Roles of Efficient Substrates in Enhancement of Peroxidase-Catalyzed Oxidations†

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Received June 20, 1996; Revised Manuscript Received October 16, 1996⊗

ABSTRACT: Efficient peroxidase substrates may have a critical role in the oxidation of secondary compounds by peroxidases. Hydrazines are often oxidized slowly by peroxidases due, in part, to hydrazine-dependent inactivation of these enzymes. Peroxidase-catalyzed oxidation of hydrazines may be dramatically affected by an efficient peroxidase substrate. We investigated this hypothesis in a model system using the well-known peroxidase substrate chlorpromazine (CPZ) and the hydrazine derivative isoniazid. CPZ stimulated isoniazid oxidation as measured by nitroblue tetrazolium (NBT) reduction and O_2 consumption. The kinetics of isoniazid and CPZ oxidation by horseradish peroxidase (HRP) in the presence of both compounds suggested CPZ was acting as an electron transfer mediator between HRP and isoniazid. Indeed, $CPZ^{\bullet+}$, the product of CPZ oxidation by HRP, was able to oxidize isoniazid. The rate constant for this pH-dependent reaction was $(2.6 \pm 0.1) \times 10^4\ M^{-1}\ s^{-1}$ at pH 4.5. In the absence of CPZ, isoniazid-dependent irreversible inactivation of HRP was observed. The inactivation process involved the formation of compound III followed by accumulation of irreversibly inactivated HRP. CPZ completely inhibited inactivation. Thus, by acting as a redox mediator and preventing HRP inactivation, CPZ stimulated isoniazid oxidation by several orders of magnitude. Similarly, other efficient peroxidase substrates, such as phenol and tyrosine, were also able to dramatically stimulate isoniazid oxidation by HRP. We suggest that the presence of efficient peroxidase substrates may potentiate the activation of isoniazid and other hydrazines. As such, these substrates may have a vital role in the pharmacological and toxicological properties of hydrazines and other compounds.

Most peroxidases are heme proteins which catalyze the reduction of H_2O_2 to H_2O and the concomitant oxidation of a wide range of reducing substrates (Dunford & Stillman, 1976; Dunford, 1982). Many reducing substrates for peroxidases are oxidized by one electron, resulting in the production of diffusible free radical intermediates (Yamazaki et al., 1960). The catalytic cycles of most peroxidases are thought to closely resemble that described for horseradish peroxidase (HRP)[1] (Chance, 1952; Dunford & Stillman, 1976; Dunford, 1982). Ferric HRP (Fe^{III}-HRP) is oxidized by two electrons by H_2O_2 to form an oxyferryl porphyrin π cation radical heme intermediate (reaction 1) known as compound I (cmpd-I). The porphyrin radical of cmpd-I accepts one electron from a reducing substrate (RH_2) to yield the corresponding substrate free radical ($RH^{\bullet}$) and the oxyferryl intermediate known as compound II (cmpd-II) (reaction 2). A subsequent one-electron reduction of cmpd-II by a second RH_2 molecule results in the formation of ferric peroxidase and a second equivalent of $RH^{\bullet}$ (reaction 3):

$$Fe^{III}\text{-HRP} + H_2O_2 \rightarrow \text{cmpd-I} + H_2O \quad (1)$$

$$\text{cmpd-I} + RH_2 \rightarrow \text{cmpd-II} + RH^{\bullet} \quad (2)$$

$$\text{cmpd-II} + RH_2 \rightarrow Fe^{III}\text{-HRP} + RH^{\bullet} + H_2O \quad (3)$$

Another interesting aspect of peroxidase catalysis is the formation of compound III, an inactive form of these enzymes. Compound III is structurally and electronically similar to oxymyoglobin and oxyhemoglobin, and for this reason, compound III is often referred to as oxyperoxidase (Wittenberg et al., 1967). The heme of peroxidase compound III is thought to exist as a resonance form between the ferrous−O_2 and ferric−$O_2^{\bullet-}$ complexes. Three pathways for the formation of peroxidase compound III have been elucidated (Yamazaki & Piette, 1963). Reaction of compound II (cmpd-II) with H_2O_2 (reaction 4) is thought to be the reason for compound III (cmpd-III) formation of peroxidases in the presence of excess H_2O_2. The second pathway involves the reaction of ferric peroxidase (Fe^{III}-Px) with $O_2^{\bullet-}$ (reaction 5). Cmpd-III can also be formed by the reaction of ferrous peroxidase (Fe^{II}-Px) with O_2 (reaction 6):

$$\text{cmpd-II} + H_2O_2 \rightarrow \text{cmpd-III} + H_2O \quad (4)$$

$$Fe^{III}\text{-Px} + O_2^{\bullet-} \rightarrow \text{cmpd-III} \quad (5)$$

$$Fe^{II}\text{-Px} + O_2 \rightarrow \text{cmpd-III} \quad (6)$$

While compound III is largely regarded as an inactive form of peroxidase, this inactivation is reversible. In the presence of excess H_2O_2, however, HRP compound III can be converted to another inactive form known as P670 (λ_{max} = 670 nm) (Adediran, 1996; Yamazaki et al., 1970). Unlike compound III, P670 is an irreversibly inactive form of HRP (Arnao et al., 1990).

It has been observed that oxidation of various compounds by peroxidases can be dramatically affected by the presence of other substrates. A number of mechanisms have been proposed to explain the role of these peroxidase substrates

† This work was supported by NIH Grant ES05056.

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⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.

[1] Abbreviations: CPZ, chlorpromazine; $CPZ^{\bullet+}$, CPZ cation radical; HRP, horseradish peroxidase; SOD, superoxide dismutase; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; NBT, nitroblue tetrazolium.

S0006-2960(96)01465-1 CCC: $14.00

in the oxidation of other chemicals. Some have suggested that the presence of efficient peroxidase substrates may prevent the accumulation of peroxidases in their inactive compound III state (Koduri & Tien, 1994; Valli et al., 1990). It has also been proposed that these substrates may participate in the conversion of inactive compound III to the active ferric form (Barr & Aust, 1994; Wariishi & Gold, 1990; Yakota & Yamazaki, 1965). Furthermore, we and other investigators have suggested that some peroxidase substrates may also act as mediators of electron transfer between the peroxidase and other compounds (Goodwin et al., 1995a; Koduri & Tien, 1995). That is, oxidation of one substrate (A) by peroxidases results in the production of a diffusible radical ($A^{\bullet+}$) which can carry out the oxidation of secondary compounds (B) (reaction 7):

$$A^{\bullet+} + B \rightarrow A + B^{\bullet+} \quad (7)$$

Clearly, the oxidation of compounds by peroxidases can become very complex in the presence of multiple substrates. Given the nonspecificity of peroxidases for reducing substrates (Dunford, 1982), it is reasonable to suggest that these enzymes may often be in an environment with multiple reducing substrates. Thus, it is important to understand the interactions between peroxidases and their substrates, particularly in environments containing a number of these compounds.

