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Redesigning the Active-site of an Acyl-CoA Dehydrogenase: New Evidence Supporting a One-base Mechanism

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Abstract—The acyl-CoA dehydrogenases are a family of related enzymes that share high structural homology and a common catalytic mechanism which involves abstraction of an α-proton from the substrate by an active site glutamate residue. Several lines of investigation have shown that the position of the catalytic glutamate is conserved in most of these dehydrogenases (the $\mathbf{E}_2$ site), but is in a different location in two other family members (the $\mathbf{E}_1$ site). Using site specific in vitro mutagenesis, a double mutant rat short chain acyl-CoA dehydrogenase (rSCAD) has been constructed in which the catalytic glutamate is moved from the $\mathbf{E}_2$ to the $\mathbf{E}_1$ site (Glu368Gly/Gly247Glu). This mutant enzyme is catalytically active, but utilizes substrate less efficiently than the native enzyme (K_m = 0.6 and 2.0 μM, and V_{max} = 2.8 and 0.3 s^{-1} for native and mutant enzyme respectively). In this study we show that both the wild-type and mutant rSCADs display identical stereochemical preference for catalysis—abstraction of the α-H_R from the substrate followed by transfer of the β-H_R to the FAD coenzyme. These results, in conjunction with molecular modeling of the native and double mutant SCAD indicate that the catalytic base in the $\mathbf{E}_1$ and $\mathbf{E}_2$ sites are topologically similar and catalytically competent. However, analysis of the ^{1}H NMR spectra of the incubation products of these two enzymes revealed that, in contrast to the wild-type rSCAD, the Gly368Glu/Gly247Glu rSCAD could not perform γ-proton exchange of the product with the solvent, a property inherent to most acyl-CoA dehydrogenases. It is evident that the base in the mutant enzyme has access to the α-H_R but is far removed from the γ-Hs. These findings provide further support for a one base mechanism of α- and γ-reprotonation/deprotonation catalysis by acyl-CoA dehydrogenases. © 1997 Elsevier Science Ltd.

Introduction

The first step in the β-oxidation of fatty acids is catalyzed by a family of flavoproteins, the acyl-CoA dehydrogenases (ACDs).[1–4] Catalysis by these enzymes involves the abstraction of the α-proton from an acyl-CoA substrate followed by the transfer of the β-hydrogen to the active-site bound flavin coenzyme (FAD), leading to the production of an α,β-enoyl-CoA.[5–7] Six distinct acyl-CoA dehydrogenases, have been isolated from diverse sources. These include short-chain (SCAD), medium-chain (MCAD), long-chain (LCAD), very long-chain (VLCAD), isovaleryl-CoA dehydrogenase (IVD), and short/branched chain acyl-CoA dehydrogenase (SBCAD). While these dehydrogenases exhibit varied substrate preferences, their catalyses have been shown to proceed via the same mechanism. Chemical labeling studies,[8–10] X-ray crystal analysis[11,12] and site-directed mutagenesis experiments[13,14] have identified an active site glutamate, E376 in MCAD of pig liver and E367 in SCAD of *Megasphaera elsdenii* (bSCAD), as the base responsible for the initial α-proton abstraction. However, despite the high sequence homology among these dehydrogenases the amino acid residue in the position corresponding to this glutamate is not conserved in mammalian LCAD and IVD. Instead, this residue is replaced by an alanine in human IVD (A375) or a glycine in human LCAD (G382) and rat IVD (G375).[15,16] Molecular-modeling and site-directed mutagenesis studies have identified the catalytic base in these later enzymes to be a glutamate at a position more than 100 residues closer to the N-terminus: E261 in human LCAD[17] and E254 in human IVD.[18] Alignment of the amino acid sequence of the various ACDs reveals that the residue at the homologous position is a threonine (T255) in pig liver MCAD[11] and a glycine (G246) in bacterial SCAD.[12] Thus, it appears that two positions ($\mathbf{E}_1$ and $\mathbf{E}_2$) are possible to accommodate the active-site base in ACDs (Fig. 1), however, in each enzyme, only one position is occupied by a glutamate.[16]

Preliminary X-ray crystal structure of rSCAD has been reported and is similar to bSCAD, having the catalytic glutamate (E368) at the $\mathbf{E}_2$ site and a glycine (G247) at the $\mathbf{E}_1$ site.[19] In an attempt to test whether these two possible positions for accommodating the catalytic glutamate are functionally equivalent, we have constructed a double mutant of rSCAD in which the catalytic glutamate at the $\mathbf{E}_2$ position has been moved to

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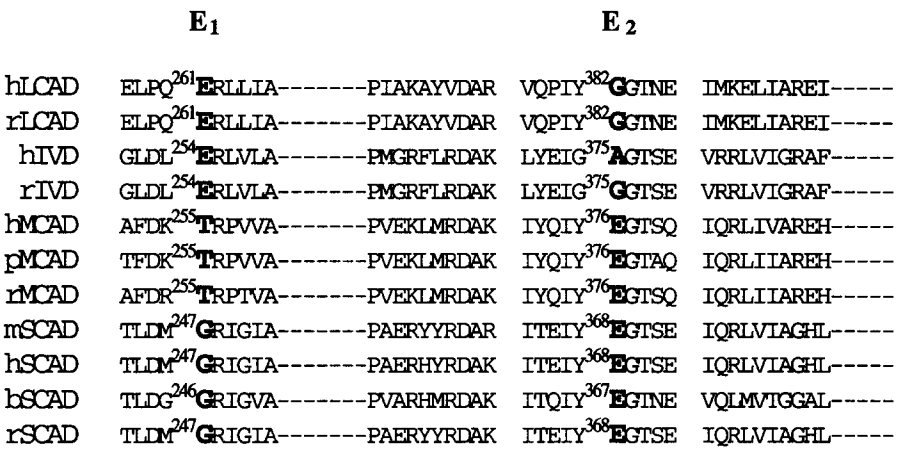


