The Biosynthesis of Cyclopropanated Mycolic Acids in Mycobacterium tuberculosis

IDENTIFICATION AND FUNCTIONAL ANALYSIS OF CMAS-2*

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Kathleen M. George‡, Ying Yuan‡, David R. Sherman§, and Clifton E. Barry III††

From the ‡Laboratory of Intracellular Parasites, Tuberculosis Research Unit, NIAID, National Institutes of Health, Rocky Mountain Laboratories, Hamilton Montana 59840 and the §Laboratory of Tuberculosis and Molecular Microbiology, PathoGenesis Corporation, Seattle, Washington 98119

The major mycolic acid produced by Mycobacterium tuberculosis contains two cis-cyclopropanes in the meromycolate chain. The gene whose product cyclopropanates the proximal double bond was cloned by homology to a putative cyclopropane synthase identified from the Mycobacterium leprae genome sequencing project. This gene, named cma2, was sequenced and found to be 52% identical to cma1 (which cyclopropanates the distal double bond) and 73% identical to the gene from M. leprae. Both cma genes were found to be reinserted in distribution to pathogenic species of mycobacteria. Expression of cma2 in Mycobacterium smegmatis resulted in the cyclopropanation of the proximal double bond in the α2 series of mycolic acids. Coexpression of both cyclopropane synthases resulted in cyclopropanation of both centers, producing a molecule structurally similar to the M. tuberculosis α-dicyclopropyl mycolates. Differential scanning calorimetry of purified cell walls and mycolic acids demonstrated that cyclopropanation of the proximal position raised the observed transition temperature by 3 °C. These results suggest that cyclopropanation contributes to the structural integrity of the cell wall complex.

An estimated 8 million persons develop tuberculosis each year, and over 30 million people are expected to die from the disease in this decade (1). Mycobacterium tuberculosis, the causative agent of tuberculosis, is an intracellular pathogen that establishes an infection in oxygen-rich alveolar macrophages of the lung (2). Mycolic acids are long chain α-alkyl-β-hydroxy fatty acids unique to mycobacteria and related taxa and represent major components of the cell wall (3). Mycolic acids are thought to contribute to both drug resistance and survival in the hostile intracellular environment of the macrophage by the formation of an impermeable asymmetric lipid bilayer (4). The biosynthetic pathway for these complex lipids is also thought to be the target for several clinically useful chemotherapeutics, including isoniazid (5). With the increasing diversification of normal fatty acid metabolism in which short chain fatty acids are extended and modified to form lipids of exceptional length (6). Mycobacterium smegmatis synthesizes three different series of α-mycolates (which lack oxygen functionalities in the meromycolate chain outside the β-hydroxy acid) shown in Fig. 1 (7). The α1 and α2 series are full-length mycolic acids extending to an average of 78 and 79 carbons, respectively (8). α1 contains two cis-olefins in the meromycolate chain, while α2 contains only a single cis-olefin and a trans-olefin with an adjacent methyl group. In addition to these three mycolates, M. smegmatis also produces a shorter α′ mycolic acid, which is 64 carbons in length as well as a full-length epoxy mycolate (9). M. tuberculosis contains only one series of α′-mycolic acids that averages 78–80 carbons in length (4) (10, 11). The tuber-bacilli also produces two oxygenated mycolic acid series, ketomycolates and methoxymycolates (not shown), which are generally of lower abundance than the α series (12). Pathogenic mycobacteria cyclopropanate a majority of their mycolic acids, whereas in saprophytic organisms, this modification is unusual (3). The functions of the various classes of mycolic acids in each of these organisms is unknown. However, we have recently shown that cyclopropanation at the distal position confers increased resistance to in vitro killing by hydrogen peroxide (13). The present studies were initiated to expand our understanding of the relationship between mycolic acid structure and function.

We have previously reported the identification of cma1, a gene whose protein product (cyclopropane mycolic acid synthase-1, CMAS-1)* catalyzed the introduction of a cyclopropane at the distal position in the meromycolate chain (13). In the course of these studies, we discovered that an unannotated related sequence had been deposited in Genbank as part of the Mycobacterium leprae genome sequencing project (accession number U00018) (14). In this paper we report that this M. leprae sequence represents a second cyclopropane synthase with a homolog in M. tuberculosis whose protein product functions distinctly from CMAS-1 to cyclopropanate the proximal cis-olefin in mycolic acid biosynthesis.

