Identification of a Nicotinamide Adenine Dinucleotide Glycohydrolase and an Associated Inhibitor in Isoniazid-Susceptible and -Resistant *Mycobacterium phlei*

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Nicotinamide adenine dinucleotide glycohydrolase (NADase) activity was demonstrated in the catalase fraction of Sephadex G-200-chromatographed sonic extracts of isoniazid (INH)-susceptible (Inh^s) and -resistant (Inh^r) *Mycobacterium phlei*. Since crude extracts had no demonstrable activity even after heating, active fractions of the NADase were purified chromatographically by removing the inhibitor with Sephadex G-200. Assays for oxidized nicotinamide adenine dinucleotide (NAD$^+$) hydrolytic activity were done by following the disappearance of NAD$^+$ by the methods of alcohol dehydrogenase or cyanide addition. The NADase activity was linear with respect to time as well as concentration of enzyme and was inhibited in the presence of 0.04 M NADP, benzoic acid hydrazide, or nicotinamide. Crude extracts or pooled concentrated Sephadex G-200 fractions eluting after the catalase inhibited NADase activity by at least 70%. Inhibitor activity was present in both the Inh^s and Inh^r strains of *M. phlei*. The activity of the partially purified inhibitors was reversible by INH or nicotinic acid hydrazide at levels between 10 and 100 mM. These findings indicate that an NADase inhibitor system which is sensitive to reversal by INH functions in both the Inh^s and Inh^r strains; however, unlike previous studies with other mycobacterial species, the enzyme is sensitive to inhibition by nicotinamide. Furthermore, the inhibitors are heat stable and sensitive to reversal by nicotinic acid hydrazide as well as INH.

Despite considerable research on metabolic functions related to elucidation of the lethal effects of isoniazid (INH) on the mycobacteria, the mechanism of action of the drug remains in question. For example, mycolic acid synthesis appears to be inhibited (14, 15, 17), but a direct relationship with cell death has not been established. Numerous other theories have also been generated from experimental results such as the potential of INH to act as an antagonist of pyrodoxal phosphate (18), to induce toxicity related to pigment production (19), and also to disrupt oxidized nicotinamide adenine dinucleotide (NAD$^+$)-related biochemical activities (1, 3). Hence, undoubtedly the interaction between this small-molecular-weight drug and the organism is a complex process with multifactorial properties, thus making the primary causes of lethality difficult to distinguish from secondary or unrelated manifestations.

An interesting and consistent observation is the direct relationship of catalase/peroxidase activity with INH sensitivity (11) and the enzymatic association of these activities with NAD$^+$ glycohydrolase (NADase) (4). In fact, a number of observations suggest that the NADase of the organism may be of considerable significance in

mediating the lethal effects of INH on mycobacteria. INH can participate in an NADase-catalyzed reaction with the nicotinamide moiety of NAD$^+$ resulting in the formation of an NAD$^+$ analog (20); however, since the highly INH-susceptible mycobacterial species *Mycobacterium tuberculosis* H$_{37}$Rv (6) and *Mycobacterium butyricum* (9) cannot manifest the reaction, it is difficult to resolve that the NADase-catalyzed reaction contributes to the mechanism of action of the drug. Yet the NADase of each of these mycobacterial species is associated with a heat-labile inhibitor which in *M. tuberculosis* H$_{37}$Rv has been shown to be sensitive to reversal by INH (13). In addition, purified peroxidase from *M. tuberculosis* H$_{37}$Rv has been shown to catalyze a reaction between INH and NAD$^+$. This reaction, known as the Y (Youatt) enzyme reaction catalyzes formation of an undefined yellow-pigmented product (4, 19).

