Comparison of Isoniazid Oxidation Catalyzed by Bacterial Catalase– Peroxidases and Horseradish Peroxidase

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Received June 22, 1995, and in revised form August 31, 1995

The physical properties and activities of the purified catalase–peroxidase hydroperoxidase I (HPI) of Escherichia coli (EcHPI) and HPI with a carboxyl-terminal extension of Mycobacterium tuberculosis (MtHPI-e) are compared to those of commercial preparations of horseradish peroxidase (HRP). The catalase–peroxidase proteins had similar absorption spectra and differed primarily in that MtHPI-e has a higher peroxidatic to catalatic activity ratio than EcHPI. Trypsin cleavage of MtHPI-e resulted in the formation of an active catalase–peroxidase lacking the carboxyl-terminal extension. The three enzymes, HRP, MtHPI-e, and EcHPI, mediated the isoniazid- and H₂O₂-dependent production of radical species, as detected by nitroblue tetrazolium reduction. A constant flux of H₂O₂, generated in situ from glucose oxidase and glucose was used. MtHPI-e was more effective at isoniazid-dependent radical production than EcHPI and HRP. Similar qualitative results were obtained by staining non-denaturing polyacrylamide gels for activity with nitroblue tetrazolium. The absorbance spectrum of HRP exhibited changes during incubation with isoniazid and H₂O₂ consistent with the formation of several typical reaction intermediates, whereas the catalase–peroxidases exhibited no distinct spectral changes. The results suggest that the sensitivity of M. tuberculosis to isoniazid may be the result of isoniazid-dependent radical formation by the catalase–peroxidase in the absence of other catalase activities to remove substrate H₂O₂.

Isonicotinic acid hydrazide (INH) is one of a number of drugs used to treat Mycobacterium tuberculosis infections. By contrast, INH is ineffective or has lower efficacy when used against Mycobacteria other than M. tuberculosis or M. bovis and is ineffective against strains of Escherichia coli even at high concentrations. It has long been recognized that catalase-deficient strains of M. tuberculosis are resistant to treatment with INH (1), but only recently has it been shown that either deletion (2) or point mutation (3) of the katG gene gives rise to such a phenotype. In M. tuberculosis, katG encodes the only catalase, which also has an associated peroxidase activity. This catalase–peroxidase is 50% identical to hydroperoxidase I of E. coli (EHPI) which in turn has been shown to be related to the superfamily of peroxidase enzymes (4).

While the molecular biology of M. tuberculosis resistance to INH involving KatG-deficiency has been demonstrated, the biochemical basis for resistance remains less clear. INH is believed to affect several intracellular targets of mycolic acid synthesis (5), including an enoyl-[acyl-carrier-protein] reductase (InhA) (6). Recently, Schultz and colleagues (7) have proposed a plausible mechanism of action for the drug based on in vitro studies of purified KatG and InhA proteins which suggest that INH must be oxidized in vivo to an electrophilic species by the catalase–peroxidase. The INH-derived electrophilic species then binds irreversibly to a cysteine residue near the active site of InhA, thereby preventing assembly of mycolic acids leading to cytotoxicity (7). If this scheme is correct, other as yet unidentified cellular components may also be inactivated or destroyed by KatG-generated free radicals through...
INH oxidation. E. coli does not synthesize mycolic acid and possesses a second monofunctional catalase enzyme (HPII) which would remove H$_2$O$_2$ before it could potentiate INH toxicity, possibly explaining why E. coli is resistant to INH. Similarly, other INH-resistant Mycobacteria have an additional catalase, providing an explanation for their resistance. Initial attempts to characterize the role of INH in KatG-mediated cell toxicity were incomplete because crude cell extracts were used, but it was demonstrated that INH was taken up by M. tuberculosis and oxidized (1).

Diaz and Wayne (8) and, subsequently, Gayathri-Devi and co-workers (9) reported the purification of KatG from M. tuberculosis and demonstrated that it had properties similar to horseradish peroxidase (HRP), including optimum pH and activities in the presence of various inhibitors and other reagents. The former report also showed that the catalase activity of KatG was inhibited in the presence of INH. More recently, it has been demonstrated that HRP (10), extracts derived from M. tuberculosis (11), and human myeloperoxidase (12) can catalyze the INH-dependent generation of radical species. Purified, plasmid-expressed M. tuberculosis KatG has also been used in mechanistic studies to confirm that oxidized derivatives of INH generated by KatG include isonicotinic acid, isonicotinamide, and pyridine-4-carboxyaldehyde (13).

