Structure-Based Mutagenesis of the Malonyl-CoA:Acyl Carrier Protein Transacylase from *Streptomyces coelicolor*†

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ABSTRACT: Malonyl-CoA:acyl carrier protein transacylase (MAT) provides acyl-ACP thioesters for the biosynthesis of aromatic polyketides, and thus is the primary gatekeeper of substrate specificity in type II PKS. A recent report described the X-ray crystal structure of the *Streptomyces coelicolor* MAT and suggested active site residues which may be important for substrate selectivity [Keatinge-Clay, A. T., et al. (2003) *Structure 11*, 147−154]. Mutants were made to test the proposed roles of these residues, and the enzymes were characterized kinetically with respect to native and non-native substrates. The activity of the MAT was observed to be greatly attenuated in many of the observed mutants; however, the K_m for malonyl-CoA was only modestly affected. Our results suggest the MAT uses an active site that is rigorously ordered around the acyl−thioester moiety of the acyl-CoA to facilitate rapid and efficient transacylation to an ACP. Our results also suggest that the MAT does not discriminate against α-substituted acyl-CoA thioesters solely on the basis of substrate size.

Polyketide synthases (PKSs)[1] are enzyme systems found in a variety of organisms that catalyze the biosynthesis of numerous medicinally important aromatic and macrocyclic natural products, including antibiotics, anticancer agents, and immunosuppressants (*1*, *2*). The biosynthesis of polyketides occurs in a manner analogous to fatty acid biosynthesis, wherein acyl-CoA esters are repeatedly condensed onto a growing enzyme-bound molecular chain (*3*, *4*). Growth of the chain is facilitated by a core set of three enzyme domains: a ketosynthase (KS), an acyl carrier protein (ACP), and an acyl transferase (AT) (*5*). Type I PKSs are large, modular enzyme systems which catalyze the biosynthesis of macrocyclic polyketide products through an "assembly line" of active sites. Individual domains act upon the growing chain and transfer it to downstream domains through a phosphopantetheine linkage (*2*). Unlike the type I PKSs, type II PKSs are responsible for the synthesis of aromatic polyketide compounds, and biosynthesize them using a set of smaller proteins with unique active sites (*3*). These proteins, including a KS, ACP, and a MAT, are used iteratively to construct the growing polyketide chain (*6*).

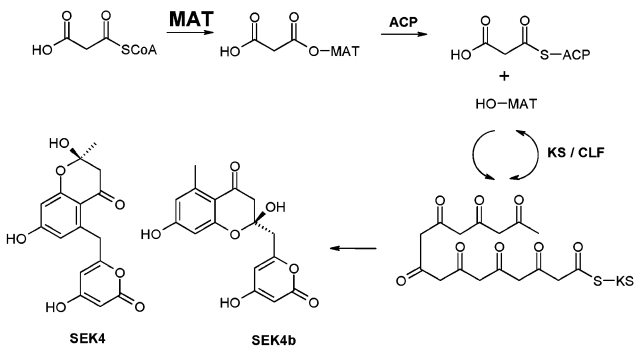

FIGURE 1: Biosynthesis of the aromatic compounds SEK4 and SEK4b using the minimal type II PKS.

The malonyl-CoA:acyl carrier protein transacylase (MAT) is part of a minimal set of enzymes required to facilitate type II polyketide synthesis (Figure 1) (*6*). The enzyme is recruited from the host's fatty acid biosynthesis pathway, and thus represents a unique example of "crosstalk" between primary and secondary metabolism (*3*, *4*, *7*, *8*). MAT catalyzes the transfer of malonyl groups onto ACPs, which are subsequently used to incorporate acyl groups into the growing polyketide chain by the ketosynthase/chain length factor (KS/CLF). In this fashion, the type of acyl-ACPs proffered to downstream enzymes is solely dictated by the MAT.

In both type I and type II PKS systems, the specificity for acyl groups incorporated into the growing chain is controlled at the level of the AT (*9*). Modification of this specificity through the replacement of native domains with foreign domains through genetic engineering has proven to be a fruitful means of obtaining polyketide compounds that contain non-native extender units (*10*−*13*). However, swapping of entire AT domains often leads to a severe reduction in the amount of polyketide produced, presumably due to

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[1] Abbreviations: ACP, acyl carrier protein; AT, acyl transferase; CD, circular dichroism; CoA, coenzyme A; DEBS, 6-deoxyerythronolide synthase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; fren, frenolicin; IPTG, isopropyl β-D-thiogalactopyranoside; KS, ketosynthase; KS/CLF, ketosynthase/chain length factor; LB, Luria-Bertani media; MAT, malonyl-CoA:acyl carrier protein transacylase; OD, optical density; PKS, polyketide synthase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SNAC, *N*-acetylcysteamine; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)-aminomethane; WT, wild type.

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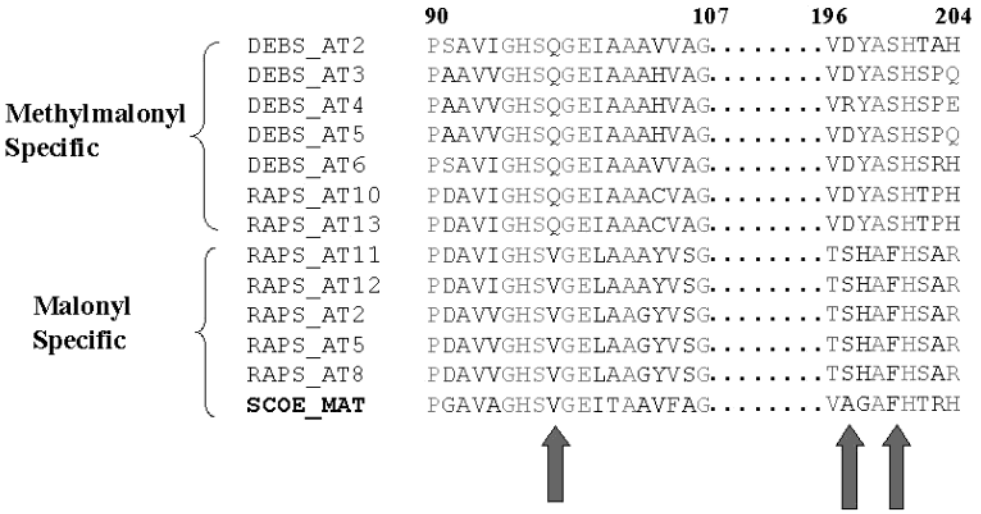

