A *Streptomyces collinus* Thiolase with Novel Acetyl-CoA:Acyl Carrier Protein Transacylase Activity[†,‡]

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ABSTRACT: Acetyl-CoA:acyl carrier protein (ACP) transacylase (ACT) activity has been demonstrated for the 3-ketoacyl-ACP synthase III (KASIII) which initiates fatty acid biosynthesis in the type II dissociable fatty acid synthases of plants and bacteria. Several lines of evidence have indicated the possibility of ACT activity being associated with proteins other than KASIII. Using a crude extract of *Streptomyces collinus*, we have resolved from KASIII an additional protein with ACT activity and subsequently purified it 85-fold in five chromatographic steps. The 45 kDa protein was shown by gel filtration to have a molecular mass of 185 ± 35 kDa, consistent with a homotetrameric structure for the native enzyme. The corresponding gene (*fadA*) was cloned and sequenced and shown to encode a protein with amino acid sequence homology to type II thiolases. The *fadA* was expressed in *Escherichia coli*, and the resulting recombinant FadA enzyme purified by metal chelate chromatography was shown to have both ACT and thiolase activities. Kinetic studies revealed that in an ACT assay FadA had a substrate specificity for a two-carbon acetyl-CoA substrate (K_m 8.7 ± 1.4 μM) but was able to use ACPs from both type II fatty acid and polyketide synthases (*Streptomyces glaucescens* FabC ACP, K_m 10.7 ± 1.4 μM; *E. coli* FabC ACP, K_m 8.8 ± 2 μM; FrenN ACP, K_m 44 ± 12 μM). In the thiolase assay kinetic analyses revealed similar K_m values for binding of substrates acetoacetyl-CoA (K_m 9.8 ± 0.8 μM) and CoA (K_m 10.9 ± 1.8 μM). A Cys92Ser mutant of FadA possesed virtually unchanged K_m values for acetoacetyl-CoA and CoA but had a greater than 99% decrease in k_{cat} for the thiolase activity. No detectable ACT activity was observed for the Cys92Ser mutant, demonstrating that both activities are associated with FadA and likely involve formation of the same covalent acetyl-*S*-Cys enzyme intermediate. An ACT activity with ACP has not previously been observed for thiolases and in the case of the *S. collinus* FadA is significantly lower (k_{cat} 3 min^{-1}) than the thiolase activity of FadA (k_{cat} 2170 min^{-1}). The ACT activity of FadA is comparable to the KAS activity and significantly higher than the ACT activity, reported for a streptomycete KASIII.

Enzymes possessing acetyl-CoA:ACP transacylase (ACT)[1] activity have long been suggested to play a role in both initiation of bacterial aromatic polyketide biosynthesis and plant and bacterial fatty acid biosynthesis (all processes catalyzed by type II dissociable synthases) (*1*−*3*).

An enzyme with ACT activity was initially purified to apparent homogeneity from *Escherichia coli* (*1*). Subsequent work revealed that such activity is associated as a minor component of 3-ketoacyl-ACP synthase III (KASIII), which appears to be the major enzyme responsible for initiation of fatty acid biosynthesis in a type II fatty acid synthase (*4, 5*). These KASIII enzymes which catalyze the condensation of acetyl-CoA or other starter units with malonyl-ACP have ACT activity which is typically 1−10% of the 3-ketoacyl-ACP synthase (KAS) activity. This observation led to a suggestion that the enzyme with ACT activity purified from *E. coli* may have been KASIII (*5*). This finding has not precluded the possibility that other *E. coli* enzymes besides KASIII may possess ACT activity. A similar possibility has been raised for plants. The developmental age of barley leaves has been shown to give rise to a change in the ratio of ACT/KASIII activities (*6*), and an avocado enzyme with ACT activity has been well resolved from KASIII (but not purified to homogeneity) (*2*). This latter observation provides the first clear evidence that these two activities are associated with two discrete enzymes in higher plants.

A similar suggestion of an ACT separate from KASIII has been made for streptomycetes. In these organisms the KASIII has broad substrate specificity (*4*) and uses acetyl-CoA, isobutyryl-CoA, and methylbutyryl-CoA to catalyze the formation of both straight-chain and branched-chain fatty acids (*7*−*9*). In both *Streptomyces collinus* and *Streptomyces glaucescens* addition of nonlethal doses of thiolactomycin, an antibiotic which is known to inhibit KASIII in vitro, has been shown to consistently lead to dramatic increases in the amount of straight-chain fatty acids relative to branched-chain fatty acids (*4, 10*). Although the reasons for this change remain obscure, one possible interpretation has been the

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[‡] The nucleotide sequence of *fadA* has been deposited in GenBank under the accession number AF411598.

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[1] Abbreviations: ACP, acyl carrier protein; FabC, a fatty acid synthase ACP; FadA, thiolase; ACT, acetyl-CoA:ACP transacylase; KASIII, 3-ketoacyl-ACP synthase III.

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presence of an acetyl-CoA-specific, TLM-insensitive ACT which continues to function when the KASIII is inhibited (*10*).

In streptomycetes and other actinomycetes an ACT activity was also thought to be responsible for initiation of aromatic polyketide biosynthesis. Such an activity was suggested to be a component of the so-called minimal polyketide synthase (PKS) (*11*). More recent studies with reconstituted actinorhodin and tetracenomycin PKSs have shown that these systems do not have ACT activity and generate the acetyl-ACP starter unit by decarboxylation of malonyl ACP (*12*, *13*). The possibility of a more direct in vivo ACT-catalyzed route to acetyl-ACP or 3-ketoacyl-ACP has yet to be explored (*14*).

Several different lines of evidence thus indicate that enzymes other than KASIII may contain ACT activity and the possibility that such activities might play a role in important biological processes. Nonetheless, no such enzyme has been purified and characterized. In this paper we describe the identification and resolution of two separate ACT activities from a *S. collinus* cell extract. Purification of the major ACT activity resulted in the isolation of a type II thiolase (FadA), rather than KASIII. The *fadA* gene has been cloned and sequenced and used to express a recombinant FadA in *E. coli*. Characterization of this enzyme has shown that it has both thiolase and ACT activities.

MATERIALS AND METHODS

Materials. *E. coli* acyl carrier protein (ACP), coenzyme A (CoA), acetoacetyl-CoA, Sephadex G-100, Sephacryl S-200, DEAE-cellulose, octyl-Sepharose CL-4B, tryptic soya broth, and Q-Sepharose were obtained from Sigma Chemical Co. (St. Louis, MO). The *E. coli* ACP (60% pure, Sigma) was purifed by anion-exchange chromatography prior to use. [γ-^{32}P]ATP, radiolabeled [^{14}C]acetyl-CoA, and isobutyric acid were obtained from ICN Pharmaceuticals, Inc. [^{14}C]-Butyryl-CoA was obtained from Moravek Biochemicals. Thiolactomycin was provided by Pfizer Inc. Tryptone and yeast extract were obtained from Difco Laboratories (Detroit, MI). All other chemicals were of reagent grade or better and were obtained from VWR Scientific (Bridgeport, CT) or Fisher Scientific (Pittsburgh, PA).

