InhA, a target of the antituberculous drug isoniazid, is involved in a mycobacterial fatty acid elongation system, FAS-II

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Most drug-resistant clinical isolates of the tubercle bacillus are resistant to isoniazid, a first-line antituberculous drug. This antibiotic was shown to act on *Mycobacterium tuberculosis* by inhibiting a 2-*trans*-enoyl-acyl carrier protein reductase, called InhA. However, the exact role played by InhA in mycobacteria remained unclear. A mycobacterial enzyme fraction containing InhA was isolated. It displays a long-chain fatty acid elongation activity with the characteristic properties described for the FAS-II (fatty acid synthetase II) system. Inhibition of this activity by InhA inhibitors, namely isoniazid, hexadecynoyl-CoA or octadecynoyl-CoA, showed that InhA belongs to the FAS-II system. Moreover, the InhA inhibitors also blocked the biosynthesis of mycolic acids, which are major lipids of the mycobacterial envelope. The data strongly suggest that isoniazid acts on the mycobacterial cell wall by preventing the FAS-II system from producing long-chain fatty acid precursors for mycolic acid biosynthesis.

Keywords: InhA, fatty acid synthetase II, mycolic acid biosynthesis, isoniazid target, *Mycobacterium*

INTRODUCTION

Tuberculosis is still a major cause of death from an infectious agent in developing countries, and constitutes a serious threat in large cities of industrialized countries (Snider *et al.*, 1994). The resurgence of tuberculosis during the last decade has been partly caused by the emergence of multi-drug resistant Koch bacilli.

Most drug-resistant *Mycobacterium tuberculosis* clinical isolates are resistant to isoniazid (INH), which is a first-line drug used to treat tuberculosis (World Health Organization, 1997). Recent investigations have shown that the InhA protein produced by this bacterium is a target of INH (Banerjee *et al.*, 1994; Dessen *et al.*, 1995; Quémard *et al.*, 1995, 1996; Zabinski & Blanchard, 1997; Rozwarski *et al.*, 1998). Mutations in the *inhA* gene, or in its putative promoter region, confer INH resistance to laboratory isolates of *Mycobacterium bovis* or *Mycobacterium smegmatis* (Banerjee *et al.*, 1994) and are probably responsible for the INH-resistant pheno-

type of a significant part of *M. tuberculosis* clinical isolates (Kapur *et al.*, 1995; Ristow *et al.*, 1995; Musser *et al.*, 1996; Basso *et al.*, 1998). The *inhA* gene product is an NADH-dependent enoyl-acyl carrier protein reductase, which catalyses the reduction of 2-*trans*-enoyl chains possessing at least 12 carbon atoms (Quémard *et al.*, 1995; Dessen *et al.*, 1995). This enzymic activity corresponds to the last step of each two-carbon-elongation round in the fatty acid biosynthesis pathway. As data presented by Banerjee *et al.* (1994) strongly suggest that the *inhA* gene is involved in the production of very-long-chain fatty acids, mycolic acids, it is reasonable to imagine that InhA is part of a fatty acid elongation system whose products represent intermediates in the biosynthesis of these unusual molecules.

Mycolic acids are α -branched β -hydroxylated fatty acids, and constitute major and specific components of the mycobacteria and related genera. They are believed to play an essential role in mycobacterial-envelope architecture, forming the junction between the capsule and the cell-wall skeleton, where they are covalently bound (Daffé & Draper, 1998). Inhibition of mycolic acid biosynthesis is the first event detected in *M. tuberculosis* treated with INH (Takayama *et al.*, 1972), and numerous observations indicate that INH treatment causes

Abbreviations: ACP, acyl carrier protein; FAS, fatty acid synthetase; FES, fatty acid elongation system; INH, isoniazid (isonicotinic acid hydrazide); NE, 2-(2,3-naphthalimino)ethyl; NE-OTf, 2-(2,3-naphthalimino)ethyl trifluoromethanesulphonate; pCMS, *p*-chloromercuriphenylsulphonic acid.

extensive damage to the envelope organization, such as loss of the acid-fast property (Middlebrook, 1952), release of abnormal amounts of proteins into the culture medium and altered ultrastructure (Bardou *et al.*, 1996).