We investigated the effect of some common peroxidase substrates, chlorpromazine (CPZ), tyrosine, and phenol, on the oxidation of a number of hydrazines by HRP. Oxidation of some of these hydrazines by peroxidases is thought to be important for both the pharmacological and toxicological effects of hydrazine-containing drugs. Our data show clearly that efficient peroxidase substrates have a dramatic stimulatory effect on oxidation of hydrazines such as isonizaid.

CPZ is oxidized by peroxidases to a stable radical which can be easily detected. Moreover, the oxidation of isoniazid by peroxidases can be easily monitored by either O_2 consumption or nitroblue tetrazolium reduction. Thus, we studied the stimulation of peroxidase-catalyzed oxidation of hydrazines by efficient reducing substrates using isoniazid and CPZ in a model system with HRP. We demonstrate using CPZ that this stimulatory effect arises for two reasons. First, CPZ acts as a mediator for electron transfer between HRP and isoniazid. Second, CPZ prevents the isoniazid-dependent and H_2O_2-dependent irreversible inactivation of HRP. We investigated both the mechanism of isoniazid-dependent inactivation and the mechanism of protection by CPZ. The implications of these reaction mechanisms in the metabolism and potential toxicity of compounds which might be metabolized by peroxidases are discussed.

MATERIALS AND METHODS

Chemicals. Hydrogen peroxide, isoniazid, nitroblue tetrazolium (NBT), HRP (type VI), SOD (from bovine erythrocytes), catalase, and DMPO were purchased from Sigma (St. Louis, MO). Buffers were prepared using purified water (Barnstead NANOpure II system; specific resistance 18.0 MΩ/cm). Buffers were then treated with Chelex 100 (BioRad, Richmond, CA). Concentrations of HRP were determined at 403 nm using an extinction coefficient of 102 000 M^{-1} cm^{-1} (Dunford, 1976).

Peroxidase Assays. Reduction of NBT to monoformazan was monitored spectrophotometrically at 560 nm using an extinction coefficient of 15 000 M^{-1} cm^{-1} (Auclair & Voisin, 1985). Oxidation of CPZ to $CPZ^{\bullet+}$ was monitored at 525 nm. All assays were performed using a Shimadzu UV2101-PC spectrophotometer.

O_2 Consumption. Changes in O_2 concentration during peroxidase-catalyzed reactions under various conditions were monitored using a Gilson 5/6 oxygraph (Middleton, WI). The instrument was equipped with a Clark-type O_2 sensitive electrode, and a 1.8 mL water-jacketed reaction chamber.

$CPZ^{\bullet+}$ Reduction by Isoniazid. The reaction between isoniazid and $CPZ^{\bullet+}$ was investigated using a three-syringe stopped-flow spectrophotometer from KinTek Instruments (State College, PA). $CPZ^{\bullet+}$ was generated in a delay line by mixing the contents of a syringe containing 100 nM HRP, and a second syringe containing 1 mM CPZ, 50 μM H_2O_2, and 1 mM acetate buffer, pH 4.5. After a 20 s delay to allow for development of $CPZ^{\bullet+}$, the contents of the delay line were then mixed with varying concentrations of isoniazid and 100 mM acetate buffer, pH 4.5. With this arrangement, ∼20 μM $CPZ^{\bullet+}$ was present at the beginning of the reaction with isoniazid. The rate of the reaction was monitored by a decrease in absorbance at 525 nm. To determine rate constants at pH values in the range from 3.0 to 5.5, $CPZ^{\bullet+}$ was generated as described above in buffer of the desired pH. This $CPZ^{\bullet+}$ solution was then mixed with varying concentrations of isoniazid in 100 mM buffer at the desired pH. In order to obtain rate constants for the reaction between $CPZ^{\bullet+}$ and isoniazid at pH 2.0 and 2.5, $CPZ^{\bullet+}$ was generated in a delay line as described above in 1 mM acetate buffer, pH 3.0. This $CPZ^{\bullet+}$ was then mixed with variable concentrations of isoniazid in 100 mM buffer at pH 2.0 or 2.5. Rate constants for this reaction at pH values ranging from 0.5 to 1.5 were obtained as previously described for $CPZ^{\bullet+}$ reduction by aminopyrine (Goodwin et al., 1996). The buffers used for these experiments were acetate buffer (pH 4.0−5.5), citrate buffer (2.5−3.5 and 6.0), and phosphate buffer (pH 2.0). HCl was used for rate constants determined from pH 0.5 to 1.5.

HRP Inactivation Studies. HRP was incubated under a variety of reaction conditions (see figure legends for details), and aliquots were removed and assayed for activity as measured by NBT reduction. Assay mixtures contained 50 mM acetate buffer, pH 4.5, 1 mM CPZ, 1 mM NBT, and 1 mM isoniazid. In order to minimize interference from incubation components in the assay, aliquots from the incubation mixtures were diluted before the assays. A concentration of 10 μM HRP in an incubation reaction resulted in a 10 nM concentration of HRP in each assay.

HRP-Monitored Stopped-Flow Kinetics. The reduction of HRP compound I to compound II by isoniazid was monitored at 411 nm, the isosbestic wavelength of ferric HRP and HRP compound II. Sequential reduction of HRP compound I to compound II and compound II to ferric HRP was monitored at 428 nm, the isosbestic wavelength for ferric HRP and compound I. The three-syringe stopped flow was used for these experiments. Formation of HRP compound I was accomplished as previously described (Goodwin et al., 1995b).