FIGURE 2: Selected regions from sequence alignments of malonyl-CoA and methylmalonyl-CoA specific type I PKS domains. The numbering of the residues is relative to the *S. coelicolor* MAT, which is included at the bottom of the alignment. Arrows denote residues conserved within their respective acyl-CoA specific domains.

the disruption of important protein−protein interactions within the PKS (*14*). Recently, a mutagenic strategy was employed to affect a relaxation in substrate specificity of the methylmalonyl specific AT4 domain of 6-deoxyerythronolide-B synthase (*15*). Sequence alignments of malonyl-CoA specific domains with domains specific for α-substituent-bearing CoA esters indicated that residues consistently different between the two may play a role in the specificity of acyl group binding (Figure 2) (*16*). Both single and multiple mutations of a number of these residues were introduced, and the engineered proteins were observed to accept both malonyl- and methylmalonyl-CoA. The most efficient catalyst in this regard accepted malonyl groups and methylmalonyl groups at a ratio of 1:1, albeit with an attenuated activity.

The X-ray crystal structure of the *Streptomyces coelicolor* MAT was recently determined to 2.0 Å (*17*). The overall fold of the enzyme is similar to its *Escherichia coli* counterpart (*18*); however, an acetate ion was observed bound to the active site in a manner which mimics malonyl binding. Using this ion as a model, the substrate binding pocket was more thoroughly defined, and residues were suggested which may be involved in substrate selectivity. Among these were residues believed to compose the oxyanion hole (V_{98}) and the "selectivity filter" (M_{126} and F_{200}) which was proposed to block binding of acyl-CoA esters bearing an α-substituent. These residues also correlate with those affecting a change in the specificity in the AT4 domain.

In this work, we have examined the proposed roles of residues in the active site of the *S. coelicolor* MAT through site-directed mutagenesis. Unlike the *E. coli* MAT, which is essential for survival of the organism and therefore difficult to manipulate via mutagenesis, the *S. coelicolor* MAT can be readily mutated in *E. coli*. We have characterized both the wild-type and mutant proteins kinetically with respect to native as well as non-native substrates. We observed that the activity of the MAT is greatly corrupted even upon subtle mutations of many of these residues. Our results suggest that the MAT catalyzes transfer of malonyl groups with an active site environment that is rigorously ordered around the acyl group. Contrary to what was previously believed, our observations also indicate that the MAT does not discriminate against α-substituent-containing CoA esters on the basis of size alone.

EXPERIMENTAL PROCEDURES

Materials. All chemicals and buffer components were purchased from Sigma unless otherwise noted. Ready-Safe scintillation fluid and 5 mL screw-capped vials were purchased from Beckman-Coulter. [2-^{14}C]Malonyl-CoA, DL-[2-^{14}C]methylmalonyl-CoA, and [1-^{14}C]acetyl-CoA were purchased from American Radiolabeled Chemicals. All mutageneses were performed using the QuikChange Site-Directed Mutagenesis Kit from Stratagene, and mutagenic oligonucleotides were purchased from Qiagen. DNA sequencing was performed by the Stanford University PAN facility. All buffers are reported at their observed pH at room temperature. Sequence alignments were constructed using Vector NTI.

Construction of Mutant MAT Genes. All mutagenesis was performed with the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions. All mutagenesis was performed on plasmid pGFL16, which contains the *S. coelicolor* fabD gene in a pET28-derived vector. Mutagenic primers were designed to have a melting temperature of ≥78 °C. The plasmids were transformed into *E. coli* XL1-Blue cells (Stratagene) and isolated, and mutations were verified by sequencing. All mutagenic primers and the resulting derivatives of pGFL16 are listed in Table 1.

Expression and Purification of Wild-Type and Mutant MAT Proteins. Wild-type and mutant MAT proteins were expressed and purified as described previously (*6*). Plasmids containing either WT MAT or mutant MATs were transformed into BL21(DE3) cells, and individual colonies were chosen for large-scale growth. One liter cultures of LB/ampicillin (100 μg/mL) were grown at 37 °C to an OD of 0.6, at which time protein synthesis was induced by the addition of IPTG (to a final concentration of 1 mM). Cultures were then grown for a further 5 h at 22 °C, or for 36 h at 13 °C for mutants which were observed to express poorly at 22 °C. Cells were harvested by centrifugation (4000 rpm, 20 min) and frozen at −80 °C until they were needed.

Table 1: Sequences of Primers Used for Mutagenesis and the Corresponding Mutant Constructs[a]