Recently, we described a gene cluster, *cmA-D*, involved in the introduction of oxygenated functions and cyclopropanes into the main chain of mycolic acids of the *M. tuberculosis* complex (Dubnau *et al.*, 1997, 1998). However, not much information is available on the formation of the main chain, called the meromycolic chain (Daffé & Draper, 1998; Lanéelle, 1989). It was postulated in the literature as early as 1982 (Winder, 1982; Takayama & Qureshi, 1984) that at least two enzyme systems are involved in the synthesis of very-long-chain precursors of mycolic acids, and this hypothesis was built up by putting together data obtained independently over the last few decades: (i) the identification, in *M. smegmatis*, of two fatty acid biosynthesis systems called FAS-I (fatty acid synthetase I) and FAS-II, FAS-I catalysing *de novo* synthesis and FAS-II producing unusually long fatty acids (Bloch, 1977); (ii) the fact that a crude soluble extract of *M. tuberculosis* can elongate palmitate, a FAS-II primer, to give up to C₃₂-saturated fatty acids and over C₄₀-monounsaturated fatty acids (Qureshi *et al.*, 1984); (iii) the demonstration that InhA, which catalyses the last step of fatty acid elongation, is the target of INH, an efficient inhibitor of mycolic acid biosynthesis (Banerjee *et al.*, 1994; Quémard *et al.*, 1995, 1996).

The above convergent data suggest that a FAS-II-like system could synthesize long-chain precursors of mycolic acids, but no experimental results directly link the above set of data to confirm this hypothesis. In this paper, we report evidence that InhA is part of the FAS-II system, and that the elongation complex is probably involved in the synthesis of mycolic acids.

METHODS

Cultures. *M. smegmatis* strain mc²155 was grown on Middlebrook 7H9 broth supplemented with 0.2% (v/v) glycerol. Culture growth was stopped at late exponential phase and cells were harvested by centrifugation.

Preparation of protein extracts containing FAS-I and FAS-II systems. The bacterial pellet was washed and suspended in 100 mM potassium phosphate buffer, pH 7.2, containing 1 mM DTT and 1 mM EDTA. The suspension was sonicated with a Branson Sonifier Cell Disrupter B15 (microtip 3, 60% duty cycle, four times with 5 s on, 40 s off). The disrupted cells were centrifuged at 43 500 g for 30 min to remove cell debris. The FAS-I and FAS-II systems were prepared from the crude supernatant obtained by centrifugation and separated as previously described (Odriozola *et al.*, 1977), except that the 35–80% (w/v) (NH₄)₂SO₄ precipitate was dissolved in 100 mM potassium phosphate buffer containing 1 mM DTT and 1 mM EDTA, and applied to a Sephadex G-200 (Pharmacia) column. The elution profile at 280 nm was similar to that obtained by Odriozola *et al.* (1977). Fraction I corresponded to the first peak (elution volume from 24.2 to 33 ml for a column of 16 × 370 mm), and fraction II to the first part of the second peak (elution volume from 39.6 to 48.4 ml). Protein fractions were concentrated either by 80% (w/v)

(NH₄)₂SO₄ precipitation or in an Amicon ultrafiltration unit (cut off, 10 kDa); similar results were obtained using either method.

Preparation of cell-wall extracts. The bacterial pellet was washed and suspended in 50 mM potassium phosphate buffer, pH 7.0, containing 3 mM β-mercaptoethanol. The cells were disrupted by two passages through a cell disrupter (Constant Systems) at 0.4 kbar. The suspension was centrifuged at 3000 g for 20 min and the upper-layer material, corresponding to cell-wall extract (Lacave *et al.*, 1990), was taken off and homogenized in 200 mM potassium phosphate buffer, pH 7.0.

Protein assays. Protein assays were performed on protein extracts or on whole bacteria by the Bio-Rad DC method (Bio-Rad), after boiling samples for 10 min in 0.5 M NaOH to solubilize insoluble proteins.

