# A Novel $\Delta^3,\Delta^2$-Enoyl-CoA Isomerase Involved in the Biosynthesis of the Cyclohexanecarboxylic Acid-Derived Moiety of the Polyketide Ansatrienin A[†,‡]

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ABSTRACT: The side chain of the antifungal polyketide ansatrienin A produced by *Streptomyces collinus* contains a cyclohexanecarboxylic acid (CHC) derived moiety. This CHC in the coenzyme A activated form (CHC-CoA) is derived from shikimic acid via a pathway in which the penultimate step is the isomerization of 2-cyclohexenylcarbonyl-CoA to 1-cyclohexenylcarbonyl-CoA. We have purified a 28 kDa 2-cyclohexenylcarbonyl-CoA isomerase (ChcB) from *S. collinus* and cloned and sequenced the corresponding *chcB* gene. The predicted amino acid sequence of ChcB showed moderate sequence identity to members of the hydratase/isomerase superfamily of enzymes. The recombinant ChcB was overexpressed in *Escherichia coli* and purified to homogeneity using metal chelate chromatography. Kinetic analysis demonstrated that recombinant ChcB had wide substrate specificity and could catalyze a double bond isomerization using 2-cyclohexenylcarbonyl-CoA ($K_m$ 116 ± 68 $\mu$M, $k_{cat}$ 3.7 ± 1.0 min$^{-1}$), *trans*-3-hexenyl-CoA ($K_m$ 39 ± 10 $\mu$M, $k_{cat}$ 12.8 ± 1 min$^{-1}$), and vinylacetyl-CoA ($K_m$ 156 ± 34 $\mu$M, $k_{cat}$ 29 ± 3 min$^{-1}$) as substrates. ChcB activity in cell extracts of *S. collinus* SP1, an insertionally disrupted *chcB* mutant, was shown to decrease by more than 99% (as compared to the wild-type strain) using all three of these substrates. The *S. collinus* SP1 strain, unlike the wild-type strain, could not produce $\omega$-cyclohexyl fatty acids but was still able to grow efficiently on methyl oleate as a sole carbon source. These observations demonstrate that the *S. collinus* ChcB is required for catalyzing the isomerization of 2-cyclohexenylcarbonyl-CoA to 1-cyclohexenylcarbonyl-CoA during CHC-CoA biosynthesis but not for degradation of unsaturated fatty acids. The *chcB* gene does not appear to be associated with the ansatrienin biosynthetic gene cluster, which has previously been shown to contain at least one gene known to be essential for CHC-CoA biosynthesis. This finding represents a notable exception to the general rule regarding the clustering of polyketide biosynthetic pathway genes.

*Streptomyces collinus* produces the polyketide antifungal antibiotic ansatrienin A (Figure 1) (*1*, *2*). Ansatrienin and the related trienomycins (*3*, *4*) are unique among polyketides in that they contain a side chain in which the cyclohexanecarboxylic acid (CHC)[1] moiety is linked via a D-alanine residue to the polyketide macrocycle. Fully saturated CHC-derived moieties are also observed in the polyketide products, such as asukamycin (*5*) and phoslactomycin (*6*), and in the $\omega$-cyclohexyl fatty acids (*7*) made by thermophilic and related bacteria. In all of these cases the CHC serves as a starter unit for the formation of the polyketide/fatty acid chain.

In *S. collinus* and *Alicyclobacillus acidcaldarius* it has been demonstrated that the CHC is derived from shikimic acid via a pathway involving a series of dehydration and double bond reduction steps (*7*, *8*) (Figure 1). 1-Cyclohexenylcarbonyl-CoA reductase (ChcA), the only enzyme in this pathway to have been studied, has been shown to catalyze in vitro at least two of the three $\alpha,\beta$-double bond reduction steps in this pathway (Figure 1) (*9*). This enzyme requires that its substrates be activated as coenzyme A thioesters, indicating that most intermediates in the CHC pathway are similarly activated (*9*).

The *chcA* gene of *S. collinus* has been cloned and sequenced (*9*) and shown to be located within the ansatrienin biosynthetic gene cluster (*10*). Insertional inactivation of *chcA* resulted in a mutant no longer able to produce cyclohexanecarboxylic acid (*9*). Recent sequence analysis of the DNA region adjacent to *chcA* has revealed open reading frames (ORFs) that have been assigned putative roles in the CHC pathway (*10*). Surprisingly, no obvious candidate gene for the enzyme catalyzing the penultimate reaction in the CHC pathway, isomerization of 2-cyclohexenylcarbonyl-CoA to 1-cyclohexenylcarbonyl-CoA, could be identified within the sequenced region of the ansatrienin biosynthetic gene cluster.

In the present paper we report the purification of a 2-cyclohexenylcarbonyl-CoA isomerase (ChcB) from *S. collinus*. The *chcB* gene has been cloned, sequenced, and used for heterologous expression of ChcB in *Escherichia coli*. The ChcB protein is able to catalyze isomerization using

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[‡] The nucleotide sequence of the *chcB* gene has been deposited in GenBank under Accession Number Af268489.

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[1] Abbreviations: CHC, cyclohexanecarboxylic acid; ChcA, 1-cyclohexenylcarbonyl-CoA reductase; ChcB, 2-cyclohexenylcarbonyl-CoA isomerase.

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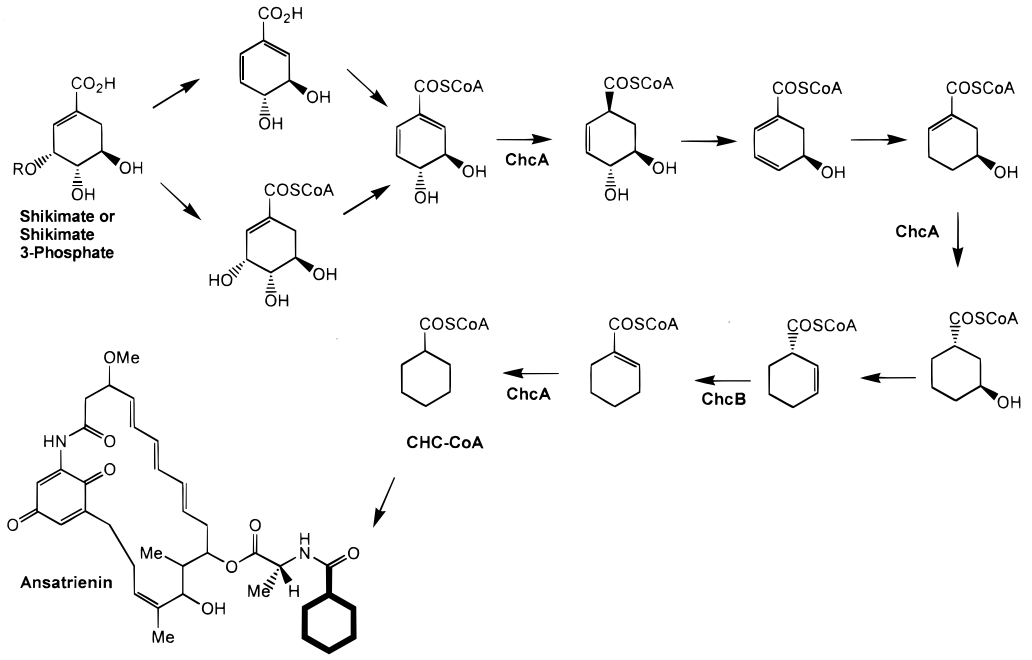


FIGURE 1: Roles of ChcA and ChcB in the biosynthesis of the cyclohexanecarboxylic acid (CHC) derived moiety of ansatrienin.

both cyclic and acyclic substrates. Inactivation of the *chcB* gene leads to a strain unable to produce $\omega$-cyclohexyl fatty acids, clearly establishing a role in the CHC pathway. The *chcB* gene is not clustered with the ansatrienin biosynthetic genes and appears to be present in a number of other streptomycetes not known to produce known CHC-derived products. This observation presents an unusual exception to the general rule that genes required for polyketide biosynthetic processes are clustered.

