Purification and Characterization of the Mycobacterium smegmatis Catalase-Peroxidase Involved in Isoniazid Activation*

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Jovita A. Marcinkevičienė, Richard S. Magliozzoš, and John S. Blanchard†
From the Departments of Biochemistry and Molecular Pharmacology, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

The unique antitubercular activity of isoniazid requires that the drug be oxidized by the katG-encoded mycobacterial catalase-peroxidase to an activated drug form. In order to quantitatively assess the catalytic capabilities of the enzyme, the native catalase-peroxidase from Mycobacterium smegmatis was purified over 200-fold to homogeneity. The enzyme was shown to exhibit both catalase and peroxidase activities, and in the presence of either hydrogen peroxide or t-butyl peroxide, was found to catalyze the oxidation of the reduced pyridine nucleotides, NADH and NADPH, as well as artificial peroxidase substrates, at rates between 2.7 and 20 s⁻¹. The homogeneous enzyme exhibited a visible absorbance spectrum typical of ferric heme-containing catalase-peroxidases, with a Soret maximum at 406 nm. Low temperature (10 K) electron paramagnetic resonance spectra in the presence of ethylene glycol revealed a high spin Fe(III) signal with g values of 5.9 and 5.6. The enzyme was very slowly (t½ = ~20 min) reduced by dithionite, and the reduced form showed typical spectral changes when either KCN or CO were subsequently added. The M. smegmatis catalase-peroxidase was found to contain 2 heme molecules per tetramer, which were identified as iron protoporphyrin IX by the pyridine hemochromogen assay. The peroxidatic activity was inhibited by KCN, NaN₃, isoniazid (isonicotinic acid hydrazide), and its isomer, nicotinic acid hydrazide, but not by 3-amino-1,2,4-triazole. The role of mycobacterial catalase-peroxidases in the oxidative activation of the antitubercular prodrug isoniazid is discussed.

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† To whom correspondence should be addressed: Dept. of Biochemistry, Albert Einstein College of Medicine of Yeshiva University, 1300 Morris Park Ave., Bronx, NY 10461. Tel.: 718-430-3096; Fax: 718-892-0703.

1 The abbreviations used are: INH, isoniazid, isonicotinic acid hydrazide; TEA, triethanolamine; EPR, electron paramagnetic resonance; ACP, acyl carrier protein.
Although many mycobacterial katG genes have been cloned and sequenced, few have been characterized, and little information about the catalytic properties of the enzyme has appeared, even in light of the central role of the enzyme in isoniazid activation.

In this report, we describe the purification of a constitutively expressed catalase-peroxidase from M. smegmatis and characterize both the spectroscopic properties of the enzyme and the catalytic reactions.

MATERIALS AND METHODS

M. smegmatis mc2155 cells (60–80 g wet weight) were suspended in 50 mM TEA buffer, pH 7.8 (50% w/v), containing protease inhibitors (typically, per liter, 2.3 mg of leupeptin, 52 mg of 1-chloro-3-tosylamido-7-amino-2-phenylheptanesulfonyl fluoride inhibitor, 1.6 mg of aprotonin, 1.1 mg of pepstatin, and 36.2 mg of phenylmethylsulfonyl fluoride). Cells were broken by sonication, and cell debris was removed by centrifugation for 45 min at 12,000 rpm. Nucleic acids were precipitated by the addition of streptomycin sulfate (1% w/v, final concentration) to the supernatant, and the solution was centrifuged for 45 min at 12,000 rpm to pellet the nucleic acids. The supernatant was dialyzed against 7 M urea, pH 7.8, containing protease inhibitors, and 50 mM TEA buffer, pH 7.8, for 3 h, during which time a precipitate formed which was removed by centrifugation at 45,000 rpm for 45 min. The clear supernatant was applied to a 400-ml Fast-Flow Q-Sepharose connective tissue matrix which had been equilibrated with 20 mM TEA, pH 7.8. The adsorbed protein was eluted using a 2-liter nonlinear 0–1 M NaCl gradient. Fractions containing peroxidatic activity eluting between 0.3 and 0.4 M NaCl were pooled, dialyzed, and applied to a 10-cm Mono Q (Pharmacia) anion-exchange column which had been equilibrated with 20 mM TEA, pH 7.8. The adsorbed protein was eluted using a 200-ml nonlinear 0–0.6 M NaCl gradient. The active fractions, eluting between 0.2 and 0.3 M NaCl, were pooled, made 1 M in ammonium sulfate by the addition of solid ammonium sulfate, and applied to a phenyl-Sepharose (Pharmacia Biotech Inc) anion-exchange column which had been equilibrated with 20 mM TEA, pH 7.8. The adsorbed protein was eluted using a 1-ml linear 0–1 M NaCl gradient. Fractions containing peroxidatic activity were pooled, concentrated (PM 10, Amicon) to 5 ml, and applied to a 1.6 × 60-cm Superdex 200 (Pharmacia) gel filtration column equilibrated with 20 mM TEA, pH 7.8, containing 50 mM NaCl. The active fractions were pooled, dialyzed, and applied to a 1 × 10-cm Mono Q (Pharmacia) high performance anion-exchange column. The enzyme was eluted using a 200-ml nonlinear 0–0.6 M NaCl gradient. The active fractions, eluting between 0.2 and 0.3 M NaCl, were pooled, made 1 M in ammonium sulfate by the addition of solid ammonium sulfate, and applied to a phenyl-Sepharose (Pharmacia) column. The protein was eluted with a 200-ml reverse gradient of ammonium sulfate (1–0 M) in 20 mM TEA, pH 7.8, and the active fractions were pooled. The enzyme solution was brownish, and displayed a single band of ~80,000 daltons on SDS-PAGE with Coomassie Blue staining (data not shown).