To investigate further the implications of mycobacterial NADase in the mechanism of action of the drug, it was of interest to determine whether the enzyme and an INH-sensitive inhibitor could be demonstrated in association with the catalase/peroxidase activity of *M. phlei*. Although *M. phlei* resistance to INH is not ordi-

narily considered to be relative to a clinical setting (16), previous work of Davis and Phillips (2) has demonstrated that *M. phlei* is susceptible to inhibition by INH at levels of 25 μg/ml and has two distinct catalases, one of which is associated with peroxidase activity and sensitive to inhibition by INH. In addition, it was shown that development of resistance to INH by *M. phlei* correlated directly with simultaneous loss of the catalase and peroxidase activities.

MATERIALS AND METHODS

Bacterial strains and growth media. *M. phlei* strain ATCC 345 (Inh[s]) was used in these studies, and a strain derived from it resistant to high levels of INH (Inh[r]) was also used. Growth media and conditions were previously described by Davis and Weber (3). The Inh[s] strain is susceptible to INH at a level of 25 μg/ml, whereas the Inh[r] strain was selected by repeated exposure of colony isolates to INH at 200 μg/ml and then maintained on INH-containing medium as described earlier (3). Other bacteria utilized included *M. tuberculosis* H[37]Ra and *Escherichia coli*. *M. tuberculosis* was grown in Middlebrook 7H9 medium (Difco) containing ADC enrichment (Difco). *E. coli* was cultivated in brain heart infusion broth.

Preparation of cell-free extracts. Cells were grown to late log phase with aeration on a New Brunswick shaker at 37°C in the appropriate medium and harvested by centrifugation. Cell-free extracts were obtained by sonication of washed cells for 3- to 5-min intervals at 4°C with a Branson Sonifier model 185 with a large probe and a power setting of 70. Cell suspensions were then clarified by centrifugation at 105,000 × g for 1 h. The supernatant fluid was collected and concentrated to one-third volume in a 40-mm stirred ultrafiltration chamber with a Pellicon ultrafiltration membrane with a nominal molecular weight limit of 25,000. The concentrated extracts were then stored at −30°C until ready for use.

Column chromatography. Column chromatography of concentrated Inh[s] and Inh[r] extracts was performed on a Pharmacia K50/100 Sephadex G-200 column equilibrated and eluted with 0.2 M potassium phosphate, pH 8.0. The column was loaded with 250 mg of protein, and fractions of chromatographed extract were collected in 1-ml portions and assayed for catalase or peroxidase activities as indicated. Fractions containing catalase/peroxidase were pooled and concentrated by ultrafiltration through a Pellicon membrane filter (Millipore Corp.). The concentrate was then filter sterilized and stored at −30°C. Other than periodic monitoring, fractions obtained from the Inh[r] strain were tested only for catalase since previous studies showed that the catalase with associated peroxidase activity is not detectable in the organism (2).

Enzyme assays. Catalase was quantified by the method of Diaz and Wayne (5).

Peroxidase was measured spectrophotometrically at an adsorbancy of 460 nm with *O*-dianisidine as the hydrogen donor (described in the Worthington Manual, Worthington Biochemical Corp., Freehold, N.J.).

NADase activity was determined by following the disappearance of NAD^{+} which was quantified by either the cyanide addition or alcohol dehydrogenase method. Aliquots of the concentrated enzyme extract were incubated for periods up to 24 h in the presence of NAD^{+} and other compounds when indicated. All reagents were filter sterilized before use, and incubations were carried out at 37°C in sterile screw-capped test tubes. Assays for the presence of NAD^{+} by the cyanide addition method were done by adding 2.3 ml of 1 M KCN to a 0.7-ml reaction mixture containing 0.42 μmol of NAD^{+} with enzyme in 0.02 M potassium phosphate, pH 8.0. At the end of the incubation period, the absorbance of the mixture was read at 325 nm, and the loss of NAD^{+} was determined by comparison with control tubes containing NAD^{+} or with other additions as indicated. The alcohol dehydrogenase method consisted of first incubating 2.8 μmol of NAD^{+} with respective enzyme preparations. The concentration of NAD^{+} remaining after the incubation period was then determined by adding 0.1 ml of the incubation mixture to 2.9 ml of 0.01 M tris(hydroxymethyl)aminomethane and ethanol containing 0.01 mg of yeast alcohol dehydrogenase (Sigma). The concentration of NAD^{+} was determined spectrophotometrically at 340 nm by its conversion to NADH and compared to controls containing NAD^{+} or with additional compounds as indicated.

