

Evidence for Isoniazid Oxidation by Oxyferrous Mycobacterial Catalase–Peroxidase

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Isoniazid (isonicotinic acid hydrazide) has been used as a first-line antibiotic for the treatment of tuberculosis for decades, yet the details of its mechanism of action are still incomplete. Studies revealed many years ago that isonicotinic acid and 4-pyridylmethanol were produced from the drug^{1,2} though the mycobacterial enzyme or enzymes responsible were not defined. The accumulation of evidence that isoniazid-resistant strains of mycobacteria have reduced activity of a hemoprotein hydroperoxidase (catalase–peroxidase)^{3–5} suggested that isoniazid is a prodrug converted by this enzyme into a bacteriocidal agent.^{6,7} Susceptibility to isoniazid could be produced in isoniazid-resistant *Escherichia coli* or *Mycobacteria smegmatis*⁴ upon introduction and expression of the *M. tuberculosis katG* gene encoding the catalase–peroxidase. The purified catalase–peroxidase from *M. smegmatis*⁸ (and other bacteria^{9–12}) contains ferric heme according to optical and/or EPR spectroscopic analyses and catalyzes classical peroxidative reactions. The *M. tuberculosis katG* enzyme produces radicals in the presence of isoniazid and H₂O₂.¹³ It is therefore unusual that *M. tuberculosis* catalase–peroxidase was reported to catalyze isoniazid oxidation *in vitro* without peroxide activation of the enzyme.¹⁴ Experimental results reported here suggest that the isoniazid oxidation mechanism involves oxyferrous catalase–peroxidase.

In the first experimental approach, optical spectroscopy was used to follow the reaction between isoniazid and resting *M. smegmatis* catalase–peroxidase. In the second, HPLC was used to quantitate the majority products in reactions catalyzed by the enzyme in the presence of hydrogen peroxide or hydrazine, a reducing agent.

The formation of a catalytically active enzyme from resting (ferric) *M. smegmatis* catalase–peroxidase in the absence of exogenous peroxide may occur as a result of iron reduction and binding of O₂ to give the oxyferrous form. In initial experi-

ments, a small increase in absorbance at 428 nm was observed immediately after mixing the enzyme (0.3 μ M) with excess isoniazid (1 mM) under CO gas, consistent with reduction of approximately 10% of the enzyme to the ferrous CO form previously characterized ($\lambda_{\text{max}} = 428 \text{ nm}$ ⁸). When this experiment was repeated using a solution of isoniazid freshly prepared from drug recrystallized from methanol, no increase in absorbance at 428 nm was observed. This result suggested that a contaminant of commercial isoniazid was able to reduce ferric catalase–peroxidase. To confirm this result and to determine if the reductant was derived from the drug molecule itself, a buffered solution (pH = 7) of isoniazid was stored for several days at room temperature. Anaerobic treatment (under CO) of resting catalase–peroxidase with this isoniazid solution yielded quantitative conversion of all the ferric enzyme to the ferrous CO form.

The aged isoniazid solution was found by HPLC to contain isonicotinic acid. The suspected presence of hydrazine was demonstrated spectrophotometrically by the formation of a chromophore equivalent to that formed from authentic hydrazine ($\lambda_{\text{max}} = 458 \text{ nm}$) using a modified Ehrlich reagent.¹⁶ The possibility that hydrazine reduces ferric catalase–peroxidase was then directly confirmed. Excess hydrazine (hydrazine monohydrate) added anaerobically to the ferric enzyme under CO immediately and quantitatively produced the optical spectrum of the ferrous CO form.