mutation	mutagenic primer sequence (5′−3′)	plasmid
Q_9A	cgtcgctcccggcGCgggcgcccagacgcccggc	pATK-I-75-4
$T_{57}V$	ccagatccgagacGTAtccgtggcccagccgc	pATK-I-36-1
$V_{98}Q$	ggcgcggtcgccggacagagcCAGggcgagatcaccgccgccg	pATK-II-39-1
$R_{122}A$	ccgccgcgctgtccctcgtacgccgtGCGggActggccatggccgaggcggcgg	pATK-I-75-12
$R_{122}K$	ccgccgcgctgtccctcgtacgccgtAAGggActggccatggccgaggcggcgg	pATK-I-75-13
$M_{126}I$	cgtcgcggcctggccatAgccgcggccgcgg	pATK-I-47-3
$M_{137}L$	ccgagaccggcTtgtcggcgctgc	pATK-I-36-9
$A_{197}D$	ccgctgaaggtggAcggcgcAttccacacccgcc	pATK-I-36-3
$G_{198}A$	cgtcccgctgaaggtggccGCCgcgttccacacccgccatatggcccccgccg	pATK-V-80-13
$G_{198}Y$	cgtcccgctgaaggtggccTACgcAttccacacccgccatatggcccccgccg	pATK-I-47-1
$F_{200}A$	ctgaaggtggccggcgcgGCCcacacccgccatatggcccccgccg	pATK-V-67-4
$F_{200}S$	ctgaaggtggccggcgcgTCCcacacccgccatatggcccccgccg	pATK-I-36-4
$F_{200}Y$	ctgaaggtggccggcgcgTACcacacccgccatatggcccccgccg	pATK-V-67-5

[a] Mutated bases are indicated in uppercase.

Generally, purification of the mutants closely paralleled the procedure for purification of the wild-type enzyme.

Frozen cells were resuspended in 10 mL of buffer A [50 mM Tris (pH 8.0)] with 1 μg/mL leupeptin and pepstatin A. Cells were lysed via sonication (3 × 30 s), and cellular debris was pelleted and removed by centrifugation (17 000 rpm, 30 min). Nickel−NTA agarose resin was added directly to the lysate (1.5 mL of resin in cells from 1 L), and the protein was allowed to bind to the resin in batch form for 45 min at 4 °C. The resin was poured into a fritted column and washed with 4.5 mL of buffer A. The column was washed with 1.5 mL of a higher-stringency wash buffer (buffer A with 5 mM imidazole) before elution of the MAT with 4.5 mL of elution buffer (buffer A with 150 mM imidazole). Eluted protein was applied directly to a Hi-Trap Q anion exchange column (Amersham-Pharmacia) and eluted using a linear gradient of buffer B (buffer A with 1 M NaCl). After this step, MAT was judged to be greater than 98% pure by SDS−PAGE. Fractions were collected, and purified proteins were dialyzed overnight into fresh buffer C [100 mM $NaHPO_4$ (pH 7.0), 2 mM EDTA, and 2 mM DTT]. Protein was then concentrated using Amicon ultra concentrators (Millipore), and sterile glycerol was added to a final concentration of 20%. The enzyme was placed into aliquots, flash-frozen, and stored at −80 °C until it was used.

Expression and Purification of Frenolicin ACP (fren ACP). The plasmid containing the frenolicin ACP (pFren) was introduced into host strain BAP1. This strain contains a chromosomal insertion of the *Bacillus subtilis* Sfp gene (*19*) and, consequently, post-translationally modifies the overexpressed ACP with a phosphopantetheine group. We observed that all ACP is expressed in the holo form. The *fren* ACP was expressed in a manner similar to that of the MAT, with postinduction growth carried out at 22 °C for 6−8 h. After being harvested by centrifugation (4000 rpm, 20 min), the cells were stored at −80 °C until they were used.

Frozen cells from a 1 L culture were resuspended in 10 mL of buffer C (buffer A with 2 mM EDTA and 2 mM DTT) supplemented with 1 μg/mL leupeptin and pepstatin A. We have observed that DTT is a requirement for all ACP purification and storage buffers, as the protein is unstable for long periods of time in its absence. Cells were lysed via sonication (3 × 30 s), and cellular debris was pelleted and removed by centrifugation (17 000 rpm, 30 min). The lysate was passed through a 0.45 μM syringe filter, loaded directly onto a Hi-Trap Q anion exchange column, and washed with 20% buffer D (buffer C with 1 M NaCl). A linear gradient from 20 to 50% buffer D was then used to elute the ACP. Fractions containing the ACP were pooled, and the protein was precipitated with ammonium sulfate (82.5%). At this point, the pH of the solution was lowered to 4.7 with glacial acetic acid, and the precipitate was collected by centrifugation (17 000 rpm, 90 min).

The protein pellet was then resuspended in a minimum amount of buffer E [100 mM $NaHPO_4$, 2 mM EDTA, 2 mM DTT, and 1.5 M $(NH_4)_2SO_4$ (pH 7.4)], passed through a 0.45 μM syringe filter, and loaded onto a phenyl-Sepharose column. The column was washed with buffer E, and elution of the ACP was accomplished with a linear gradient of buffer F [buffer E without 1.5 M $(NH_4)_2SO_4$]. Fractions containing the ACP were pooled and dialyzed overnight into fresh buffer F. If minor impurities remained after dialysis, a second round of anion exchange chromatography (Hi-Trap Q) was used in the same manner as described above. The protein was concentrated with Amicon centriprep 3 concentrators (Millipore); sterile glycerol was added to a final concentration of 20%, and the protein was divided into aliquots, flash-frozen, and stored at −80 °C until it was used. In our hands, this procedure reproducibly provides large quantities of ACP which is >99% pure by SDS−PAGE, and is free of any contaminating proteins which may be reactive toward the ACP.

Assay for MAT−ACP Transacylation. The assay we use is adapted from several previously reported procedures used in this and other laboratories (*20*, *21*). All assays were performed in buffer G [100 mM $NaHPO_4$ and 2 mM EDTA (pH 6.8)] supplemented with 1 mg/mL BSA to ensure protein stability at low concentrations. In every case, ACP and acyl-CoA thioesters were added to the solution and placed on ice, and the reaction was initiated with the addition of the MAT. Aliquots (8 μL) of the reaction mixture were removed at appropriate time points and quenched into 30 μL of ice-cold 30% TCA in an Eppendorf tube. Thirty microliters of 10 mg/mL BSA was added as a protein carrier, and the ACP was allowed to precipitate at 0 °C for 20 min. The precipitated protein was centrifuged (13000*g*, 5 min), and the supernatant was removed. Ice-cold 10% TCA (600 μL) was then added, and the solution was vortexed and centrifuged (13000*g*, 5 min). After the TCA solution had been decanted, the protein was dissolved in 100 μL of 98% formic acid. The protein solution was placed in a scintillation vial, and the Eppendorf tube was washed with 100 μL of water,

which was also combined in the vial. Five milliliters of Ready-Safe scintillation fluid was added, and the sample was vortexed. The extent of isotopic incorporation was then estimated by liquid scintillation counting.