The concentration of enzyme active sites was calculated based on the pyridine hemochromogen assay, using ε293 = 191.5 M −1 cm −1 (18). The purified protein (200 pmol) was analyzed by electrospray ionization/mass spectrometry in an API III triple-quadrupole mass spectrometer (PE SCIEX). The data were deconvoluted by computer to determine the subunit molecular weight. Automated amino-terminal sequencing was performed using an Applied Biosystem sequencer using standard Edman chemistry.

The peroxidase activity of the enzyme was determined spectrophotometrically by measuring the rate of oxidation of 0.1 M o-dianisidine at 460 nm. The protein concentration (ε460 = 11.3 M −1 cm −1) in the presence of 23 mM 1-buthyl hydroperoxide. Catalase activity was determined polarographically using a Clark electrode (Orisphere Laboratories, Geneva) by measuring the formation of molecular oxygen from H2O2. The oxygen electrode was calibrated in air saturated water at 25 °C, assuming the concentration of dissolved oxygen under these conditions is 256 μM.

Low temperature EPR spectroscopy was performed using a Varian E-112 X-band spectrometer equipped with a Varian NMR gaussmeter and a Systron-Donner frequency counter. Samples were maintained at approximately 10 K using a Heli-Tran liquid helium transfer system (Air Products). Spectral data were collected using software provided by the ESR Center of the University of Illinois (19). Experimental parameters are given in the figure legends.

Kineti parameters were determined in 50 mM phosphate buffer, pH 7.0 at 25 °C by following NADPH oxidation spectrophotometrically at 340 nm (ε340 = 6.2 M −1 cm −1), 2.2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) oxidation at 414 nm (ε414 = 36 M −1 cm −1), pyrogallol oxidation at 430 nm (ε430 = 2.47 M −1 cm −1), and 3,3'-diaminobenzidine oxidation at 416-nm (ε416 = 5.68 M −1 cm −1) using a thermostated Uvicron 9310 spectrophotometer. The concentrations of the substrates were calibrated enzymatically using excess H2O2 and horseradish peroxidase. The concentration of peroxides was determined iodometrically, using iodide in the presence of horseradish peroxidase and limiting amounts of peroxide in acetate buffer, pH 3.8. The absorbance changes were monitored spectrophotometrically at 353 nm, using ε353 = 25.5 M −1 cm −1 (20) for 15.

Initial velocity studies were performed by varying the reducing substrate concentration at several fixed concentrations of alkyl or hydrogen peroxide. Data were plotted in reciprocal form, and fitted to Equation 1, using the Fortran programs of Cleland (21):

\[ v = V[A][B]/(K_I[A] + [A][B]) \]  

(Eq. 1)

Inhibition studies were performed by varying the concentration of inhibitor versus one of the substrates at a fixed, saturating concentration of the other substrate. Data were plotted in reciprocal form, and fitted to Equation 2, which describes linear, competitive inhibition:

\[ v = V[A]/(K + [A]I_0) \]  

(Eq. 2)

