Biochemistry

 Volume 41, Number 5 February 5, 2002

Accelerated Publications

Kinetic and Mechanistic Analysis of the Malonyl CoA:ACP Transacylase from *Streptomyces coelicolor* Indicates a Single Catalytically Competent Serine Nucleophile at the Active Site†

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Received October 31, 2001; Revised Manuscript Received December 6, 2001

ABSTRACT: The source of malonyl groups for polyketide and fatty acid biosynthesis is malonyl CoA. During fatty acid and polyketide biosynthesis, malonyl groups are normally transferred to the acyl carrier protein (ACP) component of the synthase by a malonyl CoA:*holo*-ACP transacylase (MCAT) enzyme. The fatty acid synthase (FAS) malonyl CoA:ACP transacylase from *Streptomyces coelicolor* was expressed in *Escherichia coli* as a hexahistidine-tagged (His_6) fusion protein in high yield. The His_6-MCAT was purified to homogeneity using standard techniques, and kinetic analysis of the malonylation of *S. coelicolor* FAS *holo*-ACP, catalyzed by His_6-MCAT, gave K_M^∞ values of 73 (ACP) and 60 μM (malonyl CoA). A catalytic constant k_{cat}^∞ of 450 s^{-1} and specificity constants $k_{cat}^\infty/K_M^\infty$ of 6.2 (ACP) and 7.5 μM^{-1} s^{-1} (malonyl CoA) were measured. Malonyl transfer to the *E. coli* FAS *holo*-ACP, catalyzed by His_6-MCAT, was less efficient ($k_{cat}^\infty/K_M^\infty$ was 10% of that of the *S. coelicolor* ACP). Incubation of MCAT with the serine specific agent PMSF caused inhibition of malonyl transfer to FAS ACPs, and an S97A MCAT mutant was incapable of catalyzing malonyl transfer. Our results show that in the reaction with FAS *holo*-ACPs the *S. coelicolor* MCAT is very similar to the *E. coli* MCAT paradigm in terms of its kinetic mechanism and active site residues. These results indicate that no other active site nucleophile is involved in catalysis as has been suggested to explain recently reported observations.

The source of malonyl units for normal fatty acid (FAS)[1] and polyketide biosynthesis (PKS) is malonyl CoA. These three-carbon building blocks must be transferred to the phosphopantetheine (PP) prosthetic groups of acyl carrier proteins (ACPs) before they are used further by the synthases. During fatty acid biosynthesis, the transfer of malonate from CoA to ACP is catalyzed by specific malonyl CoA:ACP transacylase (MCAT) enzymes. In all MCAT enzymes that have been examined to date, this reaction is performed by

† This research was supported by EPSRC, BBSRC, and the University of Bristol. A.S. was supported by ORSAS.

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[1] Abbreviations: FAS, fatty acid synthase; PKS, polyketide synthase; CoA, coenzyme A; PP, phosphopantetheine; ACP, acyl carrier protein; MCAT, malonyl CoA:ACP transacylase; *act*, actinorhodin; *fren*, frenolicin; ESMS, electrospray mass spectrometry; FPLC, fast protein liquid chromatography; PCR, polymerase chain reaction; TCA, trichloroacetic acid; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; WT, wild type; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NAC, *N*-acetylcysteamine; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; TLC, thin-layer chromatography; IPTG, isopropyl β-D-thiogalactopyranoside.

10.1021/bi012001p CCC: $22.00
Published on Web 01/11/2002

the transfer of malonyl groups to a highly conserved active site serine nucleophile on MCAT. The malonylated MCAT then transfers the acyl group onto the PP group of *holo*-ACP (*1*).

The type I FAS proteins typical of higher organisms possess integral MCAT domains (*2*), while the type II FAS proteins of bacteria and plants use discrete MCAT proteins (*3*). Iterative and modular type I PKS systems also possess integral MCAT domains, but type II PKS systems, in contrast, do not include MCAT enzymes encoded in their gene clusters (*4*). A case in point is that of actinorhodin (*act*) biosynthesis in *Streptomyces coelicolor* where the *act* cluster contains no MCAT encoding gene. The assumed requirement for an MCAT enzyme to transfer malonyl units to ACPs led to the idea that the endogenous FAS MCAT of *S. coelicolor* must provide this essential service for the *act* PKS ACP. This idea is often termed "crosstalk" between primary and secondary metabolism, and many experiments have been performed to attempt to validate this hypothesis (*5*−*7*).

However, we observed that the *act* PKS *holo*-ACP can self-load malonyl units from malonyl CoA *without* the requirement for MCAT (*8*). This ability is possessed by other type II PKS ACPs, including those used during griseusin and oxytetracycline biosynthesis (*9*). We have extensively characterized this self-acylation reaction (*9*), and others have also observed similar reactions in related type II PKS systems (*10*, *11*). The self-acylation reaction appears to be a general property of type II PKS ACPs and can be extended from malonyl CoA to a range of other dicarboxylic and β-keto acids as either their CoA or *N*-acetylcysteamine thiolesters (*9*, *12*). FAS *holo*-ACPs, by contrast, do not undergo self-acylation reactions (*9*).