The general sequence of reactions for enzymatic turnover of horseradish peroxidase with a hydrogen donor in the presence of H$_2$O$_2$ is shown below:

$$\text{HRP(III)} + H_2O_2 \rightarrow \text{HRP compound I(V)}$$  \[1\]
$$\text{HRP compound I(V)} + AH_2 \rightarrow \text{HRP-compound II(IV)} + \cdot \text{AH}$$  \[2\]
$$\text{HRP compound II(IV)} + AH_2 \rightarrow \text{HRP(III)} + \cdot \text{AH} + H_2O,$$  \[3\]

where AH$_2$ and \cdot AH are reducing donor substrate and free radical product, respectively. The numerals in parentheses indicate formal oxidation states of HRP during enzymatic turnover. The free radical products of the reaction may undergo various reactions depending on their chemistry, such as dimerization, reaction with a neutral donor molecule, reaction with the enzyme itself, or reduction of molecular oxygen to superoxide (14, 15). Radical production under these conditions may also be monitored by including a suitable electron acceptor such as nitroblue tetrazolium (NBT) in the reaction mixture, as shown below:

$$\cdot \text{AH} + \text{NBT} \rightarrow \text{AH} + \text{reduced NBT} \text{ (formazan dye).}$$  \[4\]

Mechanism-based inactivation of HRP by radical species produced during incubation with phenylhydrazone, alkylhydrazines, and sodium azide has been shown previously (15). For catalase, catalase-peroxidase, and HRP, the reaction cycle incorporates reaction [1], but catalase enzymes preferentially reduce compound I via a one-step, two-electron reaction in which the substrate donor is usually a second molecule of hydrogen peroxide. In the case of HRP, at high H$_2$O$_2$ to donor substrate concentration ratios, as well as in the absence of donor substrates, the normal peroxidase cycle partitions at compound I to react with H$_2$O$_2$ (16) or radical species (17), yielding a reversibly inactivated HRP species called compound III, or oxyperoxidase, at an oxidation state of VI. This species may further react with radical species or other donor molecules to regenerate compound I (17). Additionally, compound I may react slowly under these conditions with H$_2$O$_2$ to yield either a second (irreversibly) inactivated form of the enzyme known as compound IV (16, 18), or to reduce compound I back to resting (ferric) HRP in a catalase (two-electron) reaction (15), though the evidence for the latter is equivocal (14).

In order to clarify the role of M. tuberculosis KatG protein in INH toxicity, we have compared properties of a M. tuberculosis KatG protein with a carboxy-terminal extension (MTHPI-e), EcHPI, and HRP. We have also compared the ability of each enzyme to produce free radicals and monitored the susceptibility of each enzyme to inactivation, during incubation with INH in the presence of low levels of H$_2$O$_2$. The results confirm that the M. tuberculosis enzyme is more effective in the generation of free radicals than either EcHPI or HRP, explaining, in part, its role in INH-mediated toxicity.

**MATERIALS AND METHODS**

Reagents. Common biochemicals, horseradish peroxidase (Types II and IX), superoxide dismutase, and glucose oxidase (Type II) were obtained from Sigma Chemical Co. Hydrogen peroxide (30%) and glucose were from Fisher Scientific, Ltd. LB media and molecular biology reagents (enzymes and buffers) were from Gibco BRL. H$_2$O$_2$ concentration was determined immediately before applicable experiments assuming an $\epsilon = 39.4$ M$^{-1}$ cm$^{-1}$ at 250 nm (19).

