Orientation of Coenzyme A Substrates, Nicotinamide and Active Site Functional Groups in (Di)enoyl−coenzyme A Reductases†

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ABSTRACT: The stereochemical course of reduction of dienoyl-coenzyme A (CoA) thiolesters catalyzed by the 2,4-dienoyl-CoA reductase from rat liver mitochondria was investigated. The configuration of the double bond in the 3-enoyl-CoA products was determined by ^{1}H NMR, and experiments to determine the stereochemical course of reduction at Cα and Cδ by use of 4-^{2}H-labeled β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), were conducted in H_2O and D_2O. Defining the diastereoselectivity of the reaction, catalyzed by the Δ^3,Δ^2-enoyl-CoA isomerase, facilitated the determination of the stereochemical course of reduction by 2,4-dienoyl-CoA reductase. The absence of solvent exchange of the proton transferred during the Δ^3,Δ^2-enoyl-CoA isomerase catalyzed equilibration of *trans*-2- and *trans*-3-enoyl-CoAs, coupled with the strong sequence homology to enoyl-CoA hydratase support the intramolecular suprafacial transfer of the *pro-2R* proton of *trans*-3-enoyl-CoA to the *pro-4R* position of *trans*-2-enoyl-CoA. The results indicate that the configuration of the double bond of the 3-enoyl-CoA product is *trans* and that a general acid-catalyzed addition of a solvent derived proton/deuteron occurs on the *si* face at Cα of the dienoyl-CoA. The addition of the *pro-4S* hydrogen from NADPH occurs on the *si* face at Cδ of *trans*-2,*cis*-4-dienoyl-CoA and on the *re* face at Cδ of *trans*-2,*trans*-4-dienoyl-CoA. The stereochemical course of reduction of InhA, an enoyl-thiolester reductase from *Mycobacterium tuberculosis*, was also determined by use of [4-^{2}H]NADH in D_2O. The reduction of *trans*-2-octenoyl-CoA catalyzed by InhA resulted in the *syn* addition of $^2H_2$ across the double bond yielding (2*R*,3*S*)-[2,3-$^2H_2$]octanoyl-CoA. In the crystal structure of the InhA ternary complex, the residue donating the proton to Cα could not be identified [Rozwarski, D. A., Vilcheze, C., Sugantino, M., Bittman, R., and Sacchettini, J. C. (1999) J. Biol. Chem. 274, 15582−15589]. The current results place further restrictions on the source of the proton and suggest the reduction is stepwise.

Enoyl-thiolester reductases are involved in many physiological processes but are primarily involved in fatty acid elongation pathways (*2*−*6*). These enzymes catalyze the NAD(P)H[1]-dependent reduction of the α,β double bond of the enoyl-thiolester to the resulting saturated acyl-thiolester and stereochemistry of these reductions has been intensively studied (*1*, *7*−*9*). Even though these enzymes all utilize a reduced pyridine nucleotide, the diastereoselectivity of the hydride transfer from the C4 of the nicotinamide ring varies among them. Likewise, the addition of a solvent-derived hydrogen occurs with varied stereospecificity, and in all previously examined cases the addition of this solvent-derived hydrogen occurs at the α-carbon (*1*). Unlike the reactions catalyzed by these reductases, the 2,4-dienoyl-CoA reductase (24DCR) catalyzes the unusual 1,4 addition of H_2 across a conjugated diene where the hydride equivalent from NADPH is presumably added to Cδ of the acyl chain.

The metabolism of most unsaturated fatty acids proceeds through a pathway requiring the 24DCR. Naturally occurring fatty acids possess cis double bonds in contrast to trans double bonds present in partially hydrogenated vegetable oils (*10*). The 24DCR is involved in the metabolism of all unsaturated fatty acids with double bonds originating at even-numbered positions (*11*, *12*) and the majority of unsaturated fatty acids with double bonds at odd-numbered positions (*13*, *14*), regardless of the stereochemical configuration of the double bonds. Consequently, 24DCR's ability to reduce both cis and trans double bonds at position 4 is of particular interest with respect to the orientation of the dienoyl-thiolester and the regiospecificity within the enzyme active site.

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[1] Abbreviations: AcAc-CoA, acetoacetyl-coenzyme A; CoA, coenzyme A; 24DCR, 2,4-dienoyl-coenzyme A reductase; ECH, enoyl-coenzyme A hydratase; ECI, Δ^3,Δ^2-enoyl-coenzyme A isomerase; HPLC, high-performance liquid chromatography; NAD^+, β-nicotinamide adenine dinucleotide; NADH, β-nicotinamide adenine dinucleotide, reduced form; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TAPS, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; *t2c*4DDCoA, *trans*-2,*cis*-4-decadienoyl coenzyme A; *t2t*4DDCoA, *trans*-2,*trans*-4-decadienoyl coenzyme A; *t2t*4HDCoA, *trans*-2,*trans*-4-hexadienoyl-coenzyme A.

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Recent studies have identified the enzymatic targets of the mainstream antimicrobial agents triclosan and isoniazid as being members of the reductase/dehydrogenase superfamily (*15*−*18*). One in particular, the InhA enoyl-thiolester reductase from *Mycobacterium tuberculosis*, has been identified as the target for the traditional drugs isoniazid and ethionamide (*15*). The crystal structures of InhA (*19*), InhA complexed with NAD^+ and isoniazid (*20*), and the ternary complex with NAD^+ and *S*-3-hexadecenoyl-*N*-acetylcysteamine (*21*) have been determined, but the stereochemical course of reduction has not been reported to date. Protein sequence alignments of the 24DCR with InhA have shown some degree of homology, especially within the active-site residues of InhA. As a result of this homology, the stereochemical course of reduction by InhA was determined so that it could be compared to the 24DCR. Determining the stereochemistry of reduction of both the 24DCR and InhA provides information about the chemical mechanism of reduction as well as information about the enzyme's active sites and the orientation of the two substrates. This information should prove useful in the rational design of potential inhibitors and therapeutics against enoyl-thiolester reductases.

EXPERIMENTAL PROCEDURES

Materials. *trans*-2,*trans*-4-Decadien-1-al, *trans*-2,*trans*-4-hexadienoic acid, formic-*d*-acid (potassium salt), and deuterium oxide (99.9 atom % D) were from Aldrich. *cis*-4-Decen-1-al was from Lancaster and *trans*-3-hexenoic acid was from Acros Organics. Coenzyme A (lithium salt) was from U.S. Biochemical Corp. Monobasic (KH_2PO_4) and dibasic (K_2HPO_4) potassium phosphate were from Fisher Scientific. *Arthrobacter* sp. and *Candida* sp. acyl-CoA oxidase, *Thermoplasma acidophilum* and *Cryptococcus uniguttulatus* glucose dehydrogenase, β-hydroxyacyl-CoA dehydrogenase (bovine liver), catalase (bovine liver), formate dehydrogenase (yeast), lactate dehydrogenase, NAD^+, $NADP^+$, NADPH, and acetoacetyl-CoA were from Sigma. [1−^{2}H]-Glucose was from Omicron Biochemicals, Inc. All other reagents were of research grade or better and were obtained from commercial sources.

Synthesis of CoA Substrates. The *trans*-2,*trans*-4-hexadienoyl-CoA (*t*2*t*4HDCoA) and *trans*-3-hexenoyl-CoA were synthesized from the corresponding fatty acid via the mixed anhydride method (*22*). The *t*2*t*4HDCoA was then purified by reversed-phase HPLC on a 10 × 250 mm Econosil C18 column (Alltech). The syntheses of *trans*-2,*trans*-4-decadienoyl-CoA (*t*2*t*4DDCoA) and *cis*-4-decenoyl-CoA were performed in a similar manner. The commercially available *trans*-2,*trans*-4-decadien-1-al and *cis*-4-decen-1-al were oxidized to their corresponding acids with Ag_2O (*23*, *24*). The acids were then used to make the corresponding acyl-CoA via the mixed anhydride method and were purified by reversed phase HPLC. After purification, *cis*-4-decenoyl-CoA and *trans*-3-hexenoyl-CoA were stored at −20 °C as lyophilized powders. The *t*2*t*4HDCoA and *t*2*t*4DDCoA were concentrated to ∼1 mM, sparged with helium, and stored frozen at −80 °C.