E. coli TG2 and BL21(DE3) pLysS strains were obtained from Novagen. Restriction enzymes were obtained from New England Biolabs, Gibco, and Promega. *S. collinus* Tü 1892 was provided by Professor Zeeck and Professor Zahner. FrnN ACP was prepared as decribed previously (*15*).

Acetyl-CoA:ACP Transacylase Assays (ACT). A standard assay consisted of 10 μM [1-^{14}C]acetyl-CoA (40−60 mCi/mmol), 180 μM *E. coli* ACP, and 0.7 μg of enzyme in 50 mM potassium phosphate buffer in a final volume of 20 μL. The reaction mixture was incubated at 30 °C for 12 min. The reaction was terminated by addition of 150 μL of ice-cold 10% trichloroacetic acid (TCA). Two microliters of a 10 mg/mL solution of bovine serum albumin (BSA) was added as a carrier in the precipitation of proteins. Precipitation was completed by incubation on ice for 10 min, and denatured proteins were collected by centrifugation at 13 000 rpm for 2 min. The pellet was washed with 10% TCA, then resuspended in 200 μL of 2% SDS in 20 mM NaOH, and counted in 5 mL scintillation cocktail.

For kinetic analyses, a 40 μL assay in 50 mM potassium phosphate buffer (pH 7.2) was carried out with 0.7 and 1.2 μg of the purified native and recombinant FadA, respectively. Substrate ranges were as follows: *S. glaucescens* FabC (2−25 μM), *E. coli* FabC (30−540 μM for the 60% pure substrate and 2−11 μM for the purified substrate), *Streptomyces roseofulvus* FrnN (5−110 μM), and [1-^{14}C]acetyl-CoA (1−30 μM). Samples were removed at 1, 2, 4, 6, 8, 10, and 12 min and assayed for radioactive acetyl-ACP generation. Nonlinear regression using Grafit 4.0 (Middlessex, U.K.) was used to determine k_{cat} and K_m values for both the ACT and thiolase activities of FadA.

ACT Inhibition Studies. Inhibition of the ACT activity of the purified native FadA by thiolactomycin was carried out as described previously for KASIII (*4*) with the exception that a 5 min preincubation period was used. Assays were carried out for 3 min in a final volume of 25 μL in 50 mM potassium phosphate buffer (pH 7.2) containing 0.2 nmol of [1-^{14}C]acetyl-CoA, and 6.6 nmol of ACP as described above. Similar methods were used to measure the inhibition of the ACT activity of FadA by iodoacetamide (using both a 5 min and no preincubation period) and unlabeled acetoacetyl-CoA (0.2−1 nmol and a 2 min preincubation period).

Acetoacetyl-CoA Thiolase Assays (FadA). A typical UV spectrophotometric assay consisted of 20 μM acetoacetyl-CoA, 20 μM CoA, and 0.2 μg of enzyme in 50 mM potassium phosphate buffer, pH 7.2, in a final volume of 1 mL. Acetoacetyl-CoA thiolase activity in the direction of thiolytic cleavage was monitored by a decrease in the absorbance at 303 nm, due to the loss of the acetoacetyl-CoA enolate chromophore. Thiolase activity was also probed using dithioerythritol and *E. coli* ACP in place of CoA. This assay was also used to investigate the effect of temperature on the thiolase activity of FadA at 15, 23, 32, 45, 50, 60, 70, and 80 °C. The stability of the substrates at these temperatures was assayed by incubating the substrates alone for 15 min at each temperature. The substrates were cooled to room temperature and used in a standard FadA assay. The stability of the enzyme at higher temperatures was assayed in a similar manner.

For kinetic analyses, a 1 mL assay in 50 mM potassium phosphate buffer (pH 7.2) was carried out with 0.2 and 0.24 μg of the purified native and recombinant FadA, respectively. Substrate ranges were as follows: acetoacetyl-CoA (1−50 μM), CoA (1−50 μM), and DTE (50 μM−3 mM).

A microassay was used to determine FadA activity using ACP (676 μM) in place of CoA. This assay was carried out essentially in the same way as described above but in a smaller volume (100 μL) and with various amounts of FadA (0.7, 1.2, 3, 5, and 10 μg).

Culture Conditions for Growth of S. collinus for Purification of FadA. Spores from a glycerol spore suspension of *S. collinus* Tü 1892 stored at −20 °C were inoculated into three 2.8 L Fernback flasks, each containing 500 mL of tryptic soy broth, pH 7.3. Following incubation for 24 h at 30 °C and 250 rpm, this 1.5 L seed culture was transferred to a 20 L Microferm fermentor containing 12.5 L of the same media (14 L total). After incubation for 20 h with aeration at 30 °C, the cells were harvested by centrifuging at 8000*g* on a Sorvall RC 5B (Dupont) centrifuge. The supernatant was discarded, and the cell pellet was washed with buffer A,

Table 1: Primers Used in This Study

primer	sequence
*Nde*IforwardI	5′-GGGGATCGCACCATATGTCTTCTGGG-3′
*Hin*dIIIreverse	5′-ACCGGAACGAAAGCTTAGGTACCGGG-3′
*Nde*IforwardII	5′-ACGAAGGAGCGCCCATATGGCCGCCACTCA-3′
*Bam*HIreverse	5′-CTCCAGATCGGTGGATCCAGCGGCGCCACC-3′
Cys92forward	5′-CGAGGCCGGAGAGGGACACCTTG-3′
Cys92reverse	5′-CAAGGTGTCCCTCTCCGGCCTCG-3′

containing 50 mM potassium phosphate buffer, pH 7.2, and 10% (vol/vol) glycerol. The cells were washed with more buffer A, divided in two equal portions, and frozen at −70 °C. The frozen cell pellet (196 g) obtained from a 7 L culture was rapidly thawed and resuspended at 4 °C in 500 mL of buffer B containing 50 mM potassium phosphate buffer (pH 7.8), 3 mM dithioerythritol, 4 mg of pepstatin, 4 mg of leupeptin, and 10% glycerol. The resulting cell suspension was broken by passage twice through a high-pressure homogenizer (Avestin, Ottawa, Canada) at 15 000 lb/in.^{2}, with efficient cooling on ice between passages. The resulting suspension was centrifuged at 30000*g* on a Beckman J2-21 centrifuge to yield approximately a 600 mL of crude cell extract. The precipitate was washed again with 100 mL of buffer B and centrifuged. The supernatant from this wash step was combined with the original cell extract (700 mL total).

Purification of FadA. A 700 mL cell extract generated from a 7 L fermentation of *S. collinus* was loaded onto a Q-Sepharose column (30 × 5 cm) and washed with 2 column volumes of 50 mM potassium phosphate buffer, pH 7.8, containing 10% glycerol and 3 mM DTT (buffer C). Bound proteins were eluted first using a 1 L linear gradient (0−0.4 M KCl) followed by a second 1 L gradient (0.4−0.8 M KCl) in buffer C. Fractions containing ACT and thiolase activities, which eluted between approximately 350 and 450 mM KCl, were combined (130 mL). A 20−80% ammonium sulfate fractionation with centrifugation at 18 000 rpm for 30 min generated a protein precipiate, which was subsequently resuspended in buffer A (8.5 mL). The majority of this solution (6.5 mL) was frozen at −70 °C and the remaining 2 mL loaded onto a Sephacryl S-200 column (50 × 2.5 cm). This column was then developed using buffer D (buffer A without glycerol). Active fractions (150−200 kDa) were pooled (24 mL) and loaded on a Pharmacia Mono-Q HR 5/5 column. This column was washed with 2 column volumes of buffer C and then developed with a linear 0−250 mM KCl gradient in buffer C (0.5 mL/min). Active fractions eluted between 110 and 130 mM KCl and were combined (3.5 mL) and concentrated to 1 mL on a Microcon YM-10 concentrator. This concentrated protein solution was then fractionated on a Pharmacia FPLC Hi Prep 16/60 S-300 column using buffer D. Active fractions (150−200 kDa range) were detected and pooled (7 mL).

