Mechanistic Studies of the Oxidation of Isoniazid by the Catalase Peroxidase from *Mycobacterium tuberculosis*

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The recent outbreaks of drug-resistant strains of tuberculosis have created a need for new drugs with improved efficacy.[1] Isoniazid (**1**) has historically been a very effective drug for the treatment of tuberculosis, but little is known about its mechanism of action.[2] A widely accepted hypothesis is that isoniazid acts on the synthesis of mycolic acids, an integral component of the mycobacterial cell wall.[3] Other theories are that free radicals generated during the oxidation of isoniazid by the catalase-peroxidase of *Mycobacterium tuberculosis* are responsible for the toxicity of the drug.[4] Recent analyses of drug-resistant strains suggest that isoniazid is converted by the catalase-peroxidase into a biologically active form.[5] In order to understand the mode of action of isoniazid and to design improved versions of the drug, a detailed understanding of the enzymatic transformation of isoniazid by the catalase-peroxidase (the *katG* gene product) from *M. tuberculosis* is needed. The overexpression and characterization of this enzyme[6] allows us now to report its catalytic properties and an investigation of the mechanism of the enzyme's reaction with isoniazid.

The bifunctional enzyme is a broad specificity peroxidase and has a high catalase activity of 2860 units/mg.[7,8] The reaction of the enzyme with isoniazid was followed by high-performance liquid chromatography (HPLC), and the products were identified by comparison with authentic samples and by mass spectroscopy.[9] At a pH of 7.5, 50 mM Na_2HPO_4 at 37 °C, the following products were identified: isonicotinic acid (**2**), isonicotinamide (**3**), and pyridine-4-carboxaldehyde (**4**), the acid **2** being the major product. Relative product yields depended upon the isoniazid concentration and changed over the course of the reaction, aldehyde **4** being eventually oxidized to acid **2**.[10] The k_{cat} and the K_M for the formation of acid **2** under the above conditions are 0.045 s^{-1} and 198 μM,[11] respectively.

In order to determine the origin of the oxygen atoms in acid **2**, the reaction of isoniazid with the enzyme was carried out under an atmosphere of $^{18}O_2$, and the isonicotinic acid **2** was isolated and analyzed by mass spectrometry.[13,14] The amount of **2** containing a single ^{18}O atom was approximately 35%. Incubation of acid **2** in $H_2{}^{18}O$ under the same conditions used for the incorporation of ^{18}O into **2** showed no exchange of the oxygen with water. The reaction of isoniazid with the enzyme in $H_2{}^{18}O$ led to the formation of **2**, in which approximately 33% of the product contained no ^{18}O; ~50%, one atom of ^{18}O; and ~17%, two atoms of ^{18}O. These results suggest the simultaneous presence of two pathways leading to the formation of acid **2**. In agreement with these data, the addition of simple amines to the reaction mixture led to the formation of the corresponding amides in up to 50% yield.[9,15,16] Oxidation of aldehyde **4** in the presence of simple amines did not lead to the formation of the corresponding amides.

The reaction products and labeling results for the reaction of isoniazid with the catalase-peroxidase and previous studies of the oxidation of hydrazides[4a] are consistent with the mechanism outlined in Scheme 1. The first step involves enzymatic oxidation of isoniazid to the corresponding acyldiimide (**5**), possibly via a hydrazide radical (**6**). Diimide **5** could then decompose via a diazenyl radical (**7**)[17] to afford the corresponding acyl radical (**8**), which could either abstract hydrogen to give aldehyde **4** or react with molecular oxygen to give the corresponding peracid (**9**). The exchange of ^{18}O into isonicotinic acid **2** from $^{18}O_2$ would occur via the radial **8**. Exchange from $H_2{}^{18}O$ and formation of amides in the presence of exogeneous amines may occur via nucleophilic addition to acyldiimide **5**,[18,19] or, alternatively, **5** could be oxidized in a second step to an electrophilic acyldiazonium ion (**10**), which then reacts with water or amine.[20]

Another interesting detail of the reaction of isoniazid with the catalase-peroxidase is the formation of amide **3**, even when ammonia is rigorously excluded from the assay mixture. Reaction of ^{15}N-labeled isoniazid with the catalase-peroxidase and analysis of the formed amide by mass spectrometry revealed that under these conditions **3** arises from the splitting of the N–N bond.[21,22]

The substrate specificity of the catalase-peroxidase was also investigated. It is known that benzoic hydrazide (**11**) and the

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(7) Catalase activity was measured at pH 7, 50 mM NaH_2PO_4, 20 °C, by following the absorbance at 240 nm.

(8) Substrates for the enzyme include *o*-phenylenediamine, 3,3'-diaminobenzidine, and *N*-(1-naphthyl)ethylenediamine.

(9) Reactions were analyzed by quenching with a solution of NH_2OH (0.13 M, pH 6.8) containing 3-nitrobenzoic acid as an external standard. Samples were analyzed on a reversed-phase C18-column (Rainin Dynamax) using a linear gradient prepared from 50 mM $(NH_4)OAc$, pH 7, acetonitrile. Concentrations were determined by integration of the HPLC profile monitored at 260 nm and comparison to the peak of 3-nitrobenzoic acid; **4** was detected as the oxime.

(10) Aldehyde **4** is a substrate of the enzyme. The amount of **3** formed strongly depends on the isoniazid concentration, ranging from below 5% at 100 μM isoniazid to ~30% relative to **2** at 2 mM isoniazid. Relative yields of **2**, **3**, and **4** (pH 7.5, 50 mM Na_2HPO_4, 37 °C, 0.3 μM enzyme, 200 μM **1**) are 8.5, 0.95, and 1.46 μM (t = 21 min) and 59, 3, and 3 μM, respectively (t = 143 min).

