**Action Mechanism of Antitubercular Isoniazid**

**ACTIVATION BY MYCOBACTERIUM TUBERCULOSIS** KatG, ISOLATION, AND CHARACTERIZATION OF InhA INHIBITOR

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**Activation of the antitubercular isoniazid (INH) by the Mycobacterium tuberculosis KatG produces an inhibitor for enoyl reductase (InhA). The mechanism for INH activation remains poorly understood, and the inhibitor has never been isolated. We have purified the InhA-inhibitor complex generated in the M. tuberculosis KatG-catalyzed INH activation. The complex exhibited a 278-nm absorption peak and a shoulder around 326 nm with a characteristic $A_{278}/A_{326}$ ratio of 0.16. The complex was devoid of enoyl reductase activity. The inhibitor noncovalently binds to InhA with a $K_d < 0.4$ nm and can be dissociated from denatured InhA for chromatographic isolation. The free inhibitor showed absorption peaks at 326 ($\epsilon_{326} = 6900 \text{ M}^{-1} \text{ cm}^{-1}$) and 260 nm ($\epsilon_{260} = 27000 \text{ M}^{-1} \text{ cm}^{-1}$). The inactive complex can be reconstituted from InhA and the isolated inhibitor. The InhA inhibitor from the KatG-catalyzed INH activation was identical to that from a slow, KatG-independent, Mn$^{2+}$-mediated reaction based on high pressure liquid chromatography analysis and absorption and mass spectral characteristics. By monitoring the formation of the InhA-inhibitor complex, we have found that manganese is not essential to the INH activation by $M. \text{tuberculosis} \text{ KatG}$. Furthermore, the formation of the InhA inhibitor in the KatG reaction was independent of InhA.

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Tuberculosis due to Mycobacterium tuberculosis infection is the leading cause of death worldwide among known infectious diseases. A sizeable increase of tuberculosis cases in the United States since 1985 (1) is followed by a decrease in more recent years. However, $13\%$ of recent cases involve $M. \text{tuberculosis}$ strains that are resistant to one or more frontline antitubercular drugs such as isoniazid (isonicotinic acid hydrazide, INH), rifampicin, and streptomycin (2–4).

INH has been the cornerstone in tuberculosis chemotherapy for almost half a century since its discovery as a potent antituberculosis drug in 1952 (5–7). INH is a prodrug, and its antituberculosis function requires activation by KatG, an enzyme with dual activities of catalase and peroxidase. The KatG-mediated activation of INH in $M. \text{tuberculosis}$ is essential to the antitubercular function of the activated INH.

Clinical $M. \text{tuberculosis}$ isolates resistant to INH were subsequently revealed to have various alterations in the katG gene (10–12). INH activation leads to inhibition of the synthesis of mycocid acid, a long chain fatty acid-containing component of the mycobacterial cell wall (13, 14). Two enzymes involved in the elongation cycle of the fatty acid biosynthesis, namely an enoyl-acyl carrier protein reductase (InhA) (15, 16) and $\beta$-ketooacyl-acyl carrier protein synthase (17), are believed to be targets of the activated inhibitor(s).

Progress made thus far notwithstanding the mechanisms of INH action and resistance are still poorly understood. Purified KatG from either $M. \text{tuberculosis}$ (18) or Mycobacterium smegmatis (19) catalyzes the in vitro inactivation of INH by INH in the presence of NADH and Mn$^{2+}$. However, the molecular nature for the INH activation by KatG and the functional role of Mn$^{2+}$ remain unclear. InhA is also inactivated in a slow nonenzymatic, Mn$^{2+}$-dependent activation of INH. The crystal structure of the resulting INhA-inhibitor complex has been determined, which shows that the bound inhibitor is an isonicotinic acyl NADH (20). It is, however, uncertain whether or not the inhibitor generated by this nonenzymatic activation is identical to that formed in the KatG-dependent process. Moreover, the inhibitor derived from INH by either the nonenzymatic or the KatG-dependent activation has never been isolated, and no simple method has been developed for the detection and quantification of the inhibitor. Consequently, biochemical or biophysical characterizations of the nature and consequences of the inhibitor binding by InhA have been greatly hindered by these limitations.

