Malonyl-CoA:ACP Transacylase from *Streptomyces coelicolor* Has Two Alternative Catalytically Active Nucleophiles†

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ABSTRACT: Fatty acids and polyketides are synthesized by mechanistically and evolutionarily related multienzyme systems. Their carbon chain backbones are synthesized via repeated decarboxylative condensations of α-carboxylated building blocks onto a growing acyl chain. These α-carboxylated building blocks are transferred from the corresponding coenzyme A thioesters onto the phosphopantetheine arm of an acyl carrier protein (ACP) by acyl transferases, which operate by a ping−pong mechanism involving an acyl-O-serine intermediate. In the course of our studies on the malonyl-CoA:ACP transacylase (MAT) from *Streptomyces coelicolor*, we observed that an active-site Ser (97) → Ala mutant retains activity as well as the ability to be covalently labeled by ^{14}C malonyl-CoA. Here we demonstrate that an alternative, catalytically competent nucleophile exists in the active site of this enzyme. Next to the active-site serine is a histidine residue that is conserved in some, but not all acyl transferases. The H96A mutant is also active and can be labeled, but an H96A/S97A double mutant is inactive and cannot be labeled. The ability of H96 to form a malonyl−imidazole adduct was confirmed by proteolysis, followed by radio-HPLC and mass spectrometric analysis of the S97A mutant enzyme. Kinetic analysis revealed that the k_{cat} of the S97A mutant was within 10-fold that of the wild-type enzyme, whereas the K_Ms of the two enzymes were comparable. Sequence comparison with the *E. coli* MAT (whose X-ray structure is known) led to the identification of H201 as the putative base in the serine-histidine catalytic dyad of the *S. coelicolor* enzyme. The absence of MAT activity in the H201A mutant and the detection of weak activity in the H201Q mutant was consistent with this proposal. The implications of this unexpected finding are discussed.

Fatty acid synthases (FASs)[1] and polyketide synthases (PKSs) are members of a superfamily of evolutionarily related multienzyme systems that catalyze the biosynthesis of fatty acids and polyketides through repetitive condensation of malonyl, methylmalonyl, and related building blocks onto an enzyme-bound growing acyl chain. Notwithstanding architectural differences, every FAS and PKS possesses one or more sets of the following three active sites: a ketosynthase (KS), an acyl carrier protein (ACP), and an acyltransferase (AT). [The only exceptions are plant and bacterial Type III PKSs, which directly utilize CoA-bound nucleophiles rather than ACP-bound nucleophiles (*1*, *2*); these will not be discussed further in this report.] As shown in Figure 1, the growing acyl chain is bound to an active-site cysteine in the KS. The AT catalyzes transfer of an α-carboxylated extender unit from the appropriate acyl-CoA to the pantetheine thiol of the ACP. A C−C bond is then formed via decarboxylative condensation between the KS-bound growing chain and the ACP-bound extender unit; this reaction is catalyzed by the KS. Following condensation, the fatty acid or polyketide chain is bound to the ACP, and its backbone has been extended by two carbon atoms. A variety of postcondensation modifications (e.g., β-keto-reduction, dehydration, and enoyl-reduction) can then occur before the growing chain is transferred back to the same KS (in the case of FASs, bacterial aromatic PKSs, and fungal PKSs) or to another KS (in the case of modular PKSs) (reviewed in ref *3*).

Bacterial aromatic PKSs represent an interesting and unusual example of crosstalk between fatty acid and polyketide biosynthesis. These PKSs are architecturally related to Type II FASs found in bacteria and plants and catalyze the biosynthesis of the polyketide skeletons of antibiotics such as actinorhodin (*act*), frenolicin (*fren*), tetracenomycin (*tcm*), doxorubicin (*dps*), and tetracycline (*tet*). Their biosynthetic gene clusters include dedicated KS and ACP genes but typically lack an AT gene. Instead, the malonyl-CoA:ACP transacylase (MAT) from the fatty acid synthase of *Streptomyces coelicolor* (actinorhodin producer) and *Streptomyces glaucescens* (tetracenomycin producer) was shown to be capable of transacylating malonyl groups on to the *act* and *tcm* ACPs as well (*4*, *5*). On the basis of this and other physiological data, it was proposed that the MAT is shared between the FAS and PKS systems in these hosts. Direct evidence in support of this hypothesis was obtained through reconstitution in vitro of the *act* PKS and the *tcm* PKS from

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[1] Abbreviations: CoA, coenzyme A; PKS, polyketide synthase; KS, ketosynthase; CLF, chain length factor; ACP, acyl carrier protein; MAT, malonyl CoA:ACP transacylase; *act*, actinorhodin; *fren*, frenolicin; TLC, thin-layer chromatography.