HRP Absorption Spectra. The effect of various reaction conditions on the visible absorption spectrum of HRP was monitored using the UV2101-PC spectrophotometer. Spectra were recorded from 700 to 500 nm with a slit width of 2 nm and a sampling interval of 0.5 nm. The formation of

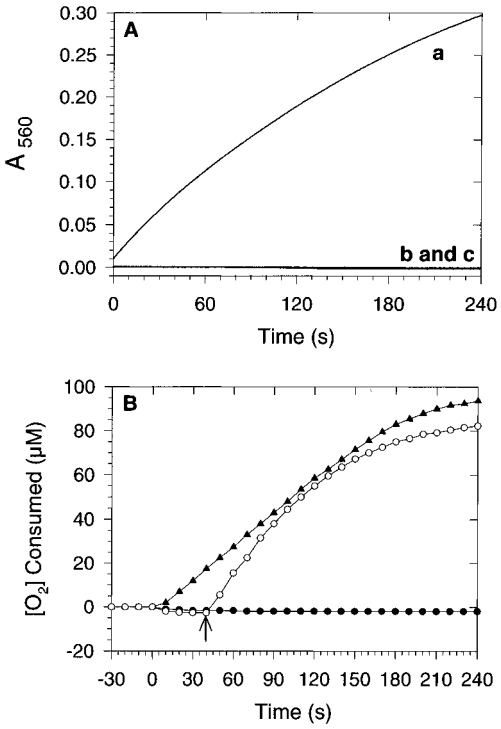

FIGURE 1: Effect of CPZ on isoniazid oxidation by HRP. Isoniazid oxidation was determined by monitoring NBT reduction (panel A) or O_2 consumption (panel B). Reactions in panel A all contained 10 nM HRP, 500 μM H_2O_2, and 1 mM isoniazid. Reactions also contained either 1 mM NBT and 1 mM CPZ (a), 1 mM NBT (b), or 1 mM CPZ (c). Reactions in panel B all contained 10 nM HRP and 500 μM H_2O_2. These reactions also contained 1 mM CPZ and 1 mM isoniazid (closed triangles), 1 mM isoniazid alone (closed circles), or 1 mM isoniazid (added at the time indicated by the arrow) and 1 mM CPZ (open circles). All reactions were initiated at time 0 by addition of H_2O_2. All reactions were carried out using 50 mM acetate buffer, pH 4.5.

the P670 intermediate of HRP with time was monitored at 670 nm.

RESULTS

It is known that peroxidase-catalyzed oxidation of isoniazid results in NBT reduction and O_2 consumption (Shoeb et al., 1985; Hillar & Loewen, 1995). Some have suggested that O_2 consumption and NBT reduction result from the formation of an organic radical that is generated during isoniazid oxidation (Shoeb et al., 1985; Hillar & Loewen, 1995). Two possible reactions are shown below (reactions 8 and 9):

$$R{-}NH{-}NH^{\bullet} + O_2 \rightarrow R{-}N{=}NH + O_2^{\bullet -} + H^+ \quad (8)$$

$$R{-}NH{-}NH^{\bullet} + NBT_{ox} \rightarrow R{-}N{=}NH + NBT_{red} \quad (9)$$

The effect of CPZ on isoniazid oxidation by HRP as determined by these two assays is shown in Figure 1. In the absence of CPZ, little or no NBT reduction was observed (Figure 1A). However, when 1 mM CPZ was present with isoniazid, H_2O_2, and HRP, the reduction of NBT was greatly stimulated. No change in absorbance at 560 nm was observed when NBT was excluded from the reaction. This indicated that absorbance changes at this wavelength were due to NBT reduction, not accumulation of $CPZ^{\bullet +}$ (λ_{max} = 525 nm). CPZ also stimulated NBT reduction at higher pH. However, the effect was less dramatic as only 5−10-fold stimulation was observed at pH 7. It has been shown previously that NBT reduction during isoniazid oxidation by

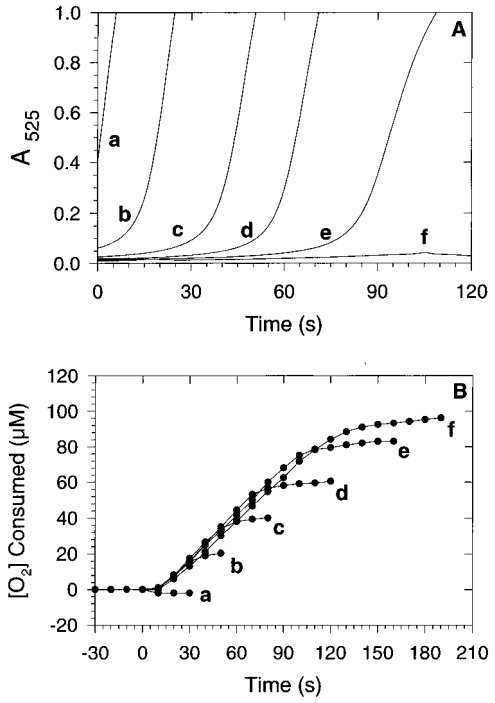

FIGURE 2: Effect of isoniazid concentration on CPZ oxidation to $CPZ^{\bullet +}$ and O_2 consumption by HRP. Panel A shows $CPZ^{\bullet +}$ formation monitored at 525 nm, while panel B shows O_2 consumption under identical reaction conditions. Reactions contained 10 nM HRP, 500 μM H_2O_2, 1 mM CPZ, and no isoniazid (a), 50 μM isoniazid (b), 100 μM isoniazid (c), 150 μM isoniazid (d), 200 μM isoniazid (e), or 250 μM isoniazid (f). All reactions were initiated at time 0 with H_2O_2. All reactions were carried out at pH 4.5 in 50 mM acetate buffer.

peroxidase is superoxide-independent (Shoeb et al., 1985; Hillar & Loewen, 1995). Likewise, addition of SOD to reactions containing HRP, isoniazid, H_2O_2, and CPZ had no effect on NBT reduction (data not shown). Phenol (1 mM) and tyrosine (1 mM), two other peroxidase substrates, also stimulated NBT reduction in the presence of isoniazid, HRP, and H_2O_2 (data not shown). The effect of the phenolic substrates at pH 7 was similar to that observed with CPZ at pH 4.5.

As observed for NBT reduction, the presence of CPZ had a dramatic effect on O_2 consumption (Figure 1B). No O_2 uptake was observed in the absence of CPZ. Addition of H_2O_2 to reactions which contained HRP, CPZ, and isoniazid resulted in high rates of O_2 consumption. A reaction containing HRP and CPZ did not result in O_2 consumption upon addition of H_2O_2. However, addition of isoniazid to this reaction 40 s after initiation with H_2O_2 resulted in rapid uptake of O_2.

Consistent with previous reports (Shoeb et al., 1985; Hillar & Loewen, 1995), the rate of isoniazid oxidation by HRP in the absence of CPZ was very slow. We obtained apparent second-order rate constants of approximately 400 M^{-1} s^{-1} and 40 M^{-1} s^{-1} for compound I and compound II reduction by isoniazid, respectively. These rate constants are considerably slower than those obtained previously for other HRP reducing substrates (Job & Dunford, 1976; Dunford & Adeniran, 1986).