The *trans*-2,*cis*-4-decadienoyl-CoA (*t*2*c*4DDCoA) was prepared immediately prior to use as follows. Initially, *cis*-4-decenoyl-CoA was dissolved in buffer [20 mM KP_i in H_2O (pH 7.6)] or D_2O (pD 7.6)) and was enzymatically converted to *t*2*c*4DDCoA by the addition of catalytic amounts of acyl-CoA oxidase (*Arthrobacter* sp.) and catalase. The progress of the reaction was monitored by observing the increase in absorbance at 296 nm and the reaction was typically complete within 15 min.

High-Performance Liquid Chromatography. HPLC purification of the acyl-CoA substrates and products were performed on an Alltech Econosil C18 reversed-phase column (10 × 250 mm). The CoA thiolesters were eluted with a methanol gradient and were detected by the ultraviolet absorbance of the adenine and dienoyl-thiolester at 260 and 296 nm, respectively.

Synthesis and Purification of [4S-^{2}H]NADPH. [4*S*-^{2}H]-NADPH was prepared enzymatically and purified in the following manner. To begin, 5 mg of $NADP^+$ was added to 2.5 mL of potassium phosphate buffer (100 mM, with 100 μM EDTA, pH 8.0) containing [1−^{2}H]glucose (∼8 mM). The reaction was initiated by the addition of glucose dehydrogenase from *C. uniguttulatus*, which adds a hydrogen to the *pro-4S* position of $NADP^+$ (*25*). The increase in absorbance at 340 nm was used to monitor the reaction. When there was no further change in the A_{340}, the enzyme was removed by vortexing the reaction solution after the addition of 50 μL of $CHCl_3$. The supernatant after centrifugation was then purified by reversed-phase HPLC using a modified method of Klaidman et al. (*26*). The [4*S*-^{2}H]-NADPH was purified on a 4.6 × 250 mm Econosil C18 column (Alltech) that had been equilibrated with buffer A (200 mM NH_4HCO_3, pH 7.6) at a flow rate of 1 mL/min. After the sample was loaded, the column was washed for 15 min at 100% buffer A, followed by a 5 min linear gradient to 15% MeOH in buffer A. The column was washed for 10 min at 15% MeOH in buffer A, followed by a second 5 min linear gradient to 30% MeOH in buffer A. Finally, the column was washed for 10 min with 30% MeOH in buffer A. The fractions with an A_{260}/A_{340} absorbance ratio less than 2.25 were pooled and desalted as follows. Initially, the concentration of the NH_4HCO_3 buffer in the pooled fractions was increased to approximately 300 mM. The [4*S*-^{2}H]-NADPH was loaded onto a 1.0 × 3.4 cm C18 column (Prep Sep, Fisher) and eluted with 20% MeOH in ddH_2O. The desalted [4*S*-^{2}H]NADPH was then pooled, lyophilized, and stored at −80°C.

^{1}H NMR Acquisition. All ^{1}H NMR spectra were acquired on a Varian Innova 600 MHz spectrometer operating at 599.908 MHz equipped with a triple-resonance probe at a 6600 Hz sweep width. The ^{1}H data were acquired in either D_2O or phosphate-buffered D_2O (20 mM, pD 7.6) at 25 °C with a solvent (H_2O) presaturation pulse 1.5 s in duration. A 90° pulse with a 7.9 μs pulse width followed by a 1.7 s acquisition time were used. At least 800 transients for all ^{1}H NMR spectra were acquired. Chemical shifts were referenced internally by use of the triplet at 2.43 ppm (t, 2H) from the 6″ methylene protons of the pantetheine backbone of CoA. The proton resonances from the coenzyme A were readily recognized and typical chemical shifts were as follows: δ 8.58 (s, 1H), 8.28 (s, 1H), 6.19 (d, 1H), 4.59 (m, 1H), 4.25 (m, 1H), 4.03 (s, 1H), 3.85 (q, 1H), 3.57 (q, 1H), 3.46 (t, 2H), 3.34 (t, 2H), 3.04 (t, 2H), 2.43 (t, 2H), 0.89 (s, 3H), 0.76 (s, 3H). These values are in agreement with those previously reported by D'Ordine et al. (*27*).

trans-2,cis-4-Decadienoyl-CoA. 7.52 (d of d, 1H, J_{trans} = 15.1 and 10.8 Hz, Cβ), 6.20 (d, 1H, J_{trans} = 15.1 Hz, Cα), 6.11 (d of d, 1H, J_{cis} = 10.3 and 10.9 Hz, Cγ), 6.06 (m, 1H, J_{cis} = 10.2 and 7.4 Hz, Cδ), 2.26 (d of t, 2H, Cϵ), 1.38 (m, 2H, Cζ), 1.25 (m, 4H), 0.84 (t, 3H).

trans-2,trans-4-Hexadienoyl-CoA. 7.15 (d of d, 1H, J_{trans} = 15.2 and 10.8 Hz, Cβ), 6.33 (m, 1H, J_{trans} = 15.2 Hz, Cδ), 6.19 (d of d, 1H, J_{trans} = 15.2 and 10.8 Hz, Cγ), 6.09 (d, 1H, J_{trans} = 15.3 Hz, Cα), 1.84 (d, 3H, Cϵ).

trans-3-Hexenoyl-CoA. 5.75 (d of t, 1H, Cγ), 5.48 (d of t, 1H, Cβ), 3.48 (d, 2H, Cα), 2.04 (m, 2H, Cδ), 0.96 (t, 3H, Cϵ).

[2,5-$^2H_2$]-trans-3-hexenoyl-CoA. 5.74 (d of d, 1H, J_{trans} = 15.0 Hz, Cγ), 5.48 (d of d, 1H, J_{trans} = 15.0 Hz, Cβ), 3.26 (d, 1H, Cα), 2.02 (m, 1H, Cδ), 0.95 (d, 3H, Cϵ).

[5-^2H] trans-3-Hexenoyl-CoA. 5.74 (d of d, 1H, J_{trans} = 15.4 Hz, Cγ), 5.48 (d of t, 1H, J_{trans} = 15.4 Hz, Cβ), 3.28 (d, 2H, Cα), 2.02 (m, 1H, Cδ), 0.95 (t, 3H, Cϵ).

[2-^2H] trans-3-Hexenoyl-CoA. 5.74 (d of t, 1H, J_{trans} = 15.3 Hz, Cγ), 5.48 (d of d, 1H, J_{trans} = 15.3 Hz, Cβ), 3.26 (d, 1H, Cα), 2.04 (m, 2H, Cδ), 0.96 (t, 3H, Cϵ).

[2-^2H]-trans-2-Hexenoyl-CoA. 6.94 (t, 1H, J = 6.39 Hz, Cβ), 2.18 (d of t, 2H, Cγ), 1.45 (m, 2H, Cδ), 0.88 (t, 3H, Cϵ).

[2,5-$^2H_2$]-trans-3-Decenoyl-CoA. 5.68 (d of d, 1H, J_{trans} = 15.1 Hz, Cγ), 5.47 (d of d, 1H, J_{trans} = 15.2 Hz, Cβ), 3.25 (d, 1H, Cα), 1.99 (m, 1H, Cδ), 1.32 (m, 2H, Cϵ), 1.24 (m, 6H), 0.84 (t, 3H).

[5-^2H]-trans-3-Decenoyl-CoA. 5.67 (d of d, 1H, J_{trans} = 15.2 Hz, Cγ), 5.46 (d of t, 1H, J_{trans} = 15.3 Hz, Cβ), 1.98 (m, 2H, Cδ), 1.32 (m, 2H, Cϵ), 1.23 (m, 6H), 0.84 (t, 3H).

Octanoyl-CoA. 2.58 (t, 2H, Cα), 1.58 (m, 2H, Cβ), 1.23 (m, 8H), 0.84 (t, 3H).

trans-2-Octenoyl-CoA. 6.95 (d of t, 1H, J_{trans} = 15.0 and 6.7 Hz, Cβ), 6.16 (d, 1H, J_{trans} = 15.0 Hz, Cα), 2.18 (d of t, 2H, Cγ), 1.41 (m, 2H, Cδ), 1.25 (m, 4H), 0.84 (t, 3H).

[3-^2H]Octanoyl-CoA. 2.57 (d, 2H, Cα), 1.56 (m, 1H, Cβ), 1.23 (m, 8H), 0.84 (t, 3H).

[2,3-$^2H_2$]Octanoyl-CoA. 2.55 (d, 1H, Cα), 1.54 (m, 1H, Cβ), 1.22 (m, 8H), 0.83 (t, 3H).

[3-^2H]-trans-2-Octenoyl-CoA. 6.18 (s, 1H, Cα), 2.18 (t, 2H, Cγ), 1.41 (m, 2H, Cδ), 1.25 (m, 4H), 0.85 (t, 3H).

