Determination of the primary target for isoniazid in mycobacterial mycolic acid biosynthesis with *Mycobacterium aurum* A⁺

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The target of the potent antituberculosis drug isoniazid was investigated in *Mycobacterium aurum* A⁺, against which isoniazid has an MIC (the minimum concentration required to give growth inhibition) of 0.3 µg/ml. Mycolic acid biosynthesis, measured by the incorporation of label from [1-¹⁴C]acetate into mycolic acids, was inhibited differentially by isoniazid in cell-wall preparations of *M. aurum* A⁺. Thus at an isoniazid concentration of 1 µg/ml, mycolic acid biosynthesis was inhibited by 80 % but concomitant biosynthesis of non-hydroxylated fatty acids was inhibited by only 15 %. Three lines of evidence identified 24:1 *cis*-5 elongase as the primary isoniazid target. First, 24:1 *cis*-5 did not restore isoniazid-inhibited mycolic acid biosynthetic activity in a crude

cell-wall preparation, suggesting that the drug acts after the formation of the Δ-5 double bond. Secondly, a 24:1 *cis*-5 elongase assay in which the product is mycolic acid is completely inhibited by isoniazid. Finally, the only intermediates that accumulate as a result of the addition of isoniazid are acids of 24 carbons. Both 24:0 and 24:1 are observed in a similar ratio whether or not isoniazid is present, even though concomitant mycolic acid biosynthesis is inhibited by isoniazid. These results are consistent with studies of the *M. tuberculosis* *InhA* protein by Dessen, Quemard, Blanchard, Jacobs and Sacchettini [(1995) *Science* 267, 1638–1641].

INTRODUCTION

It is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis*. Clinical tuberculosis develops after infection with the bacillus, notably among individuals infected with HIV, and results in some three million deaths per year worldwide [1]. Isoniazid (also referred to as isonicotinic acid hydrazide or INH) has been used almost from the outset of tuberculosis chemotherapy [2] and is a first-line agent in anti-tubercular regimens [3]. With the increasing incidence of isoniazid resistance and the emergence of multi-drug-resistant strains it is important to understand how isoniazid exerts its bactericidal effect so that new agents can be developed against the target or biochemical pathway.

The mode of action of isoniazid has not been explained satisfactorily. Isoniazid resistance might be conferred by mutations in one of two genes, *katG* [4,5] or *inhA* [6]. *katG* encodes a catalase–peroxidase, an enzyme activity long known to be required for sensitivity to isoniazid and to be associated with virulence [7,8]. Peroxide, in the presence of catalase–peroxidase, converts isoniazid to a variety of reactive intermediates ultimately leading to isonicotinic acid [9]. It is thought that one of these products binds to the target or complexes with NADH to bind to the target, with isoniazid itself being a poor inhibitor of the *inhA* gene product [10].

Isoniazid resistance can be independent of catalase–peroxidase activity in both *Mycobacterium aurum* and *M. tuberculosis*. Thus in *M. aurum* the sensitivity to isoniazid of cell-free mycolic acid biosynthesis can vary markedly between different strains, while catalase activity remains constant [11,12]. It is the most sensitive of these *M. aurum* strains, A⁺, that we use in the present study, as its MIC (the minimum concentration required to give growth inhibition) is reported to be 1 µg/ml isoniazid. In *M. tuberculosis* such resistance has been traced to the *inhA* gene [6,13]. *inhA* encodes an NADH-dependent *trans*-2-enoyl acyl-carrier protein (ACP) reductase [12], one of the constituent enzymes of a fatty

acid synthase or elongase-type complex. This finding is consistent with the observation that mycolic acid biosynthesis is inhibited by isoniazid and that fatty acids up to 24–26 carbons accumulate in its presence [11,14]. The *inhA* gene product was recently characterized as being part of an elongase [10] as it does not reduce the initial *trans*-2-enoyl product, crotonyl-CoA, of fatty acid biosynthesis *de novo*. Its preferred primers are C₁₆ to C₂₄ enoyl-ACP.

The aim of the present work was to investigate directly whether an elongase step in mycolic acid biosynthesis is the primary target of isoniazid. Further experiments determine whether isoniazid has any effect on related enzymes in mycolic acid biosynthesis, particularly desaturases which also have oxidoreductase activities. The emphasis on the metabolism of 24-carbon fatty acids in this work reflects the likely divergence of mycolic acid biosynthesis from fatty acid metabolism in general at this point (Scheme 1) with the insertion probably catalysed by a Δ-5 desaturase of a characteristic ω–19 double bond into the growing fatty acyl α-chain of mycolic acids [15,16].

