Unique Mechanism of Action of the Thiourea Drug Isoxyl on Mycobacterium tuberculosis

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The thiourea isoxyl (thiocarlide; 4,4’-disoamyoxydi-phenylthiourea) is known to be an effective anti-tuberculosis drug, active against a range of multidrug-resistant strains of Mycobacterium tuberculosis and has been used clinically. Little was known of its mode of action. We now demonstrate that isoxyl results in a dose-dependent decrease in the synthesis of oleic and, consequently, tuberculostearic acid in M. tuberculosis with complete inhibition at 3 μg/ml. Synthesis of mycolic acid was also affected. The anti-bacterial effect of isoxyl was partially reversed by supplementing growth medium with oleic acid. The specificity of this inhibition pointed to a Δ9-steayrol desaturase as the drug target. Development of a cell-free assay for Δ9-desaturase activity allowed direct demonstration of the inhibition of oleic acid synthesis by isoxyl. Interestingly, sterculic acid, a known inhibitor of Δ9-desaturases, emulated the effect of isoxyl on oleic acid synthesis but did not affect mycolic acid synthesis, demonstrating the lack of a relationship between the two effects of the drug. The three putative fatty acid desaturases in the M. tuberculosis genome, desA1, desA2, and desA3, were cloned and expressed in Mycobacterium bovis BCG. Cell-free assays and whole cell labeling demonstrated increased Δ9-desaturase activity and oleic acid synthesis only in the desA3-overexpressing strain and an increase in the minimal inhibitory concentration for isoxyl, indicating that DesA3 is the target of the drug. These results validate membrane-bound Δ9-desaturase, DesA3, as a new therapeutical target, and the thioureas as anti-tuberculosis drugs worthy of further development.

The prevalence of tuberculosis, particularly in concert with human immunodeficiency virus infection and AIDS, has been well documented (1). An equally serious public health problem is increasing multi-drug-resistant tuberculosis (2). At present only a few alternative chemotherapeutic regimens are available, resulting in poor therapeutic outcomes and high mortality rates among multi-drug-resistant tuberculosis patients (3). There is an urgent need to develop new effective antituberculosis drugs with bactericidal mechanisms different from those of the presently available agents.

It is prudent to re-examine drugs that were formerly deemed effective against tuberculosis. Isoxyl (ISO)1 (thiocarlide) (Fig. 1) is a thiourea derivative that was successfully used in the 1960s to treat tuberculosis (4–7). Recently, ISO was shown to have considerate antimycobacterial activity in vitro and to be effective against various clinical isolates of multidrug-resistant strains of Mycobacterium tuberculosis in the range of 1–10 μg/ml (8). An early note reported that ISO, like isoniazid (INH) and ethionamide (ETH), strongly inhibits the synthesis of mycolic acids (9), a result since confirmed with the demonstration that all types of mycolic acids are affected (8). In addition it was noted that ISO also inhibited shorter chain fatty acid synthesis (8–11), suggesting inhibitory effects different from those of INH and ETH and raising the prospects of novel fatty acid biosynthetic targets exploitable for new drug development against multi-drug-resistant tuberculosis.

Experimental Procedures

Bacterial Strains and Growth Conditions—Escherichia coli strain XL-1 Blue (Strategene, La Jolla, CA), cultured on Luria-Bertani broth or agar medium (Invitrogen) and containing kanamycin at a concentration of 25 μg/ml, was used for generating recombinant clones. Mycobacterium bovis BCG strain 1173P2 was used for whole-cell labeling, MIC determinations, cell-free reactions, and expression of M. tuberculosis H37Rv desA1, desA2, and desA3 genes. Liquid cultures of M. bovis BCG and M. tuberculosis H37Rv (ATCC 27711) were grown in Sauton medium containing 0.025% tyloxapol (12). Recombinant M. bovis BCG clones were selected on Middlebrook 7H11 agar supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco) (13) or on Sauton medium containing 20 μg/ml kanamycin. To determine whether the addition of this oleic acid supplement could override the effects of ISO, a series of 10-fold dilutions of M. tuberculosis was prepared from stock culture in a glycerol-alanine-salts medium with phosphate-buffered saline as diluent. An aliquot (5 μl) of each dilution was spotted on two types of agar plates, those with 7H11 agar medium