We have also observed that when purified *act holo*-ACP is incubated with *act* KS_α, KS_β (so-called CLF), and malonyl CoA alone, this minimal *act* PKS can make polyketides *without* the requirement for additional MCAT because *act holo*-ACP can self-malonate fast enough to provide the malonyl ACP required for polyketide biosynthesis in vitro (*8*, *9*). In contrast, it has been reported that a heterologous system using the frenolicin (*fren*) ACP with the *act* KS_α and KS_β has an absolute requirement for *S. coelicolor* MCAT for in vitro polyketide synthesis from malonyl CoA (*5*).

In light of these differing results, we set out to study the properties of the *S. coelicolor* MCAT using its natural substrate, the *S. coelicolor* FAS ACP, and another FAS ACP to determine its kinetic parameters, substrate specificity, and chemical mechanism. We now report the preliminary results of these studies in light of a recent report which implies the unprecedented presence of two alternative catalytic nucleophiles at the active site of this enzyme (*13*). In all other systems that have been studied to date, covalent derivatization of the active site serine results in inhibition and site-directed mutagenesis for removal of the serine results in an inactive protein (*2*, *14*, *15*).

MATERIALS AND METHODS

Expression and Purification of holo-ACPs. Escherichia coli BL21 pIJ8133 which contained the *acpP* gene encoding the *S. coelicolor* type II FAS ACP was a gift from D. Hopwood and P. Revill (John Innes Centre, Norwich, U.K.) (*16*). The protein was produced predominantly in the *apo* form, and it was purified by standard procedures (*17*) and then converted to the *holo* form by treatment with *S. coelicolor holo*-acyl carrier protein synthase (ACPS) and CoA in vitro. Conversion of the FAS ACP to the *holo* form was monitored by ESMS. The FAS *holo*-ACP was then repurified by FPLC (Q Sepharose, then desalt). *E. coli* FAS *holo*-ACP was purchased from Sigma and was purified by FPLC (Q Sepharose, NaCl gradient, followed by desalting) before use.

Cloning and Expression of S. coelicolor fabD. Standard cloning procedures were adopted unless otherwise stated. The *fab* cluster of *S. coelicolor* (*7*) was digested with *Sma*I to yield two fragments, one of which (A) contained the 5′-terminal 258 bp of the *fabD* gene and a second fragment (B) which contained the remainder of the *fab*D gene and the 5′-end of the *fabH* gene (ACP). Fragment A was ligated into a pUC vector, and an *Nde*I site was engineered at the 5′-end of the *fabD* gene by PCR, using the vector with the insert as a template, with the following synthetic oligonucleotides: a 25-mer 5′-**CATATG**CTCGTACTCGTCGCTCCCG-3′ and a universal primer based on the pUC vector. A PCR product of the expected 340 bp was precipitated from the reaction mixture, then resuspended in water, and treated with T4 polynucleotide kinase and ATP at 37 °C for 1 h. The PCR product was ligated into the dephosphorylated *Sma*I site of pBluescript using T4 DNA ligase. The sequence of one clone was confirmed by manual sequencing (Promega). This cloned vector was linearized with *Sma*I, and the 764 bp *Sma*I fragment B was ligated into the dephosphorylated *Sma*I site. The entire modified and cloned gene was excised using *Nde*I and *Bam*HI and subcloned into the pET15b vector (Novagen) between the *Nde*I and *Bam*HI sites. Positive clones were identified by restriction analysis, and a positive representative was designated pIJ2371; this was subsequently transformed into the expression host *E. coli* BL21(DE23) pLysS.

Generation of the S97A Mutant. A pair of complementary oligonucleotides (31-mers) were designed to be complementary to pIJ2371, but containing the S97A mutation (5′-CGGTCGCCGGACAC**GCT**GTCGGCGAGATCAC-3′ and 5′-GTGATCTCGCCGAC**AGC**GTGTCCGGCGACCG-3′). These were used in conjunction with the Quickchange mutagenesis kit (Stratagene). The mutated product DNA was transformed into *E. coli* XL1-Blue (Stratagene), and single colonies were isolated. Plasmid DNA from 12 colonies was isolated, and the MCAT gene was sequenced (Lark). A single clone containing the correct sequence was used for expression.

Purification of His_6-MCAT. E. coli pIJ2371 cells were grown at 37 °C and 250 rpm in Luria-Bertani liquid medium supplemented with 30 μg/mL chloramphenicol and 100 μg/mL ampicillin until a cell density (measured at 595 nm) of 0.6 was reached. The cultures were rapidly cooled to 30 °C, and then expression of His_6-MCAT was induced by the addition of IPTG to a final concentration of 1 mM, followed by growth for a further 4 h. Initial purification of the expressed His_6-MCAT was achieved by nickel(II) affinity chromatography using a 5 mL His-Bind column (Novagen) which was prepared, regenerated, and maintained following the manufacturer's instructions; buffers were as described by Novagen. All buffers and protein solutions were filtered to 0.45 μm before application to the column and were cooled