EXPERIMENTAL PROCEDURES

Materials and Strains—M. leprae DNA was provided by Becky Rivoire (Colorado State University, Ft. Collins, CO) through the NIAID, National Institutes of Health, contract NO1-AI-05074. M. smegmatis mc255 (provided by William R. Jacobs, Albert Einstein College of Medicine, NY) was grown in Middlebrook 7H9 media with albumin-dextrose-catalase supplement (ADC) (Remel, Lenexa, Kansas) containing, where appropriate, kanamycin (25 μg/ml) (Sigma) or hygromycin b.

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† To whom correspondence should be addressed: Rocky Mountain Laboratories, 903 S. Fourth St., Hamilton, MT 59840. Tel.: 406-363-9309; Fax: 406-363-9380; E-mail: kestrel@ml.niaid.ucr.niaid.nih.gov.

‡ The abbreviations used are: CMAS, cyclopropane mycolic acid synthase; PCR, polymerase chain reaction; MAME, mycolic acid methyl ester; TLC, thin-layer chromatography; kb, kilobases; MOP, mycobacterial optimal promoter; DSC, differential scanning calorimetry.
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Fig. 1. Structure of the major mycolic acids of M. tuberculosis and M. smegmatis. M. smegmatis produces three defininc α-mycolates, which average 78–79 carbons in length in α1 and α2, and 64 carbons in α3. The α1 mycolic acid contains a cis-defin in both the proximal and distal positions. The α2 mycolic acid contains a distal cis-defin and a proximal trans-defin with an adjacent methyl branch. The sole α3 mycolic acid (4) from M. tuberculosis contains two cis-cyclopropane moieties and averages 78–80 carbons in length. The exopoy mycolic acid from M. smegmatis contains an epox group at the distal position and a cis-defin at the proximal position.

(50 μg/ml) (Calbiochem). Peroxide susceptibility measurements were conducted as described previously (13).

Cloning and Sequencing cma2—PCR products from M. leprae genomic DNA were used to screen an M. tuberculosis H37Rv cosmid library. PCR product 1 displayed high (57%) homology with the M. tuberculosis cma1 gene (GenBank accession number U27357), and the M. leprae cma2 gene. This product was 459 nucleotides in length and corresponded to nucleotides 22918–23377 from the M. leprae genomic DNA. PCR product 2 (768 nucleotides) was less homologous (46%), corresponded to nucleotides 23482–24250 of the same cosmid, and was generated with the following two primers: 5'-CAGTATGCTGGGCGAATTC-3' and 5'-GCTCGGAAGAGATT-3'. PCR product 2 (768 nucleotides) was less homologous (46%), corresponded to nucleotides 23482–24250 of the same cosmid, and was generated with the following two primers: 5'-GCCACCCGGATTC-3' and 5'-CCGTTCCGGAAAGATT-3'. Colony lifts were performed of Escherichia coli containing the cosmide DNA library from H37Rv in pYUB18 with GeneScreen Plus hybridization membranes (DuPont NEN), which were then hybridized to each PCR-generated probe separately according to the manufacturer’s protocol. DNA sequencing was performed using the Sequenase 7-deaza-dGTP DNA sequencing kit (U.S. Biochemical Corp.) with synthetic universal and custom primers. Codon preferences were determined by reference to the published sequence of mycocerosic acid synthase (15) (GenBank accession number M95808).

Mycolic Acid Methyl Ester (MAME) Isolation and Purification—MAMES were isolated and purified following basic methanolic hydrolysis as described previously (13). For NMR analysis, acetonitrile/toluene precipitation was followed by a 10-cm silica gel column in 9.5:0.5 hexanes/ethylacetate. For radiolabeling, 50 μCi of sodium [1-14C]acetate was added for several hours to growing cultures of M. smegmatis containing the appropriate construct and antibiotics before purifying MAMES as above.

Two-dimensional TLC Procedure—Two-dimensional TLC analyses were performed by immersing 90% of a square silica gel 60 TLC plate (0.2 mm thickness, EM Separations, Gibbstown, NJ) into 5% aqueous silver nitrate (w/v). Following air-drying, these plates were activated as described by Kennerly (16). 14C-Labeled samples were run in the first dimension along the narrow strip without silver impregnation by developing twice with 9.5:0.5 hexanes/ethyl acetate. The plates were then dried, turned 90° and run into the silver layer by developing three times with 85:15 petroleum ether/diethyl ether. Plates were then visualized after heating at 110°C for 10 min. Plates were then hybridized to the probe separately according to the manufacturer’s protocol. DNA sequencing was performed using the Sequenase 7-deaza-dGTP DNA sequencing kit (U.S. Biochemical Corp.) with synthetic universal and custom primers. Codon preferences were determined by reference to the published sequence of mycocerosic acid synthase (15) (GenBank accession number M95808).