Expression and purification of enzymes. The HPI proteins of E. coli and M. tuberculosis were expressed from plasmids pBT22 (20) and pAH1. The latter was constructed by insertion of the 2.9-kb EcoRV-KpnI fragment of plasmid pYZ55 (2), containing the M. tuberculosis katG gene into pSK$^+$ (Stratagene) where it was under control of the lac promoter. Restriction digestion, ligation, and cloning was as described by Maniatis et al. (21). Plasmids were transformed into E. coli UM262 [recA katG::Tn10 pro leu rpsL hsdM hsdR end1 lacY] (22), lacking both HPI and HPII. EcHPI was isolated as described (23). MTHPI-e was isolated following basically the same procedure with the following modifications: (1) catalase activity was precipitated at 35 and 40% of saturation with (NH$_4$)$_2$SO$_4$, rather than at 20% (NH$_4$)$_2$SO$_4$; (2) the resuspended, pooled fractions from (NH$_4$)$_2$SO$_4$ precipitation were incubated for 45 min at 42°C and centrifuged to remove additional precipitates prior to overnight dialysis;
and (3) pooled and dialyzed fractions recovered from the anion-exchange column were loaded onto a Sephadex G-200 gel filtration column equilibrated with 50 mM potassium phosphate, pH 7.0, and eluted in 3-ml fractions. Catalase activity eluted with the first protein peak and fractions to be pooled were selected based on relative protein purity estimated by electrophoresis on SDS-polyacrylamide gels. Pooled fractions were stored frozen (−80 °C) either in potassium phosphate buffer or lyophilized. Lyophilized commercial HRP was resuspended in 50 mM potassium phosphate buffer, pH 7.0, with no further purification and stored frozen (−20 °C).

Spectrophotometry. Absorbance spectra, time courses, and peroxidatic assays were performed using a Milton Roy MR3000 spectrophotometer. All experiments were performed at room temperature in 1-ml quartz, semimicro cuvettes. Proteins were normally diluted in 50 mM potassium phosphate buffer, pH 7.0, and the same buffer was used as a reference.

Activity assays and protein determination. Catalase activity was determined by the method of Rath and Jensen (24), using a Gilson oxymeter equipped with a Clark electrode. One unit of catalase is defined as the amount that decomposes 1 μ mole of H₂O₂ in 1 min at 37 °C. Peroxidase activity was determined by the method described in the Worthington Enzyme Catalogue (Worthington Chemical Co.), modified to be carried out in 1-ml assay volumes. The average rate of H₂O₂ production from experiments employing the glucose-glucose oxidase system was estimated by determination of H₂O₂ concentration according to the method of Green and Hill (25) for duplicate or triplicate samples, at 5-min intervals, allowing 5 min for color development before making individual absorbance readings at 505 nm. Protein was estimated by the methods described by Layne (26).

An e = 15,000 m⁻¹ cm⁻¹ for the monoferric product from the reduction of NBT was used (27).

Polyacrylamide gel electrophoresis and visualization of enzymatic activities. Gel electrophoresis of purified proteins was carried out under denaturing conditions on SDS-polyacrylamide gels as previously described (28, 29). Gel electrophoresis was carried out under non-denaturing conditions according to Davis (30), except in pH 8.1 Tris·HCl. For all electrophoretic procedures, 15 × 15-cm slab gels were run in a Protein II electrophoretic chamber (Bio-Rad). Following electrophoresis, peroxidase activity was visualized by the method of Gregory and Fridovich (31) and catalase was visualized as described by Clare et al. (32), but using 20 mM H₂O₂ for better contrast. Peroxidase-mediated oxidation of INH was visualized by soaking the gels in 200 ml 50 mM potassium phosphate, pH 7.0, containing 274 mg INH, 50 mg NBT, and 60 μl 30% H₂O₂. Color development was usually complete between 30 min and 1 h, after which gels were rinsed with distilled water and soaked in 7% acetic acid, 1% glycerol overnight before mounting. Bands usually intensified slightly during incubation. Incubation of gels for 16 h in the staining solution from which NBT was omitted also resulted in band development, but the bands were much weaker than those seen using the complete staining solution.