The effect of isoniazid concentration on CPZ oxidation to $CPZ^{\bullet +}$ by HRP is shown in Figure 2A. The presence of isoniazid resulted in a lag in $CPZ^{\bullet +}$ accumulation, and the duration of the lag period was dependent on isoniazid concentration. After isoniazid was consumed, rapid ac-

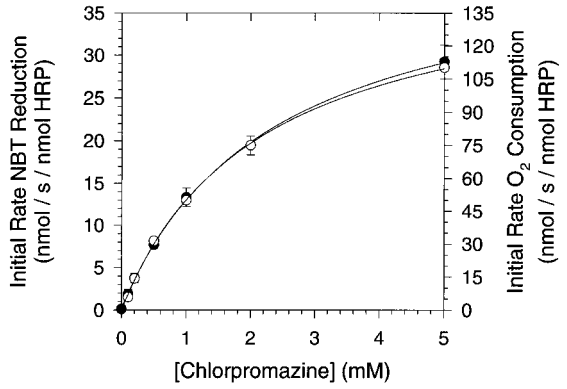

FIGURE 3: Effect of CPZ concentration on isoniazid oxidation by HRP. Isoniazid oxidation was determined by monitoring NBT reduction (closed circles) or O_2 consumption (open circles). Reactions contained 10 nM HRP, 500 μM H_2O_2, 1 mM isoniazid, and 50 mM acetate buffer, pH 4.5. NBT reduction experiments were carried out using 1 mM NBT.

cumulation of $CPZ^{\bullet+}$ ensued. We observed a similar effect when hydralazine or phenylhydrazine replaced isoniazid (data not shown). In reactions containing 250 μM isoniazid, the duration of the lag period was indefinite. This was due to the complete consumption of H_2O_2 before the complete oxidation of isoniazid. The effect of isoniazid concentration on O_2 consumption in the presence of CPZ was also investigated (Figure 2B). The rate of O_2 consumption was essentially isoniazid concentration independent. However, the extent of O_2 consumption was proportional to the initial concentration of isoniazid.

Increasing CPZ concentrations increased the rate of isoniazid-dependent O_2 consumption and NBT reduction by HRP (Figure 3). The initial rates of both of these events appeared to be saturable with respect to CPZ, and the K_M values obtained from each curve were 2.3 $\pm$ 0.2 mM for NBT reduction and 2.1 mM for O_2 consumption. These values are nearly identical to the K_M obtained previously for direct oxidation of CPZ by HRP (2.1 $\pm$ 0.2 mM) (Goodwin et al., 1996).

The kinetic data observed in Figures 2 and 3 suggested that CPZ may stimulate peroxidase-catalyzed isoniazid oxidation by acting as a redox mediator. That is, $CPZ^{\bullet+}$, generated from CPZ by HRP, may oxidize isoniazid (INH) to the corresponding radical ($INH^{\bullet}$) (reaction 10):

$$CPZ^{\bullet+} + INH \rightarrow CPZ + INH^{\bullet} + H^{+} \qquad (10)$$

The reaction between $CPZ^{\bullet+}$ and isoniazid was investigated using a stopped-flow kinetic method (see Materials and Methods). In the presence of 10-fold or greater excess isoniazid, the decrease in absorbance at 525 nm due to reduction of $CPZ^{\bullet+}$ was exponential (Figure 4). The rate of the reaction was linearly dependent upon isoniazid concentration, and a second-order rate constant of (2.6 $\pm$ 0.1) $\times$ 10^4 M^{-1} s^{-1} was determined at pH 4.5 (Figure 4, inset). We also observed that $CPZ^{\bullet+}$ rapidly oxidized hydralazine and phenylhydrazine (data not shown). The rate constant for isoniazid oxidation by $CPZ^{\bullet+}$ was pH-dependent (Figure 5). As the pH increased, the rate constant also increased. This pH dependence is consistent with the reactions of $CPZ^{\bullet+}$ and other cation radicals with a variety of reductants (Wolfenden & Willson, 1982; Pelizzetti et al., 1979).

Others have observed that HRP may be irreversibly inactivated in the presence of isoniazid and H_2O_2 (Shoeb et al., 1985; Hillar & Loewen, 1995). Our results confirmed this observation (Figure 6). HRP inactivation was dependent upon H_2O_2 concentration (Figure 6A). Moreover, isoniazid appeared to enhance H_2O_2-dependent inactivation of HRP (Figure 6B).

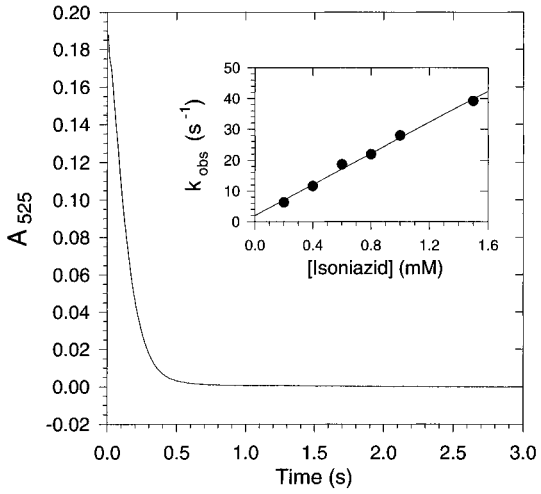

FIGURE 4: Change in absorbance at 525 nm during $CPZ^{\bullet+}$ reaction with isoniazid. The reaction contained <20 μM $CPZ^{\bullet+}$ (prepared as described under Materials and Methods), 200 μM isoniazid, and 30 mM acetate buffer, pH 4.5. The inset shows the effect of isoniazid concentration on the rate of $CPZ^{\bullet+}$ reduction at pH 4.5.

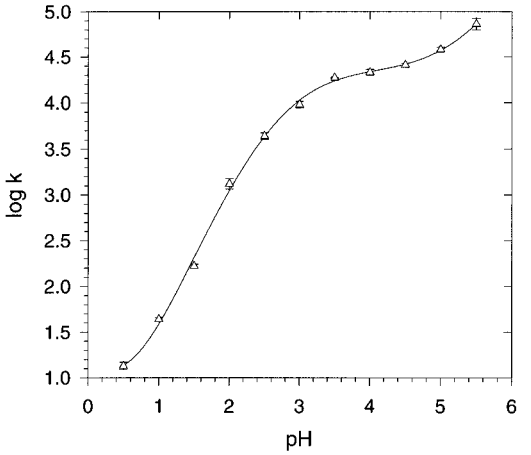

FIGURE 5: Effect of pH on the rate constant for $CPZ^{\bullet+}$ reduction by isoniazid. Rate constants were obtained for each pH as described under Materials and Methods. Points represent the mean of triplicate measurements, and the error bars represent standard deviations.

Given the stimulatory effect of CPZ on isoniazid oxidation by HRP, we investigated the effect of CPZ on the inactivation of HRP (Figure 7). Little or no peroxidase inactivation was observed in the absence of H_2O_2. Hydrogen peroxide was able to inactivate HRP in the absence of isoniazid; however, the maximal rate of HRP inactivation was observed when isoniazid was also present. Interestingly, the presence of CPZ almost completely prevented the inactivation of HRP.

