Studies on the Mechanism of Action of Isoniazid and Ethionamide in the Chemotherapy of Tuberculosis

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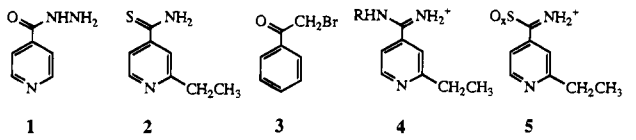
Isoniazid (**1**) has proven to be the most effective drug for the treatment and prophylaxis of tuberculosis. Ethionamide (**2**) is used concurrently for the treatment of certain drug resistant strains. Despite the importance of these two drugs, relatively



1 **2** **3** **4** **5**

little is known about their mode of action on a molecular level.[1] The study of resistant strains has shown that a single missense mutation in the *inhA* gene of *Mycobacterium tuberculosis* can confer resistance to both drugs.[2] Recently, it has been shown that the corresponding InhA protein is a NADH-specific enoyl-reductase, involved in the synthesis of fatty acids in mycobacteria.[3] Furthermore, it is known that resistance to isoniazid can also arise from mutations in or deletion of the mycobacterial *katG* gene, which encodes the catalase-peroxidase KatG.[2b,4] Studies on the mechanism of the reaction of isoniazid with KatG revealed that the oxidation of isoniazid proceeds through a number of highly reactive intermediates that are capable of acylating nucleophilic groups in proteins.[5] These observations and the report that thioacetamide *S*-oxide oxidation by rat liver microsomes leads to covalent modification of proteins[6] led us to the hypothesis that oxidation of both isoniazid and ethionamide generates electrophilic intermediates capable of inactivating InhA. We now report experiments characterizing the inactivation of InhA by the oxidation products of both drugs.

Oxidation of isoniazid by KatG in the presence of InhA leads to inactivation of InhA (Figure 1).[7] The inactivation of InhA can be inhibited by the addition of the enzyme substrate, 2-*trans*-octenoyl-ACP, indicating that an active site residue is modified.

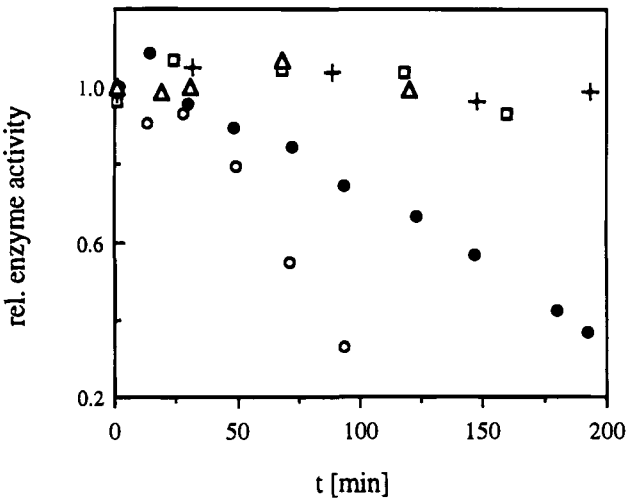


Figure 1. Inactivation of InhA (3.1 μM) during the KatG-catalyzed (1.4 μM) oxidation of isoniazid (100 μM):[7] ●, 75 μM NAD^+; ○, 70 μM NADH; □, no cofactor; △, 70 μM NADH and no KatG; +, 75 μM NAD^+ and 30 μM 2-*trans*-octenoyl-ACP.

Furthermore, inactivation of InhA could only be observed in the presence of either NADH or NAD^+,[9] suggesting that cofactor binding might induce a conformational change that renders an InhA residue more accessible to izoniazid. The enzyme lactate dehydrogenase is not inactivated under these conditions, demonstrating specificity of the drug for InhA. The oxidation of isoniazid also produces significant amounts of active oxygen species such as superoxide and hydroxyl radical.[10] However, generation of superoxide by the oxidation of xanthine with xanthine-oxidase did not lead to inactivation of InhA.[11] Neither the products of the oxidation of isoniazid, the substrates themselves, nor isonicotinoyl-ACP[13] significantly inhibit InhA.[14,15]

In order to demonstrate that InhA is covalently modified by an electrophilic drug metabolite, experiments were carried out with ^{14}C-labeled isoniazid.[16,17] After inactivation, InhA was isolated by gel filtration and was shown to contain 1.02 ± 0.1 equiv of isoniazid per molecule of InhA. Incubation of

