A New Family of Type III Polyketide Synthases in
Mycobacterium tuberculosis*

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The Mycobacterium tuberculosis genome has revealed a remarkable array of polyketide synthases (PKSs); however, no polyketide product has been isolated thus far. Most of the PKS genes have been implicated in the biosynthesis of complex lipids. We report here the characterization of two novel type III PKSs from M. tuberculosis that are involved in the biosynthesis of long-chain α-pyrones. Measurement of steady-state kinetic parameters demonstrated that the catalytic efficiency of PKS18 protein was severalfold higher for long-chain acyl-coenzyme A substrates as compared with the short-chain precursors. The specificity of PKS18 and PKS11 proteins toward long-chain aliphatic acyl-coenzyme A (C12 to C20) substrates is unprecedented in the chalcone synthase (CHS) family of condensing enzymes. Based on comparative modeling studies, we propose that these proteins might have evolved by fusing the catalytic machinery of CHS and β-ketoacyl synthases, the two evolutionarily related members with conserved thiolase fold. The mechanistic and structural importance of several active site residues, as predicted by our structural model, was investigated by performing site-directed mutagenesis. The functional identification of diverse catalytic activity in mycobacterial type III PKSs provide a fascinating example of metabolite divergence in CHS-like proteins.

Mycobacteria are classified in the phylogeny of the Actinomycetes, along with the Streptomyces bacteria. Interestingly, these two actinomycete genera have received immense attention due to their contrasting effects on human society. Whereas Streptomyces have provided a rich source of antibiotics and other therapeutic products for human diseases, Mycobacterium tuberculosis and Mycobacterium leprae have been two of humankind’s greatest scourges. Although the original phylogenetic classifications of Mycobacterium and Streptomyces were based primarily on morphology, the genome sequences of these organisms have further established their common lineage (1, 2). These genome sequences have revealed several families of genes that are common between them, which include an unusually large number of gene clusters that are homologous to polyketide synthases (PKSs). Although a polyketide product has not yet been isolated from M. tuberculosis, recent studies have implicated some of its PKS genes in the biosynthesis of complex lipids (3). In this report, we have characterized two novel polyketide synthases from M. tuberculosis, which are involved in the biosynthesis of unusual long-chain α-pyrones.

Polyketides are a diverse class of secondary metabolites that possess a broad range of biological activities (4). Despite structural diversity of these natural products, PKSs synthesize polyketides by a common chemical strategy. Initial priming by a starter molecule is followed by repetitive decarboxylative condensation of coenzyme A (CoA) analogues of simple carboxylic acids. PKS elongates the polyketide chain either by repetitively using a single active site to perform multiple condensation reactions or by using a modular assembly line mechanism (5–7). Based on protein architecture, PKSs have been classified in three distinct families. Type I PKSs are giant assemblies of multifunctional polypeptides and include both iterative and modular mechanism of biosynthesis. Type II PKSs are a large multienzyme complex of discrete proteins and resemble type II fatty acid synthases found in bacteria and plants. The type III PKS has been recently discovered in bacteria and belongs to the plant chalcone synthases (CHSs) superfamily of condensing enzymes (8). These are homodimeric proteins and are structurally and mechanistically quite distinct from type I and type II PKSs (9). Type III PKSs use free CoA substrates without the involvement of a 4′-phosphopantetheine group.

CHSs are ubiquitously present in higher plants and catalyze biosynthesis of starting materials for many flavonoids (10). Several new additions to the CHS superfamily, such as acridone synthase, 2-pyrone synthase (2PS), and benzoaceton synthase, deviate from the chalcone biosynthetic model by either utilizing non-phenylpropanoid starter units or varying the number of condensation reactions (11). Some of the plant enzymes also show different cyclization patterns, emphasizing the growing diversity of the CHS superfamily. The three-dimensional crystal structures of CHS from Medicago sativa (alfalfa) (12) and 2PS from Gerbera hybrida (daisy) (13) have led to a more comprehensive understanding to structurally rationalize the mechanistic differences between enzymes within the thiolase family. Presently, the thiolase fold enzymes can be grouped into three families (11, 14, 15). β-Ketoacyl acyl carrier protein synthase (KAS I) and KAS II proteins comprise one group, and KAS III and type III PKS form another group. The third group consists of several thiolases. These enzymes have a conserved αβαβ fold architecture that forms one side of the active site cavity. The positioning of the catalytic cysteine with respect to the dimer interface is also conserved in this family of enzymes. The differences primarily exist in the structural loops that form the other face of the cavity. The identity