## MATERIALS AND METHODS

*Materials.* Coenzyme A was purchased from Sigma, and vinylacetic acid, *trans*-3-hexenoic acid, and the chemicals for the synthesis of 2-cyclohexenecarboxylic acid were purchased from Aldrich. [$\gamma$-$^{32}$P]ATP was obtained from Amersham. The 1-cyclohexenylcarbonyl-CoA reductase (ChcA) and crotonyl-CoA reductase (CCR) expression plasmids pPW4 and pZYB3 were described previously (*9*, *11*). *E. coli* TG2 and BL21(DE3)pLysS strains were obtained from Novagen. *E. coli* GM2163 strain and plasmid pKC1139 were provided by Pfizer and Eli Lilly, respectively. Restriction enzymes were obtained from New England Biolabs, Gibco, and Promega. *S. collinus* was provided by Professor Zeeck and Professor Zahner. *Streptomyces cinnamonensis* was obtained from Eli Lilly. *Streptomyces lividans* and *Streptomyces hygroscopicus* were obtained from ATCC.

*Substrate Synthesis.* 2-Cyclohexenecarboxylic acid was prepared from 3-bromocyclohexene as described previously (*12*, *13*). The coenzyme A thioesters of 2-cyclohexenecarboxylic acid, vinylacetic acid, and *trans*-3-hexenoic acid were prepared using the mixed anhydride method (*14*).

*ChcA and CCR Purification*. Recombinant CCR was expressed in *E. coli* BL21(DE3)pLysS/pZYB3 and was purified to homogeneity as described previously (*11*). Recombinant ChcA was expressed in *E. coli* BL21(DE3)-pLysS/pPW4 and was purified to homogeneity as described previously (*9*).

*ChcB Assays*. A typical spectrophotometric coupled ChcB assay solution contained 250 $\mu$g of 1-cyclohexenylcarbonyl-CoA reductase (ChcA), 10 $\mu$L of a 10 mM NADPH solution, and 10 $\mu$L of a 6.75 mM 2-cyclohexenylcarbonyl-CoA solution in 50 mM Tris-HCl buffer (pH 7.8). This solution was incubated for 15 min at room temperature during which time residual 1-cyclohexenylcarbonyl-CoA in the 2-cyclohexenylcarbonyl-CoA preparation was reduced by ChcA. This reduction was monitored by a decrease in absorbance at 340 nm, corresponding to the oxidation of the nicotinamide cofactor NADPH. At this point a ChcB protein solution was added, bringing the total assay volume to 1.0 mL. A spectrophotometric ChcA assay at an absorbance of 340 nm then allowed the ChcB catalyzed rate of 1-cyclohexenylcarbonyl-CoA production to be monitored.

Assays of ChcB activity with 10 $\mu$L of a 10 mM *trans*-3-hexenyl-CoA substrate solution instead of 2-cyclohexenylcarbonyl-CoA were also carried out using ChcA. ChcA has previously been demonstrated to be catalyze an $\alpha,\beta$-double bond reduction with *trans*-2-hexenyl-CoA (*15*). The assessment of ChcB activity with vinylacetyl-CoA (10 $\mu$L of a 9.75 mM solution) was carried out using crotonyl-CoA reductase (CCR) (114 $\mu$g) in place of ChcA. This enzyme uses NADPH to reduce crotonyl-CoA to butyryl-CoA (*11*).

For kinetic analysis, the 1 mL coupled assay was carried out with 157 $\mu$g of recombinant ChcB (rChcB), 250 $\mu$g of ChcA, and either 2-cyclohexenylcarbonyl-CoA (16.9−270 $\mu$M) or *trans*-3-hexenyl-CoA (6.25−100 $\mu$M). Kinetic analysis using vinylacetyl-CoA (24−390 $\mu$M) as a substrate was carried out with 157 $\mu$g of rChcB and 114 $\mu$g of CCR.

An HPLC assay was also used to moniter the ChcB-catalyzed conversion of 2-cyclohexenylcarbonyl-CoA to 1-cyclohexenylcarbonyl-CoA. The typical assay contained 10 $\mu$L of 6.75 mM 2-cyclohexenylcarbonyl-CoA, 50 $\mu$L of a ChcB solution, and 940 $\mu$L of 50 mM Tris-HCl buffer, pH 7.8. Aliquots were removed at 10, 20, and 30 min intervals, and the reaction was terminated by boiling for 5

min. A 50 $\mu$L sample was then analyzed using a 4.6 × 250 mm 5-$\mu$m C18 MetaPore column (Metachem Technologies) on a Beckman System Gold HPLC. The mobile phase consisted of 50 mM sodium phosphate (pH 5.8) and methanol (60:40) at a flow rate of 0.5 mL/min. The substrate and product were detected at an absorbance of 254 nm.

The HPLC assay was used to investigate the effect of pH on ChcB in a similar fashion using 50 mM sodium phosphate (pH 6.0), 50 mM Tris-HCl (pH 7.0−9.0), and 50 mM sodium borate (pH 10.0) as assay buffers. This assay was also used to investigate the effect of temperature on the ChcB activity and was carried out in the same manner in 50 mM Tris-HCl (pH 7.8) at 25, 37, 42, 48, and 55 °C. All ChcB assays were carried out at least in duplicate.

Nonlinear regression using Grafit 4.0 (Middlessex, U.K.) was used to determine $k_{cat}$ and $K_m$ values.

*ChcB Native Molecular Weight Determination.* The native molecular wieght of ChcB was estimated by gel exclusion chromatography using a Superdex-75 HR 10/30 column. The column was eluted with 50 mM Tris-HCl, pH 7.8, at 1.0 mL/min, and 0.5 mL fractions were collected. The column was calibrated using the following $M_r$ standards: blue dextran (2 000 000), bovine serum albumin (66 000), carbonic anhydrase (29 000), and cytochrome *c* (12 300).

