ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1985, p. 399–403
0066-4804/85/030399-05$02.00/0

Vol. 27, No. 3

Peroxidase-Mediated Oxidation of Isoniazid

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Received 25 June 1984/Accepted 12 December 1984

Oxidation of isonicotinic acid hydrazide (isoniazid) by horseradish peroxidase at the expense of H_2O_2 yielded reactive species which were able to reduce nitroblue tetrazolium and bleach *p*-nitrosodimethylaniline. Nicotinic acid hydrazide oxidation did not cause these effects. At slightly alkaline pH, oxidation of isonicotinic acid hydrazide by horseradish peroxidase proceeded at the expense of molecular O_2, and the reaction was oxygen consuming. The addition of H_2O_2 abolished O_2 consumption. Bovine liver catalase enhanced the rate of nitroblue tetrazolium reduction and decreased the maximal velocity of the reaction proportionately to catalase concentration. During oxidation of isonicotinic acid hydrazide by horseradish peroxidase-H_2O_2, splitting of the heme group of horseradish peroxidase took place as shown by the disappearance of the Soret and minor bands in the visible region of the spectrum.

Peroxidase activity of *Mycobacterium tuberculosis* correlates strongly with isonicotinic acid hydrazide (isoniazid) (INH) susceptibility (3, 4). Loss of the enzyme, together with its catalase activity, causes emergence of resistance to the drug (3). Most saprophytes and atypical mycobacteria are relatively resistant to INH (12) and may acquire higher levels of resistance concomitant with the loss of their peroxidase activity and only a partial loss of catalase activity (15). The reason for these differences, as pointed out by Davis and Phillips (3), is that *M. tuberculosis* has one protein with peroxidase and catalase activity, whereas the saprophytes and atypicals have two proteins, one with peroxidase and catalase activity and the other with catalase activity only. The activities of the former enzyme are INH labile, whereas the activity of the latter is not. Therefore, although peroxidase loss in saprophytes and atypicals heightens the level of resistance, their primary relative resistance seems to be correlated with the presence of the INH-stable catalase. The mechanism by which that catalase confers protection against INH is unknown; however, a plausible explanation is its ability to scavenge H_2O_2. This does not necessarily mean that INH toxicity is mediated by H_2O_2 but rather that the removal of H_2O_2 by catalase inhibits H_2O_2-dependent peroxidase-mediated oxidation of INH. The role of peroxidase in mediating cytotoxicity in INH toward mycobacteria has been postulated to involve oxidation of INH (7, 10), with the oxidation products, isonicotinic acid (7), excited triplet-state aldehyde (16), and a yellow pigment precursor (15) being direct or indirect effectors in the mechanism of action of INH.

Of interest is the observation that although INH and its meta isomer nicotinic acid hydrazide inhibit the peroxidase enzyme activity equally, the latter has no mycobacteriocidal activity (10). This points out an important role for the pyridine ring in determining the outcome of the metabolism, probably oxidation, of the drug by the microorganism. Thus, although both isomers could bind through their hydrazine group to the enzyme (6) and undergo initial oxidation, their oxidation intermediates may differ qualitatively, quantitatively, or both ways, depending on the contribution of the pyridine ring to the reaction.

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In the present study, we demonstrate differences between the two isomers during their oxidation by peroxidase from the point of production of reactive intermediates, probably free radicals. A possible chemical explanation for these differences on the basis of the contribution of the pyridine ring by resonance stabilization to the progress of oxidation of each isomer will be discussed. Also, the contribution of the catalase enzyme to INH oxidation was studied. The observations obtained in a model system are compared with the mycobacteriocidal activity and the mechanism of action of INH as related to the mycobacterial peroxidase and catalase.