RESULTS

Enzyme Purification—The results of a typical purification of the M. smegmatis catalase-peroxidase are presented in Table I. Throughout the purification, t-butyl hydroperoxide was used for assaying the peroxidase activity in order to avoid interference from catalase activity in crude extracts. The enzyme was purified 214-fold in 30% overall yield, and ~17 mg of homogeneous enzyme can be obtained from 70 g of cells. The purified protein showed a single protein band of about ~80,000 daltons upon SDS-polyacrylamide gel electrophoresis. The native molecular mass, determined by gel filtration, was approximately 330,000 daltons, suggesting that the native enzyme exists as a homotetramer. The subunit molecular weight was determined to be 81,889 by electrospray mass spectrometry. The amino-terminal sequence for the first 20 amino acid residues was determined by sequential Edman degradation. A comparison of the amino-terminal sequence of the M. smegmatis catalase-peroxidase to that of catalases and peroxidases in the GenBank database revealed homology to the amino-terminal sequence of the previously described Mycobacterium intracellulare catalase-peroxidase (Table II). Of the 19 residues identified in the M. smegmatis enzyme, 14 were identical to the corresponding katG-encoded M. intracellulare catalase-peroxidase (22).

Optical Spectroscopy—The absorption spectrum of the M. smegmatis catalase-peroxidase exhibited a Soret band at 416 nm, and additional absorption maxima at 507 and 631 nm (Fig. 1). Anaerobic reduction of the enzyme with dithionite caused a very slow (t50 = ~20 min) decrease in the intensity of the Soret

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**TABLE I**

| Fraction* | Total protein* | Total activity* | Specific activity | Yield | Purification |
|-----------|----------------|----------------|------------------|-------|-------------|
| Crude extract | 11,800 | 29,600 | 2.5 | 100 | 1.0 |
| Q-Sepharose | 430 | 23,600 | 54 | 80 | 22 |
| Superdex 200 | 84 | 17,700 | 211 | 60 | 84 |
| Mono Q | 36 | 15,600 | 436 | 53 | 175 |
| Phenyl-Sepharose | 17 | 8,800 | 533 | 30 | 214 |

* Units equal to 1 μmol of substrate min −1 . Assay conditions are described in the text.
band, with a new band appearing at 438 nm. Peaks also appeared at 560 and 590 nm on reduction of the enzyme (data not shown). Addition of KCN to the dithionite-reduced enzyme solution resulted in a shift of the Soret band from 438 to 424 nm and the appearance of an absorption maximum at 541 nm with a shoulder at 583 nm (data not shown). Dithionite reduction of the enzyme in the presence of CO resulted in an increased intensity and narrowing of the Soret band, and a shift of the peak to 425 nm (Fig. 2), as well as the appearance of peaks at 541 and 574 nm.

The pyridine ferrohemochrome prepared from the enzyme showed a visible absorption spectrum typical for protoheme IX, with peaks at 418, 523, and 555 nm (18). The protoheme content in the enzyme was estimated to be 2.0 ± 0.2 molecules per 330,000-dalton tetramer, based on the pyridine ferrohemochrome absorbance at 418 nm. This low heme content is consistent with the low $A_{406}/A_{280}$ ratio of 0.56 for the purified protein (Fig. 1). These properties are similar to those previously reported for other bacterial catalase-peroxidases (1–5).

**EPR Spectroscopy**—Further characterization of the katG protein was achieved using EPR spectroscopy. In initial experiments, 60 μM samples of enzyme frozen in TEA or phosphate buffer had no recognizable EPR spectral features. Upon the addition of ethylene glycol (45%, v/v), a signal typical of high spin Fe(III) hemoproteins was found (Fig. 3, inset). The rhombic character of the signal is evident in the small splitting into components at $g = 5.9$ and $5.6$ (Fig. 3A). The same signal was found for the enzyme in phosphate buffer in 50% ethylene glycol (not shown). The feature expected near $g = 2.0$ was not identified due to the low concentration of heme in the samples and interference from contaminating paramagnetic species.

An EPR signal could also be elicited by the addition of sodium fluoride to the enzyme sample in TEA buffer, without the addition of ethylene glycol (Fig. 3B). This signal, which was the same in the presence of ethylene glycol (not shown), also had a very small rhombic splitting though the $g$ values ($g = 6.1, 5.8$) were different from those observed in the presence of ethylene glycol alone. No identification of features at $g = 2.0$ could be made (nor could a splitting due to $^{19}$F hyperfine interaction be resolved) although fluoride binding is evident from the observed change in $g$ values.