Western blots. Fifteen percent (w/v) polyacrylamide-SDS gels were either stained with Coomassie blue or the proteins transferred onto a nitrocellulose membrane. Polyclonal rabbit anti-InhA antibodies were used for Western blot analyses. Antibodies against pure *M. tuberculosis* H37Rv InhA protein were prepared (Eurogentec), and purified on an affinity CNBr-activated Sepharose 4B column (Pharmacia) covalently coupled with pure InhA protein. Antigen-antibody interactions were revealed by a colorimetric reaction using alkaline phosphatase conjugated to mouse anti-rabbit IgG.

Fatty acid elongation assays. FAS-type assays: The *de novo* FAS-I-type assay was based on the previously described assay (Odriozola & Bloch, 1977): 300 μM acetyl-CoA, 20 μM [2-¹⁴C]malonyl-CoA (specific radioactivity, 444 kBq μmol⁻¹), 100 μM CoA, 100 μM NADH, 100 μM NADPH, 1 μM FMN, 5 mM DTT, 100 mM potassium phosphate buffer, pH 7.2, and 200 μg proteins [or 10 μg for comparison with FES (fatty acid elongation system) assays], in a total volume of 500 μl. Reactions were incubated for 1 h at 37 °C with shaking at 200 r.p.m.

The standard FAS-II-type elongation assay was based on the previously described assay (Odriozola *et al.*, 1977): 50 μM palmitoyl-CoA, 20 μM [2-¹⁴C]malonyl-CoA (specific radioactivity, 444 kBq μmol⁻¹), 100 μM NADH, 100 μM NADPH, 40 μM ACP (acyl carrier protein), 1 mM DTT, 100 mM potassium phosphate buffer, pH 7.2, and 100 or 200 μg proteins (or 10 μg for comparison with FES assays), in a total volume of 500 μl. Reactions were incubated for 1 h at 37 °C, with shaking at 200 r.p.m. When indicated, [2-¹⁴C]malonyl-CoA was replaced with [1-¹⁴C]acetyl-CoA at the same concentration, and the same specific radioactivity.

2-Hexadecynoyl-CoA and 2-octadecynoyl-CoA (C. Vilchèzes & R. Bittman, unpublished synthesis method) were tested at 43 μM either in FAS-I or in FAS-II standard assays. *p*-Chloromercuriphenylsulphonic acid (*p*CMS) was tested at 1 mM on standard reaction mixtures, but in the absence of DTT (which is known to react with *p*CMS) in both the controls and the assays. For the assays in the presence of INH, standard reaction mixtures, without palmitoyl-CoA, malonyl-CoA and ACP for FAS-II assays, or without acetyl-CoA, malonyl-CoA, CoA and FMN for FAS-I assays, were first preincubated for 3 h at 25 °C with gentle shaking, in the presence of 100 μM INH and 2 μM MnCl₂. The control experiments were treated in the same way, except that INH was omitted. Reactions were started, after preincubation, by the addition of the missing reagents and incubated for 1 h at 37 °C.

FES-type assays: FES-I and FES-II elongation assays were based on previously described assays (Kikuchi & Kusaka,

1982; Shimakata *et al.*, 1977). Reactions were performed in a total volume of 500 μ l, with 1 mM DTT, 100 mM potassium phosphate buffer, pH 7.2, protein extract at 20 μ g ml⁻¹, and, for FES-I assays, 50 μ M octanoyl-CoA, 75 μ M [1-¹⁴C]acetyl-CoA (specific radioactivity, 444 kBq μ mol⁻¹), 200 μ M NADH, or, for FES-II assays, 50 μ M stearoyl-CoA, 20 μ M [2-¹⁴C]malonyl-CoA (specific radioactivity, 444 kBq μ mol⁻¹), 100 μ M NADH, 100 μ M NADPH and 20 μ M FMN. Reactions were incubated for 1 h at 37 °C with shaking at 200 r.p.m.

Reactions were stopped by the addition of 1 vol. of 10% (w/v) KOH in methanol/benzene (8:2, v/v) and saponification was performed at 80 °C either for 30 min for reactions with fraction I or fraction II or overnight for reactions with cell-wall extract.