on ice during column loading and washing. Cells from a 2 L culture of induced *E. coli* pIJ2371 (typically 10 g) were suspended in 50 mL of binding buffer and lysed by sonication (4 × 30 s, with cooling for 1 min on ice), and the cell debris was removed by centrifugation. The supernatant was applied to the nickel(II) column using a peristaltic pump at a rate of approximately 1.5 mL/min. The column was washed with 50 mL of binding buffer and 50 mL of wash buffer, and then the protein was eluted with 10−20 mL of elute buffer. The eluted protein was desalted into 50 mM Tris-HCl (pH 7.5) and 5% glycerol by FPLC on a Sephadex G-25 column. Protein fractions were pooled and applied to a HiLoad Q Sepharose column equilibrated in the same buffer. Protein was eluted over a 400 mL gradient from 0 to 1 M NaCl. The fractions containing His_6-MCAT (eluting at approximately 0.5 M) were pooled and desalted into 50 mM phosphate buffer (pH 8.0), and $(NH_4)_2SO_4$ was added to a final concentration of 2 M. The solution (5−10 mg of total protein) was then applied to a phenyl Superose column equilibrated in 50 mM phosphate buffer (pH 8.0) and 2 M $(NH_4)_2SO_4$. Bound protein was eluted over a 20 mL gradient from 2 to 0 M $(NH_4)_2SO_4$. Fractions containing His_6-MCAT were pooled and desalted into 50 mM Tris-HCl (pH 7.2), then applied to a Mono-Q column equilibrated in Tris-HCl (pH 7.5) and 5% glycerol, and eluted with a 25 mL gradient from 0 to 1 M NaCl. Fractions containing His_6-MCAT were pooled, frozen in 1 mL aliquots, and stored at −20 °C.

Assay of His_6-MCAT Activity and Kinetic Studies. Purified His_6-MCAT was assayed for its ability to catalyze transfer of malonate from malonyl CoA to *S. coelicolor* FAS *holo*-ACP, based on the method of Ruch and Vagelos (*3*). Typically, assay solutions contained 50 μM *holo*-ACP, 45 μM malonyl CoA, 5 μM [2-^{14}C]malonyl CoA (50 mCi/mmol), 50 mM phosphate buffer (pH 6.5), and the His_6-MCAT fraction (0.5 nM) in a final volume of 10 μL. ACP and His_6-MCAT were preincubated (without the malonyl CoA) for 5 min at 30 °C, and after addition of malonyl CoA for 2 min at 30 °C. The reaction was stopped by addition of 50% (w/v) TCA (30 μL) and 10 mg/mL BSA (30 μL). Protein was allowed to precipitate on ice for 15 min and then pelleted by centrifugation. The protein pellet was washed twice with ice-cold 10% (w/v) TCA (50 μL) and then resuspended in 50 μL of NaOH (2 M) and 50 μL of Tris base (2 M), and the amount of incorporated radioactivity was counted in liquid scintillant. Kinetic studies were performed in essentially the same manner, except that aliquots (10 μL) were removed from a 100 μL incubation at timed intervals and the reactions quenched by addition to 30 μL aliquots of ice-cold TCA (50%). BSA was added as a carrier (30 μL, 10 mg/mL), and the precipitated protein was collected and analyzed in the usual manner. All assays were repeated in triplicate, and appropriate control experiments were routinely performed.

Inhibition Studies Using Phenylmethanesulfonyl Fluoride (PMSF). His_6-MCAT (0.5 μM) was incubated in 50 mM phosphate buffer (pH 6.5) for 60 min at 30 °C with 0, 0.1, 1, and 2 mM PMSF in a total volume of 20 μL. The enzyme samples were then diluted 1000-fold and assayed for activity as described above. The reaction was quenched after 2 min and analyzed in the standard manner. To assess the ability of malonyl CoA to protect the enzyme from inhibition, 0.5 μM His_6-MCAT was incubated for 5 min at 30 °C with 50 and 100 μM malonyl CoA. The aliquots were diluted to volumes of 20 μL by addition of PMSF in buffer to the appropriate concentrations, and the inhibition experiments were repeated.

Analysis of Acylated holo-ACPs by Electrospray Mass Spectrometry. The products of the reaction catalyzed by His_6-MCAT and the substrate specificity of the enzyme were analyzed by electrospray mass spectrometry (ESMS). Typically, the assay contained 50 μM ACP, 500 μM malonyl CoA, 1 mM DTT, and 50 mM potassium phosphate (pH 7.5) in a final volume of 200 μL. The reaction was initiated by the addition of the malonyl CoA (20 μL) to the ACP (with 0.5 nM His_6-MCAT, where appropriate), which had been equilibrated for 10 min at 30 °C. Aliquots (20 μL) were removed at timed intervals up to 8 h and the reactions quenched by the addition of 5 μL of aqueous formic acid (25%). Analyses were performed immediately, and mass spectra were measured as previously described (*17*).