**Catalytic Properties**—The M. smegmatis catalase-peroxidase catalyzed the oxidation of various substrates, including NAD(P)H and synthetic dyes, and followed a steady-state Ping-Pong kinetic mechanism, as determined from the series of parallel lines observed when reciprocal velocities were plotted against reciprocal reducing substrate concentrations at various fixed peroxide concentrations (data not shown). Using o-dianisidine as reductant, the rate of o-dianisidine oxidation in the

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**Table II**

| Source | 1............ 11........ 21.... 26 | No. of amino acids | $M_r$ | Reference |
|--------|-------------------------------|-------------------|-------|-----------|
| mskatg | .SSDTSDSRP PNPDTKT.AS T...... | 7 | 81,899 | This work |
| mikatg | MSSDTSSSRP PQPDSGT.AS KSESE. | 746 | 81,385 | Morris et al. (22) |
| mtkatg | .......MPEQRIP PITETTTI.AA SNCCPV | 735 | 80,029 | Heym et al. (33) |
| mbkatg | .......MPEQPRT PITETTPGAAN SNCCPV | 740 | 80,543 | S. Cole (unpublished results) |
| eckatg | .......MST SDDHNT.TA TKGCPL | 726 | 80,009 | Triggs-Raine et al. (49) |
| stkatg | .......MST TDDHNLT.LS TKGCPL | 727 | 80,032 | Loewen and Stauffer (50) |
| bskatg | .......MENQRN.QN AAQCPF | 731 | 82,963 | Loprasert et al. (51) |

*GenBank™ accession nos.: M. intracellulare, mikatG: M86741; M. tuberculosis, mtkatG: X68081; M. bovis BCG, mbkatG: X83277; E. coli, eckatG: M21516; Salmonella typhimurium, stkatG: X53001; Bacillus stearothermophilus, bskatG: M29876.*

**Fig. 1.** Absorption spectrum of purified catalase-peroxidase from M. smegmatis.

**Fig. 2.** Absorption spectrum of the CO complex formed with the reduced catalase-peroxidase in 50 mM phosphate buffer, pH 7.0, 25°C. Time intervals between the scans were 3 min after addition of dithionite in the presence of CO. (Dotted line, oxidized enzyme.)

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* Amino-terminal sequence alignment of bacterial catalase-peroxidases
presence of hydrogen peroxide was 10 times higher than the corresponding rate in the presence of t-butyl peroxide (Table III). As expected for a Ping-Pong mechanism, neither the \( K_m \) nor the \( V/K_m \) values of the reducing substrate were dependent on the nature of the oxidant. The choice of t-butyl hydroperoxide as the standard assay substrate was made based on its aqueous solubility, and the low nonenzymatic rate of oxidation of reducing substrates. o-Dianisidine was found to exhibit the highest \( V/K_m \) and \( V_{max} \) values, and all other reduced substrates exhibited comparable \( K_m \) values (0.10 to 0.31 mM) and \( V_{max} \) values (2.7 to 8.4 s\(^{-1}\)) (Table III). With the exception of the pyridine nucleotides, the substrates listed in Table III are typical peroxidase substrates. Horseradish peroxidase oxidizes o-dianisidine more rapidly than the mycobacterial catalase-peroxidase (23), but its activity with the reduced pyridine nucleotides is 2–10% of that of the bacterial catalase-peroxidases (2).

In addition to the peroxidatic activity, the enzyme exhibited catalytic activity, which was comparable to analogous enzymes activities. The catalatic activity of the mycobacterial enzyme is significantly higher than that exhibited by the E. coli catalase-peroxidase (2380 s\(^{-1}\) compared to 563 s\(^{-1}\) (1), respectively, at pH 7.6), although the \( K_m \) values for \( H_2O_2 \) are comparable for these two enzymes (1.43 and 3.9 mM, respectively). The \( V/K_m \) value of the catalatic reaction catalyzed by the mycobacterial catalase-peroxidase (1.7 \( \times \) \( 10^6 \) M\(^{-1} \) s\(^{-1} \)) is an order of magnitude lower than that reported for bovine liver catalase (2.1 \( \times \) \( 10^7 \) M\(^{-1} \) s\(^{-1} \)) (24).

Inhibition Studies—The peroxidatic activity of the mycobacterial catalase-peroxidase was not inhibited by the prototypical catalase inhibitor, 3-amino-1,2,4-triazole, but KCN and Na\( _3 \)N\(_2 \) were effective inhibitors. KCN was a competitive inhibitor against t-butyl hydroperoxide, with a \( K_i \) of 3 \( \mu \)M. Sodium azide was a weak competitive inhibitor against o-dianisidine (\( K_i \) = 1.1 mM; Table IV). Both isoniazid (isonicotinic acid hydrazide) and its isomer, nicotinic acid hydrazide, were strong competitive inhibitors of the o-dianisidine peroxidatic reaction (Fig. 4), exhibiting \( K_i \) values of \( \sim 5 \mu M \) (Table IV). The enzyme-catalyzed peroxidatic reaction was not inhibited by nicotinamide, isonicotinamide or pyrazinamide.