EXPERIMENTAL

Chemicals

(Z)-Tetracos-5-enoic acid (24:1 *cis*-5) was synthesized as described previously [17] and used as a free acid. Additionally, this acid and also lignoceric acid (24:0) were complexed with 40 % (w/v) aqueous randomly methylated β-cyclodextrin (RAMEB-CD) (Chinodex cyclodextrins, H-1045, Budapest, Hungary) to give a final concentration of 1 mg/ml fatty acid [18]. At the concentrations used in assays, some of the fatty acid might have been displaced from the complexes by water (L. Kato, personal communication) but the complexes were used at the highest concentration possible at which marked inhibition of mycolic acid biosynthesis did not occur (see the Results section). Other chemicals, including isoniazid, were the highest grade from Sigma or BDH; solvents used with enzyme preparations were

Abbreviations used: ACP, acyl-carrier protein; MIC, the minimum concentration required to give growth inhibition.

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Scheme 1 Possible biosynthetic pathways for mycolic acids

Reactions discussed in the paper are emphasized here for *M. aurum*. *M. tuberculosis* converts double bonds to cyclopropane rings [38] and elaborates the mycolate class methoxymycolates and not ω -carboxymycolates. *InhA* is involved in elongation of acyl-ACP, but has a low affinity for acyl-CoA [10]; the carrier involved in mycolic acid biosynthesis remains to be identified directly. In this paper the target of isoniazid is identified as elongation of the 24:1 *cis*-5 moiety. Note that *trans*-2-enoyl-carrier reductases are involved in all C_2 elongation steps, but their reaction is shown here only for the elongation of 20:0-ACP to 22:0-ACP.

AnalaR grade and solvents used with HPLC were Hipersolv grade. $[1-^{14}\text{C}]$ Acetate, Na salt (54 Ci/mol) was supplied by Amersham International.

Growth of *M. aurum* A⁺

M. aurum A⁺ (Institut Pasteur Collection, Paris, France) was inoculated from a Lowenstein-Jensen slope into 66 ml of Middlebrook 7H9 medium with OADC supplement (oleic acid/albumin/dextrose/catalase; Difco) in 250 ml flasks and grown to a D_{550} of 0.4–0.6 to provide a seed culture. Seed culture (3 ml) was inoculated into 1 litre flasks containing 333 ml of the same medium and routinely grown to a D_{550} of 0.4–0.6, then harvested by centrifugation. All flasks (250 ml and 1 litre) were shaken on a rotary incubator at 200 rev./min, and cultures with a D_{550} of up to 0.8 were growing exponentially and with a D_{550} proportional to dry weight of bacteria per ml of medium. Each flask (333 ml of

suspension) took 6 days to grow to a D_{550} of 0.4–0.6 and contained 200–300 mg dry weight of bacteria.

Sensitivity tests

Samples (100 μl) of cultures used for harvesting were inoculated routinely into 10 ml of Middlebrook 7H9 with OADC supplement in 25 ml flasks containing 0, 0.1, 0.3, 1 or 3 $\mu\text{g}/\text{ml}$ isoniazid and assessed for growth at 6 or 12 days by measuring D_{550} . Isoniazid-sensitive *M. aurum* was considered to give clear sub-cultures after 6 days in the presence of 0.3 $\mu\text{g}/\text{ml}$ isoniazid.

Preparation of cell-free extracts

Predominantly cell-wall material capable of mycolic acid biosynthesis was prepared by methods previously described for *M. smegmatis* [16]. Briefly, the pellet of harvested isoniazid-sensitive *M. aurum* bacteria was washed in 0.2 M potassium phosphate buffer, pH 6.5, then resuspended in the same buffer containing 5 mM 2-mercaptoethanol and 0.5 mM PMSF at up to 100 mg dry weight of bacteria per ml. The suspension was disrupted ultrasonically [19], and a wall fraction, P60, was obtained by Percoll (60%, w/v)-density-gradient centrifugation. The extracted wall fraction, EP60, was obtained by extracting P60 at pH 4 repeatedly with hexane, a procedure designed to extract intermediates that did indeed extract fatty acids, including 24:1 acid(s) (G. S. Besra, M. R. McNeil, P. R. Wheeler and P. J. Brennan, personal communication) from the enzymically active cell-wall residue.

Enzyme assays

Three types of assay were performed at 37 °C in 50 mM potassium phosphate with 10 mM KHCO_3 , pH 6, for 30 min, as described previously [16]. Assay reactions were started by the addition of acetate. Isoniazid, when included, was added either immediately before the reaction was started, or, for preincubation with isoniazid, 30 min before starting the reaction. The assays are summarized below.

