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The Mycobacterial Cell Envelope: a Target for Novel Drugs Against Tuberculosis

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The re-emergence of mycobacterial diseases, such as tuberculosis (TB) and other atypical mycobacterioses, continues to present a formidable challenge. The World Health Organization declared TB a global health emergency in 1993, and estimated that at least 30 million people will have died from tuberculosis between 1990 and 1999, with the annual death toll expected to reach 3·5 million by the year 2000. In the USA, more than 22 000 new cases are being reported annually, and these are mainly among the elderly, minority groups, foreign immigrants and individuals co-infected with human immunodeficiency virus (HIV). The increasing prevalence of drug resistant *Mycobacterium tuberculosis* has further exacerbated the problem (Barnes et al 1991; Bloch et al 1994), such that newly infected or re-infected HIV patients with multi-drug resistant *M. tuberculosis* show a fatality rate of 40–60%, a rate equivalent to the mortality seen in untreated tuberculosis (Barnes et al 1991; Bloch et al 1994). Although anti-mycobacterial agents are available, their abuse and the historically protracted regimen have led to the emergence of multi-drug resistant (MDR) strains, and have underscored the need to develop a new series of anti-tuberculosis agents. As a consequence, the development of new therapies for treating both sensitive and especially resistant strains of *M. tuberculosis* requires a return to basic research to define the failures of existing anti-tuberculosis drugs, and to identify new drug targets. Historically, the mycobacterial cell wall has been an effective target for many drugs (Winder 1982).

Cell Wall Polysaccharides and Their Biogenesis: Targets for New Drug Development

The following discusses recent research on the structural definition and biosynthesis of the linkage unit, and arabinogalactan (AG), and puts forward a case for inhibition of the synthesis of the cell wall core.

Cell wall core

The cell wall core may be described as a cross-linked peptidoglycan to which are linked polysaccharide side chains (AG) esterified at their distal ends with long-chain α-alkyl, β-hydroxy mycolic acids, a complex which we have now termed mAGP (mycolylarabinogalactan-peptidoglycan) (Daffe et al 1990).

Peptidoglycan, which forms the backbone of the mAGP, consists of alternating units of *N*-acetylglucosamine and muramic acid (Misaki & Yukawa 1966; Adam et al 1969). The tetrapeptide side chains are attached to muramic acid which is cross-linked to AG via a phosphodiester link to position 6 of a proportion (about 10–12%) of the muramic acid residues (Lederer 1975). The tetrapeptide side chains consist of L-alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine (Petit et al 1969) with the diaminopimelic acids being further amidated (Weitzerbin-Falszpan et al 1970). This type of peptidoglycan (Alaγ) (Schleifer & Kandler 1972) is one of the most common found in bacteria. However, mycobacterial peptidoglycan differs in two ways; firstly, the muramic acid residues are *N*-acylated with glycolic acid (Azuma et al 1970), and secondly, the cross-links include a proportion of bonds between two residues of diaminopimelic acid as well as between diaminopimelic acid and D-alanine (Ghuysen 1968).

It was known even in the 1930s that the major cell wall polysaccharide is a serologically active branched-chain AG with the arabinose (Ara) residues forming the reducing termini of AG. A structural formula, which has now been shown to be incorrect, consisting of repeating units of 11 to 16 sugar residues was proposed. There was much uncertainty about the structure of the galactose (Gal) components of AG. In particular, there was dispute whether the Gal units were $\alpha 1 \rightarrow$ 4-linked Gal*p* or $\alpha 1 \rightarrow$ 5-linked Gal*f* (Vilkas et al 1973). It was also known that several mycobacteria also contain an immunologically active arabinomannan (Azuma et al 1967) which was cytoplasmic rather than cell wall polysaccharide, which has now turned out to be lipoarabinomannan (LAM) described in detail by researchers at Colorado State University (Besra & Chatterjee 1994).