[2,3-$^2H_2$]-3-Hydroxyoctanoyl-CoA. 2.75 (s, 1H, Cα), 1.45 (t, 2H, Cγ), 1.25 (m, 6H), 0.84 (t, 3H).

[3-^2H]Butyryl-CoA. 2.57 (d, 2H, Cα), 1.60 (m, 1H, Cβ), 0.88 (d, 3H, Cγ).

trans-2-Butenoyl-CoA. 6.95 (d of q, 1H, J_{trans} = 14.0 Hz, Cβ), 6.19 (d, 1H, J_{trans} = 13.9 Hz, Cα), 1.21 (d, 3H, Cγ).

Preparation of Phosphate Buffer Salts for ^1H NMR Experiments. A solution containing 14 mM K_2HPO_4 and 6 mM KH_2PO_4 was prepared and 525 μL aliquots were taken to dryness by vacuum centrifugation. The buffer salts were dissolved in 30 μL of D_2O and then taken to dryness by vacuum centrifugation. This process was repeated twice to ensure that the protons had been exchanged with deuterons in the buffer salts. Immediately prior to use, the 20 mM phosphate buffer, pD 7.6 (pH meter reading + 0.4) (*28*), was prepared by adding 525 μL of D_2O to an aliquot of the H $\rightarrow$ D exchanged phosphate salts.

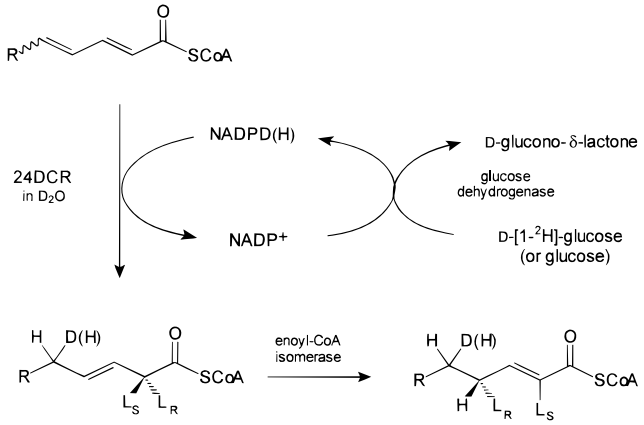
Enzymes. The recombinant functional 24DCR was overexpressed in *Escherichia coli* and was purified by the previously described protocol (*29*). The purified enzyme exhibited a single band in Coomassie Blue stained SDS−PAGE and a molecular mass of 32 413 Da was determined by electrospray ionization mass spectrometry. The purified 24DCR was used without any further manipulation. The recombinant rat liver mitochondrial enoyl-CoA hydratase was purified by the method of Wu et al. (*30*). The Δ^3,Δ^2-enoyl-CoA isomerase was a generous gift from Dr. Wilhelm Stoffel (University of Köln). The recombinant InhA enoyl-thiolester reductase from *M. tuberculosis* was overexpressed in *E. coli* and purified by following the previously described procedure (*6*). The purification of porcine heart thiolase I (broad chain length specific form) was performed following a modified procedure of Staack et al. (*31*). After the second phosphocellulose chromatography step, the pooled thiolase I was tested for thiolesterase activity against *t2t*4HDCoA and AcAc-CoA and none was detected. Since only catalytic amounts of thiolase I, without contaminating thiolesterase activity, were needed for the fatty acid oxidation reactions, the final (carboxymethyl)cellulose chromatography step was omitted and the concentrated protein was stored at −80 °C. During the purification, thiolase I activity was detected by the following assay. A typical assay was initiated by the addition of ∼10−20 μg of crude protein to a TAPS buffered solution (100 mM, pH 8.3) containing acetoacetyl-CoA (40 μM), free CoA (40 μM), and $MgCl_2$ (10 mM) at 25 °C. The decrease in absorbance at 304 nm, which corresponds to the disappearance of the Mg^{2+}-stabilized enolate ion of AcAc-CoA, was used to monitor the reaction (*32*).

Determination of the Diastereoselectivity of Hydride Transfer from NADPH. The diastereoselectivity of hydride transfer was determined by reducing *t2t*4HDCoA (1 mM) in H_2O (50 mM KP_i, pH 7.5) in the presence of [4*S*-^{2}H]-NADPH (1.5 mM) with the 24DCR. The reaction was monitored by the decrease in absorbance at 340 nm. When there was no further decrease in A_{340}, the protein was denatured by vortexing the reaction solution after the addition of 50 μL of $CHCl_3$. After centrifugation, the supernatant was removed and the 3-hexenoyl-CoA product was purified by reversed-phase HPLC. A ^{1}H NMR spectrum of the purified product was then acquired to determine the presence/absence of ^{2}H at Cδ.

Determination of the Stereochemical Course of Isomerization Catalyzed by the Δ^3,Δ^2-Enoyl-CoA Isomerase. trans-3-Hexenoyl-CoA was solubilized in D_2O buffer (20 mM KP_i, pD 7.6) and a preliminary ^{1}H NMR spectrum was acquired. Next, a catalytic amount of Δ^3,Δ^2-enoyl-CoA isomerase (ECI) was added to the NMR tube and equilibrium between *trans*-3-hexenoyl-CoA and *trans*-2-hexenoyl-CoA was established. A second ^{1}H NMR spectrum was then acquired. After an 18 h incubation a third NMR spectrum was acquired and compared to the previous spectra.

Determination of the Stereochemical Course of Reduction of Dienoyl-CoAs at Cα. The procedure for determining the stereochemical course of reduction at Cα is outlined in Scheme 1. The reductions of *t2t*4HDCoA and *t2c*4DDCoA by the 24DCR were conducted in buffered D_2O (20 mM KP_i, pD 7.6) in the presence of either NADPH or [4-^{2}H]-NADPH. Initially, the dienoyl-CoA substrates (∼1−2 mM) were prepared in D_2O buffer and a preliminary ^{1}H NMR spectrum was acquired for each. Each sample was then transferred to a clean test tube and [1-^{2}H]glucose (or [1-^{1}H]-glucose), $NADP^+$, glucose dehydrogenase (*T. acidophilum*),

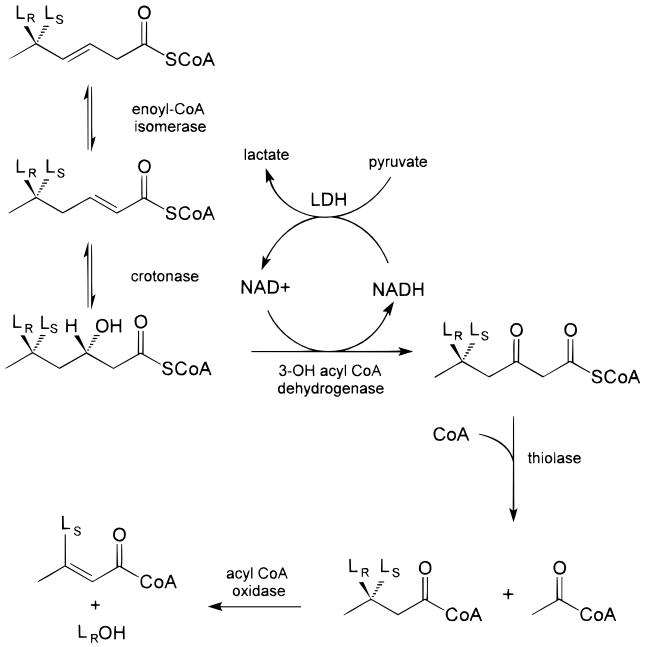
Scheme 1



and 24DCR were added to final concentrations of 4 mM, 50 μM, 10 units, and 15 μg, respectively. The reactions were incubated at room temperature and were monitored by removing a 5 μL aliquot from each reaction tube and acquiring a UV spectrum. The reaction was complete when there was no longer absorbance at 296 nm. The proteins were then denatured by vortexing after the addition of 50 μL of chloroform. The supernatant after centrifugation was removed and the 3-hexenoyl-CoA and 3-decenoyl-CoA products were purified by reversed-phase HPLC. A ^{1}H NMR spectrum of each product was acquired, followed by the addition of a catalytic amount of the Δ^3,Δ^2-enoyl-CoA isomerase to the NMR tubes. Once the isomerase catalyzed equilibrium had been established, an additional ^{1}H NMR spectrum of each sample was acquired.