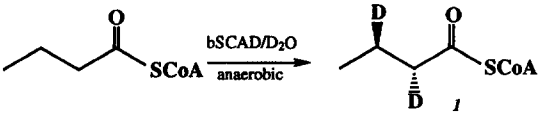
Figure 1. Alignment of the pertinent active site amino acid sequences of acyl-CoA dehydrogenases from different sources: h: human; r: rat; p: pig; m: mouse; b: bacterial (*M. elsdenii*).

the E_1 position [G247E/E368G].[20] This mutant protein proved to be active, but utilizes butyryl-CoA as substrate less efficiently than does the wild-type enzyme (K_m = 0.6 and 2.0 μM, and V_{max} = 2.8 and 0.3 s^{-1} for wild-type and mutant enzyme, respectively). It also exhibits a slightly different substrate specificity. Thus, it seems that the potential positions for the catalytic glutamate are not topologically equivalent. It should be noted that a stereochemical uniformity has been established for the catalysis of acyl-CoA dehydrogenases, that is, the bond cleavages at the α- and β-carbons are both pro-*R* specific.[21,22] If the E_1 and E_2 catalytic sites are, in fact, topologically distinct, the stereochemistry of the dehydrogenase reaction might well differ between the wild-type rSCAD and the G247E/E368G mutant. To examine this issue directly, stereospecifically labeled substrates have been synthesized and the stereochemical course of the reaction of the wild-type and mutant enzymes has been characterized. The results and implications of these studies are presented herein.

Results and Discussion

Preparation of butanoyl-CoAs stereospecifically labeled with deuterium at C-2 and/or C-3 followed the reactions delineated in Schemes 1–4. These labeled substrates (**1**–**4**) were separately incubated with the wild-type or the mutant rSCAD in 0.1 M potassium phosphate buffer prepared with D_2O (pD 7.0), and the crotonoyl-CoA product was purified by HPLC and characterized by ^1H NMR. As shown in Figure 2g, the signal of 3-Me (γ-Hs) of the standard crotonoyl-CoA is seen as a doublet at δ 1.69 (J = 7.0 Hz) and that of 3-H (β-H) at δ 6.77 is a multiplet due to the splitting by the neighboring 3-Me and 2-H (α-H) hydrogens. While, the peak of 2-H appearing as a doublet at δ 6.05 (J = 7.5 Hz) overlaps with the anomeric proton of the ribose of the CoA, the former two resonances (3-Me and 3-H) are well resolved and thus can be used as references to determine the fate of deuterium labels after enzymatic turnover. It was found that a sample of crotonoyl-CoA derived from incubation of (2*R*,3*R*)-[2,3-$^2H_2$]butanoyl-CoA (**1**) with the wild-type rSCAD is devoid of the doublet at δ 1.69 (Fig. 2a). This finding is not surprising since this class of enzymes is capable of catalyzing γ-H abstraction, thus facilitating the allylic isomerization between α,β- and β,γ-enoyl thioesters.[23] In D_2O with butyryl-CoA, complete exchange of deuterium into the methyl group of crotonoyl-CoA occurs (Scheme 5A). Figure 2a also shows that the signal of 3-H at δ 6.77 becomes a doublet (J = 15.5 Hz). This observation not only indicates that 3-H is retained in this sample, but also reveals the presence of 2-H whose resonance at δ 6.05 is partially buried. Since all isotopic labels of this sample (**1**) were lost during enzymatic incubation, the C-H bond cleavage at the C-2 and C-3 positions catalyzed by the wild-type rSCAD must be both pro-*R* stereospecific (Scheme 5A). As expected, identical spectral characteristics were also observed for the crotonoyl-CoA products derived from incubation of **2** and **3** with the wild-type enzyme (Fig. 2b, c).

The retention of both 2- and 3-Hs were again found in the crotonoyl-CoA samples isolated from the incubations of **1**, **2** and **3** with the double mutant enzyme. As can be seen in Figure 2d and e, the spectra of these samples are identical to that of the crotonoyl-CoA standard. The fact that deuterium at C-2 and/or C-3 of substrates (**1**–**3**) were selectively removed during turnover provides convincing evidence supporting a pro-*R* stereochemical specificity for the bond cleavage at both C-2 and C-3, a preference similar to the wild-type enzyme (Scheme 5B). In a separate experiment, the product derived from incubation of (2*S*)-[2-^{2}H]butanoyl-CoA (**4**) with the mutant protein had a 3-H signal at δ 6.77 (quartet, J = 7.0 Hz) and 3-Me signal at δ 1.67



Scheme 1.