Linkage unit and arabinogalactan

The polymer is unique not only in its elemental sugars but also in that, unlike most bacterial polysaccharides (Anderson & Unger 1983), it lacks any repeating oligosaccharide units, and is comprised instead of a few distinct, defined structural motifs. Partial depolymerization of the per-*O*-alkylated polysaccharide and analyses of the generated oligomers by gas chromatography–mass spectrometery (GC–MS) and fast-atom bombardment–mass spectrometry (FAB–MS) (Daffe et al 1990; McNeil et al 1987) established that the Ara and Gal residues are in the furanose form and the non-reducing termini of arabinan consist of a hexa-arabinofuranosyl motif [β-D-Ara*f*-$(1 \rightarrow 2)$-α-D-Ara*f*]$_2$-3,5-α-D-Ara*f*-$(1 \rightarrow 5)$-α-D-Ara*f*. The majority of the arabinan chains consist of 5-linked α-D-Ara*f* residues with branching introduced by 3,5-α-D-Ara*f* residues, the arabinan chains are attached to C-5 of some of the 6-linked Gal*f* residues, and approximately 2–3 arabinan chains are attached to the galactan core; the galactan regions consist of linear alternating 5- and 6- linked β-D-Gal*f* residues.

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Finally, the galactan region of AG is linked to the C-6 of some of the muramyl residues of peptidoglycan via the diglycosyl-phosphoryl bridge, α-L-Rha*p*-(1 → 3)-D-GlcNAc-(1 → P) and the mycolic acids are located in clusters of four on the terminal hexaarabinofuranosyl, but only two-thirds of these are mycolated (McNeil et al 1991).

More recently, Besra et al (1995) obtained oligosaccharide fragments containing up to 26 glycosyl residues by gentle acid hydrolysis of the per-*O*-methylated AG, converted them to fully per-*O*-alkylated oligoglycosyl alditols which were purified by high performance liquid chromatography, and the molecular weights and alkylation patterns were determined by FAB-MS and GC-MS. The extended non-reducing ends of the arabinan were shown to consist of a tricosarabinoside (23mer), with three such units attached to the galactan unit. In addition, the galactan was also isolated and was found to consist of 23 galactose residues of the repeating structure, [β-D-Gal*f*-(1 → 5)-D-Gal*f*-(1 → 6)]$_n$, devoid of any branching, thereby demonstrating that the points of attachment of the arabinan chains to galactan are close to the reducing end of galactan which itself is linked to peptidoglycan via the linker disaccharide-phosphate. As a consequence, a revised structural model is now proposed for the mAGP complex (Fig. 1).

Biosynthesis of the linkage unit and the galactan region of arabinogalactan

It would appear that the linkage unit, α-L-Rha*p*-(1 → 3)-D-GlcNAc represents the ideal target, since the whole of the mAG complex is covalently attached to peptidoglycan, via this unit, and, in addition, the Ara*f* and Gal*f* residues of AG also provide attractive targets for drug development due to their xenobiotic status in man. As a consequence, Mikusova et al (1996) recently addressed the biosynthesis of the linkage unit of the mycobacterial cell wall. Initial experiments demonstrated that freshly prepared membranes catalyzed the incorporation of radioactivity from UDP-[^{14}C]GlcNAc into a washed lipid fraction which was independent of ATP and decaprenol-phosphate. Analysis of the radio-labelled products by thin-layer chromatography revealed two glycolipids (GL 1 and GL 2) which were found to be mild-acid labile, and mild-alkali stable, a feature consistent with polyprenol-based glycolipids (Takayama et al 1973). Thus, the evidence pointed to the synthesis of two novel polyprenol containing glycolipids, much more polar than those described previously in mycobacteria (Wolucka et al 1993, 1994). When tunicamycin was added to membrane preparations, a dramatic inhibitory effect on the incorporation of [^{14}C]GlcNAc into GL 1 and GL 2 was observed, suggesting that the initial step in the synthesis of mycobacterial cell wall AG involves formation of a polyprenol-P-P-GlcNAc (GL 1) unit.

