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Inhibition of InhA Activity, but Not KasA Activity, Induces Formation of a KasA-containing Complex in Mycobacteria*

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Isoniazid (INH) remains one of the key drugs used to control tuberculosis, with the enoyl-AcpM reductase InhA being the primary target. However, based on the observation that INH-treated *Mycobacterium tuberculosis* overproduces KasA, an enzyme involved in the biosynthesis of mycolic acids, and induces the formation of a covalent complex consisting of AcpM, KasA, and INH, it has been proposed that KasA represents the primary target of INH. However, the relevance of this complex to INH action remains obscure. This study was aimed at clarifying the role of InhA and KasA in relation to INH activity. By using anti-KasA antibodies we detected the KasA-containing complex in INH-treated *Mycobacterium smegmatis*. In addition, INH-treated cells also produced constant levels of KasA that were not sequestered in the complex and presumably were sufficient to ensure mycolic acid biosynthesis. Interestingly, a *furA*-lacking strain induced the complex at lower concentrations of INH compared with the control strain, whereas higher INH concentrations were necessary to induce the complex in a strain that lacks *katG*, suggesting that INH needs to be activated by KatG to induce the KasA-containing complex. The InhA inhibitors ethionamide and diazaborine also induced the complex; thus, its formation was not specifically relevant to INH action but was because of InhA inhibition. In addition, *in vitro* assays using purified InhA and KasA demonstrated that KatG-activated INH, triclosan, and diazaborine inhibited InhA but not KasA activity. Moreover, several thermosensitive InhA mutant strains of *M. smegmatis* constitutively expressed the KasA-containing complex. This study provides the biochemical and genetic evidence. 1) Only inhibition of InhA, but not KasA, induces the KasA-containing complex. 2) INH is not part of the complex. 3) INH does not target KasA, consistent with InhA being the primary target of INH.

Isoniazid (INH)¹ remains an important first-line drug in global control strategies to treat tuberculosis. Despite its seemingly simple structure and the discovery over 50 years ago that INH is highly active against *Mycobacterium tuberculosis* (1, 2), the elucidation of its precise mode of action has been difficult. Middlebrook (3) was the first to carefully quantify the bactericidal activity of INH. Although Winder and Collins (4) demonstrated that INH inhibits mycolic acid biosynthesis, Takayama *et al.* (5) demonstrated that inhibition of mycolic acid biosynthesis correlates with cell death. Takyama *et al.* (6) further demonstrated that the inhibition of mycolic acid biosynthesis mediated by INH leads to the accumulation of a saturated C₂₆ fatty acid within 1 h of INH treatment. By using scanning electron microscopy, it was established that *M. tuberculosis* treated with INH undergoes a defined order of molecular events that leads to lysis 24 h after initiation of treatment, thus after approximately one cell generation (7). The events that lead from the inhibition of mycolic acid biosynthesis and the accumulation of a saturated C₂₆ fatty acid to cell lysis are still poorly understood. Moreover, there exists controversy as to which enzymes of the mycolic acid biosynthetic pathway are the actual targets of INH.

The mode of action of INH appears to be rather complex and requires activation by the mycobacterial catalase-peroxidase KatG (8, 9). Activation of the pro-drug leads to reactive radicals that exert a toxic effect on the tubercle bacillus (9–11). Presumably activated INH inhibits one or more targets of the mycolic acid biosynthetic pathway which eventually leads to cell death. At least two enzymes have been identified as potential targets of activated INH, the enoyl-ACP reductase InhA (12) and the β -ketoacyl-ACP synthase KasA (13). The *inhA* gene was first identified by conferring co-resistance to INH and ethionamide (ETH) after overexpression in mycobacteria (12). In addition, mutations in *inhA* confer resistance to both INH and ETH in drug-resistant *M. tuberculosis* isolates (14–17). Biochemical analyses have revealed InhA to be the NADH-

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¹ The abbreviations used are: INH, isoniazid; AcpM, acyl-carrier protein; CER, cerulenin; DZB, diazaborine; ETH, ethionamide; FAME, fatty acid methyl ester; FAS-I, type I fatty-acid synthase; FAS-II, type II fatty-acid synthase; ISO, isoxyl; KasA, β -ketoacyl-ACP synthase A; KasB, β -ketoacyl-ACP synthase B; MAME, mycolic acid methyl ester; MIC, minimal inhibitory concentration; TLM, thiolactomycin; TRC, triclosan; Ts, thermo-sensitive; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; BCG, Bacille Calmette-Guerin.

TABLE I
Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Ref./source
Strain		
<i>E. coli</i> XL-1 Blue	F':Tn10 <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> _q Δ(<i>lacZ</i>) <i>M15/recA1 endA1 gyrA96</i> (Nal ^r) <i>thi hsdR17</i> (r _k ⁻ m _k ⁺) supE44 relA1 lac	Biolabs
<i>E. coli</i> C41 (DE3)	BL21 (DE3) mutant strain	68
<i>M. smegmatis</i> mc ² 155	Hypertransformable mutant derived from <i>M. smegmatis</i> mc ² 6	69
<i>M. smegmatis</i> JS106-1	FurA-deficient mc ² 155 strain	40
<i>M. smegmatis</i> NH55	KatG-deficient mc ² 155 strain	This work
<i>M. smegmatis</i> mc ² 651	Contains a missense mutation within the <i>inhA</i> gene, resistant to both INH and ETH	12
<i>M. smegmatis</i> mc ² 2354	Merodiplid strain for the <i>ndh</i> gene	67
<i>M. smegmatis</i> mc ² 2359	<i>inhA</i> (Ts) mutant of mc ² 2354 carrying a V238F mutation within <i>InhA</i> , INH-R	23
<i>M. smegmatis</i> mc ² 2365	Revertant of mc ² 2359, carrying a F238C mutation in <i>InhA</i> , INH-S	23
<i>M. smegmatis</i> mc ² 2366	Revertant of mc ² 2359, carrying a G102C mutation in <i>InhA</i> , INH-R	23
<i>M. smegmatis</i> mc ² 2367	Revertant of mc ² 2359, carrying a F238V mutation in <i>InhA</i> , INH-S	23
<i>M. smegmatis</i> mc ² 2571	<i>inhA</i> (Ts) mutant of mc ² 155, carrying a M104G mutation in <i>InhA</i> , TRC-R	Footnote 4
<i>M. smegmatis</i> mc ² 2572	<i>inhA</i> (Ts) mutant of mc ² 155, carrying a M147T mutation in <i>InhA</i> , TRC-R	Footnote 4
<i>M. bovis</i> BCG 1173P2	Vaccine strain	WHO, Stockholm
<i>M. kansasii</i>	Wild-type	ATCC 35775
Plasmid		
pMV261	<i>E. coli</i> -mycobacteria shuttle vector	70
pMV261:: <i>inhA</i>	pMV261 containing <i>M. tuberculosis</i> H37Rv <i>inhA</i>	28
pMV261:: <i>ethA</i>	pMV261 containing the <i>M. tuberculosis</i> Rv3854c gene	46
pET28a	<i>E. coli</i> expression vector	Novagen
pET28a:: <i>kasA</i>	pET28a containing <i>M. tuberculosis</i> H37Rv <i>kasA</i>	34
pET28a:: <i>acpM</i>	pET28a containing <i>M. tuberculosis</i> H37Rv <i>acpM</i>	33
pET28a:: <i>mtfabD</i>	pET28a containing <i>M. tuberculosis</i> H37Rv <i>mtfabD</i>	33
pKAG3	KatG-expression plasmid	35