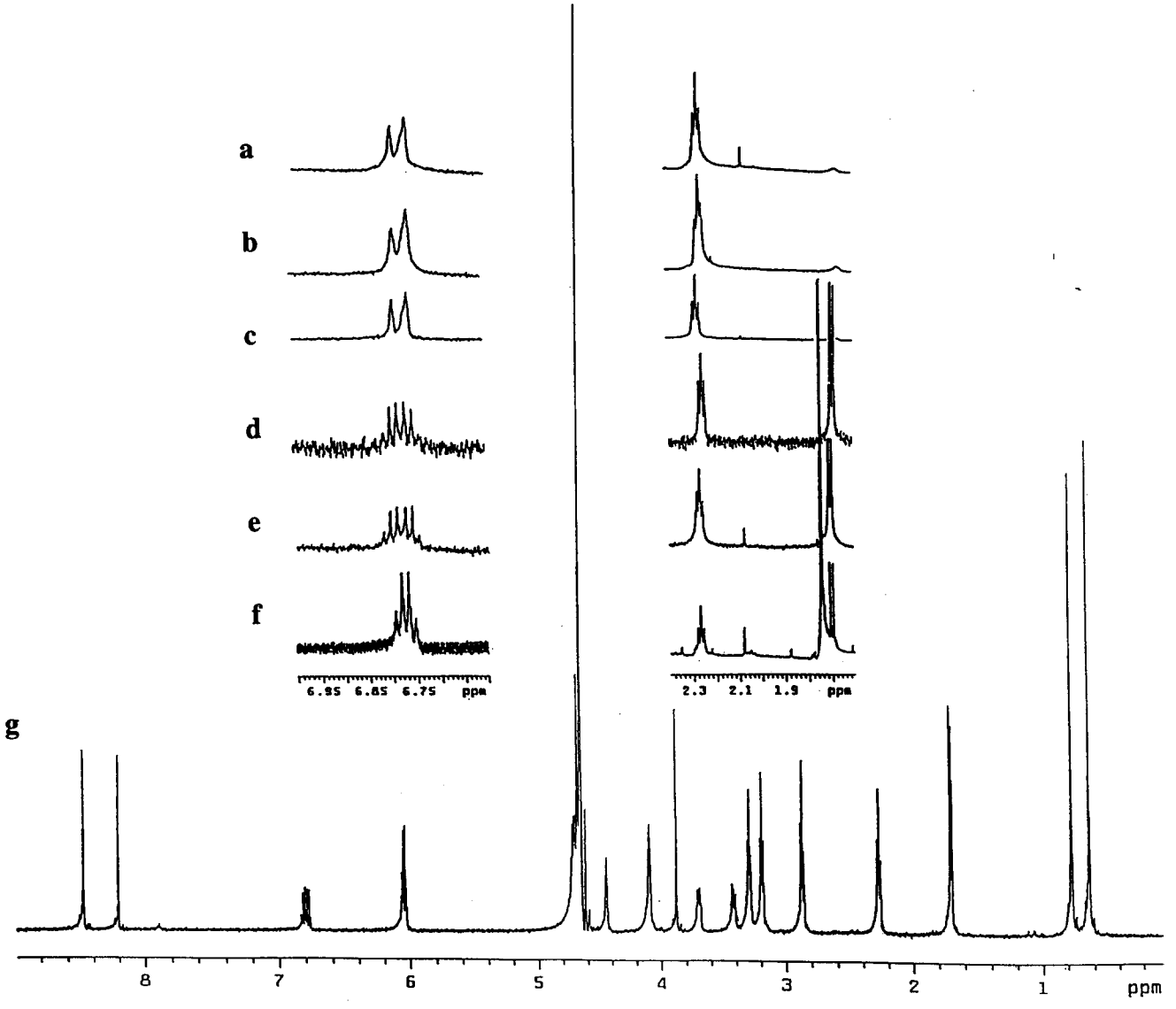
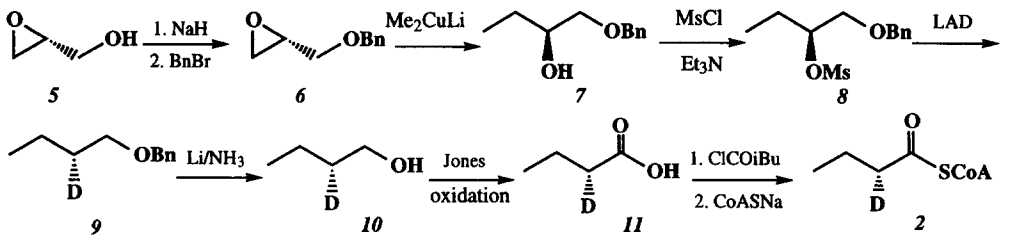


Figure 2. ^{1}H NMR spectra showing the 3-H and 3-Me regions of crotonoyl-CoAs derived from incubations of labeled substrates (**1**–**4**) and rat SCADs. (a) WT with **1**; (b) WT with **2**; (c) WT with **3**; (d) double mutant with **2**; (e) double mutant with **3**; (f) double mutant with **4**; and (g) standard crotonoyl-CoA.