To investigate the mechanism of isoniazid-dependent inactivation of HRP, and to determine the mechanism of protection by CPZ, visible absorption spectra of HRP were recorded under various reaction conditions (Figure 8). In the absence of isoniazid and H_2O_2, ferric HRP was detected (λ_{max} = 500 and 640 nm) (Figure 8A, solid line). Upon addition of H_2O_2, the spectrum changed to that of compound I with absorption maxima at 577, 622, and 651 nm (Figure 8A, dotted line). There also appeared to be a small amount of compound II present in this spectrum as well. After 6

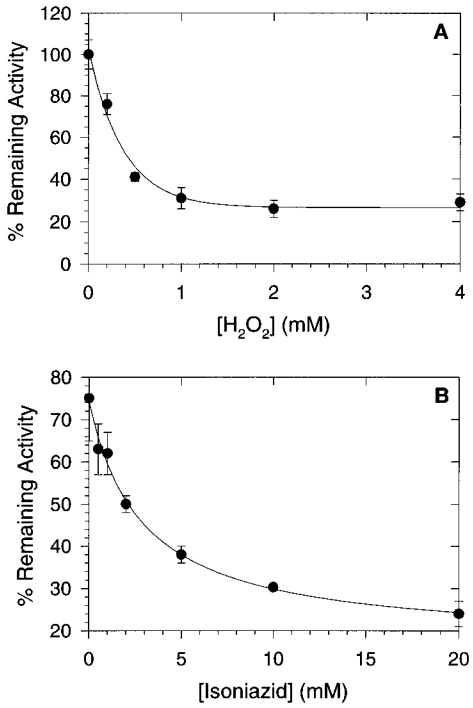

FIGURE 6: HRP activity remaining after turnover in reaction mixtures containing increasing concentrations of isoniazid or H_2O_2. Panel A reaction mixtures contained 10 μM HRP, 10 mM isoniazid, 50 mM acetate buffer, pH 4.5, and variable concentrations of H_2O_2. Panel B reaction mixtures contained 10 μM HRP, 1 mM H_2O_2, 50 mM acetate buffer, pH 4.5, and variable concentrations of isoniazid. Aliquots were removed from each reaction 2 min after initiation with H_2O_2 and assayed as described under Materials and Methods. HRP incubated for 2 min in the absence of H_2O_2 and isoniazid was taken to be 100%. Each point represents the mean of triplicate measurements, and the error bars represent standard deviations.

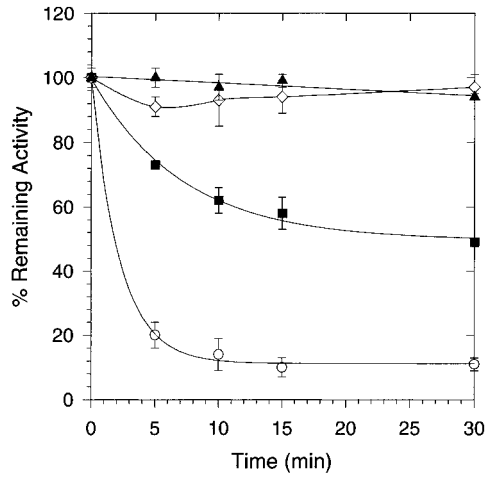

FIGURE 7: Effect of CPZ on HRP inactivation in the presence of isoniazid and H_2O_2. Reaction mixtures contained 10 μM HRP, 50 mM acetate buffer, pH 4.5: and 10 mM isoniazid (closed triangles); 1 mM H_2O_2 (closed squares); 10 mM isoniazid and 1 mM H_2O_2 (open circles); or 10 mM isoniazid, 1 mM H_2O_2, and 1 mM CPZ (open diamonds). Aliquots were removed from each reaction at various times after initiation with H_2O_2 and assayed as described under Materials and Methods. Each point represents the mean of triplicate measurements, and the error bars represent the standard deviations.

min, the spectrum had changed to yield a mixture of compound II (λ_{max} = 527 and 554 nm), compound III (λ_{max} = 546 and 580 nm), and P670 (λ_{max} = 670 nm) (Figure 8A, dashed line).

In the presence of isoniazid alone, ferric HRP was detected (Figure 8B, solid line). Upon addition of H_2O_2, the spectrum

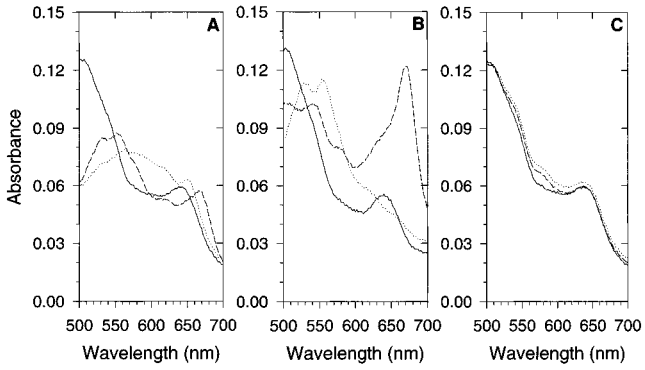

FIGURE 8: Visible absorption spectra of HRP during isoniazid oxidation. Reactions contained 10 μM HRP, 50 mM acetate buffer, pH 4.5, and 1 mM H_2O_2 alone (panel A), 1 mM H_2O_2 and 10 mM isoniazid (panel B), or 1 mM H_2O_2, 10 mM isoniazid, and 1 mM CPZ (panel C). Spectra were recorded before addition of H_2O_2 (solid lines), immediately after addition of H_2O_2 (dotted lines), and 6 min after addition of H_2O_2 (dashed lines).

changed to that of compound II (Figure 8B, dotted line). Six minutes later, the spectrum had changed further to contain a mixture of compound III and P670 (Figure 8B, dashed line). Moreover, the extent of P670 formation in the presence of isoniazid and H_2O_2 was much greater than that observed with H_2O_2 alone. In the presence of isoniazid and CPZ, HRP was observed in the ferric state. Unlike spectral changes observed in the absence of CPZ (Figure 8A/8B), very little change in the absorption spectrum was observed upon addition of H_2O_2 (Figure 8C, dotted line). Similarly, after 6 min, ferric HRP was still present as the predominant form of the enzyme (Figure 8C, dashed line).