ACT Native Molecular Mass Determination. The native molecular mass of FadA was estimated by gel exclusion chromatography using a Pharmacia FPLC Hi Prep 16/60 S-300 column. The column was preequilibrated and developed with buffer D at a flow rate of 0.5 mL/min. The column was calibrated using the following M_r standards: blue dextran (2000 kDa), thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anyhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

N-Terminal Polypeptide Sequencing. The purified protein (15 μL of a 0.138 mg/mL solution) was chromatographed on a 12.5% SDS−PAGE gel, blotted onto poly(vinylidene difluoride) membrane, and N-terminally sequenced at Pfizer Inc. (Groton, CT).

Sequencing of fadA. All recombinant DNA techniques, if not otherwise stated, followed standard methods. Using the known codon preference for *Streptomyces* (*16*) a best guess probe, 5′-TTCTCCGGCGCCGACCTGGGCGGCTTCGCCATCAAG-3′, and a degenerate probe, 5′-TTCTCSGGCGCSGACCTSGGCGGCTTCGCSATCAAG-3′, based on the N-terminal sequence of FadA were designed and synthesized (redundancies are defined as follows: S = G + C). The probes were end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and purified using a NucTrap purification column (Stratagene, La Jolla, CA). A pDual3 (*S. collinus* cosmid DNA) library (*17*, *18*) was screened for clones that hybridized (65 °C, overnight) to the *fadA* 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 60 °C for 30 min. Cosmid clones 10A6, 9G12, 18D5, 16H4, 21F5, and 21A11 positively hybridized to both probes. Cosmid clone 21A11 was digested with *Sal*I, and the resulting DNA fragments were subjected to electrophoresis on a 0.7% agarose gel. A 2.5 kb fragment containing *fadA* was identified by Southern hybridization using both *fadA* probes (*19*). DNA fragments between 2 and 3 kb in size resulting from the *Sal*I digest of 21A11 cosmid DNA were gel extracted and cloned into the *Sal*I site of plasmid pGEM5Zf(+). The resulting constructs were used to transform *E. coli* TG2, and a colony that harbored a plasmid pSL1 with the 2.5 kb insert containing a portion of *fad*A was detected by colony hybridization.

Sequencing of *fadA* was then accomplished using pSL1 and 9G12 cosmid DNA with an Applied Biosystems, Inc., Model 377 sequencer at Iowa State University's DNA sequencing facility (Des Moines, IA). The BLAST family of programs (*20*−*22*) was used to compare nucleotide and deduced amino acid sequence against the public databases.

Expression of Recombinant FadA (rFadA) in E. coli. PCR was used to amplify the 1258 bp *fad*A gene from *S. collinus* chromosomal DNA. The rightward primer *Nde*IforwardI was designed to introduce an *Nde*I restriction site (underlined) at the ATG start codon while the leftward primer *Hin*dIIIreverse was designed to introduce a *Hin*dIII site (underlined) centered 27 bases downstream of the 3′-end of *fad*A (Table 1). The PCR product was cloned into the *Nde*I and *Hin*dIII sites of pET15b, and the resulting pSL2 plasmid was used to first transform *E. coli* TG2 and then *E. coli* BL21(DE3)-

pLysS. A single colony that harbored plasmid pSL2 was used to inoculate 10 mL of LB media. The LB media used for growth of *E. coli* BL21(DE3)pLysS transformed with pSL2 or other expression plasmids (pSL12, and pSL4, described below) contained 100 μg/mL each chloramphenicol and ampicillin. Cells were grown, induced with IPTG (isopropyl β-D-thiogalactopyranoside), and harvested following standard protocols. Cells obtained from 2 × 100 mL cultures of *E. coli* BL21(DE3)pLysS/pSL2 were then suspended in 6 mL of buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and lysozyme, at pH 8.0 and 4 °C. The cells were sonicated and then centrifuged at 15 000 rpm for 15 min. A Ni−NTA−agarose spin column was used to purify the N-terminal His-tagged *FadA* following standard protocols. The rFadA was further purified using a Sephacryl S-200 gel exclusion column.

Expression of the S. glaucescens fabC in E. coli. PCR was used to amplify the *fabC* gene which encodes the ACP from *S. glaucescens*, using the 4.5 kb *S. glaucescens* FAS DNA template on plasmid pWHM194 (*23*). The rightward primer *Nde*IforwardII was designed to introduce a *Nde*I restriction site (underlined) at the ATG start codon of *fabC*. The leftward primer *Bam*HIreverse was designed to introduce a *Bam*HI site (underlined) centered 46 bases downstream of the 3′-end of *fabC* (Table 1). The resulting 313 bp PCR product was purified, digested with *Nde*I and *BamH*I, and cloned into the corresponding sites of pET15b. The resulting plasmid was passed through *E. coli* TG2 and used to generate *E. coli* BL21(DE3)pLysS/pSL4. This transformed strain was then used to generate a 1 L culture in LB broth. When the cells grown at 37 °C reached an optical density at 595 nm of approximately 1, FabC expression was induced by addition of IPTG to a final concentration of 0.5 mM. The culture was incubated at room temperature overnight at 250 rpm. The cells were pelleted by centrifugation at 5000 rpm for 15 min and resuspended in the same volume of fresh LB media without IPTG. The culture was incubated at 37 °C for another 6−7 h (*14*, *24*). The cells were pelleted by centrifugation at 5000 rpm for 15 min and stored at −70 °C. Cells were lysed, and cell extract was generated following the method described for the pSL2 transformant. A Ni−NTA−agarose column (4 × 0.8 cm) was used to purify the N-terminal His-tagged FabC following standard protocols. DTT was then added to a final concentration of 5 mM, and the FabC (5 mg) was purified further using a Sephadex G-100 gel exclusion column and an octyl-Sepharose CL-4B column (4 × 0.8 cm). The latter column was used to remove trace contaminants of the apo-ACP from the purified holo-ACP (this column specifically bound the apoACP) (*25*). Buffer D containing 5 mM DTT was used in both chromatographic steps, and fractions containing ACP were determined by chromatography on a 20% native polyacrylamide gel. Purified ACP was concentrated using a Centriplus YM-3 centrifugal filter device. ACP concentrations were determined using the Lowry assay (*26*).