(doublet, J = 7.0 Hz) (Fig. 2f). This result lends further credence to the pro-*R* stereochemical preference of this enzyme. Interestingly, with the wild-type protein, the 3-H signal of the crotonoyl-CoA product at δ 6.77 is a doublet with a coupling constant (J = 15.5 Hz) characteristic for a *trans* olefin (Fig. 2a–c); however, that of the product generated by the double mutant enzyme is a well-resolved doublet of quartets (J = 7.0, 15.5 Hz). Consistent with these results is the presence of the 3-Me signal at δ 1.66 (J = 7.0 Hz) in crotonoyl-CoA derived from the double mutant enzyme (Fig. 2d, e). Clearly, reversing the catalytically essential glutamate residue from $\mathbf{E}_2$ to $\mathbf{E}_1$ in rSCAD has impaired its ability to promote the solvent hydrogen exchange at γ-CH_3 (Scheme 5B). It is conceivable that while both α-H and γ-H are easily accessible to glutamate in the wild-type protein, the repositioned base E247 in the mutant protein can only reach the α-H of the substrate.

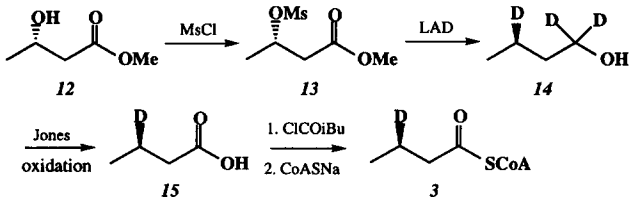
Recently, a double mutant of human MCAD (E376G/T255E) in which the catalytic glutamate has been switched from the $\mathbf{E}_2$ site to the $\mathbf{E}_1$ site was constructed.[24] This mutant protein retains approximately 20% activity of the wild-type enzyme and exhibits a much narrower substrate chain length specificity. Interestingly, the pK_a of E376 and E255 appeared to be comparable, suggesting a similar micro-environment near the reaction center in both proteins.[24] However, X-ray crystal structure analysis revealed that the distance between the E255 carboxylate and the α-H to be abstracted is greater than that of E376.[25] It was also noted that the direction in which E255 approaches the α-H of the substrate is different from that of E376. These factors are likely responsible for the intrinsic differences in the kinetic properties and the substrate specificity between the wild-type and the mutant enzyme. Although the three-dimensional structure of



Scheme 2.

the G247E/E368G mutant rSCAD is currently lacking, molecular modeling based on the X-ray crystal data for bSCAD and rSCAD has allowed the relative distance as well as the orientation of the catalytic glutamate (E247) with respect to the α-H of the substrate to be estimated.[19] Unlike the hMCAD case, our modeling results indicate that while the angle of approach of the carboxylate of E247 to the α-H to be abstracted differs markedly from that of E368; the distances between the carboxylate of the catalytic glutamate and the α-carbon of the substrate are nearly identical in the wild-type and the mutant protein. Thus, the factors that affect the catalytic efficiency of these two related mutant enzymes appear to be different.

It is worth mentioning that a dual role of the active-site glutamate residue in both α- and γ-deprotonation has long been proposed for acyl-CoA dehydrogenases. The best known example is the interconversion between vinylacetylpantetheine and crotonoylpantetheine mediated by bSCAD.[23] The inactivation of MCAD by 2-octynoyl-CoA which involves an initial rate-limiting γ-proton abstraction to afford a reactive allene intermediate is another well documented case.[8] A similar isomerization has also been established as the key step in the inactivation of these enzymes by a 3-acetylenic thioester.[23] A more recent example is the inactivation of pig kidney MCAD by 3-methyleneoctanoyl-CoA and 3-methyl-*trans*-2-octenoyl-CoA in which a common anion derived from an allylic isomerization was proposed as the reactive species responsible for flavin modification.[26] In all these cases, abstraction of a γ-proton or protonation at the γ-C is a prerequisite for the observed transformation. While it is conceivable that the α- and γ-deprotonation are mediated by two different active site basic groups, it is equally possible that these two independent events are catalyzed by a single active-site base. Direct evidence in favor of this latter hypothesis was only recently obtained by examining the consequence of mutation of this glutamate (E367) in bSCAD on the enzyme's ability to catalyze allylic isomerization.[27] In this case, it was found that the E367Q bSCAD mutant, unlike the wild-type enzyme, failed to promote γ-H exchange/abstraction of crotonoyl-CoA and 2-butynoyl-CoA with the solvent. The results from the current experiments with rSCAD provide further support for a one-base mechanism of acyl-CoA dehydrogenase catalyzed α- and γ-reprotonation/deprotonation. This conclusion is also consistent with the fact that the E376G/T255E hMCAD mutant is immune to 2-octynoyl-CoA,[24] a mechanism-based inactivator whose action is initiated by a γ-H abstraction.[8]



Scheme 3.

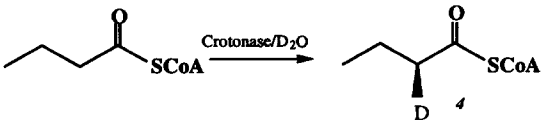
Conclusion

In summary, a G247E/E368G rSCAD mutant exhibits a stereochemical preference identical to the wild-type enzyme. Thus, the $\mathbf{E}_1$ and $\mathbf{E}_2$ sites in this acyl-CoA dehydrogenase appears to be topologically similar. However, the mutant enzyme is unusual in its inability to catalyze γ-H exchange, reflecting the geometric distinction between these two sites. Clearly, transposing the essential glutamate residue of rSCAD from its original location ($\mathbf{E}_2$) in the wild-type enzyme to a topologically similar site ($\mathbf{E}_1$) has created a new catalyst which is catalytically competent, stereochemically conserved, but functionally distinct. These results demonstrate the potential of using site-directed mutagenesis to engineer mutant proteins that have unique abilities to generate altered products and still concurrently carry out chemistry inherent to the wild-type enzyme.