Determination of the Stereochemical Course of Reduction of Dienoyl-CoAs at Cδ. The stereochemical course of reduction at Cδ was determined by reducing *t*2*t*4HDCoA, *t*2*t*4DDCoA, and *t*2*c*4DDCoA in H_2O (20 mM KP_i, pH 7.5) in the presence of [4-^{2}H]NADPH by the previously described method. The HPLC-purified 3-enoyl-CoA products were subjected to one round of fatty acid oxidation following isomerization to the corresponding *trans*-2-enoyl-CoA by the ECI. The experimental procedure is outlined in Scheme 2 and the reaction conditions were as follows. To begin, the 3-enoyl-CoA was solubilized in 2.7 mL of a TAPS-buffered solution (20 mM, pH 8.3) containing $MgCl_2$ (10 mM) to a final concentration of ~100 μM. Next, a catalytic amount of ECI was added to the cuvette and the increase in absorbance at 260 nm corresponding to the formation of the double bond at Cα was observed. When there was no longer an observable increase in A_{260}, a catalytic amount of enoyl-CoA hydratase (ECH) was added to the cuvette and the A_{260} rapidly decreased. NAD^+ and pyruvate were added to the cuvette to final concentrations of 50 and 100 μM, respectively. A catalytic amount of L-3-hydroxyacyl-CoA dehydrogenase was then added and the increase in absorbance at 340 nm corresponding to the production of NADH was monitored. When the A_{340} stopped increasing, 5 units of lactate dehydrogenase was added to the cuvette. The A_{340} slowly decreased and the reaction was incubated until no further decrease in A_{340} was observed, which indicated that this step of fatty acid oxidation was near completion. Finally, free CoA (150 μM final concentration) was added to the cuvette followed by thiolase I. The thiolase reaction was complete when the absorbance at 304 nm arising from the enolate of the 3-oxoacyl-CoA was no longer present.

Scheme 2



After the completion of the fatty acid oxidation, the reaction solution was placed in a clean test tube and the proteins were denatured by vigorous vortexing after the addition of 200 μL of $CHCl_3$. After centrifugation the supernatant was removed and the resulting acyl-CoAs were purified by reversed-phase HPLC. A preliminary ^{1}H NMR spectra of each acyl-CoA was acquired followed by the addition of acyl-CoA oxidase (*Candida* sp.) to each NMR tube. ^{1}H NMR spectra of the oxidized samples were then acquired and the results were analyzed.

Determination of the Stereochemical Course of Reduction Catalyzed by the InhA Enoyl-Thiolester Reductase. The procedure used to determine the stereochemical course of the InhA reductase was similar to that used for the 24DCR. The reduction of *trans*-2-octenoyl-CoA (500 μM) was conducted in D_2O buffer (20 mM, pD 7.6) in the presence of NAD^+ (40 μM) and [^{2}H]formate (2.5 mM). The reaction was initiated by the addition of 5 units of formate dehydrogenase and the change in absorbance at 340 nm was monitored. When the A_{340} stopped increasing, a catalytic amount of InhA was added to the reaction cuvette and the A_{340} decreased as expected. The reaction was considered complete when the absorbance at 340 nm returned to the previously observed maximum. The enzymes were then removed by centrifugal filtration (Centricon-10, Amicon) and the filtrate was divided into three equal aliquots. Acyl-CoA oxidase from *Candida* was then added to one aliquot and acyl-CoA oxidase from *Arthrobacter* sp. was added to the second aliquot. The oxidation reactions were incubated for several hours and the enzyme in the oxidized samples was denatured by vortexing after the addition of $CHCl_3$. The supernatants after centrifugation were purified with the unoxidized sample by reversed-phase HPLC. The resulting products were then analyzed by ^{1}H NMR.

RESULTS

Determination of the Stereochemical Configuration of the 3-Enoyl-CoA Products. The reductions of *t*2*t*4HDCoA,

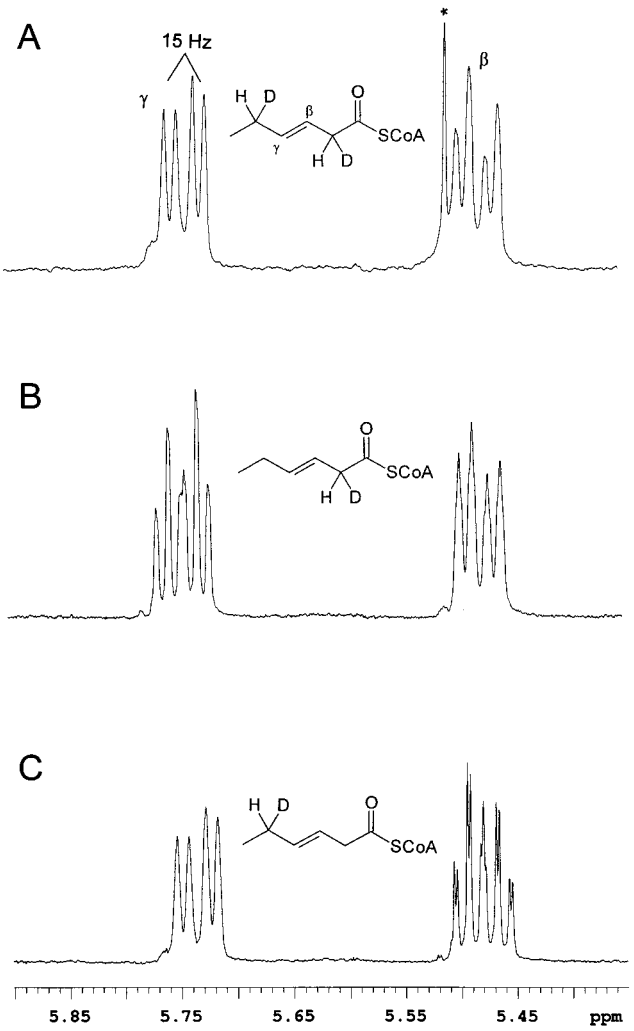


FIGURE 1: ^{1}H NMR spectra, over the region 5.9−5.35 ppm, of the *trans*-3-hexenoyl-CoA products from the 24DCR-catalyzed reduction of *trans*-2,*trans*-4-hexadienoyl-CoA in D_2O with [4*S*-^{2}H]-NADPH (**A**), in D_2O with NADPH (**B**), and in H_2O with [4*S*-^{2}H]NADPH (**C**). The 15 Hz coupling constant, $^3J_{HH}$, between the β and γ protons indicates a *trans* configuration. Also, these results demonstrate that a solvent-derived proton is added to Cα (**B**) and the hydride equivalent from NADPH is added to Cδ (**C**). The asterisk denotes a peak from an unidentified contaminant.)

*t*2*t*4DDCoA, and *t*2*c*4DDCoA by 24DCR were conducted in D_2O and H_2O in the presence of either NADPH or [4-^{2}H]-NADPH. ^{1}H NMR spectra of each were acquired and the coupling constant, $^3J_{HH}$, between the protons on Cβ and Cγ was determined to be 15 Hz for all products. This indicates that the product of reduction is *trans*-3-enoyl-CoA regardless of the stereochemical configuration of the double bond at position 4 in the dienoyl-CoA substrate (*33*). Also, these results demonstrate that a solvent-derived proton is added to Cα and the hydride equivalent from NADPH is added to Cδ (Figure 1). Additionally, the splitting pattern for the Cβ proton of the *trans*-3-decenoyl-CoA, made by reduction of *t*2*c*4DDCoA in D_2O with [4-^{2}H]NADPH, indicates that Cα is almost completely deuterated, presumably from 24DCR-catalyzed exchange (data not shown). In the absence of 24DCR, there was no observable Cα proton exchange when *trans*-3-hexenoyl-CoA was incubated in D_2O under identical conditions.

Diastereoselectivity of Hydride Transfer from NADPH. The reduction of *t*2*t*4HDCoA by the 24DCR in the presence of [4*S*-^{2}H]NADPH was conducted in H_2O. The ^{1}H NMR spectrum of the resulting *trans*-3-hexenoyl-CoA indicated that a single deuterium atom had been incorporated into the Cδ position as evidenced by the doublet of doublets splitting pattern of the Cγ proton at 5.74 ppm (Figure 1C). Thus, this result confirms that the NADPH donates the *pro-4S* hydride to Cδ of the dienoyl-CoA substrate, which is in agreement with data reported for the bovine liver enzyme (*34*).