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(1) Winder, F. G. *The Biology of Mycobacteria*; Ratledge, C., Stanford, J., Eds.; Academic Press: New York, 1982; pp 353−438.
(2) (a) Banerjee, A.; et al. *Science* **1994**, *262*, 227. (b) Heym, B.; et al. *Lancet* **1994**, *344*, 293.
(3) Dessen, A.; et al. *Science*, in press.
(4) Zhang, Y.; Heym, B.; Allen, B.; Young, D.; Cole, S. *Nature* **1992**, *13*, 591.
(5) Johnsson, K.; Schultz, P. G. *J. Am. Chem. Soc.* **1994**, *116*, 7425.
(6) Dyroff, M. C.; Neal, R. N. *Mol. Pharmacol.* **1982**, *23*, 219.
(7) Reactions were carried out in 100 mM Na_2HPO_4, pH 7.5 at 25 °C. All reactions contained 0.1 mg/mL acetylated BSA and 8% (v/v) glycerol to maintain protein stability. Aliquots were taken after defined times and analyzed for InhA activity using either 2-*trans*-octenoyl-ACP or 2-*trans*-octenoyl-CoA as substrates and NADH as a cofactor. Rates were determined by following the decrease in absorbance at 340 nm. 2-*trans*-Octenoyl-ACP was prepared via the *N*-acylimidazolide of 2-*trans*-octenoic acid according to the method of Cronan and Klages.[8] Approximately 50% of the material prepared in this way could be reduced by InhA. The activity of InhA with 2-*trans*-octenoyl-ACP as a substrate at V_{max} (pH 7.5, 25 °C) was about 6 μmol min^{-1} mg^{-1}. The activity of InhA with 2-*trans*-octenoyl-CoA as a substrate at V_{max} (pH 7.5, 25 °C) was about 7 μmol min^{-1} mg^{-1}.
(8) Cronan, J. E.; Klages, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 5440.
(9) Initial experiments with a mutant enzyme of InhA, S94A,[3] isolated from isoniazid and ethionamide resistant strains show a 1.8-fold decreased rate of inactivation by isoniazid compared to wild type enzyme at low NADH concentrations (in addition to an increased K_M for NADH[3]). Inhibition was measured under the conditions described above at equimolar concentrations of NADH and enzyme (2.3 μM).
(10) (a) Shoeb, H. A.; Bowman, B. U.; Ottolenghi, A. C.; Merola, A. J. *Antimicrob. Agents Chemother.* **1985**, *27*, 404. (b) Ito, K.; Yamamoto, K.; Kawnishi, S. *Biochemistry* **1992**, *31*, 11606.
(11) Superoxide formation was detected using ferrocytochrome C and could be inhibited by the addition of superoxide dismutase. A constant level of superoxide (4.6 μM min^{-1}) was generated using xanthine-oxidase and xanthine[12] that corresponded to about 4 times the level measured during the oxidation of isoniazid (100 μM) by KatG (1.4 μM) in the presence of 1 μM $MnCl_2$.[7]
(12) Fujimori, K.; Nakajima, H. *Biochem. Biophys. Res. Commun.* **1991**, *176*, 846.
(13) Isonicotinoyl-ACP has been proposed as a possible inhibitory species. Blanchard, J. S.; Presented at the 208th National Meeting of the American Chemical Society, Washington, DC, August 1994.
(14) The following compounds were tested as inhibitors of InhA at 200 μM concentration and a concentration of 2-*trans*-octenoyl-ACP of 40 μM: isoniazid, isonicotinic acid, isonicotinamide, 4-pyridinecarboxaldehyde, 4-pyridylcarbinol, ethionamide, 2-ethyl-4-cyanopyridine, 2-ethylisonicotinic acid, 2-ethylisonicotinamide, and 4-amidino-2-ethylpyridine. Isonicotinoyl-ACP was tested at 20 μM and isonicotinoyl-CoA at 1 mM. The measured rates were within 10% of the rate measured in the absence of the tested compounds, except isonicotinic acid, which showed an inhibition of about 20%.
(15) Isonicotinoyl-ACP and isonicotinoyl-CoA were synthesized via the *N*-acylimidazolide of isonicotinic acid and analyzed by ES-MS.
(16) Isoniazid (carbonyl-^{14}C; 59 mCi/mmol) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

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inactivated InhA in 4 M guanidinium hydrochloride in the presence of 100 mM hydroxylamine and 10 mM DTT at 37 °C for 30 min prior to gel filtration led to complete loss of the radioactive isoniazid derivative bound to InhA. Incubation with hydroxylamine in the absence of denaturant did not result in recovery of activity. The inactivated InhA was then desalted on a reversed phase column (0.1% TFA) and analyzed by ES-MS. However, the molecular weight of the isolated protein (M_r = 28 368 ± 1.6) did not show the presence of any covalently bound molecules, presumably because the covalently bound drug was lost during the workup. These data suggest that isoniazid is bound as either an ester, a thioester, or an acylimidazolide in the active site of InhA.

In order to identify the residue modified by the electrophilic drug metabolite, molecules structurally homologous to isoniazid and capable of alkylating the reactive residue were tested as irreversible inhibitors of InhA. ω-Bromoacetophenone (**3**) irreversibly inactivated InhA.[18] As with isoniazid, addition of NADH or NAD^+ to a solution of InhA and **3** greatly enhanced the inactivation of InhA. Furthermore, inactivation can be inhibited by the addition of 2-*trans*-octenoyl-CoA. Analysis of the protein by ES-MS after inactivation showed that >80% of the protein was covalently labeled with one molecule of acetophenone (M_r = 28 486 ± 2.9). These data suggest that isoniazid and ω-bromoacetophenone target the same residue in or near the active site of InhA. Digestion of the modified protein with endoproteinase LysC and subsequent peptide-mapping revealed that Cys243, which is the only Cys in InhA, is alkylated by ω-bromoacetophenone.[19] Interestingly, Cys243 shows selectivity in its reaction with electrophiles. For example, incubation of InhA with either of the cysteine specific reagents 4-vinylpyridine and methyl methanethiosulfonate in the presence of cofactor did not lead to significant inactivation of the enzyme.[20] However, addition of 1 M guanidinium hydrochloride to these reaction mixtures led to a rapid and complete loss of activity of InhA, demonstrating that Cys243 is partially shielded in the folded enzyme and that its integrity is crucial for catalytic activity.[21]

