Overexpression, Purification, and Characterization of the Catalase-peroxidase KatG from *Mycobacterium tuberculosis*

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Wild-type catalase-peroxidase KatG from *Mycobacterium tuberculosis* as well as a specific mutant (R463L) frequently found in isoniazid-resistant strains have been overexpressed in *Escherichia coli*, allowing purification of sufficient quantities of enzyme for physical and kinetic characterization. Optical absorption and EPR spectroscopies indicate that KatG is similar to a growing class of bacterial catalase-peroxidases. Optical and EPR spectra of KatG in the presence of either a strong field or weak field ligand suggest that, like horseradish peroxidase and metmyoglobin, KatG is likely to have a histidine as a proximal ligand. The wild-type enzyme functions as a highly active catalase as well as a broad specificity peroxidase. Wild-type KatG and the R463L mutant of KatG exhibit identical spectroscopic and kinetic properties. Furthermore, both enzymes are equally capable of metabolizing the important antituberculosis drug isoniazid.

Tuberculosis is a reemerging disease in the industrialized world and continues to be the single most deadly infectious disease in developing countries (1, 2). The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* (3) and the widening association of mycobacterial infection and AIDS (4) have underscored the need for research into the mechanisms of drug resistance and the design of more effective antitubercular agents (1). Introduced in 1952, isoniazid (isonicotinic acid hydrazide) is still the most widely used antituberculosis drug. Despite its importance, it has only been in the last few years, with the development of mycobacterial genetics, that an understanding of its mechanism of action has advanced (5). Analysis of isoniazid-resistant strains revealed that isoniazid is oxidized to its activated form by a mycobacterial catalase-peroxidase (KatG) (6). The ultimate target of the activated drug may either be an enoyl-acyl carrier protein reductase (InhA), an enzyme involved in mycolic acid biosynthesis (7, 8), or an as yet unidentified alternative target in the reductase (InhA), an enzyme involved in mycolic acid biosynthesis (7, 8), or an as yet unidentified alternative target in the reductase (InhA), an enzyme involved in mycolic acid biosynthesis (7, 8), or an as yet unidentified alternative target in the reductase (InhA), an enzyme involved in mycolic acid biosynthesis (7, 8), or an as yet unidentified alternative target.

There are several possible mechanisms for isoniazid resistance. One possibility is that InhA is inactivated by a specific enzyme, such as a deacetylase (9). Another possibility is that the drug is inactivated by a specific enzyme, such as a deacetylase (9). Another possibility is that the drug is not able to reach its target because of increased binding to other proteins or degradation by a specific enzyme. A third possibility is that the drug is not able to reach its target because of increased binding to other proteins or degradation by a specific enzyme. A third possibility is that the drug is not able to reach its target because of increased binding to other proteins or degradation by a specific enzyme. A third possibility is that the drug is not able to reach its target because of increased binding to other proteins or degradation by a specific enzyme. A third possibility is that the drug is not able to reach its target because of increased binding to other proteins or degradation by a specific enzyme.

It is thought that KatG is the only enzyme in *M. tuberculosis* capable of generating isoniazid susceptibility and that it therefore plays a central role in the development of at least one type of isoniazid resistance (6). Total or partial deletion of the *katG* gene, as well as mutations that render KatG inactive, all lead to isoniazid resistance (13, 30, 31). In addition, a mutation in KatG (R463L), which does not lead to a loss of peroxidase or catalase activity (30), is also thought to confer isoniazid resistance. This mutation has been identified in about 30–50% of all isoniazid-resistant clinical strains analyzed thus far but very rarely in isoniazid-susceptible strains (30–54). It has been suggested that residue 463 might interact with the substrate binding site of KatG and that Leu463 might discriminate against isoniazid (30). Furthermore, it has been found that both *Mycobacterium intracellulare*, which is naturally resistant to isoniazid, and *Mycobacterium bovis* BCG, which is less sensitive to isoniazid than *M. tuberculosis*, possess a leucine residue at position 463 of KatG (30). However, it has recently been shown that expression of either wild-type KatG or the KatG R463L mutant in an isoniazid-resistant, KatG-defective
**M. smegmatis** strain both confer isoniazid sensitivity to about the same extent (31). It is evident from these results that a clear relationship between the KatG R463L mutant and isoniazid resistance has not yet been established. We describe here the subcloning of katG from *M. tuberculosis* into an *E. coli* expression vector, the overexpression and purification of the enzyme, and its kinetic and spectroscopic characterization. Furthermore, we report the generation and characterization of the R463L mutant of KatG, including its in vitro activity toward isoniazid and its capability to mediate the inactivation of InhA.