Experiments were designed to test the hypothesis that the reduction of ferric catalase–peroxidase under aerobic conditions leads to production of the oxyferrous enzyme, which could function as an oxidant of isoniazid. Incubations of catalase–peroxidase were prepared with isoniazid, with and without hydrazine under aerobic and anaerobic conditions. The addition of hydrazine (15 μ M) to resting catalase–peroxidase (3 μ M) in the presence of 150 μ M isoniazid led to the disappearance of 44 μ M isoniazid in 2.5 h (Table 1). Isonicotinic acid and isonicotinamide accounted nearly quantitatively for the converted drug.¹⁴ No reaction occurred in an identical reaction mixture incubated under CO, demonstrating the requirement for oxygen and suggesting that the oxyferrous form of the enzyme was the oxidant formed under aerobic conditions. No reaction occurred using freshly dissolved, recrystallized isoniazid under aerobic conditions in the absence of hydrazine, consistent with the spectrophotometric results. No oxidation of the drug occurred in aerobic incubations with hydrazine in the absence of catalase–peroxidase.

In an earlier report⁸ it was demonstrated that *o*-dianisidine is oxidized peroxidatively by *M. smegmatis* catalase–peroxidase, yielding a red chromophore ($\epsilon_{460 \text{ nm}} = 11 \text{ mM}^{-1} \text{ cm}^{-1}$). This reaction was used to check for the production of hydrogen peroxide *in situ* (from autooxidation of hydrazine, as suggested by a reviewer) that could potentiate peroxidation of isoniazid by the enzyme. No peroxidation was detected optically during 2 h incubations of 3 μ M enzyme with 140 μ M *o*-dianisidine and 14 or 28 μ M hydrazine under conditions in which as little as 0.05 μ M product would have been detected. Complete peroxidation of the substrate could be demonstrated in this reaction mixture upon addition of excess peroxide after the 2 h incubation, demonstrating that the enzyme remained active.

Experiments were designed to elicit peroxidative breakdown of isoniazid by catalase–peroxidase¹⁷ by adding hydrogen peroxide to the enzyme (3 μ M) in solutions containing 150 μ M isoniazid in one or several aliquots, to final concentrations in molar excesses ranging from 100-fold to greater than 5000-fold over that of enzyme. Although low yields were found

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(15) A modification of a previously published procedure⁸ was used to purify the enzyme, which had an optical ratio $A_{408}/A_{280} = 0.56$ and specific activity greater than 500 units/mg.

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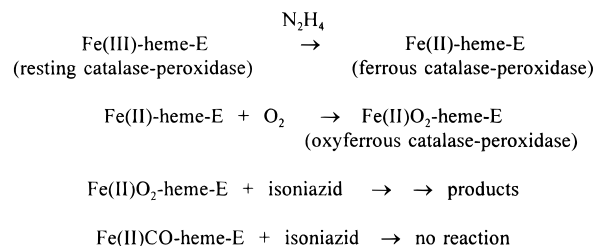
Table 1. Isoniazid Breakdown into Isonicotinic Acid and Isonicotinamide Catalyzed by *Mycobacterium smegmatis* Catalase–Peroxidase^a

reaction	isonicotinic acid, μM	isonicotinamide, μM	isoniazid reacted, μM
E/isoniazid	0	0	5
E/isoniazid/ N_2H_4 (air) ^b	15	17	44
E/isoniazid/ N_2H_4 (CO) ^c	0	5	
isoniazid/ N_2H_4 ^d	0	0	0
E/ H_2O_2 ^e	15	0	30
E/ H_2O_2 ($5 \times 0.4 \text{ mM}$) ^f	0	0	15
Mn^{2+} /isoniazid ^g	30/70	0/15	25/70
E/ Mn^{2+} /isoniazid ^h	100	20	114