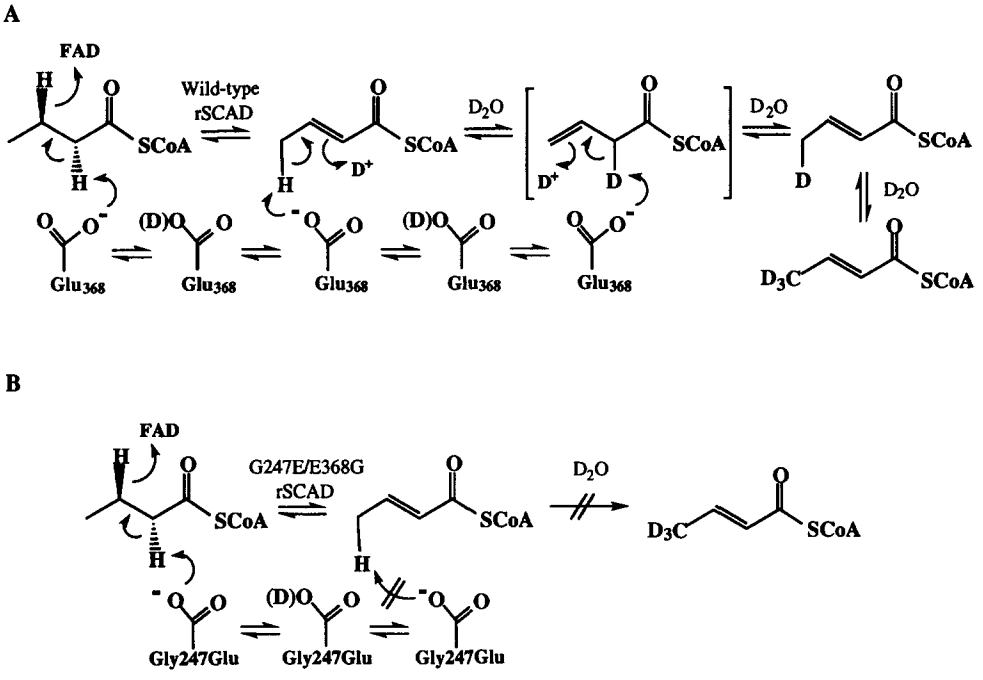
Materials and Methods

Enzymes

Bovine liver crotonase was purchased from Sigma (St Louis, MO), and bSCAD from *Megasphaera elsdenii* was purified from the overexpression construct pWTSCADT7-7 in *E. coli* BL21(DE3)pLysS according to Becker et al.[14] Wild-type and G247E/E368G mutant rSCADs were purified after overexpression in *E. coli* as



Scheme 4.



Scheme 5. Proposed mechanism for stereospecific catalysis and 1,3-allylic isomerization by (A) WT rSCAD and (B) G247E/E368G rSCAD.

previously described.[20] Briefly, induced bacterial cultures were lysed by lysozyme treatment and sonicated. After fractionation of the crude bacterial extract on DE52 resin (Whatman, Fairfield, NJ), the active fractions were pooled and reduced with sodium dithionite. Following dialysis, the rSCADs were purified to homogeneity on a 10 μM ceramic hydroxyapatite matrix (BioRad; Hercules, CA).

Enzymic incubations and product isolation

Labeled substrates (**1–4**) were incubated with both the wild-type and mutant enzyme. A typical mixture contained the labeled butanoyl-CoA (1.62 μmol), the dehydrogenase (22.0 nmol) and excess ferrocinium hexafluoride (an external electron acceptor) in 0.1 M potassium phosphate buffer in D_2O, pD 7.0. After 12 h incubation at room temperature, the cloudy solution was centrifuged to remove particulates, and the protein was removed by passing the resultant mixture through microcon-10 (Amicon, Beverly, MA). The crotonoyl-CoA product was purified on an octadecylsilica HPLC column eluted isocratically with 50 mM potassium phosphate buffer, pH 5.3, in 30% MeOH.[31] The product peak was identified by co-elution with an authentic sample of crotonoyl-CoA and the fractions corresponding to the enzymatic product were pooled. The product was then concentrated in vacuo, desalted on the same column, and lyophilized. Subsequent ^1H NMR analysis of the lyophilized white powders was performed in D_2O by a Varian NT500 spectrometer.