1 The abbreviations used are: ISO, isoxyl; ETH, ethionamide; INH, isoniazid; FAME, fatty acid methyl ester; MAME, mycolic acid methyl ester; MIC, minimal inhibitory concentration; ABC, albumin-dextrone complex; OADC, oleic acid-albumin-dextrone complex; GC, gas chromatography; RT, reverse transcription; MOPS, 4-morpholinepropanesulfonic acid.
supplemented with OADC or those with non-oleic acid-containing ADC (albumin-dextrose-catalase), prepared with the incorporation of ISO to a final concentrations of 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 μg/ml. After incubation, the plates were incubated at 37 °C for 21 days. The growth rates and bacterial effects were scored by comparing size and number of colonies on plates.

Whole-cell Radiolabeling and Analysis of the in Vivo Effects of ISO and Steric Acid on Fatty Acid and Mycolic Acid Synthesis—Mycobacteria were grown in Sauton medium at 37 °C to A560 0.250. ISO (a gift from Dr. P. Draper, National Institute of Medical Research, London, UK) was added followed by further incubation for 8 h before the addition of 1.2-14C-acetate (110 μCi/ml; PerkinElmer Life Sciences) to a final concentration of 4 μCi/ml. Cells were harvested for 24 h, harvested, washed, saponified with 15% tetrabutylammonium hydroxide at 100 °C overnight, methylated, and extracted (8). Extracts containing equal amounts of radiolabeled fatty acid methyl esters and mycolic acid methyl esters from control and ISO-treated cells were subjected to TLC on silica gel plates (silica gel 60 F254, Merck) that had been impregnated equal amounts of radiolabeled fatty acid methyl esters and mycolic acid ester standard was visualized by spraying with 10% sulfuric acid and anisaldehyde (15). Plates were developed twice in petroleum ether-acetone (90:10), radioactive bands were located by autoradiography, and the oleic acid methyl ester standard was visualized by spraying with 10% sulfuric acid and anisaldehyde (15). To analyze the effects of ISO on the synthesis of individual fatty acids, extracted methyl esters were treated with TriisO (Pierce) to silylate any free hydroxyl groups, and products were dissolved in hexane and injected onto a capillary HP-1 column (5 m by 0.25 mm inner diameter) (Supelco Inc., Bellefonte, PA) coupled to a Hewlett Packard model 5890 Series II GC with a thermal conductivity detector attached to a GC-RAM™ radio detector (Inus Systems, Tampa, FL). The initial column temperature was 80 °C, which was increased to 185 °C at a rate of 30 °C/min, followed by an increase at the rate of 5 °C/min to a final temperature of 345 °C. The eluted peaks of labeled FAMEs were identified by comparison of their retention time with those of available fatty acid methyl ester standards. Analysis of the effects of steric acid, the 9-desaturase inhibitor, on oleic acid synthesis was performed by comparing size and number of colonies on plates.

Confirmation of Position of Double Band in Mono-unsaturated Fatty Acid by Agarose Gel Electrophoresis—The effect of ISO on fatty acid synthesis was assayed as described above. The effects of sterculic acid and ISO on fatty acid synthesis were compared by argentation TLC.

Cloning and Overexpression of the M. tuberculosis desA3 Transcription Unit—The wild type—M. bovis BCG—was grown in Sauton broth as described (23), and the production of recombinant DesA1 and six-histidine-tagged DesA3 proteins was then monitored by silver staining any free hydroxyl groups, and products were dissolved in hexane and injected onto a capillary HP-1 column (5 m by 0.25 mm inner diameter) (Supelco Inc., Bellefonte, PA) coupled to a Hewlett Packard model 5890 Series II GC with a thermal conductivity detector attached to a GC-RAM™ radio detector (Inus Systems, Tampa, FL). The initial column temperature was 80 °C, which was increased to 185 °C at a rate of 30 °C/min, followed by an increase at the rate of 5 °C/min to a final temperature of 345 °C. The eluted peaks of labeled FAMEs were identified by comparison of their retention time with those of available fatty acid methyl ester standards. Analysis of the effects of steric acid, the 9-desaturase inhibitor, on oleic acid synthesis was performed by whole cell labeling with [1.2-14C]acetate followed by argentation TLC and GC analysis, as described above. The effects of steric acid and ISO on mycolic acid synthesis were compared by argentation TLC.