RESULTS AND DISCUSSION

Characteristics of MthPI-e and EchPI Compared to HRP

Purified EchPI and MthPI-e were analyzed by electrophoresis on polyacrylamide gels (Fig. 1). Under denaturing conditions (Fig. 1A), both proteins exhibited a predominant single band of protein with apparent molecular masses of 82 kDa for EchPI and 89 kDa for MthPI-e. As unmodified EchPI and MthPI polypeptides have over 50% sequence identity and differ by only 11 amino acids in length, MthPI-e was inferred to be larger due to a polypeptide extension. The N-terminal sequence of the purified protein for the first 9 amino acids corresponded to that predicted from the DNA sequence of M. tuberculosis katG, indicating that the polypeptide was larger due to a C-terminal extension, rather than an N-terminal LacZ fusion. The reason for the longer than expected terminal end is currently under investigation. Two possibilities include differences from the published sequence or aberrant translation of the G-C rich mRNA in E. coli. Translation ending at the next termination site would give rise to a 49-amino-acid extension at the carboxyl-terminal of MthPI. Under non-denaturing conditions (Fig. 1B), EchPI exhibited the normal double band of charge isoforms corresponding in size to a dimer, while MthPI-e exhibited three bands also presumed to be charge isoforms. Fortuitously, the predicted sequence of MthPI-e contains several closely spaced arginine and lysine residues in the region immediately following the usual final residue of the MthPI which proved to be a target for trypsin cleavage to generate a nearly native MthPI. As shown in Figs. 1A and 1B, a protein band with mobility comparable to EchPI under both denaturing and non-denaturing conditions was obtained from cleavage of MthPI-e with trypsin.

Staining for peroxidase activity in polyacrylamide gels may be accomplished by several techniques (31,
An inactive component, its presence should not affect the peroxidase/catalase ratio or any of the subsequent results. It should be pointed out that previous purifications of wild-type MtHPI have reported \(A_{408/280}\) ratios of 1.0 (9) and 0.37 (8); the latter value being the same as that for our purified MtHPI-e. Furthermore, the utility of a fusion protein has been demonstrated by Zhang et al. (2), who have previously shown a LacZ-MtHPI fusion to confer INH susceptibility on E. coli. This fusion protein has recently been reported purified, having a specific catalase activity of 494 U/mg and exhibiting three isoforms in nondenaturing PAGE (34).

Rate and Extent of INH-Dependent Generation of Free Radicals by HRP are Dependent on \(H_2O_2\) Concentration

HRP and myeloperoxidase catalyze a reaction dependent on the presence of INH and \(H_2O_2\) that may be followed by reduction of NBT to its purple formazan product (10, 11). Shoeb and colleagues (10) also observed that the presence of exogenous catalase caused an increase in the rate of NBT reduction by HRP and a faster termination of the reaction rather than the expected cessation of reaction as catalase removed the substrate \(H_2O_2\). They also confirmed that the presence of superoxide dismutase did not inhibit NBT reduction in the INH-dependent reaction catalyzed by HRP.

To determine if the catalase effect on HRP was solely the result of the lower \([H_2O_2]\), we varied the initial \([H_2O_2]\) and followed the INH/\(H_2O_2\)-dependent NBT reduction by HRP (Fig. 3A). Two distinct patterns of NBT reduction were observed depending on the \([H_2O_2]\). At \([H_2O_2]\) up to 100 \(\mu M\), there was a rapid reaction rate which ceased after a time proportional to the initial \([H_2O_2]\). At higher \([H_2O_2]\), the initial fast phase became progressively shorter and, eventually, indistinguishable from the second, slower phase. This change in rate at higher \([H_2O_2]\) is presumably a result of a progressive inactivation of the enzyme as \([H_2O_2]\) increases in the presence of donor substrate (15). The maximal initial reaction rate of NBT reduction with 100 \(\mu M\) \(H_2O_2\) added was estimated to be 9 nmol/min/ml. [NBT] was varied from 20 to 600 \(\mu M\) to determine whether efficiency of the reaction could be improved by reducing the probability of any side reactions of the radicals generated. Apart from a marginal increase in the rate of the initial phase of reaction at lower [NBT] (20 \(\mu M\)), varying the [NBT] had no effect over the concentration range tested. In addition, the reaction was monitored in the presence of superoxide dismutase (1.5 U), added both prior to and following the initiation of NBT reduction, to ensure that superoxide anion radical was not a significant proportion of the radicals produced. No decline in reaction rate was observed in the presence of superoxide dismutase for these experimental controls.
The effect of INH concentration on NBT reduction at an initial $[\text{H}_2\text{O}_2]$ of 250 $\mu$M is shown in Fig. 3B. The initial rate of reduction was proportional to INH concentration and there was no evidence of termination of the reduction at higher INH concentrations. This suggests that the termination of radical production seen for HRP observed in Fig. 3B is dependent upon $[\text{H}_2\text{O}_2]$, not the ratio of $[\text{H}_2\text{O}_2]$ to [donor].