Assay for nicotinamide. Determination of nicotinamide was done spectrophotometrically by using cyanogen bromide as described by Pelletier and Campbell (12). Aliquots (0.1 ml) of the NADase reaction mixtures were removed and diluted to 1 ml with 0.2 M potassium phosphate, pH 8.0. A 10% cyanogen bromide solution was added (0.5 ml), and the mixture was allowed to stand at room temperature for 25 min. Barbituric acid (2%) was then added to give a final volume of 11.5 ml, and after 5 min the absorbancy at 550 nm was determined. The concentration of nicotinamide in the reaction mixtures was calculated by comparison of absorbancy readings with those for a nicotinamide standard curve.

Protein assay. Assay for protein was by the method of Lowry et al. (10).

RESULTS

Isolation and demonstration of NADase. After Sephadex G-200 column chromatography of Inh[s] or Inh[r] concentrated cell-free extracts, NADase activity was demonstrated in the pooled-concentrated fractions containing peroxidase or catalase activity. Figure 1 shows that the catalase and peroxidase activity of Inh[s] eluted simultaneously in the early fractions following the void volume. Peak fractions were collected and concentration to one-fourth volume and sterilized by filtration through a 0.2-μm filter. The resulting preparations were clear and contained NADase activity.

The presence of NADase activity in both the Inh[s] and the Inh[r] strain was demonstrated by quantifying the amount of NAD^{+} remaining after a 24-h incubation with the enzyme. Comparison of alcohol dehydrogenase conversion of NAD^{+} to NADH with the method of cyanide
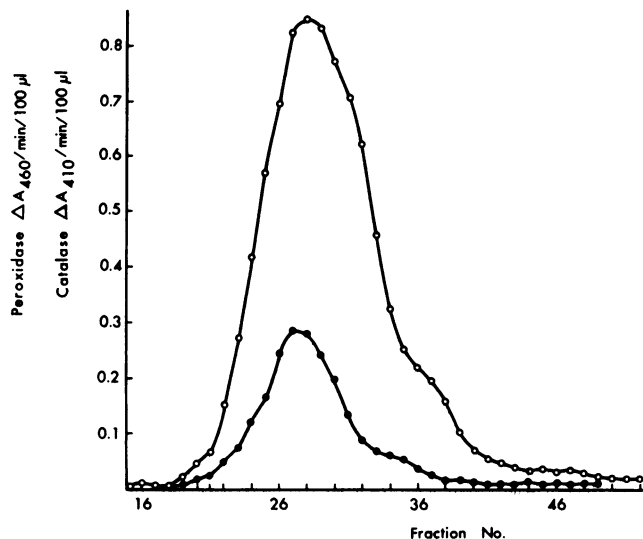
FIG. 1. *Catalase and peroxidase activity in fractions of M. phlei cell-free extract after Sephadex G-200 chromatography. Catalase and peroxidase were assayed as described in the text. Symbols:* O, *catalase activity;* ●, *peroxidase activity.*

addition showed that use of either technique reflected equivalent results for each preparation tested (Table 1). Furthermore, as shown in Table 1, each method of assay showed that the level of NADase activity from the Inh^r strain was about 25% less than that for the wild-type organism.

Although it was necessary to incubate reaction mixtures for extended periods of time to bring the levels of NAD$^+$ hydrolyzed into an easily detectable range by the methodology used, the kinetics of the reaction showed that the rate was linear with respect to time (Fig. 2A) and concentration of enzyme (Fig. 2B).