MATERIALS AND METHODS

Reduction of NBT. The reaction mixture consisted of 0.2 mM nitroblue tetrazolium (NBT) in 0.05 M phosphate buffer (pH 7.0) to which was added all or some of the following: horseradish peroxidase (HRP), H_2O_2, bovine liver catalase, INH, nicotinic acid hydrazide, isonicotinamide, isonicotinic acid, or nicotinic acid. The reactions were initiated by the addition of any of these compounds at zero time depending on the purpose of the experiment. Change in A_{560} was recorded continuously in a double-beam spectrophotometer (Beckman Instruments, Inc.). The final volume of the reaction mixtures was 3 ml, and all experiments were performed at room temperature. Scavengers for oxygen radicals, such as superoxide dismutase and benzoate, were added either before or after the reaction was initiated.

Oxygen consumption measurement. Oxygen consumption was measured with a biological oxygen monitor (Yellow Springs Instruments Co.) connected to a Sargent Welch recorder (model SRG). The temperature of the chamber was maintained at 37°C. The reaction mixture was continuously stirred with a magnetic stirrer and consisted of HRP in 0.05 M phosphate buffer (pH 7.8) in a final volume of 4 ml. The mixture was equilibrated at 37°C for 20 min before the addition of other reactants. INH and H_2O_2 were added in sequences that depended on the purpose of the experiment.

***p*-NDA bleaching.** Bleaching of the *p*-nitrosodimethylaniline (*p*-NDA) absorption peak at 440 nm was initially used as an indicator for the generation of hydroxyl radical (· OH) in the reaction of INH with HRP. The reaction mixtures contained the following: *p*-NDA in 0.01 M phosphate buffer (pH 7.0), HRP, H_2O_2, and INH or nicotinic acid hydrazide in

a final volume of 3 ml. The reactions were initiated by the addition of INH or nicotinic acid hydrazide. All experiments were performed at room temperature. The change in A_{440} was recorded continuously. Scavengers for · OH were added whenever appropriate.

Reagents. INH, nicotinic acid hydrazide, isonicotinic acid, isonicotinamide, HRP (type II), superoxide dismutase, bovine liver catalase, and NBT were purchased from Sigma Chemical Co. Hydrogen peroxide (30% [vol/vol]) was purchased from Mallinckrodt, Inc., and *p*-NDA was from Aldrich Chemical Co.

RESULTS

Reduction of NBT. NBT, an indicator for the presence of the superoxide radical (O_2^-), can also be reduced by organic radicals (2). INH oxidation at pH 7.0 by HRP at the expense of H_2O_2 resulted in the reduction of NBT (Fig. 1). This reduction was not inhibited by high concentrations of superoxide dismutase whether added before or after the initiation of the reaction. When the pH of the reaction was raised to 9.5 by replacing the phosphate buffer with 30 mM bicarbonate buffer, NBT reduction proceeded in the absence of H_2O_2 (data not shown). Nicotinic acid hydrazide at equimolar concentration did not result in NBT reduction (Fig. 1). Isonicotinamide, isonicotinic acid, and nicotinic acid did not cause NBT reduction (data not shown).

Effect of catalase on NBT reduction by the HRP-mediated oxidation of INH. A proportional increase in the rate of NBT reduction took place when bovine liver catalase in increasing concentrations was included in the reaction (Fig. 2). At the same time, a proportional decrease in the maximal level of reduced NBT was observed. Control experiments in which HRP, INH, or H_2O_2 were omitted showed no reduction of NBT, indicating that acceleration of reaction rate is not mediated by independent reactions between catalase and any of these reactants but rather by interaction with certain intermediate(s) or products of the reaction of HRP-H_2O_2 with INH.

Oxygen consumption by the reaction of INH with HRP. At slightly acidic or neutral pH levels, no or slight O_2 consumption resulted from the reaction of INH with HRP in the absence of H_2O_2. Raising the pH of the buffer to 7.8 enhanced O_2 consumption. The addition of H_2O_2 to the reaction mixture inhibited O_2 consumption (Fig. 3).

