Linking Diversity in Evolutionary Origin and Stereospecificity for Enoyl Thioester Reductases: Determination and Interpretation of the Novel Stereochemical Course of Reaction Catalyzed by Crotonyl CoA Reductase from *Streptomyces collinus*

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Abstract: The stereochemical course of reduction of crotonyl CoA by the novel crotonyl CoA reductase (CCR) of *Streptomyces collinus* was determined using a radiochemical assay. The reaction was shown to proceed with transfer of the hydrogen from the *pro*-4*S* position of NADPH to the *Re* face of the β-carbon of crotonyl CoA. This transfer represents the first exception to the observation that enoyl thioester reductases catalyze transfer of the *pro*-4*S* hydrogen of NADPH to the *Si* face of the substrate. The observation of addition of solvent hydrogen to the *Re* face of the α-carbon in the reaction catalyzed by CCR demonstrated that the overall reduction of crotonyl CoA proceeds in an *anti* fashion. The overall stereochemical outcome of the reaction catalyzed by CCR is different to the four stereochemical outcomes that have previously been observed for enoyl thioester reductases. It is significant that the predicted amino acid sequence of CCR has also been shown to be unrelated to other enoyl thioester reductases. Based on these observations it is proposed that the stereochemical course of an enoyl thioester reduction serves no mechanistic function but merely reflects the pedigree or ancestral lineage of the enzyme. Any two enoyl thioester reductases which exhibit different stereospecificities are, therefore, predicted to have different pedigrees and unrelated amino acid sequences. An evaluation of all the enoyl thioester reductases where both the stereochemical course of reduction and the predicted amino acid sequence are known is shown to be entirely consistent with these predictions.

Introduction

Enoyl thioester reductases catalyze the reduction of the α,β-double bond of enoyl thioesters utilizing either NADH or NADPH as the electron donor (Figure 1). Their typical physiological function is in primary metabolism, catalyzing the second reductive step in the cycles of either fatty acid elongation or de novo fatty acid biosynthesis.[1−5] These enzymes are therefore ubiquitous in nature and have been purified and characterized from both eucaryotes and procaryotes.[6−10] Enoyl thioester reductases are also implicated in certain secondary metabolic processes, in particular formation of the macrolide and polyether polyketide antibiotics.[11−16]

Extensive regiochemical and stereochemical studies have been reported for enoyl thioester reductases.[17−23] The regiochemical course of these reductions is conserved, proceeding with addition of the solvent hydrogen to the α-carbon of the substrate. The stereochemical course, however, is highly diverse and involves three cryptic stereochemical details: (1) the stereospecificity of hydrogen transfer from the *pro*-4*R* or *pro*-4*S* position of the cofactor to the β-carbon; (2) the face of the β-carbon to which this hydrogen is added, and; (3) the addition of solvent hydrogen to either the *Si* or *Re* face of the α-carbon. With the caveat that the regiochemistry is conserved, there are eight possible stereochemical outcomes for a reaction catalyzed by an enoyl thioester reductase (Figure 1). Four of these stereochemical

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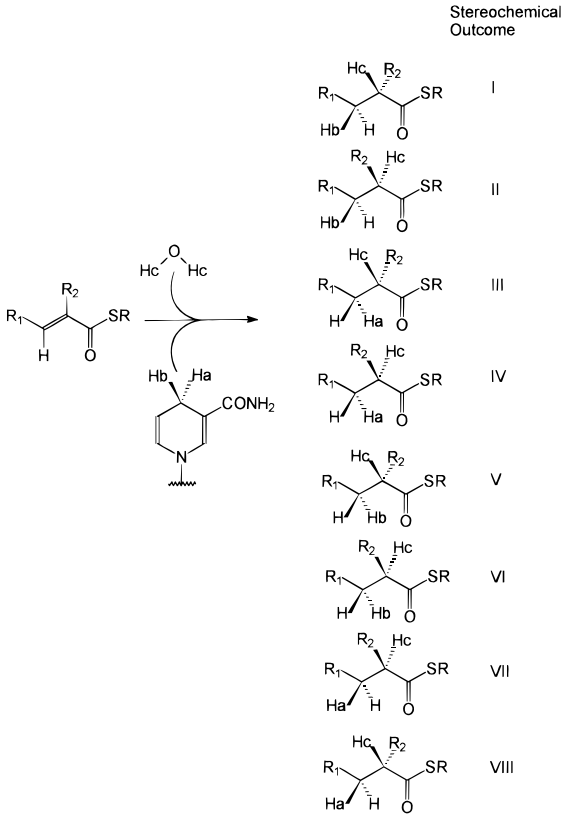

Figure 1. Eight possible stereochemical outcomes for a reaction catalyzed by an enoyl thioester reductase. In all outcomes the solvent hydrogen is added to the α-carbon (an additional eight stereochemical outcomes would be possible if addition to the β-carbon was allowed). Hb and Ha represent the the pro-4*S* and pro-4*R* hydrogens of NADPH, respectively. Hc represents the solvent hydrogen. SR represents either coenzyme A (SCoA) or an acyl carrier protein (SACP).