Radiolabeling of MCAT. WT MCAT (0.24 nmol) was incubated with [2-^{14}C]malonyl CoA (0.6 nmol). S97A MCAT (0.49 nmol) was incubated with [2-^{14}C]malonyl CoA (2 nmol). The mutant protein (1.47 nmol) was also incubated with [2-^{14}C]malonyl CoA (6 nmol). Reference samples were identical but lacked malonyl CoA. The samples were incubated at 30 °C for 4 h and then TCA precipitated, and the protein pellets were loaded on a 15% SDS−PAGE gel. The developed gel was stained in Coomassie blue and then destained overnight in a mixture of 10% methanol and 10% acetic acid. The destained gel was agitated in Amplify (Amersham) for 90 min at room temperature, rinsed thoroughly in distilled water, and dried (Flowgen gel drier) under vacuum at 80 °C. The dry gel was held in close contact with a sheet of X-ray film (Hyperfilm, AmershamPharmacia Biotech) at −80 °C for 14 days. The film was developed according to the manufacturer's instructions.

RESULTS

Catalytic Competency. S. coelicolor fabD was cloned into pET15b. This vector encodes a 20-amino acid N-terminal sequence containing a hexahistidine affinity tag and a thrombin cleavage site. In addition, the use of this leader sequence obviates the need to optimize the first few codons of the GC rich *S. coelicolor fabD* gene for expression in *E. coli*. Expression in the usual way followed by Ni^{2+} affinity chromatography afforded the expected His_6-MCAT protein in soluble form. Further purification by standard ion exchange−FPLC procedures (Figure 1A and Table 1) then afforded a highly pure protein. Analysis by ESMS (Figure 1B) confirmed the expected molecular mass (M_w) of 34 113 Da (observed, 34 114 ± 2 Da). As in previous experiments in this area, His_6-tagged proteins were used for all biochemical assays. There is no evidence that this common procedure has any effect on the activity of the proteins, although this possibility should always be kept in mind.

The *S. coelicolor fabH* ACP was also cloned, expressed, and highly purified using standard procedures. It is important to be aware that *holo*-ACPs can readily form covalent dimers by disulfide formation between the terminal thiols of the phosphopantetheine prosthetic groups. Such disulfide formation renders *holo*-ACPs inactive in PKS (*8*), FAS, and acylation assays (*18*). *Holo*-ACP proteins must therefore be

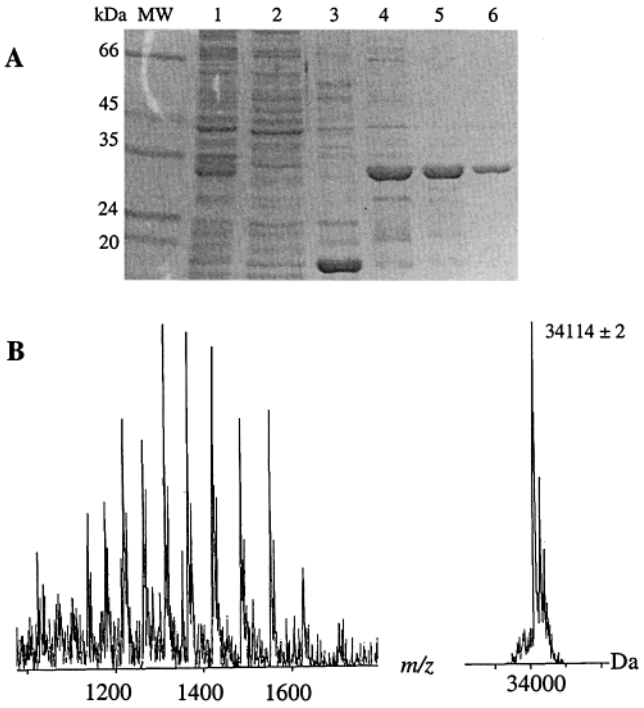

FIGURE 1: Purification of *S. coelicolor* His_6-MCAT (see Table 1). (A) Lane 1, cell free extract; lane 2, Ni^{2+} column elute; lane 3, Ni^{2+} column wash; lane 4, Q Sepharose; lane 5, phenyl Superose; and lane 6, mono-Q. (B) ESMS traces of purified WT His_6-MCAT (observed mass of 34 114 ± 2 Da, calculated mass of 34 113 Da for loss of the N-terminal methionine).

Table 1: Purification of *S. coelicolor* His_6-MCAT from *E. coli* pIJ2371[a]

lane	fraction	total protein (mg)	total activity (units)	specific activity (units/mg)	fold purification
1	cell free extract	380	84.9×10^6	223×10^3	–
2	Ni column elute	65.4	49.6×10^6	759×10^3	3.4
3	Ni column wash	–			–
4	Q Sepharose	33.0	135×10^6	4.1×10^6	18.3
5	phenyl Superose	23.9	246×10^6	10.3×10^6	46.1
6	Mono-Q	17	221×10^6	13×10^6	58.2

[a] One unit of His_6-MCAT was defined as the amount of protein required to catalyze the synthesis of 1 pmol of malonyl ACP per second at pH 6.5 and 30 °C with 100 μM ACP and 100 μM malonyl CoA.

treated with DTT to ensure the absence of disulfides, and excess DTT must be removed by desalting as we have previously reported. The disulfide dimer and free-thiol monomer cannot be distinguished by SDS−PAGE because of the strongly reducing conditions, but native PAGE and ESMS readily differentiate these species (*8*). All purified *holo*-ACPs were examined by ESMS and native PAGE before use to ensure that they were in the active form.