We are interested in the mechanisms of the INH action and resistance. This work was carried out to isolate for the first time the InhA inhibitors generated by the nonenzymatic and the $M. \text{tuberculosis}$ KatG-dependent processes and to characterize the free inhibitors and the InhA-inhibitor complexes. Evidence is also presented to show that neither Mn$^{2+}$ nor InhA is essential to the $M. \text{tuberculosis}$ KatG-mediated activation of INH.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Coenzyme A (CoA), INH, NADH, NAD$^+$, $\beta$-butyryl hydroperoxide, and H$_2$O$_2$ (30% solution) were obtained from Sigma. Octenol acid, 2,4,6-trimethylpyridine, and ethyl chloroformate were purchased from Aldrich. Escherichia coli strain UM262, a katG-deficient strain, was kindly provided by Dr. Peter C. Loewen (University of Manitoba) (21). All phosphate (Pi) buffers were at pH 7.0 and consisted of phosphates at mole fractions of 0.39 sodium monobasic and 0.61 potassium dibasic.

**Cloning**—The inhA gene (15) was cloned by the polymerase chain reaction method using $M. \text{tuberculosis}$ H37Rv DNA and primers 5′-GGAATTCATGACAGGACTGCTGGACG-3′ and 5′-TCTAGAGCAATTTGGTTGTCG-3′. The polymerase chain reaction product was cloned into pKK223–3 (Amersham Pharmacia Biotech) at the EcoRI and Smal sites to obtain the recombinant plasmid pDNA. $M. \text{tuberculosis}$ katG (9) was similarly cloned using primers 5′-GGAATTCATGACAGGACTGCTGGACG-3′ and 5′-GGATTCTCGGCACTCGCTGTC-3′.
The polymerase chain reaction product was first cloned into pKK223-3 at the EcoRI site to generate the pKKAG1 plasmid. However, the clone so obtained did not express KatG efficiently. Consequently, the katG was excised with EcoRI from pKKAG1 and inserted into the EcoRI site of a modified pET20b. The modified pET20b has a deletion so that the CATGAT of pET20b was blunt-ended at the EcoRI site of the EcoRI site. The resulting construct, pKKAG2, can be efficiently expressed in E. coli BL21 to produce a recombinant KatG with three extra amino acid residues, Met-Asn-Ser, fused to the initial N-terminal. Met residue of the wild type KatG. In or for the recombinant KatG to be expressed in E. coli UM262, the XbaI-HindIII fragment containing the katG of pKKAG2 was cloned into a 2.4-kb HindIII-BamHI fragment of the Smal and HindIII sites of pKK223-3 to get pKAG3. The recombinant KatG obtained from pKAG3-transformed E. coli UM262 cells was used throughout this study.

**Purification of Enzymes**—To obtain InhA, E. coli JM109 harboring pINA was first cultured in 6 flasks, each containing 1 liter of Luria-Bertani (LB) medium with 100 mg/l ampicillin, at 37 °C to reach about 1.0 A590, and then incubated for 8 h with 50 mg/l isopropyl-β-D-thiogalactopyranoside. To 30 g of wet cell paste, 200 ml of 25 mM P50 was added, and the suspension was sonicated for 20 min. The lysate obtained by centrifugation of the lysed cells at 8000 × g for 15 min was loaded on a 2.5 × 10-cm DEAE-Sepharose column equilibrated with 25 mM P50. The column was eluted with 50 ml of 35 mM P50 followed by 150 ml of 50 mM P50. InhA was separated by polyacrylamide gel electrophoresis pattern, by elution with water. The identity of InhA was confirmed by N-terminal amino acid sequencing.

For the expression of KatG, pKAG3-transformed E. coli UM262 cells were first grown overnight on an LB plate and were resuspended in 60 ml of water. To 0.8 ml of the InhA sample so obtained, the sample was added on a 1 × 10-cm phenyl-Sepharose column preequilibrated with 50 mM P50. The column was washed first with 50 ml of 40 mM P50, 50 ml of a linear gradient from 40–50 mM P50, and lastly 50 mM P50. InhA was recovered under a single major peak, and the constituent fractions were pooled. Four grams of ammonium sulfate were added to 100 ml of the InhA sample so obtained. The sample was loaded on a 1 × 10-cm phenyl-Sepharose column preequilibrated with 0.5 mM P50. The column was eluted first with 50 ml of 0.5 mM P50 followed by 10 ml of 50 mM P50. InhA was recovered with > 95% purity based on SDS-polyacrylamide gel electrophoresis pattern, by elution with water. The quality of the octenoyl CoA so obtained was essentially the same as that reported earlier (18).