Table 1. Stereochemical Outcome of the Reaction Catalyzed by Various Enoyl Reductases[a]

enzyme source	stereo-specificity of NAD(P)H	attack of hydrogen C-3	attack of hydrogen C-2	type of addition	stereo-chemical outcome
Brevibacterium ammoniagenes[b,c]	*pro*-4*S*	*Si*	*Si*	*anti*	II
yeast[b,c]	*pro*-4*S*	*Si*	*Si*	*anti*	II
rat[b,d]	*pro*-4*R*	*Re*	*Si*	*syn*	IV
chicken[b,d]	*pro*-4*R*	*Re*	*Si*	*syn*	IV
E. coli (chain elongation)	*pro*-4*R*	*Re*	*Re*	*anti*	III
E. coli[b]	*pro*-4*S*	*Si*	*Re*	*syn*	I
S. collinus (ChcA)[b]	*pro*-4*S*	*Si*	*Si*	*anti*	II
S. collinus (CCR)[b,e]	*pro*-4*S*	*Re*	*Re*	*anti*	V

[a] Only enoyl thioester reductases where all three cryptic stereochemical details have been elucidated are included. Roman numerals are used to correlate the observed stereochemical outcomes with those depicted in Figure 1. Most of the enzymes are enoyl ACP reductases involved in *de novo* fatty acid synthesis. The exceptions are the crotonyl CoA reductase (CCR) and 1-cyclohexenylcarbonyl CoA reductase (ChcA) of *S. collinus* and the *E. coli* enoyl thioester reductase involved in fatty acid elongation. [b] Denotes enzymes whose predicted amino acid sequence is available. [c,d] Denotes two groups of enzymes that have significant amino acid sequence similarity *within* the group. The remaining enzymes have no significant amino acid sequence similarity with each other. [e] Represents findings from this work.

outcomes have already been observed with the enoyl thioester reductases studied to date (Table 1).[17−23] A general pattern has emerged in which the nucleotide specificity, either *pro*-4*R* or *pro*-4*S*, appears to determine the stereospecificity of the hydrogen addition at the β-carbon of the fatty acid, *pro*-3*R* or *pro*-3*S*, respectively.[17,18,22] If this observation is applicable for all enoyl thioester reductases, no additional stereochemical outcomes will be observed.

Two alternative theories that explain the stereochemical dichotomy for the nucleotide specificity for enoyl thioester reductases involved specifically in fatty acid synthesis have been presented; either these enoyl thioester reductases are not homologous and have formed by convergent evolution, or the stereochemistry for transfer of the nucleotide hydrogen can diverge.[24] No attempt has been made, however, to expand these theories to include all three cryptic stereochemical details or a broader range of enoyl thioester reductases. In recent years the enoyl thioester reductase genes involved in fatty acid biosynthesis have been cloned and sequenced,[5,25−29] thereby providing an opportunity to discriminate between the two theories advanced for the observed stereochemical diversity. Furthermore, enoyl thioester reductase genes involved in other processes have also recently been cloned and sequenced[30−33] thus allowing these theories to be tested more generally for this entire class of enzymes.

Reported herein is a stereochemical analysis of crotonyl CoA reductase (CCR), a novel enoyl thioester reductase from *Streptomyces collinus*. The predicted amino acid sequence of CCR has previously been shown to have no significant similarity with other enoyl thioester reductases.[30] The stereochemical course of the reaction catalyzed by CCR is shown to be different to all previously observed stereospecificities and to represent the first exception to the observation that the nucleotide stereospecificity determines the stereospecificity of hydrogen addition at the β-carbon.[17,18,22] A proposal that stereochemical diversity reflects amino acid sequence diversity in enoyl thioester reductases is presented based on these observations. An analysis of other enoyl thioester reductases, where both the stereochemical course and amino acid sequence data are now known, is shown to be consistent with this proposal.

Experimental Methods

Materials. [1-^{14}C]Butyryl coenzyme A (4 mCi/mmol) was purchased from Moravek. [^{3}H]Water (100 mCi/g) was obtained from Dupont NEN. [1-^{3}H]Glucose (15.5 Ci/mmol), crotonyl coenzyme A, butyryl coenzyme A, coenzyme A (CoA), FAD, NADP, NADPH, glucose dehydrogenase (*Thermoplasma acidophilum*), alcohol dehydrogenase (*Thermoanaerobium brockii*), and acyl CoA oxidase (*Candida lipolytica*) were purchased from Sigma. Enzyme activity of crotonyl CoA reductase was assayed spectrophotometrically[30] and by HPLC analysis. Enzyme activities were measured in units (U), where a unit corresponds to the oxidation of 1 mmol of NADPH per min.

HPLC Analysis of Butyryl CoA and Crotonyl CoA. Butyryl CoA and crotonyl CoA concentrations were monitored at 254 nm using a Beckman HPLC system equipped with a 165 variable wavelength detector and a 4.6 × 250 mm ODS2 column (MetachemTechnologies).