specific enoyl-ACP reductase of the fatty-acid synthase II system (FAS-II), which elongates long chain fatty acids for the synthesis of mycolic acids (18–20). This enzymatic activity can be inhibited by activated INH (21, 22). X-ray crystallographic studies have revealed that the resistance-conferring mutations map to the NADH-binding pocket and that activated INH binds to NAD⁺, forming an INH-NAD adduct that specifically inhibits *InhA* (10, 19). Consistent with *InhA* being the target of INH, a combination of genetic and biochemical studies on a thermosensitive *Mycobacterium smegmatis inhA* mutant indicated that *InhA* inactivation is sufficient to cause lysis by a mechanism similar to that induced by INH treatment, as evidenced by scanning electron microscopy (23).

Despite these lines of evidence, the role of *InhA* as the primary target of INH has been questioned for three reasons. 1) Inactivation of *InhA* could not readily account for the accumulation of a saturated C₂₆ fatty acid in *M. tuberculosis* observed upon INH treatment. 2) No covalent binding of INH to *InhA* has been detected. 3) A failure to transfer INH resistance by overexpression of *inhA* in *M. tuberculosis* casted doubt on the relevance of *M. smegmatis* as a suitable surrogate host to study INH resistance in *M. tuberculosis*. Therefore, Mdululi *et al.* (24) suggested that *InhA* is not the major primary target for activated INH in *M. tuberculosis* and proposed the β-ketoacyl-AcpM synthase *KasA* as the primary target, as they found that treatment of *M. tuberculosis* with INH induced the overexpression of *KasA* and the formation of an 80-kDa complex consisting of INH, AcpM, and *KasA*. It was also suggested that *KasA* inactivation results in the accumulation of a saturated C₂₆ fatty acid (13). In addition, four mutations in the *kasA* gene were found in INH-resistant clinical isolates (13).

Numerous studies have supported that *InhA* can account for all the observations. First, a temperature-sensitive mutation in *inhA* was used to demonstrate that *InhA* inactivation leads to an accumulation of saturated FAS-I end product (23), and the three-dimensional structure of the INH-NAD⁺ adduct binding to *InhA* explained the lack of direct covalent binding of radioactive INH to *InhA* (10). Moreover, recent studies have clearly demonstrated that overexpression of *inhA* confers resistance to INH and ethionamide in *M. tuberculosis*, *Mycobacterium bovis* BCG, and *M. smegmatis*. In contrast, little evidence has accu-

mulated supporting *KasA* as a target for INH, although independent studies (25, 26) have reported that three of the four *kasA* alleles have been found in *M. tuberculosis* isolates that are fully susceptible to INH. To date, no one has performed a gene transfer experiment demonstrating that any of these mutations confer INH resistance to susceptible strains of mycobacteria. Furthermore, the failure of overexpression of *kasA* in *M. bovis* BCG (27), *M. tuberculosis*, and *M. smegmatis* (28) to confer resistance to INH is inconsistent with a previous report (29) which raises doubts as to whether *KasA* plays a relevant role in INH action. Clearly, additional *in vivo* and *in vitro* studies are required in order to determine the potential role of *KasA* and the participation of the proposed ternary AcpM-*KasA*-INH complex in INH and ETH resistance.

In this study we re-examined the potential involvement of *InhA* or *KasA* as major primary targets of activated INH, and we tested the hypothesis that *InhA* inactivation through any mechanism leads to the accumulation of the 80-kDa AcpM-*KasA* complex.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Conditions—The mycobacterial strains and plasmids used in this study are listed in Table I. Luria broth (Difco) and Luria agar containing 25 μg/ml kanamycin (Sigma) were used for the growth of transformed *Escherichia coli* strains (30). *M. bovis* BCG, *Mycobacterium kansasii*, and *M. smegmatis* were all grown in liquid Sauton medium (31) at 37 °C. Recombinant *M. smegmatis* strains were grown in Sauton medium containing 25 μg/ml kanamycin or on Middlebrook 7H10 agar supplemented with 10% oleic acid/albumin/dextrose/catalase enrichment (Difco) and containing 25 μg/ml kanamycin for 3–4 days at 37 °C. The FurA- and the KatG-defective *M. smegmatis* strains were grown in Sauton medium supplemented with 25 μg/ml kanamycin and 50 μg/ml hygromycin B (Roche Applied Science), respectively.

DNA Manipulation and Construction of a *KatG*-deficient Strain—A *KatG*-defective *M. smegmatis* strain was prepared by allelic exchange using the procedure described earlier (32). Briefly, the *katG* gene was first inactivated by the insertion of a hygromycin resistance cassette and subsequently cloned into a suicide vector containing the wild-type *rpsL* gene. This construct was then electroporated into *M. smegmatis* SMR5 strain, a derivative of mc²155, which contains a recessive mutant *rpsL* allele that confers resistance to streptomycin. The replacement of wild-type *katG* gene by *katG::hyg*, reflecting a double cross-over event, was selected by picking colonies that were resistant to both hygromycin

and streptomycin. This gene replacement was verified by PCR analysis of the recombinant clones. Restriction enzymes and T4 DNA ligase were purchased from Roche Applied Science, and Vent DNA polymerase was purchased from New England Biolabs. All DNA manipulations were performed using standard protocols, as described earlier (30).