**KatG-mediated INH Activation and InhA-Inhibitor Complex**—The catalase activity in cells from 1 ml of culture reached about 0.5 followed throughout the growth, and cells were harvested when the A590 was first grown overnight on an LB plate and were resuspended in 60 ml of water. The KatG pool was diluted with water to a conductivity range of 120–230 mΩ cm. To obtain InhA, JM109 harboring pKAG3 was transformed into E. coli UM262. The XbaI-HindIII fragment containing the katG of pKAG3 was cloned into a 2.4-kb HindIII-BamHI fragment of the Smal and HindIII sites of pKK223-3 to get pKAG3. The recombinant KatG obtained from pKAG3-transformed E. coli UM262 cells was used throughout this study.

**Other Measurements**—Protein concentrations were determined by the method of Lowry et al. (25) using bovine serum albumin as a standard.

**INH Activation**—The formation of InhA-inhibitor complex was used to assess the activation of INH by KatG. A 1-ml 50 mM P50 solution containing 2 mM NADH, 1 mM INH, 0.5 mM KatG, and 60 μM InhA was incubated for 150 min at 23 °C under aerobic condition. The reaction solution was loaded on a 0 × 40-cm Sephadex G-25 column preequilibrated with 0.4 M P50, and the gel filtration was monitored for the pool of the protein fractions. The UV-visible spectrum of the protein peak was measured. In comparison with the absorption spectrum of InhA, increases in absorbance at 326 nm and the A280/A260 ratio were used as indicators for the InhA-inhibitor complex formation.

**Isolation of INH Inhibitor**—A 10-ml 50 mM P50 solution containing 1.0 μM INH, 2.4 mM NADH, and 5 μM KatG was incubated under aerobic condition at room temperature for 150 min. The reaction solution was then loaded 2 ml/rill on a Sephadex G-25 column (1 × 40 cm) preequilibrated with 10 mM P50 and eluted with the same buffer. The protein peak containing the InhA-inhibitor complex and a trace amount of KatG was loaded on a 1 × 10-cm DEAE-Sepharose column preequilibrated with 10 mM P50. The column was first washed with 10 mM P50 until no absorbance in the range of 250 to 650 nm was present in the filtrate. The InhA-inhibitor complex, freed from KatG, was then recovered by elution with 50 ml of a linear gradient from 50–200 mM P50. The sample was heated in boiling water for 40 s. Denatured protein was separated from the inhibitor by centrifugation of the sample through Microcon 3 with a molecular weight cut-off of 3000 (Millipore). The inhibitor was concentrated and desalted to pH 7.0. The inhibitor was used for the preparation of the InhA-inhibitor complex sample to 8 μl, and the sample was loaded on a Sephadex G-25 column preequilibrated and eluted with 8 μl. Two peaks in the A280 profile of the gel filtration were observed. The first peak was denatured InhA, and the second one was the released inhibitor. The concentrated and urea-free inhibitor could be obtained by DEAE-Sepharose chromatography.

**Assay and Time Course of Inhibitor Formation**—The reactions were performed in triplicate in 4 ml of 50 mM P50 containing 0.5 μM KatG, 1 μM INH, and 0.65 μM Mn2+ at 37 °C for 4 h. Aliquots (30 μl) of the reaction were subjected to HPLC analysis using Waters HPLC system with a µBondapak C18 HPLC column. Ionotropic and INH had retention times of 4.50 and 5.56 min, respectively, when the column was eluted isocratically with 22% acetonitrile aqueous solution containing 40 mM ammonium acetate (pH 7.0). A280 was used for the detection.

**INH Oxidation**—The oxidation of INH to isoniazid acid was carried out in 1 ml of 50 mM P50 containing 100 μM INH and 0–1 μM Mn2+ at 37 °C for 4 h. Aliquots (30 μl) of the reaction were subjected to HPLC analysis using Waters HPLC system with a µBondapak C18 HPLC column. Isonicotinic acid and INH had retention times of 4.50 and 5.56 min, respectively, when the column was eluted isocratically with 22% acetonitrile aqueous solution containing 40 mM ammonium acetate (pH 7.0). A280 was used for the detection.