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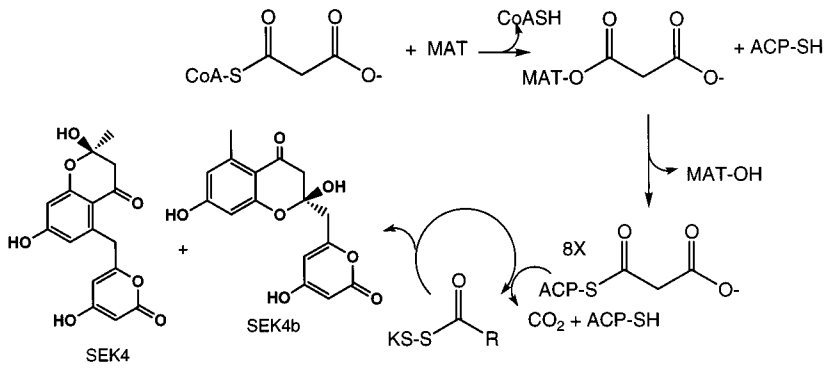

FIGURE 1: Schematic representation of chain elongation in polyketide synthesis. Malonyl units are transferred from CoA to acyl carrier protein (ACP) by the malonyl-CoA:ACP malonyl transacylase (MAT). The ketosynthase (KS), which exists as a tight heterodimer with a homologous protein- the chain length factor (CLF, not shown), utilizes malonyl-ACP units for chain priming as well as elongation. After every round of condensation, the growing acyl chain is transferred back to the KS, and a new condensation cycle can occur. The actinorhodin PKS releases the polyketide products SEK4 and SEK4b in equal ratios after seven condensation cycles.

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S.coelicolor     FTPGAVAGHS VGEITAAVFA GVLDDTAALS LVRRRGLAMA EAAAVTETGM
S.glaucescens    IAPGAVAGHS VGEITAARFA GVLDDTAALT LVRKRGLAMA EAAAITETGM
M.leprae         I....VAGHS VGEIAAYSIA GVIAADDAVA LAATRGAEMA KACAIEPTGM
E.coli           KAPAMMAGHS LGEYSALVCA GVIDFADAVR LVEMRGKFMQ EAVPEGTGAM
H.pylori         LKPVFALGHS LGEVSAVSLS GALDFEKALK LTHQRGKMMQ EACANKDASM

S.coelicolor     SALLGGDPEV SVAHLERLG. ...LTPANVN GAGQIVAAGT MEQLAALNED
S.glaucescens    SALLGGDPEV SVAHLEKLG. ...LTPANVN GAGQIVAAGT LEQLAALNED
M.leprae         SAVLGGDEAD VLNRLEELD. ...LVPANRN AAGQIVAAGR LTALEKLAED
E.coli           AAIIGLDDAS IAKACEEAAE GQVVSPVNFN SPGQVVIAGH KEAVERAGAA
H.pylori         MVVLGVSEES LLSLCQRTKN ...VWCANFN GGMQVVLAGI KDDLKALEPT

                                      *
S.coelicolor      KPEGVRKVV PLKVAGAFHT RHMAPAVDKL AE.AAKALTP ADPKVTYVSN
S.glaucescens     KPEGVRKIV PLKVAGAFHT HHMAPAVDKL AE.AARELSP ADPEVPYVSN
M.leprae          PPARAR.VR ALDVAGAFHT EFMTAALDGF AE.AAASITI AEPIAMLLSN
E.coli           CKAAGAKRAL PLPVSVPSHC ALMKPAADKL AVELAKFITF NAPTVPVVNN
H.pylori         LKEMGAKRVV FLEMSVASHC PFLEPMTFKF QELLEKSLK. DKFHFEIISN
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FIGURE 2: Polypeptide sequence comparison of the active site of different bacterial MATs. Asterisks mark conserved residues H96, S97, and H201 (numbering in *S. coelicolor*).

purified components (*6*, *7*), where polyketide synthesis was found to depend on the presence of the KS, the ACP, and the MAT.