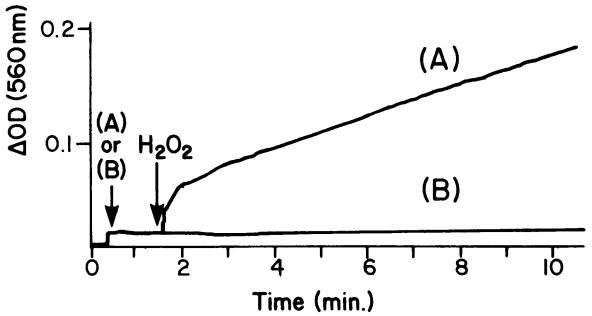

FIG. 1. NBT reduction by the reaction of INH or nicotinic acid hydrazide with HRP-H_2O_2. The reaction mixture at room temperature consisted of 0.2 mM NBT, 33 μg of HRP per ml, and 100 μM H_2O_2 in 3 ml of 0.05 M phosphate buffer (pH 7.0). The reaction was initiated by the addition of 9.6 mM of either INH (A) or nicotinic acid hydrazide (B), and the change in A_{560} was recorded. Reference cuvettes contained the same concentrations of NBT, HRP, and INH.

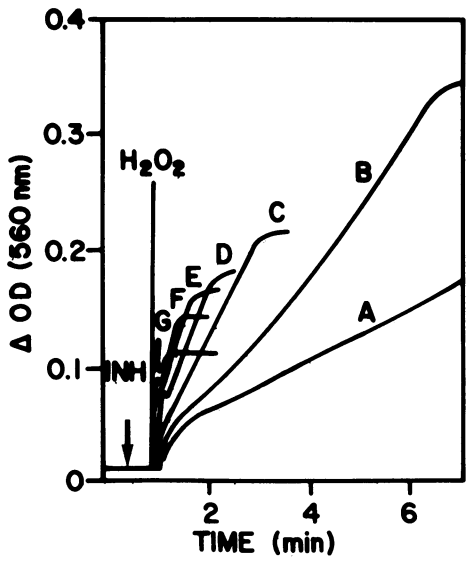

FIG. 2. Effect of catalase on NBT reduction by the INH-HRP-H_2O_2 system. The reaction mixture at room temperature consisted of 0.2 mM NBT, 33 μg of HRP per ml, 100 μM H_2O_2, and 0, 10, 40, 80, 120, 160, or 300 μg of bovine liver catalase (curves A to G, respectively) in 3 ml of 0.05 M phosphate buffer (pH 7.0). The reaction was initiated by the addition of 9.6 mM INH, and the change in A_{560} was recorded. Reference cuvettes contained the same concentrations of NBT, HRP, catalase, and INH.

***p*-NDA bleaching by the reaction of INH with HRP.** *p*-NDA may be bleached by the reaction with · OH as well as with organic radicals (1). INH, but not nicotinic acid hydrazide, at equimolar concentrations reacted with HRP in the presence of H_2O_2 to produce certain species that could bleach *p*-NDA (Fig. 4). The hydroxyl-radical scavengers sodium benzoate and mannitol did not affect the reaction even at relatively high concentrations (data not shown). Bovine liver catalase moderately enhanced the reaction rate only in bicarbonate buffer (pH 7.2) (data not shown). Other pyridine derivatives, isonicotinamide, isonicotinic acid, and nicotinic acid, did not induce *p*-NDA bleaching (data not shown).

Changes induced by INH in HRP absorption spectrum. The addition of INH to HRP-H_2O_2 (compound I) at pH 7.0 caused the gradual disappearance of the Soret band of compound I at 410 nm and was accompanied with a gradual development of a new absorption peak at 335 nm (Fig. 5). In addition, bleaching of minor adsorption peaks of compound

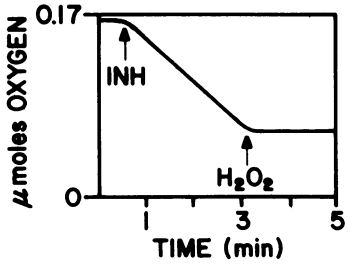

FIG. 3. Effect of H_2O_2 on INH-HRP-dependent oxygen consumption. The reaction mixture at 37°C consisted of 50 μg of HRP in 4 ml of 0.05 M phosphate buffer (pH 7.8). The reaction was initiated by the addition of 2.4 mM INH. At the indicated time, 100 μM H_2O_2 was injected into the reaction mixture.

