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Differentiation of Catalases in *Mycobacterium phlei* on the Basis of Susceptibility to Isoniazid: Association with Peroxidase and Acquired Resistance to Isoniazid

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Mycobacterium phlei contains two catalase activities and a single peroxidase activity. The latter is associated with one of the catalases. The single catalase-peroxidase enzyme accounted for 75% of the total catalase activity and was lost upon acquisition of resistance to the antitubercular drug isoniazid (INH). Heat-treated (68°C) wild-type cells showed similar decreases in catalase activity as well as complete loss of peroxidase activity. Catalase activity in the INH-resistant strain of *M. phlei* (Inh^r) was unaffected by heating. The heat-sensitive catalase of the wild-type *M. phlei* was completely inhibited by 0.1 M INH, and Cu^{2+} enhanced this inhibitory effect by 100-fold. No inhibition of activity was found with the heat-stable enzyme. Equivalent inhibition of catalase was also observed with nicotinic acid hydrazide and benzoic acid hydrazide. Peroxidase activity was also completely inhibited by any one of the three hydrazides, either INH, benzoic acid hydrazide, or nicotinic acid hydrazide at 10^{-3} M. The presence of two catalase activities and the loss of one (catalase-peroxidase) on acquiring INH resistance or heating wild-type cells was confirmed by acrylamide gel electrophoresis of the cell-free extracts.

A loss of catalase-peroxidase activity has been consistently observed in *Mycobacterium tuberculosis* upon acquisition of resistance to isoniazid (INH) (9). However, the precise role of catalase and/or peroxidase in the mechanism of action of the drug remains obscure. It is of further interest that atypical and saprophytic mycobacteria may generally be considered clinically resistant to INH because they require higher minimal inhibitory concentrations than does *M. tuberculosis* (18). As a result, the antimicrobial therapy of atypical mycobacterial infections is a serious problem.

Most of the saprophytic and atypical mycobacteria show only a partial loss of catalase activity but complete loss of peroxidase upon acquiring resistance to INH (17, 18). *M. tuberculosis* possesses a single heat-labile catalase in which also resides peroxidase activity, as demonstrated by polyacrylamide gel electrophoresis and association of both activities during purification (4, 5). Atypical and saprophytic mycobacteria possess two catalases, one of which is heat labile and lost upon acquisition of resistance to INH (1, 12). Catalase activity in cell-free extracts of *M. tuberculosis* has been shown to be inhibited by nearly 100%, whereas in saprophytic or atypical mycobacteria only 70% inhibition has been attained (5, 7, 16). These data suggest that a catalase-peroxidase common to both *M. tuberculosis* and the atypical mycobacteria may have a role in mediating the action of INH. To further investigate this hypothesis, studies on the susceptibility of saprophytic mycobacterial catalase to INH were undertaken in *M phlei*. *M. phlei* was used as a prototype since it was inhibited by high levels of the drug (25 mg/ml), and it could develop resistance associated with a partial decline in catalase activity and complete loss of peroxidase.

The present study elucidates the basis of the incomplete INH inhibition of catalase activity in mycobacteria of the atypical variety by demonstrating that one of the two catalases present in *M. phlei* is selectively inhibited by INH and other cyclic hydrazides. It further identifies the INH-susceptibility catalase in *M. phlei* as corresponding exclusively to the heat-labile enzyme and as the sole source of peroxidase activity in the cells. The possible significance of this INH-susceptible catalase-peroxidase with regard to susceptible of the organism to the drug is discussed.

MATERIALS AND METHODS

Bacterial strains and growth media. A wild-type (ATCC 345) and INH-resistant strain (Inh^r) of *M. phlei* were used in these studies. Growth of wild-

type *M. phlei* was completely inhibited by 25 μg of INH per ml when the inoculum size was 10^5 cells per ml. The Inh^r strain was obtained by repeated exposure of low-inoculum (10^5 cells per ml) cultures to increasing levels of INH up to 200 μg/ml. An isolated colony growing on media containing 200 μg of INH per ml was used to prepare stock cultures. The Inh^r strain reverted to INH susceptibility if stored in the absence of INH, and, therefore, cultures were periodically transferred in both media containing 200 μg of INH per ml. Growth media and conditions have been described (2).

Preparation of cell-free extract. Cells grown for 48 h at 37°C with vigorous aeration on a New Brunswick gyratory incubator shaker were harvested by centrifugation, washed twice, and then suspended to a level of 1 g (wet weight) of cells per ml in 0.1 M potassium phosphate (pH 7.5). The resulting cell suspension was subjected to sonic oscillation and centrifuged at 5,000 × *g* for 20 min. The resulting supernatant fluid was centrifuged at 105,000 × *g* for 1 h to remove membranes and other particulates, and the resulting supernatant fluid (sonic extract) was sterilized by filtration and stored at −30°C.