The effect of isoniazid concentration on the rate of P670 formation was examined (Figure 9A). Increasing isoniazid concentration increased the rate of P670 formation far above that observed in the presence of H_2O_2 alone. SOD did inhibit the rate of isoniazid-dependent P670 accumulation by 10%; however, the same effect was also observed with boiled SOD (data not shown). NBT, which reacts with isoniazid radicals, was able to inhibit irreversible inactivation of HRP. Increasing concentrations of NBT decreased the rate of P670 accumulation, but CPZ was more effective, as only 1 mM CPZ completely prevented accumulation of P670.

Detection of P670 in reactions with HRP, H_2O_2, and isoniazid suggested that HRP compound III may be formed as an intermediate in HRP inactivation. To test this hypothesis, we monitored changes in the visible absorption spectrum of HRP during stepwise addition of single equivalents of H_2O_2 in the presence of isoniazid (Figure 10A). With each addition of H_2O_2, the absorbance increased at 546 and 580 nm and decreased at 500 and 640 nm with isosbestic points at 600 and 530 nm. These data are consistent with the accumulation of HRP compound III. When CPZ was present with HRP and isoniazid, stepwise addition of H_2O_2 only resulted in minor changes in the absorption spectrum (Figure 10B). This indicated that little or no compound III was formed in the presence of CPZ. Similarly, NBT was able to prevent HRP compound III formation (data not shown).

To investigate the pathways involved in isoniazid-dependent compound III formation, reactions were carried out as in Figure 8B in the presence and absence of carbon monoxide (CO). In the absence of CO, a spectrum similar to that observed in Figure 8B (dashed line) was observed 6 min after the addition of H_2O_2 (Figure 11, solid line). When the

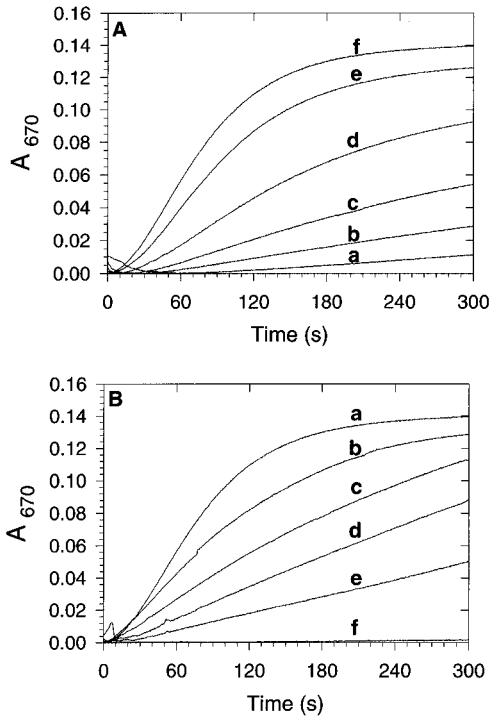

FIGURE 9: Effect of isoniazid, NBT, and CPZ on change in absorbance at 670 nm during reaction of HRP with isoniazid. Reactions represented in panel A contained 10 μM HRP, 1 mM H_2O_2, 50 mM acetate buffer, pH 4.5, and no isoniazid (a), 500 μM isoniazid (b), 1 mM isoniazid (c), 2 mM isoniazid (d), 5 mM isoniazid (e), and 10 mM isoniazid (f). Reactions represented in panel B contained 10 μM HRP, 1 mM H_2O_2, 10 mM isoniazid, 50 mM acetate buffer, pH 4.5, and no NBT or CPZ (a), 500 μM NBT (b), 1 mM NBT (c), 2 mM NBT (d), 3 mM NBT (e), or 1 mM CPZ (f).

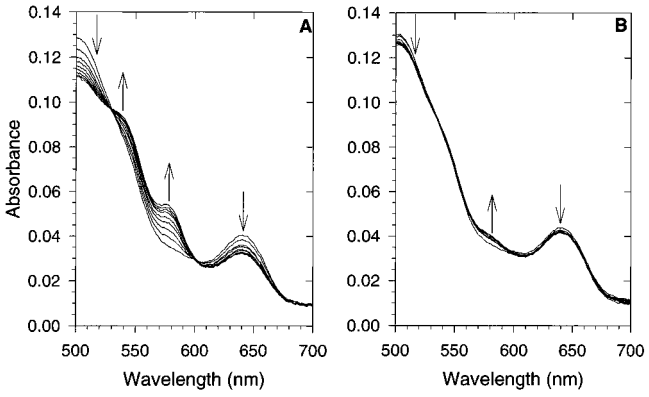

FIGURE 10: Effect of CPZ on changes in the visible absorption spectrum of HRP during stepwise addition of H_2O_2 in the presence of isoniazid. Each reaction contained 10 μM HRP, 10 mM isoniazid, 50 mM acetate buffer, pH 4.5, and no CPZ (panel A), or 1 mM CPZ (panel B). The first spectrum in each panel was obtained prior to addition of H_2O_2. H_2O_2 was added in 10 μM increments, and a spectrum was recorded after each addition. The directions of change in absorbance with each H_2O_2 addition are indicated by the arrows.

reaction was carried out in the presence of CO, peaks were observed at 540, 573, and 630 nm (Figure 11, dotted line). Peaks at 540 nm and 573 nm were indicative of the ferrous HRP−CO complex. The substantial peak at 630 nm was evidence that P670 was also produced. It has been shown that P670 is converted to another species in the presence of CO. This species (P630) has an absorbance maximum at 630 nm (Yamazaki et al., 1970). A comparison of the intensities of the peaks at 670 nm (solid line) and 630 nm (dotted line) and the relative extinction coefficients of these species indicated that CO inhibited some formation of

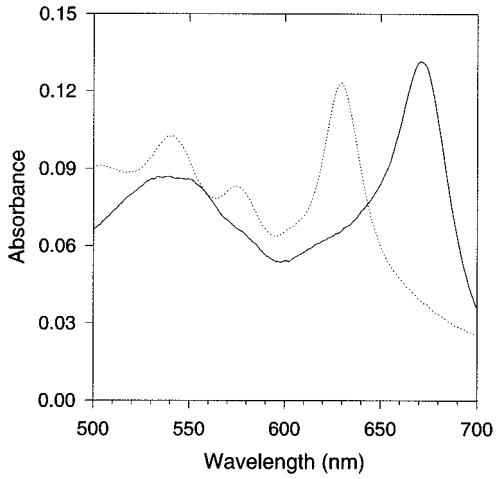

FIGURE 11: Visible absorption spectra of HRP during isoniazid-dependent irreversible inactivation in the presence and absence of CO. Reactions contained 10 μM HRP, 1 mM H_2O_2, and 10 mM isoniazid. The reaction without CO is indicated by the solid line, and the reaction with CO is indicated by the dotted line. When present, CO was added by bubbling CO through the reaction mixture for 30 min prior to addition of HRP and an additional 5 min prior to initiation with H_2O_2. All reactions were carried out in 50 mM acetate buffer, pH 4.5. All spectra were recorded 6 min after initiation with H_2O_2.

irreversibly inactivated HRP. Interestingly, when the same experiment was performed using 10-fold less H_2O_2, CO was able to completely prevent the formation of irreversibly inactivated HRP (either P670 or P630) (data not shown). Additionally, a small amount of ferrous−CO complex was detected in place of compound III.