Site-Directed Mutagenesis of FadA and Expression of the FadA Mutant in E. coli. A four-primer site-directed mutagenesis system was used to create a Cys92Ser substitution within *fadA.* Amino acid substitution was obtained using two pairs of primers, *Nde*IforwardI−Cys92reverse and Cys92-forward−*Hin*dIIIreverse, to amplify two overlapping fragments from chromosomal DNA. The resulting two fragments were mixed and further amplified using *Nde*IforwardI and *Hin*dIIIreverse. The resulting PCR product contained the desired mutant of *fadA* and was cloned into the *Nde*I and *Hin*dIII sites of pET15b to give pSL12. This insert was sequenced to confirm that only the desired mutation was present. The FadA was subsequently expressed and purified from *E. coli* BL21(DE3)pLysS/pSL12 following the protocol described for the rFadA.

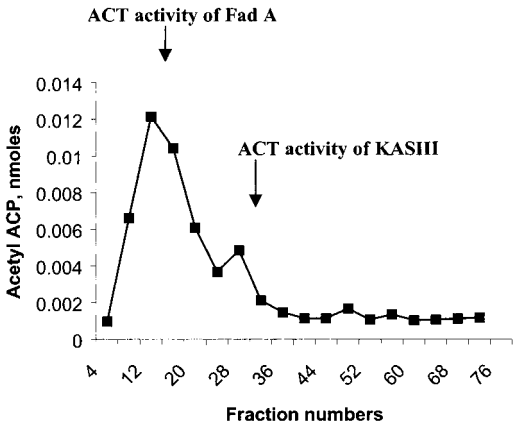

FIGURE 1: Gel exclusion chromatogram of an enzyme with a unique acetyl-CoA:ACP transacylase (ACT) activity separated from KASI-II. The ACT activities are calculated as the nanomoles of product acetyl-ACP formed per minute in a standard transacylase assay.

RESULTS

Gel Exclusion Chromatography Separates an Enzyme with ACT Activity from a KASIII. The possibility of multiple streptomycete enzymes possessing ACT activity was probed by fractionation of an *S. collinus* cell extract using gel exclusion chromatography. A standard ACT activity using radiolabeled acetyl-CoA and ACP consistently revealed two distinct peaks (Figure 1). KASIII assays using malonyl-ACP (as opposed to ACP) revealed significantly greater activity (as compared to ACT) with the second peak and no detectable activity with the first peak (data not shown). ACT assays were also carried out with [^{14}C]butyryl-CoA in place of [^{14}C]acetyl-CoA as a substrate. Again, only fractions assayed from the second ACT peak were observed to have butyryl-CoA:ACP transacylase activity, which had previously been reported for a *Streptomyces* KASIII (*4*). These observations and a predicted native molecular mass of 72 ± 3 kDa are all in agreement with the previously observed properties for a *Streptomyces* KASIII and indicated that the larger ACT activity peak, with a predicted native molecular mass of 185 ± 35 kDa, was that of a protein distinct from KASIII. A five-step chromatographic purification protocol was then developed to purify this enzyme to near homogeneity from a cell extract of *S. collinus* Tü 1892 (Table 2).

Purification of a Thiolase with Novel ACT Activity. A 14 L fermentation culture of *S. collinus* Tü 1892 cells was divided in two batches of 7 L each. One 7 L batch was processed for purification of the enzyme with ACT activity. Five chromatographic steps produced a protein sample shown by SDS−PAGE (Figure 2) to contain a single major band with a molecular mass of 45−48 kDa. After the third purification step (ammonium sulfate precipitation), only 2 mL of a total of the 8.5 mL protein solution generated was used in further purification steps. The N-terminal peptide

Table 2: Purification of FadA with both Thiolase and ACT Activities from *S. collinus* Extracts[a]

purification steps[b]	total protein (mg)	total thiolase activity (μmol/min)	% thiolase activity retained	specific activity for thiolase (μmol min^{-1} mg^{-1})	*x*-fold purification	total ACT activity (μmol/min)	% ACT activity retained	specific activity for ACT (μmol min^{-1} mg^{-1})	*x*-fold purification
crude extract[b]	1295	514		0.4		0.35		0.00027	
Q-Sepharose[b]	138	92	17.9	0.67	1.68	0.037	10.6	0.00027	1.00
$(NH_4)_2SO_4$ precipitation	88.4	43.2	8.4	0.49	1.23	0.036	10.1	0.0004	1.5
Sephacryl S-200	28.4	90	17.5	3.2	8	0.067	19.4	0.0024	9.0
FPLC Mono-Q	2.5	30.4	5.91	12.2	30.5	0.011	3	0.0042	15.8
FPLC S-300	1.0	35.1	6.83	36.6[b]	91.5	0.023	6.4	0.022[b]	83

[a] The values in the purification table were generated from the second 7 L batch of the total 14 L *S. collinus* culture. [b] Represents the purification steps where the total protein and total thiolase and ACT activities were adjusted accordingly (×2/8.5) to account for only 2 mL of the 8.5 mL protein solution obtained in the ammonium sulfate precipitation step used in further purification steps. The specific ACT and thiolase activities of FadA were calculated on the basis of the micromoles of products acetyl-ACP and acetyl-CoA formed in the respective assays.

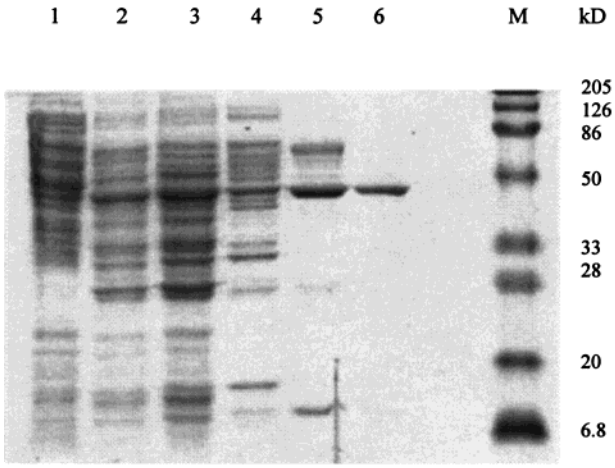

FIGURE 2: SDS−PAGE of pooled fractions from the purification steps of FadA from *S. collinus*. Lanes: 1, crude extract of *S. collinus*; 2, after Q-Sepharose fractionation; 3, after ammonium sulfate precipitation (20−80%);4, after Sephacryl S-200 fractionation; 5, after FPLC Mono-Q fractionation; 6, after FPLC Hi Prep 16/60 S-300 fractionation; M, broad-range molecular mass markers.

sequence analysis of the 45−48 kDa protein (Ser-Ser-Gly-Thr-Thr-Ser-Ser-Val-Iso-Val-Ala-Gly-Ala-Arg-Thr-Pro-Met-Gly-Arg-Leu-Leu-Gly-Ser-Leu-Lys-Ser-Phe-Ser-Gly-Ala-Asp-Leu-Gly-Gly-Phe-Ala-Iso-Lys-Ala-Ala-Leu) purified from the first 7 L batch showed significant (68%) sequence identity to an acetoacetyl-CoA thiolase (*fadA4*) from *Mycobacterium tuberculosis* (*27*) and other thiolases from different sources (*28*−*30*). A standard thiolase assay was then used to demonstrate such activity with acetoacetyl-CoA, CoA, and the purified native protein.