*Determination of the Presence of ChcB Activity in Different Streptomycetes.* Seed cultures were prepared by transferring spore suspensions of *S. cinnamonensis*, *S. lividans*, and *S. hygroscopicus* to 500 mL Erlenmeyer flasks containing 100 mL of tryptic soy broth. These flasks were then incubated at 30 °C and 280 rpm for 48 h. A 50 mL sample of this fermentation was then used to inoculate 500 mL of tryptic soy broth in a 2.8 L flask. After a 24 h fermentation period the cells were harvested by centrifugation at 8000*g* for 30 min. The supernatant was discarded, and the cells were washed with buffer A, which contained 50 mM Tris-HCl (pH 7.8), 1 mM dithioerythritol (DTE), 1 mM EDTA, 0.1 mM PMSF, and 10% (v/v) glycerol. The cells were pelleted by centrifugation. The supernatant was discarded, and the wet cell paste was either frozen at −70 °C or used directly for the preparation of cell extracts. The cell pellet was suspended in buffer A (2:1 ratio of cells to buffer) and ruptured by passage through a high-pressure homogenizer (Avestin) at 12 000−15 000 psi. The resulting suspension was centrifuged at 30000*g* on a Beckman J2-21 centrifuge to yield a crude cell extract suitable for ChcB assays.

*Purification of ChcB.* A 758 mL cell extract generated from 12 L fermentation of *S. collinus* CD1075 was loaded onto a DEAE-Trisacryl column (5 × 12.5 cm) and washed with 2 column volumes of 50 mM Tris-HCl, pH 7.8 (buffer B). Proteins were eluted using a 600 mL linear gradient of 0−350 mM KCl in the same buffer. Fractions containing ChcB activity, which eluted between 100 and 200 mM KCl, were combined (222 mL) and diluted to 420 mL with buffer B. The resulting solution was loaded onto a green agarose column (1.5 × 8 cm). ChcB activity did not bind to this column, was collected in the wash, made up to 5% ammonium sulfate saturation, and subsequently loaded onto a phenyl-Sepharose CL-4B column (2.5 × 8 cm). The column was first washed with buffer B and then developed with a linear gradient from 50 mM Tris-HCl, pH 7.8, to 1 mM Tris-HCl, pH 7.8, over 150 mL. Fractions containing ChcB activity eluted between 25 mM Tris-HCl, pH 7.8, and 1 mM Tris-HCl, pH 7.8, and were combined (196 mL). This protein solution was applied to a Pharmacia Mono-Q HR 5/5 column that was equilibrated with buffer B. The column was washed with 2 column volumes of the same buffer and then developed using a 0−300 mM KCl gradient in buffer B, at a flow rate of 1.0 mL/min. ChcB active fractions eluted as a peak centered around 220 mM KCl and were combined (15 mL). The protein solution was then concentrated to approximately 3 mL using a Centriplus concentrator (Amicon), loaded onto a G-100 Sephadex column (2.5 × 100 cm), and eluted with buffer B. The resulting active fractions (14 mL) were pooled.

*N-Terminal Polypeptide Sequencing.* The purified ChcB protein solution was chromatographed on a 12.5% polyacrylamide−SDS gel using a solution containing 25 mM Tris−base, 192 mM glycine, and 0.4% SDS (pH 8.3) as a running buffer. The gel was wetted in 10 mM CAPS (pH 10.4) and then blotted onto a poly(vinylidene difluoride) membrane with a Hoefer SemiPhor transfer blotter (36 mA, 1 h). After blotting, the membrane was stained for 10 s with Coomassie and was destained slowly using 50 mL of acetic acid/50 mL of methanol/400 mL of $H_2O$. Two protein bands were cut from the membrane and used for N-terminal amino acid sequence analysis (Pfizer Inc.).

*Cloning and Sequencing of ChcB.* All recombinant DNA techniques, if not otherwise stated, followed standard methods (*16*). Using the known codon preference for *Streptomyces* (*17*), a degenerate probe 5′ GCS GAC ACS GTS CTS TAC GAG GTS TCS GAC 3′ based on the N-terminal sequence of ChcB was designed and synthesized (redundancies are defined as follows: S = G + C). This probed was end labeled using [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase and purified using a NucTrap purification column (Stratagene, La Jolla, CA).

A *S. collinus* cosmid library was screened (*18*) for clones that hybridized (55 °C) to the *chcB* probe. Membranes were washed once with 200 mL of 4× SSC (0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 35 °C for 30 min and once with 200 mL of 4× SSC and 0.1% SDS at 50 °C for 30 min. Cosmid clone 21D6 which positively hybridized to the probe was digested with *Pst*I, and the resulting DNA fragments were separated on a 0.6% agarose gel. The DNA was transferred, and a 4.5 kb fragment containing *chcB* was identified by Southern hybridzation using the ChcB probe. DNA fragments between 4 and 7 kb were gel extracted and cloned into the *Pst*I site of pUC119. The resulting constructs were used to transform *E. coli* TG2, and a colony which harbored a plasmid pSP3 (containing a portion of *chcB*) was detected by colony hybridization. The *chcB* gene was subsequently sequenced using both pSP3 and cosmid DNA from the 21D6 cosmid clone as templates. DNA sequencing was carried out on a Model 377 ABI Prism sequencer at Iowa State University, Core Facility. The BLAST family of programs was used to compare nucleotide and deduced amino acid sequence against the public sequence databases (*19*, *20*).

*Expression of ChcB by E. coli.* PCR was used to amplify the *chcB* gene from the 21D6 cosmid clone. The rightward primer 5′-CGTCACCAGCCGCTGGATCCGG-3′ was designed to introduce a *Bam*HI restriction site (underlined) centered 9 bases upstream of the 5′ end of *chcB*. The leftward

primer 5′-CGCGTTGATGCAAGCTTGCGG-3′ was designed to introduce a *Hin*dIII restriction site (underlined) centered 77 bases downstream of the 3′ end of *chcB*. The resulting 922 bp PCR product was purified, digested with *Bam*HI and *Hin*dIII, and cloned into the *Bam*HI and *Hin*dIII sites of pET30a. The resulting plasmid pSP7 was used to transform *E. coli* TG2. Subsequently, pSP7 was isolated from *E. coli* TG2 and was used to transform *E. coli* BL21(DE3)-pLysS. A single colony that harbored pSP7 was obtained and used for induction and detection of ChcB activity. This colony was used to inoculate a 1 mL overnight culture of LB with 50 $\mu$g/mL kanamycin. Half of the culture (500 $\mu$L) was used to make a glycerol stock of *E. coli* BL21(DE3)-pLysS/pSP7, and the remaining was used to inoculate 50 mL of LB containing 50 $\mu$g/mL kanamycin. When the cells reached an optical density at 595 nm of approximately 0.6, they were induced by addition of IPTG (isopropyl $\beta$-D-thiogalactopyranoside) to a final concentration of 1.0 mM and grown for an additional 3 h (*21*).

*Purification of rChcB.* After 3 h, the cells were lysed and a Ni−agarose column (4 × 1 cm) was used to purify the N-terminal His-tag-recombinant ChcB (rChcB) following standard protocols. The resulting protein solution was precipitated with ammonium sulfate, resuspended in 2 mL of buffer B, and further purified using a Superdex-75 gel exclusion column.