**Analyzes of INH Inhibitor**—Inhibitor samples from the KatG- and Mn2+-mediated INH activation were each analyzed by HPLC using the same system as described above. The column was eluted isocratically with 10% methanol aqueous solution at a rate of 0.6 ml/min, and A280 was monitored. Both inhibitor samples were also analyzed by mass spectrometry. For each purified inhibitor, 10 μl of the inhibitor sample with A280 of about 0.5 was injected into a Hewlett Packard 1100 MSD mass spectrometer and carried by a mixture of 2% formic acid, 98% acetonitrile containing 0.1% trifluoroacetic acid at a rate of 0.5 ml/min. The spectra were obtained by scanning the m/e range of 120–1000 after ionization with atmospheric pressure electrospray and fragmentation voltage of 200 V. The spectra were analyzed in the negative mode.
KatG-mediated INH Activation and InhA-Inhibitor Complex

**RESULTS**

**Recombinant KatG**—About 20 mg of homogeneous KatG were obtained from 6 liters of culture without isopropyl-β-D-thiogalactopyranoside induction. The purified enzyme had an A_{326}/A_{280} ratio of 0.70 and, based on protein content, an extinction coefficient ε_{326} of 2 mg/cm°·cm⁻¹ or 12.5 mmol°·cm⁻¹ (for KatG dimer). In the determination of heme content by the pyridine hemochrome assay, the ratio of A_{326} of the purified KatG over A_{414} developed in the assay was found to be 0.60 ± 0.04 yielding an ε_{418} of 115 ± 8 mmol°·cm⁻¹ for the heme. These measurements gave 1.9 ± 0.1 mol heme/dimer. The KatG possesses catalase and peroxidase activities similar to those of KatG from *E. coli* expressing the wild type katG gene (28). The KatG catalase activity displayed a K_m of 2.4 mm for H_2O_2 and a k_cat of 1.3 × 10^6 s^-1, yielding a k_cat/K_m of 5.4 × 10^5 M^-1 s^-1. The specific peroxidase activity of KatG was 0.17 unit/mg under the conditions described under "Experimental Procedures."

**INH Activation by KatG**—A limiting amount of KatG was incubated with InhA, INH, and NADH for 150 min, and the proteins in the reaction were then separated from free small molecules by gel filtration. The UV-visible spectrum of the protein sample so obtained displayed substantial absorption in the range of 315–350 nm and a smaller absorption peak at 408 nm due to the KatG-bound heme (Fig. 1, *dashed curve*). The 315–350 nm absorption was absent in control samples obtained the same way but without the addition of KatG (Fig. 1, *dotted curve*). The extra absorption in the 315–350 nm range was, as will be shown later, associated with an InhA inhibitor formed in this INH activation. Therefore, A_{326} provides a convenient indicator for following the INH activation and the inhibitor formation. The inhibitor was apparently bound by InhA very tightly. Repeated gel filtrations easily removed other small molecules such as NADH but not the inhibitor from the protein samples.

**InhA-Inhibitor Complex**—A series of experiments were carried out to establish the formation of an InhA-inhibitor complex and to characterize the absorption spectral properties of the free and bound inhibitor. First, the protein fraction from the KatG reaction was subjected to DEAE-Sepharose chromatography to separate InhA from KatG. The InhA so obtained showed an absorption peak at 278 nm and a pronounced shoulder around 326 nm (Fig. 2A, *dashed curve*), giving an A_{326}/A_{278} ratio of 0.16 ± 0.03. Second, the inhibitor was released from InhA by heat treatment and was isolated. The free inhibitor exhibited absorption peaks at 260 and 326 nm with an A_{326}/A_{260} ratio of 0.255 (Fig. 2A, *solid curve*). The bound inhibitor showed no significant absorption at ≥ 350 nm, whereas the free inhibitor had a pronounced absorption at 350 nm. Apparently, there was a blue shift of 326-nm peak of the inhibitor upon binding to InhA. The inhibitor was also obtained after dissociation from InhA by 8 M urea and purified by DEAE-Sepharose chromatography. The inhibitor so isolated showed an absorption spectrum identical to that of the free inhibitor obtained by the heat treatment.

Reconstitution of the complex was performed using InhA and the isolated inhibitor. Mixing equal molar of InhA and the isolated inhibitor resulted in a spectrum that was significantly different from the sum of the spectra of the two individual constituents but identical to that of the InhA-inhibitor complex. A difference spectrum ([A_inhibitor + A_inhA] – A_inhibitor) was obtained with peaks at 259 and 353 nm and troughs at 301 and 405 nm (Fig. 2B). The trough at 301 nm and the peak at 353 nm are consistent with the blue shift of a 326-nm peak of the free inhibitor upon binding to InhA as shown in Fig. 2A. Again, the inhibitor binding by InhA was apparently very tight. In a subsequent gel filtration of the mixture, the absorption spectrum of the reconstituted complex remained unchanged.