Vectors and Constructs—pYUB18 was a gift of W. R. Jacobs, Albert Einstein College of Medicine, NY (17). pMV206 and pMV261 were provided by MedImmune, Inc., Gaithersburg, MD. pMH29 was derived from pMV206 with the cosmid DNA library from H37Ra in pYUB18 with GenBank accession number U27357. pMH29 was provided by C.K. Stover and M.J. Hickey (PathoGenesis Corp., Seattle, WA). pMV206_Hyg and pMH29_Hyg were constructed by placing it with a hygromycin resistance cassette consisting of a 1.3-kb BspHI to Smal fragment from pl61R1 (provided by Douglas Young, Wright Flemming Institute, London) (19). pYUB-cma1 contains the tac promoter with the cma1 open reading frame and upstream region as described previously (13) cloned into the BamHI site of pYUB18. pYUB-cma2 contains a 35-kb chromosomal fragment from M. tuberculosis H37Ra cloned at the BamHI site of pYUB18. pMH29_Hygcma1 contains the 1.5-kb BamHI to PstI fragment cloned into the same sites in pMH29_Hyg, resulting in expression from PstI. pMH29_Hygcma2 contains a 1.2-kb fragment constructed by digesting the 3.9-kb cma2-containing insert with NruI followed by ligation of XbaI linkers and digestion with both BamHI and XbaI. The gel-purified insert was ligated to BamHI, XbaI-digested pMH29_Hyg. pMV206_Hyg—
**RESULTS**

Cloning and Characterization of the cma2 Gene from M. tuberculosis—Two factors led us to conclude that cma1 and the unannotated cosmId sequence from M. leprae, although both clearly cyclopropane synthases, were not coding for homologous proteins with identical function. First, sequences surrounding the cma1-coding region were nonhomologous to the sequences surrounding the coding region of the M. leprae open reading frame. Second, the relatively low similarity of the two protein sequences (53% identity) seemed anomalous compared with the relatedness of other proteins between the two pathogens (for example, the RecA proteins share 92% identity (20) and the relatedness of other proteins between the two pathogens (for example, the RecA proteins share 92% identity (20) and the relatedness of other proteins between the two pathogens (for example, the RecA proteins share 92% identity (20)).

**CoMase-2 Expression in M. smegmatis—**In order to study the function of the cma2 gene product CoMase-2, the pYUB18 cosmId containing this gene was introduced into M. smegmatis, and MAMEs were purified as described under “Experimental Procedures.” Analysis of the total MAMEs by 500 MHz $^1$H NMR revealed the presence of resonances characteristic of cyclopropane ring hydrogens ($\delta ~ 0.33$ ppm, multiplet; $\delta ~ 0.56$ ppm, multiplet; $\delta ~ 0.64$ ppm, broad multiplet). By integration of these resonances and comparison with the corresponding integration of the signal for the terminal methyl groups ($\delta ~ 0.88$ ppm), it was estimated that expression of cma2 in this system resulted in monocyclopropanation of 10% of the total mycolic acids. In this same analysis, control samples of wild-type M. smegmatis showed <2% cyclopropanation.

To confirm that this transformation was due to the putative cma2 sequence as well as to improve the extent of conversion, a 1.2-kb NruI BamHI fragment containing the cma2 reading frame was subcloned into pMH29_Hyg. pMH29_Hyg is a derivative of pMV261 that contains a hygromycin resistance marker in place of kanamycin. Hygromycin selection has much lower background and allows much faster recovery times than kanamycin for transformed mycobacteria. This vector also contains a synthetic MOP in place of the Hsp60 promoter region. CoMase-2 produced from this construct was capable of converting 25% of the total mycolates to the cyclopropanated type as determined by NMR.

Identification of the CoMase-2 Protein and Co-expression of CoMase-1 and 2—For comparison purposes, cma1 was placed in pMH29_Hyg to give a CoMase-1-overproducing system. In addition, cma1 and cma2 were both cloned with several hundred nucleotides of upstream sequence into pMV206_Hyg, a derivative of pMV261 that has a hygromycin cassette replacing the kanamycin resistance gene. pMV206_Hyg is promoterless, allowing expression of both genes from their own promoter regions. MAMEs from M. smegmatis transformed with each of