S. coelicolor FAS ACP (50 μM, Figure 2A), prepared as described above, was incubated with the purified His_6-MCAT (5 nM) in the presence of malonyl CoA (200 μM). After 30 min, examination by ESMS showed very substantial conversion to malonyl ACP (Figure 2B) (expected mass of 9211 Da, observed mass of 9211 ± 3 Da). In the absence of MCAT, no production of malonyl ACP could be detected.

Kinetic Mechanism. E. coli FAS MCAT has been purified and investigated kinetically and structurally. The enzyme has been shown to process its two substrates in a classic ping-pong bi-bi mechanism in which MCAT first binds malonyl

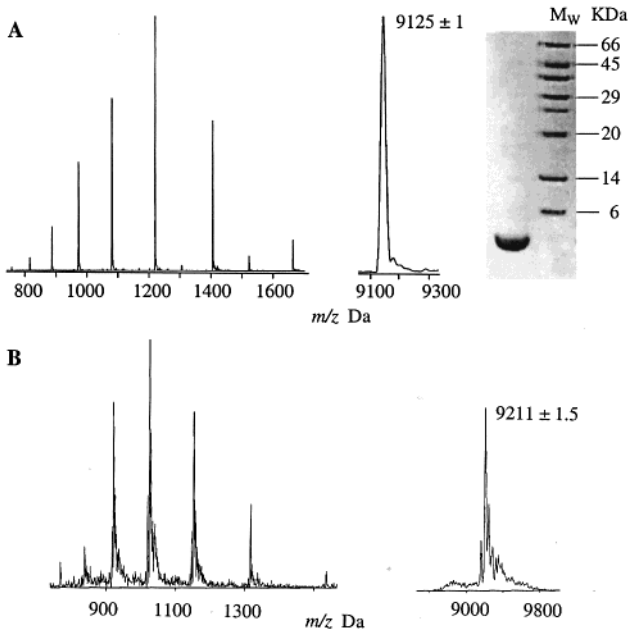

FIGURE 2: ESMS spectra and transforms of purified *holo*-ACP monomer substrates and malonyl ACP products. (A) *S. coelicolor holo*-ACP, also showing a 15% SDS−PAGE of the protein. Expected M_w of 9125 Da. (B) *S. coelicolor* malonyl-ACP. Expected M_w of 9211 Da.

CoA, transferring the malonyl unit to an active site hydroxyl side chain of a serine residue (*19*). The malonyl unit is then transferred in a second step to the terminal thiol of the phosphopantetheine of *holo*-ACP. Derivatization by hydroxyl specific reagents such as phenylmethanesulfonyl fluoride (PMSF) inhibits the *E. coli* MCAT by blocking the active site hydroxyl.

In a recent study of *S. coelicolor* MCAT, it was reported that attempts to establish a kinetic assay for the direct transfer of malonate from malonyl CoA to ACP were unsuccessful (*13*). This appears to be surprising given that this is the biochemical reaction catalyzed by MCAT. An indirect assay in which transfer to the simple thiol *N*-acetylcysteamine was followed by TLC autoradiography was used instead. In our hands, however, the transfer of malonyl groups from CoA to properly purified monomeric *holo*-ACP was simple to observe.

We used two assays to follow malonyl transfer. The first assay involved the incubation of MCAT (0.5 nM) with *holo*-ACP (50 μM) and malonyl CoA (50 μM). ESMS can then be used to directly examine the malonyl ACP (Figure 2). If need be, the malonyl ACP can be purified by HPLC and differentiated from other acyl ACP species as previously described (*20*). The second assay we used involved following the transfer of ^{14}C-labeled malonyl groups from [^{14}C]malonyl CoA to *holo*-ACP. This assay is quantitative and can be used for kinetic analysis of the transfer reaction (Figure 3A).

With the assay tools in hand, we then set out to examine the reaction catalyzed by MCAT between malonyl CoA and *S. coelicolor* FAS *holo*-ACP. By varying the malonyl CoA and *holo*-ACP concentrations in a systematic manner and measuring the initial rates of the transfer reaction (first 5% of the reaction), we were able to obtain K_M^∞ and k_{cat}^∞ data for this reaction (Table 2). In addition, the assay allowed us to confirm that, like the reactions of all other MCAT enzymes studied to date, the reaction can be classified as a ping-pong