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The mobile phase typically consisted of 50 mM potassium phosphate (pH 4.0) and methanol (75:25) at a flowrate of 1.0 mL/min. For radiolabeled analysis, eluent from the column was collected in 0.5 mL fractions and mixed with 5 mL of scintillation fluid (Scintisafe Econo 1 from Fisher Scientific). Both ^{3}H and ^{14}C levels were determined using a Beckman LS5801 Liquid Scintillation Counter. The ratio of ^{3}H/^{14}C was calculated based on the counts per min (CPM) determined for each individual isotope in the standard manner. Crotonyl CoA and butyryl CoA concentrations were determined by comparisons of the peak areas from the HPLC analysis with injections of standards. Specific activities of these compounds was calculated based on this peak area and the radioactivity associated with the peak (CPM were converted to disintegrations per minute based on a counting efficiency of 38% and 74% for ^{3}H and ^{14}C, respectively).

HPLC Analysis of NADP and NADPH. An HPLC analysis was also used for monitoring concentrations of NADP and NADPH. A mobile phase of 50 mM potassium phosphate (pH 4.0) and methanol (91.5:8.5) at a flowrate of 1.0 mL/min allowed baseline separation of NADP and NADPH with retention times of 3.2 and 5.2 min, respectively. The specific activity of NADP and NADPH was determined in the manner described above.

Synthesis of [4*R*-^{3}H]- and [4*S*-^{3}H]NADPH. [4*S*-^{3}H]NADPH was prepared enzymatically by incubating NADP (8 mg, 10 mmol) with glucose dehydrogenase (1 μL, 3.5 units), glucose (2.0 mg, 11 mmol), and [1-^{3}H]glucose (0.1 mCi, approximately 0.007 mmol) in a 50 mM potassium phosphate solution (0.6 mL) at pH 7.0 for 2 h at 30 °C.[34] The solution was freeze dried.

[4*R*-^{3}H]NADPH was prepared from NADP through a series of three enzymatic steps.[35] Initially [4*S*-^{3}H]NADPH was generated with glucose dehydrogenase in the manner described above. Acetone (16 mg, 0.28 mmol) and alcohol dehydrogenase (5 units) were added to the reaction mixture (0.2 mL). Incubation of the resulting solution for 2 h at 30 °C produced [4-^{3}H]NADP. [4-^{3}H]NADP was reduced in volume and purified by HPLC (fractions with retention times from 3.0−3.5 min were collected and freeze dried). The resulting material was dissolved in water (1 mL), and the pH was adjusted to 7.0 with K_2HPO_4 (1 M). Glucose (0.5 mg, 2.8 mmol) and glucose dehydrogenase (1 μL, 3.5 units) were added into the solution. The reaction mixture was incubated for 5 h at 30 °C and subsequently freeze dried.

Preparation of Crotonyl CoA Reductase. Crotonyl CoA reductase was purified as a recombinant protein from *Escherichia coli* as previously described.[30]

Determination of the Stereochemical Course of Hydrogen Transfer from NADPH in the Crotonyl CoA Reductase Reaction. Purified crotonyl CoA reductase (1 μL, 3.5 units) was added to 600 μL of 50 mM potassium phosphate solution (pH 7.0) containing crotonyl CoA (0.7 mg, 0.84 mmol) and [4*S*-^{3}H]NADPH (3 mCi, 0.7 mg, 0.9 mmol), and the reaction mixture was incubated overnight at 25 °C. Analysis by HPLC indicated in this and subsequent experiments with crotonyl CoA reductase almost a complete conversion of crotonyl CoA to butyryl CoA.

The reaction mixture was heated for 5 min at 100 °C to inactivate crotonyl CoA reductase and treated with [1-^{14}C]butyryl CoA, acyl CoA oxidase from *Candida lipolytica* (0.2 units) and FAD (20 μL of 1 mM solution). A 16-h incubation of the reaction mixture in the dark (30 °C) resulted in virtually complete conversion of butyryl CoA to crotonyl CoA. The reaction mixture was fractionated by HPLC and analyzed as described above.

Reduction of crotonyl CoA in the presence of [4*R*-^{3}H]NADPH was performed in a similar manner. Thus, 200 μL of 50 mM, pH 7.0 potassium phosphate solution containing crotonyl CoA (0.03 mg, 0.04 μmol) and [4*R*-^{3}H]NADPH (0.1 μCi, 0.04 μmol) was treated with purified crotonyl CoA reductase (1 μL, 3.5 units).

Determination of the Stereochemical Course of Solvent Hydrogen Addition in the Crotonyl CoA Reductase Reaction. A solution containing 100 μL of [^{3}H]water (10 mCi), 80 μL of potassium phosphate buffer (50 mM, pH 7.0), crotonyl CoA (2 mg, 2.4 μmol), and NADPH (2.5 mg, 3 μmol) was treated with the recombinant crotonyl CoA reductase (2 μL, 7 units) overnight at 25 °C. The solution was treated with 150 μL of water and freeze dried. This washing process was repeated twice. The dried reaction mixture was resuspended in water (200 μL) and analyzed by HPLC. This solution was combined with [1-^{14}C]butyryl CoA and FAD and treated with acyl CoA oxidase as described above.