1,3,6,8-tetrahydroxynaphthalene; THNS, 1,3,6,8-tetrahydroxynaphthalene synthase; ESI, electrospray ionization; MS, mass spectrometry; HPLC, high pressure liquid chromatography.
and the number of the key catalytic amino acid residues might account for the observed differences in the catalytic machinery. The crystal structures also provide a framework for developing structure-based approaches for characterizing other CHS homologues. Over the past several years, CHS homologues have also been reported in bacteria. The type III PKS involved in the biosynthesis of 1,3,6,8-tetrahydroxynaphthalene (THN) has now been characterized from S. griseus (16), S. erythraea (17), S. antibioticus (18) and S. coelicolor (19). All of these THN synthases (THNSs) use malonyl-CoA as starter and extender units. The chain elongation proceeds through successive decarboxylative condensations of C-2 units to form pentaketide, which is then cyclized into THN. The THNSs from S. griseus (also known as RppA) has been shown to possess broad substrate specificity to yield a wide variety of products (20). In several strains that produce vancomycin group of natural products, a CHS-like protein performs the biosynthesis of 3,5-dihydroxyphenylglycine by using several molecules of malonyl-CoA as substrate (21–23). The precursor to the broad spectrum antimicrobial agent 2,4-diacetylphloroglucinol is produced by a CHS homologue (phiD) in many strains of fluorescent Pseudomonas sp. (24). The PhlD protein uses acetoacetyl-CoA and two molecules of malonyl-CoA to form monoacetyl phloroglucinol, which is then converted to 2,4-diacyltetruglucinol.

In this study, we have characterized two type III PKS proteins, PKS18 and PKS11, from M. tuberculosis that are involved in the biosynthesis of long-chain e-lysines. Kinetic analysis indicated that the catalytic efficiency of PKS18 protein to use long-chain acyl-CoA substrates is severalfold higher than that for small-chain substrates. This remarkable specificity of mycobacterial proteins is unprecedented in the family of CHS-related proteins. Molecular modeling studies suggested that these proteins might have evolved by combining the active sites of CHS and KAS proteins, the two mechanistically related members of the thiolase fold enzymes. The catalytic and structural importance of several active site residues, as predicted by members of the thiolase fold enzymes. The catalytic and structural importance of several active site residues, as predicted by members of the thiolase fold enzymes. The catalytic and structural importance of several active site residues, as predicted by members of the thiolase fold enzymes. The catalytic and structural importance of several active site residues, as predicted by members of the thiolase fold enzymes.

EXPERIMENTAL PROCEDURES

Materials—BAC genomic library of M. tuberculosis was obtained from Prof. Stewart Cole of Pasteur Research Institute (1). [2,14C]Malonyl-CoA (58.40 mCi/mmol) was procured from PerkinElmer Life Sciences, and nonradioactive acyl-CoA starter substrates were purchased from Sigma. Plant-specific starter substrates were kindly donated by Joseph P. Noel’s group. [2,14C]Lauroyl-CoA (57 mCi/mmol) was synthesized using an acyl-CoA synthetase protein.2

Cloning, Expression, and Purification—Type III PKS genes, pks10, pks11, and pks18, were amplified by PCR from the BAC genomic library of M. tuberculosis using gene-specific primers and cloned into pBlue-script SK (Stratagene). The authenticity of clones was confirmed by automated nucleotide sequencing. These genes were subcloned into the pET21c vector system (Novagen) and expressed as C-terminal hexahistidine-tagged proteins in the BL21(DE3) strain of Escherichia coli. Optimum soluble expression was achieved by growing cultures at low temperature. Whereas induction at 22 °C with 0.5 mM isopropyl-thio-β-D-galactopyranoside led to expression of PKS18 in soluble form, PKS11 protein (~20%) was obtained in the supernatant by growing noninduced cultures at 18 °C for 24 h. Under the experimental conditions tested, PKS10 persistently expressed as inclusion bodies and could not be rescued in the soluble form. Proteins were partially purified using Ni2+–nitrilotriacetic acid-agarose (Qiagen) and further resolved on a Resource Q anion exchange column (Amersham Biosciences).

Mutagenesis—PKS18 mutants A148T, A148S, A148F, L348S, and K318A were generated using the QuikChange site-directed mutagenesis kit (Stratagene). Mutagenesis reaction was carried out in accordance with the manufacturer’s protocol. Mutant clones were screened by restriction endonuclease digestion and confirmed by automated DNA sequencing. All of the mutant proteins were expressed and purified analogous to the wild type PKS18 protein. A148T, A145S, and A148S mutants expressed as inclusion bodies and could not be expressed in soluble form.