Mycolic acid biosynthesis assays. For assays with whole bacteria, an aliquot of the bacterial suspension used to prepare the cell-wall extract was removed before cell disruption (see above). Mycolic acid biosynthesis was tested on cell-wall extracts or on whole bacteria in reactions with 100–125 μ g protein ml⁻¹ in 100 mM potassium phosphate buffer, pH 7.0, in a total volume of 1.5 ml. INH was tested at 100 μ M in the presence of 10 μ M MnCl₂, with a 3 h preincubation at 25 °C in the reaction mixture. Octadecynoyl-CoA was tested at 43 μ M, with or without a 30 min preincubation at 25 °C. Reactions were started by the addition of 50 μ M [1-¹⁴C]acetate (specific activity, 2.11 MBq μ mol⁻¹) and incubated for 1 h at 37 °C with shaking at 160 r.p.m. Reactions were stopped by the addition of 1 vol. 10% (w/v) KOH in methanol/benzene (8:2, v/v), and saponification performed overnight at 80 °C.

Analysis of the reaction products. Lipids were extracted from the reaction mixtures with diethyl ether. Total lipid labelling was measured by scintillation counting on an aliquot of each extract. For TLC analysis, fatty acids were methylated with diazomethane. Samples were analysed on Silicagel G60 plates eluted with dichloromethane. Radiolabelling of TLC-separated components was measured by a radioscaner (Automatic TLC linear analyser LB 2832; Berthold), and lipids were revealed by spraying with 20% (v/v) sulphuric acid and charring.

To determine the chain lengths of the fatty acids produced by fraction II, the alkali-labile lipid extracts of five standard FAS-II reactions were pooled. To be able to detect the fatty acids by spectrophotometry, 2-(2,3-naphthalimino)ethyl (NE) ester derivatives were prepared by modifying the method of Yasaka *et al.* (1990), as follows. Known amounts of unlabelled fatty acid standards were added to the radiolabelled lipids to facilitate the detection of the fatty esters separated by HPLC and to measure the yield of derivatization of each fatty acid in the mixture. Eighty nanomoles of total fatty acids were solubilized in 4.8 ml CHCl₃ and the following reagents were added: 800 μ l 18-Crown-6 (1 mM in CHCl₃), 40 mg anhydrous potassium fluoride, and, after vortexing, 80 μ l 2-(2,3-naphthalimino)ethyl trifluoromethanesulphonate (NE-OTf; Pierce) (1 mM in acetonitrile). Reactions were incubated in sealed tubes overnight at room temperature with vigorous shaking. After the reaction, fatty acyl-NE esters were separated from the small amount of low-*R_F* radiolabelled compounds (observed on thin-layer silica gels) on a Sep-pak silica cartridge (Waters). Reaction mixtures were dried under nitrogen and loaded onto the cartridge in petroleum ether/dichloromethane (7:3, v/v); fatty acyl-NE esters were eluted with the same solvent mixture, nitrogen-dried, and dissolved in acetonitrile. Each sample was analysed by HPLC on a Chromosorb 30DS2 column (Chromato-Sud) eluted at 1 ml min⁻¹ with the following conditions: a 10 min gradient from

90 to 100% (v/v) CH₃OH in water, followed by a 50 min step at 100% (v/v) CH₃OH. Acyl-NE derivatives were detected at 259 nm and collected separately. The quantity of acyl-NE in each fraction was calculated by measuring absorbance at 259 nm in CH₃OH (ϵ_{259} = 62000 M⁻¹ cm⁻¹), and radioactivity was measured by scintillation counting in Lipoluma scintillant (Lumac, LSC). The amount of radioactivity of each acyl-NE compound was corrected for the yield of derivatization obtained for the standards.

RESULTS

Isolation of an enzyme fraction containing InhA

FASs have been classified into three types (I, II and III; Harwood, 1986). The type I synthetases are high-molecular-mass multifunctional proteins containing covalently bound ACP and occur mainly in animal cells and yeast; the type II synthetases consist of several monofunctional enzymes and are present in plants and bacteria. Both type I and type II systems catalyse *de novo* fatty acid synthesis, whereas the type III synthetases, which are found in animals and plants, elongate preformed acyl chains to give long- or very-long-chain molecules (Harwood, 1986). Mycobacteria are very unusual in that they contain several types of fatty acid synthetase. Four different straight-chain fatty acid biosynthesis systems have been described in *M. smegmatis* by two independent groups: FAS-I, which is a type I synthetase responsible for the synthesis of medium-length fatty acids (C₁₆, C₂₄) (Bloch, 1977), and FAS-II (Bloch, 1977), FES-I (Shimakata *et al.*, 1977) and FES-II (Kikuchi & Kusaka, 1982), which are elongation systems composed of monofunctional enzymes, and which resemble the type III rather than the type II synthetases. The enzyme system which was the most likely to contain