Drug Susceptibility Testing of *M. smegmatis*—The susceptibility to INH, ETH, cerulenin (CER), diazaborine (DZB), triclosan (TRC), and isoxyl (ISO) on *M. smegmatis* was determined by performing serial 10-fold dilutions of the cultures on Middlebrook 7H10 agar containing 10% oleic acid/albumin/dextrose/catalase enrichment and increasing concentrations of the drugs. The minimal inhibitory concentration (MIC) was defined as the minimal concentration required to completely inhibit 99% of mycobacterial growth after incubation at 37 °C for 3–4 days.

Production and Purification of Recombinant *M. tuberculosis* Proteins—Recombinant His-tagged KasA, mtFabD, holo-AcpM, and palmitoyl-AcpM were produced in *E. coli* C41(DE3) containing the relevant expression vectors and purified by affinity chromatography using a His-Trap column (Amersham Biosciences) as described previously (33, 34). KatG was purified from pKAG3-transformed *E. coli* UM262 as described elsewhere (35). Purified *M. tuberculosis* InhA was kindly provided by GlaxoSmithKline.

Preparation of Anti-KasA and Anti-InhA Immune Sera—Rat anti-KasA antibodies were obtained as reported previously (36). Rabbit anti-InhA antibodies were produced as follows. A His-tagged version of InhA (10) was prepared by His-trap Ni²⁺-chelating chromatography. After this chromatographic step, InhA was further purified to near-homogeneity by size exclusion chromatography (Amersham Biosciences) on a Superdex-200 column. Purified InhA was dialyzed against Tris-HCl (pH 7.6), containing 150 mM NaCl. 1 mg of His-tagged InhA (1 mg/ml) was mixed with 1 ml of TitermaxR gold adjuvant (Sigma). A rabbit was immunized subcutaneously with 400 µg of His-tagged InhA (100 µg of protein per site) in a final volume of 400 µl. The first booster was given 21 days after the primary immunization. During the first booster 200 µg of protein (50 µg per site) was injected in the animal. Sera were collected 10 days after the first booster, and the presence of anti-InhA antibodies was verified by immunoblot analysis. A second booster was given (200 µg of protein) 15 days after the first booster. The rabbits were bled, and sera were prepared. Preimmune sera were collected before immunization.

Immunoblotting—Ten-milliliter aliquots of culture were harvested at mid-log phase. Cells were then resuspended in 0.8 ml of phosphate-buffered saline (PBS; 20 mM K₂HPO₄, pH 7.5, 0.15 M NaCl) and disrupted for 10 min using a Branson Sonifier 450. Protein concentrations were determined using the BCA Protein Assay Reagent kit (Pierce). Equal amounts of proteins (20 µg) were then separated on a SDS-PAGE as described by Laemmli (37). After electrophoresis, the proteins were transferred onto a Hybond-C Extra membrane (Amersham Biosciences). The membrane was then saturated with 5% dry milk in PBS, 0.1% Tween 20, incubated overnight with either rat anti-KasA antibodies (1:500 dilution) or rabbit anti-InhA antibodies (1:30,000 dilution), washed, and incubated with anti-rat antibodies or anti-rabbit antibodies conjugated to alkaline phosphatase (1:7000 dilution; Promega), respectively.

In Vivo Effects of INH on Mycolic Acid Synthesis—*M. smegmatis* was grown to mid-log phase, and INH was added at various concentrations followed by incubation at 37 °C for 4 h. 1 µCi/ml of [1,2-¹⁴C]acetate (50–62 mCi/mmol, Amersham Biosciences) then was added to the cultures followed by further incubation at 37 °C for 4 h. The ¹⁴C-labeled cells were harvested by centrifugation at 2000 × *g* and washed with PBS. Extraction of the ¹⁴C-labeled fatty acid methyl esters (FAMES) and mycolic acid methyl esters (MAMES) has been reported previously (34). Equal amounts of counts were subjected to TLC as described previously (34) and exposed overnight to a Kodak X-Omat film.

β-Ketoacyl-AcpM Synthase Assay—Mycobacterial KasA assays were performed according to the condensation assays developed for the *E. coli* β-ketoacyl-ACP synthases (38, 39) and recently adapted to the mycobacterial β-ketoacyl-ACP synthase (34). Initially, assay components were mixed together in a batch fashion. The amounts stated correspond to those for a single reaction, which was scaled up proportionately to the number of assays performed. Holo-AcpM (40 µg) was incubated on ice for 30 min with β-mercaptoethanol (0.5 µmol) in a total volume of 40 µl. [2-¹⁴C]Malonyl-CoA (6.78 nmol, 0.045 µCi; Amersham Biosciences), mtFabD (40 ng), and 25 µl of 1 M potassium phosphate buffer, pH 7.0, were added. The reaction mixture was held at 37 °C for 20 min to allow mtFabD to catalyze transacylation of holo-AcpM with malonyl-CoA to provide malonyl-AcpM (33). C₁₆-AcpM (22.5 µg) was then added to afford a final volume of 89 µl. The reaction was then

TABLE II
MICs (µg/ml) of INH against *M. smegmatis* strains determined on 7H11 supplemented with OADC

Strain	INH
mc ² 155 (pMV261)	5
mc ² 155 (pMV261:: <i>inhA</i>)	400-600
mc ² 651	300-400
JS106-1	0.5-1
NH55	15

dispensed according to single assay mixtures into 1.5-ml microcentrifuge tubes. The drugs were then immediately added, 1 µl per reaction. All drugs were dissolved in Me₂SO apart from TRC and CER, which were dissolved in 1 M NaOH and ethanol, respectively. Control reactions containing 1 µl of the appropriate solvent were carried out. When KatG-activated INH was used, KatG was diluted to 80 µg/ml in 30 mM PIPES, pH 6.8, 0.05% bovine serum albumin, containing 150 µM NADH. INH was added at the same time, and the mixture was held at ambient temperature for 150 min before assaying, and a 10-µl sample of this reaction mixture was incorporated into the assay.

A 10-µl aliquot of KasA (0.25 µg) was added to the reaction mixture that was incubated at 37 °C for 1 h. The reaction was then quenched by the addition of 2 ml of NaBH₄ reducing solution converting the β-keto-[¹⁴C]₁₈-AcpM product into a [¹⁴C]₁₈-1,3-diol. The reducing solution was prepared freshly and consisted of 5 mg/ml NaBH₄ in 0.1 M K₂HPO₄, 0.4 M KCl, and 30% (v/v) tetrahydrofuran (38, 39). The reaction was incubated at 37 °C for at least 1 h. The completely reduced [¹⁴C]β-ketoacyl product was extracted twice with 2 ml of water-saturated toluene. The combined organic phases from both extractions were pooled and washed with 2 ml of toluene-saturated water. The organic layer was removed and dried under a stream of nitrogen in a scintillation vial. The reduced product was subsequently quantified by liquid scintillation counting using 5 ml of EcoScintA (National Diagnostics).

