initial exchange reaction there is no epimerization of the substrate. [e] C2−HD refers to the hydroxybutyryl C2 methylene group in which one of the protons has been replaced by deuterium. [f] k_{exc} is the rate of disappearance of the C2−HD resonance due to solvent exchange. [g] k_{RHB} is the rate of formation of 3(*R*)-hydroxybutyryl-CoA from the 3(*S*) enantiomer. [h] C4−H refers to the hydroxybutyryl C4 methyl group. [i] The coupling constant of C4−H does not change during the experiment. [j] k_{exc} is the rate of decrease of the C4−H resonance.

the equilibrium for hydration of *cis*-2-crotonyl-CoA lies more in favor of hydration than for the *trans* isomer, as originally reported by Wakil.[1]

3(*S*)-Hydroxyacyl-CoA dehydrogenase was used to test for formation of 3(*R*)-hydroxybutyryl-CoA. Oxidation of 3(*S*)-hydroxybutyryl-CoA to acetoacetyl-CoA by the dehydrogenase was driven to completion by coupling the reaction to the lactate dehydrogenase-catalyzed reduction of pyruvate. Subsequent analytical HPLC enabled resolution of acetoacetyl-CoA from any remaining hydroxybutyryl-CoA. The inset to Figure 2 shows an HPLC trace generated from the reaction mixture, following complete α-proton exchange that demonstrates the separation of acetoacetyl-CoA (peak 1) from unreacted hydroxybutyryl-CoA (peak 2). This indicates that at the end of the experiment there is a 50:50 mixture of 3(*S*) and 3(*R*)-hydroxybutyryl-CoA.

To determine the rate of formation of 3(*R*)-hydroxybutyryl-CoA, 300 μM 3(*S*)-hydroxybutyryl-CoA was incubated with 14 μM enoyl-CoA hydratase in H_2O phosphate buffer, pH 7.4. Samples were taken from the reaction mixtures at various times during the incubations and analyzed using the coupled assay and the ratio of the two HPLC peaks was then plotted against time. Curve fitting and data analysis yielded a first-order rate constant for the formation of 3(*R*)-hydroxybutyryl-CoA (k_{RHB}) of $(4.4 \pm 0.6) \times 10^{-3}$ s^{-1} (Table 2). This value is ~6.5-fold faster than the rate of α-proton exchange observed in D_2O using NMR spectroscopy (k_{exc} 6.8×10^{-4} s^{-1}; Table 2). To reconcile these differences, we measured the rate of formation of 3(*R*)-hydroxybutyryl-CoA in D_2O phosphate buffer pD 7.4, and obtained a value of k_{RHB} of $(7 \pm 1) \times 10^{-4}$ s^{-1}, identical to the previously determined k_{exc} (Table 2). We conclude that there is a solvent isotope effect on the formation of 3(*R*)-hydroxybutyryl-CoA. The value of k_{RHB} obtained in H_2O is 4×10^5-fold slower than k_{cat} for the normal hydration reaction (k_{cat} 1790 s^{-1}), but still at least 1.6×10^6-fold faster than the non-enzyme-catalyzed reaction (vide infra).

To confirm that unreacted product in the coupled assay was hydroxybutyryl-CoA, the unreacted peak was isolated, lyophilized, and redissolved in D_2O. This compound had an identical NMR spectrum to authentic hydroxybutyryl-CoA except that no C2−H peak was present due to deuterium exchange. 3(*R*)-hydroxybutyryl-CoA was also prepared from the 3(*S*) isomer in H_2O resulting in appearance of the expected C2−H resonance at 2.77 ppm in the spectrum of the 3(*R*) isomer.