Nicotinamide generation. To insure that the loss of NAD$^+$ was due to NADase activity, assays were done to determine whether a stoichiometric increase in nicotinamide occurred. Duplicate alcohol dehydrogenase assays showed that of the added NAD$^+$, 1.59 mg was hydrolyzed during a 24-h incubation period. Assuming that on a molar ratio the nicotinamide constituent of NAD$^+$ was 1 to 5.43, the theoretical amount of nicotinamide expected was calculated to be 0.29 mg. The amount of nicotinamide actually generated in the reaction mixtures, assayed as described in Materials and Methods, was 0.222 mg.

Identification of NADase inhibitor activity. The presence of a heat-labile inhibitor in *M. tuberculosis* (6) and *M. butyricum* (9) was first demonstrated by observations that boiling cell-free extracts resulted in manifestation of NADase activity. Similar heat treatment of Inh^s or Inh^r extracts to demonstrate NADase activity

TABLE 1. *Assay of NADase activity in chromatographed extracts of M. phlei and Inh^r by the methods of alcohol dehydrogenase and cyanide addition*

Source of *M. phlei* extract	NAD$^+$ hydrolyzed (ng/min per mg of protein)^a	
	Alcohol dehydrogenase	Cyanide addition
Inh^s	1.50	1.56
Inh^r	1.17	1.17

^{a} Protein in reaction mixtures containing *M. phlei* NADase was 1.21 mg/ml, and that for Inh^r was 1.0 mg/ml.

were unsuccessful; however, after demonstration of NADase activity after Sephadex G-200 chromatography of the extracts, it was deemed possible that a heat-stable inhibitor was present. It was found that addition of crude extract to reaction mixtures resulted in inhibition of the NADase activity. Thus, pooled Sephadex G-200 fractions of the Inh^s and Inh^r extracts eluting in one void volume immediately after the catalase fractions were pooled, and concentrated by ultrafiltration. After sterilization by filtration through a membrane filter (0.25 μm; Millipore Corp.), the concentrated preparations were tested for inhibitor activity. As shown in Fig. 3, a maximal inhibition of 73% of the *M. phlei* wild-type NADase activity resulted at an inhibitor-versus-NADase protein ratio of 1:1.

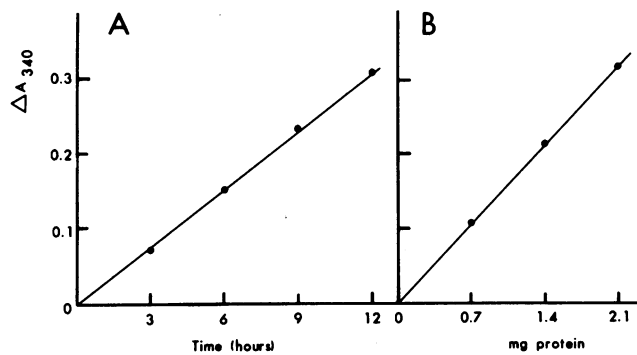The Inh^s and Inh^r NADase activity also appeared to be species specific, but not strain spe-

FIG. 2. *Kinetics of M. phlei NADase assay. M. phlei NADase activity was measured by the alcohol dehydrogenase method. (A) Reaction mixtures contained 1 mg of protein of M. phlei NADase. (B) Assay mixtures contained M. phlei NADase at the concentration of protein indicated and were assayed for NAD^+ after a 6-h incubation period.*
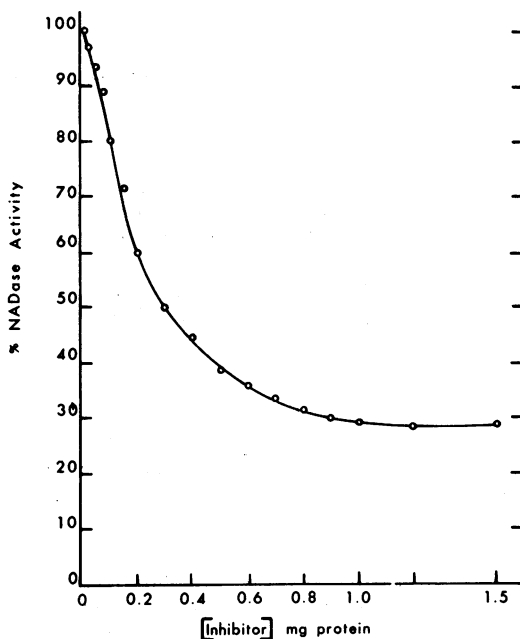