To confirm that the purified enzyme was indeed a thiolase (FadA) with ACT activity, a second (82−92-fold) purification using both assays was undertaken (Table 2). The same procedures and chromatographic steps in the initial purification of FadA were used and resulted in the isolation of almost 1 mg of protein from 25% of cells obtained from a 7 L *S. collinus* fermentation (Table 2). Thiolase and ACT activities overlapped in every enzyme purification step except for the first Q-Sepharose step. The ratio of thiolase to ACT activity at most of the remaining purification steps was constant (thiolase activity was approximately 1000-fold greater than the ACT activity), indicating that one enzyme is responsible for both activities. The activity ratio was 2-fold higher in protein samples obtained from the Q-Sepharose and Mono-Q steps. This increase is presumably due to the reversible inhibition of FadA by salts such as potassium chloride and ammonium sulfate. The 40 μL ACT assay requires much less dilution of the protein sample with buffer than the 1 mL thiolase assay and is thus more sensitive to salt inhibition.

Fractions containing thiolase activity but no ACT activity were observed only in the initial Q-Sepharose step, suggesting the existence of other thiolases in *S. collinus* with no ACT activity. The purified *S. collinus* FadA thus appears to be the only thiolase with novel ACT activity.

Cloning of the S. collinus fadA Gene Encoding an Enzyme with ACT and Thiolase Activities. Two oligonucleotide probes, based on a portion of the N-terminal peptide sequence (Phe-Ser-Gly-Ala-Asp-Leu-Gly-Gly-Phe-Ala-Iso-Lys) of FadA, were used to screen a cosmid library of *S. collinus* DNA in *E. coli* (*31*). Cosmid clone 21A11 identified in this manner was used to generate pSL1 containing a 2.5 kb *Sal*I *S. collinus* DNA fragment of *fad*A gene with the exception of 30 bps encoding the C-terminus. 9G12 cosmid clone DNA similarly identified was then used as a template to complete the sequencing and obtain the entire open reading frame (1200 bp) of *fadA*.

The *fadA* open reading frame, which established patterns of GC bias and preferred codon usage for *Streptomyces* (*16*, *32*), encodes a 399 amino acid protein with a predicted molecular mass of 44 400 Da, which compared well with the observed molecular mass of 45−47 kDa for the purified native FadA from *S. collinus*. The protein sequence predicted from the sequenced *fadA* gene matched exactly to the N-terminal protein sequence obtained from purified native FadA. The deduced amino acid sequence of *fadA* was shown to be highly homologous to both known and putative thiolases (Figure 3).

Expression and Purification of the Recombinant fadA. An *E. coli* expression plasmid pSL2 was constructed by cloning a 1237 bp PCR fragment containing *fadA* into *Nde*I and *Hin*dIII restriction sites of the pET15b vector, such that it was under the control of the inducible T7*lac* promoter. A recombinant N-terminal His-tagged FadA (rFadA) protein was obtained by induction of an *E. coli* BL21(DE3)pLysS/pSL2 culture using standard procedures. This protein was then purified approximately 35-fold to apparent homogeneity (Figure 4) and shown to have ACT and thiolase activities comparable to that of the native enzyme *S. collinus* FadA (see below).

Kinetic Analysis of the Purified Native and Recombinant FadA. A K_m of 4.3 ± 0.6 μM for acetyl-CoA, 488 ± 74 μM

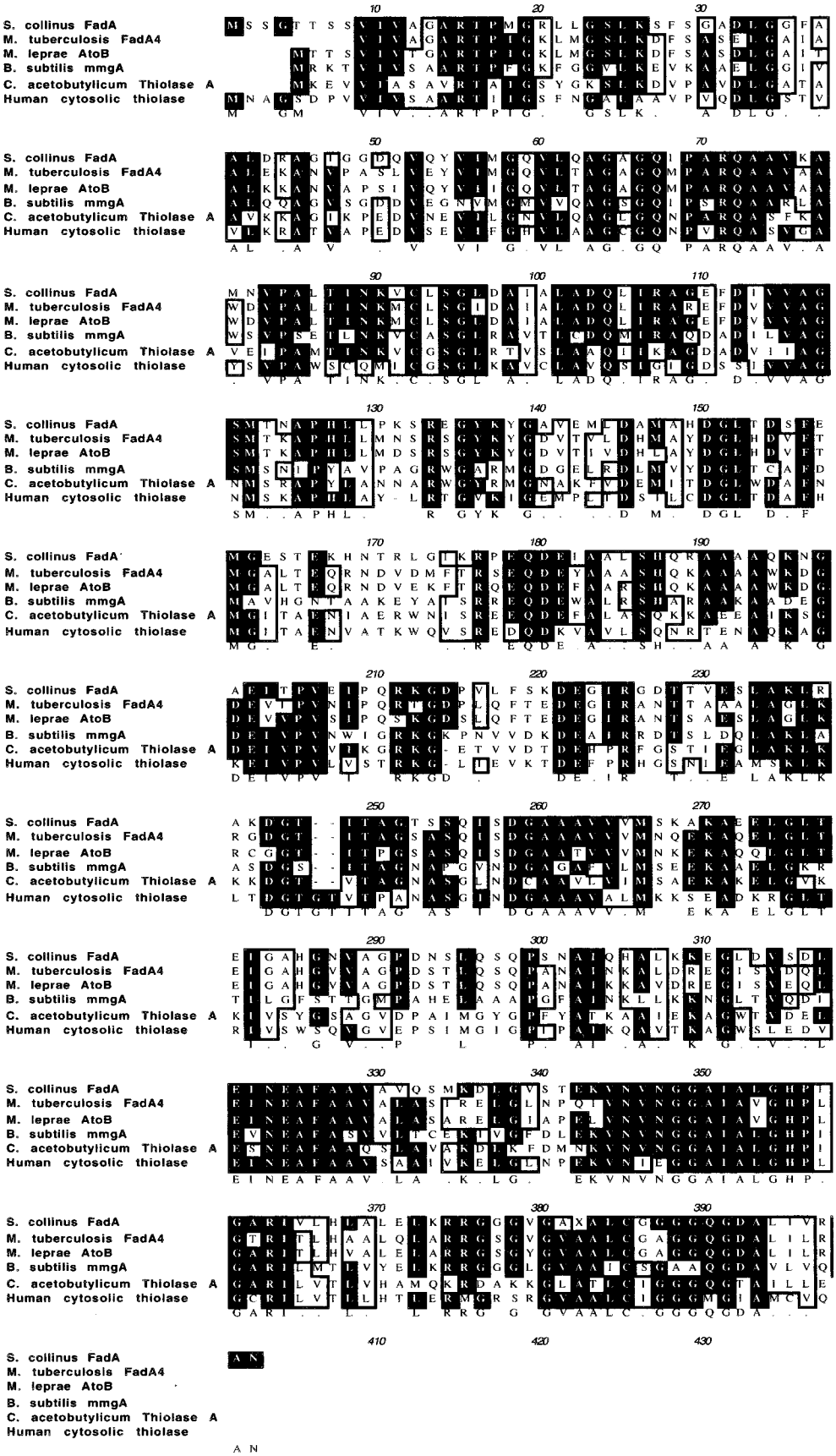

FIGURE 3: Multiple protein sequence alignment of the *S. collinus* acetoacetyl-CoA thiolase (FadA) with members of the acetoacetyl-CoA thiolase/β-ketoacyl-CoA thiolase superfamily. Sequence identity of 68% to a putative acetoacetyl-CoA thiolase (FadA4) from *M. tuberculosis* (strain H37RV) (*27*), 67% to a putative acetyl-CoA *C*-acetyltransferase (AtoB) from *M. leprae* (accession number P46707, EMBL/GenBank/DDBJ databases), 52% to a putative acetyl-CoA acetyltransferase (*mmgA*) from *B. subtilis* (*45*), 49% to an acetyl-CoA *C*-acetyltransferase (thiolase A) from *Clostridium acetobutylicum* (*30*), and 45% to a cytosolic acetyl-CoA acetyltransferase from humans (*46*) was obtained. Identical residues present in at least four of the six sequences are in white font framed on a black background. Conserved residues are framed in black.