General

GC-MS analysis was performed on an HP 5890A gas–liquid chromatograph and a VG 7070E-HF spectrometer. Ultraviolet–visible spectroscopy was recorded on a Shimadzu UV-160, or a Hewlett–Packard 8452A spectrophotometer. High-performance liquid chromatography analysis and/or purification were conducted with either a Hewlett–Packard 1090A instrument equipped with an HP3392 integrator or a Beckman 110B instrument. ^1H NMR and ^{13}C NMR spectra were recorded on an IBM NR/200 or NR/300 or Varian U-300 or U-500 spectrometer. Chemical shifts are reported on the δ scale relative to the appropriate solvent peak with coupling constants given in hertz. Flash column chromatography was performed on columns of various diameters with J. T. Baker (230–400 mesh) silica gel by elution with the solvents reported. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 G-254 plates (25 mm) and developed with the solvents mentioned. TLC spots were visualized either with UV light or by dipping into the staining solutions of $KMnO_4$ (1%), vanillin: ethanol:H_2SO_4 (1:98:1), or phosphomolybdic acid (7% EtOH solution) and then heating. Solvents, unless otherwise specified, were of analytical reagent grade or the highest quality commercially available. For anhydrous reactions, the solvents were pretreated prior to distillation as follows: tetrahydrofuran was dried over sodium and benzophenone, methylene chloride, dimethyl sulfoxide, and dimethyl formamide were dried over calcium hydride, pyridine and triethylamine were dried over KOH.

Synthesis

(2*R*,3*R*)-[2,3-$^2H_2$]butanoyl-CoA (1). A modification of the literature procedure[28] was adapted for the synthesis of compound **1**. In an anaerobic reaction flask was added an aliquot of bSCAD (200 μL, 22.8 nmol), in 0.1 M potassium phosphate buffer prepared in D_2O, pD 7.0. In the side-arm chamber was added a solution of butyryl-CoA (1.0 mL, 20 μmol) in D_2O. This enzyme and butyryl-CoA had been previously dialyzed and lyophilized, respectively in D_2O. The system was sealed and connected to a schlenk line apparatus to replace air with an inert gas (N_2). The two solutions were then mixed and incubated at room temperature for 12 h. The reaction was quenched by heat denaturation of the enzyme. The precipitated protein was removed by passing the cloudy solution through a Centricon-10 (Amicon, Beverly, MA), and the filtrate was lyophilized. The residue was redisolved in a minimal amount of water and the product was purified by HPLC to give the title compound as a white powder; 16.5mg, 98% yield. ^1H NMR (D_2O, signal of the acyl moiety given in italics) δ 8.40, 8.14 (1H each, s, adenine Hs), 6.02 (1H, d, J = 5.4, ribose anomeric H), 4.71 (2H, m), 4.43 (1H, br s, ribose H), 4.05 (2H, br s, $C(Me)_2CH_2O$), 3.86 (1H, s, $HOCHCMe_2$), 3.64, 3.39 (1H each, m, ribose Hs), 3.28 (2H, t, J = 6.3), 3.07 (2H, m), 2.79 (2H, t, J = 7.0), *2.35* (1H, d, J = 8.0, 2-H), 2.25 (2H, t, J = 7.0), *1.48* (1H, m, 3-H), 0.72 (3H, s), *0.68* (3H, d, J = 7.5, 3-Me), 0.59 (3H, s).

(*S*)-glycidylbenzylether (6). The literature protocol of Lai et al.[29] was adapted to synthesize the title compound; 3.8 g, 85% yield. ^1H NMR ($CDCl_3$) δ 7.40–7.26 (5H, m, Ar Hs), 4.62, 4.55 (1H each, d, J = 11.9, CH_2Ph), 3.77 (1H, dd, J = 11.4, 3.0, one of side chain -OCH_2-), 3.44 (1H, dd, J = 11.4, 5.8, one of side chain -OCH_2-), 3.18 (1H, m, ring -OCH), 2.80 (1H, dd, J = 4.9, 4.3, one of ring -OCH_2-), 2.62 (1H, dd, J = 4.9, 2.7, one of ring -OCH_2-).

(2*S*)-benzyloxy-2-butanol (7). To a solution of 0.2 mol of lithium dimethylcuprate[30] in 30 mL of diethyl ether was added dropwise a solution of 1 g (6.1 mmol) of (*S*)-glycidylbenzyl ether (**6**) dissolved in 30 mL of diethyl ether at −20 °C. The reaction was stirred at 0 °C for 12 h, and the resultant green solution was hydrolyzed by adding 20 mL of saturated NH_4Cl solution. The mixture was stirred at room temperature for an additional hour, and the aqueous layer was separated and extracted with two 25 mL portions of diethyl ether. The combined ether layers were washed with saturated NaCl solution and dried over anhydrous $MgSO_4$. The crude product was purified by flash chromatography (25% ethyl acetate in hexanes) to give **7** (0.9g, 82%). ^1H NMR ($CDCl_3$) δ 7.37–7.27 (5H, m, Ar Hs), 4.55 (2H, s, $PhCH_2$), 3.75 (1H, m, 2-H), 3.51 (1H; dd, J = 9.3, 3.1, 1-H), 3.32 (1H, dd, J = 9.3, 8.1, 1-H), 2.34 (1H, bs, OH), 1.48 (2H, dq, J = 7.5, 7.5, 3-Hs), 0.95 (3H; t, J = 7.5, 3-Me); ^{13}C NMR ($CDCl_3$) δ 138.0, 128.5, 127.8 (Ar Cs), 74.3 (C-2), 73.3 ($PhCH_2$), 71.8 (C-1), 26.1 (C-3), 9.9 (Me).