Results and Discussion

Stereochemical Course of the Crotonyl CoA Reductase Reaction. An enoyl CoA reductase, believed to be involved in supplying butyryl CoA for primary and secondary metabolic processes, has been isolated from *S. collinus*.[30] This enzyme specifically requires NADPH as the sole electron donor and a four carbon substrate, crotonyl CoA. The complete stereochemical course of reduction of crotonyl CoA has been determined using a direct radiochemical approach. Previous approaches to analyzing stereochemical course of reduction of crotonyl CoA or crotonyl ACP (acyl carrier protein) have used indirect methods that require hydrolysis of the butyryl CoA and crotonyl CoA thioester bonds prior to analysis. Such methods require harsh conditions and result in hydration of crotonyl CoA to hydroxybutyrate and a potential for hydrogen exchange at the α-carbon.[19,36] An alternative approach involving direct derivatization with benzylamine has also been reported.[22] All of these methods have proven problematic in our experience and led to the development of a radiochemical assay that allows direct analysis of butyryl CoA and crotonyl CoA.

To investigate the addition of hydrogen at the β-carbon of crotonyl CoA, [4*R*-^{3}H]- and [4*S*-^{3}H]NADPH were prepared enzymatically. Crotonyl CoA was incubated overnight with purified crotonyl CoA reductase in the presence of [4*R*-^{3}H] and [*S*-^{3}H]NADPH. The butyryl CoA from the enzyme reaction was then separated from unreacted crotonyl CoA and NADP^{3}H using reverse phase HPLC and subsequently analyzed. The analysis clearly demonstrated that the incubation in the presence of [4*S*-^{3}H]NADPH (3.4 ± 0.3 mCi/mmol, determined from triplicate injections) resulted in the production of tritiated butyryl CoA (2.2 ± 0.2 mCi/mmol, determined from HPLC analysis from three separate experiments) (Figure 2). Conversely, incubation of the enzyme in the presence of crotonyl CoA and [4*R*-^{3}H]NADPH (2.6 ± 0.3 mCi/mmol, determined from triplicate injections) resulted in the production of butyryl CoA with no detectable radiolabel (Figure 2). In experiments using both the isomers of labeled NADPH, almost complete conversion of crotonyl CoA to butyryl CoA was observed. In both sets of experiments the HPLC analyses was conducted using comparable butyryl CoA quantities. These experiments clearly demonstrated that the enzyme catalyzed reaction proceeds with transfer of the *pro*-4*S* hydrogen of NADPH to crotonyl CoA.

The tritiated butyryl CoA formed in these experiments was combined with [1-^{14}C]butyryl CoA to yield a ^{3}H/^{14}C ratio of 1.1. This butyryl CoA (with a specific activity for ^{14}C of 0.98 mCi/mmol) was converted to crotonyl CoA by incubation with an acyl CoA oxidase from *Candida lipolytica*. This enzyme stereospecifically catalyzes the removal of the *pro*-2*R* and *pro*-3*R* hydrogens of the acyl CoA substrate.[37] Under the experimental conditions HPLC analysis indicated that almost a complete conversion of butyryl CoA to crotonyl CoA was obtained (Figure 3). Consistent with this observation was the loss of all ^{14}C and ^{3}H radioactivity associated with the butyryl CoA peak (Figure 3). The resulting crotonyl CoA had a specific activity for ^{14}C of 0.84 ± 0.04 mCi/mmol (determined from three separate experiments) but no detectable levels of ^{3}H (a ^{3}H/^{14}C ratio of 0) indicating that the reaction proceeded with 100% loss of tritium. This result is entirely consistent with an initial transfer of tritium from the *pro*-4*S* hydrogen of NADPH to the *Re* face of the β-carbon of crotonyl CoA (addition of hydrogen from the cofactor to the α-carbon in enoyl thioester reductions is unprecedented and would contravene a clear