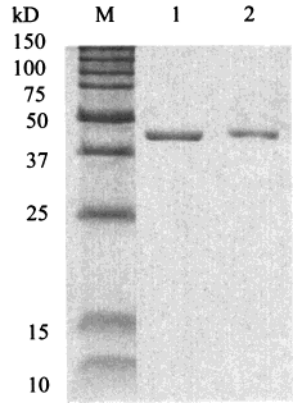

FIGURE 4: SDS−PAGE of the purified Cys92Ser mutant of FadA (lane 1) and rFadA (lane 2).

Table 3: Kinetic Parameters for ACT and Thiolase Activities of FadA Purified from *S. collinus* Tü 1892, the Recombinant His-Tagged *S. collinus* FadA (rFadA), and the His-Tagged Cys92Ser Mutant of FadA Purified from *E. coli* (BL21)pLysS[a]

enzyme	activity	substrate	K_m (μM)	V_{max} (units/mg of protein)[b]	k_{cat} (min^{-1})
FadA	ACT	acetyl-CoA	4.3 ± 0.6	0.064	3.05
		E. coli ACP (crude)	488 ± 74		
		FrnN ACP	44 ± 12		
	thiolase	acetoacetyl-CoA	3.8 ± 0.5	43.8	2170
		CoA	5.8 ± 0.6		
rFadA	ACT	acetyl-CoA	8.7 ± 1.4	0.028	1.35
		S. glaucescens FabC ACP	10.7 ± 1.4		
		E. coli ACP (purified)	8.8 ± 2.1		
	thiolase	acetoacetyl-CoA	9.8 ± 0.8	50.1	2404
		CoA	10.9 ± 1.8		
Cys92Ser	ACT	*S. glaucescens* FabC	ND[c]		
	thiolase	acetoacetyl-CoA	6.2 ± 1.9	0.1	4.64
		CoA	10.5 ± 3.1		

[a] The k_{cat} values for the thiolytic reaction of FadA reflects the generation of two molecules of product acetyl-CoA, as compared to only one molecule of product acetyl-ACP generated in an ACT reaction. [b] Activity is defined as the formation of 1 μmol of product acetyl-ACP/min in an ACT assay and as the formation of 1 μmol of product acetyl-CoA/min in a thiolase assay. [c] Not detectable.

for partially purified *E. coli* ACP (60% pure, Sigma), and 44 ± 12 μM for frenolicin ACP (FrnN) was determined for the ACT activity of the native *S. collinus* FadA (Table 3). No significant transacylase activity was observed with other acyl-CoA substrates such as butyryl-CoA and isobutyryl-CoA. FadA also did not exhibit any β-ketoacyl-ACP synthase III (KAS III) activity (KAS assay conditions were identical to that of the ACT assay, only the substrate ACP was substituted for malonyl-ACP). A K_m of 8.7 ± 1.4 μM for acetyl-CoA, 10.7 ± 1.4 μM for *S. glaucescens* ACP (FabC), and 8.8 ± 2 μM for purified (Sigma) *E. coli* ACP was obtained in an ACT assay using the rFadA. The K_m values obtained for the purified ACPs were thus similar, suggesting no significant ACP specificity in the ACT activity of FadA. The only significant difference was a higher K_m (488 ± 74 μM) obtained for 60% pure *E. coli* ACP (Sigma) with the native purified FadA, as compared to a much lower K_m (8.8 ± 2 μM) obtained for purified *E. coli* ACP (Sigma) with the recombinant FadA. This difference most likely could be attributed to the presence of impurity/impurities in the 60% pure *E. coli* ACP (Sigma) sample.

A K_m of 3.8 ± 0.5 μM for acetoacetyl-CoA and 5.8 ± 0.6 μM for CoA was obtained (Table 3) for the acetoacetyl-CoA thiolase activity of FadA. Steady-state kinetics were observed with the rFadA where a K_m of 9.8 ± 0.8 μM for acetoacetyl-CoA and 10.9 ± 1.8 μM for CoA was obtained for the thiolase activity. The thiolase activity (k_{cat} = 2170 min^{-1}) was significantly greater than the ACT activity (k_{cat} = 3 min^{-1}). The coenzyme A substrate in the thiolase assay could be replaced, albeit poorly, with other thiol-containing compounds such as dithiothreitol (K_m 1.51 mM) and *E. coli* ACP.

Native Molecular Mass Determination. The native molecular mass (M_r) of the purified native and recombinant FadA from *S. collinus* Tü 1892 was estimated by gel filtration chromatography on a FPLC Hi Prep 16/60 S-300 column and was found to be 185 ± 35 and 187 ± 20 kDa, respectively. SDS−PAGE analysis of the recombinant FadA (Figure 4) and native FadA (Figure 2) purified from *S. collinus* Tü 1892 revealed a protein band with a mobility that corresponded to an M_r of approximately 46−48 and 45−47 kDa, respectively. These data are consistent with a homotetrameric structure for *S. collinus* FadA.

Temperature Dependence. The temperature profile of FadA was analyzed by assaying thiolase activity between 15 and 60 °C. The thiolase activity increased in a linear fashion between 25 and 60 °C. The actual thiolase activity above 60 °C could not be assayed due to the apparent instability of substrates acetoacetyl-CoA and CoA at higher temperatures. These substrates, when incubated for 15 min at temperatures above 40 °C and analyzed in thiolase assays, were found to be highly unstable (data not shown). The FadA was comparatively more stable at high temperatures (53% activity retained after 15 min at 60 °C).

Inhibition Studies with the Type II FAS Inhibitor Thiolactomycin. Thioactomycin is a known inhibitor of the ketoacyl-ACP synthase III which initiates fatty acid biosynthesis in a type II FAS. Alterations in fatty acid profiles obtained by growing streptomycetes in the presence of thiolactomycin have raised the possibility of a KASIII-independent, ACT-catalyzed pathway for initiation of straight-chain fatty acid biosynthesis that is less sensitive to thiolactomycin. The IC_{50} for inhibition of FadA by thiolactomycin was 0.36 ± 0.04 mM, which is at least 10-fold less sensitive to inhibition by thiolactomycin compared to a *Streptomyces* KASIII (IC_{50} 20 μM) (*4*).