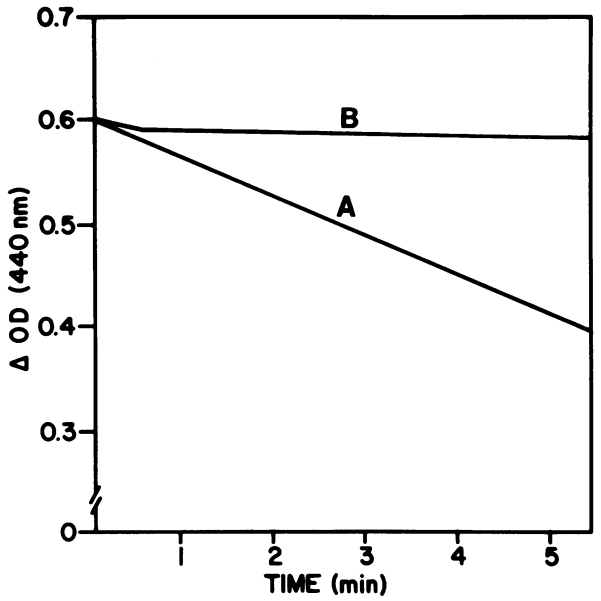

FIG. 4. *p*-NDA bleaching by INH and nicotinic acid hydrazide reaction with HRP-H_2O_2. The reaction mixture at room temperature consisted of 0.2 mM *p*-NDA, 8 μg of HRP per ml, and 100 μM H_2O_2 in 3 ml of 0.05 M phosphate buffer (pH 7.0). The reaction was initiated by the addition of 2.4 mM INH (A) or nicotinic acid hydrazide (B), and the change in A_{440} was recorded.

I took place (Fig. 6). This bleaching may indicate heme splitting as a result of an interaction with INH oxidation products. The same results were obtained in the absence of H_2O_2 only at alkaline pH.

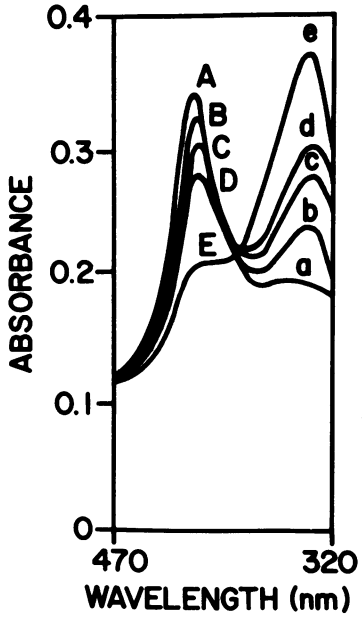

FIG. 5. INH-induced changes in the spectrum of HRP compound I. HRP (0.8 mg/ml) in 0.05 M phosphate buffer (pH 7.0) was incubated with 100 μM H_2O_2. After 5 min of incubation at room temperature, 9.6 mM INH was added to a final volume of 3 ml, and change in spectrum was recorded at 0 (A,a), 10 (B,b), 15 (C,c), 20 (D,d), and 80 (E,e) min. The reference cuvette contained the same concentration of INH.

DISCUSSION

The potential of INH to mediate the production of species that are able to reduce NBT and bleach *p*-NDA may be an indication that these species involve organic free radicals during HRP-catalyzed oxidation of INH at the expense of H_2O_2. Superoxide and · OH radicals could not be incriminated as the agents causing NBT reduction and *p*-NDA bleaching, respectively. This is not in agreement with the results of Sinha (11), who was able to spin trap · OH in the INH-HRP-H_2O_2 system at pH 7.4. It is interesting that in our study of *p*-NDA bleaching by that system, benzoate and mannitol did not inhibit the reaction, whereas ethyl alcohol in a concentration which does not diminish the activity of HRP in oxidizing substrates, such as *o*-dianisidine, inhibited bleaching completely (unpublished results). This raises the question whether we were dealing with a bound · OH or · OH-equivalent species that was shielded by a hydrophobic region. This might also be applicable to the spin-trapped · OH by Sinha (11); that is, the electron spin resonance signal he recorded might have been for a · OH-equivalent species, for example, the ferryl ion that might have resulted from the heme group of HRP upon reaction with INH and H_2O_2.