Enzyme Assay and Product Characterization Using Mass Spectrometry—The standard reaction conditions involved 100 μM starter molecule and 50 μM malonyl-CoA (inclusive of 9.12 μM [2-14C]malonyl-CoA (58.40 mCi/mmol)). Reactions were carried out with 45 μg of protein at 30 °C for 10–60 min and quenched with 5% acetic acid. Products were extracted with 2 × 300 μl of ethyl acetate and dried under vacuum. Radiolabeled products were resolved on silica gel 60 F254 TLC plates (Merck) in ethyl acetate/hexanes/acetic acid (65:27.5:5, v/v/v). Reaction products were resolved on reverse phase columns (C18). A linear gradient of 30% CH3CN in H2O (each containing 2% acetic acid) to 60% CH3CN in H2O over 30 min was used for separating hexanoyl-CoA-primed reaction products. Products of lauroyl-CoA-primed reaction were separated using a solvent system of 20 mM ammonium acetate, pH 5.4 (solvent A), and CH3CN/H2O/CH3OH (42:5.7:5, v/v) (solvent B). Polyketide products were characterized using nanospray electrospray ionization (ESI)-mass spectrometry (MS) API QSTAR Pulsar i MS/MS, Applied Biosystems).

Determination of Kinetic Parameters—A standard reaction volume of 75 μl contained 100 μM malonyl-CoA, inclusive of 9.12 μM [2-14C]malonyl-CoA (58.40 mCi/mmol), 45 μg of protein, 50 μM Tris, pH 7.5, 1 mM MgCl2, 10% glycerol and varied (0.5–20%) was obtained in the supernatant by growing one phase condensations of C-2 units to form pentaketide, which is then cyclized into THN. The THNSs from S. griseus (also known as RppA) has been shown to possess broad substrate specificity to yield a wide variety of products (20).

M. tuberculosis. Two of these genes, pks10 and pks11, are annotated putative chalcone synthases, whereas pks18 (which shows similar sequence similarity) is surprisingly listed as a conserved hypothetical protein in the revised M. tuberculosis genome annotations (30). The pks10

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and pks11 genes are clustered in an unusual organization with other PKS genes that encompass 18 kb of genomic DNA. The pks18, on the other hand, is not flanked by any PKS-related genes. All three genes are completely conserved in M. bovis BCG, suggesting their functional relevance in mycobacteria.

Comparative analysis of protein sequences of plants and microbial type III PKSs have previously led to the clustering of these proteins based on species. The dendrogram based on complete protein sequences had two major branches corresponding to plants and microbial PKSs; further categorization within the plant family was not feasible (8, 17). Since protein sequence diversity between plant and bacterial type III PKSs correlates with functional differences between the two systems, it is possible that the active site residues in the catalytic pocket can facilitate more reliable clustering. Similar active site sequence analyses of acyl transferase domains from modular PKSs (31) and adenylation domain of nonribosomal peptide synthetases (32) have indicated much better correlation with their functions. Fig. 1 shows a dendrogram of all microbial and some of the plant type III PKSs, constructed by using 32 active site residues that have been identified in the three-dimensional structure of CHS. The product biosynthesized by the corresponding PKS is drawn next to its branch (proteins and their products are drawn in the same color). It can be noticed that all the plant polyketide synthases cluster together. The eight CHS proteins that have been selected randomly have identical sets of active site residues. Interestingly, the plant PKSs associated with divergent products appear at different branches in the dendrogram, which was not the case when analysis was done with the entire protein sequence. The three distinct types of bacterial type III PKSs that have been experimentally characterized also cluster in three distinct branches. Here again we observe that active site residues are completely conserved among their family members. This trend of complete conservation of active site residues may be a manifestation of biased data set, since most of the sequences are available from the same genus. There are presently seven protein sequences in the THN synthase family, and six of them have been experimentally characterized. It can be predicted with reasonable confidence that the seventh sequence would also be involved in THN biosynthesis, which was also recently suggested by Austin and Noel (11). This protein was probably wrongly annotated as PhD homologue during S. avermitilis genome sequencing (33). Several PhD homologues from Pseudomonas fluorescens also possess an identical set of active site residues, indicating that they are involved in the biosynthesis of the same metabolite. The other bacterial type III proteins of unknown function are present in a separate branch in the dendrogram. Although several important catalytic and structural residues are conserved, these CHS-like enzymes from bacterial genomes have significant variation at several amino acid positions. Our sequence comparison analysis therefore could not predict putative function of mycobacterial type III PKSs. In order to characterize the function of these mycobacterial type III PKSs, we have used two distinct approaches. In the genetic-based approach, the pks10-pks11 cluster of mycobacteria has been
knocked out by using double recombination. The metabolic profiles for the wild type and mutant Mycobacteria can reveal the function of PKS genes. The results of this study would be discussed elsewhere.