To determine the effect of isoniazid on cell-free mycolate biosynthesis, P60 was incubated in a final volume of 1 ml with 0.2 mM acetate and isoniazid concentrations ranging from 0 to 10 $\mu\text{g}/\text{ml}$. $[1-^{14}\text{C}]$ Acetate (1 μCi) was included except when HPLC determination of products was done; in that case 10 μCi was used.

In experiments to determine whether 24-carbon acids could restore isoniazid-inhibited mycolic acid biosynthesis, both the established two-phase (water/hexane) assay [16] and a one-phase aqueous assay using fatty acids complexed with cyclodextrin were used. In two-phase assays the aqueous phase (144 μl) contained P60, isoniazid at concentrations ranging from 0 to 3 $\mu\text{g}/\text{ml}$, 1 μCi of $[1-^{14}\text{C}]$ acetate (0.2 mM), and the hexane phase (1 ml) contained 1 mg of 24:0 or 24:1 *cis*-5 or no added fatty acid in controls. The one-phase aqueous-only assays were in a final volume of 1 ml and contained P60, isoniazid at concentrations ranging from 0 to 3 $\mu\text{g}/\text{ml}$, 1 μCi of $[1-^{14}\text{C}]$ acetate (0.2 mM) plus 5 μg of 24:0 or 24:1 *cis*-5 complexed in 2 mg of RAMEB-CD. In controls with no added fatty acid, 2 mg of RAMEB-CD was included.

For both the one-phase and two-phase assays, restoration of activity was expressed as a percentage (% restoration), calculated as follows:

$$100 \times \frac{\left[\text{d.p.m. (isoniazid with } C_{24} \text{ acid}) - \text{d.p.m. (isoniazid with no added acid)} \right]}{\left[\text{d.p.m. (no isoniazid } \pm C_{24} \text{ acid}) - \text{d.p.m. (isoniazid with no added acid)} \right]}$$

where d.p.m. is the radioactivity incorporated from $[1-^{14}\text{C}]$ acetate into mycolic acid. The presence of isoniazid and C_{24} acid in the assay mixtures is indicated in parentheses. The ' $\pm C_{24}$ ' in the denominator reflects the fact that there was no significant difference in radioactivity incorporated between assays with or without added acid.

The third assay was essentially the 24:1 *cis*-5 elongase assay [16], in which activity was measured as d.p.m.(with 24:1 *cis*-5) minus d.p.m.(with no added acid) in the two-phase (aqueous/hexane) assay described above, but with EP60 in place of P60 as the enzyme source.

Analysis of products

Enzyme reactions were stopped by the addition of tetrabutylammonium hydroxide [16]. Then methyl esters of the fatty acid and mycolic acid products were made by phase-transfer catalysis [20]. Alternatively, when UV-absorbing derivatives were required, pentafluorobenzyl esters were prepared by replacing methyl iodide with an equimolar quantity of pentafluorobenzyl bromide.

To determine the radioactivity in mycolic acid and non-hydroxylated fatty acids, their methyl esters were separated by high-performance TLC developed with hexane/acetone/triethylamine (95:5:1, by vol.) and quantified by scintillation counting [16]. To determine whether intermediates accumulated in the presence of isoniazid, pentafluorobenzyl esters were prepared from assay products of assays with 10 μ Ci of [14 C]acetate. The esters were separated by TLC developed with hexane/acetone/triethylamine (95:5:1, by vol.). The [14 C]non-hydroxylated fatty acid esters were scraped from the TLC plate, eluted with hexane, filtered and separated on a Waters μ Bondapak C_{18} 10 μ m reversed-phase HPLC column (3.9 mm $\times$ 150 mm). C_{18} – C_{36} esters were separated with a 0–70 % (v/v) 1,4-dioxan gradient in acetonitrile [21] at a flow rate of 2.2 ml/min. Esters up to C_{24} were separated isocratically with acetonitrile at a flow rate of 1.4 ml/min. Esters were detected by absorbance at 254 nm and by their radioactivity: eluate from the UV cell was mixed with 2 volumes of Ecocint A (National Diagnostics, Atlanta, GA, U.S.A.) in an LKB-Wallace precision mixer and radioactivity was detected in an LKB-Wallace 1208 Betacord radioactivity monitor (Reeve detector). Retention times for the detectors were synchronized by detecting peaks of UV and radioactivity for [14 C]18:0 and 24:0 pentafluorobenzyl esters [prepared by phase-transfer catalysis of tetrabutylammonium salts prepared from 10 μ mol of fatty acid including 1 μ Ci; [14 C]18:0 was obtained from Amersham (18:0) and 24:0 was obtained from CEA (through Fluorochem, Glossop, Derbyshire, U.K.)]. Unlabelled pentafluorobenzyl esters were also prepared from 10 μ mol of 24:1 *cis*-5 and 30:0.