To account for the difference in activities between INH and its meta isomer, a difference in their oxidation pathway by peroxidase might exist. Autooxidation of linear hydrazide in the presence of a strong base has been elucidated by Rapaport et al. (9). It involves deprotonation of and electron transfer from the hydrazine group to O_2^- in two steps, yielding N_2 and a carbonyl radical together with two O_2^- molecules. At this point, the hydrazide has gone through a two-step, one-electron transfer. Peroxidase mediates most oxidations of various electron donors by a two-step, one electron transfer. Therefore, it is plausible that hydrazides such as INH might be oxidized by the peroxidase in the same manner. A difference between the base-catalyzed autooxidation and the peroxidase-catalyzed oxidation of INH may be in the nature of the oxidant, i.e., H_2O_2 or O_2. Thus, whereas base-catalyzed oxidation of INH utilizes O_2 as oxidant in which deprotonation of the hydrazine group must take place before electron transfers occur, HRP-catalyzed oxidation utilizes either O_2 at alkaline pH or H_2O_2 at neutral pH, with O_2^- produced in the former. This means that at alkaline pH and in the absence of H_2O_2, the reaction

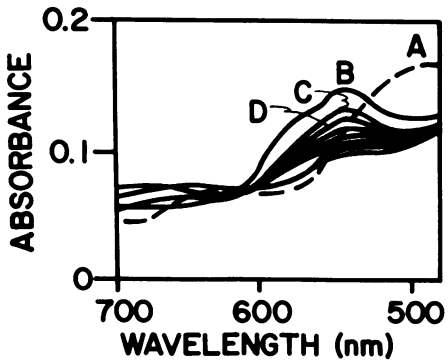

FIG. 6. INH-induced changes in the far visible region of HRP compound I spectrum. The concentrations of reactants and the sequence of additions were the same as in Fig. 5. The spectrum was recorded before H_2O_2 addition (A) and 30 s after H_2O_2 addition (B). Curves C, D, and the subsequent curves were recorded at 5.5-min intervals after INH addition.

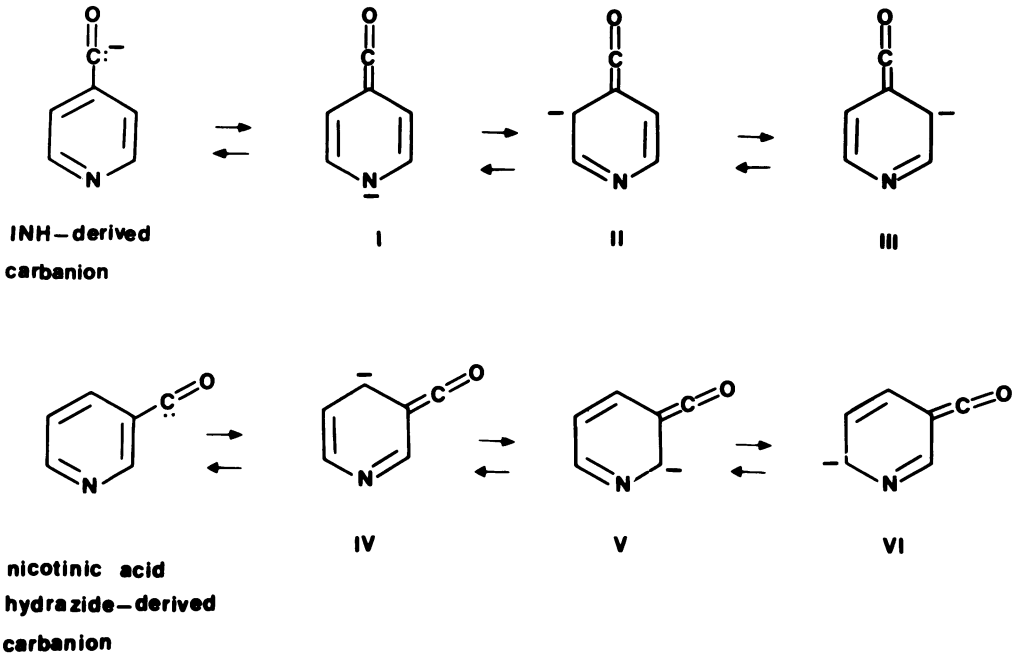

FIG. 7. Proposed resonance structures stabilizing the carbanion during oxidation of INH (I, II, and III) contrasted with that of nicotinic acid (IV, V, and VI).