To confirm that interconversion of the 3(*S*) and 3(*R*) enantiomers was reversible, 3(*R*)-hydroxybutyryl-CoA was incubated with enzyme and was shown to form 3(*S*)-hydroxybutyryl-CoA. Following the procedure described for 3(*S*)-hydroxybutyryl-CoA, the exchange of the 3(*R*)-hydroxybutyryl-CoA C2−H protons in D_2O was followed by NMR spectroscopy and samples were analyzed at various times using the 3(*S*)-hydroxyacyl-CoA dehydrogenase coupled assay. Figure 3 shows the partial ^{1}H NMR spectrum of 3(*R*)-hydroxybutyryl-CoA (1.94 mM) that had been enzymatically prepared in H_2O.

The triplet at 2.43 ppm is assigned to the CoA 6″-methylene group, while the hydroxybutyryl C2−H and C4−H resonances are observed at 2.77 and 1.21 ppm, respectively. Following addition of 73 μM enoyl-CoA hydratase, there is a rapid decrease in the C2−H resonance due to the enzyme-catalyzed interconversion of hydroxybutyryl-CoA and crotonyl-CoA (Figure 3a and 3b). However, unlike the experiment with the 3(*S*) enantiomer, the initial decrease in the C2−H resonance is not accompanied by a change in intensity of the hydroxybutyryl-CoA C4−H resonance, and there is no evidence for the formation of *cis*-2-crotonyl-CoA (C4−H 2.02 ppm). These data indicate that the equilibrium between *cis*-2-crotonyl-CoA and 3(*R*)-hydroxybutyryl-CoA must lie strongly in favor of hydration. In agreement with this, incubation of 3(*R*)-hydroxybutyryl-CoA with 1 μM enzyme did not result in any appreciable change in absorbance at 260 nm. An increase in absorbance would have been expected if a measurable amount of crotonyl-CoA were formed, as occurs in the reaction of 3(*S*)-hydroxybutyryl-CoA with enoyl-CoA hydratase.

As observed for the 3(*S*) enantiomer, deuterium exchange in 3(*R*)-hydroxybutyryl-CoA is accompanied by an upfield shift in the C2−H resonance to 2.75 ppm (Figure 3b). However, ^3J is 8.3 Hz for the C2(D)−H resonance, in contrast to the ^3J of 4 Hz observed for the 3(*S*) compound (Table 2). The difference in ^3J for the monodeuterated hydroxybutyryl-CoAs can be rationalized on the basis of the angle between the remaining C2−H proton and the proton at C3.[14−17] The 3(*S*)-hydroxybutyryl group is expected to exist predominantly in a staggered conformation in which the *pro*-2*R* proton and C3−H are *anti* to each other. Consequently, since we expect the *pro*-2*R* proton to be the first proton exchanged for both 3(*S*)- and 3(*R*)-hydroxybutyryl-CoA, then the remaining proton at C2 (*pro*-2*S*) will be *syn* or *anti* to the C3−H for the *S* and *R* enantiomers, respectively. The difference in ^3J thus arises from the angular dependence in the coupling constant and the ^3J for the diprotio C2−H resonance is an average of the individual ^3J for the two α-protons. Thus, the difference in ^3J for the mono-deuterohy-

(14) Karplus, M. *J. Chem. Phys.* **1959**, *30*, 11−15.
(15) Alberty, R. A.; Bender, P. *J. Am. Chem. Soc.* **1959**, *81*, 542−546.
(16) Gawron, O.; Glaid, A. J.; Fondy, T. P. *J. Am. Chem. Soc.* **1961**, *83*, 3634−3640.
(17) Mohrig, J. R.; Vreede, P. J.; Schultz, S. C.; Fierke, C. A. *J. Org. Chem.* **1981**, *46*, 4655−4658.