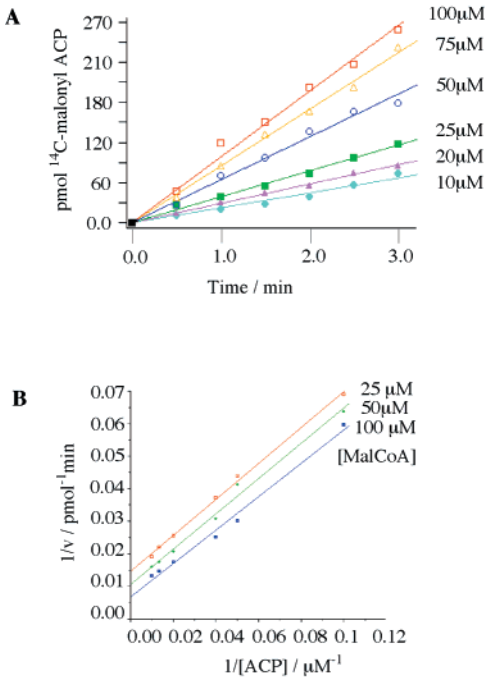

FIGURE 3: Typical kinetic analysis of malonyl transfer from CoA to *S. coelicolor* FAS *holo*-ACP catalyzed by His$_6$-MCAT. (A) Raw rate data showing precipitated [^{14}C]malonyl ACP concentration vs time with increasing ACP concentrations at a fixed malonyl CoA concentration (50 μM). Similar data were collected at varying ACP and malonyl CoA concentrations. (B) Lineweaver−Burk treatment of the rate data.

Table 2: Comparison of Absolute Kinetic Constants for the Malonyl Transfer Reactions Catalyzed by *S. coelicolor* His$_6$-MCAT and *E. coli* MCAT

kinetic constant	His$_6$-MCAT (*S. coelicolor* FAS ACP)	His$_6$-MCAT (*E. coli* ACP)	*E. coli* MCAT (*E. coli* ACP) (*3*)
K_M^∞ for MalCoA (μM)	60	327	250
K_M^∞ for ACP (μM)	73	776	351
k_{cat}^∞ (s^{-1})	450	420	1580
$k_{cat}^\infty/K_M^\infty$ for MalCoA (μM^{-1} s^{-1})	7.50	1.28	6.32
$k_{cat}^\infty/K_M^\infty$ for ACP (μM^{-1} s^{-1})	6.16	0.54	4.50

bi-bi one (Figure 3B). The kinetic values that were obtained were similar to those obtained by Ruch and Vagelos (*3*), who examined the reaction catalyzed by *E. coli* MCAT. We used the same assay to study the reaction of purified *E. coli* *holo*-ACP and obtained similar results. The figures obtained for *S. coelicolor* MCAT interacting with *S. coelicolor* FAS *holo*-ACP are approximately 5-fold lower than the figures measured for *E. coli* MCAT interacting with *E. coli* FAS ACP, although the specificity constant ($k_{cat}^\infty/K_M^\infty$) is remarkably similar. Interactions between *S. coelicolor* MCAT and the heterologous *E. coli* FAS ACP are unsurprisingly weaker, with the specificity constants being 6−11-fold lower.

Active Site Residues. We probed the active site of *S. coelicolor* MCAT by incubating it with the hydroxyl specific agent PMSF. Preincubation of MCAT with PMSF at 2 mM caused significant inhibition as measured by ^{14}C-labeled malonyl transfer to *S. coelicolor* FAS ACP (Table 3). However, incubation in the presence of the substrate, malonyl CoA, reduced the level of inhibition, indicating the likelihood

Table 3: Inhibition of His$_6$-MCAT Activity by PMSF[a]

inhibitor concentration (mM)	preincubation with malonyl CoA (μM)	malonyl ACP (pmol)	percentage inhibition
0	−	5.80	0
1	−	2.44	58
2	−	1.77	69
2	50	2.72	53
2	100	3.05	47

[a] See Materials and Methods for details.

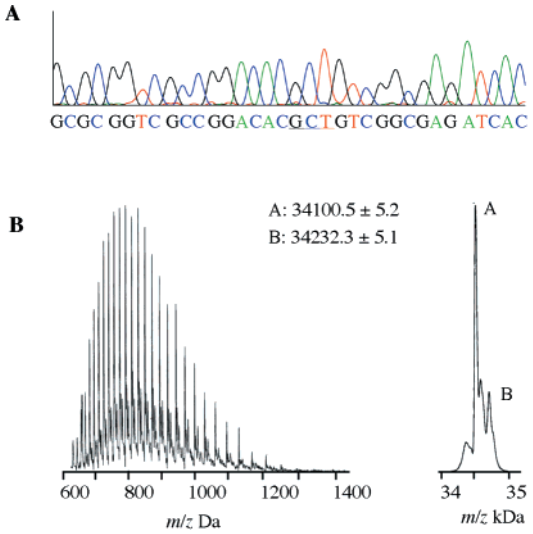

FIGURE 4: Production of S97A MCAT. (A) Sequence analysis in the region of the S97A mutation (underlined). (B) ESMS analysis of S97A His$_6$-MCAT (found mass of 34 100.5 ± 5.5 Da, calculated mass of 34 097 Da for loss of the N-terminal methionine; also shown is the methionine-bearing protein).