In the structure-based approach, we have probed the differences in the initiation/elongation cavities of all three mycobacterial CHS homologues. The chimeric models of PKS10, PKS11, and PKS18 were constructed by replacing variable amino acids in the catalytic pocket of alfalfa CHS (Protein Data Bank code 1CGZ) by analogous residues in these proteins. For example, the PKS18 chimeric model was constructed by replacing 11 of 32 residues that constituted the initiation/elongation cavity of CHS. The amino acid replacements were carried out by the rotamer library approach using the SCWRL program. This comparative modeling approach is based on the premise that the overall fold as well as the active site geometry of protein would be similar. In one such successful attempt, CHS was transformed into 2PS by introducing bulkier side chains at three crucial positions. The changes of Tyr and Cys are compensatory in terms of changes of the cavity volume for THNS. In PKS10 and PKS11, Gly256 of CHS is changed to Tyr and Trp, respectively. Although there is a reduction in the volume of the cavity compared with CHS, substrate preference of these proteins is not apparent from these homology-modeled chimeric structures. In contrast, the PKS18 homology model shows remarkable constriction in the active site cavity. The steric bulk from three active site residues, Asn208, Leu266, and Leu348, significantly affects the active site volume. We therefore predicted that PKS18 protein might resemble 2PS and may catalyze biosynthesis of pyrones. In order to improve our predictability for PKS10 and PKS11, various CHS-related plant products were docked in the initiation/elongation cavity. Most of these docked structures resulted in steric clashes with the protein side chains and backbone, suggesting that p-coumaroyl-CoA-like starter units may not be a substrate for these proteins.

Characterization of Mycobacterial Type III PKSs—Three mycobacterial type III PKSs showed 40–45% similarity with both plant and bacterial CHS-like sequences. These genes were cloned from the H37Rv BAC genomic library and were then expressed in E. coli using the T7 expression system. Although overexpression for all three proteins, PKS10, PKS11, and PKS18, was observed, most of the protein was present in in-

![Fig. 2. Comparison of the active site cavities of mycobacterial PKS10, PKS11, and PKS18 proteins with the experimentally determined CHS initiation/elongation cavity complexed with resveratrol (1CGZ). Some of the crucial active site residues along with the catalytic Cys (in red) are shown here. Chimeric models of PKS10, PKS11, and PKS18 were generated by amino acid replacements using the SCWRL amino acid rotamer library. The 2PS and THNS chimeras were constructed for comparison. 2PS cavity resembles the crystallographically elucidated structure validating the homology-based active site predictions. Since active site cavity is buried, two residues were removed for clarity.](image)
Mycobacterial Type III Polyketide Synthases

Remarkable Substrate Specificity for PKS18 Protein

Although our modeling studies predicted that mycobacterial proteins would not be able to use plant-specific CoA-esters, we investigated the function of these proteins with different CoA-esters due to their sequence similarity with plant PKSs. Several plant-specific CoA-esters were incubated with PKS18 and PKS11 proteins along with radiolabeled malonyl-CoA. Analysis of the reaction mixture by TLC showed one faint radioactive band for PKS18 reactions with an $R_f$ value different from that of the expected plant products. Further examinations with longer time incubations clearly showed that PKS18 protein could synthesize this product from malonyl-CoA itself. Since 2PS uses acetyl-CoA as a starter substrate, we incubated PKS18 protein with acetyl-CoA and [2-13C]malonyl-CoA. Under the assay condition used in this study, there was no significant change in the product formation. Since PKS11 protein did not show any appreciable activity under these conditions, we proceeded to identify the PKS18 reaction product.

**Remarkable Substrate Specificity for PKS18 Protein—** Type III PKSs such as CHS and THNS have been shown to have rather promiscuous starter unit specificity (20, 35). However, in most cases the incorporation of suboptimal moiety has often adversely affected the catalytic activity, both in terms of formation of end products and in the amount of products. Since PKS18 protein did not exhibit any activity with plant-specific acyl-CoA substrates, we used several medium- and long-chain fatty acid CoA-thioesters as starter substrates. Fig. 3 shows radio-TLC of a 10-min assay performed using several starter units and [2-13C]malonyl-CoA as extender substrate with PKS18 protein. It can be noticed that several significant bands appear on the TLC plate, particularly for the long-chain acyl-CoA esters. There is a systematic increase in the $R_f$ value of the products with increasing length of the fatty acyl chain, clearly indicating that PKS18 protein is able to use medium- and long-chain aliphatic acyl-CoAs as substrates. Whereas incubation of PKS18 protein with hexanoyl-CoA shows one major product, incubation with long-chain acyl-CoAs (C12 to C20) produces two significant products. Under the assay conditions used in this study, no activity was detected for small-chain, aromatic, and branched-chain acyl-CoA analogues. These studies clearly suggested that PKS18 protein can extend long-chain acyl-CoA substrates by using malonyl-CoA as an extender molecule to synthesize polyketide products.