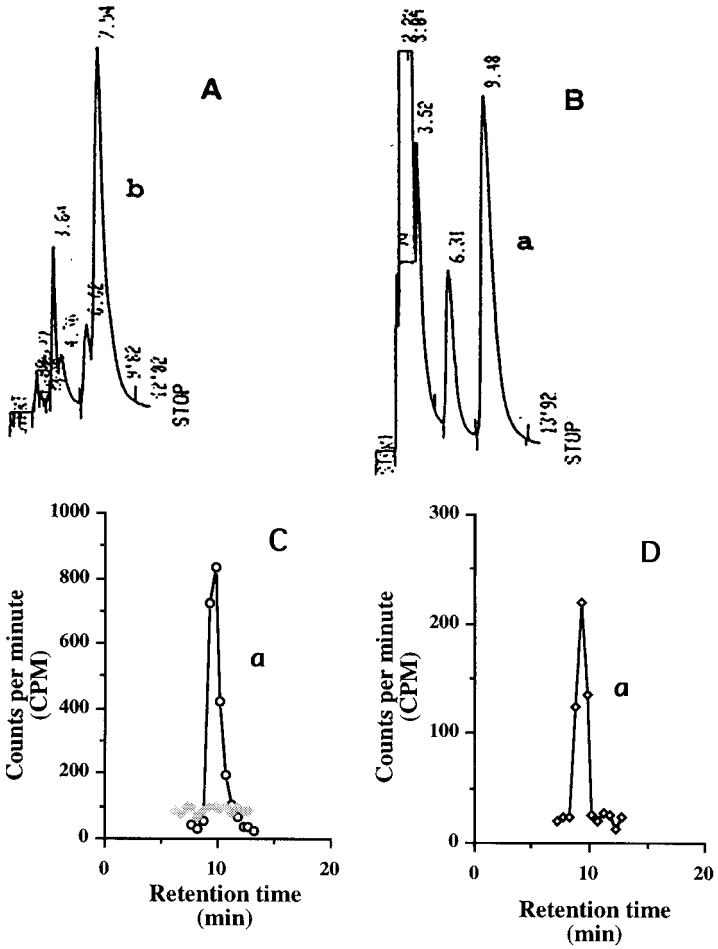

Figure 2. Determination of the NADPH stereospecificity in the reaction catalyzed by crotonyl CoA reductase (CCR). A and B represent a typical HPLC analysis of an incubation of crotonyl CoA and NADPH with CCR at time zero and 16 h, respectively. HPLC analyses were conducted using 50 mM potassium phosphate (pH 4.0) and methanol (70:30) at a flow rate of 1 mL/min. Crotonyl CoA (b) and butyryl CoA (a) had retention times of 7.5 and 9.5 min, respectively. C represents overlaid results of the HPLC-radiochemical analyses of the butyryl CoA formed when incubations of CCR were carried out with crotonyl CoA and either [4*S*-^{3}H]NADPH (open circles) or [4*R*-^{3}H]NADPH (grey circles). D represents the radiochemical analyses of butyryl CoA formed when the crotonyl CoA was reduced by CCR in the presence of tritiated water.

mechanistic imperative) (Table 1). This result is unusual since reduction reactions that proceed with transfer of the *pro*-4*S* hydrogen of NAD(P)H have previously been shown to always result in addition of hydrogen to the *Si* face of the β-carbon (Table 1).[17,18,22]

To determine the stereochemistry of hydrogen transfer from solvent to the α-carbon of crotonyl CoA, the crotonyl CoA reductase was incubated in ^3H_2O (1 mCi/mmol in the reaction mixture) in the presence of NADPH and crotonyl CoA in an overnight incubation. A series of freeze drying steps were used to remove the tritiated water. Butyryl CoA with a specific activity 56 μCi/mmol was obtained in these reactions, reflecting a significant isotope effect. The reaction mixture was combined with [1-^{14}C]-butyryl CoA to provide a sample containing butyryl CoA with a ^{3}H/^{14}C ratio of 0.8 (a specific activity of approximately 56 μCi/mmol for ^{3}H and 40 μCi mmol for ^{14}C). This reaction mixture was then incubated with the acyl CoA dehydrogenase from *C. lipolytica*. Under the experimental conditions HPLC analysis again revealed a complete conversion of butyryl CoA to crotonyl CoA (Figure 3). Consistent with this observation was the loss of ^{14}C and ^{3}H radioactivity associated with the butyryl CoA peak (Figure 3). The resulting crotonyl CoA was determined to have a specific activity of 33 ± 3 μCi mmol for ^{14}C (determined from three separate experiments) and to have no significant levels of ^{3}H above background (a ^{3}H/^{14}C ratio of 0) (Figure 3). These results indicate that the reaction proceeded with a 100% loss of tritium. This result is consistent with the location of a tritium label in the *pro*-2*R* position. Therefore, the crotonyl CoA reductase reduces crotonyl CoA in an *anti* fashion with addition of solvent hydrogen to the *Re* face of the α-carbon (Table 1).

Interpretation of Stereochemical Diversity for Enoyl Thioester Reductases. The interpretation of stereospecificity in enzyme catalyzed processes has attracted considerable interest and controversy for several decades.[24,38−47] Of particular interest has been the interpretation of cases where stereospecificity is either highly conserved or highly diverged for enzymes cata-