Stereochemical Course of Isomerization Catalyzed by the Δ^3,Δ^2-enoyl-CoA Isomerase. The stereochemical course of reduction at Cα was determined by reacting the *trans*-3-enoyl-CoA products with the Δ^3,Δ^2-enoyl-CoA isomerase (ECI) and analyzing the resulting 2-enoyl-CoA products by ^{1}H NMR. Prior to conducting these experiments, the stereochemical course of the ECI-catalyzed isomerization needed to be determined. The ^{1}H NMR of an ECI-catalyzed equilibrium between *trans*-3- and *trans*-2-hexenoyl-CoA in D_2O is shown in Figure 2A. The splitting pattern of the β proton of *trans*-2-hexenoyl-CoA did not change after extended incubation, which indicates that there was no solvent-derived ^{2}H incorporation. This demonstrates that the ECI catalyzes the conserved intramolecular transfer of a hydrogen from Cα to Cγ. This observation agrees with previous reports for the ox liver vinylacetyl-CoA isomerase, which catalyzes a similar reaction (*35*). The Δ^3,Δ^2-enoyl-CoA isomerase is a member of the hydratase/isomerase enzyme superfamily and shares a degree of homology with enoyl-CoA hydratase (ECH). Most importantly, both enzymes share a conserved glutamate residue at the active site that is necessary for catalysis, E164 in ECH and E165 in ECI (*36*). The stereochemistry of the ECH-catalyzed addition of H_2O to the double bond of *trans*-2-enoyl-CoA has been previously determined where the conserved glutamate 164 adds a proton to the *pro-2R* position (*37*). Similarly, the vinylacetyl-CoA isomerase from *Clostridium kluyveri* transfers the *pro-2R* hydrogen to the *re* face at Cγ of the vinylacetyl-CoA (*38*), demonstrating that the vinylacetyl-CoA reaction is a suprafacial proton-transfer process (*39*). On the bases of the homology between ECI and ECH, the similarity to the vinylacetyl-CoA isomerase and the absence of exchange with solvent, we conclude that the ECI catalyzes the suprafacial transfer of the *pro-R* proton from Cα of *trans*-3-enoyl-CoA to the *pro-R* position at Cγ of *trans*-2-enoyl-CoA.

Stereochemical Course of Proton Addition at Cα Catalyzed by 24DCR. The *trans*-3-enoyl-CoA 24DCR products were isomerized with ECI and ^{1}H NMR spectra were acquired. The ^{1}H NMR spectrum of the isomerized *trans*-3-hexenoyl-CoA made from the reduction of *t*2*t*4HDCoA in D_2O with NADPH is shown in Figure 2C. The results display a triplet centered at 6.95 ppm (J_{HH} 7 Hz), which corresponds to the proton at Cβ of the *trans*-2-hexenoyl-CoA. This splitting pattern and the 7 Hz coupling constant indicate that there are two protons at the Cγ position and a deuterium atom at the Cα position of the *trans*-2-hexenoyl-CoA. On the basis of the ECI stereochemistry shown in Scheme 1, we conclude that the proton at the *pro-2R* position of the *trans*-3-hexenoyl-CoA was transferred to the *pro-4R* position, leaving behind a single deuterium atom at Cα. This deuterium atom was originally in the *pro-2S* position, indicating the 24DCR adds a solvent-derived proton/deuteron to the *si* face at Cα of the dienoyl-CoA substrates.

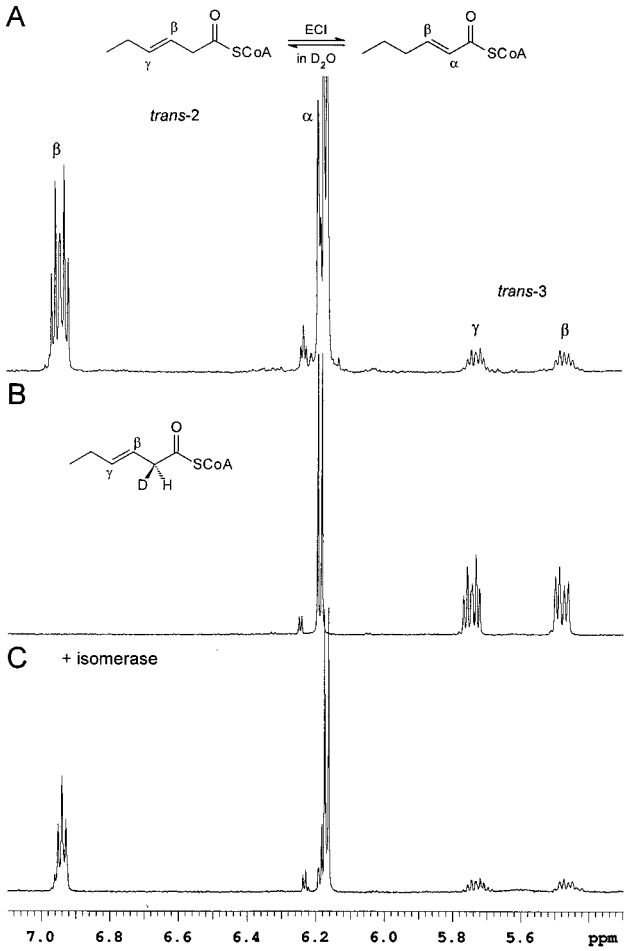


FIGURE 2: ^{1}H NMR spectra, over the region 7.1−5.3 ppm, of the enoyl-CoA isomerase-catalyzed equilibrium between *trans*-2- and *trans*-3-hexenoyl-CoAs in D_2O (**A**). The splitting pattern of the β proton did not change after extended incubation, which indicates that there was no solvent-derived ^{2}H incorporation into the Cα or Cγ positions. The ^{1}H NMR spectra of the *trans*-3-hexenoyl-CoA made from the reduction of *trans*-2,*trans*-4-hexadienoyl-CoA in D_2O with NADPH before (**B**) and after (**C**) isomerization with the enoyl-CoA isomerase. The results display a triplet centered at 6.94 ppm ($^3J_{HH}$ 7 Hz) which corresponds to the β proton of *trans*-2-hexenoyl-CoA. This splitting pattern, the 7 Hz coupling constant, and the absence of a Cα proton resonance in **C** indicate that there are two protons at the Cγ position and a deuterium atom at the Cα position of the *trans*-2-hexenoyl-CoA.

Determination of the Stereochemical Course of Reduction at Cδ Catalyzed by 24DCR. The reductions of *t*2*t*4HDCoA, *t*2*t*4DDCoA, and *t*2*c*4DDCoA by the 24DCR were conducted in H_2O in the presence of [4-^{2}H]NADPH, and the *trans*-3-enoyl-CoA products were taken through one round of fatty acid oxidation as illustrated in Scheme 2. The resulting acyl-CoA was shortened by two carbon atoms and the original Cδ position was now at Cβ which facilitated the determination of the absolute configuration at this position.

The chain-shortened fatty acid oxidation products, butyryl-CoA and octanoyl-CoA, were dehydrogenated by the acyl-CoA oxidase from *Candida* sp. *Candida* acyl-CoA oxidases catalyze the *anti* elimination of the *pro-2R* and *pro-3R* hydrogens of the acyl-CoA (*40*). The ^{1}H NMR spectra of the crotonyl-CoA and 2-octenoyl-CoA products are shown in Figure 3 and the resulting absolute stereochemistry at Cδ is shown in Scheme 3. The presence of a proton at Cβ in the dehydrogenated chain-shortened products of *trans*-2,*trans*-4-dienoyl-CoAs indicates that the addition of the deuteride from NADPD occurs on the *re* face at Cδ resulting in (5*R*)-[5-^{2}H]-*trans*-3-enoyl-CoA. The absence of the Cβ proton in the dehydrogenated chain-shortened product of *trans*-2,*cis*-4-decadienoyl-CoA indicates that deuteride addition occurs on the *si* face at Cδ, resulting in (5*S*)-[5-^{2}H]-*trans*-3-decenoyl-CoA.