**Identification of Novel Reaction Products—** Polyketide products biosynthesized from purified enzymes were separated on reverse phase HPLC and characterized by nanospray ESI MS. Since hexanoyl-CoA-primed reaction showed a reasonable amount of product on radio-TLC assays, we proceeded to characterize this product. The HPLC profile showed a single major peak a at 280 nm (Fig. 4A). Incubation of PKS18 with [2-13C]malonyl-CoA along with hexanoyl-CoA as starter gave one major radioactive product that co-migrated with the peak a. This product was then subjected to nanospray MS analysis, and a molecular ion peak (M-H)~ at m/z 181.08 was observed. Based on our understanding of polyketide biosynthesis, this molecular mass is possible for a cyclized triketide synthesized from one molecule of hexanoyl-CoA and two molecules of malonyl-CoA. In order to characterize products from lauroyl-CoA, a ternary solvent system was developed, where the two main products could be separated as shown in Fig. 4B. Nanospray mass spectrometric analysis showed molecular ion peaks (M-H)~ at m/z 265.17 for c and m/z 307.20 for b, which could be expected from triketide and tetraketide products, respectively, of lauroyl-CoA reactions with extensions from malonyl-CoA.

Tetraketide products of type III PKSs are known to cyclize in three distinct ways, where different polyketide products are formed from aldol condensation, Claisen condensation, and lactonization of the reaction intermediate (10, 11). The structural elucidation of PKS18 reaction products was completed by using tandem mass spectrometry. Fig. 4C shows the fragmentation pattern for the molecular ion (M-H)~ at m/z 307.20 (obtained for b), which yielded a fragment of m/z 265.17 corresponding to (C9H14O2)~ and m/z 263.21 corresponding to (M-CO2H)~ ion. This fragmentation pattern has been previously reported for the tetraketide $\alpha$-pyrones biosynthesized by RppA from S. griseus (20). The molecular ion peak (M-H)~ at m/z 265.17 for c yielded a (M-CO2H)~ peak at m/z 221.18, which indicated the presence of an $\alpha$-pyrone ring (Fig. 4D). Products biosynthesized by PKS18 protein with other acyl chains were similarly characterized by nanospray MS and determined to be triketide and tetraketide $\alpha$-pyrones.

The starter unit specificity of PKS18 was investigated by performing steady state kinetic analysis using C2-C20 acyl-CoA substrates. PKS18 protein obeyed saturation kinetics in response to the increasing concentration of acyl-CoA substrates, which is summarized in Table I. It can be observed that the $k_{cat}$/$K_m$ values for the biosynthesis of tetraketide $\alpha$-pyrones of long-chain acyl-CoA substrates are severalfold higher than that observed for small-chain substrates. Specificity of PKS18 pro-
teins for long-chain acyl-CoA starters, as reflected in the $K_m$ values, is similar for C$_{12}$ to C$_{20}$ acyl chains. Aliphatic CoA substrates longer than C$_{20}$ were not investigated in this study. This unusual specificity toward long-chain aliphatic fatty acids is unparalleled in the CHS family of condensing enzymes.

Previous studies have shown octanoyl-CoA to be the largest starter unit extended by CHS and THNS (20, 35). Based on this unusual ability of CHS-like proteins to use long-chain substrates, reactions were carried out with PKS11 protein using medium- and long-chain acyl-CoA units. Fig. 5 shows the radio-TLC of reactions of PKS11 and PKS18 proteins. As can be visualized, various different products were obtained when the proteins were incubated for 1 h with these acyl-CoA starters and [2-14C]malonyl-CoA. Like PKS18, PKS11 readily accepted these starter units and formed pyrone products, as confirmed by nanospray mass spectrometric analysis (data not shown).