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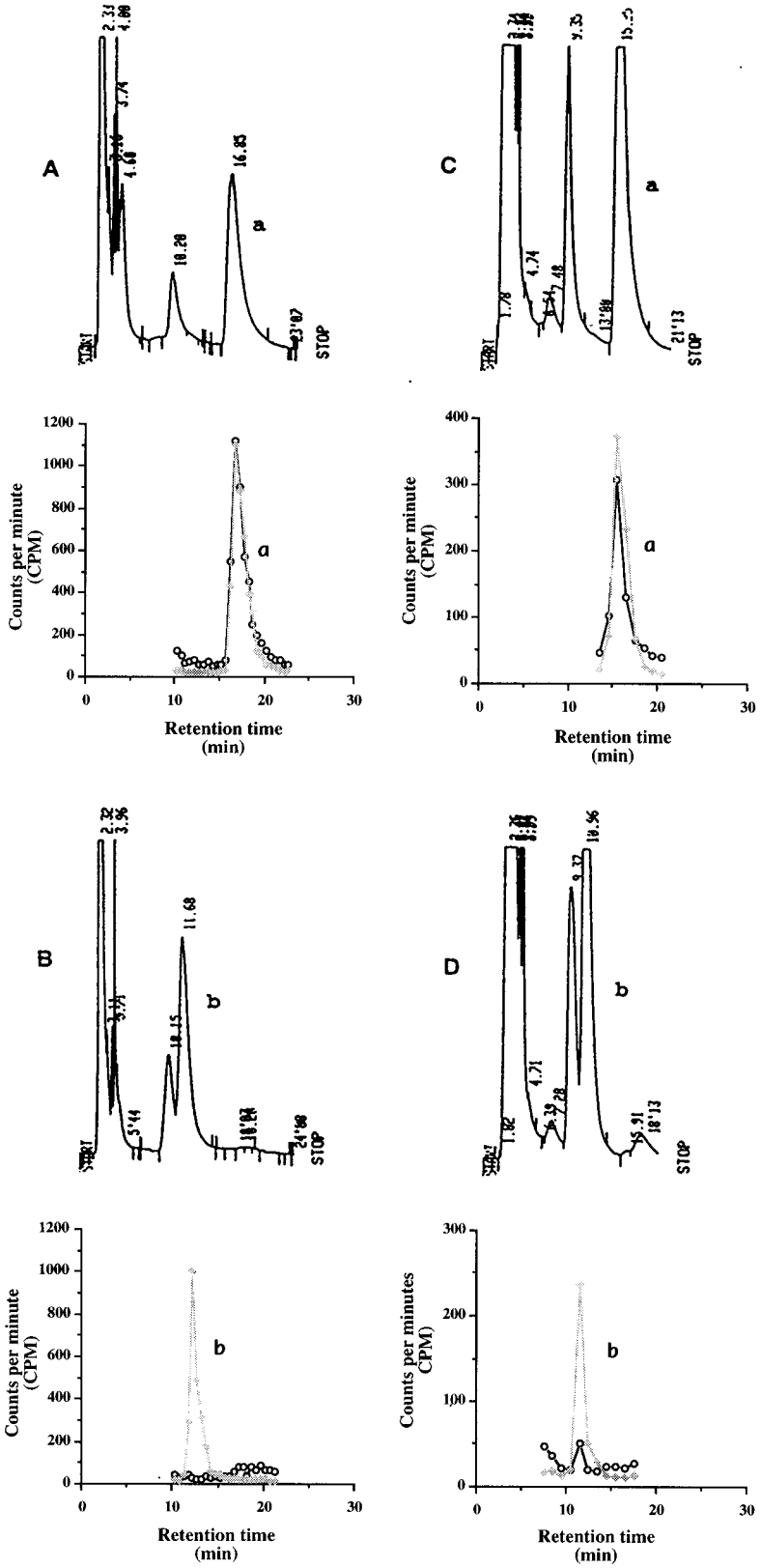

Figure 3. Determination of the overall stereochemical course of reduction of the α,β double bond of crotonyl CoA by crotonyl CoA reductase (CCR). A represents an HPLC-radiochemical analysis of a mixture of ^{3}H butyryl CoA (formed from an incubation CCR with crotonyl CoA and [4*S*-^{3}H]NADPH) and ^{14}C butyryl CoA. B represents an HPLC-radiochemical analysis of this same reaction mixture after an overnight incubation with an acyl CoA oxidase. C represents an HPLC-radiochemical analysis of a mixture of ^{3}H butyryl CoA (formed from an incubation CCR with crotonyl CoA, NADPH and tritiated water) and ^{14}C butyryl CoA. D represents an HPLC-radiochemical analysis of this same reaction mixture after an overnight incubation with an acyl CoA oxidase. HPLC analyses were conducted using 50 mM potassium phosphate (pH 4.0) and methanol (75:25) at a flow rate of 1 mL/min. Crotonyl CoA (b) and butyryl CoA (a) had approximate retention times of 11.7 and 16.8 min, respectively. The ^{14}C and ^{3}H radiochemical analyses are depicted using grey diamonds and open circles, respectively.

lyzing analogous reactions. Historical and functional models have both been presented to rationalize cases where enzymes catalyzing analogous reactions exhibit extreme conservatism in their stereospecificity.[38,39,47] In the historical model, stereospecificity is a nonselected trait which is conserved during divergent evolution.[24,40,43] In the functional model the conserved

nature of the stereospecificity is dictated by a mechanistic imperative.[47] In the most concise form "the basis for stereochemical conservation may be embedded in the reaction mechanism or the perpetuation of a segment of protein structure that defines certain stereochemical imperatives in the active site".[47] Only historical models have been presented to rationalize enzymes that catalyze analogous or identical reactions with differing stereospecificities. In these cases the observed stereochemical diversity reflects either that the stereospecificity is not a functional trait which alters during divergent evolution or that the enzymes being compared are unrelated.[24,39]