To test the hypothesis that ethionamide could also be metabolized to an electrophilic intermediate, the oxidation of ethionamide and its *S*-oxide at pH 7.5 by H_2O_2 or horseradish peroxidase (HRP) and H_2O_2 was studied. Under these conditions oxidation yields the corresponding amide and nitrile as the major products.[22] Addition of simple amines such as ethylamine or aniline as nucleophilic scavengers to the oxidation reaction led to the formation of the corresponding amidines **4** as the major products. These experiments suggest that ethionamide could be metabolized to a higher *S*-oxide **5** (possibly the corresponding sulfinate) capable of undergoing addition reactions to nucleophilic protein side chains. Moreover, initial experiments confirm that InhA can be inactivated by intermediates generated during the oxidation of ethionamide *S*-oxide.[23] However, more detailed studies require identification of the enzyme responsible for activation of ethionamide.

In conclusion, these experiments suggest for the first time, since the introduction of these drugs some 40 years ago, a molecular mechanism of action. Both drugs are prodrugs that are activated by different mycobacterial enzymes. Isoniazid is activated by KatG; ethionamide or its *S*-oxide, by an enzyme that remains to be identified. The activated forms of these drugs then react with Cys243 of InhA, leading to irreversible inactivation of InhA. An understanding of the mode of action is not only valuable for the design of improved versions of these drugs but is also essential for the understanding of the phenomena of drug resistance.

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Supplementary Material Available: Spectra (ES-MS) of modified InhA and isonicotinoyl-ACP, a graph showing the inactivation of InhA by **3**, and an elution profile of gel filtration of InhA after inactivation with ^{14}C-labeled **1** (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(17) InhA (8.2 μM) was incubated with isoniazid (100 μM, 59 mCi/mmol), $MnCl_2$ (1 μM), NAD^+ (70 μM), KatG (1.4 μM) in 100 mM Na_2HPO_4, pH 7.5 at 25 °C. After 25 min (<5% initial activity of InhA) InhA was isolated with a Superose 12 HR sizing column, and fractions were analyzed for radioactivity, protein concentration, and catalase activity.

(18) Reactions with **3** were carried out in 100 mM Na_2HPO_4, pH 7.5 at 25 °C; InhA was 1.2 μM, and **3** was 100 μM. The second-order rate constant for inactivation of InhA under these conditions was ~120 M^{-1} min^{-1}.

(19) InhA (25 μg) was inactivated by **3** and isolated by HPLC. The alkylated protein was lyophilized and taken up in 60 μL of 25 mM Tris, 1 mM EDTA, pH 8.5. LysC (0.4 μg, Boehringer Mannheim, sequencing grade) was added, and the reaction mixture was incubated for 22 h at 37 °C. The digest was analyzed by HPLC, and fragments were identified by ES-MS. Fragment 241−269 was shown to be alkylated by **3** (M_r = 3134.17 ± 0.7). Cys243 in alkylated fragment 241−269 was protected against reaction with 4-vinylpyridine, and sequencing stopped after amino acid 242, presumably because the α-amino group of Cys243 reacts with the keto group of acetophenone to form the corresponding cyclic imine.

(20) InhA (2.5 μM) was incubated with either 4-vinylpyridine (1 mM) or methyl methanethiosulfonate (1 mM) for 30−60 min in the presence of NADH (70 μM).[7] No significant decrease in activity of InhA was detected (±20%).

(21) After incubation of InhA in 1 M guanidinium hydrochloride in the absence of any cysteine-specific reagents, about 50% of the enzyme activity could be recovered (measured after 70-fold dilution of InhA). InhA had no measurable activity in 1 M guanidinium hydrochloride.

(22) Reactions were carried out in 50 mM Na_2HPO_4, pH 7.5 at 25 °C. Substrate concentrations were in the range 100−300 μM. Samples were analyzed on a reversed-phase C18 column, and products were identified by comparison to authentic samples. KatG was shown not to oxidize ethionamide. The *S*-oxide was synthesized according to a procedure of Walter and Curts: Walter, W.; Curts, J. *Chem. Ber.* **1960**, *93*, 1511.

(23) The oxidation of ethionamide *S*-oxide (100 μM) by HRP (0.34 μM) and H_2O_2 (100 μM) also led to inactivation of InhA.[7] The inactivation can be partially inhibited by the addition of octenoyl-ACP to the reaction mixture. NADH or NAD^+ does not need to be present for the inactivation of InhA in contrast to the results with isoniazid.