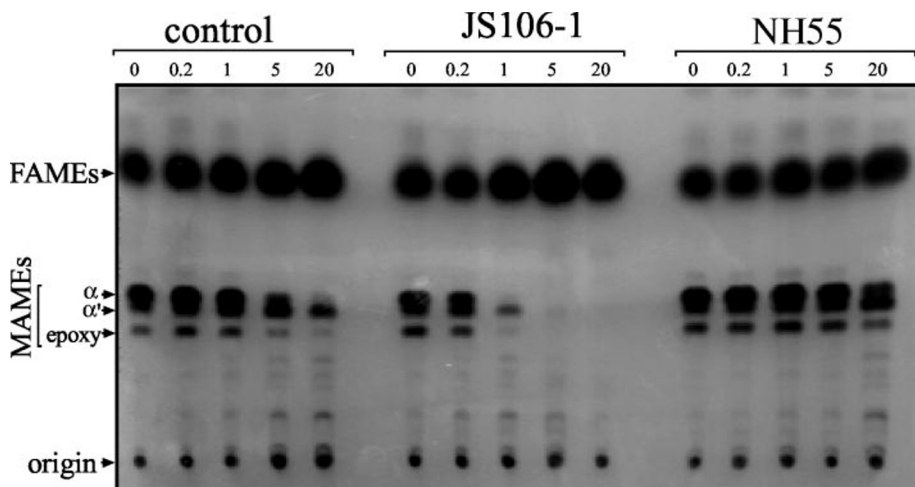
Enoyl-CoA Reductase Assay—InhA activity was assayed according to the method developed by Quémar *et al.* (18). The standard assay was carried out in 1 ml of 30 mM PIPES, pH 6.8, 0.05% bovine serum albumin, containing 150 µM NADH and 4 µg of InhA as well as the drugs to be tested. These were dissolved in Me₂SO, apart from TRC and CER, which were dissolved in 1 M NaOH and ethanol, respectively. A maximum of 10 µl of drug solution was added to each reaction mixture, and the control reactions contained 10 µl of the appropriate solvent. Unless otherwise stated, reactions were initiated after 5 min by the addition of 215 µM 2-*trans*-dodecenoyl-CoA. The rates of NADH oxidation were measured spectrophotometrically at 340 nm for 1 min using a Hitachi U-2001 UV-visible spectrophotometer.

RESULTS

Induction of the KasA-containing Complex by Activated INH—Because activation of INH requires the mycobacterial catalase-peroxidase KatG (8), and FurA represses *katG* expression (40), we determined the MICs against INH of *M. smegmatis* strains containing a deletion in either *katG* or *furA* and compared them to that of wild-type *M. smegmatis* (Table II). As expected, *M. smegmatis* NH55 (the KatG-deficient strain) was more resistant to INH than the control strain, whereas *M. smegmatis* JS106-1 (the FurA-deficient strain) was highly sensitive to INH. To evaluate the effect of INH on mycolic acid and fatty acid synthesis in these mutants, the cultures were treated with increasing INH concentrations and labeled with [¹⁴C]acetate. MAMES and fatty acid methyl esters (FAMES) were extracted and analyzed by TLC. As shown in Fig. 1, INH affects essentially the synthesis of α and epoxy mycolates, as described previously (41). In agreement with the MIC data, this inhibitory effect was apparent at lower concentrations in JS106-1, whereas the synthesis of mycolates was found to be more resistant to INH in NH55 (Fig. 1).

Several studies reported that INH induces overproduction of KasA in *M. tuberculosis* (13, 29, 42) and that this enzyme was sequestered within a ternary complex that involves INH-AcpM and KasA (13). The authors further speculated that because KasA was part of the complex induced by INH treatment, active KasA enzyme was not available to ensure FAS-II activity

FIG. 1. Dose-response of INH on mycolic acid synthesis in various *M. smegmatis* strains. The inhibitory effect on the incorporation of [1,2-¹⁴C]acetate was assayed by labeling *M. smegmatis* carrying pMV261 (control strain), *M. smegmatis* JS106-1 (FurA-deficient), or *M. smegmatis* NH55 (KatG-deficient) in the presence of increasing concentrations of INH ($\mu\text{g/ml}$) and terminated by the addition of 15% tetrabutylammonium hydroxide at 100 °C overnight. The corresponding FAMES and MAMES were isolated, and equal amounts of counts (100,000 cpm) were subjected to TLC and exposed overnight to a Kodak X-Omat film.



and consequently mycolic acid biosynthesis (13). We therefore treated *M. smegmatis* with various concentrations of INH and analyzed the presence of KasA alone or associated with other proteins by immunoblotting using rat anti-KasA antibodies. As shown in Fig. 2A, a 45-kDa protein corresponding to KasA was readily detected in untreated cells. Slower migrating anti-KasA reactive material, running at ~ 80 kDa, was also observed in *M. smegmatis* untreated cells, suggesting that the formation of the KasA-containing complex described by Mdluli *et al.* (13) occurs without INH exposure. However, this complex was highly induced upon INH treatment (Fig. 2A) and was also detected by anti-AcpM antibodies,² consistent with its assignment as an AcpM-KasA complex. In contrast to previous speculations (13), suggesting that mature mycolates could no longer be produced upon INH treatment because of KasA sequestering in the complex induced by INH, the level of unbound KasA remained unchanged, regardless of the concentration of INH used. Even at concentrations of INH exceeding the MIC, the levels of unbound KasA remained unchanged.

To determine whether induction of the complex is restricted to *M. smegmatis*, or whether it also occurs in other mycobacteria, we treated various mycobacterial species, including *M. kansasii* and *M. bovis* BCG, with INH and analyzed the presence of both KasA and the KasA-containing complex by immunoblotting. As shown in Fig. 2B, both *M. kansasii* and *M. bovis* BCG contained the KasA-containing complex upon treatment with INH. In agreement with Mdluli *et al.* (13), similar results were found with *M. tuberculosis* H37Rv.² These results suggest that activated INH induces the formation of the KasA-containing complex in a wide range of mycobacterial species.