Kinetic parameters were determined by fixing either ACP or acyl-CoA at an appropriate concentration to ensure saturation while varying its cognate substrate, if possible. Initial rate (<5% conversion) data obtained from these experiments were fit using the standard Michaelis−Menten equation. In cases where saturation was not possible, the concentration of both substrates was varied in a 4 × 4 matrix, and the resulting initial rates were fit to a two-parameter Michaelis−Menten equation. All analyses were performed in duplicate at five or six concentrations of varied substrate.

Radioactive Labeling of MAT. MAT (6.25 μM) and either 45 μM [2-^{14}C]malonyl-CoA (100 μCi/mL, 55 mCi/mmol), DL-[2-^{14}C]methylmalonyl-CoA (10 μCi/mL, 60 mCi/mmol), or [1-^{14}C]acetyl-CoA (50 μCi/mL, 50 mCi/mmol), both in the presence and in the absence of 20 μM ACP, were incubated in a total volume of 8 μL for a maximum of 2 min. The reaction was quenched by the addition of 4 μL of Laemmli buffer (lacking a reducing agent). Time course labeling reactions were performed in the same manner, with 8 μL aliquots removed from a larger reaction volume at 2, 5, and 10 min. Following quenching, samples were heated at 100 °C for 2 min and separated by SDS−PAGE. The gel was then vacuum-dried and analyzed by phosphorimaging.

Circular Dichroism Spectroscopy. The CD spectra of wild-type and mutant MAT proteins were measured from 190 to 250 nM in a buffer of 20 mM Tris (pH 8.0) and 20% glycerol using an Aviv 3DS CD spectrophotometer. The spectra were recorded at an enzyme concentration of 3.75 μM at 4 °C.

RESULTS

Selection, Construction, and Expression of Mutant MATs. The recently determined crystal structure of the wild-type MAT was used (*17*), in conjunction with sequence alignments of malonyl-CoA and methylmalonyl-CoA specific domains, to identify residues which were believed to impart substrate specificity to the MAT. In a fashion similar to that previously reported for the production of unnatural DEBS analogues (*15*), our strategy was to mutate residues in the malonyl-CoA specific MAT to the corresponding consensus residues in methylmalonyl-CoA specific domains. These mutations were made to residues in three groups. The first group is composed of residues M_{126}, G_{198}, and F_{200}, and is predicted to form the selectivity filter which was hypothesized to prevent binding of CoA esters with α-substituents. Mutations to residues in the selectivity filter of DEBS AT4 afforded the highest proportion of incorporated nonnatural substrate. The second group involves residues V_{98} and Q_9, the backbone amides of which are predicted to form the oxyanion hole for stabilization of developing charge on the substrate carbonyl in both catalyzed half-reactions. Manipulation of residues in the oxyanion hole of DEBS AT4 also had an effect on alteration of the substrate specificity. The third group involves active site residues which are conserved among malonyl specific domains, but whose role was not explicitly clear from the crystal structure. This group contains residues T_{57}, M_{137}, and A_{197}. We chose to introduce and characterize single mutations in each of the groups before constructing multiple mutations in an effort to more thoroughly determine the residues that are important for specificity. Additionally, we tested the proposed role of residue R_{122} and the side chain of Q_9, which are conserved throughout all domains and are in position to hydrogen bond to the malonyl-CoA carboxylate in the structure.

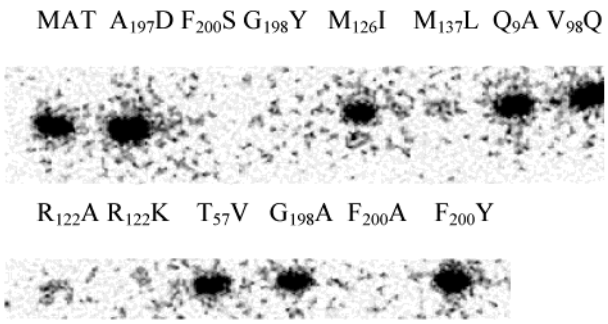

FIGURE 3: Self-acylation of wild-type and mutant MATs with malonyl-CoA. Solutions of MAT (6.25 μM) and [2-^{14}C]malonyl-CoA (45 μM) were allowed to react at 0 °C for 2 min, and the reactions were quenched with the addition of Laemmli buffer without DTT and quantified by SDS−PAGE radiography. Reactions were initiated by the addition of enzyme to a solution of radiolabeled acyl-CoA in reaction buffer.

Mutagenesis was performed, and the presence of all mutations was confirmed by gene sequencing. In most cases, overexpression of the mutant MATs followed the same procedure as that of the wild type. Several mutants (G_{198}Y, F_{200}S, R_{122}A, and R_{122}K) required modified overexpression conditions (13 °C and 24 h), and resulted in low protein yields. Although this observation is often interpreted as an indication of improper protein folding, the CD spectra of all the mutants that were examined, including those requiring a lower induction temperature, were not markedly different from that of the wild-type enzyme.