Evidence for the Involvement of the Same Covalent Acetyl-S-Cys$_{92}$ Intermediate in the ACT and Thiolase Reaction Mechanisms of FadA. FadA catalyzes the CoA-dependent thiolysis of its substrate acetoacetyl-CoA. The thiolase reaction mechanism involves the formation of an acetyl-*S*-Cys enzyme intermediate with a nucleophilic cysteine residue in the active site of FadA (*33*, *34*). The same intermediate could form in the transacylation (ACT) reaction of FadA (Figure 5). Iodoacetamide is known to form covalent bonds with nucleophilic cysteine residues and prevents formation of an acetyl-*S*-Cys intermediate (*35*). Accordingly, the ACT activity of FadA was inhibited more than 50% when the

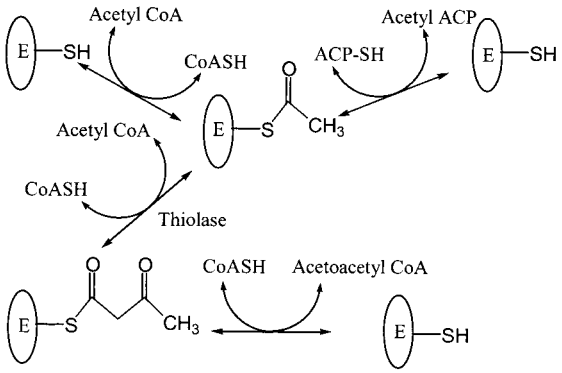

FIGURE 5: Proposed mechanisms for ACT and thiolase activities of the *S. collinus* FadA.

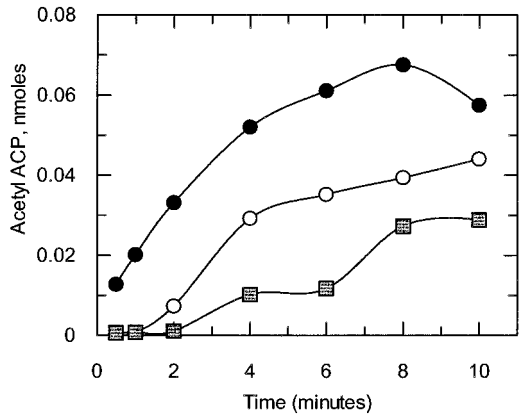

FIGURE 6: Decreased rate of FadA-catalyzed [1-^{14}C]acetyl-ACP formation from [1-^{14}C]acetyl-CoA by preincubation with acetoacetyl-CoA. The ACAT activity of FadA was calculated as the nanomoles of product [1-^{14}C]acetyl-ACP formed at various time points in the assay. Each assay consisted of a 2 min preincubation at 30 °C of FadA with 0 mM (closed circle), 25 mM (open circles), or 50 mM acetoacetyl-CoA (squares).

enzyme was preincubated (12 min) with 40 μM iodoacetamide. Higher iodoacetamide (80 μM) concentrations led to almost complete inhibition of ACT activity. The FadA substrate acetyl-CoA (20 μM) offered complete protection from inhibition for up to 100 μM iodocetamide. Iodoacetamide was also found to inhibit the thiolase activity of FadA in a similar fashion, suggesting that the same active site nucleophilic cysteine residue might be involved in both processes (Figure 5). Additional support for this hypothesis was provided by performing an ACT assay after preincubating FadA with 25 μM acetoacetyl-CoA. Under these conditions a decrease in the rate of radioactive acetyl-ACP product formation from [1-^{14}C]acetyl-CoA was observed (Figure 6). Addition of higher concentrations of acetoacetyl-CoA (50 μM) led to a further decrease in this rate, consistent with both substrates acetoacetyl-CoA and [1-^{14}C]acetyl-CoA, being used to generate the same acetyl-ACP product in the ACT assay, presumably via the common acetyl-*S*-Cys enzyme intermediate. In these competition experiments, preincubation with acetoacetyl-CoA generates an unlabeled acetyl-*S*-Cys enzyme intermediate which must be cleaved by ACP before the radioactive acetyl-CoA can be used, leading to the observed lag in [1-^{14}C]acetyl-ACP production (Figure 6).

Analysis of a multiple sequence alignment (Figure 3) of the *S. collinus* FadA with other known thiolases identified Cys_{92} as the residue likely involved in formation of this *S*-acetyl-enzyme intermediate. A Cys92Ser FadA mutant was prepared and purified to apparent homogeneity (Figure 4). An apparent K_m of 6.2 $\pm$ 1.9 μM for acetoacetyl-CoA and 10.5 $\pm$ 3.1 μM for CoA, obtained for the thiolase activity of the Cys92Ser mutant, was comparable to that of rFadA (Table 3). The k_{cat} value for this mutant, 4.64 min^{-1}, was 0.2% of that for the rFadA (2404 min^{-1}). A similar large decrease in k_{cat} values but not K_m values has been observed with a similar Cys89Ser mutant of the β-ketothiolase from *Zoogloea ramigera* (*34*). An effect on catalysis rather than substrate binding is consistent with the change from a cysteine sulfur nucleophile to a poorer serine oxygen nucleophile. No detectable ACT activity was observed with experiments with the Cys92Ser mutant. These observations unequivocally demonstrate that FadA is responsible for both ACT and thiolase activities and that an acetyl-*S*-Cys_{92} is likely an enzyme intermediate common to both processes.

Thiolase Activity of FadA Using ACP in Place of Coenzyme A. A common acetyl-*S*-Cys_{92} intermediate predicts that the thiolase activity of FadA should also be observed using ACP as a substrate. Thiolase activity of FadA with ACP as a substrate could be observed but was at least 3 orders of magnitude slower as compared to a standard thiolase assay using CoA (Table 3). Thiolase activity was only observed using significantly higher FadA and ACP concentrations.

DISCUSSION

Purification of the major ACT activity in this study produced a thiolase with strong amino acid sequence identity (45−68%) to the thiolase superfamily (*27*−*30*). Thiolases form a ubiquitous family of enzymes, found in both prokaryotes and eukaryotes, which catalyze the reversible thiolytic cleavage of 3-ketoacyl-CoA substrates into acyl-CoA and acetyl-CoA (*36*). Thiolases can be classified as being type I or type II on the basis of their substrate specificity and function. Thiolase-I (commonly referred to as the 3-ketoacyl-CoA thiolase) has broad chain length specificity, being able to cleave both acetoacetyl-CoA and also molecules with longer fatty acid tails (C_4 to C_{16}). This thiolase has been proven to be essential in the β-oxidation pathway for the degradation of fatty acids (*37*−*41*) and is often part of a multifunctional enzyme dedicated to this process. The *S. collinus* FadA shows the greatest amino acid sequence similarity to the type II (EC 2.3.1.9) or acetoacetyl-CoA thiolases, which are monofunctional and not part of the multifunctional enzyme (*39*). While these enzymes can cleave acetoacetyl-CoA, their main function is the synthesis of this compound from two molecules of acetyl-CoA, in a Claisen condensation reaction, and are thus important in several biosynthetic pathways. The *S. collinus* FadA also exhibits the acetyl-CoA/acetoacetyl-CoA substrate specificity associated with type II thiolases (*39*).