Concentration of sonic extracts. For acrylamide gel electrophoresis sonic extracts were concentrated to one-third volume in a 40-mm ultrafiltration cell (Millipore Corp.) by using a Pellicon ultrafiltration membrane with a nominal molecular weight limit of 25,000.

Enzyme assays. Catalase activity was assayed by the method of Diaz and Wayne (5). To test the effect of INH and other compounds on catalase activity, extract and the appropriate additions, as indicated, were preincubated with 0.1 M potassium phosphate (pH 8.0) in a final volume of 1 ml. The reaction was started after 5 min of preincubation by the addition of 2.0 ml of H_2O_2 (50 μg/ml). All assays were carried out at 37°C. Reactions were stopped by the addition of 5×10^{-3} M titanium tetrachloride, and the absorbancy was read at 410 nm.

Peroxidase activity was determined by the method described in the *Worthington Manual* (Worthington Biochemical Corp., Freehold, N.J.) using *O*-dianisidine as the hydrogen donor. Activity was monitored spectrophotometrically by measuring the rate of color development at 460 nm.

Acrylamide gel electrophoresis. Acrylamide gels, 100 mm in length, were prepared by a modification of the method of Weber and Osborn (14). The gels were prepared in sodium phosphate buffer without the addition of sodium dodecyl sulfate. Stacking gels were not used. The particle-free extracts were made 0.2 M with respect to sucrose. Bromophenol blue was added, and a 100-μl sample of the mixture was layered on the acrylamide gels under sodium phosphate buffer. Electrophoresis was performed in the cold at 4 mA per gel for 20 h. Location of the catalase-active areas on acrylamide gels was determined by the technique previously described by Gruft and Gaafar (6), except that gels incubated with INH were immersed in 1.2% H_2O_2. Peroxidase-active areas were identified by the method of Shannon, Kay, and Lew (10).

Protein determination. Protein was estimated by the procedure of Lowry et al. (8).

Reagents. INH and other hydrazides were obtained from Aldrich Chemical Co. Hydrogen peroxidase and *O*-dianisidine were purchased from Sigma Chemical Co. Acrylamide and bisacrylamide were obtained from Eastman.

RESULTS

Catalase and peroxidase levels in sonic extracts of wild-type and Inh^r *M. phlei*. To determine the presence of and quantify the relationship between heat-stable and -labile catalases and peroxidase in INH-susceptible and -resistant *M. phlei*, the respective activities were compared in cell-free sonic extracts. As shown in Table 1, the catalase activity of the resistant strain was 74% less than that observed in the extract of the wild-type strain. Heating wild-type cells for 20 min at 68°C before sonic treatment resulted in about 80% loss of catalase activity demonstratable in their sonic lysates (Table 1). Heating Inh^r cells at 68°C before sonic oscillation had no significant effect on the level of catalase activity in the sonic extracts. However, heating lysates of either strain at 68°C for 20 min resulted in complete destruction of enzymatic activity, indicating that the heat-stable enzyme requires cell association for stability. Similar observations were made by Bartholomew on *M. smegmatis* (1).

With regard to peroxidase, the data in Table 1 show that either heat treatment of *M. phlei* at 68°C or acquisition of resistance to INH causes a complete loss of peroxidase activity. Devi et al. (4) and Diaz and Wayne (5) have shown that the peroxidase and catalase activities of *M. tuberculosis* can be attributed to a single enzyme present in *M. tuberculosis*. Hence, as in the data for *M. tuberculosis*, the data in Table 1 provide evidence that the heat-sensitive cata-

TABLE 1. *Comparison of levels of catalase and peroxidase and their thermal stability in wild-type and INH-resistant M. phlei*[a]

Strain	Catalase activity[b]		Peroxidase activity[c]	
	37°C	68°C	37°C	68°C
Wild type	0.88 ± 0.12	0.15 ± 0.10	1.4	NA[d]
Inh^r	0.23 ± 0.08	0.20 ± 0.11	NA[d]	

[a] Catalase and peroxidase activities in sonic extracts were determined as described in the text. Heat treatment consisted of heating twice-washed cells suspended in 0.2 M potassium phosphate (pH 6.7) at 68°C for 20 min before sonic oscillation.

[b] At an absorbancy at 410 nm per minute per milligram of protein.

[c] At an absorbancy at 460 nm per minute per milligram of protein.