Incorporation of dTDP-[^{14}C]Rha, which is no longer commercially available, was dependent on the enzymatic synthesis of dTDP-[^{14}C]Rha from dTDP-[^{14}C]Glc using a crude extract of *E. coli* B (Okazaki et al 1962). The specific activity of the resulting dTDP-[^{14}C]Rha was low, and, because of its lesser specific activity, incorporation was considerably less than in the case of UDP-[^{14}C]GlcNAc. However, it was quite clear from autoradiography that the incorporation of [^{14}C]Rha took

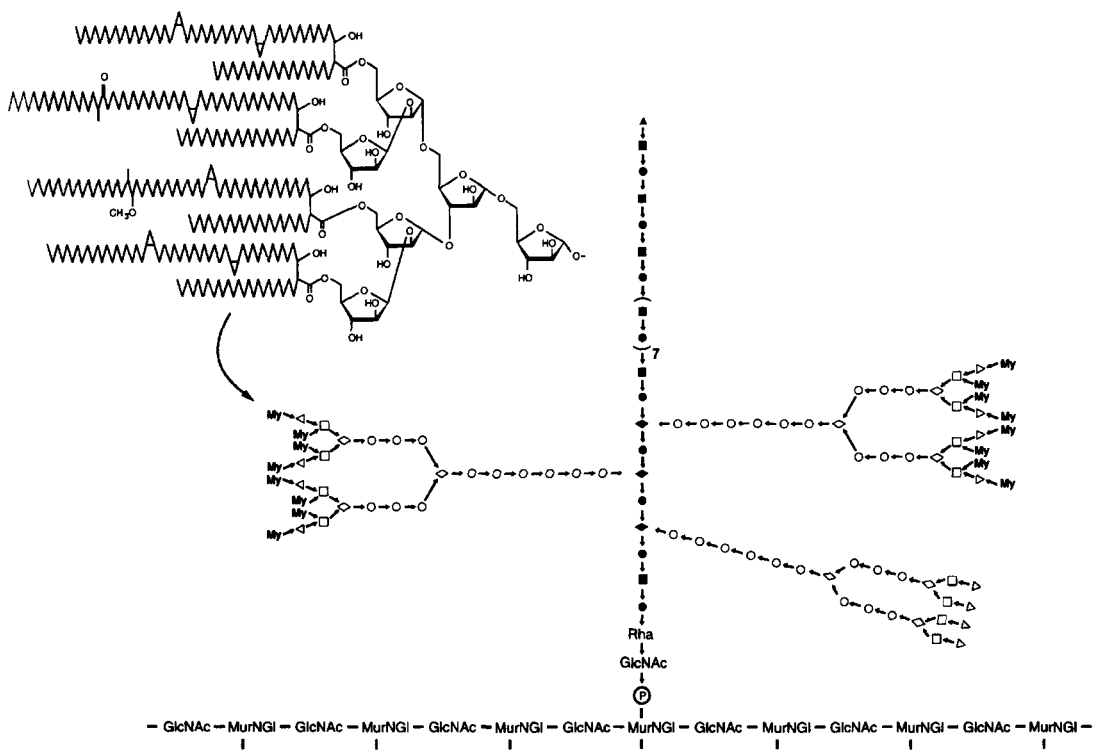

FIG. 1. An illustration of one of the ways in which the macro structural motifs of the cell wall arabinogalactan (AG) may be assembled. My mycolic acid, △ T-β-D-Ara*f*, □ 2-α-D-Ara*f*, ○ 5-α-D-Ara*f*, ◇ 3,5-α-D-Ara*f*, ▲ T-β-D-Gal*f*, ■ 6-β-D-Gal*f*, ● 5-β-D-Gal*f*, ◆ 5,6-β-D-Gal*f*.

place only in GL 2, and into the apolar glycopeptidolipids (GPLs), thus suggesting that GL 2 was a polyprenol-P-P-GlcNAc-Rha, which was confirmed later chemically by radio-GC analysis of its NaB[^{2}H]$_4$-reduced, per-*O*-methylated, per-*O*-acetylated product with an authentic standard prepared from the cell walls of *M. bovis* BCG.