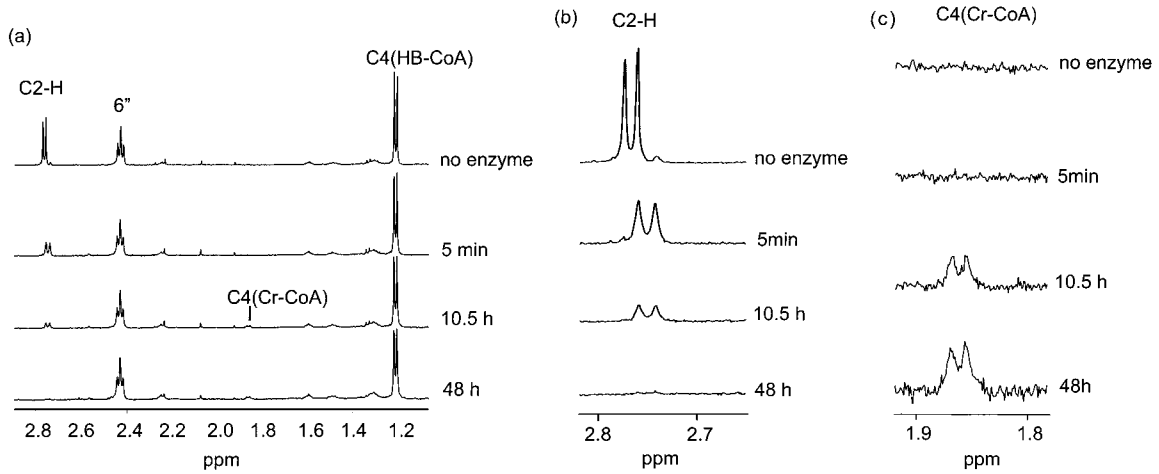


Figure 3. Selective ^{1}H NMR spectra of 3(*R*)-hydroxybutyryl-CoA over the region from 2.89 to 1.06 ppm. Conditions and peak assignments are the same as those in Figure 1. Peaks due to impurities in the substrate can be observed at 2.24, 2.07, 1.59, 1.49, and 1.31 ppm. These peaks did not change in intensity following addition of enzyme or during the experiment. (a) 1.94 mM 3(*R*)-hydroxybutyryl-CoA incubated with 73.0 μM enoyl-CoA hydratase with the incubation time shown at the right-hand side of each spectrum. The top spectrum was obtained prior to addition of enzyme. (b) Enlargement of the region from 2.65 to 2.82 ppm. (c) Enlargement of the region from 1.78 to 1.92 ppm. The enlargement factor is 4-fold larger than that used for (b).

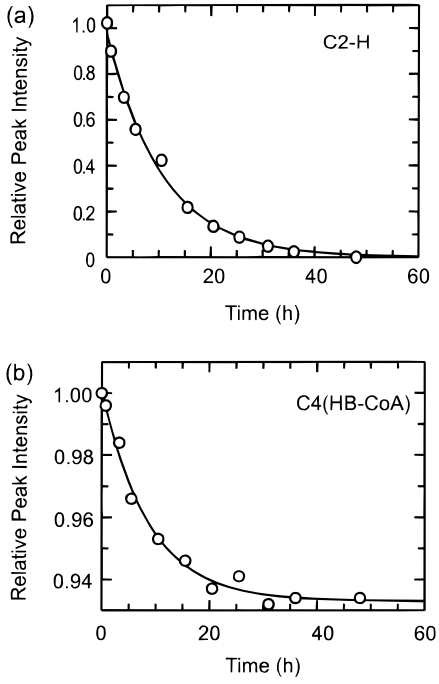


Figure 4. (a) Plot of peak intensity of the *pro*-2*S* proton as a function of time for the sample in Figure 3. All peak intensities were normalized to that of the first data point. The data were fit to eq 1 giving k_{obs} 0.098 $\pm$ 0.003 min^{-1}. (a) Plot of the peak intensity of the hydroxybutyryl-CoA methyl resonance (C4−HB) as a function of time for the sample in Figure 3. All peak intensities were normalized to that of the first data point. The data were fit to eq 1 giving k_{obs} 0.12 $\pm$ 0.01 h^{-1}, and the total change in peak intensity was 6.5%.

droxybutyryl-CoAs supports the proposal that the stereochemistry at C3 is different.