Qualitative and Quantitative Analysis of Enzyme Activity with Malonyl-CoA. The activity of the wild-type and mutant MATs was analyzed in two ways. First, we measured the ability of the proteins to self-acylate with malonyl groups from [^{14}C]malonyl-CoA. As shown in Figure 3, most mutants were efficiently labeled after 2 min. Interestingly, this probably does not reflect maximal occupancy of label for all of these enzymes, as the MAT and many of the mutants are observed to label efficiently at very short reaction times (10 s) (data not shown), and most likely have begun to hydrolyze some of the ES intermediate after 2 min. The mutants M_{137}I and F_{200}A required a slightly longer time (5 min) to be efficiently labeled, while G_{198}Y, F_{200}S, R_{122}A, and R_{122}K exhibited little to no self-acylation. The absence of a detectable label in the latter mutants might suggest either that the protein is incapable of reacting with malonyl-CoA or that the resulting acyl−enzyme intermediate is rapidly hydrolyzed in the absence of an ACP. Although the mutations impair the enzyme's ability to react with malonyl-CoA, we believe the latter case is more likely, as turnover of these mutants in the presence of ACP is easily quantifiable. Interestingly, the intensity of the label in many of the mutants was seen to persist for several minutes at room temperature. This is in stark contrast to the wild-type MAT, which loses its label rapidly, presumably to hydrolysis, even on ice.

Second, we measured the steady-state kinetic parameters of the wild-type and mutant enzymes with respect to

Table 2: Steady-State Kinetic Parameters for Malonyl-CoA and Mutant ACP Proteins

enzyme	k_{cat} (s^{-1})	$K_m^{malonyl-CoA}$ (μM)	K_m^{ACP} (μM)	$k_{cat}/K_m^{malonyl-CoA}$ ($s^{-1}\,\mu M^{-1}$)	k_{cat}/K_m^{ACP} ($s^{-1}\,\mu M^{-1}$)
WT	100 ± 7	19 ± 3	120 ± 20	5.3	0.83
$V_{98}Q$	95 ± 25	32 ± 10	120 ± 50	3.0	0.79
$A_{197}D$	70 ± 7	34 ± 3	130 ± 30	2.1	0.54
$T_{57}V$	25 ± 3	400 ± 25	570 ± 150	0.06	0.04
$R_{122}K$	11 ± 4	700[a]	3500[a]	0.015	0.003
$M_{126}I$	3 ± 0.7	23 ± 13	150 ± 70	0.13	0.02
Q_9A	0.7 ± 0.1	200 ± 30	920[a]	0.004	0.0008
$G_{198}Y$	0.40 ± 0.08	60 ± 30	130 ± 15	0.007	0.003
$R_{122}A$	0.35 ± 0.06	150 ± 10	1100[a]	0.002	0.0003
$M_{137}L$	0.30 ± 0.04	15 ± 4	100 ± 60	0.02	0.003
$F_{200}S$	0.15 ± 0.03	50 ± 25	100 ± 60	0.003	0.0015

[a] These values lie outside of the range of substrate concentrations accessible to our assay and should thus be interpreted as estimates.

Table 3: Steady-State Kinetic Parameters for Malonyl-CoA and ACP for Selected Mutations

enzyme	k_{cat} (s^{-1})	$K_m^{malonyl-CoA}$ (μM)	K_m^{ACP} (μM)	$k_{cat}/K_m^{malonyl-CoA}$ ($s^{-1}\,\mu M^{-1}$)	k_{cat}/K_m^{ACP} ($s^{-1}\,\mu M^{-1}$)
$G_{198}Y$	0.40 ± 0.08	60 ± 30	130 ± 15	0.007	0.003
$G_{198}A$	12 ± 5	40 ± 20	150	0.3	0.08
$F_{200}S$	0.15 ± 0.03	50 ± 25	100 ± 60	0.003	0.0015
$F_{200}Y$	36 ± 6	95 ± 15	75 ± 25	0.38	0.48
$F_{200}A$	0.94 ± 0.20	83 ± 30	240 ± 100	0.011	0.0040

malonyl-CoA. Although we have previously described a convenient assay for MAT activity which involves transacylation of malonyl groups onto the surrogate thiol acceptor *N*-acetylcysteamine (SNAC) (*22*), in this study we chose to measure the kinetics of transacylation onto a holo-ACP directly for two reasons. First, the greater sensitivity of the ACP assay is advantageous for the measurement of kinetic properties of mutants with low activity, and of unnatural substrates. Second, we wished to test the effect of the mutations on ACP binding, which is intrinsically more difficult using the SNAC assay. As noted previously (*22*), reaction equilibrium is rapidly reached in such an ACP assay; however, the assay has been modified to obtain initial rates.

The results of our kinetic analyses are summarized in Table 2. Although all of the mutants suffer an impaired ability to transacylate malonyl groups, interestingly, the K_m for malonyl-CoA is only modestly affected in most cases. This observation may suggest that the rate-limiting step in transacylation exists after the binding of malonyl-CoA. Of the mutants which exhibit a drastic increase in the K_m for malonyl-CoA, both R_{122} and Q_9 were predicted to interact directly with the carboxylate on malonyl-CoA. The loss of activity relative to the wild type observed in $R_{122}A$, and the subsequent restoration of moderate amounts of activity in $R_{122}K$, support the hypothesis that this residue is involved in a salt bridge interaction with the carboxylate. Thus, an increase in the observed K_m for malonyl-CoA for this mutant can be explained by the presence of disruptive interactions of the lysine on residues in the active site. A previous report documented a significant alteration of substrate specificity (from malonyl- to acetyl-CoA) via an analogous R to A mutation within a eukaryotic MAT (*23*). We, however, did not observe the transacylation of acetyl-CoA by $R_{122}A$ or any of the enzymes examined in this report. We also wished to test the role of Q_9 in the binding of malonyl-CoA. While Q_9A exhibited an expected drop in activity and an increase in K_m, we were unable to express soluble Q_9N. Residue T_{57} in the wild-type enzyme is proposed to form a hydrogen bond with Q_9. The loss of this bond may allow Q_9 more flexibility, which, in turn, may result in poorer binding to the malonyl carboxylate, which is reflected in the increase in the K_m for malonyl-CoA. Additionally, T_{57} and Q_9 are in the active site proximal to the proposed site of binding of the phosphopantetheine arm of ACP, and the elevated K_m for ACP in mutants of these residues may reflect a disruption of interactions with this moiety.