Interestingly, addition of the cell wall enzyme preparation (Percoll-60) to the standard enzyme preparation containing UDP-[^{14}C]GlcNAc resulted in the emergence of two more polar glycolipids, GL 3 and GL 4. These higher homologues, GL 3 and 4, were also faintly evident in experiments using dTDP [^{14}C]Rha. More significantly, the inclusion of UDP-[^{14}C]Gal*p* resulted in exclusive labeling of GL 3 (trisaccharide) and GL 4 (tetrasaccharide), with Gal indicating the growth of the galactan chain on the polyprenol-P-P-GlcNAc-Rha unit. Glycosyl linkage analysis of [^{14}C]Gal-labeled glycolipids 3 and 4 showed two products coincident with t-Gal*f* and 5-linked Gal*f*, consistent with the proposed structure of AG and suggesting the presence of a contractase enzyme within our membrane preparations which converts UDP-Gal*p* to UDP-Gal*f* (Nassu et al 1996) before transfer to GL 2 and further extension to higher homologues (GL 3 and 4). Pulse-chase experiments established that a precursor-product relationship existed between GL 1–2 and GL 3–4.

Isolation of the polymerized product resulting from labelling using either UDP-[^{14}C]GlcNAc or UDP-[^{14}C]Gal*p* by differential solvent extraction also resulted in the emergence of even higher intermediates, GL 5, 6, and so on, eventually resulting in a polymer which possessed the characteristics of being mild acid-labile, mild-alkali stable, soluble in organic solvents, and excluded on a Bio-Gel P-100, suggesting a highly polymerized lipid-linked version of GL 1–4. Following mild-acid treatment and subsequent analysis by size-exclusion chromatography (Bio-Gel P-100), the polymer was found to consist of approximately 35–50 residues, and the glycosyl linkage analysis of the polymer by radio-GC demonstrated that the polymer contained t-Gal*f*, 5-linked Gal*f*, 6-linked Gal*f*, and 5,6-linked Gal*f*, thus demonstrating that the in-vitro synthesized product contained a full complement of galactan which is branched, presumably using endogenous arabinose-monophosphoryldecaprenol (DPA), which was recently implicated as a donor of Ara in the biogenesis of AG and LAM or from an unidentified sugar nucleotide.

Biosynthesis of the arabinan regions of arabinogalactan

Unfortunately, the low stability and poor biological availability through metabolic whole cell labeling of [1-^{14}C]-β-D-arabinofuranosyl-1-monophosphoryldecaprenol have hindered studies of arabinan metabolism in AG and LAM. As a consequence, a synthetic strategy was recently developed (Lee et al 1995) based on, firstly, the application of the phosphoramidite-phosphite triester methodology and, secondly, a novel *tert*-butyl dimethylsilyl arabinofuranosyl protection strategy, which allowed for regioselective C-1 acid hydrolysis and final deprotection with ammonium fluoride under mild conditions. Using synthetically derived DPA, a basic arabinosyl transfer assay was developed and incorporation into wall material was assayed using descending paper chromatography in a similar manner to that described by Yokoyama et al (1988) for the incorporation of galactosyl phosphorylpolyprenols into the lipoteichoic acids of *Bacillus coagulans*. Incorporation of radioactivity was boosted by co-incubation with a variety of sugar nucleotides and was linear with respect to membrane protein and acceptor P60 concentration. In addition, in a related time-course experiment, the α-anomer was found to be inactive. More recently, the DPA assay has been refined through the use of simple di- and trisaccharide hydrophobic acceptors which has clearly demonstrated that DPA is the donor of 2-linked and 5-linked Ara*f* residues within AG and LAM.

Mode of action of ethambutol

Recently, ethambutol, a widely used antimycobacterial agent, was shown to decrease the arabinose content of the cell wall (Mikusova et al 1995), however, the precise mode of action was never determined. Ethambutol was found to inhibit arabinosyl transferase activity in the DPA assay described by Lee et al (1995) to a residual activity of 40% at 50 $\mu g\ mL^{-1}$, suggesting that DPA is a substrate to more than one arabinosyl transferase within AG and LAM and that the primary site of action of ethambutol is as an inhibitor of one or more arabinosyl transferase. Mikusova et al (1995) demonstrated that ethambutol also primarily inhibits the synthesis of the arabinan components of AG, while inhibition of the corresponding arabinan components of LAM occurs later, suggesting a secondary target and separate pathways for AG and LAM biosynthesis. While the synthesis of these arabinans was normal in an ethambutol-resistant isogeneic strain, the addition of ethambutol to the resistant strain resulted in partial inhibition of the synthesis of the arabinan of LAM, thus resulting in the emergence of a novel, truncated form of LAM (Khoo et al 1996), indicating partial susceptibility of the resistant genes and suggesting an array of extramembranous arabinosyl transferases with various degrees of sensitivity to ethambutol.