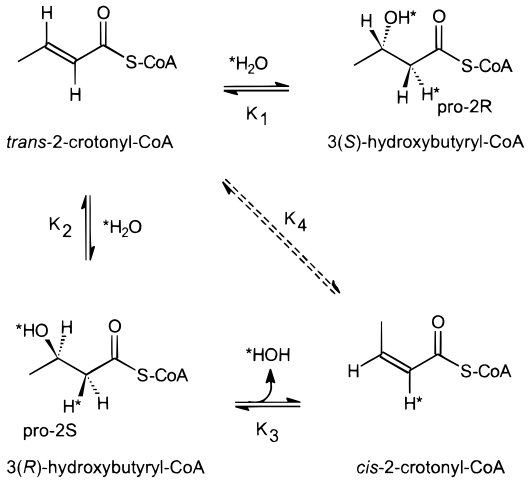
Following the initial α-proton exchange, the C2−H resonance at 2.75 ppm decreases over time (Figure 4a) with an observed first-order exponential decay of 0.098 $\pm$ 0.003 min^{-1}, giving k_{exc} (7.2 $\pm$ 0.2) $\times$ 10^{-4} s^{-1} (Table 2).

This value of k_{exc} is the same, within experimental error, of the value of 6.8 $\times$ 10^4 s^{-1} observed for 3(*S*)-hydroxybutyryl-CoA. As the C2−H resonance decreases, a peak at 1.86 ppm appears (Figure 3c) and the hydroxybutyryl methyl resonance at 1.21 ppm decreases by 6.5%. The latter observations are consistent with the formation of 3(*S*)-hydroxybutyryl-CoA that subsequently dehydrates to *trans*-2-crotonyl-CoA. Evidence for the time-dependent formation of 3(*S*) enantiomer was obtained by running the coupled assay at various times and demonstrating that the amount of acetoacetyl-CoA formed in the coupled assay increased from 0 to 50% of the total hydroxyacyl-CoA present, during the course of *pro*-2*S* proton exchange. The first-order rate constant for the decrease in the methyl resonance at 1.21 ppm is (8.5 $\pm$ 0.9) $\times$ 10^{-4} s^{-1} (Figure 4b) is identical within experimental error to that observed for the decrease in the C2−H resonance at 2.75 ppm, thus supporting the hypothesis that proton exchange and the formation of *trans*-2-crotonyl-CoA are coupled (Table 2). This observation confirms that the interconversion of the 3(*S*) and 3(*R*) enantiomers is reversible. However, the data suggest that the equilibrium constant between *cis*-2-crotonyl-CoA and 3(*R*)-hydroxybutyryl-CoA is substantially larger than the value of 4.3 originally reported by Wakil.[1] Since we had inferred but not proven the formation of *cis*-2-crotonyl-CoA from (*R*)-hydroxybutyryl-CoA, we undertook to synthesize *cis*-2-crotonyl-CoA and characterize its reaction with the enzyme.

Reaction of *cis*-2-Crotonyl-CoA. The ^{1}H NMR spectrum of *cis*-2-crotonyl-CoA differs from that of the *trans* isomer with C4−H at 2.02 ppm, C3−H at 6.24 ppm and C2−H at 6.13 ppm. Upon addition of a catalytic amount of enoyl-CoA hydratase, *cis*-2-crotonyl-CoA is rapidly converted to 3(*R*)-hydroxybutyryl-CoA as evidenced by appearance of the hydroxybutyryl methyl and C2−H resonances at 1.21 and 2.77 ppm, respectively (data not shown). The hydroxybutyryl-CoA that was formed was confirmed to be the 3(*R*) enantiomer by the inability of 3(*S*)-hydroxyacyl-CoA dehydrogenase to convert the product to acetoacetyl-CoA. However, prolonged incubation of the 3(*R*)-hydroxybutyryl-CoA with higher concentrations of enoyl-CoA hydratase resulted in formation of a 50:50 equilibrium mixture of the 3(*R*) and 3(*S*) product enantiomers.