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ison, WI). To locate DesA3 within the bacterial cell, Western blotting was performed using subcellular fractions prepared from the DesA3-His-tagged recombinant strain. The cytosol was recovered as the supernatant after centrifugation of cell lysate at 200,000 \( \times g \) for 1 h, and the pellet represented the cell membrane fraction. Aliquots (100 \( \mu \)g) of protein from each fraction were analyzed by immunoblotting as described above. Alignment of amino acid sequences was achieved with Multalin (www.toulouse.inra.fr/multalin.html).

**Purification of Recombinant His-tagged M. tuberculosis DesA3**—The recombinant \( M. \) \textit{bovis} BCG containing pVV16/desA3 were grown to mid-log phase \((A_{600} 0.7) \) at 37 °C in Sauton medium containing 20 \( \mu \)g/ml kanamycin and 20 \( \mu \)g/ml FeSO\textsubscript{4}. Cells were harvested and washed once in 50 mM MOPS buffer, pH 8. The cell pellet (20 g wet weight) was subsequently resuspended in 30 ml of buffer A (50 mM MOPS, pH 8, 10 mM MgCl\textsubscript{2}, 10% glycerol, and 500 mM NaCl) and sonicated for 10 cycles (60 s on; 90 s off) on ice. The cell lysate was centrifuged at 27,000 \( g \) for 30 min, the resulting supernatant was collected and loaded onto a 1-ml metal BD-Talon\textsuperscript{TM} resin-chelating column (BD Biosciences Clontech, Palo Alto, CA) previously equilibrated with 20 ml of buffer A. Column washing was performed with 25 ml of buffer A followed by 25 ml of buffer B containing 5 mM imidazole. Proteins were eluted using buffer C containing 50, 100, or 200 mM imidazole. The elution fractions were checked by SDS-PAGE (12% gel) and Western blot. The 200 mM imidazole fraction, which contained His-tagged \( M. \) \textit{tuberculosis} DesA3, was subsequently adjusted to a volume of 2.5 ml with buffer B (10 mM MOPS pH 8, 1 mM MgCl\textsubscript{2}, 10% glycerol), desalted using a PD-10 column (Amerham Biosciences), and concentrated, and the protein concentration was measured before the determination of the enzymatic function of the purified protein.

**Determination of the Effect of Overexpression of desA3 on the MIC of ISO**—The microplate alamar blue assay as modified by Yajko et al. (24) was used to determine the MIC of ISO. Inocula were prepared from \( M. \) \textit{bovis} BCG and the recombinant strain harboring the pVV16/desA3 construct. Cells were grown in Sauton medium containing 0.025% Tyloxapol to a turbidity equal to that of a No. 1 McFarland standard \((-4.5 \times 10^{8} \) colony-forming units/ml). Serial dilutions of ISO from a stock solution in Me\textsubscript{2}SO were prepared in Sauton medium. Duplicated treatments with different concentrations of ISO were conducted. Four controls were included that contained medium-only, culture-only, medium plus Me\textsubscript{2}SO, and culture plus Me\textsubscript{2}SO. At the outset, 10 \( \mu \)l of ISO in Me\textsubscript{2}SO and 10 \( \mu \)l of culture were added to each of the wells in an opaque 96-well microtiter plate. Plates were further incubated, and the color in each treated culture was recorded. MIC was determined as the lowest concentration of ISO for which the blue dye in the treated culture did not change color.

**RESULTS**

**ISO Inhibits the Synthesis of Unsaturated Fatty Acids and Mycolic Acids**—Previous studies with [1,2,14C]acetate as a precursor of fatty acid synthesis showed that ISO inhibited the synthesis of both mycolic acids and shorter chain fatty acids in \( M. \) \textit{tuberculosis} (8). In this latter respect, ISO differed from INH and ETH, suggesting a different mode of action of ISO. To evaluate the effects of ISO on the synthesis of saturated fatty acids, unsaturated fatty acids, and mycolic acids, extracts containing 14C-labeled fatty acid and mycolic acid methyl esters were prepared from untreated and ISO-treated \( M. \) \textit{tuberculosis} cultures and analyzed by autoradiography. In the presence of ISO at concentrations as low as 1 \( \mu \)g/ml, there was a apparent decrease in the synthesis of unsaturated fatty acids concomitant with a partial increase in the synthesis of saturated fatty acids (Fig. 2). Measurement of the radioactivity in the relevant bands established that ISO at 1.0 \( \mu \)g/ml inhibited unsaturated fatty acid synthesis by 60%. The effect of ISO on mycolic acid synthesis, reported previously (8), can also be seen by this simple analysis; clearly, ISO inhibited the incorporation of [1,2-14C]acetate into all types of resolved mycolic acids (Fig. 2).