### Table I

<table>
<thead>
<tr>
<th></th>
<th>HRP</th>
<th>EcHPI</th>
<th>MthHPI-e</th>
<th>EcHPI-14</th>
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<tr>
<td>Catalase (units/mg)</td>
<td>&lt;0.6</td>
<td>1568</td>
<td>963</td>
<td>197</td>
</tr>
<tr>
<td>Peroxidase (units/mg)</td>
<td>6405 ± 170</td>
<td>4.6 ± 0.2</td>
<td>5.1 ± 0.5</td>
<td>1.1 ± 0.1</td>
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<tr>
<td>$A_{407/280}$</td>
<td>3.1</td>
<td>0.60</td>
<td>0.37</td>
<td>0.28</td>
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</table>

*Ratio reported for HRP is $A_{404/280}$.*

MtHPI-e, EcHPI, and HRP Have Different Rates of NBT Reduction under Conditions of Constant $\text{H}_2\text{O}_2$ Generation

Our initial attempts at evaluating the capacity of MtHPI-e and EcHPI to mediate the oxidation of INH, via the NBT reduction assay used for HRP, were confounded by the catalase activities of the MtHPI-e and the EcHPI obscuring their peroxidatic activities under conditions of single peroxide additions where the catalase activity would rapidly remove $\text{H}_2\text{O}_2$ from the medium. In order to generate a constant source of $\text{H}_2\text{O}_2$, glucose oxidase and glucose were added to the system, resulting in the generation of $\text{H}_2\text{O}_2$ at a rate of approximately 7 nmol/ml/min. The results of exposing MtHPI-e, EcHPI, and HRP to this constant peroxide flux in the presence of INH and NBT are shown in Fig. 4. It is apparent that MtHPI-e was much more effective than EcHPI and HRP in mediating INH oxidation and NBT reduction compared to catalyzing peroxidation of $\text{o}$-dianisidine. Comparison of the kinetics for INH oxidation of the catalase–peroxidases based on the amount of NBT reduced under these assay conditions yielded $K_m$ of 1.24 and 1.49 mm and $k_{cat}$ values

![FIG. 3](image1.png)

**FIG. 3.** Time courses for HRP-mediated oxidation of INH followed by NBT reduction. Experiments done at room temperature in semimicro quartz cuvettes containing 1-ml final volumes of NBT (0.2 mM), HRP (30 $\mu$g), and potassium 50 mM phosphate buffer, pH 7.0. (A) In the presence of 9 mM INH, $\text{H}_2\text{O}_2$ addition initiated the reaction at the time indicated by the arrow at the following initial concentrations: (a) 5 $\mu$M, (b) 10 $\mu$M, (c) 50 $\mu$M, (d) 100 $\mu$M, (e) 1 mM, (f) 500 $\mu$M, (g) 250 $\mu$M. (B) $\text{H}_2\text{O}_2$ addition (250 $\mu$M) initiated the reaction (arrow) in the presence of the following initial concentrations of INH: (a) 87.5 $\mu$M, (b) 440 $\mu$M, (c) 875 $\mu$M, (d) 4.4 mm, (e) 17.5 mm, and (f) 52.5 mm.

![FIG. 4](image2.png)

**FIG. 4.** Comparison of INH oxidation rates of catalase–peroxidases and HRP. Experiments done at room temperature in semimicro quartz cuvettes containing 1-ml final volumes of NBT (0.2 mM), INH (9 mM), glucose oxidase (5 mg), and either 1 U peroxidase (0.2 mg) of EcHPI (dotted line), 1 U peroxidase (0.2 mg) of HRP (broken line), or 1 U peroxidase (0.2 mg) of MtHPI-e (solid line). Addition of glucose (4 mm) initiated the reaction at the time indicated by the arrow.
of 0.092 and 0.58 s\(^{-1}\) for EcHPI and MtHPI, respectively. The rate of NBT reduction by MtHPI-e increased with the addition of more enzyme, indicating that the rate of \(\text{H}_2\text{O}_2\) formation was not limiting under these conditions. However, the addition of more than 100 peroxidase units of HRP did not elicit further increases in the initial rate of reaction. Similar to the slowing of the NBT reduction rate upon single addition of \(\text{H}_2\text{O}_2\) (Fig. 3), the rates of NBT reduction also declined over time in the presence of a continuous flux of \(\text{H}_2\text{O}_2\). As the rate of generation of \(\text{H}_2\text{O}_2\) was confirmed to be stable for at least 15 min under the conditions employed, this phenomenon may be attributed to either a progressive inhibition of the radical generating reaction(s) or the establishment of a steady state of formation for the radical species present.