### Structural Analysis of Long-chain Specificity for Mycobacterial Proteins

The analysis of the structural models of PKS10, PKS11, and PKS18 proteins clearly indicated that the putative CHS active site cannot accommodate pyrone rings with long aliphatic side groups, suggesting the possibility of an altered binding pocket in these type III PKS proteins from *M. tuberculosis*. It is interesting to note that some members belonging...
to the thiolase fold family possess the capability to use long aliphatic chains as substrates (15, 36, 37). The analysis of the three-dimensional crystal structure of KAS III enzyme (1HZP) from \textit{M. tuberculosis} (referred to as mtFabH) has revealed the mechanism by which it can catalyze the decarboxylative condensation of bulky myristoyl-CoA with malonyl-acyl carrier protein. Apart from the overall thiolase fold, KAS III and CHS-like proteins also share the Cys-His-Asn catalytic triad. The structural superposition of 1HZP on 1CGZ, as shown in Fig. 6A, gives a root mean square deviation of 2.9 Å at a Z score of 6.8. The core of the two structures superposes quite well, and the differences are restricted to the loop regions. The catalytic triad also superposes with a root mean square deviation of 0.54 Å. It is interesting to note from Fig. 6A that the two active site cavities (red dots for CHS and green dots for KAS III) are oriented differently with respect to the CoA-binding tunnel. Whereas the KAS III orients the starter units to the right and toward the dimer interface, CHS orients them toward the left. Unlike CHS, mtFabH enzyme can elongate long-chain aliphatic chains and has been shown to be specific for C8 to C20 aliphatic substrates (36). The structural basis of the substrate specificity for six priming ketosynthases was investigated recently (36, 38). These studies indicated that the selectivity and primer unit size are primarily controlled by the side chains of three residues from the loops present close to the dimer interface. In mtFabH, all of the side chains of the cavity-limiting residues (Asn\textsuperscript{110}, Thr\textsuperscript{113}, and Glu\textsuperscript{200}) are either short or point outwards, resulting in a long tunnel. A recent review article has discussed the structural and mechanistic implications of various members of the thiolase family of enzymes (11).

Since mycobacterial type III PKSs biosynthesize products that have characteristics of both CHS and KAS III proteins, we set out to test the hypothesis that these enzymes would have evolved by combining the two catalytic centers of CHS and KAS III. PKS18 displays a homology of 42% with alfalfa CHS over the entire protein sequence. As described earlier, a comparative model of PKS18 based on 1CGZ structure was constructed, which demonstrated a constricted CHS initiation/elongation cavity. In the CHS-related structural family of condensing enzymes, several proteins such as mtFabH use long-chain substrates. The attempt to model long-chain aliphatic substrate in the putative active site of PKS18 was tested by using 1HZP as template. Sequence similarity of PKS18 to mtFabH is restricted to a small region consisting of amino acids 111–239. The C-terminal domain does not show any homology with PKS18 in sequence comparisons. Since threading methods are capable of detecting structural similarity at low sequence identities, PKS18 was threaded using the 3D-PSSM server (39). The threading results indicated the plant CHS structure (1CGZ) to be the highest scoring fold, although the structure of mtFabH (1HZP) was also suggested to be a possible template. Upon detailed inspection of the alignments given by the threading servers, it was found that 3D-PSSM gives a lower score for 1HZP due to poor alignment of the 90 N-terminal residues. Deletion of 90 N terminus residues improved the threading score (3D-PSSM E-value from 1.17 to 0.037) and thus provided an optimal alignment of PKS18 sequence on the structural fold of mtFabH. However, plant CHS structure was still the highest scoring fold. We decided to use 1HZP as a template for homology modeling, since this protein is known to bind long-chain aliphatic substrates. Using the program ACSITE (40), an elongated cavity was identified in mtFabH along the dimer interface, indicating that elongated substrate can be accommodated in the active site of the 1HZP fold. Since the mtFabH coordinates complexed with substrate are not available in the Protein Data Bank, lauric acid was modeled in the active site of PKS18 using the information from the crystal structure of lauric acid–KAS I complex (1EK4) (37). Although KAS I proteins have no detectable sequence homology to the CHS family of condensing proteins, crystal structure analysis has revealed a conserved thiolase fold (41). Superposition of the substrate-bound 1EK4 on 1HZP indicated that the transformed substrate fits in the

![Comparative TLC of radiolabeled products of PKS11 and PKS18 proteins. Lanes 1 and 2, hexanoyl-CoA-primed reactions; lanes 3 and 4, lauroyl-CoA-primed reactions. [2-14C]malonyl-CoA was used as an extender unit in all of the reactions. Chemical structures of the major products are indicated beside the radioactive bands.](image)
cavity of 1HZP. These transformed coordinates were then used as starting orientation in the active site of 1HZP fold. The amino acids of 1HZP that were in contact with the lauric acid were replaced with equivalent residues of PKS18 based on threading alignment. The carboxyl carbon of the lauric acid was covalently attached to the thiol of active site cysteine, and the complex was energy-minimized using the CVFF force field (42). The energy-minimized structural model of PKS18 complexed with covalently tethered lauric acid is shown in Fig. 6B. The residues in contact with the substrate form a hydrophobic acyl-binding channel, and no steric clashes were observed. These observations offer a structural model for understanding the unusual substrate specificity of PKS18 protein.