To investigate whether the induction of the KasA-containing complex depends on INH activation, immunoblot analyses were carried out on the KatG-deficient and the FurA-deficient strains. The results shown in Fig. 2A (middle and right panels) revealed that the complex formation was induced at 15 $\mu\text{g/ml}$ in NH55 and at 0.1–0.5 $\mu\text{g/ml}$ in JS106-1, which correspond to the respective MIC values of these strains (Table II). These results strongly suggest that the formation of the KasA-containing complex is induced in the presence of activated INH.

Induction of the KasA-containing Complex by *InhA*-inhibitory Drugs—We examined the formation of the complex following treatment of mycobacterial cells with various FAS-II inhibitors. Aside from INH or ETH, other agents inhibiting FAS-II enzymes in bacteria or plants were used, including DZB, CER, ISO, and TLM (43). ETH is a structural analog of INH and is

used as a useful second-line antitubercular drug for the treatment of multidrug-resistant strains (44). ETH and INH display nearly identical effects on the inhibition of mycolic acid biosynthesis (45). In addition, a single mutation in the *inhA* gene confers resistance to both INH and ETH (12). As shown in Table III, overexpression of *inhA* in *M. smegmatis* leads to a 20-fold increase in resistance to ETH, in agreement with a recent study (28) demonstrating that overexpression of *inhA* in *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* increases resistance against both INH and ETH. These observations suggest that the mode of action of both drugs is identical, although their modes of activation differ. Whereas INH is activated by KatG, ETH is activated by a monooxygenase, designated EthA (46–48). To investigate whether ETH also induces the KasA-containing complex, immunoblot analyses were carried out on crude lysates of *M. smegmatis* treated with increasing concentrations of ETH. Probing the immunoblots with anti-KasA antibodies clearly showed the formation of the complex upon treatment of the mycobacteria with ETH, mainly at the MIC concentration (MIC = 20 $\mu\text{g/ml}$, Table III) or higher (Fig. 3A). To assess whether ETH requires activation by EthA to induce the formation of the complex, an *M. smegmatis* strain overproducing EthA was used. Overproduction of EthA results in hypersensitivity to ETH and inhibition of mycolic acid synthesis at lower concentrations than those required to inhibit mycolic acid synthesis in control strains (46). As anticipated, the strain harboring pMV261::*ethA* was able to produce the KasA-containing complex at lower ETH concentrations than the control strain carrying the empty plasmid (Fig. 3A). An immunoreactive band was already detectable at 0.1–1 $\mu\text{g/ml}$ corresponding to the MIC value of this strain (46), indicating that ETH requires activation by EthA in order to induce the formation of the KasA-containing complex.

We then determined the ability of other *InhA* inhibitory drugs to induce the KasA-containing complex. DZBs are a class of boron-containing compounds that are only used experimentally, due to the inherent toxicity of boron. Davis *et al.* (49) synthesized DZB analogs carrying different side chains that displayed enhanced activity against *M. tuberculosis*. Although the MIC value of DZB for *M. smegmatis* (pMV261) was significantly higher (MIC = 100 $\mu\text{g/ml}$) compared with that of other drugs, *M. smegmatis* carrying pMV261::*inhA* showed a reproducible 2-fold increase in resistance against this drug over the strain carrying the empty vector (Table III). This suggests that *InhA* may be a target of DZB, in agreement with the fact that DZB inhibits the *E. coli* enoyl-ACP reductase encoded by the *fabI* gene (50–52). Immunoblot analyses of *M. smegmatis*

² L. Kremer, H. R. Morbidoni, W. R. Jacobs, Jr., and G. S. Besra, unpublished results.

FIG. 2. Induction of a *KasA*-containing complex by activated INH in mycobacteria. A, *M. smegmatis* strains were grown to mid-log phase and treated with increasing concentrations of INH for 6 h. B, *M. kansasii* ATCC 35775 and *M. bovis* BCG were treated with 0.5 $\mu\text{g/ml}$ INH for 24 h. Cells were then harvested, resuspended in PBS, and disrupted by sonication. Equal amounts of crude lysates (25 μg) were then loaded onto a 12% acrylamide gel subjected to electrophoresis and transferred onto a membrane for immunoblot analysis. Detection of *KasA* and of the 80-kDa *KasA*-containing complex was performed using rat anti-*KasA* antibodies raised against *M. tuberculosis* *KasA*.

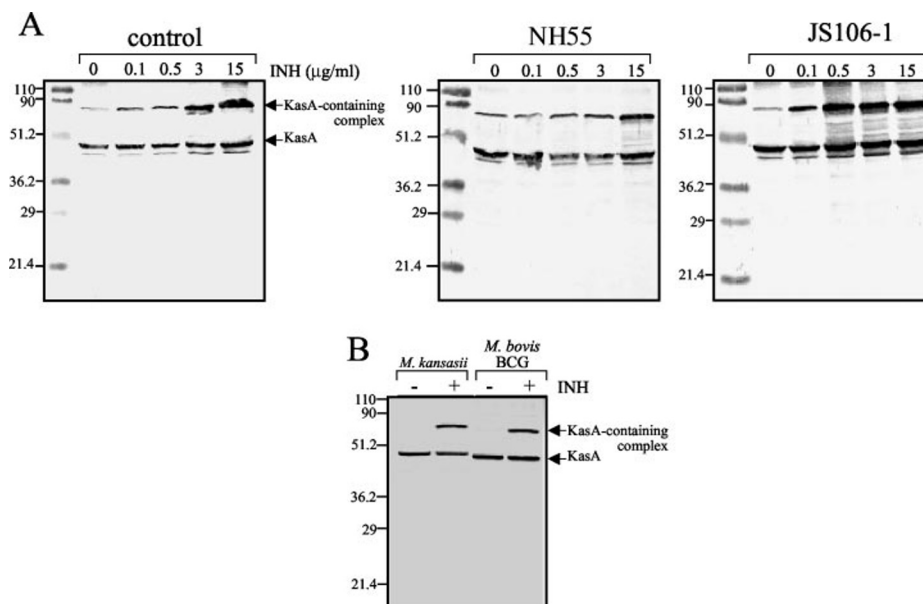


TABLE III
MICs ($\mu\text{g/ml}$) of various antimycobacterial inhibitors against *M. smegmatis* overproducing *inhA* determined on 7H11 supplemented with OADC

Inhibitor	mc ² 155 (pMV261)	mc ² 155 (pMV261:: <i>inhA</i>)
INH	5	400-600
ETH	20	400
TRC	5	20-40
DZB	100	200
CER	0.5	0.5
ISO	600	700

treated with 250 $\mu\text{g/ml}$ of DZB revealed the presence of the *KasA*-containing complex (Fig. 3A).