The dehydroquinases are one of the clearest recent examples of enzymes that catalyze reactions with opposite stereospecificities. The type I and type II dehydroquinases catalyze a 1,4-elimination reaction with dehydroquinate in a *syn* and *anti* fashion, respectively.[48] Analysis of the predicted amino acid sequences of these type I and II dehydroquinases have shown they are unrelated and have arisen by convergent evolution.[49] Thus these enzymes represent examples where the observation of stereochemical diversity is an indicator of diversity in evolutionary origin. Mohrig and co-workers have presented direct evidence that the *syn*−*anti* dichotomy observed for the entire class of hydratase−dehydratase enzymes is based on a historical contigency rather than a mechanistic imperative.[50]

Enoyl thioester reductases represent an unusual case where both a functional and a historical model must be invoked to interpret the observed stereospecificities. In all cases examined to date the solvent hydrogen is added to the α-carbon.[18,19,22−23] This observation is consistent with a mechanistic imperative associated with the polarization of the α,*β*-double bond. With the inclusion of the *S. collinus* crotonyl CoA reductase, five of the eight stereochemical outcomes possible for enoyl CoA thioester reductases have now been observed (Table 1). It is now clear that the nucleotide stereospecificity does not determine the stereospecificity of hydrogen addition at the *β*-carbon and suggests that eventually examples of all eight possible stereochemical outcomes may be observed for these enzymes. In the case of the dehydroquinases, a difference in the stereochemical outcome correlates with a difference in evolutionary origin. In an analogous fashion, the five different stereospecificities of the enoyl thioester reductases may correlate with a minimum of five different evolutionary origins (i.e., the enzymes that exhibit different stereospecificities are not related, while those exhibiting the same stereospecificities may or may not be related). Alternatively, all enoyl thioester reductases may have the same evolutionary origin, so long as the stereospecificity can alter during divergent evolution. The phenomenal diversity observed in both the predicted amino acid sequence and putative evolutionary origin of enoyl thioester reductases presents clear evidence contrary to the latter of these proposals.[26−33] Evidence consistent with the former of these proposals, however, is provided by examination of the stereospecificity and amino acid sequence of CCR.[30] The CCR has a different stereospecificity with other enoyl thioester reductases where the overall stereospecificity is known (Table 1). Analysis of the amino acid sequence of this CCR with these enoyl thioester reductases, where the sequence is known, reveals no obvious similarity (some minor sequence similarity centered around the putative NAD(P)H binding domain has been noted).[30] In fact the CCR appears to be most closely related to some alcohol dehydrogenase members of the quinone oxidoreductase superfamily. Thus crotonyl CoA reductase, like dehydroquinase, is an example where stereospecific diversity correlates with diversity in evolutionary origin. As described below, this observation can be extended for other enoyl thioester reductases.

The enoyl thioester (ACP) reductases involved in fatty acid biosynthesis exhibit different stereospecificities (Table 1). The *E. coli* enoyl ACP reductase involved in *de novo* synthesis exhibits different stereospecificities to the eukaryotic enoyl ACP reductases. Consistent with this observation, the predicted amino acid sequence data of *fabI* (the gene that putatively encodes this enzyme) shows similarity to some alcohol dehydrogenases but not with other enoyl ACP reductase.[29] In contrast, an 83% identity has been observed for the enoyl ACP reductase sites in the chicken and rat fatty acid synthases (FAS).[5] Overall these two synthases exhibit 67% identity.[5] Consistent with this observation the chicken and rat FAS enoyl ACP reductases exhibit the same stereospecificity (Table 1). The yeast and *Brevibacterium ammoniagenes* have recently been shown to have significant sequence homology (30% and 46% identity along the entire sequence for FAS1 and FAS2, respectively).[25] This observation would predict that the same stereochemical outcome would be observed for the reaction catalyzed by these enzymes. The initial stereochemical evaluation of the enoyl ACP reductase catalyzed step in *B. ammoniagenes* reported[17] that the reaction proceeded with a *syn* addition of the solvent hydrogen to the *Re* face of the α-carbon (differing to the *syn* addition observed for the yeast FAS) and is inconsistent with this prediction. It is significant that a reevaluation of this reduction has suggested that the reaction proceeds with an *anti* addition of the solvent hydrogen to the *Re* face of the α-carbon.[20] Consistent with our predictions, the same stereochemical course are now reported for the yeast and *B. ammoniagenes* enzymes (Table 1). Also consistent with our predictions is the lack of any clear amino acid sequence similarity between the yeast/*B. ammoniagenes* and the chicken/rat FAS enoyl ACP reductases which catalyze reactions with different stereochemical outcomes. It has even been suggested that the yeast and chicken FAS diverged from a common evolutionary pathway at a very early time or were obtained via a different evolutionary pathway.[5] These data do indicate, albeit from a small data set, that the origin of the diversity in stereospecificity in the fatty acid biosynthetic enoyl ACP reductases can now be attributed to either highly diverged genes or different ancestral genes.[24]