Probing the Active Site of PKS18 Protein by Site-directed Mutagenesis—Structural analysis has provided a testable model to probe into the functional relevance of catalytic and structural residues of mycobacterial PKSs. Our modeling stud-

ies in conjunction with the analysis of the substrate specificity for priming ketosynthases suggested that amino acid residue Ala\textsuperscript{148} from PKS18 may be a crucial residue. CHS contains a Met\textsuperscript{137} residue at the equivalent position, which is contributed from the second monomer and lines the active site cavity. It can be observed from Fig. 6A that the Met\textsuperscript{137} amino acid restricts the extension of the CHS cavity (red dots) further into the acyl-chain binding pocket (green dots). The Ala\textsuperscript{148} residue in PKS18 was mutated to bulkier side chains of threonine, methionine, and phenylalanine to restrict the extension of the cavity. Similar strategy was previously successfully employed, where the KAS II specificity was modulated to favorably accept shorter acyl-chain length substrates (43). Mutation of the Ala\textsuperscript{148} residue of PKS18 protein resulted in expression of three proteins in inclusion bodies. Despite our best efforts, these mutant proteins could not be recovered in the soluble form. Although we could not directly probe the functional significance of this residue, it can be inferred that Ala\textsuperscript{148} may be a structurally important residue.

Another residue predicted to be involved in the formation of active site cavity, Leu\textsuperscript{348} of PKS18, was investigated by performing mutagenesis studies. Previous mutagenesis studies of the equivalent residue in CHS (Ser\textsuperscript{338}) (Fig. 6A) have implicated its role in starter unit selectivity and determination of the chain length of the growing polyketide (13). Mutation of the analogous Ala\textsuperscript{305} residue to a bulkier isoleucine residue in THNS protein abolished the synthesis of tetraketide pyrones, and only triketide pyrones were observed (18). In PKS18, Leu\textsuperscript{348} was mutated to a smaller serine residue. Biochemical characterization carried out with [2-\textsuperscript{14}C]malonyl-CoA and the various starter substrates resulted in a relative increase in the synthesis of the tetraketide products. Fig. 7 shows the nanospray tandem mass spectrometric analysis of the tetraketide pyrone of the hexanoyl-CoA-primed reaction of L348S mutant. The wild type PKS18 protein does not synthesize this product. An increase in the tetraketide product for the L348S mutant suggested that the Leu\textsuperscript{348} residue might be located in the elongation/cyclization cavity of PKS18 protein, consistent with our homology model.

CHS condensing enzymes contain three conserved basic amino acid residues that are located at the outer rim of the CoA-binding tunnel for electrostatic interactions with CoA phosphates (12). Bacterial type III PKSs contain an extra conserved basic amino acid residue (Lys\textsuperscript{318} in PKS18) in proximity to the predicted CoA-binding tunnel (11). Plant CHSs have a conserved alanine residue at this position, as shown in Fig. 6A. The equivalent residue is arginine in several KAS III enzymes, where the guanidino side chain facilitates the binding of acyl carrier protein (46). It has been speculated that some bacterial type III PKSs may also have specificity for ACP-thioester substrates (11). In an attempt to elucidate the role of this conserved residue, Lys\textsuperscript{318} of PKS18 was mutated to alanine. The K318A mutant was unable to synthesize any polyketide products in our standard PKS18 assay. Biophysical analysis of the mutant protein showed no change in the overall secondary and tertiary structure as compared with wild type PKS18 protein. The inactivity of the mutant protein was probed by carrying out binding assays with radiolabeled starters and extender units. Fig. 8 shows the autoradiogram of the SDS-PAGE gel containing wild type PKS18 protein and K318A mutant protein incubated independently with [2-\textsuperscript{14}C]lauroyl-CoA and [2-\textsuperscript{14}C]malonyl-CoA. It can be observed that the wild type PKS18 protein can be radiolabeled with both malonyl-CoA and lauroyl-CoA (Fig. 8A). In contrast, the K318A mutant retains the ability to accept the starter lauroyl-CoA but could not bind to the extender malonyl-CoA (Fig. 8B). Further mechanistic investiga-
tation suggested that this mutant protein was severely impaired in malonyl-CoA decarboxylation (Fig. 8C).