### Table III

| Reaction (electron donor) | \( K_m \) | \( V_{max} \) | \( V_{max}/K_m \) |
|--------------------------|----------|--------------|------------------|
| Catalatic                 |          |              |                  |
| M. tuberculosis           | 0.6 ± 0.2| 460 ± 100    | 7.6 ± 0.9 \( \times \) 10\(^5\) |
| M. smegmatis              | 1.4 ± 0.2| 2400 ± 430   | 1.7 ± 0.01 \( \times \) 10\(^6\) |
| Peroxidatic               |          |              |                  |
| M. tuberculosis           | 0.14 ± 0.04| 2.1 ± 0.6   | 1.6 ± 0.0 \( \times \) 10\(^4\) |
| o-Dianisidine\( ^a \)    | 0.15 ± 0.02| 20.0 ± 2.0   | 138 ± 0.5 \( \times \) 10\(^4\) |
| o-Dianisidine\( ^b \)    | 0.19 ± 0.03| 190 ± 20     | 98 ± 8 \( \times \) 10\(^4\) |
| Pyrogallol\( ^a \)       | 0.31 ± 0.03| 43 ± 0.4     | 1.4 ± 0.1 \( \times \) 10\(^4\) |
| 2,2’-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)\( ^a \) | 0.11 ± 0.01| 2.7 ± 0.3    | 2.6 ± 0.3 \( \times \) 10\(^4\) |

\( ^a \) Reactions performed at four concentrations of t-butyl peroxide.

\( ^b \) Reactions performed at four concentrations of hydrogen peroxide.

### Table IV

| Inhibitor\( ^a \) | Variable substrate | \( K_i \) | Inhibition pattern |
|-------------------|--------------------|---------|-------------------|
| NaN\(_3 \)        | o-Dianisidine      | 1100 ± 200 | C                 |
| KCN               | t-Butyl HP         | 3.0 ± 0.2 | C                 |
| Isonicotinic acid | o-Dianisidine      | 4.3 ± 0.7 | C                 |
| hydrazide         |                    |         |                   |
| Nicotinic acid    | o-Dianisidine      | 5.3 ± 1.5 | C                 |

\( ^a \) The enzyme was not inhibited by nicotinamide, pyrazinamide, isonicotinamide, or 3-amino-1,2,4-triazole.

**Fig. 3. EPR spectra of M. smegmatis catalase-peroxidase.** Inset at top shows the x-band EPR spectrum of the resting enzyme recorded at 10 K. A, low field region of spectrum in inset; B, low field region after addition of excess of NaF to sample in A. Experimental conditions: microwave power, 10 mW; modulation amplitude, 10 G; microwave frequency, 9.308 GHz. A baseline correction was performed digitally for the data in B.

**Fig. 4. Isoniazid inhibition versus o-dianisidine.** 50 mM phosphate buffer, pH 7.0, 25 °C. The fit of the data yield a calculated \( K_i = 4.3 ± 0.7 \mu M \).

**DISCUSSION**

A wide variety of bacteria have been shown to express heme-containing hydroperoxidases capable of performing both catalatic and peroxidatic chemistries (1–5). The katG-encoded catalase-peroxidases from several mycobacterial species have been cloned and sequenced, and the M. intracellulare katG gene has been expressed in E. coli and shown to exhibit both catalase and peroxidase activities (22). However, no detailed substrate specificity studies, nor spectroscopic characterization, has been performed on any mycobacterial catalase-peroxidase. Our interest in these enzymes stems from the unique role of the enzyme in oxidative stress management, virulence...
and the unique susceptibility of mycobacteria, especially M. tuberculosis, to isoniazid.