*Insertional Inactivation of chcB in S. collinus.* PCR was used to amplify an internal fragment of *chcB* ORF, using the cosmid clone 21D6 as template. The rightward primer 5′-CTCGTCCTCCTTCTCAAGCTTCTCCGCGAGCG-3′ was designed to introduce a *Hin*dIII restriction site (underlined). The leftward primer 5′-ATCGGGCTTCTAGACCAGGAC-CGGGAGACCG-3′ was designed to introduce an *Xba*I restriction site. The 506 bp PCR product was purified, digested with *Hin*dIII and *Xba*I, and cloned into the *Hin*dIII and *Xba*I restriction sites of pKC1139 to give pSP13. This construct was subsequently used to transform *E. coli* TG2, reisolated, and used to transform *E. coli* GM2163. The pSP13 isolated from transformed *E. coli* GM2163 was used to transform *S. collinus* following standard procedures (*22*). *S. collinus*/pSP13 colonies were selected by growth at 30 °C on R2YE plates, supplemented 12 h after protoplast transformation with 50 $\mu$g/mL apramycin sulfate. The *S. collinus* SP1 strain was generated by growing *S. collinus*/pSP13 on the same solid media at 39 °C. The vector pKC1139 contains both an apramycin resistance gene and a temperature-sensitive origin of replication that, when grown at temperatures above 37 °C, prohibits replication of the plasmid (*23*). Apramycin-resistant colonies obtained at 39 °C can only be retained by homologous recombination of pSP13 onto the chromosome. Integration of pSP13 into the chromosome of *S. collinus* SP1 was confirmed by hybridization analysis. Total DNA from *S. collinus* SP1 and *S. collinus*/pSP13, along with pSP13 and pKC1139 DNA, were digested with *Pst*I and separated on an agarose gel. The DNA was transferred to a Nytran membrane. The membrane was then probed overnight at 68 °C with radiolabeled pKC1139 (this probe was generated using the Prime-It random primer kit of Stratagene, La Jolla).

*Phenotype of the S. collinus SP1.* Fatty acid profiles of the *S. collinus* wild-type strain and SP1 strain were collected using standard protocols (*24*). Growth on methyl oleate as a sole carbon source was evaluated by inoculating spore suspensions (100 $\mu$L) of each strain into 10 mL of media containing methyl oleate (0.5 g/L), $K_2HPO_4$ (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), and $(NH_4)_2SO_4$ (2 g/L) at pH 7.0. Cells were grown for 48 h at 39 °C and 280 rpm. The rates of growth of both cells were evaluated visually. The fermentation broth for the *S. collinus* SP1 mutant was acidified with 6 N hydrochloric acid and extracted with hexane. The hexane layers were combined, reduced in volume (approximately 1.0 mL), and treated with diazomethane. Excess diazomethane was removed, and the sample was analyzed by GC−MS for the presence of methyl *cis*-dodec-3-enoate and other fatty acid degradation intermediates.

## RESULTS

*Assay Development for Analysis of ChcB Activity.* Two different ChcB assays were developed. The direct conversion of 2-cyclohexenylcarbonyl-CoA to 1-cyclohexenylcarbonyl-CoA could be readily followed by HPLC using conditions where baseline resolution of the substrate (16.1 min) and product (18.7 min) was accomplished (Figure 2). A coupled spectrophotometric ChcB assay was also developed in which the 1-cyclohexenylcarbonyl-CoA product was reduced to cyclohexylcabonyl-CoA by the NADPH-dependent ChcA. In this assay the small quantity (less than 4%) of contaminating 1-cyclohexenylcarbonyl-CoA in the 2-cyclohexenylcarbonyl-CoA was removed by preincubating the substrate with ChcA and NADPH. In all coupled assays the second enzyme was present in suitable excess such that under all conditions the ChcB-catalyzed step was rate limiting.

*Purification of 2-Cyclohexenylcarbonyl-CoA Isomerase (ChcB).* Cell extracts of *S. collinus* CD1075 (*9*), a strain in which a region of the *chcA* gene has been replaced with the *ermE* resistance marker, were used to purify ChcB. This strain has no detectable ChcA activity, enabling both the HPLC and spectrophotometric coupled ChcB assays to be used at all steps during the purification process. Five chromatographic steps (Table 1) produced a protein sample shown by SDS−PAGE analysis to contain two major proteins, 45 and 29 kDa. SDS−PAGE analyses and ChcB assays of individual fractions from the last two chromatographic steps indicated that ChcB was the smaller 29 kDa protein. The ChcB activity was reversibly inhibited by ammonium sulfate, potassium chloride, and imidazole. The Mono-Q purification step consistently resulted in significant and irreversible loss of activity but proved to be essential for the purification of ChcB. A range of alternative purification schemes in which this step was eliminated or replaced were all shown to be less efficient.

The N-terminal peptide sequence of the 45 kDa protein did not demonstrate significant sequence identity to any proteins within the databases. The N-terminal peptide sequence (Ala-Asp-Thr-Val-Leu-Tyr-Glu-Val-Ser-Asp-Gly-Leu-Ala-Thr-Iso-Thr-Leu-Asn-Arg-Pro) for the 29 kDa ChcB protein exhibited 66% amino acid sequence identity to a probable enoyl-CoA hydratase from *Rhodobacter capsulatus* as well to a dihydroxynaphthoate synthase from *Bacillus subtilis* (57% identity) (*25*).

*Characterization of ChcB.* A ChcB $K_m$ of 100 ± 56 $\mu$M was obtained for the substrate 2-cyclohexenylcarbonyl-CoA.