Silver nitrate TLC was performed as described previously [19], except that dichloromethane replaced carbon tetrachloride to develop plates.

Catalase assay

Assays were performed as described previously [19].

RESULTS

Inhibition of growth of *M. aurum* A⁺ by isoniazid

We chose *M. aurum* A⁺ for these studies on the action of isoniazid because the MIC has been reported to be low [11]. We confirmed and extended the report of a MIC of 1 μ g/ml, showing that after 6 days of incubation of *M. aurum* A⁺ in the presence of isoniazid (0.3 μ g/ml) cultures remained effectively clear (Figure 1). Thus 6-day cultures were used throughout this study. The isoniazid sensitivity of each subculture used in the experiments in this study was established because *M. aurum* A⁺ apparently results from a point mutation [11], so it was necessary to be certain to avoid using any resistant revertants in this work.

Mycolic acid biosynthesis by the P60 fraction

Before starting inhibition studies it was necessary to establish an effective enzyme system capable of mycolic acid biosynthesis. The specific activity (d.p.m./min per mg of protein per μ Ci of acetate supplied) of incorporation of label into all mycolic acids

Figure 1 Inhibition of growth of *M. aurum* by isoniazid

Symbols: ■, cultures at 6 days; ●, cultures at 12 days. Cultures were grown in the presence of a range of isoniazid concentrations. Growth conditions and inoculum size are described in the Experimental section.

was 991 ± 236 (S.E.M., 11 determinations) in an extract made from *M. aurum* A⁺ grown for 6 days. When older cultures were used, the specific activity was much lower, providing a second reason for using 6-day cultures routinely. In P60 from 12-day ($D_{550} = 1.3$) and from 20-day ($D_{550} = 1.7$) cultures the specific activities were 286 ± 103 (S.E.M., four determinations) and 34 ± 6 (S.E.M., two determinations) respectively. Radioactivity was distributed between the types of mycolic acid as follows: α -mycolate/ketomycolate/carboxymycolate, 57:26:17.

Effect of isoniazid on the biosynthesis of mycolic acids and fatty acids

The incorporation of [14 C]acetate into mycolic acids by *M. aurum* A⁺ P60 was strongly inhibited by isoniazid (Figure 2). Isoniazid exerted a greater effect on mycolic acid biosynthesis than on fatty acid biosynthesis (Figure 2).

No intermediates of mycolic acid biosynthesis accumulated in the presence of isoniazid that were not observed without

Figure 2 Inhibition of cell-free mycolic acid biosynthesis by isoniazid

Wall material P60 was incubated with [14 C]acetate for 30 min in the presence or absence of isoniazid, and label was traced into α -mycolates (▲), oxygenated (i.e. keto- and ω -carboxy)-mycolates (▼), and non-hydroxylated fatty acids (●). Specific activities (d.p.m./min per mg of protein per μ Ci) were respectively 565, 426 and 1522.

Figure 3 Representative HPLC trace of non-mycolic acids synthesized by P60

HPLC of non-hydroxylated fatty acyl pentafluorobenzyl esters obtained by incubating *M. aurum* A⁺ P60 with 10 μ Ci of [1-¹⁴C]acetate. Peaks a–d are interpreted in Table 1; in this Figure a peak containing 15 000 d.p.m. would give a full-scale deflection (from the baseline to the top of the y-axis) at the initial, lower sensitivity (see below) used in the trace. Events (along the x-axis): 1, 0 min, 0% 1,4-dioxan; 2, 5 min, sensitivity increased 8-fold and chart speed decreased from 1.5 to 5 min/cm; 3, 30 min, 70% 1,4-dioxan (this concentration was maintained for a further 5 min).

isoniazid. Very little radioactivity, typically 300–1000 d.p.m. in total, was incorporated from [1-¹⁴C]acetate into mycolic acids, even in the presence of 1 μ g/ml isoniazid. Isoniazid appeared to inhibit the biosynthesis of different types of mycolate equally because there was no apparent difference in the distribution of radioactivity between the types of mycolate, with 31–66% (mean $\pm$ S.E.M., $53 \pm 5\%$, eight determinations) being in α -mycolate.

There was no observable difference in the effect of isoniazid, either on mycolic acid biosynthesis or in experiments on the restoration of mycolic acid biosynthesis (reported below), whether it was added immediately before mycolic acid biosynthesis was started in assays or whether the drug was pre-incubated for 30 min with P60 or EP60.