We also measured kinetic constants for the reaction of *cis*-2-crotonyl-CoA with enoyl-CoA hydratase. At 25 °C in pH 7.4 20 mM phosphate buffer (H_2O), k_{cat} and K_m for the hydration of the *cis* isomer were determined to be 152 s^{-1} and 5 μM, respectively (Table 1). This contrasts with a k_{cat} of 1790 s^{-1}

Scheme 2



and a K_m of 5 μM for *trans*-2-crotonyl-CoA;[3] thus, k_{cat} is 12-fold slower for the *cis* isomer than for the *trans* isomer.

The observation that the enzyme rapidly exchanges one of the 3(*R*)-hydroxybutyryl-CoA α-protons with solvent deuterium is at odds with a recent report in which enoyl-CoA hydratase was shown to catalyze α-proton exchange for the 3(*S*) but not the 3(*R*) enantiomer of hydroxybutyryl-CoA.[18] At the relatively high enzyme concentrations used in our study (73−80 μM), exchange of one α-proton in both the 3(*R*) and 3(*S*) enantiomers is complete before acquisition of the first NMR spectrum. However, the difference in the rate of dehydration of 3(*R*)- and 3(*S*)-hydroxybutyryl-CoA could be sufficiently large such that, under the conditions of the experiments described by Xiang et al.,[18] the 3(*S*) has completely exchanged one α-proton before there has been any detectable exchange into the 3(*R*) α-proton.

Mechanism of Epimerization. The data presented above indicate that enoyl-CoA hydratase catalyzes the facile interconversion of 3(*R*)-hydroxybutyryl-CoA and *cis*-2-crotonyl-CoA, in addition to the well-characterized reaction involving 3(*S*)-hydroxybutyryl-CoA and *trans*-2-crotonyl-CoA. Since no evidence is available for epimerization via deprotonation/reprotonation at the hydroxybutyryl C3 position, we favor an epimerization mechanism that involves the hydration of *trans*-2-crotonyl-CoA directly to 3(*R*)-hydroxybutyryl-CoA. This can be most readily envisaged by addition of H_2O to the opposite face of *trans*-2-crotonyl-CoA than occurs normally. This would result in the 3(*R*) enantiomer of the product in which the *pro*-2*S* proton is derived from solvent. The subsequent rapid interconversion of the 3(*R*)-hydroxybutyryl-CoA with *cis*-2-crotonyl-CoA would then result in the exchange of the second (*pro*-2*R*) hydroxybutyryl proton with solvent. Thus, we propose that loss of the second C2−H proton observed in the NMR experiments is directly linked to the formation of the incorrect hydroxybutyryl enantiomer (Scheme 2). Since deuterium is also incorporated into the *pro*-2*S* proton of 3(*S*)-hydroxybutyryl-CoA, this requires that the formation of 3(*R*)-hydroxybutyryl-CoA from *trans*-2-crotonyl-CoA must be reversible. In keeping with this expectation, incubation of 3(*R*)-hydroxybutyryl-CoA with enzyme results in the formation of 3(*S*)-hydroxybutyryl-CoA (see above).

Modeling studies suggest that sufficient space exists in the active site such that water could approach the crotonyl-CoA double bond from the opposite face. However, recent Raman studies in this lab indicate that *trans*-2-crotonyl-CoA is likely bound to the enzyme in two conformations, either *s-cis* or *s-trans* about the crotonyl C1−C2 single bond (unpublished data). Hydration of the *s-cis* conformer results in formation of 3(*S*)-hydroxybutyryl-CoA, while hydration of the *s-trans* conformer, in which the opposite face of the ethylenic double bond is oriented toward the water molecule, yields 3(*R*)-hydroxybutyryl-CoA. The rapid formation of 3(*S*)-hydroxybutyryl-CoA compared to that of the 3(*R*) enantiomer results from the fact that the *s-cis* conformer of *trans*-2-crotonyl-CoA is optimally positioned with respect to the catalytic machinery, whereas the *s-trans* conformer is not. Thus, the observed stereochemistry of the hydration reaction is hypothesized to result from the preferential hydration of one of two bound substrate conformers rather than from preferential binding of a single substrate conformer. The first-order rate constant for the formation of 3(*R*)-hydroxybutyryl-CoA of 4.4×10^{-3} s^{-1} is 4×10^5-fold slower than k_{cat} for the formation of 3(*S*)-hydroxybutyryl-CoA in the normal hydration reaction (1790 s^{-1}; Table 1). Thus, the stereospecificity for the enzyme-catalyzed hydration of *trans*-2-crotonyl-CoA is 1 in 4×10^5. This value is similar to the limit estimated previously based on exchange experiments using lower concentrations of enzyme.[13]