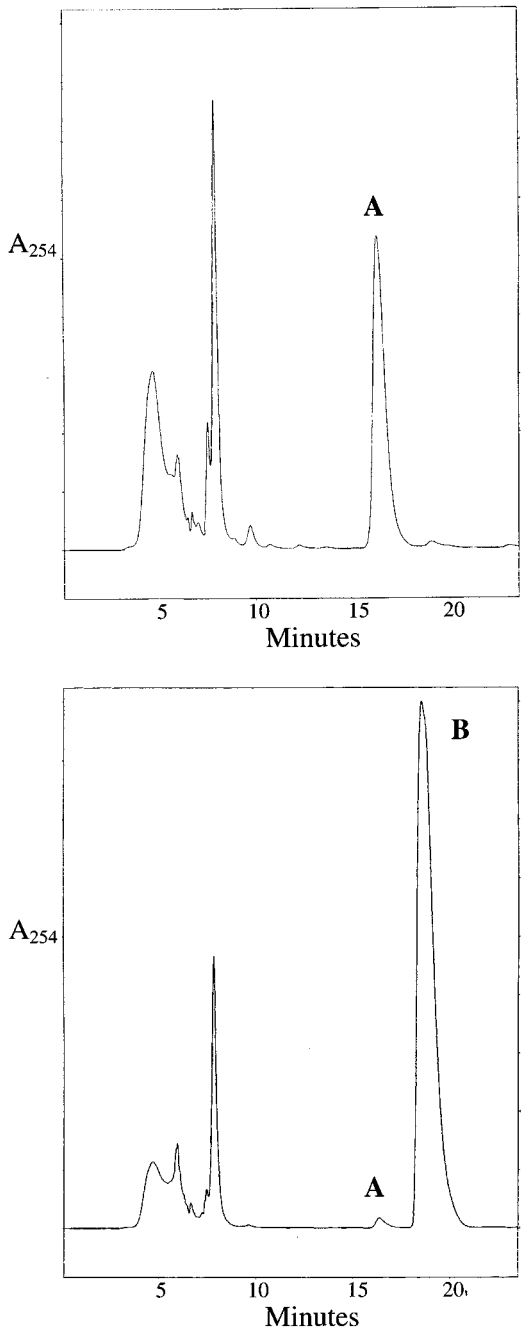


FIGURE 2: HPLC analysis of 1-cyclohexenylcarbonyl-CoA (A) and 2-cyclohexenylcarbonyl-CoA (B). The 2-cyclohexenylcarbonyl-CoA substrate (bottom panel) contains trace levels of 1-cyclohexenylcarbonyl-CoA.

The pH dependence of the ChcB between pH 6.0 and pH 10.0 was also analyzed using an HPLC assay. ChcB was active throughout the entire range and displayed a bell-shaped curve with an optimum around 8.0 (Figure 3). The temperature dependence of the enzyme between 25 and 55 °C was also analyzed using an HPLC. ChcB was active throughout the entire range and displayed an increase in activity in the temperature range of 25−40 °C. The ChcB activity dramatically decreased at higher temperature, most likely due to a thermal denaturation process (Figure 4). Control experiments carried out in the absence of ChcB demonstrated that the substrate was stable under both the pH and temperature conditions used in these analyses.

Table 1: Purification Table for 2-Cyclohexenylcarbonyl-CoA Isomerase Purified from *S. collinus*

| chromatographic step | total protein (mg) | total activity (milliunits)[a] | specific activity (milliunits/$\mu$g)[a] | purification factor |
|---|---|---|---|---|
| crude | 1 970 800 | 553 | $2.8 \times 10^{-4}$ | |
| DEAE-Trisacryl | 1 021 200 | 321 | $3.0 \times 10^{-4}$ | 1.1 |
| green agarose | 714 000 | 630 | $8.8 \times 10^{-4}$ | 3.0 |
| phenyl-Sepharose | 74 480 | 225 | $3.0 \times 10^{-3}$ | 10 |
| Mono-Q | 6 250 | 10 | $1.6 \times 10^{-3}$ | 5.7 |
| G-100 | 1 400 | 4.7 | $3.4 \times 10^{-3}$ | 12.1 |

[a] One unit of enzyme activity is defined as the production of 1 $\mu$mol of 1-cyclohexenylcarbonyl-CoA/min. All assays were conducted in 50 mM Tris-HCl buffer, pH 7.8, at 25 °C.

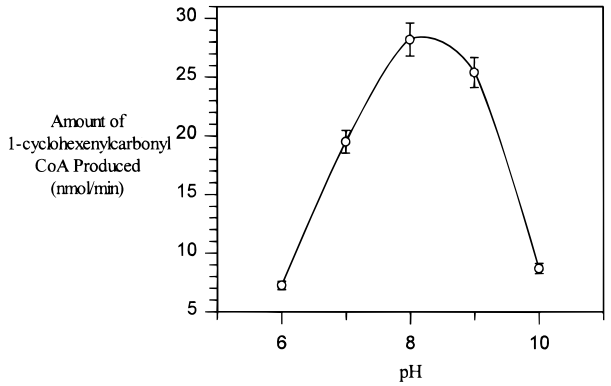


FIGURE 3: Effect of pH on the activity of 2-cyclohexenylcarbonyl-CoA isomerase.

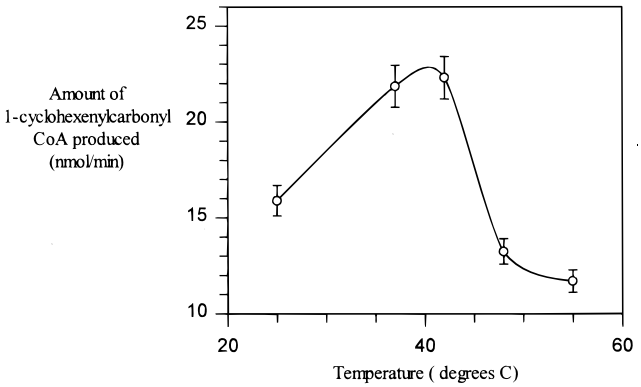


FIGURE 4: Effect of temperature on the activity of 2-cyclohexenylcarbonyl-CoA isomerase.

*Cloning of the S. collinus chcB Gene Encoding 2-Cyclohexenylcarbonyl-CoA Isomerase.* An oligonucleotide probe based on a portion of the N-terminal peptide sequence (Ala-Asp-Thr-Val-Leu-Tyr-Glu-Val-Ser-Asp) of ChcB was used to screen a cosmid library of *Xba*I-digested *S. collinus* DNA. A cosmid clone, 21D6, that hybridized to this *chcB* probe was identified and was digested with *Pst*I. A 4 kb *Pst*I fragment of *S. collinus* DNA was identified by Southern hybridization using the *chcB* probe and was subsequently cloned into the *Pst*I site of pUC119 to give pSP3. The majority of the *chcB* gene with the exception of the 3′ end was contained on pSP3. Using pSP3 and 21D6 as templates the entire 813 bp *chcB* gene was sequenced. The *chcB* open reading frame which exhibited the established patterns of GC bias and preferred codon usage for *Streptomyces* (*17*) encodes a 270 amino acid protein with a predicted molecular