Non-hydroxylated fatty acids synthesized from [1-¹⁴C]acetate by P60 were separated as their pentafluorobenzyl esters by an HPLC system designed to resolve acids of 18–56 carbons. In most instances, four peaks were obtained irrespective of whether isoniazid was included in the assays (Figure 3). The peaks were referred to as a, b, c and d. Three of these, a, b and c, corresponded closely to 18:0, 24:1 and 24:0 standards respectively (Table 1). Peak d, which was not always observed, might be meromycolic acid, about 52 to 56 carbons, as judged by comparison with the previously published HPLC of fatty acid derivatives from mycobacteria [21]. Peak a, which eluted 1.2 min after acetate in the isocratic system and 0.7 min in the gradient system, was poorly resolved and might contain 14–18 carbon acids [16], which this system was not designed to resolve. Peaks b and c correspond closely to 24:1 and 24:0 respectively. Because isoniazid might exert its effect by binding to or modifying NADH and subsequently interfering with mycolate biosynthesis, it has been argued that it might act on a 24:0 desaturase (discussed further in [22]) just as conceivably as InhA protein. To test whether such a desaturase is affected (note that there is no

Table 1 HPLC of non-hydroxylated (i.e. non-mycolic) fatty acyl pentafluorobenzyl esters

Abbreviations: n.d., not done; n.a., not applicable.

Sample peak* or standard	Retention time (min)	
	Gradient (0–70% 1,4-dioxan) (2.2 ml/min)	Isocratic (acetonitrile) (1.4 ml/min)
18:0	2.22	3.17
a	2.00–2.10	3.12
b	3.02–3.07	4.89
c	3.68–3.74	5.9
24:1 <i>cis</i> -5	2.99	4.65
24:0	3.53	5.8
30:0	6.5	n.d.†
d	30.5	n.a.†

* Lettering corresponds to Figure 3.

† Long-chain (above C₃₀) esters are not resolved in the isocratic system (note that the column might be cleaned with 1,4-dioxan after the end of the run).

Table 2 Effect of isoniazid on non-mycolic acid, fatty acid biosynthesis

Label from [1-¹⁴C]acetate (10 μ Ci) was incorporated by P60 into fatty acids which were converted into pentafluorobenzyl esters after the enzyme reaction was stopped. The non-hydroxylated fatty acid esters were purified by HPTLC, then further fractionated by isocratic HPLC and AgNO₃-TLC. The distribution of radioactivity in three individual experiments is shown.

Isoniazid (μ g/ml)	Fatty acid*	Radioactivity (d.p.m.) in fractions			
		Peak a†	Peaks b and c, saturated acids‡	Peaks b and c, unsaturated acids‡	Percentage unsaturated acids in peaks b and c
0	161 800	16 670	8960	1120	11.1
3	189 800	36 200	15 610	3590	18.7
0	124 500	24 815	12 410	2582	17.2
1	441 800	65 740	36 950	5120	12.2
3	431 100	54 740	40 230	4763	10.6
0	221 040	47 750	13 880	1943	10.3
3	139 340	26 190	16 100	2824	14.9

* HPTLC fraction.

† HPLC peak a (see Figure 3 and Table 1).

‡ HPLC peaks b and c, collected together then separated by AgNO₃-TLC.

direct assay), material was collected from isocratic HPLC and the fraction containing C₂₄ acids was analysed for saturation by AgNO₃ TLC (peak b might contain 22:0, but AgNO₃ TLC analysis can identify unsaturated esters unambiguously). Thus peak a was collected for retention times (see Table 1) between 2.7 and 4.2 min, and peaks b and c together were collected between 4.2 and 7.3 min. Results with three different batches of P60 are shown in Table 2. The total radioactivity incorporated from 10 μ Ci of [1-¹⁴C]acetate into all non-hydroxylated fatty acids was inhibited in some experiments and stimulated in others by isoniazid at 1 or 3 μ g/ml, in agreement with the finding of no significant effect ($P > 0.10$) shown for a total of nine determinations at these two concentrations (Figure 2). The effect of inhibition or stimulation of total fatty acid biosynthesis in individual P60 preparations was reflected in peak a (Table 2). However, the appearance of radioactivity in peaks b and c was always stimulated by isoniazid (Table 2). Isoniazid had no consistent effect on the production of unsaturated fatty acids in peaks b and c. In the presence of up to 3 μ g/ml isoniazid, and

Table 3 Restoration of mycolic acid biosynthesis by 24:1 *cis*-5

24:1 *cis*-5 was added to two-phase (water/hexane) assays including 1 μ Ci of [1- 14 C]acetate in the absence or presence of isoniazid, and the incorporation of label into all mycolic acids was analysed. Results are means $\pm$ S.E.M.; *n* is the number of determinations.