Equilibrium Constant for the Interconversion of 3(*R*)-Hydroxybutyryl-CoA and *cis*-2-Crotonyl-CoA. Early studies gave the equilibrium constants for the hydration of *trans*- and *cis*-2-crotonyl-CoA as 2 and 4.3, respectively.[1] These two equilibria are labeled K_1 and K_3, respectively, in Scheme 2. Although the reported K_1 is lower than our measured value (7.5), the published values are qualitatively consistent with our hypothesis that the overall equilibrium between crotonyl-CoA and hydroxybutyryl-CoA increases during the NMR experiment as 3(*R*)-hydroxybutyryl-CoA is formed.

To obtain a lower limit for K_3, we estimated that the limit of our ability to detect changes in NMR peak integrals was ∼1%. Since the addition of 1 μM enoyl-CoA hydratase to a solution of 3 mM 3(*R*)-hydroxybutyryl-CoA failed to elicit any observable change in the intensity of the hydroxybutyryl C4−H resonance, this gave 100 as a lower limit for K_3. Additionally, K_3 can be calculated if the relative stabilities of *cis* and *trans*-2-crotonyl-CoA (K_4, Scheme 2) are known. Previous ab initio calculations had determined that the *cis* isomer of (*S*)-ethyl thiocrotonate was 12 kJ mol^{-1} less stable than the *trans* isomer.,[19] giving K_4 0.0079 at 25 °C. Since 3(*S*)-hydroxybutyryl-CoA and 3(*R*)-hydroxybutyryl-CoA have the same energy, the equilibrium for hydration of *trans*-2-crotonyl-CoA to 3(*R*)-hydroxybutyryl-CoA (K_2) must be the same as that for the hydration of *trans*-2-crotonyl-CoA to 3(*S*)-hydroxybutyryl-CoA (K_1, 7.5). This is supported by the HPLC data that demonstrate equal concentrations of the two stereoisomers following completion of α-proton exchange (see above). This enables a K_3 of 0.001 to be calculated using the relationship $K_3 = K_4/K_2$. K_3 is the equilibrium constant for the dehydration of 3(*R*)-hydroxybutyryl-CoA to *cis*-2-crotonyl-CoA and thus the equilibrium constant for the hydration of *cis*-2-crotonyl-CoA is 1000, which is 130-fold more favorable than the hydration of *trans*-2-crotonyl-CoA (7.5). This value of K_3 is substantially larger than that reported by Wakil (4.3).[1] However the *cis*-2-crotonyl-CoA (*iso*crotonyl-CoA) used in the previous study contained 30% of the *trans* isomer, which would have affected calculation of the equilibrium constant for hydration of the *cis* isomer if it were not corrected for.

By using K_1, K_2, and K_3 the equilibrium concentrations of the different species can be calculated. The two hydroxybutyryl-

(18) Xiang, H.; Luo, L. S.; Taylor, K. L.; Dunaway-Mariano, D. *Biochemistry* **1999**, *38*, 7638−7652.

(19) Fausto, R.; Tonge, P. J.; Carey, P. R. *J. Chem. Soc., Faraday Trans.* **1994**, *90*, 3491−3503.

CoA stereoisomers have equal concentrations, each 46.8% of the total; *trans*-2-crotonyl-CoA is 6.3%, and *cis*-2-crotonyl-CoA is 0.05%. Immediately after the enzyme is added, the concentration of *trans*-2-crotonyl-CoA is 12% of the total, and therefore the concentration of *trans*-2-crotonyl-CoA is expected to decrease by 50% during the time course of the experiment. This is in agreement with the change in the integrated intensity of the crotonyl-CoA methyl resonance at 1.86 ppm (see above).