**Inhibition of InhA Activity**—As detailed under "Experimental Procedures," the InhA-inhibitor complex was isolated from a reaction solution containing InhA, NADH, INH, and KatG. Similarly, a control InhA sample was also isolated from the same reaction solution but without KatG. The control InhA, at 3.6 μM, was similarly active in catalyzing the oxidation of NADH, with octenoyl CoA as a cosubstrate, in the absence and presence of KatG (Fig. 3A, ○ and ●, respectively). No activity was observed in the assay without octenoyl CoA (Fig. 3A, △). In comparison with InhA, the InhA-inhibitor complex was only about 3% active in the presence of NADH and octenoyl CoA (Fig. 3A, ▲). To test whether the InhA-inhibitor complex dissociated at lower concentrations in the presence of the substrates, 0.36 μM of the complex and the control InhA were tested for their activities under conditions identical to that described for Fig. 3A. The rates of NADH oxidation were decreased by 10-fold for both samples with the inhibition level of the diluted complex remaining at 97% (Fig. 3B). No apparent increase of activity in the diluted InhA-inhibitor sample was observed after 10 min of standing. Therefore, the 3% activity of the InhA-inhibitor complex samples at 3.6 and 0.36 μM was probably due to the presence of a trace amount of free InhA, and no significant dissociation was observed at 0.36 μM InhA-inhibitor complex. Conservatively, the dissociation constant of the InhA-inhibitor complex at 23 °C could be estimated to be less than 0.4 nM. Reconstituted InhA-inhibitor complex was also inactive in the enoyl reductase assay.

The degree of inhibition increased with the increase of A_{326}/A_{278} of the isolated InhA-inhibitor samples and reached nearly 100% when the A_{326}/A_{278} was around 0.16 at which the InhA must be saturated by the inhibitor. Assuming a 1:1 stoichio-
metry for the binding of inhibitor by InhA and based on spectral differences between bound and free inhibitor, the $\varepsilon_{320}$ and $\varepsilon_{260}$ of the free inhibitor can be estimated to be 6900 and 27,000 M$^{-1}$ cm$^{-1}$, respectively.

**Identity of InhA Inhibitor**—The InhA-inhibitor complex and the free inhibitor were also isolated from the Mn$^{2+}$-mediated activation of INH as described under "Experimental Procedures" and compared with that generated by KatG activation. The inhibitors or their complexes with InhA from the two activation reactions had identical absorption spectra. In the HPLC analysis, the free inhibitors from both activation reactions also had the same retention time. As shown in Fig. 4, the isolated free inhibitor samples from KatG- (A) and Mn$^{2+}$- (B) mediated INH activation were identical in their mass spectra. Both spectra show the same three dominant peaks at m/e 664.1, 769.1, and 791. The peak of m/e 769 corresponded to the (M-H$^-$) peak of isonicotinic acyl-NADH, which has a molecular mass of 770 daltons. The peak of m/e 664 can be correlated with the loss of the positively charged isonicotinic carbonyl group from the inhibitor. The peak m/e 791 corresponded to the complex of one sodium and the dibase form of the inhibitor. These spectra are fully consistent with the structure of isonicotinic acyl-NADH determined for the InhA-bound inhibitor from the nonenzymatic Mn$^{2+}$ activation of INH (20). Taken all analyses together, the KatG-generated inhibitor was apparently identical to the inhibitor, isonicotinic acyl NADH, from the Mn$^{2+}$-mediated INH activation.

**Nonrequirement of InhA for Inhibitor Production**—Two possible modes for the inhibitor production were considered. First, KatG itself can catalyze the formation of the inhibitor, which then interacts with InhA. Second, InhA is involved in the addition to KatG in the inhibitor-producing reaction. To distinguish these two possibilities, INH was first incubated with NADH and KatG for 150 min in the presence and absence of
InhA. The reaction mixture without InhA was freed from KatG, and InhA was added to the same level as that in the sample that contained InhA during the INH activation. After standing at room temperature for 10 min, both samples were twice passed through Sephadex G-25 columns, and the formation of the InhA-inhibitor complex was quantified by absorption measurements. We found that the KatG activation of INH produced the inhibitor at about the same level in the presence and absence of InhA.