Recent studies by Belanger et al (1996), through the use of target overexpression by a plasmid vector as a selection tool, have reported cloning of the *M. avium* ethambutol region, which contains three open reading frames, *embR*, *embA* and *embB*, which render the otherwise susceptible *M. smegmatis* resistant to ethambutol. Interestingly, the DPA assays described above demonstrated that *embA* and *embB* are associated with high level ethambutol-resistant arabinosyl transferase activity and that *embR* appears to modulate this in-vitro level activity. In summary, it would appear that *embA* and *embB* encode the drug target of ethambutol, and represent the putative arabinosyl transferases associated with AG synthesis.

Mycolic Acid Biosynthesis: Targets for New Drug Development

The mycolic acids are a complex family of long-chain fatty acids (Fig. 2) and frequently contain modifications such as double bonds, cyclopropyl rings or oxygen functions (Minnikin 1982). Takayama and colleagues investigated mycolic acid biosynthesis in the late 1970s and proposed a pathway (Fig. 3) involving four distinct stages (Takayama & Qureshi 1984). Stage 1 involves the synthesis of C_{24}–C_{26} straight chain saturated fatty acids to provide C–1 and C–2 atoms and the short alkyl chain; stage 2, the synthesis of C_{40}–C_{60} meromycolic acids to provide the main carbon backbone; stage 3, the modification of this backbone to introduce other functional groups (for instance, the introduction of the distal (Yuan et al

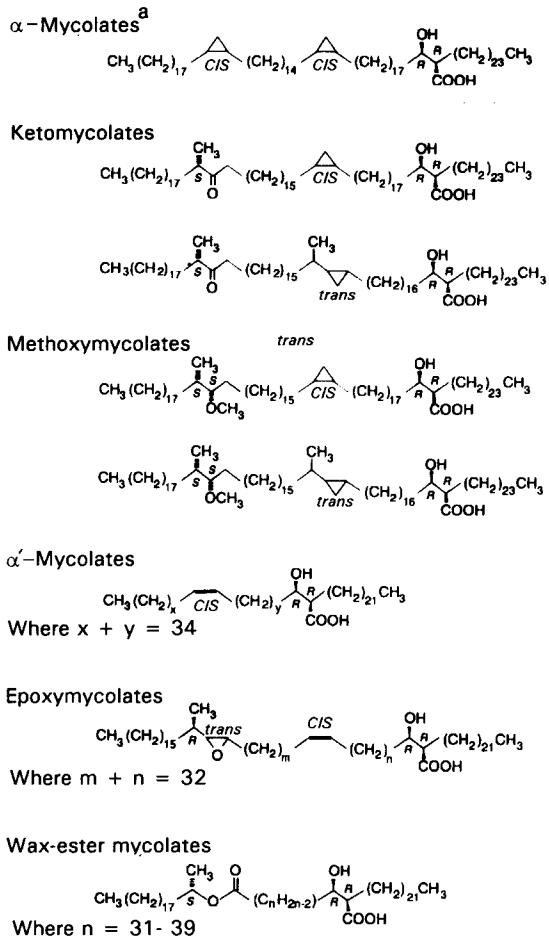

FIG. 2. Structures of mycobacterial mycolic acids. [a]In addition to α-mycolates, certain mycobacteria produce similar diunsaturated mycolates.

1995) and proximal (George et al 1995) cyclopropane rings has recently been ascribed to two genes, *cma1* and *cma2*, which show homology to the cyclopropane fatty acid synthase from *Escherichia coli* (Wang et al 1992)) and stage 4, the final mycolic acid condensation step. The cell-free system described by Takayama and co-workers (Takayama & Qureshi 1979; Takayama et al 1980) did not produce complete mycolic acids, but did provide evidence for an elongation system leading to meromycolates. The next development in our present-day understanding of mycolic acid biosynthesis came from the Toulouse group. Lacave et al (1990) developed a cell-free system capable of synthesizing whole mycolic acids from [^{14}C]acetate as a labelled precursor based on an insoluble enzymatic fraction from disrupted mycobacteria. Wheeler et al (1993) later extended this assay to examine the role of putative intermediates involved in mycolic acid biosynthesis.