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2 Y. Yuan and C. E. Barry III, unpublished results.
these constructs were prepared following labeling with [1-14C]acetate and were analyzed by two-dimensional TLC. In this experiment, the first dimension was normal silica gel, and the second dimension was silica gel impregnated with silver ions. Such argentation TLC allows the selective retardation of components containing cis-olefins, while components with either trans double bonds or cyclopropanes are less affected or unaffected in their mobility (18). Analysis of wild-type M. smegmatis by this technique allowed the identification of all the major mycolates (Fig. 3A) whose structures are shown in Fig. 1. Introduction of cma1 in this system (Fig. 3B) results in production of a single spot of high mobility in the argentation dimension, which we have previously identified as a hybrid mycolate containing a distal cyclopropane and a proximal trans double bond with an α-methyl branch (13). pMH29H_cma2 also results in production of a new mycolate that is retarded more strongly than the cma1 product by silver ions (compare Fig. 3B, spot 1, with Fig. 3C, spot 2). This product (2) appears to result from conversion of the α2-mycolate. In addition, the epoxy mycolate series (spot e in Fig. 3C) also appears to change retention time on argentation chromatography in a manner consistent with cyclopropanation at the proximal position to produce spot 3.

Introduction of both cma genes into M. smegmatis resulted in a pronounced change in the radio-TLC profile, which contained both the CMAS individual products as well as a unique MAME, which was unaffected by silver ion impregnation (Fig. 3D). This MAME exactly co-elutes with the major α-mycolic acid from M. tuberculosis (data not shown). To confirm these structural predictions, MAMEs were purified from 1 liter of M. smegmatis containing pMV206_Hyg-cma1+cma2 and separated by preparative argentation TLC. 500 MHz of 1H NMR analysis of spot 4 (Fig. 4A) showed that this spot had no olefinic resonances but had cyclopropane resonances (δ = 0.33, 0.56, and 0.65 ppm). These were present in a 4:6 ratio with terminal methyl groups, indicating that this M. smegmatis mycolate corresponded to structure 4, which is the major mycolate from M. tuberculosis (Fig. 1). The mycolate corresponding to spot 1 in Fig. 3D showed 1H NMR resonances (Fig. 4B) corresponding to a trans-olefin at δ 5.3 ppm (J = 15 Hz) as well as a doublet corresponding to an α-methyl group at δ 0.93 ppm and cyclopropane resonances as
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**TABLE I**
Proportion of total mycolic acids cyclopropanated in the proximal and distal positions in CMAS-1 and CMAS-2 overexpressing recombinant M. smegmatis

|       | proximal | distal | % Total Mycolic Acids |
|-------|----------|--------|-----------------------|
| pYUB18 |          |        |                       |
| pM629H_cma1 |        |        |                       |
| pM629H_cma2 |        |        |                       |
| pMV206H_cma1+c2 |       |        |                       |

|       | proximal | distal | % Total Mycolic Acids |
|-------|----------|--------|-----------------------|
| 23    | 19       | 8      | 8                     |
| 45    | 22       | 39     | 10                    |
| 1     | 33       | 5      | 34                    |
| 6     | 6        | 29     | 17                    |
| 1     | 1        | 2      | 17                    |

Table entries indicate the percentage of mycolic acid cyclopropanated in the proximal and distal positions in CMAS-1 and CMAS-2 overexpressing recombinant M. smegmatis. 

In this work, we have used a homologous sequence from the M. leprae genome sequencing project to identify the protein involved in construction of the cyclopropane from M. tuberculosis. This enzyme is the fourth identified member of a family of proteins to catalyze the transfer of a methylene group from S-adenosyl-L-methionine to the double bond of a fatty acid substrate. The three mycobacterial members of this family are closely related to one another, with the cma2 genes from M. leprae and M. tuberculosis being more closely related to one another (73% identity) than to the cma1 gene of M. tuberculosis (52% identity). Heterologous expression of cma2 in M. smeg-
matis results in a proportion of the α-mycolates becoming cyclopropanated at the proximal position. Expression of cmd1 results in cyclopropanation at the distal position, while coexpression of both genes results in the production of a dicyclopropyl mycolate nearly identical to the major mycolic acid produced by M. tuberculosis.

The cyclopropanation of the epoxy mycolates by CMAS-2 in M. smegmatis suggests that the enzyme is either insensitive to substituents occurring toward the ω end of the chain or that CMAS-2 acts on a precursor meromycolate, which can become either cyclopropanated to form the dicyclopropyl mycolate or further oxidized to form the epoxy series. CMAS-2 activity is unchanged upon coexpression of both cyclopropane synthases with about 30% of the total mycolates cyclopropanated at the proximal position (Table I). Total CMAS-1 activity, however, increases upon coexpression from 30 to 50% cyclopropanation of the distal position. One interpretation of this result is that the distal cyclopropane is formed after the proximal cyclopropane with CMAS-1 preferentially recognizing the proximal cyclopropanated precursor as a substrate.