DISCUSSION

It is clear that CPZ stimulates HRP-catalyzed isoniazid oxidation. Due to the magnitude of stimulation that is observed, it is important to address the role of CPZ and other efficient peroxidase substrates in stimulating the oxidation of hydrazines and other chemicals. In our model system with isoniazid, CPZ, and HRP, this effect arises due to the ability of CPZ to act as a mediator of electron transfer between HRP and isoniazid. In other words, $CPZ^{\bullet+}$, generated by reaction of HRP with CPZ, oxidizes isoniazid to the corresponding radical. This is consistent with the ability of $CPZ^{\bullet+}$ to act as a powerful oxidant ($E^\circ = 0.78$ V) (Pelizzetti et al., 1979), and the observation that hydrazines are, in general, good reductants (Juchau & Horita, 1972). As peroxidases are able to convert many substrates to highly oxidizing radicals, it is reasonable to suggest that any number of compounds may be able to act as redox mediators. One would expect, therefore, that redox mediation might be a common phenomenon in peroxidase catalysis. Moreover, the participation of this mechanism in a particular system should be readily observable through a few key kinetic measurements.

The role of CPZ as a redox mediator in our model system is evident from the complete inhibition of $CPZ^{\bullet+}$ accumulation in the presence of isoniazid, the dependence of the rate of isoniazid oxidation on CPZ, and the independence of the rate of isoniazid oxidation on its concentration. These kinetic features are typical of redox mediation in peroxidase-catalyzed oxidation (Goodwin et al., 1995a). They indicate that the rate-limiting step of isoniazid oxdiation in the presence of the mediator (CPZ) is the turnover of the

peroxidase. Thus, the rate of isoniazid oxidation in the presence or absence of CPZ is strictly dependent on HRP turnover. In light of the comparative rate constants for HRP compound II reduction by CPZ (4.5×10^5 M^{-1} s^{-1}) and isoniazid ($\sim$40 M^{-1} s^{-1}), an explanation of the stimulation of isoniazid oxidation by CPZ becomes clear. The ability of CPZ to turn HRP over at much higher rates than isoniazid, coupled with the fact that $CPZ^{\bullet+}$ can oxidize isoniazid at high rates, results in the dramatic stimulation of HRP-catalyzed isoniazid oxidation in the presence of CPZ. Thus, the slow rate of isoniazid oxidation by HRP contributes to the dramatic effect of CPZ and the other efficient peroxidase substrates. In this regard, it is interesting to note that bacterial catalase/peroxidase enzymes also oxidize isoniazid rather slowly with apparent k_{cat} values ranging from approximately 0.1 to 0.65 s^{-1} (Hillar & Loewen, 1995). It is possible that an efficient substrate may have a similar effect on isoniazid oxidation by these enzymes.

Stimulation of isoniazid oxidation by CPZ also results from the ability of CPZ to completely prevent the isoniazid-dependent inactivation of HRP. It is important to first discuss the mechanism of peroxidase inactivation by isoniazid. It has been shown that the oxidation of various hydrazine-containing compounds results in the formation of radicals which covalently modify the heme group (Ator & Ortiz de Montellano, 1987; Ator et al., 1987; Ortiz de Montellano et al., 1988). Others have suggested that irreversible inactivation of HRP during isoniazid oxidation may proceed by a similar mechanism (Hillar & Loewen, 1995; Shoeb et al., 1985). Our results suggest that although the mechanism of isoniazid-dependent peroxidase inactivation is similar to that observed for other hydrazines, there are some noteworthy differences. Unlike inactivation with other hydrazines, isoniazid caused the accumulation of HRP as P670. The P670 intermediate has been associated with the H_2O_2-dependent irreversible inactivation of HRP in the absence of a reducing substrate (Adediran, 1996; Arnao et al., 1990). Isoniazid enhanced the accumulation of P670 only in the presence of H_2O_2. Furthermore, NBT (which reacts with isoniazid radical) inhibited the accumulation of P670. Thus, similar to inactivation by other hydrazines, isoniazid radical was implicated in the inactivation mechanism. The accumulation of P670, however, suggested that the interaction of the isoniazid radical with the peroxidase was different than that observed for other hydrazines.

The formation of P670 suggested that compound III may be an intermediate in the isoniazid-dependent inactivation mechanism (Adediran, 1996). Indeed, we observed the formation of HRP compound III in the presence of isoniazid upon the addition of single equivalents of H_2O_2. The minute concentrations of H_2O_2 present in these experiments suggested that reaction of excess H_2O_2 with compound II, if involved, was not the only mechanism for compound III formation. The inability of SOD to prevent inactivation eliminated the reaction of $O_2^{\bullet-}$ with ferric HRP as the mechanism of compound III formation. We propose that compound III is formed by the initial reduction of ferric HRP to the ferrous state by isoniazid radical. The subsequent rapid reaction of ferrous HRP with O_2 yields compound III (Figure 12A). The ability of both NBT and CO to inhibit formation of compound III and irreversibly inactivated HRP supports this proposed mechanism. The detection of ferrous HRP−CO complex in the presence of HRP, H_2O_2, INH, and CO also supports this hypothesis. This mechanism of HRP

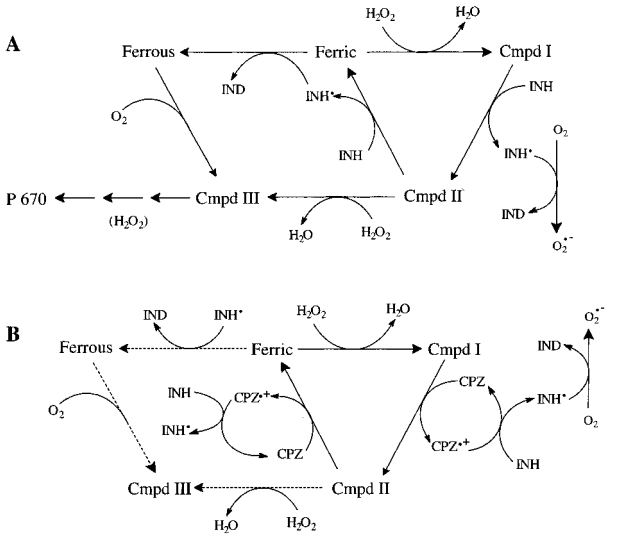

FIGURE 12: Scheme representing the proposed mechanism for isoniazid-dependent HRP inactivation and the mechanism of stimulation of isoniazid oxidation by CPZ. The proposed mechanism in the absence of CPZ is shown in panel A, and the mechanism in the presence of CPZ is shown in panel B. Cmpd I, Cmpd II, and Cmpd III represent HRP compound I, compound II, and compound III, respectively. Ferric and ferrous refer to ferric HRP and ferrous HRP, respectively. Isoniazid is represented by INH, and the isoniazid radical is shown as $INH^{\bullet}$. IND represents isonicotinic acid diimide. The dashed arrows indicate reactions which are minimized in the presence of CPZ.

inactivation appears to be distinct from that observed with other hydrazines. However, it is important to recognize that in the case of each hydrazine a reactive radical is generated in close proximity to the heme group.