Fig. 1. Western blots using anti-InhA antibodies. (a) Analysis of fraction I and fraction II, (b) analysis of cell-wall extract. Lanes: 1, pre-stained molecular standards; 2, pure InhA; 3, total sonication extract; 4, fraction I; 5, fraction II; 6, cell-wall extract. 25 μ g protein from each sample and 5 ng pure InhA were used for SDS-PAGE. Anti-InhA antibodies were previously purified on an affinity column.

Table 1. FAS-I and FAS-II-type enzymic assays with fraction I and fraction II

Two hundred micrograms of proteins were used for reactions with fraction I or fraction II. Assays were repeated in two independent experiments, and representative results are shown.

Elongation unit...	Radioactivity (d.p.m.)					
	FAS-II reaction*				FAS-I reaction†	
	[2- ¹⁴ C]Malonyl-CoA		[1- ¹⁴ C]Acetyl-CoA		[2- ¹⁴ C]Malonyl-CoA	
	I	II	I	II	I	II
Fraction...	I	II	I	II	I	II
	3059	32 152	787	1740	30 986	3206

* Primer was palmitoyl-CoA, cofactor ACP.

† Primer was acetyl-CoA, cofactor FMN.

InhA was the FAS-II system, since it shares properties with the InhA protein, such as its specificity for substrates bearing at least 12 carbons, its preference for around 16-carbon molecules, and its ACP dependence (Bloch, 1977; Quémard *et al.*, 1995). Thus, we first isolated from *M. smegmatis* mc²155 a protein fraction containing FAS-II to test for the presence of InhA. FAS-I and FAS-II, which are both present in the 35–80% (w/v) ammonium sulphate precipitate, were separated on a Sephadex G-200 column. Two pools of fractions, called fraction I and fraction II, assumed to contain the FAS-I and the FAS-II systems, respectively (Odrizola *et al.*, 1977), were analysed by Western blotting, using polyclonal rabbit anti-InhA antibodies (Fig. 1a). The total protein extract obtained after sonication showed a faint band staining for InhA, while fraction II appeared highly enriched in this protein. In contrast, no positive signal corresponding to InhA was detected in fraction I.

Fatty acid elongation activity in the InhA-containing fraction

To confirm that fractions I and II contain FAS-I and FAS-II systems, respectively, these fractions were then tested for elongation activity (Table 1). Indeed, the optimum reaction conditions for fraction II corresponded to those described for FAS-II (Odrizola *et al.*, 1977): elongation of palmitoyl-CoA by [2-¹⁴C]malonyl-CoA, in the presence of ACP. Omission of ACP resulted in a decrease of 85% ($\pm 11\%$) of radioactive incorporation into total lipids, and a complete inhibition of fatty acid biosynthesis. In FAS-I-type reactions, only fraction I led to strong labelling of alkali-labile lipids (Table 1). Furthermore, by TLC and radioscanning (see below), fraction II displayed no significant fatty acid biosynthesis activity under the optimum reaction conditions described for the elongation systems FES-I (primer, octanoyl-CoA; elongation unit, [¹⁴C]acetyl-CoA; Shimakata *et al.*, 1977) and FES-II (primer, stearoyl-CoA; elongation unit, [¹⁴C]malonyl-CoA; cofactor, FMN; Kikuchi & Kusaka, 1982).

Fig. 2. Chain lengths of the fatty acids synthesized by fraction II. Acyl-NE derivatives of the radiolabelled fatty acids produced by fraction II in FAS-II assays were prepared and separated by HPLC. The percentage of labelling of each fatty acid relative to the total radioactivity of all fatty acids is displayed. Radioactivity values varied from 2×10^3 to 3×10^4 d.p.m.