All known acyl transferases involved in fatty acid and polyketide biosynthesis are homologous. Mutational, biochemical, and structural studies have established the centrality of two universally conserved residues: a serine residue (corresponding to S97A in the *S. coelicolor* protein) and a histidine residue (corresponding to H201 in the *S. coelicolor* protein). The catalytic cycle of ATs involves a ping−pong mechanism in which an acyl group is first transferred from a CoA thioester onto the serine residue, followed by transesterification of this acyl group onto the pantetheine arm of an ACP (*8*). Consistent with this prediction, X-ray crystallographic analysis of the MAT from the *Escherichia coli* fatty acid synthase revealed that the conserved serine and histidine residues lie in the active site of the enzyme and are hydrogen bonded in a fashion similar to various serine hydrolases (*9*). Finally, mutagenesis of AT domains in modular PKS (*10*) and FAS systems (*11*) has confirmed the requirement of this serine for AT activity. Surprisingly however, during the course of our kinetic analysis of the actinorhodin PKS from *S. coelicolor*, we observed that the S97A mutant of the *S. coelicolor* MAT was able to support PKS activity (*12*). Moreover, the mutant enzyme could be radiolabeled by [^{14}C]malonyl-CoA, suggesting that a second nucleophile exists in the MAT.

Here we have investigated the mechanism of acyl transfer by the *S. coelicolor* MAT using site-directed mutagenesis and biochemical analysis. In this study, we were guided by the strong similarity between primary amino acid sequences of several members of this family of proteins (Figure 2) and by the known crystal structure of the *E. coli* MAT (*9*).

MATERIALS AND METHODS

Materials. Silica gel plates Si250F for thin-layer chromatography were obtained from J. T. Baker. [^{14}C]Malonyl CoA (50 μCi/mL, 56 mCi/mmol) was purchased from Movarek Biochemicals. All other chemicals were from Sigma Chemical Co. and of the highest available grade.

Construction of MAT Mutants. The construction of the MAT S97A mutant has been described earlier (*12*). For all other mutants, the template used was pANS512, a construct with the *S. coelicolor fab*D gene in pET21c (*12*). MAT H96A mutant was constructed by replacing the CAC codon for H96 with GCT. The MAT H96AS97A double mutant was constructed by replacing the CAC AGC codons for H96−S97 by GCT GCT. The MAT H201A and MAT H201Q mutants were constructed from pANS512 by replacing the CAC codon for H201 with GCC and CAA, respectively. All

mutant genes were sequenced prior to biochemical analysis of their encoded products.

Enzyme Purification. The actinorhodin (*act*) ketosynthase-chain length factor (KS/CLF) heterodimer was purified from *Streptomyces coelicolor* CH999/pSEK38 as described earlier (*12*). The frenolicin (*fren*) holo-ACP was expressed in *E. coli* and purified as described in ref *12*. Wild-type and mutant MATs were purified as before on Ni-NTA agarose (Pharmacia) taking advantage of the C-terminal hexa-histidine tags on these proteins (*12*).

In Vitro Polyketide Assay. The production of SEK4 and SEK4b by the *act* PKS was followed in vitro using ^{14}C malonyl-CoA (3.5 mM, 0.71 mCi/mmol) as a substrate (*6*, *13*). A standard assay (100 μL reactions in 100 mM $NaPO_4$, pH 7.3, 2 mM DTT, and 2 mM EDTA) contained 0.8 μM KS/CLF (calculated as heterodimers), 20 μM ACP, and 100 nM MAT. Reactions were quenched by adding solid NaH_2PO_4, and reaction products were separated by thin-layer chromatography and quantified on an Instant Imager 2024 (Packard).