between INH and peroxidase should consume O_2. Also, the addition of H_2O_2 should inhibit O_2 consumption due to the preferential use of H_2O_2 as oxidant by peroxidase over O_2. This fact was demonstrated in Fig. 3.

After the formation of carbonyl radical, peroxy and carboxylate radicals are formed (9). Any of these radicals may mediate both NBT reduction and *p*-NDA bleaching. In the whole oxidation pathway, a rate-determining step seems to be a carbanion (acyl anion,

$$\begin{matrix} O \\ \| \\ RC:^- \end{matrix},$$

where R is an aliphatic or aromatic group) species which is the precursor of the carbonyl radical. This species might explain the role of the pyridine ring in the oxidation outcome of both INH and its meta isomer nicotinic acid hydrazide. In the case of INH, the carbanion should be stabilized by the resonance structure in which the two electrons are accommodated by the strong electronegative nitrogen of the pyridine ring. In the case of nicotinic acid hydrazide-derived carbanion, all the resonating structures have the two electrons accommodated by the weak electronegative carbons of the ring (8), as proposed in the equations in Fig. 7.

Among these structures, structure I is the most stable. As a result of its stability, a lower transition energy is achieved, and the reaction will proceed to produce carbonyl radical much faster than in the case of nicotinic acid hydrazide. This might indicate that the NBT-reducing and *p*-NDA-bleaching molecule(s) is present either in or after the carbanion formation step but not in the hydrazine oxidation stage. Furthermore, if this assumption is extended to the mycobacterial peroxidase, this might indicate that the resulting radicals and excited states of INH might play a role in the mechanism of killing of mycobacteria by the drug due to the difference in oxidation pathway of INH and its meta isomer. It follows that the peroxidase enzyme might be participating in killing rather than in the uptake of the drug.

More evidence supporting the presence of this pathway of INH oxidation came from the finding that INH oxidation by compound I resulted in heme splitting. Although heme splitting might be caused in various ways, one of the mechanisms seems to involve peroxyradicals. Examples are HRP-catalyzed oxidation of indol-3-acetate (12) and arachidonic acid cyclooxygenase pathway (5). In both cases, heme splitting takes place, and a peroxy-radical intermediate seems to be involved in or at least accompanies the event.

The effect of catalase on the peroxidase-catalyzed oxidation of INH is interesting since it might parallel the catalase and peroxidase situation in mycobacteria when both enzymes are present and their inhibition by INH is related to susceptibility or resistance. In the presence of two catalases, one of which is noninhibitable by INH, as in the case of saprophytes and atypicals, destruction of H_2O_2 will inhibit the oxidation of INH by peroxidase. In the present study, this situation corresponds to the inhibitory effect of catalase on the maximal level of reduced NBT, although in the presence of only the catalase inhibitable by INH, as is the case of *M. tuberculosis*, only low-level residual catalase activity may have the same enhancing effect on INH oxidation by peroxidase as that observed in the present study at low catalase concentrations. On the other hand, lack of catalase and peroxidase in INH-resistant strains of *M. tuberculosis* should confer absolute resistance to the drug due to failure to oxidize the drug. A general assumption may then be that INH-susceptible *M. tuberculosis* strains occupy an intermediate position, with respect to catalase and peroxidase content and inhibition by INH, between the INH-resistant *M. tuberculosis* strains at one end of the scale, where they lack both enzymes, and the INH-resistant saprophytes and atypicals at the other end of the scale, where they contain two catalases and a peroxidase. Varia-

tions on the scale will result in more or less susceptible strains depending on these two enzymes.

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