The cellular fatty acids synthesized under present conditions by \( M. \) \textit{tuberculosis} and \( M. \) \textit{bovis} BCG are palmitic acid, stearic acid, oleic acid, and tuberculostearic acid (Fig. 3, A and C), in accord with earlier reports (25, 26). In \( M. \) \textit{tuberculosis} and \( M. \) \textit{bovis} BCG treated with ISO at 2.0 \( \mu \)g/ml, the incorporation of [1,2-14C]acetate into oleic acid and tuberculostearic acid was almost totally eliminated as determined by radio-GC (Fig. 3, B and D). Similar results were obtained at 1 \( \mu \)g/ml ISO.

**The Effects of Oleic Acid Supplement on Partial Rescue of \( M. \) \textit{tuberculosis} from ISO**—The observations that ISO inhibited oleic acid synthesis in \( M. \) \textit{tuberculosis} and \( M. \) \textit{bovis} BCG led to the concept that supplementation of the growth medium with oleic acid should rescue cells from the killing effect of ISO. The addition of oleic acid to the TH11 agar medium in the form of the OADC partially reversed the bactericidal effect of ISO; there was comparable growth on the OADC-supplemented medium with or without ISO; however, the albumin-dextrase complex without oleic acid could not support growth in the presence of ISO (Fig. 4).

**Demonstration of Δ9 Desaturase Activity in Cell-free Extracts and the Effects of ISO**—The specific inhibition of oleic acid synthesis by ISO suggested that the drug target is the aerobic desaturation system responsible for the synthesis of oleic acid from stearoyl-CoA in mycobacteria (17, 18, 27, 28). To test this hypothesis \( M. \) \textit{bovis} BCG was grown in iron-supplemented medium, sonicated, and centrifuged at low speed, and both the cell wall pellet and the supernatant containing both cytosol and plasma membrane were assayed for Δ9 fatty acid desaturase activity. The assay was performed as originally described by Fulco and Bloch (18) with minor modifications. For instance, ferrous ions were added to the culture medium rather than to the reaction mixture itself because iron was shown to be required at the terminal stage of synthesis and/or assembly of the desaturase system (17). Only the supernatant allowed the conversion of stearoyl-CoA to the oleic acid derivative in the presence of atmospheric oxygen and NADPH. Consistent with the findings of Fulco and Bloch (18), in the absence of NADPH in the assay mixture, no oleic acid was detected. ISO consistently inhibited the incorporation of radioactivity into the oleic acid, but not into stearic acid, by 7% at 0.1 \( \mu \)g/ml and 61% at a concentration of 1.0 \( \mu \)g of ISO/ml in one typical experiment.
Overexpression of M. tuberculosis desA1, desA2, and desA3 Genes in M. bovis BCG and Evidence That desA3 Encodes a Fatty Acid Desaturase—Three open reading frames in the genome of M. tuberculosis are annotated as putative fatty acid desaturases: desA1, desA2, and desA3 (20). All three open reading frames of desA were amplified by PCR, cloned in-frame into the mycobacterial expression vector pMV261 or pVV16, and constitutively expressed under the control of the hsp60 promoter in M. bovis BCG. Production of all three proteins was confirmed the function of the DesA3 desaturase as responsible for the synthesis of oleic acid. Analysis of the radiolabeled fatty acid methyl esters by radio-GC confirmed this conclusion (Fig. 5). Consistent with these results, comparison over time of the δ9-desaturase activity exhibited by cell-free extracts of M. bovis BCG wild type and the M. bovis BCG/pVV16desA3 clone over time showed an almost 50% increase in enzyme activity in the latter strain at the longer incubation periods (Fig. 6).

That the C18 unsaturated product formed in the whole cells corresponded to oleic acid (C18:1Δ9) was checked by subjecting the methyl esters to OsO4 oxidation, trimethylsilylation, and GC-mass spectrometry analysis (16). The characteristics of the EI-mass spectrometry spectrum of C18:1Δ9 are a major fragment at m/z 257 and minor fragments at m/z 217 and 185. However, C18:1Δ9 yields typical fragments at m/z 259 and 217. The sole product identified in whole cells of wild type M. bovis BCG and M. bovis BCG/pVV16desA3 was C18:1Δ9 (data not shown).