Pathogenic mycobacteria, including M. tuberculosis, M. leprae, and the M. avium-M. intracellulare complex, are phagocytized by macrophages and can survive and proliferate inside macrophage phagosomes. One of the reasons mycobacteria can survive the high levels of reactive oxygen species, particularly H$_2$O$_2$ generated by the macrophage, is that they can efficiently remove this injurious oxidant by the action of intracellular catalases and peroxidases. M. tuberculosis contains only a constitutively expressed katG-encoded catalase-peroxidase (8), while other mycobacteria may contain an additional katE-type catalase (9), as does E. coli (7). Mycobacterial catalase-peroxidases (katG-encoded proteins) are highly homologous, and are additionally homologous to other bacterial catalase-peroxidases. Though a certain amount of structural information is appearing for these enzymes, little is known about their catalytic properties. The paucity of mechanistic information is surprising considering the critical role of the mycobacterial catalase-peroxidase in the action of the potent antitubercular drug, isoniazid (see below). This encouraged us to undertake the purification and characterization of the constitutively expressed catalase-peroxidase from M. smegmatis.

The enzyme purified from M. smegmatis was found to share a number of structural and spectroscopic properties with other bacterial catalase-peroxidases. It is a tetramer of 81,889-dalton subunits, containing two ferric protoporphyrin IX prosthetic groups, and exhibits a high spin ferric heme optical spectrum. The iron could be slowly reduced by dithionite and converted to a low spin ferrous form in the presence of either CN$^-$ or CO. Dithionite reduction distinguishes the catalase-peroxidases from the typical catalases, which are not reduced by dithionite, and typical peroxidases, which are rapidly reduced by dithionite. The rate of dithionite reduction is atypical for both catalases and peroxidases.

The EPR results indicate that ethylene glycol stabilizes high spin ferric heme iron in the catalase-peroxidase enzyme. The absence of EPR signals in frozen solutions without added ethylene glycol or fluoride ion may arise from a freezing artifact similar to that previously described for two other heme peroxidases, cytochrome c peroxidase (25) and lactoperoxidase (26). In these cases, freezing induces the formation of low spin species, which for cytochrome c peroxidase has been reported to involve coordination of an endogenous strong field ligand (25). The formation of a mixture of species upon freezing dilute samples of catalase-peroxidase in buffer alone could similarly be responsible for the loss of resolvable intensity of either high or low spin forms.

The EPR signal observed in ethylene glycol/buffer mixtures are atypical for classical catalases, such as bovine liver catalase (27) and plant or bacterial peroxidases such as horseradish peroxidase (28) and cytochrome c peroxidase (29), all of which exhibit larger rhombic splittings, with the lowest field g value ranging from 6.6 to 6.3. In contrast to these, plant lignin peroxidase (30) and high spin globins (27) exhibit no rhombic splitting and a g value near 5.8. The EPR spectrum of catalase-peroxidase from Streptomyces has recently been reported, although the multiple g features at g ~ 5–7 make comparison to our results difficult (31). The small rhombicity found for the M. smegmatis catalase-peroxidase, usually encountered for high spin globins (27), is considered to be an indicator of the presence of six-coordinated ferric heme containing a proximal imidazole as the fifth ligand to iron and water as sixth ligand. This idea is supported by alignment of the amino acid sequences of cytochrome c peroxidase (for which a high resolution crystal structure is available), bacterial catalase-peroxidases (32) and mycobacterial catalase-peroxidases (22, 33). There is a highly conserved region in the NH$_2$-terminal domain (RMGMDEETVALIAGGHTLGKTH), and the underlined residue His-175 of cytochrome c peroxidase is a proximal ligand of the heme iron and is conserved in other catalase-peroxidases (His-267 in E. coli, His-269 in M. tuberculosis, and His-277 in M. intracellulare). This homology is not apparent in the amino-terminal region where, with the exception of the M. intracellulare (Table II), divergent sequences are observed between bacterial catalase-peroxidases and especially between catalase-peroxidases and cytochrome c peroxidase, although these differences were not considered catalytically significant, as judged by activity staining (34).

Additional characteristics shared with other bacterial catalase-peroxidases include the inhibition by CN$^-$ and N$_3^-$ and the lack of inhibition by the catalase inhibitor, 3-amino-1,2,4-triazole (35). The linear, competitive nature of the inhibition by CN$^-$ versus peroxide suggests that the heme iron atom of the catalase-peroxidase is the site of both peroxide and cyanide binding, as is the case for other peroxidases (36).

In contrast to cyanide inhibition, azide was found to be a linear, competitive inhibitor versus dianisidine, suggesting that it binds to the reducing substrate binding site of catalase-peroxidase. It has been proposed that electron donors bind to the enzyme at a hydrophobic site 7–8.5 Å from the heme iron (37). In horseradish peroxidase, azide inhibits the enzymatic activity yet does not bind at neutral pH to the prosthetic heme iron atom (38). The one-electron oxidation of azide is catalyzed by chloroperoxidase, myeloperoxidase, and lactoperoxidase (39), and the inhibition of these enzymes, as well as the mycobacterial catalase-peroxidase, may be due to heme modification by the product of the one-electron oxidation of azide.