(2*S*)-4-benzyloxy-2-(butyl)methanesulfonoate (8). To a solution of **7** (0.8 g, 4.44 mmol) in 25 mL of dry methylene chloride was added Et_3N (4.88 mmol, 0.68 mL) at 0 °C. The solution was aged for 5 min followed by the addition of mesyl chloride (8.88 mmol, 0.69 mL) in a dropwise manner. The reaction mixture was stirred vigorously at room temperature, and the progress of the reaction was monitored by TLC. The reaction was complete within 1 h. Excess MsCl was quenched by the addition of water (10 mL). The organic layer was separated and extracted with two 25 mL portions of methylene chloride. The pooled organic layer was throughly washed with water (3 × 25 mL each) and dried over anhydrous $MgSO_4$. The excess solvent was removed in vacuo to give essentially pure product (1.1 g) in 95% yield. ^1H NMR ($CDCl_3$) δ 7.37–7.29 (5H, m, Ar Hs), 4.75 (1H, m, 2-H), 4.57, 4.52 (1H each, d, J = 11.8; $PhCH_2$), 3.58 (2H, m, 1-Hs), 3.01 (3H, s, CH_3SO_3-), 1.70 (2H, dq, J = 7.5, 7.5, 3-Hs), 0.99 (3H, t, J = 7.5, 3-Me).

(2*R*)-[2-^{2}H]benzyloxybutane (9). To a solution of **8** (1.1 g, 4.26 mmol) in 25 mL of dry diethyl ether was added solid lithium aluminum deuteride (0.27 g, 6.39 mmol) portionwise at 0 °C. After stirring overnight, the reaction was quenched at 0 °C by the addition of 274 μL of 15% NaOH solution followed by 822 μL of water. The precipitated lithium salt was filtered and the filtrate was mixed with water. The organic layer was separated, washed with water (20 mL) and dried over anhydrous $MgSO_4$. The excess solvent was removed in vacuo to yield essentially pure **9** (0.6g, 87%). The extent of deuterium incorporation was estimated to be 98% based on ^1H NMR analysis. ^1H NMR ($CDCl_3$) δ 7.36–7.26 (5H, m, Ar Hs), 4.50 (2H, s, $PhCH_2$), 3.47 (2H, d, J = 6.5, 1-Hs), 1.62–1.54 (1H, m, 2-H), 1.39 (2H, dq, J = 10.9, 10.9, 3-Hs), 0.91 (3H; t, J = 10.9, 3-Me); ^{13}C NMR ($CDCl_3$) δ 138.0, 128.5, 127.8 (Ar-Cs), 72.9 ($PhCH_2$), 70.2 (C-1), 31.5 (J_{C_2H} = 19.2, C-2), 19.3 (C-3), 13.9 (C-4).

(2*R*)-[2-^{2}H]butanol (10). Dry-ice condensor was mounted on a three-necked round bottom flask and flame dried. Both the condensor and the round-bottom flask were brought to −40 °C and anhydrous ammonia solution (15 mL) was condensed. Small pieces of lithium (0.4 g, 57.6 mmol) were added to the flask and the resultant blue colored solution was stirred for 15 min. To this solution, compound **9** (1.0 g, 6.06 mmol) in 30 mL of diethyl ether was added dropwise under anhydrous conditions. The resultant solution was vigorously stirred and the temperature was allowed to gradually increase to 10 °C during the course of 12 h. The reaction was quenched by the addition of saturated NH_4Cl (20 mL) at −78 °C. The product was extracted into diethyl ether (150 mL each; 2 ×), dried over anhydrous $MgSO_4$ and excess solvent was carefully removed in vacuo. Due to its high volatility, the crude alcohol was used in the next

reaction immediately. ^{1}H NMR ($CDCl_3$) δ 3.62 (2H, d, J = 6.5, 1-Hs), 1.59 (1H, bs, OH), 1.54–1.49 (1H, m, 2-H), 1.36 (2H, dq, J = 7.3, 7.3, 3-H), 0.92 (3H, t, J = 7.3, 3-Me).

(2*R*)-[2-^{2}H]butanoic Acid (11). To a solution of **10**, from above, in acetone (10 mL) at 0 °C was added dropwise the Jones oxidation reagent (prepared by mixing 26.72 g of CrO_3 with 23 mL of concentrated H_2SO_4 and dilution with water to a final volume of 100 mL) until a red color was sustained. After the reaction was complete (1 h), the excess oxidant was quenched with 2-propanol (1 mL). Acetone was removed in vacuo, and the resultant green precipitate was redissolved in water (5 mL). The product was extracted with chloroform (3 × 20 mL) and the organic portions were pooled, washed with brine, and dried with anhydrous $MgSO_4$. The solvent was removed by rotary evaporation and the oily residue was purified by flash column chromatography (20% ethyl acetate in hexanes) to yield **11** (0.27g, 50%, two steps) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 2.31 (1H, m, 2-H), 1.65 (2H, dq, J = 7.4, 7.4, 3-Hs), 0.96 (3H, t, J = 7.4, 3-Me); ^{13}C NMR ($CDCl_3$) δ 177.6 (C-1), 35.6 (t, J_{C^2H} = 19.5, C-2), 18.1 (C-3), 13.5 (C-4).