Because the overexpression of desA3 in whole BCG cells could have indirectly stimulated the production of oleic acid, recombinant DesA3 purification was achieved (Fig. 7A). Although the purified enzyme itself showed no activity in the desaturase assay, presumably due to missing cofactors or components of the desaturase system (e.g. cytochrome c reductase or ferredoxin, flavoprotein) (18, 28), the addition of the purified enzyme to the standard Δ9 cell-free assay resulted in an approximate 2-fold increase in specific activity of the enzyme (Fig. 7B). The enzyme activity was dependent on the amount of recombinant DesA3 added to the assay mixture (results not shown) and the incubation time (Fig. 7B). These results confirmed the function of the DesA3 desaturase as responsible for the synthesis of oleic acid. Finally, a desA3 transcript was detected by RT-PCR in M. tuberculosis H37Rv during the exponential growth (data not shown), confirming the expression of this gene in vivo and emphasizing the importance of the enzymatic product and function.

Overexpression of the M. tuberculosis desA3 Increased the Resistance of M. bovis BCG to ISO—The MIC of ISO against...
the parent *M. bovis* BCG as determined by the microplate alamar blue assay was 3.0 \( \mu \text{g/ml} \) compared with 6.0 \( \mu \text{g/ml} \) for the *M. bovis* BCG/pVV\text{-}desA3-overexpression strain. Thus the terminal enzyme of the \( \Delta 9 \) desaturation system encoded by \textit{desA3} is very likely to be the target of the drug.

Comparative Effects of ISO and the Known \( \Delta 9 \) Desaturase Inhibitor, Sterculic Acid—The naturally occurring cyclopentenediene fatty acid, sterulic acid (9,10-cyclopropenoid acid), is a potent and specific inhibitor of the \( \Delta 9 \) desaturation of stearic to oleic acid (29–32) and, therefore, provided a useful means to compare the effects of ISO to those of an established inhibitor of acyl-CoA \( \Delta 9 \)-desaturase. The effect of sterulic acid on the growth of *M. bovis* BCG was determined by the broth dilution technique (8) at concentrations of 10, 25, 50, and 100 \( \mu \text{g/ml} \). Consistent with the fact that oleic acid is synthesized by a stearoyl-CoA utilizing \( \Delta 9 \)-desaturase in mycobacteria, *M. bovis* BCG was found to be sensitive to sterulic acid. Sterulic acid at 50 \( \mu \text{g/ml} \) was sufficient to inhibit the growth of the cells by about 80%, and complete inhibition of 1.0 \( \times 10^6 \) colony-forming units/ml was obtained at a concentration of 100 \( \mu \text{g/ml} \). Under present growth conditions employing the broth dilution technique, sterculic acid had a MIC value between 50 and 100 \( \mu \text{g/ml} \). The inhibitory effect of sterulic acid on the \( \Delta 9 \)-desaturase activity was also evaluated in intact cells of *M. bovis* BCG by measuring the incorporation of [1,2-\( ^{14} \text{C} \)]acetate into oleic acid and analyzing the extractable lipids by radio-GC. At 50 \( \mu \text{g/ml} \), there was complete inhibition of the synthesis of oleic acid (Fig. 8), and the effects of ISO and sterulic acid on oleic acid synthesis were similar in this respect. However, at this concentration, sterulic acid, unlike ISO, had no effect on mycolic acid synthesis, whereas under these conditions, 1 \( \mu \text{g} \) ISO/ml resulted in inhibition of synthesis of \( \alpha \)- and \( \beta \)-mycolates by 64 and 27%, respectively (results not shown).
conclusion that the production of oleic acid and mycolic acids is not linked, a conclusion reinforced by the fact that sterculic acid, unlike ISO, does not result in the accumulation of long chain fatty acids (C24:0 to C26:0) (Fig. 8). This latter phenomenon is also seen under conditions of INH treatment (10), suggesting that long chain fatty acid accumulation is a consequence of the inhibition of mycolic acid synthesis and provides further proof that the effects of ISO on mycolic acids are distinct from those on oleic acid synthesis.