Epimerase Activity Is Not Due to a Bacterial Contaminant and Is Catalyzed by the Active Site. Our initial experiments were performed using recombinant enoyl-CoA hydratase purified by ethanol recrystallization and a CoA affinity column.[3] However, since bacterial cells contain an endogenous epimerase, we needed to confirm that the activity we were monitoring was not due to a contaminant in our enzyme preparation. Consequently, we subcloned the enoyl-CoA hydratase gene into a pET15(b) vector downstream of a $(His)_6$ amino acid tag. Subsequent metal-affinity purification of the enzyme using the N-terminal His-tag followed by removal of the His-tag using thrombin yielded enzyme with identical epimerase activity to the protein purified by more conventional means. This effectively eliminated the possibility that the observed epimerase activity resulted from a contaminant co-purified with the enzyme. In addition, to confirm that 3(*R*)-hydroxybutyryl-CoA was catalyzed by the active site, incubations were performed in the presence of 300 μM 4-dimethylaminocinnamoyl-CoA. The rate of epimerization was reduced by 25-fold.

Uncatalyzed Rate of Hydration. A final question that required attention concerned the uncatalyzed rate of hydration of crotonyl-CoA. Conceivably, the apparent epimerase activity we were observing could result from the non-stereospecific non-enzyme-catalyzed hydration of crotonyl-CoA. Bearne and Wolfenden have described methods to measure the non enzymatic hydration of fumarate, which involved incubating fumarate at 100−150° for several hours.[20] However, since crotonyl-CoA contains a labile thiolester bond, we expected that hydrolysis would compete with hydration.[21] Consequently, to estimate an upper limit for the nonenzymatic hydration of crotonyl-CoA, we dissolved crotonyl-CoA in pH 7.4 D_2O buffer and obtained ^1H NMR spectra over a period of 60 days. After 60 days there was no significant change in the intensity of the crotonyl-CoA peaks, and there was no evidence for formation of 3-hydroxybutyryl-CoA. If we assume that the limit of detection in the NMR experiments was a 1% change in the crotonyl-CoA peak intensities, then we can estimate an upper limit of the uncatalyzed hydration reaction of 2.7×10^{-9} s^{-1}. This value is 1.6×10^6-fold slower than the rate of formation of 3(*R*)-hydroxybutyryl-CoA that occurs in the presence of enzyme (4.4×10^{-3} s^{-1}), confirming that the latter reaction is catalyzed by the enzyme.

(20) Bearne, S. L.; Wolfenden, R. *J. Am. Chem. Soc.* **1995**, *117*, 9588−9589.

(21) Mohrig, J. R.; Moerke, K. A.; Cloutier, D. L.; Lane, B. D.; Person, E. C.; Onasch, T. B. *Science* **1995**, *269*, 527−9.

Conclusions

In summary, we have shown that enoyl-CoA hydratase catalyzes the hydration of crotonyl-CoA with a stereospecificity of 1 in 4×10^5. Formation of the incorrect stereoisomer likely occurs via *syn* addition of water to the incorrect face of the *trans*-2-crotonyl-CoA double bond. To account for the exchange of the hydroxybutyryl *pro*-2*S* proton, the enzyme must also catalyze the dehydration of 3(*R*)-hydroxybutyryl-CoA to *cis*-2-crotonyl-CoA. Thus, the enzyme is capable of catalyzing the epimerization of hydroxybutyryl-CoA. There is no evidence that epimerization occurs via deprotonation/reprotonation at the hydroxybutyryl C3 center. To corroborate our model, we have synthesized *cis*-2-crotonyl-CoA and determined that it is hydrated to 3(*R*)-hydroxybutyryl-CoA ~12-fold more slowly than the corresponding hydration of *trans*-2-crotonyl-CoA to 3(*S*)-hydroxybutyryl-CoA.

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