Mn$^{2+}$ Is Not Essential for INH Activation by KatG—Manganese ion has been reported to be essential to the INH activation by the M. smegmatis KatG (19), but its requirement was not tested for the M. tuberculosis KatG. In this work, the time courses of the InhA inhibitor formation in reaction solutions containing M. tuberculosis KatG, NADH, and INH without and with the addition of 1 mM Mn$^{2+}$ were compared. The assay for the inhibitor developed for such measurements was sufficiently sensitive to detect at least as low as 1 mM inhibitor. As shown in Fig. 5, a time-dependent formation of the InhA inhibitor was observed over 180 min for the sample containing both KatG and 1 mM Mn$^{2+}$ for INH activation. Importantly, the omission of Mn$^{2+}$ resulted in only about a 30% decrease in the amounts of inhibitor formed over the same period. As a control, activation of INH by 1 mM Mn$^{2+}$ but without KatG generated very low levels of the inhibitor. These results indicated that Mn$^{2+}$ enhanced the efficiency of but was not essential to the activation of INH by the M. tuberculosis KatG.

A decisive conclusion would require the proof of a lack of a significant amount of Mn$^{2+}$ in the so-called Mn$^{2+}$-free reaction solution. In the absence of KatG and InhA, INH can be oxidized to isonicotinic acid (INA) in a Mn$^{2+}$-dependent reaction. This reaction provides a test of the levels of Mn$^{2+}$ in our reaction solutions. INH was incubated in 50 mM P$_i$ buffer with 0–1 mM exogenously added Mn$^{2+}$ at 37 °C for 4 h under aerobic conditions, and the reaction solutions were subjected to HPLC analysis. As the Mn$^{2+}$ level increased from 0.1 to 1 mM, increasing amounts of INH were converted to INA (Fig. 6). However, INA was formed in the 50 mM P$_i$ buffer without Mn$^{2+}$ addition in an amount about 30% of that with 0.1 mM Mn$^{2+}$ added (Fig. 6). This suggested a possible maximum concentration of 0.044 µM Mn$^{2+}$ in 50 mM P$_i$. If this were the case, more efficient INA formation should be obtained using a higher concentration of the P$_i$ buffer. However, no increase in INA formation was observed when the P$_i$ concentration was raised from 50 to 200 mM (Fig. 6), thus arguing against any significant contamination of Mn$^{2+}$ in the P$_i$ buffer. Because the InhA inhibitor was formed with similar efficiencies with and without InhA during the INH activation by KatG, InhA samples could not be a source of Mn$^{2+}$ contamination. The KatG sample was also tested for Mn$^{2+}$ content. To 0.3 ml of KatG containing 0.14 mM heme, 0.3 ml of distilled 100% HNO$_3$ was added. The sample was gently heated until the precipitates formed upon the addition of HNO$_3$ disappeared. Water was then added to a total volume of 4.0 ml. This KatG sample and a 4.0-ml standard containing 10 µM MnCl$_2$ in 10% HNO$_3$ were each examined by inductively coupled plasma atomic emission at 257.61 nm for the detection of Mn$^{2+}$. Three independent series of measurements were made, and seven readings were recorded for each set of measurements. The averages were calculated after excluding the highest and lowest data from each set of measurements. The standard of 10 µM MnCl$_2$ gave an emission of 0.233 ± 0.001 in comparison with a blank for −0.005 ± 0.0005. Our KatG sample contained 10.5 µM heme at a ratio of 1.9 heme/KatG homodimer and was expected to produce an emission of 0.245 if KatG contained tightly bound Mn$^{2+}$ at a 1:1 molar ratio with the heme. Rather, the KatG sample yielded an emission of only 0.005 ± 0.009, indicating no significant content of Mn$^{2+}$.