Analysis of the *S. coelicolor* genome (*S. coelicolor* sequencing project, www.sanger.ac.uk) revealed a homologue with 92% amino acid sequence identity to the *S. collinus* FadA. Putative assignments have also been made for six or more additional thiolases which exhibit 30−40% amino acid sequence identity with FadA. Biochemical evidence for multiple thiolases in streptomycetes was also provided by the first Q-Sepharose step of purification of FadA from *S.*

collinus cell extracts, where fractions with thiolase activities but no detectable ACT activities were identified. The role of these different thiolases in fatty acid degradation and other metabolic processes, such as synthesis of butyryl-CoA (*42*), remains to be determined. Interestingly, as many as six different thiolases have also been indicated from sequencing of the *M. tuberculosis* genome (*27*). A similar analysis of *E. coli* reveals only two thiolases.

A multiple sequence alignment (Figure 3) of the *S. collinus* FadA with other known thiolases revealed that Cys_{92} was homologous to the known active site cyteine in other thiolases (*33*, *34*). The alignment also revealed highly conserved residues centered not only around Cys_{92} but also around Cys_{385} and His_{355}. These conserved amino acid residues have been identified as critical for thiolase activity on the basis of X-ray crystallographic studies carried out on a 3-ketoacyl-CoA thiolase from *Saccharomyces cerevisiae* (*33*) and site-directed mutagenesis studies carried out on a 3-ketoacyl-CoA thiolase from *Z. ramigera* (*34*). A consensus sequence for the active site of prokaryotic and eukaryotic 3-ketoacyl-CoA thiolases has been proposed: Asn-Arg-aa-Cys-aa-Ser-aa-aa-Gln (where aa is an amino acid and Cys is the cysteine involved in forming the acyl enzyme intermediate) (*29*). The glutamine residue has been suggested to be related to the broad substrate specificity of 3-ketoacyl-CoA type I thiolases as this position is occupied by a basic residue (arginine or lysine) in type II acetoacetyl-CoA thiolases (*28*, *30*). This rule does not apply universally as this position in the type II *S. collinus* FadA, as well as the *M. tuberculosis* FadA4 and the *Mycobacterium leprae* AtoB, is occupied by an acidic aspartate residue (Figure 3).

While transacylase activity is a well-established property of thiolases, there have been no previous reports of such activity using ACP as the thiol group. Attempts to observe such activity with the *E. coli* FadA (*43*) using ACP were unsuccessful. The radioactive ACT assay used to detect acetyltransferase activity using the ACP substrate used in the current study is significantly more sensitive than standard thiolase assay. As the ACT activity of the *S. collinus* FadA is significantly less than the thiolase activity, it is possible that ACT activity in other thiolases could have been missed. On the other hand, it is possible that the *S. collinus* FadA is unusual in possessing significant ACT activity. This possibility is consistent with the observation of other thiolase activities in the first step of FadA purification (anion-exchange column), which did not display measurable amounts of ACT activity. Furthermore, all evidence to date indicates that the only detectable ACT activity in *E. coli* cell extracts is attributable to KASIII, despite the presence of at least two thiolases.

In the FadA-catalyzed thiolytic reaction, a molecule of acetoacetyl-CoA is converted into two molecules of acetyl-CoA (Figure 5). This reaction is reversible, and FadA can also catalyze the synthesis of acetoacetyl-CoA from two molecules of acetyl-CoA. The crystal structure of β-ketoacyl-CoA thiolase from *S. cerevisiae* has revealed that a cysteine residue (Cys_{125}) in the active site reacts with the C3 carbon of acetoacetyl-CoA (in the degradative reaction), or the C1 carbon of acetyl-CoA (in the biosynthetic reaction), to form a common covalent acetyl-*S*-Cys enzyme intermediate (*33*). In the case of the *Z. ramigera* β-ketoacyl-CoA thiolase (*34*) mutagenesis studies have shown that this intermediate is formed using Cys_{89}. The properties of the Cys92Ser FadA mutant constructed in the current study are consistent with the same Cys92 being involved in generating the analogous enzyme intermediate in both the thiolase and ACT-catalyzed reactions. In the thiolase assay the release of this intermediate by coenzyme A is 3 orders of magnitude faster than with an ACP. The same difference in rate is also observed between the ACT and thiolase activity of the *S. collinus* FadA. Thus, ACP release of an acetyl group from the acetyl-*S*-Cys_{92} enzyme intermediate appears to be the slow step in FadA-catalyzed generation of acetyl-ACP from either acetyl-CoA or acetoacetyl-CoA.

While the ACT activity of FadA is significantly lower than its corresponding thiolase activity, the activity (64 nmol min^{-1} mg^{-1}) is comparable to the reported KAS activity (using the acetyl-CoA substrate) of the KASIII from *S. glaucescens* (11.50 nmol min^{-1} mg^{-1}), *E. coli* (494 nmol min^{-1} mg^{-1}), and *Bacillus subtilis* (14 and 113 nmol min^{-1} mg^{-1}) (*4*, *44*). Thus, depending upon availability and relative concentrations of substrates acetyl-CoA, ACP, and coenzyme A in vivo, the *S. collinus* FadA could contribute to the initiation of straight-chain fatty acid biosynthesis by generating acetyl-ACP. Within this context it is noted that K_m values for FadA with purified *E. coli* ACP (8.8 ± 2.1 μM) are similar to those observed for the ACP (6 μM) (Florova and Reynolds, unpublished results) and malonyl-ACP (3.7 μM) substrates of the streptomycetes FAS enzymes, malonyl-CoA: ACP transacylase A (FabD) and KASIII, respectively (*4*). As described above, the possibility of such a KASIII-independent pathway for straight-chain fatty acid biosynthesis in streptomycetes has been raised (*10*). Such a pathway, it has been argued, would be specific to acetyl-CoA and less sensitive to thiolactomycin than KASIII, properties observed for *S. collinus* FadA. Additional experimentation, currently underway, is needed to establish what role, if any, the *S. collinus* FadA has in fatty acid biosynthesis.

Acetyl-ACP is well established as the starter unit in numerous streptomycetes aromatic polyketide biosynthetic processes (*13*). In this case a dedicated ACP, distinct from the FAS ACP, is used. In vivo experiments with reconstituted aromatic PKS systems have shown that this acetyl-ACP is generated from the decarboxylation of malonyl-ACP (*12*, *13*). The possibility that FadA with ACT activity catalyzes a more direct conversion from acetyl-CoA to acetyl-ACP in vivo, bypassing the malonyl-ACP intermediate, cannot be ruled out. However, while the *S. collinus* FadA did process an ACP (FrnN) from the frenolicin PKS of *S. roseofulvus*, a higher K_m value (44 ± 12 μM) was observed than with the FAS ACP (10 ± 1.4 μM). While the intracellular concentrations of PKS ACPs are not yet determined in a streptomycete, there is no reason to suspect that they are as high as the concentrations of either the FAS ACP or coenzyme A (two thiols which can also release the acetyl group from the acylated FadA intermediate) (*23*). Thus it seems unlikely that significant quantities of the desired acetyl-ACP for priming PKS biosynthesis could be obtained from FadA.

In conclusion, the *S. collinus* FadA represents the first thiolase to be characterized from a streptomycete and is also the first documented thiolase with ACT activity. The availability of the *fadA* gene and the knowledge of the catalytic properties of this enzyme now afford an opportunity to probe the role of this enzyme in a range of important

cellular processes such as fatty acid biosynthesis/degradation and polyketide biosynthesis.

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