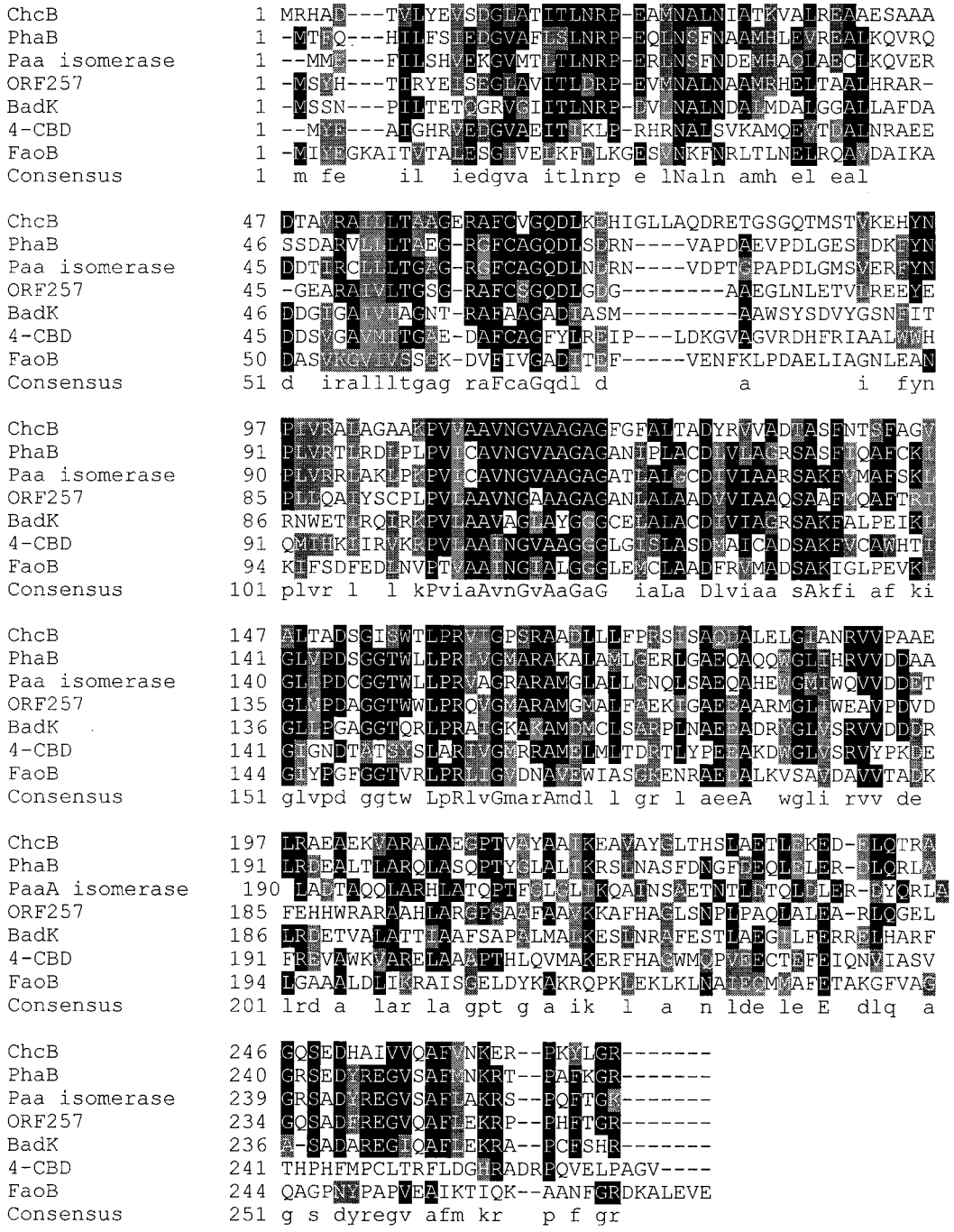

FIGURE 5: Alignment of 2-cyclohexenylcarbonyl-CoA isomerase (ChcB) to members of the hydratase/isomerase superfamily. PhaB is a phenylacetic acid catabolic enzyme from *P. putida* (*26*); Paa isomerase is a putative enoyl-CoA isomerase involved in phenylacetaldehyde degradation in *E. coli* (*27*); ORF257, a hypothetical protein from *R. capsulatus*, has high homology with the N-terminus of FadB; BadK is a *Rhodopseudomonas palustris* 1-cyclohexenylcarbonyl-CoA hydratase involved in benzoate degradation (*51*); 4-CBD is a 4-chlorobenzene dehydrogenase from *Pseudomonas* sp. CBS-3 (*52*); and FaoB is the N-terminus of the α subunit of the *P. fragi* fatty acid oxidation complex (*32*). Identical residues present in at least four of the sequences are framed in black. Conservative exchanges are framed in gray.

mass of 28 201 Da. The predicted and experimentally determined N-terminal sequences of ChcB match exactly. The deduced amino acid sequence of ChcB was compared to sequences available in public databases and had 43% identity to the *Pseudomonas putida* PhaB (a putative enoyl-CoA hydratase in the phenylacetic acid catabolic pathway) (*26*), 45% identity to the probable enoyl-CoA hydratase from *R. capsulatus* (*25*), and 42% identity to the putative PaaA from *E. coli* (a putative enoyl-CoA isomerase involved in 2-phenylethylamine catabolism (*27*)) (Figure 5). Many of the proteins that provided BLAST scores that were statistically significant were members of the hydratase/isomerase superfamily of enzymes (Figure 5).

Sequencing at both the 5′ and 3′ ends of *chcB* revealed open reading frames with homology to DNA−3-methyladenine glycosidase and glycolate oxidase, respectively. Southern hybridization analysis indicated that the cosmid clone containing *chcB* did not contain a polyketide synthase gene, nor overlap with previously identified cosmid clones which contain the ansatrienin biosynthetic genes (*10*).

*Presence of ChcB Activity in Various Streptomycetes.* The genetic evidence that indicated that *chcB* is not located with

Table 2: 2-Cyclohexenylcarbonyl-CoA Isomerase Activity in Different Streptomycete Extracts

| organism | activity relative to *S. collinus* CD1075[a] |
|---|---|
| *S. collinus* CD1075 | 1.0 |
| *S. cinnamonensis* | 2.1 |
| *S. lividans* | 1.9 |
| *S. hygroscopicus* | 2.1 |

[a] Cultures were harvested after 24 h of incubation in tryptic soy broth and assayed for ChcB activity using 2-cyclohexenylcarbonyl-CoA as a substrate.

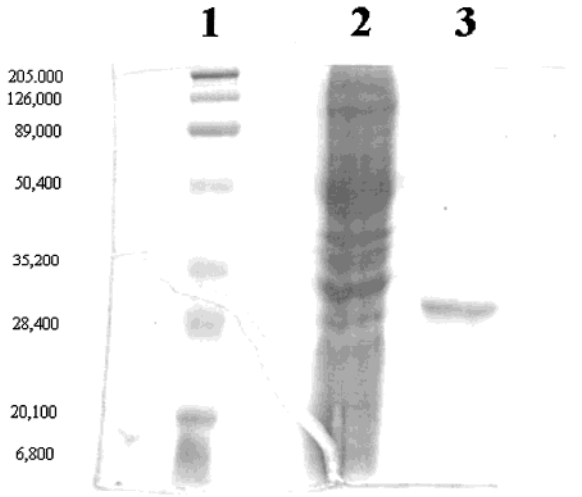


FIGURE 6: SDS−PAGE analysis of 2-cyclohexenylcarbonyl-CoA isomerase (ChcB) purified from *E. coli* BL21(DE3)pLysS/pSP7. Lanes: 1, broad-range molecular weight markers; 2, crude *E. coli* BL21(DE3)pLysS/pSP7 pool; 3, purified rChcB.

the ansatrienin biosynthetic genes suggested that it may not be specific to *S. collinus*. Assays with cell-free extracts from a number of streptomycetes indicated the presence of ChcB activity in all cases tried (Table 2). In contrast, ChcA activity can only be found in the cell extracts of the *S. collinus* wild-type strain. Clear hybridization between the *chcB* probe and chromosomal DNA from the various streptomycetes provided preliminary evidence that the observed ChcB activity in streptomycetes is a result of the presence of *chcB*.