**DISCUSSION**

An InhA-inhibitor complex can be obtained by a slow INH activation reaction that is Mn$^{2+}$-mediated but KatG-independent; the identity of the bound inhibitor as isonicotinic acyl NADH has been established from the determination of the crystal structure of such a complex (20). An InhA inhibitor can also be obtained by a rapid KatG-dependent activation of INH. However, it is not clear whether these two inhibitors are the same. Moreover, the inhibitor derived from either activation process has never been isolated in solution. In this work, procedures were developed for the isolation and quantification of the InhA-inhibitor complex and the free inhibitor using either the M. tuberculosis KatG-dependent or the Mn$^{2+}$-mediated

**FIG. 5.** INH Activation catalyzed by KatG in the absence and presence of Mn$^{2+}$. Reactions were carried out in triplicate, each in 4 ml of 50 mM P$_i$, containing 0.5 mM KatG, 1 mM NADH, and 1 mM INH without (●) or with 1 mM Mn$^{2+}$ (○). The reaction with 1 µM Mn$^{2+}$ but without KatG (▲) was carried out as a control. At the indicated times, 1 ml of the reaction solution was taken for the detection of the InhA inhibitor formed as described under “Experimental Procedures.”

**FIG. 6.** Evidences for the absence of Mn$^{2+}$ contamination in P$_i$ buffer. The oxidation of INH was carried out by shaking 1 ml of 100 µM INH in 50 mM P$_i$ containing 0–1 µM Mn$^{2+}$ at 37 °C for 4 h. Aliquots of reaction mixtures were analyzed by HPLC as described under “Experimental Procedures.” The heights of the INH (○) and INA (▲) peaks are shown as a function of the concentration of Mn$^{2+}$ added to the reaction solution. The INH and INA peaks were normalized by setting the peak of the unreacted INH and the highest INA peak at 1. Also shown are the yields of INA in samples in which no Mn$^{2+}$ was added, but the phosphate concentration was varied from 50 to 200 mM (●), thus arguing against any significant contamination of Mn$^{2+}$ in the P$_i$ buffer. Because the InhA inhibitor was formed with similar efficiencies with and without InhA during the INH activation by KatG, InhA samples could not be a source of Mn$^{2+}$ contamination. The KatG sample was also tested for Mn$^{2+}$ content. To 0.3 ml of KatG containing 0.14 mM heme, 0.3 ml of distilled 100% HNO$_3$ was added. The sample was gently heated until the precipitates formed upon the addition of HNO$_3$ disappeared. Water was then added to a total volume of 4.0 ml. This KatG sample and a 4.0-ml standard containing 10 µM MnCl$_2$ in 10% HNO$_3$ were each examined by inductively coupled plasma atomic emission at 257.61 nm for the detection of Mn$^{2+}$. Three independent series of measurements were made, and seven readings were recorded for each set of measurements. The averages were calculated after excluding the highest and lowest data from each set of measurements. The standard of 10 µM MnCl$_2$ gave an emission of 0.233 ± 0.001 in comparison with a blank for −0.005 ± 0.0005. Our KatG sample contained 10.5 µM heme at a ratio of 1.9 hemes/KatG homodimer and was expected to produce an emission of 0.245 if KatG contained tightly bound Mn$^{2+}$ at a 1:1 molar ratio with the heme. Rather, the KatG sample yielded an emission of only 0.005 ± 0.009, indicating no significant content of Mn$^{2+}$.
process for the INH activation. The InhA-inhibitor complex obtained by the KatG-dependent activation of INH was apparently identical to its counterpart derived from the Mn$^{2+}$-mediated activation with respect to absorption spectra and the lack of enoyl reductase activity. The free inhibitors obtained from these two complexes were also identical in their absorption spectra, HPLC retention times, and mass spectra. These results indicate that the same InhA inhibitor was generated by either the slow Mn$^{2+}$-mediated or the fast KatG-catalyzed INH activation.

Both the free and the InhA-bound inhibitor were sufficiently stable to allow the isolation and the subsequent characterization of the inhibitor. The complexes derived from both activation processes were indistinguishable from those reconstituted from InhA and the isolated free inhibitor with respect to absorption spectra. The binding of the inhibitor to InhA was apparently very tight with a dissociation constant estimated to be lower than 0.4 nM. The free inhibitor has absorption peaks at 260 and 326 nm (Fig. 2A). In comparison with NADH, the 326-nm peak of the inhibitor is stronger in absorptivity (ε$_{326}$ about 6900 M$^{-1}$ cm$^{-1}$) and in a shorter wavelength range than the NADH 340-nm peak (ε$_{340}$ 6200 M$^{-1}$ cm$^{-1}$). Upon binding to InhA, the inhibitor 326-nm peak was blue-shifted, but the complex still showed a pronounced shoulder around 320 nm (Fig. 2A). The characteristic absorption around 320 nm provides a very useful reporting signal for monitoring the formation and isolation of the inhibitor. The spectral changes resulting from the inhibitor binding by InhA, especially the ΔA$_{326}$ (Fig. 2B), also provide a convenient means for investigating the binding of the inhibitor by InhA. Using this method, the KatG S315T mutant frequently encountered in INH-resistant M. tuberculosis isolates has been shown to fail to catalyze the formation of the InhA inhibitor. We believe that the methodology developed in this report can also be applied to a recently identified INH target KasA (17) for further verification and investigation of the inactivation mechanism.