FIG. 3. *M. phlei NADase inhibitor activity. Concentrated fractions of Sephadex G-200-chromatographed M. phlei extracts eluting immediately after catalase/peroxidase activity were diluted to the indicated protein concentrations and incubated with 1 mg of protein of M. phlei NADase for 1 h and assayed for NADase activity by the alcohol dehydrogenase method. Activity is expressed as the percent of the control not containing inhibitor.*

cific with respect to inhibitor. Crude extracts of Inh⁸ or Inhʳ were equally effective in bringing about inhibition of the NADase activity from either organism, whereas the crude extract obtained from *M. tuberculosis* H₃₇Ra had no significant inhibitory effect when added at an equivalent protein concentration (Table 2). The

TABLE 2. *Specificity of inhibitor activity[a]*

Inhibitor source[b]	% Inhibition	
	M. phlei NADase	Inhʳ NADase
Inh⁸	73.4	74.6
Inhʳ	71.4	73.2
M. tuberculosis H₃₇Ra	1.3	1.9
E. coli	0.6	3.7

[a] Inhibitor consisted of cell-free extracts added to reactions to give a concentration of 30 mg/ml. Protein for the extracts containing NADase for *M. phlei* and Inhʳ were 1 mg/ml.

[b] Inhibitor sources were incubated with the respective NADase at 37°C for 1 h before the addition of NAD^+. Assay for NADase was by the alcohol dehydrogenase method as described in the text.

specificity of the Inh⁸ inhibitor action was also further demonstrated by the absence of comparative inhibition of the NADases by a cell-free extract obtained from *E. coli*.

Prevention of NADase inhibitor activity by INH. The inhibition of the Inh⁸ NADase activity could be partially reversed by INH. As shown in Fig. 4, incubation of the inhibitor fraction with increasing concentrations of INH before addition to the NADase reaction mixture resulted in a significant decrease in the NADase inhibitor effect at INH concentrations as low as 0.08 M; however, higher concentrations of INH did not enhance the ability of the drug to prevent the inhibitor action on the NADase. Although the data are not shown, INH also prevented inhibitor activity similarly on respective Inhʳ preparations at the same concentrations.

The specificity of INH in preventing NADase inhibitor activity was tested with several analogs of the drug. In addition to INH, only nicotinic acid hydrazide effectively reduced the inhibition of NADase activity brought about by the con-
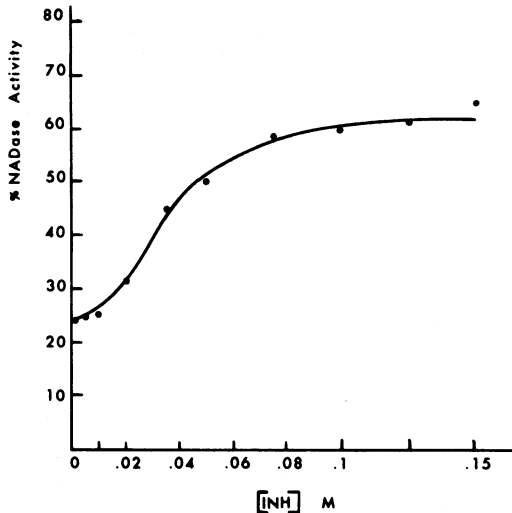
FIG. 4. *Prevention of M. phlei NADase inhibitor activity by INH. M. phlei NADase inhibitor (1 mg of protein) was incubated at 37°C with INH for 1 h before addition to NADase reaction mixtures containing 1 mg of protein of NADase. NADase activity was determined by the alcohol dehydrogenase method as described in the text.*