^a Reactions initially contained 3 μM heme plus 150 μM isoniazid added from a freshly prepared stock solution (10 mM in water) in 15 mM potassium phosphate buffer, pH = 7. Products were analyzed by reversed phase HPLC chromatography after 2.5 h incubation (or at the indicated times). Enzyme (E) was removed at the end of the incubations by ultrafiltration (Centricon 30), and the HPLC analysis was performed on the retentate. No other reagents were added and compounds were quantitated on the basis of the areas of peaks measured in chromatograms of freshly prepared standard solutions of isonicotinic acid, isonicotinamide and isoniazid in 15 mM potassium phosphate buffer. The unreacted isoniazid and the two breakdown products quantitated here were resolved by elution with the aqueous phase (50 mM ammonium acetate, pH = 7). An acetonitrile gradient eluted additional material that was not identified or quantitated since most of the lost isoniazid could be accounted for by the yield of isonicotinic acid and isonicotinamide. Some loss of isoniazid occurs during manipulation of samples and due to binding of isoniazid to the enzyme. This fraction was subtracted from the starting concentration to calculate amounts reacted. Determinations are within $\pm 10 \mu\text{M}$. ^b Hydrazine concentration, 15 μM . ^c The solution of enzyme plus isoniazid was degassed and equilibrated under CO before the anaerobic addition of 15 μM hydrazine. ^d Conditions: 15 μM hydrazine, 150 μM isoniazid, no enzyme. ^e Hydrogen peroxide (16 mM) was added in one aliquot, and the reaction was incubated for 1.5 h. ^f Hydrogen peroxide was added in 5 aliquots over a 2 h period. ^g Isoniazid (150 μM) was incubated with 2 μM Mn^{2+} ; entries are for analyses after 4 and 6.5 h. ^h After 4 h preincubation of isoniazid with Mn^{2+} , enzyme (3 μM) was added to the reaction mixture followed by incubation for 2.5 h.

based on the concentration of peroxide added (Table 1, selected results) the cleavage of the drug may suggest that a complex analogous to horseradish peroxidase compound I^{18,19} is active as an oxidant. However, the oxyferrous form of catalase–peroxidase assumed to be the catalytically competent form of the enzyme generated in the presence of hydrazine, may also be generated in the presence of high concentrations of peroxide. In support of this suggestion is the evidence that large excesses of H_2O_2 favor the formation of compound III (oxyperoxidase) from resting (ferric) horseradish peroxidase.^{18–20}

The use of Mn^{2+} has been reported in experiments demonstrating inhibition of the mycobacterial fatty acyl-ACP (acyl carrier protein) enoyl reductase (*inhA* gene product) by isoniazid with catalase–peroxidase,^{21,22} and inhibition of the reductase has been implicated in the bacteriocidal mechanism of the drug.^{22,23} Mn^{2+} has also been reported to catalyze the aerobic decomposition of isoniazid in a radical-mediated mechanism.²⁴ Here, incubation of isoniazid (150 μM) with 2 μM Mn^{2+} resulted

Scheme 1

in extensive decomposition of the drug in the absence of catalase–peroxidase (Table 1). Isoniazid was nearly quantitatively converted into isonicotinic acid and isonicotinamide after an additional 2.5 h incubation in the presence of catalase–peroxidase. These observations indicate that nonenzymatic Mn^{2+} -catalyzed reactions of isoniazid produce good yields of the same products as the enzyme-catalyzed reactions.

The evidence that oxyferrous *M. smegmatis* catalase–peroxidase may be catalytically competent as an oxidant of isoniazid, as outlined in Scheme 1, may be related to oxidations catalyzed by oxyperoxidase^{25–27} and to the catalytic mechanism of the enzyme tryptophan 2,3-dioxygenase.²⁸ Analogy may also exist with the mechanism of reduction of methemoglobin by phenylhydrazine²⁹ and its reaction with oxyhemoglobin.^{30,31}

The antimycobacteriocidal action of isoniazid has a known requirement for oxygen in cell cultures.^{32,33} That observation, along with the present results, may be especially relevant to the mechanism of action of the drug since the level of peroxide in cultured mycobacteria may never be appropriate for initiation of peroxidative reactions involving catalase–peroxidase and isoniazid. The presence of a hydrazidase activity in mycobacteria¹⁶ leads to the possibility that, *in vivo*, the *bona fide* oxidant of isoniazid is oxyferrous catalase–peroxidase generated upon hydrazinolysis of the drug and reaction of hydrazine with the ferric enzyme under aerobic conditions.

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