[d] NA, No activity detectable.

lase enzyme of *M. phlei* also corresponds to the peroxidase activity.

Inhibition of catalase and peroxidase activity by INH and other hydrazides. The absence of peroxidase and the heat-labile catalase activity in the Inh^r strain and the concurrence of these data with a single catalase present in *M. tuberculosis* (5, 10) prompted investigation of the susceptibility of *M. phlei* catalase to INH and other hydrazides. As shown in Fig. 1, the catalase activity in the sonic extract of the wild type was inhibited up to 75% by concentrations of INH between 10^{-3} and 0.1 M. No further inhibition of the 25% residual activity was observed at INH levels above 0.1 M. The maximal inhibitory level of INH could be reduced 100-fold in the presence of 0.05 M $CuSO_4$ (Fig. 1). It is of interest that the same effects obtained for INH were observed with nicotinic acid hydrazide (NAH) and benzoic acid hydrazide (BZH). All of these findings support those of Hawkins and Steenken (7). However, maleic acid hydrazide had no inhibitory effect. The residual activity observed in the presence of high levels of INH (0.1 M) was shown to be enzymatic since it was destroyed upon boiling and, as shown in Figure 1B, demonstrated linear rate kinetics as a function of enzyme concentration. Neither the activity of the Inh^r strain nor the residual activity of heat-treated wild-type cells was inhibited by any level of INH, NAH, or BZH tested even in the presence of copper (Fig. 1).

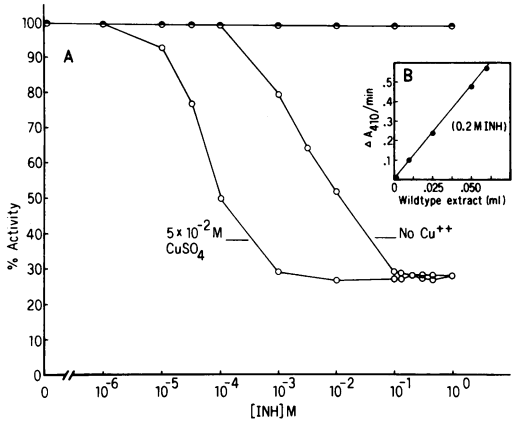

FIG. 1. *INH inhibition of catalase activity in sonic lysates of wild-type and Inh^r M. phlei. (A) Catalase assays were carried out as described in the text: (○) wild-type catalase activity in the presence or absence of $CuSO_4$ as indicated; (●) catalase activity from Inh^r strain and from wild-type strain treated at 68°C. (B) Wild-type extract at the indicated volume was incubated in the presence of a maximal inhibitory level of INH (0.2 M) for 5 min followed by addition of H_2O_2. Absorbancy at 410 nm was determined after 5 min of incubation as described in the text*

Isoniazid concentrations as low as 10^{-3} M totally inhibited the peroxidase activity of the wild-type strain. Controls containing the hydrogen donor, *O*-dianisidine, and INH in the presence of H_2O_2 were included to rule out the possible nonspecific interference between INH and the hydrogen donor. NAH inhibited peroxidase as well as INH at 10^{-3} M levels. Since 0.1 M INH is required to give maximal inhibition of catalase, these data indicate that *M. phlei* peroxidase activity is considerably more susceptible to INH than is catalase with regard to both drug concentration and degree of inhibition.

Comparison on acrylamide gels of catalase and peroxidase activities from INH-susceptible and -resistant *M. phlei*. Two catalases have been demonstrated on acrylamide gels in a number of atypical and saprophytic mycobacterial species (1, 6); however, only a single catalase enzyme has been shown for the highly INH-susceptible species *M. tuberculosis* and *M. bovis* (13).

Comparison of the bands of catalase activity on acrylamide gels from wild-type, heat-treated wild-type, and Inh^r *M. phlei* cell-free extracts can be seen in Fig. 2. Two bands of catalase activity (A and B) appeared in the wild-type extract, whereas, a single band was found in the Inh^r extract, the latter corresponding to the more rapidly migrating enzyme (B) from the wild type. Wild-type, heat-treated extracts contained only the single enzyme found in the Inh^r strain. Incubation of the gels containing wild-type lysate with INH resulted in selective and complete inhibition of the slower migrating (A) band. No inhibition by INH of the faster migrating enzyme was detected, nor was there any INH inhibition of the catalase on the gels containing the heat-treated wild-type or Inh^r extracts.

Further evidence that INH had no effect on the faster migrating catalase of the wild type was obtained by eluting the enzymes from the gels and quantitating the eluted activities.