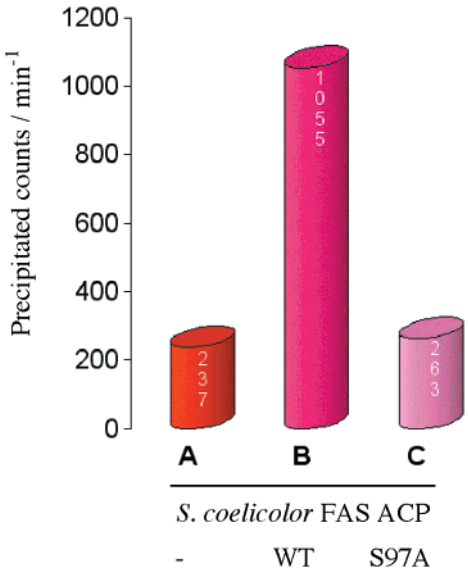

FIGURE 5: Activity of WT MCAT and S97A MCAT with *S. coelicolor* FAS *holo*-ACP. See Materials and Methods for experimental details: (A) ACP and malonyl CoA, (B) ACP, WT MCAT, and malonyl CoA, and (C) ACP, S97A MCAT, and malonyl CoA.

of a nucleophilic hydroxyl at the active site. ESMS analysis of PMSF-inhibited MCAT revealed multiple acylations of the protein.

S. coelicolor MCAT is highly homologous to its *E. coli* counterpart (44% similar and 33% identical). Pile-up analysis of the two proteins indicates that S97 of *S. coelicolor* MCAT

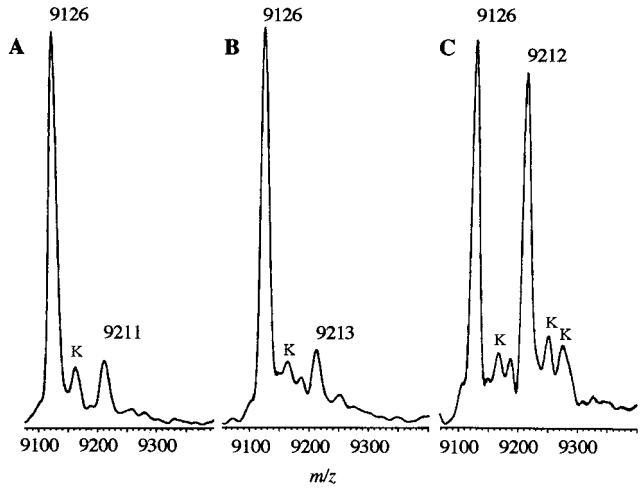

FIGURE 6: ESMS spectra of ACP components from MCAT-catalyzed reactions between ACP and malonyl CoA: (A) *S. coelicolor* FAS *holo*-ACP and malonyl CoA, (B) *S. coelicolor* FAS *holo*-ACP, malonyl CoA, and S97A MCAT, and (C) *S. coelicolor* FAS *holo*-ACP, malonyl CoA, and WT MCAT. K denotes potassium adducts.

corresponds to the active site S92 of *E. coli* MCAT. We therefore prepared an S97A mutant of the *S. coelicolor* MCAT using standard methods. The site of mutation was confirmed by double-strand sequencing (Figure 4A). The protein was expressed and purified in the usual way and examined by ESMS which yielded the expected M_w [34 100 ± 5.2 Da (found), 34 097 Da (calculated); Figure 4B].

We then examined the activity of this mutant MCAT in malonyl transfer reactions. *S. coelicolor* FAS *holo*-ACP was incubated with [^{14}C]malonyl CoA in the presence and absence of MCAT and S97A MCAT (0.5 nM). After 60 min, the protein was precipitated, the excess malonyl CoA was removed, and the protein pellets were resuspended in scintillant and counted (Figure 5). In a control reaction (Figure 5A), FAS *holo*-ACP was incubated with malonyl CoA alone. As expected, in the presence of WT MCAT, significant malonyl transfer occurred (Figure 5B). However, in the presence of S97A MCAT, only the background number of counts was observed (Figure 5C).

The reactions of the S97A MCAT were also studied using ESMS analysis of reaction mixtures. When *holo*-FAS ACP was incubated with 1 mM malonyl CoA for 1 h, ~5% malonyl ACP was observed (Figure 6A). The same results were observed in the presence of S97A MCAT at a concentration of 0.5 nM (Figure 6B), but in the presence of 0.5 nM WT MCAT, ~50% malonyl ACP was observed (Figure 6C).

Prevention of malonyl transfer by the S97A mutant could be caused at either of the two catalytic steps. To determine whether *binding* of malonyl groups by MCAT or *transfer* of malonyl groups to *holo*-ACP is inhibited, we incubated both WT MCAT and the S97A mutant with [^{14}C]malonyl CoA. The proteins were then run on a nondenaturing electrophoresis gel (Figure 7A) which was used to prepare an autoradiograph (Figure 7B). WT MCAT is clearly labeled with ^{14}C malonyl groups, whereas the S97A mutant is not.

DISCUSSION

For the first time, a systematic investigation of the kinetic mechanism and substrate specificity of the *S. coelicolor* MCAT has been carried out using both native and heterologous ACP substrates. The enzyme appears to function kinetically in a manner very similar to that of the *E. coli* MCAT, an enzyme to which it is 44% similar and 33% identical. Full kinetic analysis leads to the conclusion of a ping-pong bi-bi mechanism identical to that catalyzed by the *E. coli* enzyme. In terms of substrate affinity, the *S. coelicolor* enzyme closely resembles the *E. coli* MCAT when interacting with its native substrate (*S. coelicolor* FAS ACP). However, interaction between *S. coelicolor* MCAT and the heterologous *E. coli* ACP is ~10-fold less efficient (Table 2).