To characterize the compounds radiolabelled by fraction II in a standard FAS-II-type assay, lipids extracted after alkaline treatment of the reaction medium were first analysed by TLC, argentation-TLC and radio-scanning. Most of the radioactivity coming from [2-¹⁴C]malonyl-CoA was incorporated into saturated, straight-chain fatty acids (data not shown). The chain lengths of the labelled fatty acids were then determined by HPLC. Highly fluorescent NE ester derivatives of the fatty acids were prepared from NE-OTf. Although the method has been optimized to improve the derivatization yield of long-chain fatty acids, which are less soluble, the yield sharply decreased with increasing chain length. To solve this problem, derivatization was performed with internal standards, i.e. in the presence of known amounts of C₁₆–C₃₀ saturated fatty acids, and the derivatization yield was calculated for each chain length (see Methods). The corrected values of radioactivity corresponding to each HPLC peak showed that fraction II synthesizes saturated fatty acids from C₁₈ to C₃₀ (Fig. 2). The major products are C₁₈–C₂₆ fatty acids. The

Fig. 3. Effect of inhibitors on fraction II elongation activity or on mycolic acid biosynthesis. Inhibitors were tested in FAS-II standard assays (a) or in mycolic acid biosynthesis assays (b). For testing pCMS, DTT was omitted. pCMS, 2-hexadecynoyl-CoA (HDC) and 2-octadecynoyl-CoA (ODC) were tested either without preincubation or with a 30 min preincubation in the presence of the protein extract; INH was preincubated for 3 h in the presence of the protein extract (see Methods). Inhibition levels (%) were calculated as labelling ratios of assays versus control experiments performed in parallel in the absence of inhibitor. Means and standard deviations of at least two independent experiments are displayed.

weak labelling of C₁₆-NE is probably due to the contamination of commercial palmitoyl-CoA by myristoyl-CoA (C₁₄), which can also be used as primer by FAS-II.

Thus fraction II, which contains the InhA protein, exhibits the same properties as those described for the FAS-II system (Bloch, 1977), i.e. an ACP-dependent enzymic activity which promotes elongation of palmitoyl-CoA into C₁₈–C₃₀ fatty acids, using malonyl-CoA as the elongation unit.

Is InhA involved in the elongation activity of FAS-II?

The effects of inhibitors on the incorporation of radioactivity into saturated fatty acids were analysed by scintillation counting and radioscanning on TLC. As expected, pCMS, which is a thiol-selective reagent, strongly inhibited both the elongation activity of fraction II (by 90%, Fig. 3a) and the synthesis activity of fraction I (by 71 ± 4%), since systems involved in fatty acid biosynthesis bear thiol groups in their active site. 2-Hexadecynoyl-CoA and 2-octadecynoyl-CoA are structural analogues of 2-*trans*-hexadecenoyl-CoA and 2-*trans*-octadecenoyl-CoA, two substrates of the InhA protein. These analogues are strong competitive inhibitors, versus enoyl-CoA substrates, of InhA activity (A. Quémard, J. S. Blanchard & W. R. Jacobs, Jr, unpublished data). Addition of hexadecynoyl-CoA or of octadecynoyl-CoA at 43 µM, a concentration that totally abolishes InhA activity (unpublished data), inhibited the FAS-II activity by more than 90% (Fig. 3a), while it inhibited the *de novo* FAS-I activity only partially (by 52 ± 4%). Further evidence for the involvement of InhA in FAS-II activity was obtained by testing the effect of INH in the presence of MnCl₂, which catalyses the activation of the drug (Zabinski &

Blanchard, 1997). After a 3 h preincubation with fraction II, the activated INH inhibited the elongation reaction by 60% (Fig. 3a). The same inhibition rate was obtained for the test of INH on purified *M. tuberculosis* InhA under similar experimental conditions, after a 3 h preincubation (Zabinski & Blanchard, 1997). In contrast, Mn²⁺-activated INH had no effect on FAS-I activity.

Is FAS-II involved in mycolic acid biosynthesis?