To investigate whether the ability to induce the *KasA*-containing complex was specific for drugs that target *InhA*, we examined the ability of CER to induce the formation of the complex. CER is a drug that is produced naturally by *Cephalosporium caerulens* and exhibits potent activity against a wide variety of yeast, fungi, and bacteria (53) through inhibition of both FAS-I and FAS-II (43, 54). Several studies (34, 43, 55, 56) have demonstrated that it specifically targets β -ketoacyl-ACP synthases by covalently attaching to the active site cysteine. CER is known to inhibit *KasA* activity *in vitro* (34, 56). However, this drug was not able to inhibit *InhA* activity *in vitro* (Table IV), and the MIC of CER for a strain overproducing *InhA* was found to be comparable with that for the control strain (Table III), confirming that *InhA* is not inhibited by CER. As shown in Fig. 3A, cells treated with CER do not induce the *KasA*-containing complex, even at a concentration exceeding 20-fold the MIC value.

To extend these observations to other drugs, we treated *M. bovis* BCG with ISO, which is known to inhibit the Δ^9 -desaturase DesA3,³ and thiolactomycin (TLM), a drug produced by *Nocardia* sp. that has been shown to inhibit mycobacterial growth (41) by targeting both β -ketoacyl-ACP synthases *KasA* and *KasB* (27, 56). *M. bovis* BCG was chosen because it is more sensitive to ISO and TLM than *M. smegmatis*, the MIC value of ISO for *M. smegmatis* is 600 $\mu\text{g/ml}$ (Table III), and it corresponds to 0.5 $\mu\text{g/ml}$ for *M. bovis* BCG (57). Fig. 3B shows that treatment

of *M. bovis* BCG with high concentrations of ISO or TLM does not lead to the induction of the *KasA*-containing complex.

In summary, from the drugs used in these studies, only those capable of inhibiting *InhA* induce the formation of the *KasA*-containing complex. Therefore, induction of this complex is a consequence of *InhA* inhibition.

In Vitro InhA and KasA Assays—In light of the discrepant reports regarding the primary target of INH, we tested the inhibitory action of INH in *in vitro* assays using purified *InhA* and *KasA*. As shown in Table IV, KatG-activated INH efficiently inhibited *InhA* activity, consistent with the findings of Johnsson *et al.* (21). Similarly, DZB was also able to inhibit *InhA* activity, although to a lesser extent than INH. The IC₅₀ was greater than 1000 μM , a value consistent with the high MIC of this compound against *M. smegmatis*. TRC, a widely used biocide (58), was found to be very active against *InhA* with an IC₅₀ <1 μM , which is in agreement with previous findings demonstrating that TRC inhibits mycobacterial *InhA*, both *in vitro* and *in vivo* (59, 60).

However, activated INH did not inhibit the β -ketoacyl-AcpM synthase activity of *KasA*, ruling out the possibility of *KasA* being a target for INH. Similarly, DZB and TRC did not inhibit *KasA* activity (Table IV), whereas CER was found to be highly active against *KasA* but not against *InhA* (Table IV).

Genetic Evidence That Inhibition of InhA Leads to the Induction of the KasA-containing Complex—Vilch ze *et al.* (23) have isolated a thermosensitive (Ts) *inhA* mutant of *M. smegmatis* (mc²2359) that is resistant to INH, and they demonstrated that thermal inactivation of *InhA* results in mycolic acid biosynthesis inhibition. Two novel Ts mutants resistant to TRC (mc²2571 and mc²2572) have been characterized recently, and the mutations were found to map within the *inhA* gene.⁴ We used these three Ts strains to decipher the mechanism of induction of the *KasA*-containing complex. As expected, in the control strain mc²155, complex formation was barely detectable, regardless of the growth temperature (Fig. 4A, upper panel). However, all three Ts mutants grown at 42 $^{\circ}\text{C}$ produced the complex, which confirms that thermal inactivation of *InhA* leads to the formation of the *KasA*-containing complex. However, these strains also produced the complex at 30 $^{\circ}\text{C}$, albeit at a lower level, suggesting that even at the permissive temperature, some *InhA* inhibition occurs.

³ B. Phetsuksiri, M. Jackson, D. C. Crick, M. R. McNeil, V. Vina, G. S. Besra, A. R. Baulard, R. A. Slayden, A. E. Sampson, C. E. Barry, III, and P. S. Brennan, unpublished results.

⁴ H. R. Morbidoni and W. R. Jacobs, Jr., unpublished results.

FIG. 3. Induction of the KasA-containing complex by treatment of *M. smegmatis* with *InhA*-inhibitory drugs. A, *M. smegmatis* carrying either pMV261 or pMV261::*ethA* was treated with increasing concentrations of ETH (left panels). *M. smegmatis* cultures were treated with either 250 μ g/ml DZB or with increasing concentrations of CER (right panels). B, *M. bovis* BCG was treated with INH, ISO, or TLM. Cultures were incubated with the drugs for 6 h for *M. smegmatis* or for 24 h for *M. bovis* BCG. Cells were then harvested, resuspended in PBS, and disrupted by sonication. Equal amounts of crude lysates (20 μ g) were then loaded onto a 12% acrylamide gel, subjected to electrophoresis, and transferred onto a membrane for immunoblot analysis using the rat anti-KasA antibodies. Drug concentrations are expressed in μ g/ml.

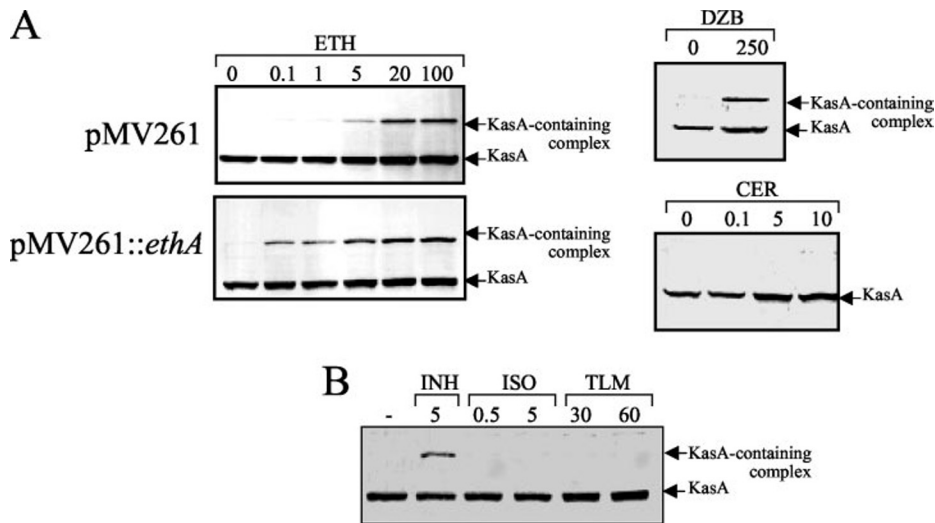


TABLE IV
Inhibitory effect of various antitubercular drugs against *M. tuberculosis* *InhA* and *KasA*

NI denotes that no inhibition was observed in any of the assays.