(2*R*)-[2-^{2}H]butanoyl-CoA (2). Synthesis of the CoA derivative of **11** was achieved by a previously reported[31] mixed anhydride coupling procedure; 46.4 mg, 85% yield. ^{1}H NMR (D_2O, signal of the acyl moiety given in italics) δ 8.40, 8.11 (1H each, s, adenine H's), 6.02 (1H, d, J = 5.4, ribose anomeric H), 4.71 (2H, m), 4.45 (1H, br s, ribose H), 4.10 (2H, br s, $C(Me)_2CH_2O$), 3.86 (1H, s, $HOCHCMe_2$), 3.71, 3.42 (1H each, m, ribose Hs), 3.32 (2H, t, J = 6.3), 3.17 (2H, m), 2.82 (2H, t, J = 7.0), *2.38* (1H, d, J = 7.5, 2-H), 2.28 (2H, t, J = 7.0), *1.44* (2H, dq, J = 7.5, 7.5, 3-Hs), *0.74* (3H, t, J = 7.5, 3-Me), 0.74 (3H, s), 0.59 (3H, s).

(3*S*)-methyl 3-methanesulfonyloxybutanoate (13). The synthetic procedure of **13**, starting with 1.0 g (8.46 mmol) of **12**, 1.30 mL (9.31 mmol) of Et_3N, and 1.31 mL (16.93 mmol) of mesyl chloride, was identical to **8**. The desired product was obtained in 91% (1.5g) yield. ^{1}H NMR ($CDCl_3$) δ 5.13 (1H, m, 3-H), 3.72 (3H, s, OMe), 3.02 (3H, s, CH_3SO_3), 2.78 (1H, dd, J = 16.6, 8.3, 2-H), 2.57 (1H, dd, J = 16.6, 4.6, 2-H), 1.49 (3H, d, J = 6.3, 3-Me); ^{13}C NMR ($CDCl_3$) δ 172.0 (C-1), 75.8 (OMe), 52.0 (Ms), 41.0 (C-3), 38.3 (C-2), 21.5 (C-4).

(3*R*)-[1,1,3-$^2H_3$]butanol (14). Reduction of **13** (1.1 g, 5.61 mmol) by lithium aluminum deuteride (0.27 g, 6.39 mmol) followed the same procedure as described for the preparation of **9**. The yield was 87% (0.4 g) and the extent of deuterium incorporation was estimated to be 98% based on ^{1}H NMR analysis. ^{1}H NMR ($CDCl_3$) δ 1.57 (1H, bs, OH), 1.54 (2H, d, J = 7.7, 2-Hs), 1.35 (1H, m, 3-H), 0.91 (3H, d, J = 7.3, 3-Me).

(3*R*)-[3-^{2}H]butanoic acid (15). Oxidation of the alcohol **14** (0.5 g, 6.5 mmol) was effected following the same procedure as that of compound **11**; 0.6g, 97% yield. ^{1}H NMR ($CDCl_3$) δ 2.3 (2H, d, J = 7.5, 2-Hs), 1.65 (1H, qt, J = 7.5, 2.0, 3-H), 0.96 (3H, d, J = 7.5, 3-Me); ^{13}C NMR ($CDCl_3$) δ 180.3 (C-1), 35.9 (C-2), 17.7 (J_{C^2H} = 19.2, C-3), 13.5 (C-4).

(3*R*)-[3-^{2}H]butanoyl-CoA (3). Synthesis of the CoA derivative of **11** was achieved by a previously reported[31] mixed anhydride coupling procedure; 47 mg, 86% yield. ^{1}H NMR (D_2O, signal of the acyl moiety given in italics) δ 8.40, 8.14 (1H each, s, adenine Hs), 6.02 (1H, d, J = 5.4, ribose anomeric H), 4.71 (2H, m), 4.43 (1H, br s, ribose H), 4.12 (2H, br s, $C(Me)_2CH_2O$), 3.86 (1H, s, $HOCHCMe_2$), 3.70, 3.42 (1H each, m, ribose Hs), 3.28 (2H, t, J = 6.3), 3.15 (2H, m), 2.82 (2H, t, J = 7.0), *2.41* (2H, d, J = 7.5, 2-H), 2.27 (2H, t, J = 7.0), *1.46* (1H, m, 3-H), 0.72 (3H, s), *0.68* (3H, d, J = 7.5, 3-Me), 0.59 (3H, s).

(2*S*)-[2-^{2}H]butanoyl-CoA (4). Compound **4** was prepared enzymatically (enoyl-CoA hydratase) in near quantitative yield as previously described.[32] ^{1}H NMR (D_2O, signal of the acyl moiety given in italics) δ 8.40, 8.15 (1H each, s, adenine Hs), 6.02 (1H, d, J = 5.4, ribose anomeric H), 4.71 (2H, m), 4.43 (1H, br s, ribose H), 4.04 (2H, br s, $C(Me)_2CH_2O$), 3.81 (1H, s, $HOCHCMe_2$), 3.64, 3.39 (1H each, m, ribose Hs), 3.25 (2H, t, J = 6.3), 3.07 (2H, m), 2.79 (2H, t, J = 7.0), *2.33* (1H, t, J = 7.5; 2-H), 2.22 (2H, t, J = 7.0), *1.39* (2H, dq, J = 7.5, 7.5, 3-Hs), *0.74* (3H, d, J = 7.5, 3-Me), 0.74 (3H, s), 0.59 (3H, s).

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