TABLE 3. *Specificity of INH in reversing M. phlei NADase inhibitor activity*

Compounds[a] tested	% NADase activity[b]	
	−Inhibitor	+Inhibitor[c]
No addition	100	37.2
INH	100	75.6
NAH	100	83.7
BAH	55.2	30.2
INA	100	36.0
α-NADH	100	38.9
Nicotinamide	16.2	ND[d]
NADP	19.2	ND

[a] Activity is expressed as the percentage of the control containing no inhibitor or added compound (1.17 mμg NAD hydrolyzed per min per mg of protein). NAH, nicotinic acid hydrazide; BAH, benzoic acid hydrazide; INA, Isonicotinic acid.

[b] Compounds were added at a final concentration of 0.04 M.

[c] Inhibitor was added to give a protein ratio of 1:1 with respect to NADase extract. In the presence of indicated additions compounds were incubated with inhibitor for 1 h before addition to reaction mixtures.

[d] No activity detectable.

centrated inhibitor fractions (Table 3). A significant amount of direct inhibition of the Inh[s] NADase activity in the absence of inhibitor was observed with nicotinamide, NADP, and benzoic acid hydrazide, thereby abrogating the ability to assay the effect of these compounds on the inhibitor's activity.

DISCUSSION

The major findings of these studies were that *M. phlei* and a strain resistant to high levels of INH possess an NADase sensitive to inhibition by nicotinamide and apparently regulated by an inhibitor. The inhibitor differs from previously described forms of NADase inhibitor systems in other mycobacterial species in that the *M. phlei* inhibitor is heat stable and sensitive to reversal by not only INH but also nicotinic acid hydrazide.

Since heat treatment of *M. phlei* cell-free extract as a means for manifesting NAD⁺ hydrolytic activity is not effective, Sephadex G-200 chromatography is required to remove the inhibitor from the enzyme. Thus, the heat stability of the *M. phlei* inhibitor assumes particular significance among other described mycobacterial NADase inhibitor complexes as an important distinguishing physical characteristic.

It appears from these studies that the regulatory properties of the *M. phlei* inhibitor are not related to the INH susceptibility of the organism. This is evidenced by observations that the inhibitor from either the Inh[s] or Inh[r] strain of *M. phlei* was equally effective in bringing about inhibition of the NADase activity of either strain. In a similar regard, the significance of inhibitor properties in the mechanism of action of INH is further questioned in that prevention of the NADase inhibitory effect by inhibitor, whether obtained from the INH-susceptible or -resistant strain, could be maximally accomplished at equivalent concentrations of INH. However, it should be noted that these findings are not considered to be surprising, since other investigators have shown that among several INH-resistant mutants of *M. tuberculosis*, only in a few did the NADase inhibitor have a diminished sensitivity to reversal by INH (13). Although it has also been shown that exposure of *M. tuberculosis* to INH results in a decrease in the level of cellular NAD⁺ (1), suggesting INH activation of the organism's NADase, Jackett et al. (8) conducted studies which indicated that the extent of NAD⁺ depletion was not sufficient to result in cell death. Finally, the observation that nicotinic acid hydrazide, a nonlethal INH analog, is effective in preventing the NADase inhibitor action adds further evidence invalidating the direct role of the *M. phlei* NADase inhibitor in the drug's mechanism of action.

The NADase activities of *M. butyricum* (9) and *M. tuberculosis* H₃₇Rv (7) can be distinguished from mammalian NADases by their insensitivity to nicotinamide. Thus, the inhibition of the *M. phlei* NADase by nicotinamide suggests an important biological differential from

other mycobacterial NADases. It is apparent that studies on the effect of nicotinamide on the *M. phlei* NADase reaction and further characterization of the nature of the enzyme as compared to that of *M. tuberculosis* are required. Such studies may reveal essential information necessary to more clearly explain the difference in the so-called natural susceptibility to INH between these mycobacterial species and subsequently to a better understanding of the mechanism of action of the drug.

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