Protein Labeling Assay. A total of 10 pmol of MAT was incubated with 5 μL of [^{14}C]malonyl-CoA (50 μCi/mL, 56 mCi/mmol) for 15 min at room temperature in the presence or absence of 500 pmol of *fren* holo-ACP. Proteins were separated by 10% SDS−PAGE, and labeled species were detected by autoradiography on an X-ray film.

Identification of Labeling Sites. The locations of covalent attachment of malonyl groups in the active sites of wild-type and mutant MAT proteins were deduced by a combination of labeling, proteolysis, and liquid chromatography/mass spectrometry (LC/MS). Specifically, each protein (300 μL, 20 μM) was incubated with malonyl CoA (15 μL, 170 μM) at 37 °C for 8 min in 100 mM sodium phosphate buffer at pH 7.3. The labeling reaction was quenched, followed by lyophilization. The pellet was dissolved in a buffer containing 2 M urea and 0.1 M NH_4HCO_3, acidified to pH 2 using HCl, and incubated with pepsin (Sigma; MAT:pepsin = 25:1 w/w) at 37 °C for 1 h. The pepsin digest mixture was analyzed by LC/MS.

Mass spectra were recorded on a Finnigan LCQ Quadrupole ion trap mass spectrometer (Thermofinnigan, San Jose, CA) equipped with an electrospray source. Peptides were separated by reversed-phase HPLC on a Michrom Magic C18 column (Michrom, 1 × 50 mm) equilibrated with solvent A (solvent A: 0.1% formic acid and 0.025% trifluoroacetic acid in water). Elution of the peptides was accomplished using a gradient (2−42%) of solvent B over 30 min, followed by 95% solvent B for 3 min (solvent B, 0.085% formic acid and 0.022% trifuoroacetic acid in acetonitrile). Solvents were pumped at a constant flow rate of 200 μL/min. Spectra were obtained in full scan mode (m/z 200−2000) with data dependent zoom scan; the fragmentation patterns of peaks of interest were analyzed via tandem MS/MS.

Kinetic Analysis of Wild-Type and Mutant MATs. The normal reaction catalyzed by the *S. coelicolor* MAT is the transfer of malonyl groups from malonyl-CoA to a holo-ACP. Despite several attempts, we were unable to establish a kinetic assay for this reaction, presumably due to limitations associated with the reaction equilibrium at typical ACP concentrations (1−100 μM). However, *N*-acetylcysteamine (SNAC) could be used as a surrogate thiol acceptor in lieu of a holo-ACP. Although the K_M for this substrate is significantly higher than that for an ACP (see Results, below), in the presence of high SNAC concentrations the forward reaction could be driven to completion, thereby facilitating the measurement of steady-state kinetic parameters for the wild-type and mutant enzymes.

FIGURE 3: Labeling of MAT. Purified MATs were incubated with ^{14}C malonyl CoA as described in the text. Reaction products were separated on 10% SDS−polyacrylamide gels and analyzed by autoradiography. Lane 1, wild-type MAT; lane 2, S97A; lane 3, H96A; lane 4, H96A S97A double mutant; lane 5, H201A; lane 6, H201Q.

Assays were performed at room temperature (25 °C) in a total volume of 15 μL. The composition of the reaction mixture was as follows: 2 μM wild-type MAT or 4 μM mutant MAT, 1.0 mM SNAC, varying malonyl-CoA concentrations (10, 20, 30, 40, and 50 μM) in 100 mM sodium phosphate buffer at pH 7.3 containing 2 mM DTT and 2 mM EDTA. At appropriate time points, individual reactions were quenched with 10 μL of cold acetone, applied to silica gel TLC plates, and developed using a solvent system comprising of 89% ethyl acetate, 10% 2-propanol and 1% trichloracetic acid (TCA). The R_f of malonyl-SNAC was 0.59, as compared to an authentic reference standard of this compound (*14*). Product formation was quantified by counting radioactivity associated with malonyl-SNAC on an Instant Imager 2024 (Packard).

RESULTS

Qualitative Assays for Comparing the Activity of Wild-Type and Mutant MATs. Five mutants of the *S. coelicolor* MAT were constructed, expressed in *E. coli*, and purified as described in the Materials and Methods. They included S97A, H96A, H201A, H201Q, and a H96A/S97A double mutant. S97 and H201 correspond to the nucleophile and general base, respectively, in the serine protease-like active sites of all known acyl transferases from the PKS/FAS superfamily. H96 is present in most, but not all ATs; its role was unclear from the crystal structure of the *E. coli* enzyme (*9*). The qualitative properties of the wild-type and mutant enzymes were compared using three assays, described below.