	IC ₅₀		KasA
	InhA	KasA	
		μ M	
INH	7.3		NI
DZB	>1000		NI
TRC	<1		NI
ISO	NI		NI
CER	NI		0.67

The expression level of *InhA* was similar in the control strain mc²155 and in the three Ts strains independent of the growth temperature (Fig. 4A, lower panel). This rules out an effect of differential expression of *inhA* in these strains and supports the notion that mutations conferring a Ts phenotype by affecting the enzymatic activity of *InhA* are responsible for the KasA-containing complex formation. These observations also imply that resistance to INH or to TRC in these strains cannot be ascribed to increased levels of *InhA*, as reported earlier for *Staphylococcus aureus* strains resistant to TRC (61). In addition, no immunoreactive band corresponding to the KasA-containing complex could be detected with the anti-*InhA* antibodies, suggesting that *InhA* is not part of this complex.

The presence of the complex was examined in several revertants of mc²2359 containing mutations within the *inhA* gene (23). *M. smegmatis* mc²2354 from which mc²2359 (V238F) originated as a control strain. Revertant mc²2367, containing a F238V mutation conferring susceptibility to INH, is therefore genetically identical to strain mc²2354. As expected, no significant induction of the complex was detected by immunoblot analysis in *M. smegmatis* mc²2367 (Fig. 4B). Similarly, no significant induction was observed in the INH-sensitive revertant mc²2365, containing an F238C mutation. More surprising was the presence of the KasA-containing complex in the INH-resistant revertant mc²2366 containing a G102C mutation. Vilchèze *et al.* (23) showed that residue 238 is located in the NADH binding pocket of *InhA* and that the V238F mutation likely reduces the binding of the INH-NAD adduct to mediate resistance. It is therefore tempting to speculate that the intramolecular suppressor G102C mutation, found in mc²2366, may also affect NADH binding of *InhA* and *InhA* activity, although this remains to be further investigated.

DISCUSSION

Since the discovery of its potent bactericidal activity against *M. tuberculosis* (2), INH has been quickly adopted as a front-line antitubercular drug. However, until recently, the mechanism of INH activity remained unclear. Field strains of *M. tuberculosis* were found to naturally contain mutations in *oxyR*, a gene encoding a key regulator of the peroxide stress response, which may account for the exceptionally high sensitivity of *M. tuberculosis* to INH (62). Studies conducted over the last decade have led to the isolation and characterization of the molecular target of INH in mycobacteria (44). Through genetic and biochemical approaches two different genes, *inhA* and *kasA*, have been postulated to encode the primary target of INH (12, 13). Due to conflicting reports, the mode of action of INH and the structurally related drug ETH has recently been re-examined. A recent study (28) showed that overexpression of *inhA*, but not of *kasA*, confers resistance to INH and ETH in *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis*, and it was therefore concluded that *InhA* represents the primary target of action of INH and ETH in all three species. On the other hand, *KasA* was found to be covalently associated with AcpM and a low molecular weight molecule, which was postulated to be a reactive intermediate of INH, to form an 80-kDa complex in *M. tuberculosis*, leading to the proposal that *KasA* represents the primary target of INH (13). Although the conclusions of this latter study were based on the identification of proteins acquiring the label from [¹⁴C]INH, direct proof of the labeling of the AcpM-*KasA* complex was not shown. In addition, no conclusive evidence was provided to demonstrate that INH inhibits *KasA* activity. In light of these conflicting conclusions, we re-examined the possibility of *KasA* and/or *InhA* being the primary target(s) of INH.

By using anti-*KasA* antibodies, we found that, in addition to being part of the 80-kDa complex, *KasA* was present as a "free" enzyme in INH-treated *M. smegmatis* at levels similar to those found in non-treated cells. Therefore, INH treatment does not result in significant *KasA* sequestering, which can thus not be used as evidence for *KasA* to be a major target for INH.

It has been criticized that *M. smegmatis* does not constitute a suitable host to isolate drug targets and may thus not be biologically relevant as a surrogate (63). We therefore extended the *kasA* expression analyses to different mycobacterial species treated with INH, including strains of the *M. tuberculosis* complex. Unbound *KasA* as well as the *KasA*-containing complex were present in all species analyzed, including *M. kansasii*, *M. bovis* BCG, and *M. tuberculosis*, suggesting that with respect to the effect of INH on *KasA* steady state levels and