We have also shown that the transfer of malonyl groups to FAS ACP is inhibited when MCAT is incubated with PMSF, implicating an active site serine. We have also prepared the S97A mutant MCAT, and we find that it cannot transfer malonate to FAS ACPs, suggesting that in common with all other previously studied MCAT enzymes, serine is the sole active site nucleophile (*2*, *14*, *15*). A recent paper reports that the removal of the active site serine 97 of MCAT does not inhibit the functioning of a minimal PKS in vitro and, by implication, that S97 is not essential to catalysis by *S. coelicolor* MCAT (*5*). The same group has subsequently

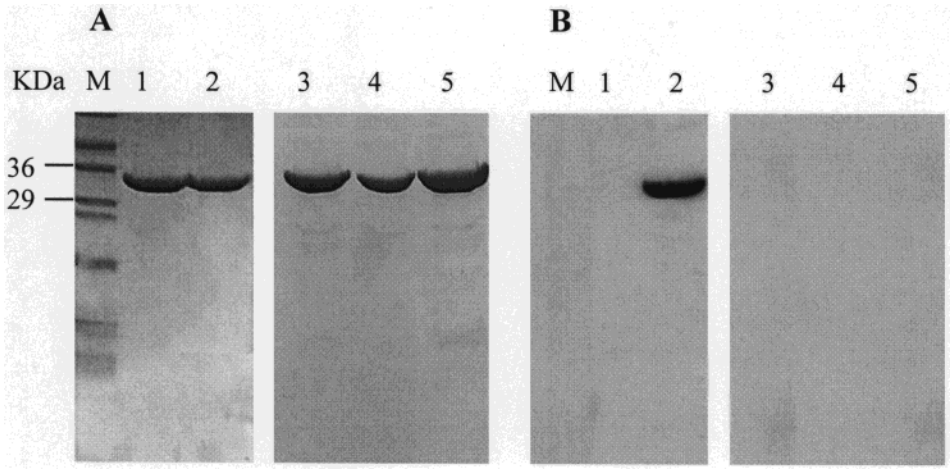

FIGURE 7: (A) SDS−PAGE (15%) and (B) autoradiograph of *S. coelicolor* His_6-MCAT and *S. coelicolor* S97A His_6-MCAT incubated with [2-^{14}C]malonyl CoA: lane M, molecular mass markers; lane 1, His_6-MCAT reference; lane 2, His_6-MCAT and [2-^{14}C]malonyl CoA; lane 3, S97A His_6-MCAT reference; lane 4, S97A His_6-MCAT and [2-^{14}C]malonyl CoA; and lane 5, S97A His_6-MCAT (3-fold higher concentration) and [2-^{14}C]malonyl CoA.

reported that, inter alia, S97A and H96A mutants are labeled upon incubation with [^{14}C]malonyl CoA and are able to transfer malonate from malonyl CoA to the heterologous *fren* ACP (*13*). The corresponding double mutant H96A/S97A was unlabeled itself by [^{14}C]malonyl CoA but, surprisingly, did transfer malonate to the *fren* ACP. It was suggested that this could be explained by contamination of the mutant protein with the *E. coli* MCAT.

On the basis of these reported observations, it was proposed that H96 can function as a second "surrogate" or alternative catalytically competent nucleophile for malonyl transfer via a malonyl−imidazole adduct instead of the normal serine−*O*-malonyl intermediate. The evidence presented for the formation of this unprecedented adduct came from mass spectral analysis of protein digests of the mutant and WT enzymes. However, examination of the observed and calculated molecular masses for the peptide parent and fragment ions reveal discrepancies of 1−20 Da. In particular, a parent ion of 826.4 Da was ascribed to a malonylated fragment from the mutant protein, GAVAGH(malonyl)AVG. This would have an expected M_w of 823.4 Da (MH^+ = 824.4), whereas a fragment derived from the WT protein, AVAGHSVGE, has a calculated mass of 825.4 Da (MH^+ = 826.4).

We are not aware of any precedent for the formation of a malonyl−histidine adduct which would undoubtedly be highly active as an acyl transfer agent, but which appears to be surprisingly stable to the proteolytic (pH 2, 37 °C, 60 min) and chromatographic procedures involved prior to the mass spectrometric analysis. *N*-Acylimidazole derivitization of protected histidine has been detected by NMR analysis of crude reaction mixtures, but these species are reported to be rapidly hydrolyzed under both mild acid and base conditions (*21*).

Overall, we find that the *S. coelicolor* MCAT is remarkably similar in both its kinetic and chemical mechanism to the *E. coli* MCAT, a protein to which it is 33% identical and 44% similar. The protein possesses an essential active site serine nucleophile, the removal of which (by mutation or PMSF labeling) abolishes activity. We, thus, find no reason to postulate the need, or evidence for, a putative "extra" and unprecedented active site histidine nucleophile.

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BI012001P