DISCUSSION

Previous observations demonstrated that ISO inhibits the synthesis of both mycolic acids and shorter chain fatty acids (8–10). In contrast, both INH and ETH inhibit mycolic acid synthesis (10, 11) and result in the accumulation of fatty acids (10). Cross-resistance between ISO and ETH suggests that these two drugs share a common step in their action, and a recent genetic and chemical study revealed that cross-resistance to ISO and ETH is associated with the activation of the thiocarbonyl moiety (Fig. 1) shared by these two drugs (33, 34). The product of the resistance-conferring gene was identified as a putative regulator controlling the expression of a flavin-containing monoxygenase that catalyzes the S-oxidation of the thiocarbonyl to S-oxide electrophilic intermediates (34, 35).

The present work serves to characterize the site of action of this activated form of ISO. Following the earlier observation of an inhibition of short chain fatty acids, efforts were focused in this area of metabolism, resulting in the conclusion that ISO is unique in its ability to inhibit the synthesis of oleic acid and tuberculostearic acid (Figs. 2 and 3). Oleic acid is the most abundant unsaturated fatty acid in Mycobacterium spp. (36) and is a vital constituent of mycobacterial membrane phospholipids (37, 38), where it apparently plays an essential role in membrane physiology (39). At physiological temperatures, phospholipids containing only saturated fatty acids cannot form a lipid bilayer, but the introduction of the appropriate unsaturated fatty acids decreases the transition temperature from gel to liquid crystalline phases and provides membranes with the necessary fluidity for physiological function (40–43). The vital functions of oleic acid lead to the conclusion that inhibition of its synthesis results in cell death, and thus, the enzymes involved in oleic acid synthesis are probably lethal targets for drug therapy.

ISO also had a dramatic effect on the synthesis of the C19-monomethyl-branched stearic acid, tuberculostearic acid. Typically, mycobacteria contain a large amount of methyl-branched fatty acids (44, 45), mainly tuberculostearic acid and multimethyl-branched acids such as phthienoic (2,4,6-trimethyloctacosanoic) acids (46, 47) and mycocerosic (2,4,6,8-tetramethylheptacosanoic) acids (48). The mycocerosic acids are confined to the phthiocerol-containing cell wall lipids and, although apparently implicated in the pathogenesis of M. tuberculosis (49, 50), are not essential for viability. However, tuberculostearic acid is a key ingredient of membrane phospholipids. It appears likely that the inhibition of tuberculostearic acid by ISO is a consequence of its effect on oleic acid synthesis, since tuberculostearic acid arises by direct methylation of oleic acid by S-adenosylmethionine once oleic acid is first esterified in the form of oleoylphosphatidylethanolamine (51, 52). The indirect effect of ISO on tuberculostearic acid probably also extends to the synthesis of the phosphatidylinositol mannosides and even lipomannan and lipoarabinomannan, since tuberculostearic acid is a major constituent of all of the mycobacterial phosphoinositides (53).

This evidence suggested at once that ISO acts by inhibiting stearoyl desaturase, the enzyme responsible for the insertion of the double bond at carbon 9 of stearic acid. The existence of such a desaturation system catalyzing the formation of oleic acid in mycobacteria has actually been demonstrated using Mycobacterium phlei as a model strain and shown to involve a particulate terminal desaturase enzyme that desaturates ste-
aroyl-CoA to form octadec-9-enoic acid (oleic acid) in the presence of NADPH as the reducing agent, NADPH-cytochrome c reductase, iron for electron flow, and oxygen as the final acceptor (17, 18, 27, 28). A desaturase assay based on that described by Fulco and Bloch (18) and conducted with cell-free extracts of M. bovis BCG in the presence or absence of ISO clearly demonstrated the ISO inhibits Δ9-desaturase activity in vitro. Furthermore, the Δ9-desaturase of the present system appears to be encoded by the putative acyl-CoA desaturase gene, desA3, as overexpression of the M. tuberculosis desA3 gene in M. bovis BCG, but not those of the other putative desaturase genes of the genome, desA1 or desA3, resulted in increased synthesis of oleic acid and Δ9-desaturase activity in the recombinant strain (Figs. 5 and 6). Our inability to associate Δ9-desaturase activity with the purified DesA3 enzyme is not surprising in light of its membranous nature and complex cofactor requirements. Instead, we had to rely on demonstrating a boosting effect on the endogenous activity of the wild type strain (Fig. 7). The overexpression of desA3 in M. bovis BCG also resulted in increased MIC of ISO in BCG/pVV16desA3 as compared with the parent strain, strongly suggesting that the terminal desaturase enzyme of the desaturation system is the target of the drug. Incidentally, Obukowicz et al. (54) show that a urea-derived inhibitor with a structure not unlike that of ISO selectively inhibits the stepwise Δ5 desaturase synthesis of arachidonic acid in rat and offers promise as an anti-inflammatory agent. This work further underlines the role of urea-based products as inhibitors of membrane and CoA requiring desaturases.