*Expression and Purification of ChcB.* The ChcB was expressed with an N-terminal $His_6$ tag in *E. coli* using pET30a. The recombinant ChcB (rChcB) protein was purified in two steps using nickel−agarose affinity and a gel filtration chromatography and migrated on SDS−PAGE with an apparent molecular mass of ~33 kDa, consistent with the molecular weight of ChcB with the accompanying N-terminal histidine tag sequence provided by pET30a (Figure 6). A $64 \pm 6$ kDa mass obtained for the rChcB using gel exclusion chromatography (Superdex-75) indicated that the protein is homodimeric. A similar conclusion was drawn using this same analysis with the native ChcB.

Using the appropriate coupled enzyme assays the rChcB was shown to be able to catalyze an enoyl-CoA isomerization with both cyclic (2-cyclohexenylcarbonyl-CoA) and acyclic substrates (*trans*-3-hexenylcarbonyl-CoA and vinylacetyl-CoA). Steady-state $k_{cat}$ and $K_m$ values for each substrate were obtained (Figure 7, Table 3), and the $k_{cat}/K_m$ values indicated the following descending order of reactivity: *trans*-3-hexenoyl-CoA, vinylacetyl-CoA, and 2-cyclohexenoyl-CoA.

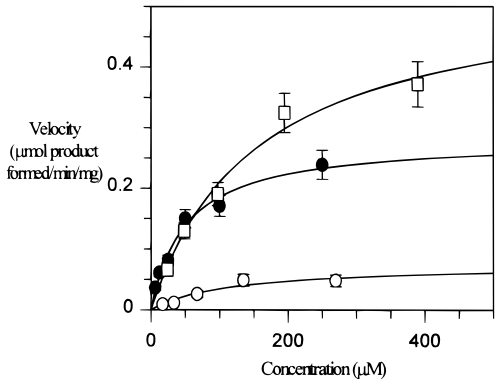


FIGURE 7: Kinetic analysis of the recombinant 2-cyclohexenylcarbonyl-CoA isomerase using 2-cyclohexenylcarbonyl-CoA (○), *trans*-3-hexenylcarbonyl-CoA (●), and vinylacetyl-CoA (□) as substrates.

Table 3: Calculated Kinetic Values for Recombinant 2-Cyclohexenylcarbonyl-Isomerase for Vinylacetyl-CoA, 3-*trans*-Hexenylcarbonyl-CoA, and 2-Cyclohexenylcarbonyl-CoA

| substrate | $K_m$ ($\mu$M) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ ($M^{-1}/min^{-1}$) |
|---|---|---|---|
| *trans*-3-hexenyl-CoA | $39 \pm 10$ | $12.8 \pm 1$ | $3.2 \times 10^5$ |
| vinylacetyl-CoA | $156 \pm 34$ | $29 \pm 3$ | $1.8 \times 10^5$ |
| 2-cyclohexenylcarbonyl-CoA | $116 \pm 68$ | $3.7 \pm 1.0$ | $3.2 \times 10^4$ |

The $k_{cat}$ value for the rChcB was at least 2 orders of magnitude higher than an estimated $k_{cat}$ for the native enzyme, most likely reflecting significant inactivation of the latter during the purification process. Supporting evidence for this hypothesis was provided by the observation that introduction of a Mono-Q purification step in the rChcB purification reduced specific activity by at least 99%.

*Disruption of the chcB Gene in S. collinus.* A PCR 500 bp internal fragment of *chcB* was cloned into pKC1139 to generate pSP13. This plasmid was shuttled through *E. coli* GM2163 and used to transform *S. collinus*. Growth of the resulting *S. collinus*/pSP13 at 39 °C on apramycin sulfate allowed the selection of *S. collinus* SP1 in which the integration of the pSP13 into the genome had resulted in the insertional inactivation of *chcB*. Insertion of pSP13 onto the chromosome was confirmed by Southern blot hybridization analysis of *Bam*HI-digested total DNA from the *S. collinus* wild-type strain, *S. collinus*/pSP13, and *S. collinus* using radiolabeled pKC1139 as a probe. A 7 kb hybridizing band was observed for *S. collinus*/pSP13 corresponding to the plasmid pSP13. Only a 20 kb (approximately) hybridizing band could be observed for the *S. collinus* SP1 strains (no band was observed for the wild-type *S. collinus*), indicating integration of pSP13 into the chromosome.

*Phenotype of the chcB Disrupted Strain.* Analysis of cell extracts of *S. collinus* SP1 showed a greater than 99% reduction in ChcB activity as compared to extracts of an *S. collinus* wild-type strain generated under similar conditions. This loss in activity was observed not only with 2-cyclohexenylcarbonyl-CoA as a substrate but also using *trans*-3-hexenyl-CoA and vinylacetyl-CoA. This observation clearly demonstrated that under the conditions used ChcB is the enzyme primarily responsible for the enoyl-CoA isomerization with all three of these substrates.

The presence of ChcB activity in a number of streptomycetes and the demonstrated ability of the enzyme to

preferentially catalyze isomerization using acyclic enoyl-CoA substrates were suggestive of a role in the degradation of unsaturated fatty acids. The *S. collinus* wild-type and SP1 strains were shown to grow in an efficient and identical manner in media containing methyl oleate as a sole carbon source, while analysis by GC−MS revealed no evidence of the buildup of any fatty acid degradation intermediates in the fermentation broth. These observations clearly demonstrated that catabolism of methyl oleate is not blocked by insertional inactivation of *chcB* in the *S. collinus* SP1.

Fatty acid profiles of the *S. collinus* SP1 grown at 30 °C exhibited no detectable levels of $\omega$-cyclohexyl fatty acids. No detectable levels of ChcB activity were observed in cell extracts of the SP1 strain grown under these conditions for 48 h. Trace levels of $\omega$-cyclohexyl fatty acid, amounting to as much as $1.0 \pm 0.3\%$ of total fatty acids, were consistently observed when the wild-type *S. collinus* was grown under similar conditions. Neither strain produced detectable levels of $\omega$-cyclohexyl fatty acids when grown at 39 °C.

## DISCUSSION

*ChcB, a Streptomycete $\Delta^3,\Delta^2$-Enoyl-CoA Isomerase with Broad Substrate Specificity.* The *S. collinus* ChcB protein is able to catalyze an enoyl-CoA isomerization with a range of different 3-enoyl-CoA substrates. The enzyme was able to utilize substrates with both *trans* double bonds (*trans*-3-hexenoyl-CoA) and *cis* double bonds (2-cyclohexenylcarbonyl-CoA) (the double bond in these substrates is numbered differently but has the same relationship to the CoA-activated carboxyl residue). The preferential use of acyclic 3-enoyl-CoA substrates over 2-cyclohexenylcarbonyl-CoA indicates that the latter is unlikely to be the physiological substrate and that CHC biosynthesis is not the primary role of the *S. collinus* ChcB. This hypothesis is supported by evidence that ChcB is present in streptomycetes which do not appear to have a CHC biosynthetic pathway.