To assess the role of the residues that comprise the proposed selectivity filter, G_{198} was mutated into an Ala or Tyr residue, and F_{200} was mutated into an Ala, Ser, or Tyr residue. As shown in Table 3, a marked activity was seen for $G_{198}A$ relative to $G_{198}Y$, which may suggest the alanine mutation is less disruptive to the local fold of the enzyme than tyrosine. Similarly, $F_{200}Y$ retains levels of activity comparable to those of the wild-type MAT. The $F_{200}A$ mutation, however, is similar to its serine mutant counterpart. Together, this suggests that F_{200} may have structural contacts that are important for the interior of the active site, and that activity is largely affected without retaining these contacts with the presence of an aliphatic residue.

Qualitative and Quantitative Analysis of Enzyme Activity with Methylmalonyl-CoA. The ability of the mutant enzymes to transacylate non-native acyl groups, specifically methylmalonyl groups, onto holo-ACP was investigated using the same techniques. To our surprise, we observed that the wild-type MAT is capable of self-acylating and transferring a methylmalonyl group onto a holo-ACP, albeit at very low levels. As shown in Figure 5, the wild-type enzyme and three mutants ($A_{197}D$, $V_{98}Q$, and Q_9A) exhibited some ability to self-acylate with methylmalonyl-CoA. Remarkably, in the absence of an acceptor thiol such as phosphopantetheine, the methylmalonyl label persisted for several minutes at room temperature. At 4 °C, the intensity of the self-acylation, as measured by phosphorimaging, displayed a measurable increase with time to a maximum occupancy (approximately 5 min), followed by loss through hydrolysis. This observation is in contrast to that for malonyl-CoA, as the MAT and these

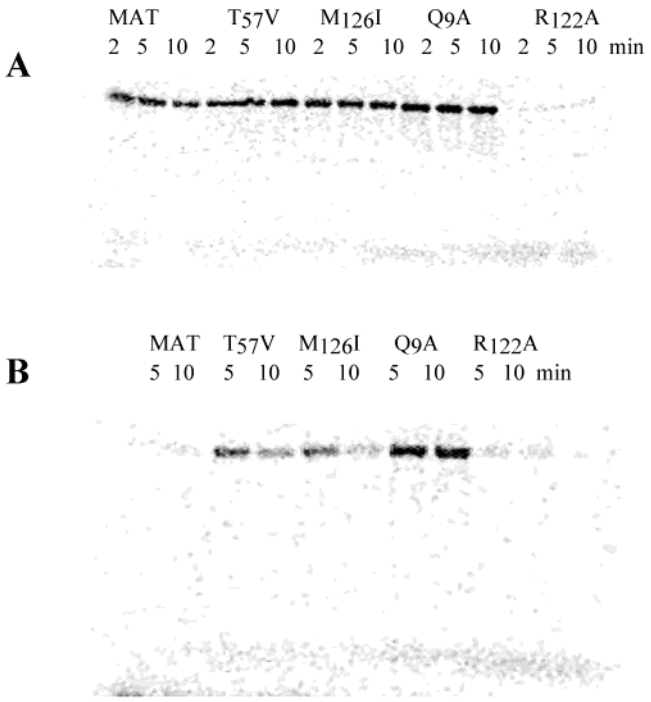

FIGURE 4: Persistence of the malonyl−enzyme intermediate in wild-type and selected mutant enzymes at 0 (A) and 22 °C (B). Solutions of MAT (6.25 μM) and [2-^{14}C]malonyl-CoA (45 μM) were allowed to react for either 2, 5, or 10 min (0 °C) or 5 and 10 min (22 °C), and the reactions were quenched with the addition of Laemmli buffer without DTT and quantified by SDS−PAGE radiography. Reactions were initiated by the addition of enzyme to a solution of radiolabeled acyl-CoA in reaction buffer.

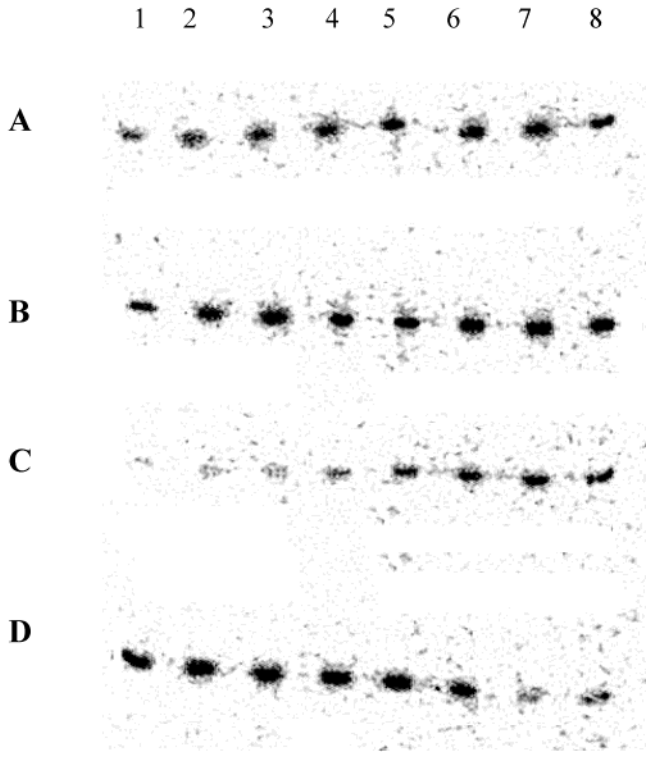

FIGURE 5: Self-acylation of wild-type and mutant MATs with methylmalonyl-CoA for (A) the wild type, (B) $A_{197}D$, (C) Q_9A, and (D) $V_{98}Q$. Solutions of MAT (6.25 μM) and [2-^{14}C]methylmalonyl-CoA (45 μM) were allowed to react before the reactions were quenched by the addition of Laemmli buffer without DTT and quantified by SDS−PAGE radiography. Reactions were initiated by the addition of MAT to a solution of radiolabeled acyl-CoA in reaction buffer. Lanes are as follows: (1) 1 min after initiation of the reaction, (2) 2 min after, (3) 3 min after, (4) 5 min after, (5) 10 min after, (6) 15 min after, (7) 20 min after, and (8) 30 min after.

mutants were observed to label rapidly to the maximum observed occupancy (~10 s) followed by a hydrolytic loss of label.