The biological significance of lipid cyclopropanation has been most extensively studied in E. coli; however, the lack of any dramatic phenotype associated with either cyclopropane fatty acid synthase null mutants or cyclopropane fatty acid synthase overexpressors has left the role cyclopropanation plays in cellular metabolism unclear (28, 29). A large increase in the synthesis of cyclopropane-containing plasma membrane fatty acids has been shown to accompany the transition from log to stationary phase, which suggests that cyclopropanation offers some protective advantage to stationary cultures (27). E. coli, which have been grown on cyclopropane fatty acids, are more resistant to killing by hyperbaric oxygen treatment, suggesting that cyclopropanes do have a stabilizing or rigidifying effect on the membrane (30). This is confirmed by the increased susceptibility to killing by freezing observed in cyclopropane fatty acid synthase mutants of E. coli (29). It has also been shown by examining the 2H NMR of specifically deuterated cyclopropane-containing lipids, that cyclopropanated membranes enhance stability by suppressing segmental mobility of hydrocarbon chains, thus providing increased rigidity with respect to external stress (31). These studies consistently support the position that cyclopropanation of membrane lipids, although a rather subtle modification, does contribute to increased structural integrity of membranes containing short chain fatty acids (32). In addition, cyclopropanation is intermediate in fluidity effects between the more fluid cis-olefin and the less fluid trans-olefin as measured by DSC (33).

Recent work on the structure of the mycobacterial cell wall suggests that the proximal cyclopropane lies at the boundary of what Minnikin (3, 26) has referred to as the structural permeability barrier. A dramatic high temperature phase transition has recently been demonstrated to occur at 60 °C in purified cell walls of M. chelonei by DSC (4). The temperature of this transition suggests that at physiologically relevant temperatures, much of the cell wall exists in a state of exceptionally low fluidity. Cyclopropanation of mycolic acids, in addition to rendering lipids less susceptible to peroxidation, may decrease the actual fluidity even more, thus contributing to the overall impermeability of the cell wall. We examined the effect of substitution of a cis-olefin with a cis-cyclopropane in mycolic acids on cell wall thermochemistry and showed, with either purified cell wall or MAMEs, that proximal cyclopropanation increased the observed temperature of the transition by approximately 3 °C. The magnitude of this change seems quite reasonable since substitution of a cis-cyclopropane for a cis-olefin in the much shorter palmitoleate (C16:1), raises the observed temperature of phase transition by 15.6 °C (33), and only about 30% of the mycolates are converted to the cyclopropanated form. The distal cyclopropane had no such effect, possibly reflecting the role of this cyclopropane in interacting with other lipids that form a less tightly associated region that is not observed by DSC of detergent-extracted cell walls. In fact, our M. smegmatis cell wall preparations gave significantly lower melting temperatures than purified cell walls from M. smegmatis prepared without detergent extraction presumably due to the loss of ancillary lipids during the Triton X-114 extraction.

The impermeability of the mycobacterial cell wall is a hallmark of the organism. In the case of slow growing and pathogenic mycobacteria such as M. tuberculosis, it seems likely that high durability of mycolic acids would be essential, especially in the face of environmental and host-initiated oxidative stress in its intracellular habitat (13, 16). Dicyclopropyl mycolic acids are the major species found in many slow growing and pathogenic strains of mycobacteria including M. avium, Mycobacterium kansasii, M. marinum, M. leprae, Mycobacterium paratuberculosis, and M. tuberculosis (3). In contrast fast growing saprophytic mycobacteria such as M. smegmatis, Mycobacterium phlei, and Mycobacterium chelonae appear to possess primarily diunsaturated mycolic acids with an abundance of cis-olefins (34). In the case of the distal cyclopropane, we have previously demonstrated that expression in M. smegmatis results in significant protection from hydrogen peroxide (13). In the case of the proximal cyclopropane, we have been unsuccessful in demonstrating a similar role in protection from oxidative stress (data not shown). This may be related to the largely internal and less accessible location of the proximal cyclopropane.

Cyclopropanation of fatty acids only occurs in a small number of related taxa of bacteria. Among mycobacteria, this modification is limited to the slow growing pathogens. Mammals do not cyclopropane unsaturated lipids. Thus, enzymes catalyzing this unique modification constitute a viable target for the design of new chemotherapeutic agents against pathogenic mycobacteria, as well as providing the tools for understanding the biosynthesis, regulation, and function of these complex lipids.

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