Although HRP may be inactivated by the above mechanism, other inactivation pathways may also be important, especially in the presence of high concentrations of H_2O_2. The rate constants for HRP compound I and compound II reduction by isoniazid indicate that compound II would be the steady-state intermediate for isoniazid oxidation. The reaction of compound II with isoniazid is relatively slow (about 40 M^{-1} s^{-1}). Once compound II is initially formed, its steady-state concentration will remain high until the H_2O_2 has been consumed. In the presence of high concentrations of H_2O_2, this will allow for the substantial accumulation of compound III and P670. Isoniazid would also be expected to accelerate this mechanism of inactivation. The reaction of isoniazid with compound I may result in the more rapid generation of compound II than is possible in its absence. This accelerated rate of compound II formation would result in the accelerated formation of compound III and P670.

We suggest that both mechanisms are involved in isoniazid-dependent HRP inactivation. The fact that CO is a more effective inhibitor of irreversible inactivation at lower H_2O_2 concentrations suggests that a reduction of ferric HRP by isoniazid radical dominates under these conditions. Conversely, at high H_2O_2 concentrations, CO only prevents some irreversible inactivation, suggesting that the reaction of compound II with H_2O_2 dominates.

The ability of CPZ to completely prevent isoniazid-dependent HRP inactivation indicates that this substrate effectively interferes with both inactivation mechanisms. This ability of CPZ appears to arise from its role as a redox mediator and efficient peroxidase substrate. It seems paradoxical that CPZ, which enhances the generation of isoniazid radical by acting as a redox mediator, protects the peroxidase

from inactivation. However, the proposed mechanism for isoniazid-dependent inactivation requires that the reactive isoniazid radical be generated in close proximity to the heme. The formation of the diffusible $CPZ^{\bullet+}$ by HRP would allow for generation of isoniazid radical at a much greater distance from the heme. We suggest that isoniazid radical generated in this manner is not formed close enough to the heme to initiate the formation of compound III and P670 (Figure 12B). Given the similar mechanisms of suicide inactivation by isoniazid and other hydrazines, it is reasonable to suggest that a redox mediator such as CPZ may protect peroxidases from inactivation by a range of hydrazine-containing compounds.

An efficient peroxidase substrate, like CPZ, is also ideally suited to prevent HRP inactivation by excess H_2O_2. The high rate of HRP turnover in the presence of CPZ results in the rapid consumption of H_2O_2. Moreover, the ability of CPZ to react efficiently with compound II prevents the alternative reaction of compound II with H_2O_2 to form compound III.

It is important to point out that the functions ascribed to CPZ in this mechanism need not be restricted to this compound alone. Many other peroxidase substrates may have the same stimulatory effect as CPZ on isoniazid oxidation. The rate constants for oxidation of many phenolic (Dunford & Adeniran, 1986) and phenothiazine (Kelder et al., 1994) substrates are such that these compounds could stimulate the oxidation of isoniazid. Provided that the redox potentials of the radicals produced upon oxidation of these substrates are higher than that of isoniazid, these compounds are likely to make efficient mediators of isoniazid oxidation. Indeed, we have observed that two phenolic substrates, phenol and tyrosine, were also able to enhance the oxidation of isoniazid by HRP. Moreover, the oxidation of many compounds other than isoniazid may be greatly enhanced in the presence of a redox mediator.

The role of peroxidases in the metabolism of drugs and other xenobiotics has become an area of intense investigation. Some have suggested that peroxidases may activate a number of foreign compounds to reactive intermediates, leading to a variety of consequences (Mason, 1982; Aust et al., 1993; Uetrecht, 1992; Kalyanaraman & Solne, 1985). In this regard, isoniazid may be of particular interest. Isoniazid is an important antibiotic used in the treatment of tuberculosis, and it has been proposed that isoniazid is activated by bacterial catalase/peroxidases to yield its antibiotic effects (Devi et al., 1975; Zhang et al., 1992; Johnsson et al., 1995; Johnsson & Schultz, 1994). Unfortunately, isoniazid is also associated with a number of serious side effects. Metabolism of isoniazid results in hepatic injury in humans, and this compound is a suspect carcinogen (Juchau & Horita, 1972). Isoniazid is also known as a cause of drug-induced lupus, and this side effect of isoniazid is thought to arise from activation of the drug by myeloperoxidase (Jiang et al., 1994; Uetrecht, 1992). In light of the role of peroxidases both in the therapeutic effect of isoniazid as well as in its harmful side effects, it is important to investigate the factors which affect the rate of peroxidase-catalyzed isoniazid oxidation. We have shown here that the presence of an efficient peroxidase substrate such as CPZ, phenol, or tyrosine may have a dramatic stimulatory effect on isoniazid oxidation. The role of these substrates in stimulating isoniazid activation is 2-fold. First, they are able to mediate electron transfer between the heme of the peroxidase and isoniazid. Because these efficient substrates are able to turn the peroxidase over much more rapidly than isoniazid, the net result is a high rate of isoniazid oxidation. Second, these substrates are also able to prevent the isoniazid-dependent irreversible inactivation of the peroxidase. Thus, not only is the initial rate of isoniazid activation stimulated due to the mediator effect, but the peroxidase turnover is also extended for a much greater period of time. The combined effect is that both the rate and extent of isoniazid activation are greatly enhanced in the presence of these substrates. These observations may have many implications for the peroxidase-catalyzed activation of isoniazid necessary not only for the antibiotic effects of isoniazid on *M. tuberculosis* but also for the immunotoxic side effects of this drug.

ACKNOWLEDGMENT

We gratefully acknowledge Randy Booth and Curtis Takemoto for excellent technical assistance, and Terri Maughan for excellent secretarial assistance.

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BI961465Y