All four proteins were capable of transferring the label onto holo-ACP, and the three most active enzymes are shown in Figure 6. Steady-state rates of turnover, however, proved to be unattainable. Apparent k_{cat}/K_m measurements were obtained from time course reactions. The values for the wild type, $A_{197}D$, and $V_{98}Q$ were $(1.1 \pm 0.3) \times 10^{-5}$, $(3.0 \pm 1) \times 10^{-5}$, and $(0.6 \pm 0.1) \times 10^{-5}$ s^{-1} μM^{-1}, respectively. Transacylation catalyzed by Q_9A was too slow to be assessed accurately.

DISCUSSION

The incorporation of non-native acyl groups into a growing polyketide chain by domain swapping has proven to be an effective means of generating "unnatural" natural products via type I PKS (*24*). This technique has been hindered by low yields of the polyketide product, as the introduction of foreign domains can disrupt important protein−protein interactions in the PKS (*14*). Recently, the substrate selectivity of an isolated AT domain was altered using mutagenesis, and the biosynthesis of non-native products was demonstrated to occur much more efficiently than it does for domain-swapped PKS systems (*15*).

The MAT is the primary gatekeeper of substrate specificity in type II PKS systems, and the reaction catalyzed by the MAT dictates the type of acyl-ACPs available to the KS/CLF. Modulation of MAT selectivity is therefore important for the process of producing unnatural aromatic PKS in an appropriate host. The X-ray crystal structure of the *S. coelicolor* MAT, along with sequence alignments and the aforementioned type I mutational data (*17*), has suggested that substrate specificity is dictated by several active site residues. We have examined the effects of mutating these residues, along with others that are postulated to play a more direct role in substrate binding.

Previous observations by this and other laboratories has verified that S_{97} and H_{201} are the catalytic residues in the WT enzyme (*25*). The residue corresponding to R_{122} in the *S. coelicolor* enzyme had been hypothesized to form a salt bridge with the substrate carbonyl group in the MAT (*23*). Mutation of this residue to alanine in a eukaryotic MAT effected a marked change in selectivity for acetyl-CoA relative to malonyl-CoA. In our hands, mutating R_{122} to alanine results in a severe loss in k_{cat} relative to that of the wild-type enzyme, which is partially recovered upon mutation of R_{122} to lysine. These data support the postulated role of R_{122} in binding malonyl-CoA. The carbonyl group is also positioned to interact with another active site residue, Q_9. A loss of activity is also noted upon mutation of Q_9 to alanine; however, the corresponding mutation of Q_9 to Asn did not yield a soluble protein. We believe the R_{122} and Q_9 mutations have secondary effects on the overall structure of the active site in addition to the loss of malonyl-CoA binding affinity, as K_m values for both ACP and malonyl-CoA are increased dramatically relative to that of the wild type, and the enzymes overexpress poorly using conditions suitable for the wild-

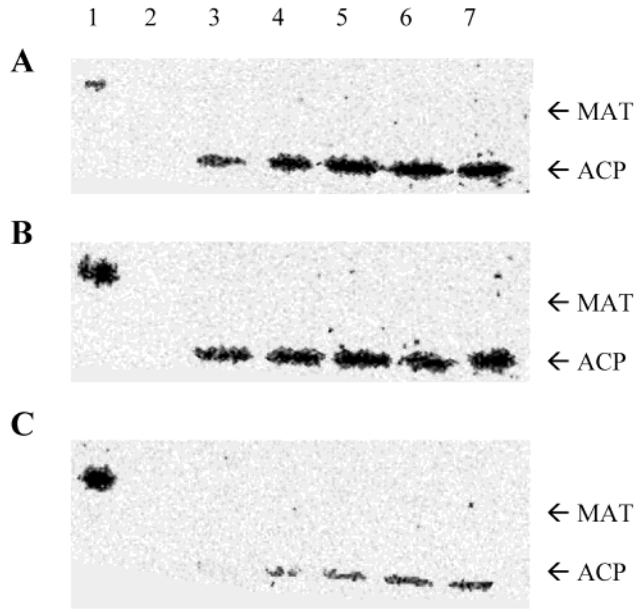

FIGURE 6: Transacylation of methylmalonyl groups onto holo-ACP for (A) the wild type, (B) $A_{197}D$, and (C) $V_{98}Q$. Solutions of MAT (6.25 μM), ACP (20 μM), and [2-^{14}C]methylmalonyl-CoA (45 μM) were allowed to react before the reactions were quenched by the addition of Laemmli buffer without DTT and quantified by SDS−PAGE radiography. Reactions were initiated by the addition of MAT to a solution of radiolabeled acyl-CoA and ACP in reaction buffer. A separate control reaction to verify the incorporation of methylmalonyl groups onto MAT is provided in lane 1. Lanes are as follows: (1) no ACP, (2) reaction mix prior to addition of MAT (0 min), (3) 2 min after addition of MAT, (4) 4 min after, (5) 6 min after, (6) 8 min after, and (7) 10 min after.

type MAT. A similar effect may be seen upon mutation of T_{57}. T_{57} was proposed to help orient Q_9 through a hydrogen bond. The K_m values for both malonyl-CoA and ACP are increased with the loss of this hydrogen bond through mutation to valine, and we believe this may also be attributable to a secondary effect. Neither the wild type nor any of the mutants we examined were active toward acetyl-CoA, which may highlight the differences in active site structure between vertebrate and bacterial MATs.