The finding that all three enzymes mediate the oxidation of INH to yield radical species is not surprising in view of their common peroxidase activity. What is of more immediate interest is the finding that the MtHPI-e supports a higher rate of radical generation than do either HRP or EcHPI under conditions of constant peroxide flux. The catalytic activity responsible for the INH-dependent generation of radicals captured by NBT is not directly proportional to the o-dianisidine peroxidase activity among the three enzymes. MtHPI has a 10% higher peroxidase specific activity than EcHPI, but causes NBT reduction to a threefold higher level, which could, in part, explain the ability of MtHPI to enhance INH sensitivity of Mycobacteria and confer INH sensitivity on \(E.\) coli (2). Early in the reaction there will be a partition of the enzyme between catalatic and peroxidatic modes at compound I (the enzyme–\(\text{H}_2\text{O}_2\) complex) resulting in the eventual establishment of a steady state. In the case of MtHPI-e, the proportion of peroxidatic (and derived radical formation) to catalatic activity will be greater than for EcHPI. However, a simple disproportion of peroxidatic to catalatic activities is not a sufficient explanation because an \(E.\) coli HPI mutant with a peroxidatic to catalatic activity ratio similar to MtHPI-e (Table I, 22) was no more effective in the INH-dependent generation of radicals than the wild-type MtHPI. It may also be argued that the three enzymes examined mediate production of different types of radical species, resulting in different rates of NBT reduction. The products of reaction of INH with MtHPI have been identified as isonicotinic acid, isonicotinamide, and pyridine-4-carboxaldehyde, consistent with a scheme of enzymatic oxidation of INH potentially involving hydroxazide radical, diazenyl radical, and an acyl radical (13). As NBT reduction cannot be used to distinguish between these radicals, the relative reaction rates of the individual radicals with the acceptor cannot be estimated. However, the catalase–peroxidases examined in this study exhibit many similarities in their physicochemical properties, which we believe provide good support for the likelihood that they produce similar radical species. Two further explanations for the greater radical production by MtHPI-e include the possibility that INH is a more effective substrate for MtHPI than for EcHPI, and the possibility that EcHPI is more susceptible to inactivation by INH-derived radicals. The latter possibility will be discussed below.

**Spectral Evaluation of INH Oxidation Mediated by MtHPI-e, EcHPI, and HRP**

The incubation of the compound II form of HRP with INH resulted in spectral shifts, including a bleaching of the Soret band, suggestive of heme splitting which would be synonymous with inactivation (10). It was unclear whether the decline in radical production in the presence of a constant flux of \(\text{H}_2\text{O}_2\) (Fig. 4) was the result of the establishment of a steady state of formation or of a progressive inhibition of the reaction(s) involved in the generation of radicals possibly by enzyme inactivation. To address this, we monitored both the spectra and the peroxidase activities of MtHPI-e, EcHPI, and HRP before and after exposure of the proteins to the peroxide-generating system, in the presence and absence of INH.

The absorption spectra of the enzymes before and after a 10-min incubation with the constant flux of \(\text{H}_2\text{O}_2\) from glucose oxidase/glucose with and without INH are shown in Fig. 5. In the absence of INH, there is a red shift of the Soret band of HRP to 418 nm, combined with the disappearance of the band at 639 nm and appearance of maxima at 550 and 580 nm (Fig. 5A). These spectral shifts are indicative of the formation of compound III or oxyperoxidase, a reversibly inactivated form of the enzyme that is typically observed at high peroxide–enzyme ratios or in the presence of certain electron donors (16). In the presence of INH, however, the Soret band was markedly reduced in intensity as well as red-shifted to 418 nm, and a broad band at 530 nm with a shoulder at 550 nm, as well as a peak at 670 nm, appeared. The maximum at 670 nm is indicative of the formation of compound IV or P670, a second, irreversibly inactivated form of the enzyme (15), while the peak with the shoulder at 530 nm suggests the presence of HRP compound II, or ferryl form of the enzyme. The decline in the intensity of the Soret peak is indicative of a major perturbation of the electronic structure of the heme, attributable to either heme modification or degradation. Indeed, the heme of HRP has been shown to be susceptible to reaction with phenylhydrazine-derived radicals, resulting in a similar spectral change (35).