Data published by Banerjee *et al.* (1994) strongly suggested that InhA is involved in mycolic acid biosynthesis. Thus, it was important to characterize the involvement of this enzyme, and therefore of the FAS-II system, in this pathway. Mycolic acid biosynthesis has been shown to take place in a membrane-containing cell-wall fraction (Lacave *et al.*, 1990). The presence of InhA in *M. smegmatis* cell-wall extracts was investigated by Western blot analysis, using purified anti-InhA antibodies, and a positive signal was observed (Fig. 1b). The action of InhA inhibitors on the biosynthesis of mycolic acids in a *M. smegmatis* cell-wall extract was then tested by following the incorporation of [1-¹⁴C]acetate (Fig. 3). Both octadecynoyl-CoA and MnCl₂-activated INH inhibited the labelling of mycolic acids. A 30 min preincubation of the cell-wall extract in the presence of octadecynoyl-CoA was necessary to reach the level of inhibition observed for the FAS-II system. This may be due to the low accessibility of enzymes in the insoluble cell-wall extract.

In parallel, the effect of octadecynoyl-CoA on mycolic acid biosynthesis by whole *M. smegmatis* cells was tested under the same conditions as for the cell-wall extract. Even after a 30 min preincubation in the presence of bacteria, octadecynoyl-CoA had absolutely no effect on the incorporation of [1-¹⁴C]acetate into either mycolic acids or the total alkali-labile lipidic compounds. This experiment showed that the activity of mycolic acid biosynthesis in our mycobacterial cell-wall extracts was completely independent of any potential presence of unbroken bacteria.

DISCUSSION

The picture of the course of action of INH against *M. tuberculosis* has long resembled a jigsaw puzzle, but important pieces have recently been added. We know that INH passively diffuses through the mycobacterial envelope (Bardou *et al.*, 1998), is activated by MnCl₂ (Zabinski & Blanchard, 1997) and the catalase-peroxidase KatG (Quémard *et al.*, 1996), possibly into an isonicotinoyl radical or anion, which then inhibits the InhA enzyme through covalent attachment to NADH within the active site of the protein (Rozwarski *et al.*, 1998). InhA has been shown to preferentially catalyse the NADH-dependent reduction of 2-*trans*-enoyl-ACP molecules with 16 or more carbons (Quémard *et al.*, 1995; Dessen *et al.*, 1995). This reaction corresponds to the last step of fatty acid elongation. Furthermore, INH inhibits the biosynthesis of mycolic acids, which are

extremely long fatty acids, specific to mycobacteria and related genera. Strong evidence suggests that this pathway is the primary target of INH (Takayama *et al.*, 1972; Winder, 1982; Quémard *et al.*, 1991). A link between the inhibition of InhA and the inhibition of mycolic acid synthesis is provided by the fact that a mutation in the *inhA* gene, which confers resistance to INH, also leads to resistance of mycolic acid biosynthesis to INH (Banerjee *et al.*, 1994). However, several pieces of the puzzle are still missing between InhA and mycolic acid synthesis. The present work allowed us to find one of these pieces.

We first sought for the presence of InhA in the known fatty acid biosynthesis complexes. The FAS-II system was the best candidate since it shares several important properties with InhA: specificity for relatively long chain substrates having ~16 carbons, and for ACP. Indeed, we have shown that InhA eluted in the mycobacterial protein fraction previously identified to contain FAS-II (Odriozola *et al.*, 1977), the enzyme system that catalyses the malonyl-CoA and ACP-dependent elongation of palmitoyl-CoA into C₁₈–C₃₀ fatty acids. Furthermore, inhibition of this fatty acid synthesis by inhibitors of InhA activity, namely hexadecynoyl-CoA, octadecynoyl-CoA or MnCl₂-activated INH, strongly suggests that the InhA protein is part of the FAS-II system.

On the *M. tuberculosis* chromosome, the *inhA* gene forms an operon with the *mabA* gene, which probably encodes a β -ketoacyl-ACP reductase (Banerjee *et al.*, 1994, 1998). The MabA protein most likely represents the other reductase of the FAS-II complex. Furthermore, in the reported *M. tuberculosis* genome sequence (Cole *et al.*, 1998), three genes have been predicted as encoding a β -ketoacyl-ACP synthase; two of them, *kasA* and *kasB*, are adjacent to genes encoding ACP and a malonyl-CoA-ACP transacylase. Therefore, the KasA and KasB proteins most likely belong to a malonyl-CoA- and ACP-dependent fatty acid biosynthesis system, and may catalyse the first step of the FAS-II system elongation cycle. KasA has been proposed to be an INH target in *M. tuberculosis* (Mdluli *et al.*, 1998), on the basis of the detection of an interaction between INH and an ACP molecule, which is itself linked to KasA. However, neither direct interaction between KasA and INH nor inhibition of KasA activity by the drug have been shown.