First, we assayed the ability of the MAT to transfer malonyl groups from malonyl-CoA to itself by incubation with ^{14}C-malonyl-CoA, followed by SDS−PAGE and autoradiography. As shown in Figure 3, lane 1, the wild-type MAT was labeled, presumably at the S97 active site. Likewise, as shown in lanes 2 and 3, both the S97A and H96A mutants were labeled. [Labeling of the S97A mutant is consistent with our earlier observation that prompted us to initiate this study (*12*).] In contrast, the H96A/S97A double mutant was not labeled. Together, these results suggest that both H96 and S97 are sufficiently nucleophilic so as to displace CoASH from malonyl-CoA. The H201A mutant (lane 5) was weakly labeled, suggesting that presence of this basic residue is not essential for the nucleophilicity of either the S97 hydroxyl or the H96 imidazole (or both). In contrast, the H201Q mutant was not labeled (lane 6). This might suggest that either this protein is incapable of reacting with malonyl-CoA or the resulting malonyl−enzyme intermediate

Table 1: Mass Spectrometric Analysis of the Proteolytic Products Derived from Wild-Type MAT and the S97A Mutant to Which a Malonyl Group Was Found Attached

MAT	molecular weight	sequence
wild-type	1982.6 (parent ion)	TGPGFTPGAVAGHS(-malonyl)VGEITAA
	1321.8 (fragment ion)	TGPGFTPGAVAGHS(-malonyl)
	1420.4 (fragment ion)	TGPGFTPGAVAGHS(-malonyl)V
	1477.4 (fragment ion)	TGPGFTPGAVAGHS(-malonyl)VG
	831.4 (fragment ion)	S(-malonyl)VGEITAA
S97A	826.4 (parent ion)	GAVAGH(-malonyl)AVG
	711.2 (fragment ion)	AVAGH(-malonyl)AV
	599.2 (fragment ion)	GAVAGH(-malonyl)

[a] Both parent ion and the major fragment ion derived from the parent ions are reported. As shown in Figure 2, the active site serine (S97) of the *S. coelicolor* MAT is flanked by the sequence VAGHSV.

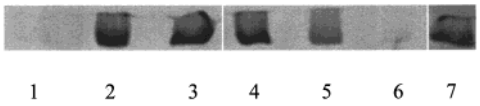

FIGURE 4: Labeling of ACP by wild-type and mutant MATs. ^{14}C label is transferred from ^{14}C malonyl-CoA to ACP by MAT in reactions described in the text. Lane 1, ACP only; lane 2, ACP + wild-type MAT; lane 3, ACP + MAT S97A; lane 4, ACP + MAT H96A; lane 5, ACP + MAT H96AS97A; lane 6, ACP + MAT H201A; lane 7, ACP + MAT H201Q.

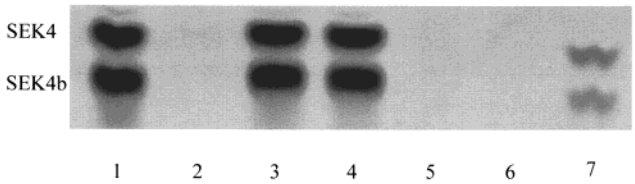

FIGURE 5: Activity of MATs in polyketide synthesis in vitro. Polyketide products are synthesized from ^{14}C malonyl-CoA, separated by TLC and visualized by autoradiography. All reactions contain *act* KS/CLF, *fren* ACP and MAT: lane 1, wild-type MAT; lane 2, no MAT; lane 3, MAT S97A; lane 4, MAT H96A; lane 5, MAT H96A S97A; lane 6, MAT H201A; lane 7, MAT H201Q.

is rapidly hydrolyzed in the absence of an ACP. As discussed below, the latter hypothesis is likely to be the case.