Because a fast peroxidatic mode of reaction was not observed for HRP and because compound III and IV forms of the enzyme were observed, the involvement of
INH as an electron donor may therefore result in the partition of HRP compound II into the oxidatic mode and to accelerate the decay of compound III to compound I for reinitiation of the cycle. This may protect the HRP from further inactivation but may also be the reason for the significantly slower generation of radicals from INH compared to the MtHPI-e protein.

The spectra obtained for EcHPI and MtHPI-e (Figs. 5B and 5C) in presence of the peroxide-generating system, with and without INH, did not show spectral changes as distinct as those seen for HRP. In the absence of INH, the Soret peak and the band at 639 nm intensified slightly, while the 500-nm broad band declined in intensity. A similar type of spectral shift has been observed in the visible region of the spectrum for purified recombinant ascorbate peroxidase, when a stoichiometric amount of H$_2$O$_2$ was added to the enzyme (36). The presence of INH resulted in the formation of a broad band around 510 nm and a slight decline in the intensity of the peak at 639 nm for both enzymes. MtHPI-e showed no change in intensity of the Soret peak, whereas EcHPI showed a slight decline in the intensity of the same peak. These spectral changes suggest that the hemes of the catalase-peroxidases do not undergo major electronic perturbations or modification during reaction with H$_2$O$_2$ and INH under the conditions chosen. It should be pointed out, however, that the partition between the one-step, two-electron reduction of compound I via the catalatic cycle and the two-step, one-electron reductions of compound I via the peroxidatic cycle favors the former cycle's steady-state intermediate(s). This caveat requires these spectral results to be interpreted with appropriate caution.

As noted above, one possible explanation for the lower rate of INH-dependent radical production by

![Figure 5](image)

**FIG. 5.** HRP spectral changes during incubation with INH in presence of constantly generated H$_2$O$_2$. Conditions as described in the legend to Fig. 4. Spectra for each hemoprotein were obtained prior to initiation of H$_2$O$_2$ generation (solid lines), 10 min following addition of glucose (dotted lines), and 10 min following addition of glucose in presence of INH (9 mM) (broken lines). Insets show visible regions of the spectrum at scales expanded by the factors shown. (A) Spectra of HRP (30 μg). (B) Spectra of MtHPI-e (0.2 mg). (C) Spectra of EcHPI (0.2 mg).

an oxidatic mode may be inferred (17). The oxyperoxidase intermediate of the oxidase cycle has an extremely slow rate of decay and is usually formed at high [H$_2$O$_2$], and its function has been hypothesized to rescue the enzyme from irreversible inactivation. The presence of

### TABLE II

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>0 min</th>
<th>5 min</th>
<th>10 min</th>
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<tr>
<td>HRP</td>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EcHPI</td>
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<td>MtHPI-e</td>
<td>100</td>
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Note. Incubations were done under conditions described in the legend to Fig. 5. At the times indicated, aliquots were withdrawn from the incubation mixtures and assayed for peroxidatic activity as outlined under Materials and Methods.

Data are expressed as the percentage of o-dianisidine activity remaining compared to the activity before the addition of glucose.
The biochemical bases for INH resistance in a significant proportion of M. tuberculosis clinical isolates is thus also apparently realized. It is estimated that 25–50% of INH-resistant M. tuberculosis isolates are also catalase–peroxidase negative, and that another 20–25% of resistant isolates have mutations in the inhA locus (6). It would therefore appear that either modification of the gene for the target protein such that INH fails to covalently bind to INH-derived radicals, or inactivation of the gene for the prodrug activator (MtHPI) such that production of INH-derived radicals is eliminated or reduced, are two of the major determinants in strains of M. tuberculosis acquiring INH-resistant phenotypes.

ACKNOWLEDGMENTS

We thank Stewart Cole and Judah Rosner for the gifts of plasmid pYZ55 and various strains.

REFERENCES