Like other peroxidases and catalase-peroxidases, the mycobacterial enzyme oxidized artificial electron donors. However, its ability to use both NADH and NADPH as reductants is a unique property of bacterial catalase-peroxidases (2, 4) and distinguishes this class of hydroperoxidases from classical peroxidases and catalases. It is likely that reduced pyridine nucleotides are the in vivo reductants for the mycobacterial catalase-peroxidases, and the enzyme may thus serve physiologically to oxidize the reduced coenzymes, influencing the rates of metabolic pathways which are sensitive to this ratio. In contrast, mammalian catalases have been shown to contain four tightly bound molecules of NADPH (40), although the function of the bound nucleotide is not clear. It has been proposed that the bound nucleotide protects the enzyme from inactivation, both by preventing, and reversing, the formation of compound II, an inactive form of catalase (40, 41).

Perhaps the most intriguing feature of the mycobacterial catalase-peroxidases is their role in the activation of the antitubercular drug, isoniazid. There is compelling evidence that the antimycobacterial activity of isoniazid is dependent on the catalase-peroxidase (15) and M. smegmatis catalase-peroxidase,2 is also a potent, competitive inhibitor of the peroxidatic reaction, competing for the substrate binding site. The lack of inhibition using other aromatic ring containing inhibitors (Table IV) suggests that the

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2 A. Quemard, A. Dessen, M. Sugantiono, W. R. Jacobs, J. C. Sacchettini, and J. S. Blanchard, submitted for publication.
The 10-fold lower peroxidatic activity of the M. tuberculosis catalase-peroxidase compared to that of M. smegmatis is unexpected, since if sensitivity to isoniazid only involves rates of conversion of the prodrug, isoniazid, into an activated form, then one would expect that M. smegmatis would be more sensitive to isoniazid than M. tuberculosis. This is clearly not the case, since reported values for the minimal inhibitory concentration of isoniazid are 0.02 and 8 μg/ml for M. tuberculosis H37Rv and M. smegmatis, respectively (47, 48). Possible reasons for the greater sensitivity of M. tuberculosis to isoniazid may be related to prodrug uptake, target sensitivity to inhibition, or other interspecies differences. These possibilities are under investigation.