Assay contents	Specific activity*	<i>n</i>	Restoration (%)
P60	607 $\pm$ 172	10	
EP60	63 $\pm$ 9	7	
EP60 + 24:1 <i>cis</i> -5	200 $\pm$ 34	7	25.3
P60 + isoniazid (1 μ g/ml)	122 $\pm$ 10	3	
P60 + isoniazid (1 μ g/ml) + 24:1 <i>cis</i> -5	103 $\pm$ 72	4	-3.9
P60 + isoniazid (3 μ g/ml)	68 $\pm$ 7	7	
P60 + isoniazid (3 μ g/ml) + 24:1 <i>cis</i> -5	66 $\pm$ 23	7	-0.7

* Expressed as d.p.m. of 14 C incorporated into mycolic acids/min per mg of protein from 1 μ Ci of [1- 14 C]acetate.

with the concomitant greater than 80% inhibition of mycolic acid biosynthesis, 12.2–18.7% of the radioactivity remained in the unsaturated acid fraction.

Restoration of mycolic acid biosynthesis by 24:1 *cis*-5

First it was established that restoration of mycolic acid biosynthesis by 24:1 *cis*-5 could be shown in an enzyme preparation from *M. aurum* A⁺ (Table 3) to a degree comparable with that previously obtained with *M. smegmatis* EP60 [16]. Isoniazid decreased mycolic acid biosynthesis in P60 from *M. aurum* A⁺ to a specific activity similar to that in EP60 (Table 3), obtained by the repeated extraction of P60 with hexane. However, mycolic acid biosynthesis could not be restored by 24:1 *cis*-5 when isoniazid was present (Table 3).

In a development of the assay, fatty acids were introduced as complexes with a cyclodextrin so that aqueous single-phase assays could be performed (Table 4). Again, 24:1 *cis*-5 significantly ($P < 0.02$) restored mycolic acid biosynthesis in EP60 from *M. aurum* but not mycolic acid biosynthesis inhibited by isoniazid (Table 4). The saturated acid 24:0, when complexed with cyclodextrin, did not restore mycolic acid biosynthesis in either EP60 or P60 in the presence of isoniazid (Table 4). The concentration of RAMEB-CD (2 mg/ml) and RAMEB-

Table 5 Effect of isoniazid on 24:1 *cis*-5 elongase activity

Results are means $\pm$ S.E.M.; *n* is the number of determinations.

Assay contents	Specific activity*	Inhibition (%)	<i>n</i>
EP60 + 24:1 <i>cis</i> -5	200 $\pm$ 34	0	8
EP60	63 $\pm$ 9		
0.1 μ g/ml isoniazid + EP60 + 24:1 <i>cis</i> -5	163 $\pm$ 9	22	2
0.1 μ g/ml isoniazid + EP60	56 $\pm$ 12		
0.3 μ g/ml isoniazid + EP60 + 24:1 <i>cis</i> -5	149 $\pm$ 10	25	2
0.3 μ g/ml isoniazid + EP60	46 $\pm$ 16		
1 μ g/ml isoniazid + EP60 + 24:1 <i>cis</i> -5	73 $\pm$ 10	75	4
1 μ g/ml isoniazid + EP60	39 $\pm$ 9		
3 μ g/ml isoniazid + EP60 + 24:1 <i>cis</i> -5	42 $\pm$ 25	101	6
3 μ g/ml isoniazid + EP60	44 $\pm$ 7		

* Expressed as d.p.m. of 14 C incorporated into mycolic acids/min per mg of protein from 1 μ Ci of [1- 14 C]acetate.

CD–fatty acid complex used was close to the maximum concentration permissible, because higher concentrations (8 mg/ml) inhibited by 80% incorporation of 14 C into mycolic acids from [1- 14 C]acetate by P60 from *M. aurum* A⁺.

Inhibition of 24:1 *cis*-5 elongase activity by isoniazid

Elongase activity was estimated as the difference in mycolic acid biosynthesis by using EP60 from *M. aurum* A⁺ with and without 24:1 *cis*-5 in an assay in hexane/water (7:1, v/v) (Table 5). Essentially the method is an adaptation of classical fatty acid elongase assays established previously in work with *M. smegmatis* [16]. The products are mycolic acids, because there is almost no accumulation of fatty acids between C₂₆ and mycolic acids [16] (confirmed for *M. aurum* A⁺ in Figure 3). Isoniazid inhibited elongase activity in a concentration-dependent manner and gave complete inhibition at 3 μ g/ml (Table 5).