The association between catalase–peroxidase, isoniazid susceptibility and resistance, and mycolic acid biosynthesis has been the subject of much discussion in the old literature (Winder 1982). Recently, it has been demonstrated that both isoniazid susceptibility and resistance are mediated by the *katG* gene which encodes a 80-kDa protein containing haeme and structural motifs characteristic of several bacterial catalase–peroxidases (Zhang et al 1992; Heym et al 1993). The *M. tuberculosis katG* gene was found to restore sensitivity to isoniazid in a resistant mutant of *M. smegmatis*, and deletion of the *katG* gene resulted in isoniazid resistance in two clinical isolates of *M. tuberculosis* (Zhang et al 1992; Heym et al 1993). More recently, it has been proposed that AhpC, a homologue of the thioredoxin-dependent alkyl hydroperoxide reductase, interacts directly with activated isoniazid and that AhpC compensates for the loss of *katG* peroxidase in isoniazid-resistant isolates of *M. tuberculosis* (Sherman et al 1996).

The primary biochemical effect of isoniazid is on mycolic acid synthesis (Takayama et al 1980). Recently, Banerjee et al (1994) isolated a novel gene, InhA, which, through point mutations within the *inhA* gene and in the 5′-regulatory region, were found to confer resistance to both isoniazid and ethionamide among clinical isolates. The inhA protein has now been shown to catalyze the NADH-specific reduction of long-chain (C_{12}–C_{24}) 2-trans-enoyl ACP intermediates (Quemard et al 1995) involved in fatty acid elongation consistent with its involvement in the early stages of mycolic acid biosynthesis. However, Barry & Mdluli (1996) recently suggested that isoniazid may specifically inhibit the insertion of a Δ5-double bond into a C_{24} fatty acid. Although Takayama originally suggested a Δ5-desaturase as the molecular target of isoniazid, he also suggested that isoniazid may be involved in some aspects of the elongation of C_{30}–C_{56} meromycolates based on the inhibitory effects of isoniazid on the synthesis of fatty acids and hydroxy lipids in a cell-free preparation of *M. tuberculosis* H37Ra (Takayama & Qureshi 1979; Takayama et al 1980). The addition of NADH and NADPH appeared to neutralize the inhibitory effects of isoniazid in this cell-free system, consistent with the recent identification of the InhA protein as the molecular target of isoniazid (Takayama & Qureshi 1979; Quemard et al 1995).

From our own perspective, we have recently investigated the mechanism of ethionamide and isoniazid on fatty acid and mycolic acid synthesis in *M. smegmatis* (unpublished results). In-vivo and in-vitro studies demonstrated that short-chain fatty acid synthesis by fatty acid synthase (FAS) complexes I and II were not inhibited by isoniazid, while the mycolic acid synthase (MAS) were affected in a differential fashion; inhibition of epoxymycolate and α-mycolate synthesis; no inhibition of α′-mycolates. In addition, the selection of ethionamide resistant mutants, still sensitive to isoniazid, demonstrated that, although epoxymycolates provide a primary site of action to ethionamide, α-mycolate synthesis provided the crucial target. Moreover, the study furnished other key information, in particular, the inhA target for isoniazid and ethionamide must be on the pathway leading to α-mycolate synthesis beyond the putative C_{24} Δ5-fatty acid intermediate and possibly after the synthesis of the C_{36}–C_{42} α′-meromycolate utilized in α–α′-mycolate synthesis, consistent with the earlier findings of Takayama and co-workers (Takayama & Qureshi 1979, Takayama et al 1980).