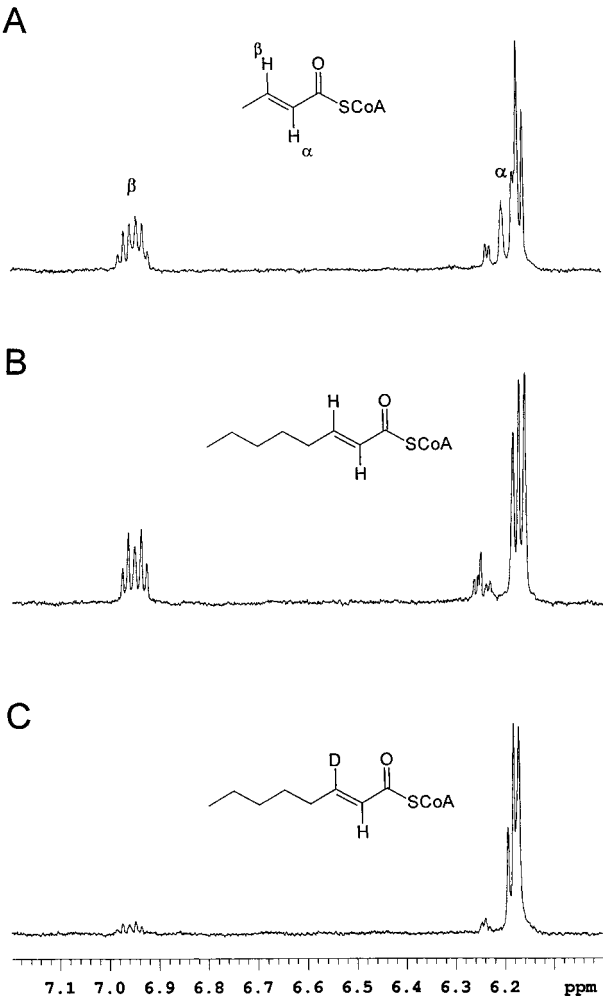
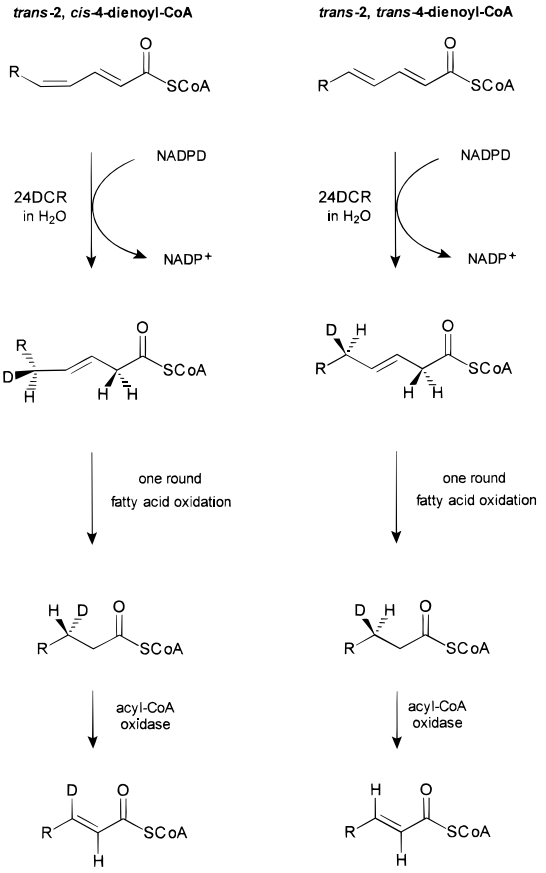


FIGURE 3: ^{1}H NMR spectra, over the region 7.2−6.0 ppm, used to determine the stereochemical course of reduction at Cδ. (A) ^{1}H NMR spectrum of the [4-^{2}H]NADPH-reduced *trans*-2,*trans*-4-hexadienoyl-CoA, chain-shortened by fatty acid oxidation and dehydrogenated by *Candida* acyl-CoA oxidase. (B) ^{1}H NMR spectrum of the [4-^{2}H]NADPH-reduced *trans*-2,*trans*-4-decadienoyl-CoA, chain-shortened by fatty acid oxidation and dehydrogenated by *Candida* acyl-CoA oxidase. (C) ^{1}H NMR spectrum of the [4-^{2}H]NADPH-reduced *trans*-2,*cis*-4-decadienoyl-CoA, chain-shortened by fatty acid oxidation and dehydrogenated by *Candida* acyl-CoA oxidase. The presence of β protons in the oxidized chain-shortened products of *trans*-2,*trans*-4-dienoyl-CoAs (**A** and **B**) indicates that the addition of the deuteride from [4-^{2}H]NADPH occurs on the *re* face at Cδ, resulting in (5*R*)-[5-^{2}H]-*trans*-3-enoyl-CoA. The absence of the β proton in the oxidized chain-shortened product of *trans*-2,*cis*-4-decadienoyl-CoA (**C**) indicates that the deuteride addition occurs on the *si* face at Cδ, resulting in (5*S*)-[5-^{2}H]-*trans*-3-decenoyl-CoA. The residual β protons that appear in panel **C** are a result of the first round of the NADPH recycling process used during the reduction of the dienoyl-CoA.

Reduction of trans-2-Octenoyl-CoA Catalyzed by InhA. The reduction of *trans*-2-octenoyl-CoA by InhA was performed in D_2O in the presence of [4-^{2}H]NADH. The ^{1}H NMR spectrum of the resulting [2,3-$^2H_2$]octanoyl-CoA verified the presence of deuterium atoms at the Cα and Cβ

Scheme 3



positions. This verification was done by comparing the splitting patterns of the protons at Cα and Cβ of the [2,3-$^2H_2$]octanoyl-CoA versus a fully protonated octanoyl-CoA standard (data not shown).

The ^{1}H NMR spectrum of the octanoyl-CoA product that had been oxidized by the *Candida* acyl-CoA oxidase showed only the presence of a proton at Cα (singlet at 6.16 ppm) and a trace amount of protons at Cβ (multiplet at 6.95 ppm) (Figure 4). The trace amount of β protons originated from the nucleotide recycling process where the 4-hydrogen of the catalytic NAD$^+$ is the initial proton transferred to the product acyl-CoA (*41*). The presence of deuterium at Cβ was confirmed by the splitting pattern of the protons at Cγ (triplet, 2.18 ppm). Finally, the presence of a single proton at Cα was confirmed by addition of a catalytic amount of ECH to the NMR tube. Another ^{1}H NMR spectrum of the hydrated product was acquired and a singlet at 2.75 ppm corresponding to a single Cα proton was observed while no resonance for the Cβ proton was detected. Therefore, the reduction of *trans*-2-octenoyl-CoA by InhA in D_2O with [4-^{2}H]NADH results in (2*R*, 3*S*)-[2,3-$^2H_2$]octanoyl-CoA (Scheme 4).

The determination of the absolute stereochemistry of the InhA reductase also facilitated the confirmation of the stereochemistry of oxidation by the *Arthrobacter* sp. acyl-CoA oxidase, which has not been reported to date. The ^{1}H NMR spectrum of the InhA product, oxidized by the *Arthrobacter* sp. oxidase, was identical to the spectrum of the InhA product oxidized by the *Candida* oxidase. From these results, we conclude that the *Arthrobacter* sp. oxidase catalyzes the identical reaction as the *Candida* oxidase, the

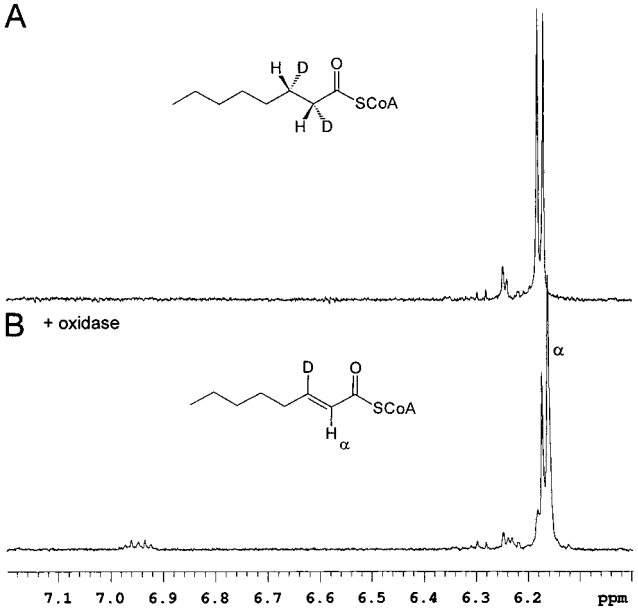
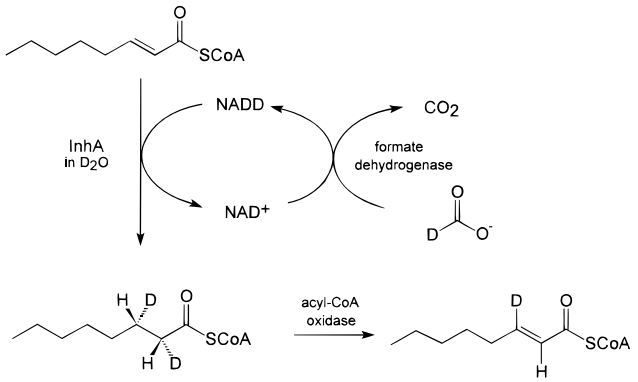


FIGURE 4: ^{1}H NMR spectra, over the region 7.2−6.0 ppm, of the octanoyl-CoA InhA product before (**A**) and after (**B**) oxidation by the *Candida* acyl-CoA oxidase. The presence of the α proton of the *trans*-2-octenoyl-CoA (singlet at 6.18 ppm) and the absence of a strong β proton resonance indicate that the reduction of *trans*-2-octenoyl-CoA by InhA in D_2O with [4-^{2}H]NADH results in (2*R*,3*S*)-[2,3-$^2H_2$]octanoyl-CoA. The trace amount of β protons in panel **B** originated from the first round of the NADH recycling process.