Catalase

Catalase activity was measured in wall preparations because of its implication in isoniazid sensitivity. This enzyme was detected both in P60 at (1.63–4.3) $\times 10^{-4}$ unit/mg of protein and in EP60

Table 4 Restoration of mycolic acid biosynthesis by 24:1 *cis*-5

24:1 *cis*-5 was added to one-phase (water/hexane) assays including 1 μ Ci of [1- 14 C]acetate in the absence or presence of isoniazid, and the incorporation of label into all mycolic acids was analysed. Results are means $\pm$ S.E.M.; *n* is the number of determinations.

Assay contents	Specific activity*	<i>n</i>	Restoration (%)
P60	991 $\pm$ 236	11	
P60 + RAMEB-CD	783 $\pm$ 133	7	
P60 + RAMEB-CD + 24:1 <i>cis</i> -5	754 $\pm$ 76	6	
P60 + RAMEB-CD + 1 μ g/ml isoniazid	211 $\pm$ 55	4	
P60 + RAMEB-CD + 1 μ g/ml isoniazid + 24:1 <i>cis</i> -5	156 $\pm$ 131	3	-10.0
P60 + RAMEB-CD + 3 μ g/ml isoniazid	91 $\pm$ 28	7	
P60 + RAMEB-CD + 3 μ g/ml isoniazid + 24:1 <i>cis</i> -5	99 $\pm$ 33	8	1.4
P60 + RAMEB-CD + 3 μ g/ml isoniazid + 24:0	93 $\pm$ 5	2	0.3
EP60† + RAMEB-CD	118 $\pm$ 15	3	
EP60† + RAMEB-CD + 24:1 <i>cis</i> -5	252 $\pm$ 9	3	20.2
EP60† + RAMEB-CD + 24:0	107 $\pm$ 22	2	-1.7

* Expressed as d.p.m. of 14 C incorporated into mycolic acids/min per mg of protein from 1 μ Ci of [1- 14 C]acetate.

† Hexane used for extraction was dried off with N₂ before EP60 was used in these assays.

at $(0.53\text{--}4.2) \times 10^{-4}$ unit/mg of protein A unit of catalase is defined as $(1/t) \times \ln(S_0/S_t)$, where t is the incubation time in seconds and S_0 and S_t are the concentrations of H_2O_2 at zero time and the end of the incubation respectively [19].

DISCUSSION

The A⁺ strain of *M. aurum* shares with the human pathogen *M. tuberculosis* complete growth inhibition by isoniazid at a concentration of 1 µg/ml. Therefore we chose the saprophytic and relatively rapid-growing *M. aurum* A⁺ for this study on the effect of isoniazid on mycolic acid biosynthesis. Cell-free mycolic acid biosynthesis was inhibited by 80–90% by 1–3 µg/ml isoniazid (Figure 1), 3–10 times the concentration that similarly inhibited growth (Figure 2). As mycobacteria concentrate isoniazid approx. 20-fold from their environment at these concentrations [23], the effect of isoniazid on mycolic acid biosynthesis occurs at pharmacological concentrations.

At 1–3 µg/ml, isoniazid exerts selective inhibition over the biosynthesis of mycolic acids compared with fatty acids synthesized in the same assay mixture. The only intermediates that accumulated by the action of isoniazid were 24-carbon acids. The ratio of unsaturated to saturated fatty acid was not affected by isoniazid. Together these observations suggest that isoniazid acts on the elongation of C₂₄ acids rather than the desaturation of 24:0. Because 24:1 *cis*-5 elongase activity in hexane-extracted enzyme material is also strongly inhibited by isoniazid at 1–3 µg/ml, and adding 24:1 *cis*-5 to unextracted wall preparations failed to overcome isoniazid inhibition, it is concluded that the primary target for isoniazid is the elongation of 24:1 *cis*-5 by an elongase enzyme complex to give the characteristic ω-19 α-acyl chains in mycolic acids. As non-hydroxylated fatty acids continue to be synthesized in the presence of isoniazid, it was possible that isoniazid might block the pathway from mycolic acid biosynthesis at a later stage than 24 carbons, producing a feedback inhibitor to inhibit 24:1 *cis*-5 elongase. There are many places to block, such as the insertion of a second double bond into α-mycolates and the introduction of an oxygen function into ketomycolates [24]. However, no such intermediate accumulated as a result of the action of isoniazid and there would have been no difficulty in detecting them because background intermediates were not observed in the absence of isoniazid (Figure 3). Thus the results argue strongly for a direct effect of isoniazid or a metabolite of isoniazid on 24:1 *cis*-5 elongase. Note that all preparations used in this work contained catalase, the enzyme thought to be responsible for isoniazid activation within the bacterial cell [7,25].