**EXPERIMENTAL PROCEDURES**

**Materials**—Plasmid pYZ55 containing katG was provided by Dr. Stewart Cole (Pasteur Institute) (6). *E. coli* strain UM262 (recA katG::Tn10 pro leu rpsL hsdM hsdR endl lacY) was provided by Dr. John Blanchard (Albert Einstein College of Medicine) (8). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, Inc. Sequenase was obtained from Amersham Life Science Inc. T7 DNA polymerase was obtained from Promega. Isoniazid was obtained from Aldrich and was recrystallized from ethanol. All synthetic DNA oligomers were synthesized with an Applied Biosystems 391 DNA synthesizer. All other chemicals were the highest quality available and used without further purification.

**Subcloning Methods**—The katG coding region of pYZ55 was amplified by polymerase chain reaction using the following primers: 5'-GGA GAT ATG TCG ATG CCC GAA CAA CCA CCC ATT ACA GAA-3', consisting of a ClaI restriction site followed by the sequence encoding the 10 N-terminal amino acids of KatG; and 5'-CAG GTC AAG AAG CTT TCA GCG CAC GTC GAA CCT GTC GGT-3', consisting of the final eight C-terminal amino acids of KatG and a TGA termination codon that extends over the termination codon of the coding region of katG. pKAT was digested with ClaI and HindIII, yielding a 2.4-kilobase pair ClaI fragment and a 1.68-kilobase pair HindIII fragment. The fragments were ligated into the plasmid pRG (36), also digested with ClaI and HindIII, yielding the plasmid pKAT. Sequencing of pKAT indicated that two fragments had been introduced by polymerase chain reaction. To correct these mutations, pY55S was digested with NsiI and NheI, yielding a 2.4-kilobase pair NheINsiI fragment that extends over the termination codon of the coding region of katG. pKAT was digested with NheI and HindIII, and the digested plasmid was gel-purified. The NheINsiI fragment and a NheIHindIII double-stranded oligomer (containing an internal KpnI site) were ligated into the digested plasmid. The resulting plasmid pKATII was used to transform competent *E. coli* UM262. The transformants were screened for the KpnI site, and the sequence of the gene was subsequently verified. An ApII site at Leu<sup>A</sup> and a BamHI site at Val<sup>A</sup> allowed the introduction of a synthetic oligonucleotide 5'-TT AAG AGC CAG ATC TCG GCA TCG GGT CTC ACT GTC TCA-3' bearing the mutation R463L. Introduction of the mutation R463L was confirmed by sequencing.

**Bacterial Growth and Purification of the Catalese-Peroxidase**

**KatG**— *E. coli* UM262 pKATII and *E. coli* UM262 pKATII R463L were grown in M9 medium (Sigma) supplemented with 30 µg/ml Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>. Bacterial growth was typically done on a 6-liter scale (6 × 1 liter) at 37 °C with vigorous agitation and were induced at midlog phase by the addition of β-indolacrylic acid (40 mg/liter). Cells were grown for an additional 10 h after induction and collected by centrifugation. All of the following purification procedures were performed at 0–4 °C. Cells were lysed with DNase I according to standard proce-

dures (37). Solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to the crude cell lysate to make a 40% saturated solution at 4 °C. After 15 min, the solution was centri-

**fig. 1. Proposed reaction pathways of catalase-peroxidases.**

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The abbreviations used are: MOPS, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.
Expression and Purification of KatG—The mycobacterial katG gene was subcloned into the plasmid pBG to afford the plasmid pKATII, placing the wild-type katG downstream of the E. coli tryptophan promoter. The mutant pKATII R463L was subsequently prepared by a standard site-directed mutagenesis technique as described under “Experimental Procedures.” E. coli UM262, a katG-deficient strain, was transformed with both pKATII and pKATII R463L, and a successful transformant of each was identified and grown on a 6-liter scale. Analysis of cell extracts after induction of expression showed that KatG migrates as two bands (termed KatGI and KatGII) on nondenaturing PAGE (Fig. 2). It has been previously observed that nondenaturing PAGE of cell extracts from M. tuberculosis cultures shows two bands with KatGI as the major component (6, 30). The ratio between KatGI and KatGII in cell extracts after induction of expression (determined by nondenaturing PAGE and activity staining) depends on the amount of supplementary Fe(NH₄)₂(SO₄)₂ added to the medium, with KatGI being the predominant band above a concentration of 0.5 mg/liter. The amount of catalase activity observed in these cell extracts also increased approximately 10-fold as the supplementary iron concentration was increased from 0 to 5 mg/liter Fe(NH₄)₂(SO₄)₂.