DISCUSSION

The identification of diverse catalytic activity in mycobacterial type III PKSs provides an intriguing example of metabolic divergence in CHS-like proteins. Interestingly, the plant prototype CHS protein has not yet been characterized in bacteria. In this article, we discuss the characterization of two novel type III polyketide synthases from \textit{M. tuberculosis}. PKS11 and PKS18 enzymes have remarkable specificity to use long-chain aliphatic-CoA analogues (C12 to C20) as starter units to biosynthesize \( \alpha \)-pyrones. The reaction catalyzed by PKS18 protein is summarized in Fig. 9. The mechanism of PKS18 protein to synthesize pyrones was examined to explore whether the pyrone formation was a result of hydrolysis followed by nonenzymatic cyclization or if this was produced by intramolecular lactonization. The pyrone products of PKS18 were extractable with organic solvent even without acidification of the reaction mixture, suggesting that the acidic conditions were not required for pyrone formation. Although acidification does promote spontaneous lactonization, it could also stabilize and promote the extraction into organic solvents of an enzymatically formed pyrone. Moreover, pyrone products with long aliphatic chains would enhance extractability of these products in organic solvents and can complicate the interpretation of results. The nanospray ESI-MS analysis did not detect any hemiacetal or carboxylic acid compounds, indicating that the release of final product proceeded by intramolecular lactonization. A similar mechanism of ring closure has been previously proposed for RppA protein (20).

Pyrone synthases have been previously characterized in plants (47), although such proteins have not been identified from the microbial world. Several plant and bacterial type III PKSs are also known to synthesize lactones as derailment products upon incorporation of a suboptimal starter moiety (20, 35). However, this often results in relatively less activity. Whereas the broad starter molecule specificity of PKS18 and PKS11 enzymes obscures the assessment of \textit{in vivo} biological function, the general preference for the type of starter units (preference of aliphatic or aromatic, smaller or larger, straight-chain or branched) provides clues about the likely physiological substrate. This fact combined with the \textit{in vivo} availability of CoA-thioesters should determine the biological function. The notable preference of mycobacterial type III PKSs for long-chain acyl-CoAs makes it tempting to speculate that these are the preferred substrates \textit{in vivo}, although such pyrone-containing long-chain derived metabolites have not been isolated from \textit{Mycobacterium}. It should be noted that \textit{M. tuberculosis} contains a rich pool of a remarkable array of long-chain and very long-chain fatty acids (48). Moreover, the \textit{M. tuberculosis} genome also includes 36 copies of \textit{fadD} homologues that are typically involved in the biosynthesis of acyl-CoA substrates (1).

Comparative modeling studies suggest that the unusual capability to accept long-chain acyl-CoA substrates might have evolved by combining the catalytic machinery of CHS and KAS proteins. Apart from a constricted CHS active site initiation/elongation cavity, an additional acyl group binding pocket (typically observed in KAS-related proteins) has been identified along the dimer interface in these mycobacterial proteins. Since homology modeling studies at low sequence identity cannot give unambiguous results, three-dimensional crystal struc-

![Fig. 7. Tandem mass spectrometric analysis of hexanoyl tetra-ketide pyrone synthesized by L348S mutant. The molecular ion peak (M-H\(^{-}\)) at m/z 223 fragments into m/z 125 corresponding to (C_{6}H_{12}O_{3})\(^{-}\) and m/z 179 corresponding to (M-CO_{2}H\(^{-}\))\(^{-}\) ion.](image)

![Fig. 8. Biochemical analysis of PKS18 mutant. The autoradiographs of substrate binding assays are shown for wild type PKS18 (A) and K318A mutant (B). The substrate used for labeling is indicated above the lane. C shows the TLC-based analysis of malonyl-CoA decarboxylation assay carried out for wild type and mutant protein (K318A).](image)
The starter molecule and its corresponding component in the polyketide parallel lysine residue from protein was incapable of decarboxylating malonyl-CoA. Similar dicarboxylic malonyl-CoA as a substrate. The K318A mutant monocarboxylic starter units but lost the capability of using characteristics, where the mutant protein could load the iterations (13, 18). The K318A mutant showed rather interest-
ketide pyrone product. This catalytic profile corroborated with L348S mutant protein showed increased biosynthesis of tetra-
binding that are permitted within the type III PKS superfamily intermediates. The presence of an additional positively charged possible that there are variations in the binding of extender boxylic acid extender units. Although malonyl-CoA is an ex-
dynamic and structural consequences of mutations in the hy-

dictating long-chain substrate specificity. It is possible that the studies had suggested that this residue may be important in binding Leu to a smaller Ser residue. The characteristics of this L348S mutant protein showed increased biosynthesis of tetra-
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REFERENCES


Mycobacterial Type III Polyketide Synthases

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