There are some debates about whether InhA is a primary target of activated INH in M. tuberculosis (29), in part due to the infrequency of mutation in the inhA gene in INH-resistant M. tuberculosis strains. Particularly, the S92A mutation of InhA, which confers high INH resistance in M. smegmatis (15), has not been encountered in M. tuberculosis isolates from INH-resistant patients. Our findings support InhA as a primary target of INH action in M. tuberculosis. KatG catalyzes the formation of the InhA inhibitor. The extremely high affinity of the inhibitor to InhA (K$_D$ < 0.4 nM) is consistent with the high susceptibility of M. tuberculosis to INH. There are two possibilities for the lack of the S92A InhA allele in M. tuberculosis. One is that the selection pressure is not high enough for the mutation. M. smegmatis (which does not cause tuberculosis) and M. tuberculosis are sensitive to INH with minimum inhibitory concentrations of > 5 and 0.01–0.02 μg/ml, respectively. The M. smegmatis InhA S92A mutant confers INH resistance of minimum inhibitory concentration of > 50 μg/ml, whereas most of clinical INH-resistant M. tuberculosis isolates have minimum inhibitory concentrations of 1–5 μg/ml. In clinical treatments, INH was absorbed by the gut to reach peak levels of 3–7 μg/ml in 1–2 h after a usual oral dosage of 300 mg (30). At such an in vivo level of INH, the InhA S92A mutation of M. tuberculosis would not be effectively selected. The other possibility is that, because the InhA enzymes from M. tuberculosis and M. smegmatis have 11.7% nonidentity in their amino acid sequences, the M. tuberculosis InhA S92A mutant may be distinct from the corresponding M. smegmatis mutant in remaining sensitive to the inhibitor. This latter possibility is under current investigation.

The M. smegmatis KatG has been shown to require Mn$^{2+}$ for the activation of INH for the inhibition of InhA (19), possibly by converting Mn$^{2+}$ to Mn$^{3+}$, which in turn oxidizes INH (31). We found that, although Mn$^{2+}$ enhanced the INH activation by M. tuberculosis KatG, the M. tuberculosis KatG can efficiently activate INH without exogenously added Mn$^{2+}$. We did not find any detectable amount of Mn$^{2+}$ in the P$_i$ buffer, the purified InhA, or the purified KatG used in the activation reaction. Therefore, Mn$^{2+}$ is apparently not essential to the activation of INH by M. tuberculosis KatG. The two KatG enzymes from M. tuberculosis and M. smegmatis are thus different in their modes of the INH activation. Such a difference might be related to the differential susceptibilities of M. tuberculosis and M. smegmatis to INH.

Previous in vitro experiments of INH activation all included INH in the reaction. It is not clear whether InhA is required in addition to KatG for INH activation. A similar level of the InhA inhibitor was generated in the KatG reactions with or without InhA. Therefore, the simultaneous presence of InhA and KatG is not required for the inhibitor production.

KatG used in this report had a Met-Asn-Ser tripeptide fused to the first residue Met of the wild type KatG. In comparison with the wild-type KatG, the purified modified enzyme exhibited essentially the same A$_{320}$/A$_{280}$ ratio, kinetic parameters, the ability to activate INH, and blue shift of the Soret band A$_{408}$ (32) upon INH binding (not shown). The extra peptide apparently does not significantly change the structure or function of KatG. This is in contrast with another fusion enzyme that had a Met-Glu-Val tetrapeptide fused to the second residue Pro. This latter modified KatG bound only about 0.5 heme/dimer, in comparison with 2 heme/dimer by the wild type and our modified KatG, and thus had a much lower enzyme activity (33). A constitutive expression was adopted to slowly accumulate our enzyme while the other KatG fusion was overexpressed with an inducible system. The difference in the expression strategy could lead to the difference in the incorporation of the heme cofactor.

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