Scheme 4



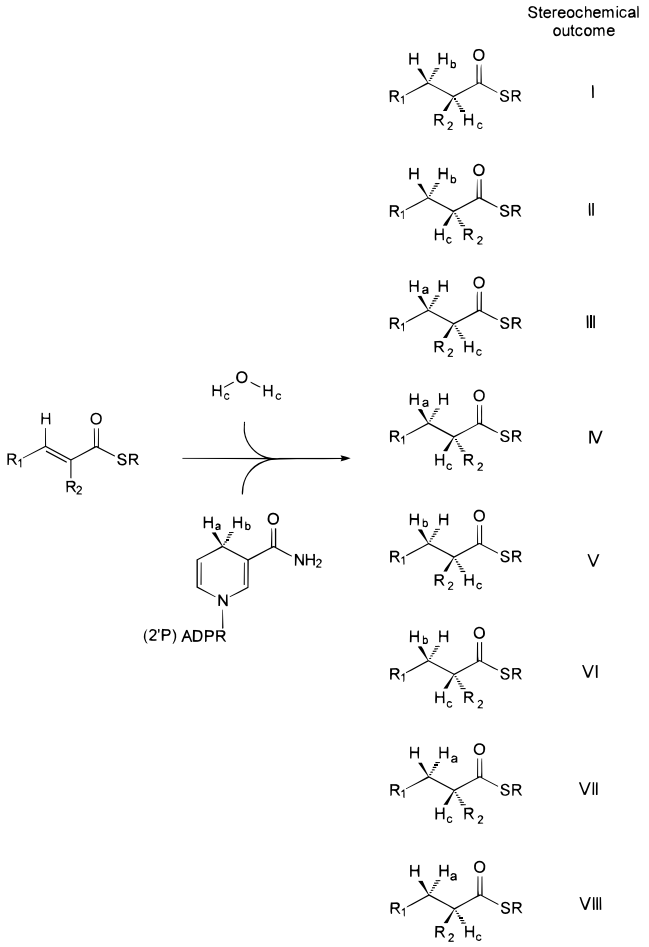
stereospecific dehydrogenation by *anti* elimination of the *pro-2R* and *pro-3R* hydrogens.

DISCUSSION

Relative Stereochemistry of InhA and 24DCR. The stereochemical course of the reduction of enoyl-thiolesters by NAD(P)H involves three stereochemical centers; consequently there are eight possible stereochemical combinations. These eight possibilities were enumerated by Reynolds[2] (*1*) as shown in Scheme 5 and the results of prior studies were reviewed. Of the eight possible stereochemical courses, five of them have been previously observed. The InhA results correspond to class I and are identical to those determined for FabI, the *E. coli* enoyl-thiolester reductase. The results with 24DCR, however, correspond to class VI, a previously

[2] The scheme previously presented (*1*) had an error in the drawing of the structure of the nicotinamide (Reynolds, personal communication), so the corrected scheme is presented here.

Scheme 5



unobserved stereochemical reaction course. Classes I and VI share two characteristics; they both transfer the *pro-4S* hydrogen of the nicotinamide ring to the electrophilic carbon of the conjugated thiolester, and the addition of the two hydrogen atoms occurs in a *syn* fashion. The two classes differ, in the face of the carbon−carbon double bond where the chemistry occurs.

There is only a small amount of primary sequence homology between the class I enoyl-thiolester reductases and 24DCRs. The homologous region of the sequences is shown in Figure 5. The conservation of this sequence is too great to be random. The sequence includes two residues, Lys-165 and Thr-196 identified by asterisks, that interact with the nicotinamide nucleotide. This Lys residue in InhA was originally proposed to be the residue that acted as an electrophilic catalyst by polarizing the carbonyl of the thiolester (*6*), which would enhance the electrophilic character of the β-carbon. In the crystal structure of the ternary NAD^+•*N*-acetyl-*S*-hexadecenoylcysteamine •InhA complex, this Lys residue is H-bonded to the 3′-hydroxyl of the nicotinamide ribose. Similarly, the homologous Lys residue in FabI is also within H-bonding distance of the 3′-hydroxyl in the ternary structures with either triclosan (*16*) or thienodiazaborine (*42*). Consistent with this assignment, mutation of Lys-165 to alanine or methionine abolishes NADH binding, but mutation to the H-bond donors glutamine or arginine have minimal effects on the activity of the enzyme (*43*).

The conserved threonine is within H-bonding distance of the 3-carboxamide of the nicotinamide. Since this interaction orients the face of the nicotinamide ring, its conservation is consistent with the observed transfer of the *pro-4S* hydrogens from NADH. Thus the conserved sequence shown in Figure 5 appears to be responsible for binding and orienting the nicotinamide ring for transfer of the *pro-4S* hydride.

The orientation of the nicotinamide, CoA substrates, and active-site functional groups that catalyze the *syn* addition of H_2 to the defined face of the conjugated thiolester are illustrated in Figure 6. In the InhA reaction, the thiolester substrate adapts the *s-cis* conformation about the C1−C2 bond (i.e. the torsion angle O═C−C_α−$C_\beta \approx 0$). This is the lowest energy solution conformation for conjugated thiolesters (*44*). Furthermore, in every other crystal structure of enzymes that utilize enoyl-thiolesters, including medium-chain acyl-CoA dehydrogenase (*45*) and enoyl-CoA hydratase (*46*), the enoyl-thiolester is also bound at the active site in the *s-cis* conformation, suggesting this will be a common feature of these enzymes. Thus the relative spatial orientation of the nicotinamide ring, the active site proton donor to Cα, and the residues that interact with the carbonyl of the thiolester are all defined. In 24DCR, the σ bond between Cβ and Cγ must be *s-trans* as shown because the product of the reduction is the *trans*-3-enoyl-CoA. The conjugated carbon−carbon double bonds must be flipped 180° relative to the nicotinamide ring when compared to InhA, as the stereochemical results require the addition of the hydride and proton to the opposite face of the conjugated system. There are two choices: either the carbonyl−C1 bond remains *s-cis* or it rotates 180° to become *s-trans*. The stereochemical results cannot differentiate between these two possibilities. Most NAD(P)H-dependent dehydrogenases have an active-site electrophilic catalyst that polarizes the double bond that is to be reduced. In the enoyl-thiolester reductases, it has been presumed that the carbonyl will be polarized to stabilize an intermediate enolate (*43*). If 24DCR shares enough structural homology with InhA that the relative orientation of the electrophilic catalyst(s) that interact with the carbonyl is conserved, then the *s-trans* conformation would be favored. However, the InhA crystal structure has suggested that Tyr-158 interacts with the carbonyl of the thiolester; the mutagenic results are ambiguous on this point, as the Y158F mutant is inactive but the Y158S mutant retains wild-type activity (*43*). As shown in the homology scheme, Tyr-158 is not conserved between InhA and 24DCR, although there are several Ser residues in the 24DCR sequence that with a minimal gap could be aligned with Tyr-158 in the InhA sequence. The alternative *s-cis* conformation is favored by the noted preference for enoyl-CoAs to retain the lower energy *s-cis* conformation.