The hypothetical schemes presented in Fig. 3 raise several intriguing possibilities. The first concerns the role of a unique phospholipid, Myc-phospholipid, which has the attributes of a mycolyl carrier which is equivalent to R1 in Fig. 3. The other carrier group R2 is presumably an acyl carrier protein (ACP) or

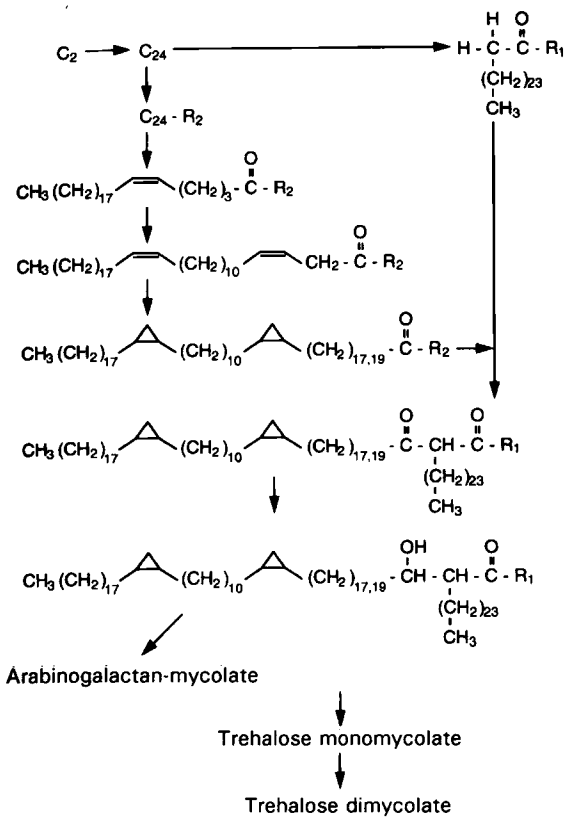

FIG. 3. Proposed biosynthetic pathway for mycolic acids.

membrane bound protein. Secondly, we speculate that Myc-phospholipid is a precursor of trehalose monomycolate. Finally, the fully formed mycolic acid is transferred to AG to form the mAG-complex either from Myc-phospholipid or trehalose monomycolate. We have previously demonstrated that Myc-phospholipid is a precursor in the transfer of mycolic acids to AG, trehalose monomycolate, and trehalose dimycolate. However, the precise role and transfer of mycolic acids, including the inter-relationship of Myc-phospholipid and trehalose dimycolate, are still unresolved.

It now appears, that we have isolated a mycolyltransferase that in a cell-free system catalyzes the transfer and exchange of mycolic acids from trehalose monomycolate to free [^{14}C]trehalose to yield both [^{14}C]trehalose monomycolate and [^{14}C]trehalose dimycolate (Belisle, unpublished results). We then observed that the *N*-terminal amino acid sequence of this mycolyltransferase was closely related to the antigen 85 (Ag85) complex. The three proteins comprising the Ag85 complex of *M. tuberculosis* (85A, B, and C) were purified to homogeneity and assessed for mycolyltransferase activity. All three exhibited substantial activity: the 85A and 85C yielded similar specific activities of $18{\cdot}2 \times 10^6$ and $25{\cdot}8 \times 10^6$ counts min^{-1} (mg of protein)$^{-1}$, respectively; however, the specific activity of 85B was only 20% of that observed for 85C. Analysis of the amino acid sequences of the Ag85 complex revealed the presence of a highly conserved carboxylesterase consensus sequence, GXSXG. To test the hypothesis that this sequence was essential for mycolyltransferase activity, site-directed mutagenesis was performed on the recombinant *ag85c* gene. Initially, a gene fragment encoding the mature Ag85C was cloned into the *E. coli* expression vector pET 23B and expressed as a C-terminal His-Tag fusion product. This recombinant product demonstrated significant mycolyltransferase activity. However, when the gene was altered so that a Ser → Ala mutation occurred in the carboxylesterase consensus sequence, all mycolyltransferase activity was lost. The definition of the active sites of the mycolyltransferase by site-directed mutagenesis should ultimately allow for the rational development of analogues that would inhibit this key enzymatic step.

Conclusions

The uniqueness of the mycobacterial cell wall which incorporates features of both Gram-positive and Gram-negative bacteria is built on an array of mycolic acids, regions of arabinan and galactan, and, indeed, the crucial linker disaccharide unit, some of which have been at the fore in current anti-mycobacterial therapy without never being fully understood, provide attractive targets in new drug design. As a consequence, the topic of mycobacterial cell wall biogenesis should provide valuable new tools that may eventually pave the way for the development of high-throughput screens to identify novel anti-tuberculosis agents or lead to the isolation of key enzymes and genes that encode them that may serve as new molecular targets for selective antimycobacterial chemotherapy.

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