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REFERENCES

1. Claiborne, A., and Fridovich, I. (1979) J. Biol. Chem. 254, 4245-4252
2. Hochman, A., and Goldberg, I. (1991) Biochim. Biophys. Acta 1077, 299-307
3. Brown-Peterson, N. J., and Salin, M. L. (1993) J. Bacteriol. 175, 4197-4202
4. Levi, E., Eyal, Z., and Hochman, A. (1992) Arch. Biochem. Biophys. 299, 321-327
5. Yumoto, I., Fukumori, Y., and Yamanaka, T. (1990) J. Biochem. 108, 583-587
6. Jackott, P. S., Abber, V. R., and Lowrie, D. B. (1978) Gen. Microbiol. 104, 37-45
7. Lowen, P. C., Swatland, J., and Triggs-Raine, B. L. (1985) Arch. Biochem. Biophys. 243, 144-149
8. Diaz, G., and Wayne, L. G. (1974) Am. Rev. Respir. Dis. 110, 312-319
9. Bartholomew, W. R. (1968) Am. Rev. Respir. Dis. 97, 710-712
10. Gruft, H., and Gaafar, H. A. (1971) Chromatogr. 56, 169-171
11. Middelbroe, G., Cohn, M. L., and Schaefer, W. B. (1954) Ann. Rev. Microbiol. 10, 70-827
12. Zhang, Y., Heym, H., Allen, B., Young, D., and Cole, S. (1992) Nature 358, 591-593
13. Zhang, Y., Garbe, T., and Young, D. (1993) Mol. Microbiol. 8, 521-523
14. Wilson, T. M., de Lisle, G. W., and Collins, D. M. (1995) Mol. Microbiol. 15, 1009-1015
15. Johnson, K., and Schultz, P. G. (1994) J. Am. Chem. Soc. 116, 7425-7427
16. Johnson, K., King, D. S., and Schultz, P. F. (1995) J. Am. Chem. Soc. 117, 5009-5010
17. Quemard, A., Sacchettini, J. C., Dessen, A., Vilcheze, C., Bittman, R., Jacobs, W. R., and Blanchard, J. B. (1996) Biochemistry 35, 8235-8241
18. Falk, J. K. (1964) in Porphy ris and Metallopor phyrins (Smith, K. M., ed) pp. 804-807, Elsevier Publishing Co., New York
19. Morse, P. D., II (1987) Biophys. J. 51, 440a
20. Cotton, V. L., and Dunford, H. B. (1973) Can. J. Biochem. 51, 582-587
21. Cleland, W. W. (1979) Methods Enzymol. 63, 103-138
22. Morris, S. L., Nair, J., and Rouse, D. A. (1992) J. Gen. Microbiol. 138, 2363-2370
23. Lebedeva, O. V., Ugurova, N. N., and Bereizin, I. V. (1977) Biokhimia 42, 1372-1379
24. Kirkman, H. N., Galiano, S., and Gaetani, G. F. (1987) J. Biol. Chem. 262, 660-666
25. Yonetani, T., and Anni, H. (1989) J. Biol. Chem. 264, 3342-3355
26. Blumberg, W. E., Peisach, J., Wittenberg, B. A., and Peisach, J. (1968) J. Biol. Chem. 243, 1854-1862
27. Wittenberg, B. A., Kampa, L., Wittenberg, J., Blumberg, W. E., and Peisach, J. (1968) J. Biol. Chem. 243, 1863-1870
28. Anderson, L. A., Renganathan, V., Chiu, A. A., Loehr, T. M., and Gold, M. H. (1985) J. Biol. Chem. 260, 6080-6087
29. Yoon, H. D., Yim, Y. I., Kim, K., Hah, Y. C., and Kang, S. O. (1995) J. Biol. Chem. 270, 13740-13747
30. Welinder, K. G. (1991) Biochim. Biophys. Acta 1000, 215-220
31. Heym, B., Zhang, Y., Poulet, S., Young, D., and Cole, S. T. (1993) J. Bacteriol. 175, 4255-4259
32. Zhang, Y., Heym, B., Allen, B., Young, D., and Cole, S. T. (1992) Nature 358, 591-593
33. Margolishi, E., Novogradsky, A., and Schejter, A. (1960) Biochim. J. 74, 339-348
34. Dunford, H. B. (1991) in Peroxidases in Chemistry and Biology (Everse, J. E., Everse, K. E., and Grisham, H. B., eds) Vol. 2, pp. 1-24, CRC Press, Boca Raton, FL
35. Schejter, A., Lanir, and Epstein, N. (1976) Arch. Biochem. Biophys. 174, 36-44
36. Morishima, I., Ogava S., Inubushi, T., Yonezawa, T., and Iizuka, T. (1977) Biochemistry 16, 5109-5115
37. Kajyanaraman, B., J. anzen, E. G., and Mason, R. P. (1985) J. Biol. Chem. 260, 4003-4006
38. Kirkman, H. N., and Gaetani, G. F. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 4343-4347
39. Kirkman, H. N., Galiano, S., and Gaetani, G. F. (1987) J. Biol. Chem. 262, 660-666
40. Shoeb, H. A., Bowman, J. B. U., Ottolenghi, A. C., and Merola, A. J. (1985) Antimicrob. Agents Chemother. 27, 399-403
41. Shoeb, H. A., Bowman, J. B. U., Ottolenghi, A. C., and Merola, A. J. (1985) Antimicrob. Agents Chemother. 27, 404-407
42. Heym, B., Alzari, P. M., Honore, N., and Cole S. T. (1995) Mol. Microbiol. 15, 235-245
43. Stockl, Y. M., Guan, L., and Rieger, N. (1993) J. Infect. Dis. 168, 1063-1065
44. Banerjee, A., Dubnau, E., Quemard, A., Balsubramanian, V., Um, K. S., Wilson, T., Collins, D., de Lisle, G., and Jacobs, W. R. (1994) Science 263, 227-230
45. Young, D. B. (1994) Curr. Biol. 4, 351-353
46. Wallace, R. J., Nishi, D. R., Tsukamura, M., Blacklock, Z. M., and Slixox, V. A. (1988) J. Infect. Dis. 158, 52-59
47. Triggs-Raine, B. L., Nosh, B. W., Mulvey, M. R., Sorby, P. A., and Loewen, P. C. (1988) J. Bacteriol. 170, 4415-4419
48. Loewen, P. C., and Stauffer, G. V. (1990) Mol. Gen. Genet. 224, 147-151
49. Lopresti, S., Negro, S., and Okada, H. (1989) J. Bacteriol. 171, 4871-4875