Enolic Intermediates in Enoyl-CoA-Utilizing Enzymes. The *syn* addition to the double bond of the enoyl-CoA substrates of InhA has mechanistic implications. If the crystal structure of the NAD^+•*N*-actyl-*S*-hexadecenoyl cysteamine•InhA ternary structure is a correct representation of the active complex prior to hydride transfer, then there is no proton donor capable of simultaneously donating a proton to Cα. The *re* face of Cα is even occluded by the nicotinamide ring preventing the approach of a proton donor without a rearrangement of the nicotinamide ring or CoA thiolester. While initially this orientation of the nicotinamide ring was

FIGURE 5: Sequence alignment of the conserved region of enoyl-thiolester reductases. The residues identified by asterisks interact with the nicotinamide nucleotide in InhA and FabI. The Lys residue H-bonds to the 3′-hydroxyl of the nicotinamide ribose, and the Thr interacts with the 3-carboxamide.

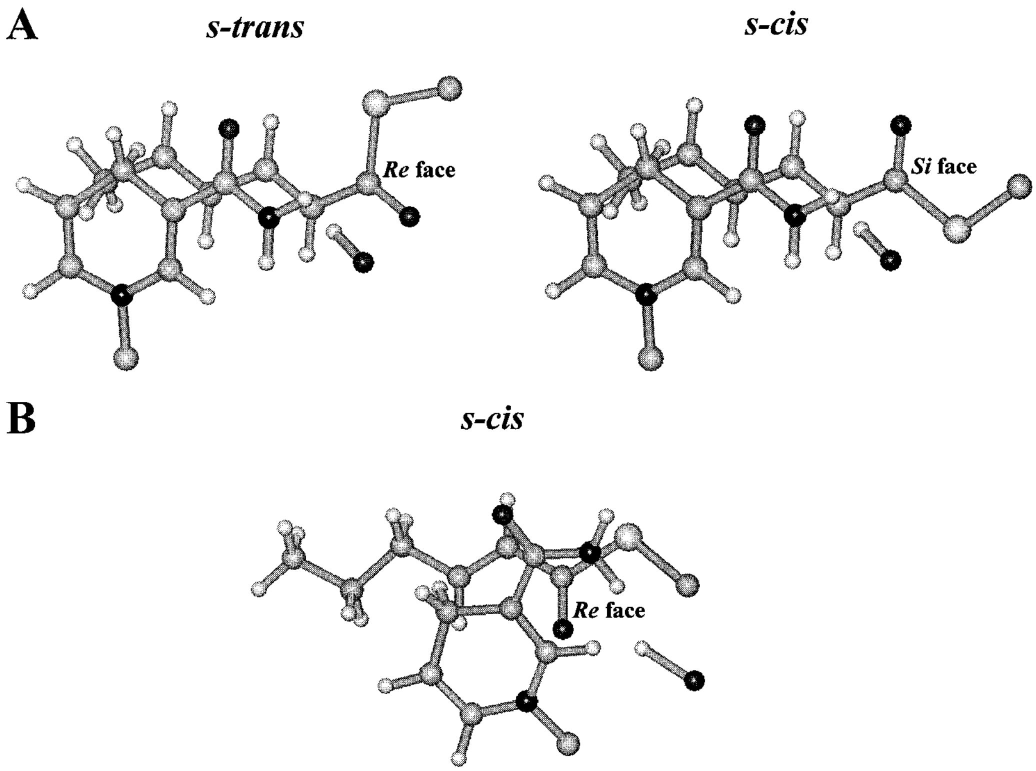
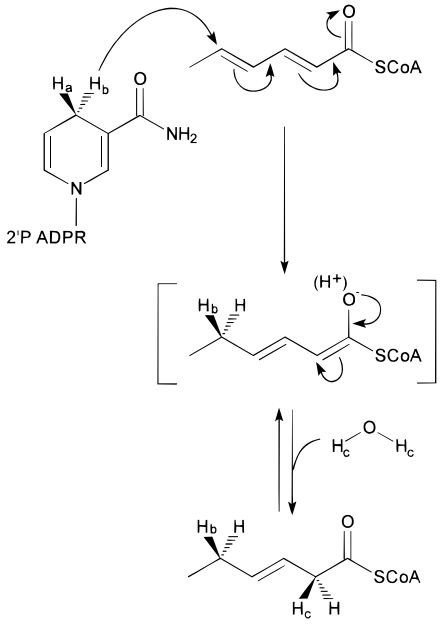


FIGURE 6: Reduction of (**A**) *trans*-2,*trans*-4-dienoyl-CoA the 24DCR and (**B**) *trans*-2-enoyl-CoA by InhA. The nicotinamide ring has been positioned so that the *pro-4S* hydrogen is facing the appropriate C=O face of the conjugated thiolester. For presentation purposes, the CoA was truncated at C9″ (*27*) and the NAD(P)H was truncated at the glycosidic carbon of the nicotinamide ribose. (***A***) Binding of the dienoyl-CoA in the *s-trans* or *s-cis* conformation orients the *pro-4S* hydrogen of the nicotinamide ring toward the *re* and *si* faces of the C=O, respectively. Addition of the hydride occurs on the *re* face at Cδ of *trans*-4 double bonds and on the *si* face of Cδ for *cis*-4 double bonds. Addition of a solvent-derived proton occurs on the *si* face at Cα. (**B**) Binding of *trans*-2-enoyl-CoA to InhA in the *s-cis* conformation orients the *pro-4S* hydrogen of the nicotinamide ring toward the *re* face of the C=O bond. Hydride addition occurs on the *si* face at Cβ while protonation occurs on the *re* face at Cα. The orientation of the nicotinamide ring was adapted from the crystal structure of the InhA ternary complex (*21*).

puzzling, the positive charge generated on the pyridinium N would serve to most effectively stabilize the enolate intermediate. The high degree of stereospecificity in the protonation identified by these studies (estimated at greater than 95%) indicates that the protonation occurs at the enzyme active site and not in solution following release of an enolic intermediate. The protic group closest to the *re* face of the Cα carbon is the 2′-hydroxyl group of the NAD^+ cofactor. The 2′-hydroxyl group has previously been suggested to play a role in the proton transfers in alcohol dehydrogenases (*47*). An alternative would be for an unidentified active-site water molecule to be the proton donor. A similar addition to the 5,6 double bond of dUMP catalyzed by thymidylate synthase has been studied where the source of the proton, and consequently the identity of the presumed general acid, could not be identified (*48*). The implied existence of an enolic intermediate is consistent with the isotope effects on InhA, where both primary isotope effects on the transfer of the hydride and the solvent-derived proton are observed, and the presence of ^2H in the solvent reduces the primary isotope effect on hydride transfer to unity (*43*).

The observation of retention of transfer of ^2H from Cα of 3-enoyl-CoA to Cγ of *trans*-2-enoyl-CoA catalyzed by enoyl-CoA isomerase again suggests the presence of an enolic intermediate. The simplest explanation of this result is that the active-site base, Glu-165, abstracts the proton, forming an enolic intermediate. The initial prediction is that the intermediate will be ionized and not protonated. The electrophilic catalysts in the enoyl-CoA hydratase/isomerase family are two amide protons (*46*, *49*). The proton affinity of an amide (pK_a > 15) is greater than the proton affinity of an enolate (*50*). Reprotonation of the intermediate enolate at either carbon occurs more rapidly than exchange of the carboxylic acid proton. The reaction then would be an

Scheme 6



intramolecular 1,3-proton transfer as observed for all previously studied allylic isomerases (*39*).

In the 24DCR reaction the identities of potential electrophilic catalyst(s) interacting with the carbonyl oxygen of the thiolester or the active-site general acid that would donate the proton to Cα are unknown. However, the intermediate that would be formed in the 24DCR reaction is a dienolate, as shown in Scheme 6. This dienolate intermediate has a lower proton affinity (i.e., a more easily formed base, pK_a ∼ 10.5) than the enolate intermediates present in the InhA and enoyl-CoA isomerase reactions due to the presence of the additional conjugated carbon−carbon double bond (*51*). Therefore, the 24DCR reaction is likely to proceed through a dienolate intermediate.

An interesting question remaining to be answered is why 24DCR catalyzes the protonation of the proposed dienolate intermediate at Cα rather than at Cγ. Protonation at Cγ would generate the thermodynamically favored 2-enoyl-CoA and obviate the requirement for the additional action of the enoyl-CoA isomerase during oxidation. This direct reduction of 2,4-dienoyl-CoAs to 2-enoyl-CoAs is the reaction catalyzed by the *E. coli* flavin containing 24DCR (*33*). In solution studies of ketonization of 1,3-dienolates, however, protonation at C2 is favored kinetically (*51*). In the liver 24DCR, then, the enzyme has followed the kinetically preferred pathway to the less stable 3-enoyl-CoA isomer.

ACKNOWLEDGMENT

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