(11) At high isoniazid concentrations (>200 μM), a decrease in catalase activity was observed during the oxidation of isoniazid.[12] Rates were determined over periods during which no significant decrease in activity was observed.

(12) Oxidation of hydrazines and hydrazides can lead to the inactivation of catalase-peroxidases and cytochrome P450. Ortiz de Montellano, P. R. In *Cytochrome P450*; Ortiz de Montellano, P. R., Ed.; Plenum Publishing Corp. New York, 1986; pp 273–314.

(13) $^{18}O_2$ (98.1%) was purchased from Isotec Inc., NY; $H_2{}^{18}O$ (95%) was purchased from Aldrich Chemical Co.

(14) Samples in the $^{18}O_2$ and $H_2{}^{18}O$ exchange experiments were analyzed after >80% completion. Analysis of the oxidation under $^{18}O_2$ at 0.2 mM isoniazid and >90% completion and at 1 mM isoniazid and ~40% completion showed the same amount of ^{18}O in acid **2**.

(15) Chemical oxidation of hydrazides yields electrophilic intermediates.[16a,b] Furthermore, it has been reported that oxidation of acetylated hydrazides in the liver leads to covalent binding of the acetyl portion to proteins.[16c]

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(18) Depending on its lifetime, **5** might get hydrolyzed and/or exchange the oxygen with solvent,[19] providing an explanation for acid **2** with two atoms of ^{18}O. Another explanation for the formation of **2** with two atoms of ^{18}O would be enzymatic oxidation of the hydrate of **4**.

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(20) (a) Aylward, J. B. *J. Chem. Soc. (C)* **1969**, 1663. (b) Misra, H. P.; Fridovich, I. *Biochemistry* **1976**, *15*, 681.

(21) ^{15}N-Labeled isoniazid was synthesized by condensing the corresponding methyl ester with ^{15}N-labeled hydrazine sulfate (99% isotopic purity, Cambridge Isotope Laboratories).

(22) A mechanism in agreement with the strong concentration dependence of the amide formation is the disproportionation of the hydrazide. Coucouvanis, D.; Mosier, P. E.; Demadis, K. D.; Patton, S.; Malinak S. M.; Kim, C. G.; Tyson, M. A. *J. Am. Chem. Soc.* **1993**, *115*, 12193.

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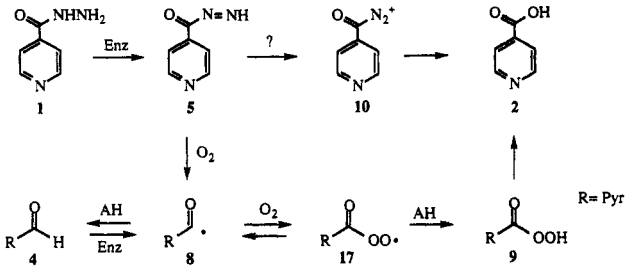
Scheme 1. Proposed Mechanism for the Enzymatic Oxidation of Isoniazid



isomeric substrate nicotinic hydrazide (**12**) show very little activity as antituberculosis drugs compared to isoniazid.[23] In accordance with these data, **11** and phenylacetic hydrazide (**13**) reacted with the catalase-peroxidase at rates below 2% that of isoniazid. The picture with nicotinic hydrazide **12**, however, is more complex. The reaction of the enzyme with **12** proceeded at a rate of approximately 55% of that of the reaction of isoniazid with the enzyme, yielding the following products: nicotinic acid (**14**), nicotinamide (**15**), and pyrimidine-3-carboxaldehyde (**16**). In contrast to the reaction with isoniazid, however, aldehyde **16** is not a substrate of the enzyme.[24] Another interesting difference from the reaction with isoniazid is the much faster inactivation of the enzyme during the oxidation of nictonic hydrazide **12**,[12,25] providing a possible explanation for the fact that **12** is an antagonist of isoniazid.[23] Furthermore, the rate of oxidation showed a strong dependence on the reaction conditions. For example, addition of 20 mM ammonium acetate to the reaction of nicotinic hydrazide **12** with enzyme led to a 50-fold decrease in the rate of formation of the acid relative to isoniazid, which also might account for their different biological activities.[26]

The mechanism proposed above provides a number of possibilities for the mode of action of isoniazid. The drug is converted *in vivo* into a number of highly reactive species capable of either oxidizing or acylating groups in proteins. One attractive hypothesis is that an enzyme involved in the biosynthesis of fatty acids in *M. tuberculosis*[27] might be one of the targets of these active agents, leading to a covalent and irreversible modification of the protein.

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Supplementary Material Available: A table of the concentration of isoniazid and the products as a function of time during the enzymatic reaction, a graph showing the decrease in catalase activity during the reaction of the catalase-peroxidase with isoniazid and nicotinic hydrazide, and a Lineweaver-Burk plot for the determination of kinetic constants (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(24) The ratio of acid **14** to aldehyde **16** (ratio ~ 2) remained constant during the enzyme-catalyzed oxidation.

(25) At a substrate concentration of 500 μM **1** or **12** at pH 7.5, 50 mM Na_2HPO_4, 37 °C, the catalase activity during the reaction with **12** fell to 18% of the value at $t = 0$ within 1.5 h, whereas the reaction with **1**, the catalase activity only fell to 72% during that time.

(26) Ethylamine and glycinamide showed similar effects. The concentration of amino groups in living cells should exceed 20 mM.

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