The ChcB has significant isomerase activity. Using vinylacetyl-CoA as a substrate the $k_{cat}$ value of ChcB (30 $min^{-1}$) is the same order of magnitude as the $\Delta^3,\Delta^2$-enoyl-CoA isomerase activity found in the rat mitochondrial L-specific hydratase/isomerase dehydrogenase $\beta$-oxidation multifunctional enzyme (*28*). Significantly higher $k_{cat}$ (>7000 $min^{-1}$) values are observed for some monofunctional mitochondrial $\Delta^3,\Delta^2$-enoyl-CoA isomerases (*29*, *30*). It has been suggested that some of the differences in the $k_{cat}$ values for these various enzymes might represent substrate preferences imposed by the in vitro enzyme assays (*29*). The ChcB-catalyzed process may be even more efficient with different substrates which more closely resemble the physiological substrate.

$\Delta^3,\Delta^2$-Enoyl-CoA isomerases play an important role in the degradation of unsaturated fatty acids. In yeast there is one oleate-inducible monofunctional enzyme which is required for the degradation of unsaturated fatty acids (*29*). In mammals, which have numerous overlapping but distinct fatty acid oxidation processes, $\Delta^3,\Delta^2$-enoyl-CoA isomerases are found in both monofunctional and multifunctional enzymes (*31*). In contrast, bacteria such as *E. coli* and *Pseudomonas fragi* all have the $\Delta^3,\Delta^2$-enoyl-CoA isomerase activities primarily associated with the multifunctional fatty acid degradation enzyme (*30*, *32*, *33*). The ChcB represents a novel monofunctional bacterial $\Delta^3,\Delta^2$-enoyl-CoA isomerase. Insertional inactivation of the *chcB* gene did not affect the ability of the resulting *S. collinus* SP1 strain to grow on methyl oleate as a sole carbon source, clearly indicating the presence of an additional $\Delta^3,\Delta^2$-enoyl-CoA isomerase. Such an enzyme might be inducible by growth on methyl oleate or might simply be a multifunctional enzyme complex similar to those found in *E. coli* and *P. fragi*. Indeed, sequencing of the *Streptomyces coelicolor* genome (www.sanger.ac.uk) has recently found a gene encoding a 726 amino acid protein with significant sequence identity (33%) to the $\alpha$ subunit of the *P. fragi* fatty acid oxidation complex. In either case, the $\Delta^3,\Delta^2$-enoyl-CoA isomerase that plays a role in methyl oleate degradation must use *cis*-3-dodecenyl-CoA as a substrate. This enzyme does not appear to be able to use 2-cyclohexenylcarbonyl-CoA, vinylacetyl-CoA, or *trans*-2-hexenoyl-CoA significantly, as no $\Delta^3,\Delta^2$-enoyl-CoA isomerase activity was observed in these substrates in the cell extracts of *S. collinus* SP1.

Members of the 2-enoyl-CoA hydratase/isomerase superfamily, which include ChcB, have been shown to have a common active site which comprises a coenzyme A binding site, an expandable acyl binding pocket, and an oxyanion hole for binding/polarizing the thioester carbonyl (*34*). Strategic positioning of acidic and basic amino acid side chains in the active site then allows for proton shuffling (*34*). In the case of ChcB an amino acid residue acting as a base should abstract the $\alpha$ proton of the 3-enoyl-CoA substrate while a residue acting as an acid should donate a proton to the $\gamma$ carbon. Previous studies of ChcB in a cell extract of *S. collinus* have shown that a deuterium label at the $\alpha$ carbon of 2-cyclohexenylcarbonyl-CoA is transferred to the $\gamma$ carbon, indicating that the same amino acid residue catalyzes both steps of the process (*13*). In the case of the mitochondrial enoyl-CoA hyratase/isomerase mutagenic studies have indicated that the Glu164 side chain is responsible for abstracting the $\alpha$ proton of the 3-enoyl-CoA substrate (*30*). Similarly, the structure of the rat dienoyl-CoA isomerase has revealed that the position of the structurally equivalent Glu196 in the active site is consistent with a direct role in abstracting the $\alpha$ proton. A multiple sequence alignment of ChcB with these and other members of the hydratase/isomerase reveals several conserved glutamate and aspartate residues but none that align with either of these specific glutamate residues (*35*). Thus it remains to be determined which amino acid residue of ChcB is responsible for catalyzing the reaction.

*The chcB Gene, Located Outside of the Ansatrienin Biosynthetic Gene Cluster, Is Required for CHC Biosynthesis.* While the physiological role of the novel $\Delta^3,\Delta^2$-enoyl-CoA isomerase ChcB in streptomycetes remains to be determined, the lack of $\omega$-cyclohexyl fatty acid formation in the *S. collinus* SP1 strain indicates that the ChcB-catalyzed conversion of 2-cyclohexenylcarbonyl-CoA to 1-cyclohexenylcarbonyl-CoA is required for CHC biosynthesis. This finding is surprising because polyketide biosynthetic gene clusters invariably contain all of the genes required for the biosynthesis of polyketide-specific precursors. This has clearly been demonstrated for the genes required for biosynthesis of unusual starter units, such as the 3-amino-5-hydroxybenzoic acid (AHBA) that is used in the biosynthesis of ansatrienin, naphthomycin, rifamycin, and mitomycin C (*10*, *36*, *37*). The same has been made for genes involved in making the range

of different sugars that attached to both type I and type II polyketide aglycons (*38*−*40*), although there are examples of exceptions to this rule (*41*, *42*).

In the current example, ChcB is required for production of the unusual CHC-CoA precursor used in ansatrienin biosynthesis, yet the corresponding gene is not contained within the corresponding biosynthetic gene cluster. Furthermore, evidence indicates that ChcB is present in other streptomycetes, indicating that its primary role is other than CHC biosynthesis. Similar observations have been drawn for the streptomycete crotonyl-CoA reductase (CCR), which is important for generating butyryl-CoA for monensin biosynthesis but whose primary metabolic role remains to be determined (*43*). Butyryl-CoA, unlike CHC-CoA, is an intermediate in primary metabolism. Even with this difference it appears that numerous gene clusters involved in production of polyketides which utilize the butyryl-CoA precursor contain *ccr* homologues (*44*, *45*).

It remains to be determined how many other cases there are where primary metabolic enzymes are essential for catalyzing a step in the biosynthesis of a polyketide-specific precursor. We have recently demonstrated that only five genes from the ansatrienin biosynthetic gene cluster are required for introducing a functional CHC-CoA biosynthetic pathway into other streptomycetes (*46*). As this pathway involves a minimum of at least nine catalytic steps from shikimic acid, it is possible that primary metabolic enzymes in addition to ChcB may be involved. Primary metabolic enzymes conceivably might also catalyze steps in the conversion of shikimic acid to the dihydroxycarboxylic acid precursor used for rapamycin, FK506, and ascomycin biosynthesis (*47*, *48*). No candidate genes for the first steps in this process have been identified from analysis of the corresponding biosynthetic gene clusters (*49*, *50*).

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