Consistent with its demonstrated membrane localization and affinity for acyl-CoA substrates, DesA3 shares signature motifs of membrane acyl-lipid and acyl-CoA desaturases characterized by three regions of primary conserved sequences, HX3 or 4H, HX2 or 3H, and HX2 or 3HH, designated as region Ia, Ib, and II (39, 55). However, the alignment of the conserved "membrane desaturase" regions of DesA3 with some representative membrane Δ9 acyl-CoA desaturases from rat, yeast, and mouse revealed certain distinctions of some variable amino acids. In region Ia, all representative Δ9-desaturases contain the sequence HX4H, whereas M. tuberculosis DesA3 contains the sequence HX3H. In region Ib, the representatives of the Δ9-desaturases contain the sequence HX2HH, whereas M. tuberculosis DesA3 has HX3HH. In region II, the same representative Δ9-desaturases have a second occurrence of HX2HH, whereas DesA3 from M. tuberculosis has a second occurrence of HX3HH (Fig. 9). In these respects it is interesting that DesA3 shares closer sequence identity with the Δ6 acyl-CoA desaturase of Rattus norvegicus (Rat), Saccharomyces cerevisiae (Yeast), and Mus musculus (Mouse).
lipid of cyanobacteria and higher plants than with Δ9 acyl-CoA and acyl-acyl-desaturases of yeasts, yeasts, higher plants, and cyanobacteria (39). The hydropathy analysis of the secondary structure of DesA3 provided further evidence that DesA3 contains up to three hydrophilic domains, sufficient to span the membrane bilayer several times. Also, computer-assisted predictions (www.ch.embnet.org/software/TPRedForm.html) suggest the presence of at least two transmembrane domains starting at positions 104 and 194, and the three His regions are within hydrophilic loops. All of the His motifs (amino acids 90–94, 125–130, and 303–308) are located in the hydrophilic regions of the primary sequences as is the case in all desaturases, suggesting that the catalytic site of DesA3 is located on the cytosolic face of the membrane (55). Altogether, the presence of His conserved motifs and transmembrane domains supports the conclusion that DesA3 is a membrane desaturase, a conclusion reinforced by Western blotting, demonstrating directly that DesA3 is localized in the bacterial membrane fraction (results not shown).

The use of sterolic acid proved to be invaluable in demonstrating the parallel between its action as a known inhibitor of oleic acid synthesis and that of ISO. First, this comparison demonstrated that the inhibition of oleic acid synthesis is sufficient to kill M. bovis BCG and, thus, that the enzymes involved in the synthesis of this fatty acid are indeed lethal targets in mycobacteria. Second, the use of sterolic acid demonstrated that there is likely no relationship between the two effects of ISO on oleic acid and mycolic acid synthesis, i.e. mycolic acid probably does not originate in oleic acid. Rather, the results raise the possibility that ISO exerts an additional effect, possibly on the specialized enzymes, dehydratases and desaturases, that provide precursors for mycolic acid synthesis (56). Although ISO inhibits the formation of both oleic acid and mycolic acids, the data from this study support the concept that the inhibition of the growth of mycobacteria results primarily from effects on oleic acid rather than mycolic acid synthesis. This conclusion is drawn primarily from the observation of the rescue of ISO-treated M. tuberculosis with oleic acid and the high MIC value of BCG expressing desA3. The mechanism by which ISO inhibits mycolic acid synthesis has yet to be determined, but clearly it is not merely an effect on oleic acid synthesis.

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REFERENCES

38.草地に生息するバクテリア(Mycobacteria)の生物学とその治療に関する研究
39. 薬理学の進歩とその応用
40. 寄生菌に対する薬物療法の進歩
41. 薬物療法の進歩とその応用
42. 薬物療法の進歩とその応用
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