Mycobacteria contain a number of fatty acid biosynthesis systems (Kolattukudy *et al.*, 1997; Cole *et al.*, 1998), a property reflected by the abundance and the great variety of unique lipids in their envelope. Bloch attempted to explain the presence in mycobacteria of several enzymic machineries providing straight-chain fatty acids by postulating that FAS-I and FAS-II act sequentially, FAS-I producing palmitoyl-CoA which is then transferred to FAS-II for further elongation (Bloch, 1977). One can imagine that the long-chain fatty acids produced by the FAS-II system serve as precursors for the formation of the meromycolic acids, which represent the C₄₀–C₆₀ main chain of mycolic acids. Indeed, the

presence of the InhA protein in a mycobacterial cell-wall extract that synthesizes mycolic acids, and the fact that inhibitors of the InhA and FAS-II activities also inhibit mycolic acid synthesis strongly suggest that InhA and FAS-II participate in the mycolic acid pathway. Other experimental evidence favours the involvement of a fatty acid elongation system like FAS-II in the synthesis of the meromycolic chain (Winder, 1982). In particular, Qureshi *et al.* (1984) showed that a total soluble-protein extract from *M. tuberculosis* was able to incorporate radiolabelled palmitate in the presence of malonyl-CoA into saturated C₁₈–C₃₂ fatty acids but also into mono-unsaturated fatty acids bearing from 18 to more than 40 carbons. This suggests that a mycobacterial enzyme system may be able to further elongate the products of the FAS-II system, after introduction of a double bond. Also, the possibility remains that the FAS-II system, on its own, is able to synthesize molecules which have more than 30 carbons. Wheeler & Anderson's work (1996) suggests that the INH-sensitive fatty acid elongation system involved in mycolic acid biosynthesis, therefore FAS-II, uses (Z)-tetracos-5-enoic acid as precursor.

After comparing the effects of antibiotic thiolactomycin on fatty acid elongation and on mycolic acid biosynthesis in *M. smegmatis*, the work of Slayden *et al.* (1996) led to the hypothesis that at least two ACP-dependent elongation systems would be involved in the synthesis of mycolic acids, one INH-sensitive and one thiolactomycin-sensitive. However, the present work shows, in disagreement with the assumptions of Slayden *et al.*, that the InhA-containing FAS-II system inhibited by INH, and which is involved in mycolic acid biosynthesis, is the FAS-II system described by Bloch (1977). It is difficult to come to a conclusion about the Slayden *et al.* experiments which were performed on a crude cytosolic fraction that contains several different fatty acid biosynthesis complexes, without any identification of the assayed elongation activity. Furthermore, the experimental conditions for the testing of INH on their crude extract, from which they conclude that FAS-II is not inhibited by INH, are not given.

Thus, the present work, as well as previous reports (Winder, 1982), leads to the conclusion that the FAS-II system described initially by Bloch is involved in mycolic acid biosynthesis, and that the antituberculosis drug INH prevents mycolic acid biosynthesis by inhibiting the FAS-II system activity. The precursors used by FAS-II are most likely supplied by the only *de novo* fatty acid synthetase known in mycobacteria, FAS-I. However, our knowledge of the mycolic acid biosynthetic pathway still remains incomplete. The following questions should be answered: (i) is FAS-II sufficient to elongate the chain to the final size of meromycolic chains (around C₆₀); and (ii) what is the mechanism of introduction of double bonds into the meromycolic chain? Genes encoding the proteins responsible for the introduction of oxygenated functions (Dubnau *et al.*, 1997) and cyclopropane rings (Dubnau *et al.*, 1997; Yuan *et al.*, 1995; George *et al.*, 1995) into the meromycolic chain have been recently identified. Investigations into the properties of other

enzymes necessary for building the complete backbone common to all types of mycolic acid will require the isolation of mutants defective in the mycolic biosynthesis pathway.

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