The final enoyl thioester reductase for which both the complete stereochemical course of reaction and predicted amino acid sequence are known is the 1-cyclohexenylcarbonyl CoA reductase (ChcA) of *S. collinus*.[23,51] The stereospecificity of ChcA matches only the yeast FAS enoyl ACP reductase. Significantly, the amino acid sequence of ChcA shows no sequence similarity with other enoyl thioester reductases (in particular the *S. collinus* crotonyl CoA reductase or the enoyl ACP reductases involved in fatty acid biosynthesis) but rather with members of the short-chain alcohol dehydrogenase superfamily.[51]

All of these observations are consistent with the suggestion that the observation of diversity in stereospecificity correlates with diversity in evolutionary origin for enoyl thioester reductases. Furthermore, the case of the yeast FAS enoyl ACP reductase and ChcA demonstrates that the observation of the same stereospecificity does not necessarily indicate that two enoyl thioester reductases are related. We therefore propose that conserved stereospecificity *may* indicate related enzymes, whereas diverse stereospecificity *must* indicate unrelated or highly diverged enzymes. As shown below, these proposed

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interpretations of stereochemical diversity can be used in a highly successful predictive manner with other enoyl thioester reductases where only one or two stereochemical details have been determined.

In *S. collinus* it has been shown that a pathway from shikimic acid to cyclohexanecarboxylic acid (CHC) likely proceeds with three separate enoyl thioester reductions, each proceeding in an *anti* fashion with addition of solvent hydrogen to the *Si* face of the α-carbon (this *in vivo* analysis did not allow the nucleotide specificity to be determined).[23,52,53] Based on this observation, it has been suggested that one enoyl thioester reductase is responsible for catalyzing all three of these steps.[52,53] Recent enzymatic and genetic analysis of ChcA has proven consistent with this prediction.[51]

Two enoyl thioester reduction steps have also been implicated for the conversion of shikimic acid to dihydroxycyclohexanecarboxylic acid (DHCHC), used as a starter unit in the biosynthesis of ascomycin and rapamycin in various strains of *Streptomyces hygroscopicus*.[54] Analysis from *in vivo* incorporation experiments with the ascomycin producer indicated that the second of these reductions steps occurs in an *anti* fashion, while the first likely occurs in a *syn* fashion (this *in vivo* analysis also did not allow the nucleotide specificity to be determined).[54] Two unrelated enoyl thioester reductases would, therefore, be predicted to catalyze these two reactions. Recent analysis of the DNA sequence encoding the rapamycin biosynthetic genes suggests that the second enoyl thioester reduction is catalyzed by an enoyl thioester reductase that is part of a multifunctional enzyme (RAPS1) involved in rapamycin biosynthesis.[31] There is no information at this time concerning the enzyme involved in the first reduction. The location of a coenzyme A ligase adjacent to the enoyl thioester reductase in the rapamycin gene cluster[31] suggests, however, that the first reduction may not even occur at the level of the thioester.

The enoyl thioester reductions involved in the biosynthesis of CHC and the final step of the DHCHC pathway proceed with opposite absolute stereochemistry[52,54] and would be predicted to be catalyzed by unrelated enzymes. No amino acid sequence similarity of ChcA with the enoyl thioester reductase of RAPS1 is observed.

Finally, collected findings with fungal systems have shown that within an organism the enoyl thioester reductase reactions that occur in both fatty acid and polyketide biosynthesis proceed with opposite stereospecificities from the perspective of solvent hydrogen addition at the α-carbon.[12,14,15] These enzymes would, therefore, be predicted to have unrelated or highly divergent amino acid sequences. Although insufficient data are available at this time to test this prediction, some support is provided by the growing consensus that amino acid sequence identity is greater between primary and secondary metabolic enzymes if genes are compared between organisms, rather than in the same organism.[55] The one exception to the findings with fungal polyketide and fatty acid biosynthesis has been the biosynthesis of averufin and fatty acids in *Aspergillus parasiticus*, where the first three enoyl thioester reduction steps proceed with solvent hydrogen addition to the same face of the growing polyketide and fatty acid.[13] Again, the observation of the same stereospecificity may indicate a reaction catalyzed by related enoyl thioester reductases. In fact, the stereochemical observation has previously been used to argue that the first three addition reactions in the polyketide biosynthetic process are catalyzed by a fatty acid synthase rather than a polyketide synthase.[13,56] These predictions are supported by recent genetic analysis of the averufin polyketide synthase PKS gene cluster which reveals a FAS-like gene essential to the biosynthetic process.[57]

Conclusion

In summary, analysis of the available database of enoyl thioester reductases has demonstrated that different stereospecificities can be attributed to proteins that have unrelated amino acid sequences. The stereospecificity of an enoyl thioester reductase, with the exception of solvent addition to the α-carbon, is not a functional trait and is, therefore, reflective of the evolutionary origin of the protein. Furthermore, there is no strong evidence that stereospecificity can alter with divergent evolution. Conclusive evidence for these interpretations cannot be obtained. Rather, it is the accumulation of supporting data and the absence of any data to the contrary that lends credence to the interpretation of enoyl thioester stereospecificity presented herein. It would be interesting to determine in the light of recent published amino acid sequences whether a similar historical model, rather than the controversial functional model, can be used to interpret dehydrogenase stereospecificity[24,39−45]

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