Second, we assessed the ability of the MAT to transfer the enzyme-bound malonyl group to the ACP by SDS−PAGE and autoradiography. As shown in Figure 4, lane 2, incubation of malonyl-CoA (45 μM), holo-ACP (5 μM), and MAT (0.1 μM) for 15 min at room temperature resulted in appearance of an intensely labeled malonyl-ACP band. In the absence of the MAT (Figure 4, lane 1), the ACP was not labeled. It could be concluded that the MAT catalyzes transfer of a malonyl group to the pantetheine arm of the ACP, since apo-ACP was not labeled under identical conditions (data not shown). The efficiency with which both the S97A (lane 3) and the H96A (lane 4) mutants label the ACP is comparable to the wild-type enzyme, whereas the H201A mutant (lane 6) was unable to label the ACP. H201Q (lane 7) transferred the labeled malonyl group onto the ACP, albeit with reduced efficiency compared to the wild-type protein. Interestingly, although the H96A/S97A double mutant was unlabeled in the earlier assay (Figure 3), it was able to transfer malonyl groups onto the ACP (lane 5) at a low level. These results confirm that both the S97A mutant and the H96A mutant can catalyze both half-reactions (i.e., acyl transfer onto the MAT and subsequently onto the ACP).

Finally, we determined whether the MAT was able to support polyketide synthesis in an in vitro assay using reconstituted holo-ACP and the *act* KS/CLF. As shown in Figure 5, lanes 1 and 2, the presence of the MAT is essential for polyketide synthesis. Likewise, both the S97A (lane 3) and H96A (lane 4) mutants of MAT are able to support SEK4 and SEK4b production, whereas within experimental limits, neither the H201A mutant (lane 6) nor the H96A/S97A double mutant (lane 5) appear to do so. H201Q (lane 7) shows low but measurable ability to support polyketide synthesis. Together, the results shown in Figures 3−5 support the well-established role of S97 as the nucleophile and H201 as a general base in the active site of the MAT. However, most unexpectedly, they suggest that the active site of the enzyme harbors a residue, H96, that can serve as an alternative catalytically active nucleophile in the absence of S97. To obtain direct evidence for this unprecedented finding, we performed additional protein chemical and kinetic experiments, described below.

Identification of Covalent Attachment Sites on Wild-Type MAT and the S97A Mutant. To identify the sites at which malonyl-CoA derived malonyl groups were covalently attached, wild-type MAT and the S97A mutant were subjected to pepsin digestion followed by LC/MS, as described in the Materials and Methods. The results of mass spectrometric analysis of these two labeled protein digests are summarized in Table 1. The labeled fragment S*VGEITAA (mass 831.4) in the fragmentation spectrum of wild-type MAT demonstrates that Ser 97 is the primary nucleophile in this protein. In S97A mutant, a labeled fragment GAVAGH*AVG was identified (malonyl group covalently attached to H96). These results confirm the above proposal that H96 serves as an alternative nucleophile in the S97A mutant of the *S. coelicolor* MAT.

Kinetic Analysis of Wild-Type and Mutant MAT Enzymes. To quantify the impact of the S97A and the H96A mutations on the catalytic activity of the *S. coelicolor* MAT, the steady-state kinetic parameters of the wild-type enzyme and the single mutants were measured as described in the Materials and Methods. In the presence of SNAC as the acceptor thiol, the k_{cat} of the wild-type enzyme was 0.41 min^{-1}, whereas the k_{cat} of the S97A mutant was 0.056 min^{-1}. The K_M values of the two enzymes for malonyl-CoA were relatively unchanged, 20.8 ± 4.8 μM for wild-type MAT and 23.9 ± 1.3 μM for the S97A mutant. The k_{cat} and k_{cat}/K_M values for the H96A mutant were too low to be measured accurately.

DISCUSSION

Although many enzymes are known to possess reactive functional groups in their active sites that are not associated with their normal catalytic cycle, the existence of "spurious"

catalytically active residues in the active site of an enzyme is a relatively rare phenomenon. Our studies on the FabD protein of *S. coelicolor* have revealed the existence of such a residue in its active site.