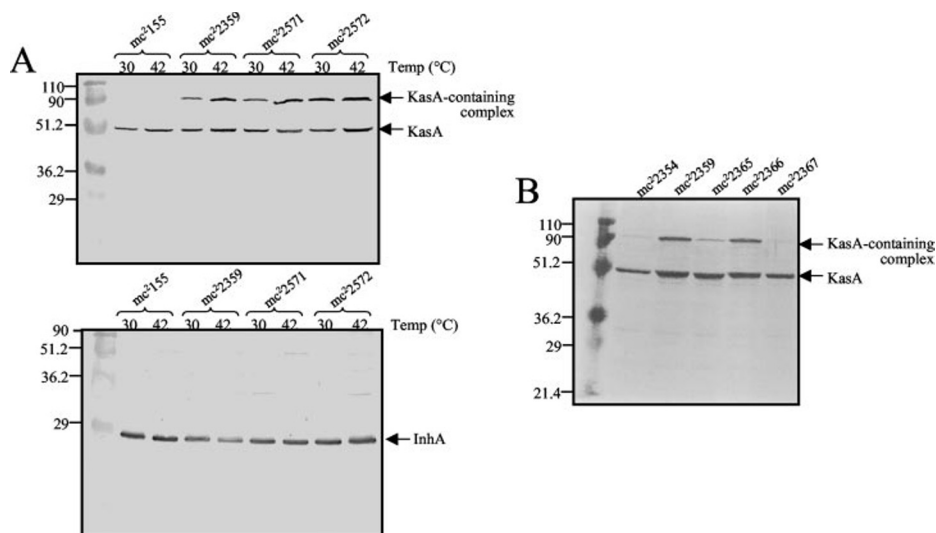


FIG. 4. Induction of the KasA-containing complex in *InhA* thermosensitive mutants (*inhA*(Ts)) and *inhA*(Ts) revertants. A, Ts mutants resistant either to INH (mc²2359) or to TRC (mc²2571 and mc²2572) were grown either at the permissive temperature (30 °C) or at the non-permissive temperature by shifting the temperature to 42 °C for 3 h. Cells were then harvested, resuspended in PBS, and disrupted by sonication. Equal amounts of crude lysates (25 μ g) were then loaded onto a 12% acrylamide gel, subjected to electrophoresis, and transferred onto a membrane for immunoblot analysis using rat anti-KasA antibodies (upper panel) or rabbit anti-*InhA* antibodies raised against *M. tuberculosis* *InhA* (lower panel). B, various *inhA*(Ts) revertants (mc²2365, mc²2366, and mc²2367) of the parental strain mc²2359 were analyzed by immunoblotting using the rat anti-KasA antibodies. Cells were grown at the permissive temperature (30 °C), harvested, resuspended in PBS, and disrupted by sonication. Equal amounts of crude lysates (25 μ g) were then loaded onto a 12% acrylamide gel, subjected to electrophoresis, and transferred onto a membrane for immunoblot analysis. Strain mc²2354 was used as a negative control.

complex formation, *M. smegmatis* behaves similarly to the other mycobacterial species.

We have recently developed a KasA *in vitro* assay using AcpM and C₁₆-AcpM and have shown that KasA expresses β -ketoacyl-AcpM synthase activity (34). By using this assay, we measured the condensation activity of KasA in the presence of various drugs. Activated INH did not inhibit KasA activity *in vitro*, whereas CER, a known inhibitor of β -ketoacyl-ACP synthases (34, 64), inhibited the activity very efficiently. In contrast, and as reported by others (65), KatG-activated INH strongly inhibited *InhA* activity. Thus, both the *in vivo* and the *in vitro* results demonstrate that INH does not target KasA.

In contrast to the report of Mdluli *et al.* (13), we were unable to detect the presence of radiolabeled INH associated with the KasA-containing complex by autoradiography.² Furthermore, our study reveals that not only INH, but also activated ETH and DZB, induces the formation of the KasA-containing complex. In addition, treatment of *M. smegmatis* with TRC, another *InhA* inhibitor, also revealed the presence of the KasA-containing complex.⁴ The induction of the complex was also found in Ts mutants of *M. smegmatis* containing amino acid substitutions in *InhA*. Thermal inactivation of *InhA* *in vivo* in three different *inhA*(Ts) strains at 42 °C induced the complex without any drug treatment. The presence of the complex found in these mutants even at the permissive temperature suggests that, even at 30 °C, *InhA* activity is somehow altered. This is consistent with *in vitro* studies performed with the purified *InhA*V238F from *M. tuberculosis* that showed complete inactivation at 42 °C and a significant reduction of the catalytic activity at a lower temperature (23). Overall, the use of *InhA*-inhibitory drugs combined with the results from the *inhA*(Ts) strains indicate that inhibition of *InhA* activity leads to the induction of the KasA-containing complex. Altogether, these results indicate that the complex does not include INH, which is supported by the fact that it is produced, albeit at low levels, even in non-treated mycobacteria and is also produced constitutively in *inhA*(Ts) strains.

Because all the *InhA*-inhibitory drugs tested in this work induce the formation of the KasA-containing complex, even

though they are structurally unrelated to each other, we conclude that it is very unlikely that they all covalently bind to AcpM and/or KasA. Moreover, neither DZB nor TRC require activation, but both trigger the formation of the KasA-containing complex, indicating that there is no involvement of reactive intermediates in linking AcpM to KasA, as was suggested for INH (13). Thus, the identity of the different constituents of the complex needs to be re-evaluated. Anti-AcpM antibodies are able to recognize the complex in TRC-treated cells, indicating that, as reported by others (13), AcpM is covalently bound to the complex.⁴ However, anti-*InhA* antibodies did not recognize the complex. Purification of the complex will be required to determine its precise nature. On the basis of the high sensitivity of the KasA-containing complex to alkaline pH (13),⁵ it is tempting to hypothesize that the AcpM phosphopantetheine prosthetic group is directly involved in the covalent linking of AcpM and KasA. The phosphodiester group that links the prosthetic group to Ser-41 of AcpM⁵ is the most chemically sensitive bond in the protein. This bond is alkali-sensitive; thus, at high pH the phosphopantetheine group is removed, converting the Ser-41 to a dehydroalanine residue (66).

Our results also show that the complex is synthesized in small amounts in non-treated cells, suggesting that it may play a functional role in mycobacterial physiology, although no function has been assigned to this complex yet. Structural studies will help us to understand the putative role of the complex *in vivo* and its relationship to the regulation of mycolic acid biosynthesis. Alternatively, low levels of the complex in non-treated cells may represent a marker of cell lysis within the culture, but this remains to be investigated.

Although it is now clear that inhibition of *InhA* activity either by drugs, such as activated INH, or by amino acid substitutions results in the induction of the KasA-containing complex and in the inhibition of mycolic acid synthesis, many questions remain to be answered regarding the events that lead to mycobacterial lysis following INH treatment. It remains

⁵ L. Kremer *et al.*, unpublished results.

to be established whether the accumulation of the *KasA*-containing complex participates in cell death or whether the accumulation of the FAS-I end product is sufficient to induce lysis. Alternatively, it is conceivable that the lysis of the mycobacteria is induced by alterations of the NADH/NAD ratio within the cells (67). It will also be important to know whether inhibition of any enzyme of FAS-II system is sufficient for cell lysis or whether it is specific for *InhA*. The failure of *KasA* inhibition to induce the complex might suggest that, whereas inactivation of *KasA* is bacteriostatic, it may not be bactericidal. Knowledge of the precise events conducting to cell lysis following INH treatment should result in the development of improved drugs to kill *M. tuberculosis* as well as in the development of drugs that synergize with INH or overcome INH resistance mechanisms.

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