Although most of the mutations we introduced included residues not specifically involved in catalysis, many mutations affected a severe loss in the activity of malonyl-CoA transacylation. Unexpectedly, this loss in activity is largely not reflected in an increase in K_m for either malonyl-CoA or ACP. Moreover, CD spectroscopy suggested that none of these mutants had a tertiary structure markedly different from that of the wild-type MAT. We therefore believe these kinetic problems may be explained if precise organization in the MAT active site is required for rapid transacylation. Minor modifications may not hinder the capacity of the active site for acyl-CoA binding, but would allow for more motion of the acyl−thioester moiety of malonyl-CoA, or the E−S intermediate. This in turn could result in a lower rate of transacylation if interaction of the thioester carbonyl with the oxyanion hole occurs less efficiently than it does in the wild-type enzyme. This hypothesis is supported by experiments designed to test the self-acylation abilities of the mutant MATs. The wild-type enzyme self-acylates rapidly, and subsequently deacylates (hydrolyzes) within seconds in the absence of an ACP even at reduced temperatures. However, many self-acylated mutants retain the label for minutes at room temperature. This observed capacity for hydrolysis could be viewed as an approximation of E−S reactivity. One could envision a scenario wherein substrate binding is primarily mediated by contacts to the CoA nucleotide or phosphopantetheine arm, but acyl group transfer is mediated by efficient orienting of the acyl ester by the active site residues. Conservative mutations performed on or near residues in the selectivity filter also support this model, as $F_{200}Y$ and $G_{198}A$ have activities and acylation profiles more comparable to those of the wild type than mutations which introduce drastically different side chains ($F_{200}S$, $F_{200}A$, and $G_{198}Y$).

Our results also indicate that, contrary to what was previously assumed, the MAT does not rigorously exclude all CoA esters with α-substituents. This property seems somewhat unique to the MAT, as type I extender AT domains do not self-acylate or transacylate with non-native substrates (*26*). The wild-type enzyme and three mutants were observed to possess some ability to both self-acylate with methylmalonyl groups, as well as to transfer the labeled methylmalonyl group onto an ACP, albeit at a greatly decreased capacity relative to the native substrate. We were unable to observe steady-state rates of transacylation of ACP with methylmalonyl-CoA, so we obtained the apparent bimolecular rate constant for this substrate through a rate versus substrate plot (*27*). It is important to note that the slope of this plot will only yield a true approximation of k_{cat}/K_m if the observed rates are not influenced by a burst of product formation. Both self-acylation alone and transacylation of methylmalonyl groups from the CoA ester to ACP are observed to occur on roughly the same time scale. Additionally, intercepts of product versus time plots at varying enzyme concentrations are independent of the amount of added protein. Given this, we have no evidence to suggest that a burst exists with the MAT and methylmalonyl-CoA. A rate versus substrate plot for methylmalonyl-MAT acylation also has a slope similar to that for methylmalonyl-ACP transacylation (data not shown). Of the mutants capable of methylmalonyl transacylation, only the A_{197} mutant has a specificity constant larger than that of the wild-type enzyme. On the basis of the crystal structure, this residue is not located in the active site. We believe that the mutation may afford a slightly larger pocket for methylmalonyl binding without perturbing the contacts in the active site necessary for efficient acylation.

Our explanation of the decreased activity toward malonyl-CoA observed in the mutants may also be invoked to explain the sluggishness of the enzyme toward methylmalonyl-CoA. As shown in Figure 5, the methylmalonyl label on MAT persists after 30 min at room temperature. Additionally, the methylmalonyl moiety is transacylated from the corresponding CoA thioester at a greatly diminished rate relative to malonyl-CoA. It is conceivable that the α-methyl group present on methylmalonyl-CoA prevents efficient orientation in the active site to allow for rapid acylation, whether that is to form the E−S complex, or to form methylmalonyl-ACP. An analogous acylation reaction in serine proteases is believed to occur using both active site chemistry and conformational isomerization of the active site (*28*, *29*). Perhaps the MAT uses a similar catalytic strategy, and the methyl group in methylmalonyl-CoA could possibly impede this isomerization.

An obvious question would be why the mutations made in the context of a modular AT yield a domain with relaxed specificity, whereas the same mutations in the *S. coelicolor* MAT do not. The answer may lie in the manner in which acyl-CoA substrates are bound in the active site. In the case of the methylmalonyl specific AT4, the non-native substrate, malonyl-CoA, has room to bind in the methylmalonyl pocket, but most likely also has considerable motional freedom and does not orient into the oxyanion hole efficiently. Mutations to shrink the methylmalonyl pocket serve to better orient the malonyl-CoA ester into the oxyanion hole, and to facilitate transacylation to the ACP. Additionally, orientation of methylmalonyl-CoA into the oxyanion hole in the shrunken pocket is also more difficult. In the case of MAT, the interaction of methylmalonyl-CoA with the oxyanion hole could not be mediated by restricting movement in the active site via mutagenesis. More significant active site reorganization is required to facilitate efficient interaction with the oxyanion hole, and this strategy is inherently more difficult, as multiple mutations may damage the active site architecture as a whole. Indeed, no multiple mutant examined in this report was capable of self-acylation or transacylation with any acyl-CoA.

In summary, guided by the X-ray crystal structure of the *S. coelicolor* MAT, we have prepared and analyzed a series of mutant enzymes. Our findings highlight the importance of several apparently noncatalytic residues in the active site for maintaining the high velocity of malonyl-CoA:ACP transacylase activity of the enzyme. They also suggest that re-engineering MAT specificity toward unnatural acyl-CoA substrates would not just require alterations that allow the acyl group to bind in the active site, but would also need to orient this acyl group precisely to facilitate transesterification by this ping-pong enzyme.

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