Transfer of acyl chains from CoA thioesters to acyl carrier proteins is a crucial step in fatty acid synthesis as well as polyketide synthesis. We have shown that polyketide synthesis by the *act* PKS in *S. coelicolor* requires the *fab*D gene product (*6*). FabD is a malonyl CoA:ACP transacylase (*4*, *5*, *15*, *16*). Acyl transfer by this family of enzymes occurs through a ping−pong mechanism (*8*) where the acyl group is transiently attached to a universally conserved serine residue in the active site of the enzyme via an oxoester linkage (Figure 2). Mutational analysis in type I PKS and FAS systems has confirmed the crucial role of this serine (*10*, *11*). In an attempt to construct a null mutant of the *S. coelicolor* MAT, we therefore mutated this serine into an alanine (*12*). To our surprise, the S97A mutant was capable of supporting polyketide synthesis by the *act* PKS. We therefore speculated that an alternative nucleophile could substitute for S97 in the ping−pong mechanism of this enzyme. Sequence comparison of several MAT proteins led to the identification of two conserved histidine residues, H96 and H201 (Figure 2). Both are located close to the active-site serine in the three-dimensional structure of a closely related protein, the *E. coli* MAT (*9*). Here we show via mutagenesis (Figures 3−5) and direct protein chemical analysis (Table 1) that S97 is the primary nucleophile in the active site of the wild-type *S. coelicolor* MAT, whereas H96 is a surrogate nucleophile that is catalytically active in the S97A mutant. We also show via mutagenesis that H201 plays the role of a general base in the serine−histidine catalytic dyad of the enzyme (Figures 3−5). In the absence of either S97 or H96, the enzyme is able to catalyze acyl transfer; however, in the absence of both residues activity is inadequate to support polyketide biosynthesis (Figure 5). The k_{cat} of the S97A mutant is within 10-fold that of the wild-type enzyme and the K_M is unperturbed, suggesting that the substrate binding pocket in the mutant remains intact and that H96 is a fairly potent nucleophilic catalyst. In contrast, the activity of the H96A mutant is significantly lower (even though it can support polyketide biosynthesis at substoichiometric concentrations relative to the KS and the ACP), suggesting that the integrity of the catalytic triad is influenced by the orientation of H96. In this context it is noteworthy that X-ray crystallographic studies of the wild-type MAT from *E. coli* had revealed that the histidine corresponding to H96 points away from the catalytic dyad (*9*). Structural analysis of the wild-type and mutant forms of the *S. coelicolor* MAT should therefore provide useful insights into the mechanistic basis for this unusual flexibility in the choice of nucleophilic residues in an enzyme active site.

Two other results of the mutagenesis experiments reported here deserve comment. First, it is intriguing that, although the double mutant H96A/S97A cannot support polyketide biosynthesis at a detectable level, it appears to transacylate malonyl groups from malonyl-CoA to the ACP at a very low level (Figure 4). The reason for this result is unclear, but is likely to be due to contamination of our preparations of the H96A/S97A double mutant by trace amounts of the *E. coli* MAT. Second, the importance of H201 as a general base is underscored by the fact that H201A has no detectable catalytic activity, whereas H201Q is very weakly active (Figures 4 and 5). Thus, not only does H201 enhance the nucleophilicity of S97 but it also enhances the electrophilicity of the corresponding oxoester intermediate. (Alternatively, it may aid in the departure of the serine leaving group from the tetrahedral intermediate.)

Why did a surrogate nucleophile evolve in the active site of a MAT from a type II PKS/FAS? Sequence analysis reveals that, whereas S97 and H201 are universally conserved among all known acyl transferases belonging to the FAS/PKS superfamily, H96 is absent in certain type II MATs such as the *mdc*G gene product in the malonate utilizing pathway of *Klebsiella pneumoniae* (PID no. g1314815; 37% identity to *E. coli* MAT; active-site sequence is GLSIG, where S is the catalytically active serine). Perhaps a surrogate nucleophile is required in organisms such as *S. coelicolor*, where a single MAT is shared by several biosynthetic pathways. Its presence may emphasize the importance of fatty acid biosynthesis, both as a primary metabolic activity and as a resource for the evolution of novel bioactive substances via secondary metabolism. Alternatively, it may simply represent an opportunistic role for H96 which ordinarily optimizes the role of S97 as the nucleophile in the wild-type MAT.

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