There is a growing body of work that supports the view that isoniazid or its metabolite(s) act on a target in mycolic acid biosynthesis [10,22,26,27]. The *inhA* gene, for which variants encode isoniazid resistance, has recently been shown to encode one of the enzymes of a fatty acyl elongase complex. The individual InhA enzyme is a *trans*-2-enoyl ACP reductase in which a difference in NADH binding between wild-type and mutant InhA might account for the difference in isoniazid sensitivity [10]. Of particular interest in the context of our work is its chain-length preference, because the ability to elongate acids beyond C₂₄ is required for involvement in mycolic acid biosynthesis. In the work on InhA [10], 2-enoyl-CoAs, of chain length 12–24 carbons, were compared at 1 µM; reaction velocities were highest for C₁₆ and C₂₀, then C₂₄. Enoyl-CoA had a K_m 100 times that of the corresponding enoyl-ACP, indicating that the target is in fact a *trans*-2-enoyl ACP reductase.

Thus it should be expected that the isoniazid-sensitive target in P60 described in the present work is an elongase containing the

isoniazid-sensitive *trans*-2-enoyl ACP reductase. Indeed P60 from both *M. smegmatis* [16] and *M. aurum* (Figure 3) synthesize a range of fatty acids up to C₂₄ as well as mycolates and thus the elongase in P60 that acts on 24:1 *cis*-5 might act on a variety of primers, an activity shown in soluble preparations of mycobacterial fatty-acyl carrier elongases [28–30]. However, in the tightly coupled wall-associated P60 it was not possible to show any effect with fatty acids, including 16:0, 24:0 or 24:1 *cis*-15 (nervonic acid) and CoA esters [16] (only 24:0 was tested with *M. aurum*), except for 24:1 *cis*-5. Similar preparations from *M. aurum*, but made without the Percoll gradient step by the group that originally obtained cell-free mycolate biosynthesis [11,12,31], had similar properties to our P60. Their wall-based preparations also used acetate and produced long-chain fatty acids, including mycolates, required no cofactors to be added during the 30–90 min incubations used and had isoniazid-sensitive mycolate biosynthesis. The insoluble, wall-associated nature of the mycolate-synthesizing systems has so far discouraged any attempt at isolation of individual enzymes or enzyme complexes from them.

In contrast, the *trans*-2-enoyl ACP reductase that has been cloned and over-expressed [10] is a soluble enzyme. In that respect it is related to *trans*-2-enoyl carrier reductases that have been isolated from soluble native elongases and synthases [32]. In contrast with wall-associated systems such as P60, the soluble elongases and synthases require CoA or ACP esters, only use acetate or malonate if supplied with cofactors even in crude preparations [33] and do not synthesize complete mycolic acids [22]. Our results predict that a reaction characteristic of mycolic acid biosynthesis for its selective inhibition should be catalysed by an isoniazid-sensitive elongase; thus it should be possible to demonstrate isoniazid-sensitive reduction of *trans*-2-*cis*-5-tetracosdienoyl-ACP (Scheme 1).

The biosynthesis of the *trans*-2-*cis*-5-tetracosadienoyl moiety remains a mystery (Scheme 1). Both fatty acyl desaturases [34] and elongases [28,29] have been long known in mycobacteria. However, Δ-5 desaturases have been sought but never detected [35]. In addition, despite the fact that products of mycobacterial elongases have been extensively analysed [21,30], there is no evidence for the alternative mechanism, anaerobic desaturation [36] in which instead of reduction of *trans*-2-docosaenoyl-carrier by an InhA-like enzyme there is isomerization to *cis*-3-docosaenoyl-carrier followed by a round of elongation to give *cis*-5-tetraecosenoyl-carrier. Although 22:1 *cis*-3 is a minor component of fatty acids from mycobacterial extracts in which 24:1 *cis*-5 is a major component [37], these acids were unlabelled and might equally have been breakdown products rather than biosynthetic intermediates. Series of labelled mono-unsaturated fatty acids with an undefined position for the double bond have been obtained from incubation of mycobacterial extracts with [¹⁴C]C₂ donors; however, some series started at 24:1, whereas the series that included 22:1 started at 16:1, and it may be that the acids with the least carbons were primers for elongases in each series of acids [21].

Thus, although the present work adds to the evidence that the target of isoniazid in a highly susceptible mycobacterium is a fatty acyl elongase in mycolic acid biosynthesis, much remains to be shown. Ultimately, the isolation of individual enzymes should allow the correct pathway to be established (Scheme 1) and the binding of isoniazid to its target to be demonstrated directly.

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