It was also of interest to determine the location of peroxidase with regard to the two catalases of the wild type on acrylamide gels. As seen in Fig. 2, the peroxidase activity corresponded to the slower migrating catalase enzyme (A). Further identification was observed when gels that had been used to develop the catalase assay were subsequently placed in a solution of the hydrogen donor *O*-dianisidine. The characteristic brownish band resulting from peroxidase activity appeared imposed on the clear zone, representing the slower migrating catalase enzyme (A) of the wild type. In

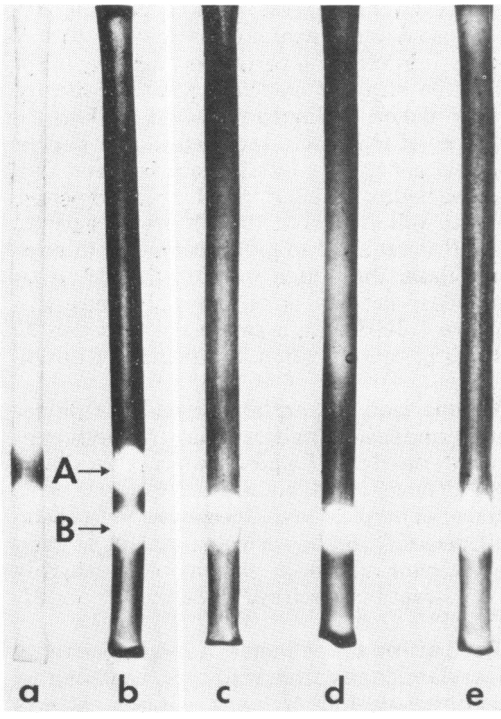

FIG. 2. *Acrylamide gel electrophoresis of sonic lysates. (a) Peroxidase activity stain, wild-type extract; (b) catalase, wild-type extract; (c) catalase, Inh^r extract; (d) catalase activity in the presence of 0.2 M INH, wild-type extract; (e) catalase, heat-treated wild-type cell extract. Extracts shown in (c) and (e) were concentrated by ultrafiltration as described in the text. Migration is from top to bottom.*

addition, peroxidase activity could be demonstrated only after elution of band A.

Hence, these data corroborate the kinetic findings in Table 1 in that peroxidase activity corresponds to the heat-labile, INH-susceptible catalase of the wild type, which is lost upon acquisition of INH resistance.

DISCUSSION

These studies indicate that a requirement among the mycobacteria necessary for susceptibility to INH may be a catalase-peroxidase that is inhibited by the drug.

An important question is what is the significance of the INH inhibition of the heat-labile catalase-peroxidase. Devi et al (3, 4) showed that the catalase-peroxidase of *M. tuberculosis* catalyzes a reaction between INH and NAD^+ that results in the production of a colorless product, which turns yellow upon acidification (Y-enzyme reaction). Hence, it is proposed that the observed inhibition of the catalase-peroxidase by INH in *M. phlei* is an inconsequential but necessary effect for a similar reaction to occur, with the resulting product providing the impetus for the lethal action of the drug. In fact, the inhibition of the catalase-peroxidase by NAH and BZH, which are not considered to be lethal for mycobacteria, supports the consideration that the inhibitory interaction of INH with a catalase-peroxidase is the first step in a sequence leading to cell death. A plausible explanation in this regard for the observed BZH and NAH inhibition of the catalase-peroxidase is that, although they inhibit the catalase-peroxidase activity, they cannot be utilized in such a way to participate in the subsequent activities necessary for the lethal action of the drug. As previously proposed by Wimpenny (15), an alternate explanation for the role of catalase-peroxidase in the mechanism of action of INH may be that the enzyme acts as a binding and/or transport protein for INH. Once bound or inside the cell, the lethal manifestation may occur through inhibition of pathways such as mycolic acid synthesis, as proposed by Takayama (11).

The basis for the differential in susceptibility to INH among the mycobacteria remains to be defined. The heat-stable INH-susceptible catalase, which is not found in *M. tuberculosis*, may be a key factor; however, comparison of INH-binding affinities on purified INH-susceptible catalase-peroxidases from mycobacteria of varying susceptibility to INH may also provide useful information regarding a transport role of this enzyme in conferring the degree of susceptibility of the organism to the drug. Further studies presently in progress regarding possible Y enzyme-associated activity with the INH-susceptible catalase-peroxidase might be profitable in relating the lethal action of INH on *M. phlei* to *M. tuberculosis* and, therefore, help establish a basis for elucidating the role of this enzyme in the mechanism of action of INH.

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