Expression of KatG and subsequent purification by standard protein purification techniques yielded approximately 3 mg ofKatG/ liter of shake flask culture (Table I). Protein purification techniques yielded approximately 3 mg ofKatG/liter of shake flask culture (Table I). Purification from a 6-liter shake flask culture of M. tuberculosis strain, was transformed with plasmid pKATII, placing the wild-type katG downstream of the E. coli tryptophan promoter. The mutant pKATII R463L was subsequently prepared by a standard site-directed mutagenesis technique as described under “Experimental Procedures.” E. coli UM262, a katG-deficient strain, was transformed with both pKATII and pKATII R463L, and a successful transformant of each was identified and grown on a 6-liter scale. Analysis of cell extracts after induction of expression showed that KatG migrates as two bands (termed KatGI and KatGII) on nondenaturing PAGE (Fig. 2). It has been previously observed that nondenaturing PAGE of cell extracts from M. tuberculosis cultures shows two bands with KatGI as the major component (6, 30). The ratio between KatGI and KatGII in cell extracts after induction of expression (determined by nondenaturing PAGE and activity staining) depends on the amount of supplementary Fe(NH₄)₂(SO₄)₂ added to the medium, with KatGI being the predominant band above a concentration of 0.5 mg/liter. The amount of catalase activity observed in these cell extracts also increased approximately 10-fold as the supplementary iron concentration was increased from 0 to 5 mg/liter Fe(NH₄)₂(SO₄)₂.

Expression of KatG and subsequent purification by standard protein purification techniques yielded approximately 3 mg of active enzyme (KatGI/liter of shake flask culture (Table I). KatGI was resolved from KatGII in the final high resolution chromatographic step of the purification protocol and possessed a significantly higher RZ (A₂₈₀/A₃₃₂ = 0.69) compared with samples composed of predominantly KatGII (A₂₈₀/A₃₃₂ = 0.4), suggesting that the heme content of KatGII is significantly lower. The sequence of the first five N-terminal amino acids was confirmed by Edman degradation and showed that the N-terminal methionine is cleaved from the protein. Nondenaturing PAGE of the KatG preparation stained for peroxidase activity is shown in Fig. 2. The enzyme runs as a single band of approximately 82 kDa (purity >90%) on SDS-PAGE (Coomassie Blue staining). In addition to the major 82 ± 3-kDa band, a fragment about 5 kDa smaller was seen in SDS-PAGE of samples composed of predominantly KatGII, presumably proteolytically degraded KatG. These results suggest that KatGII arises from insufficient in vivo concentrations of cofactor and/or proteolytic degradation. All the following physical and kinetic characterization of KatG was done with samples composed of KatGI.

Expression levels and purification properties of the KatG R463L mutant were similar to wild-type KatG.

Physical Characterization of KatG—The native molecular weight of KatG was determined by gel filtration chromatography on Sephadex G200 and Superose 12HR columns. The measured apparent molecular mass was 175 and 161 kDa, respectively, indicating that the protein forms a dimer in solution. This is in agreement with the value obtained for KatG isolated from cell extracts from M. tuberculosis (42). The heme content of native KatG was determined through the pyridine hemochrome spectrum, which exhibited the typical peaks of iron protoporphyrin IX, with maxima at 419, 525, and 556 nm (data not shown). Based on a value of $f_{556} = 33,200 \text{ M}^{-1} \text{ cm}^{-1}$ for pyridine hemochrome (40), the heme content of the enzyme was calculated to be 0.98 heme/dimer. This stoichiometry is consistent with the relatively low $A_{408}/A_{280}$ ratio of 0.69 and agrees well with other catalase-peroxidases of this class.

The visible absorption spectrum of native KatG exhibited maxima at 408, 490, and 627 nm (Fig. 3). These values for the Soret and overlapping $\alpha$ and $\beta$ absorption bands are similar to those of horseradish peroxidase (43, 44) and aquomet myoglobin (45), both high spin heme proteins with histidine as the proximal ligand. In addition, the absorption band at 627 nm is a more general characteristic of high spin ferric heme proteins, including catalase (46) and cytochrome P450_{cama} (47). The native protein can be reduced with sodium dithionite, yielding new maxima at 440 and 559 nm (Fig. 3). Reduction by sodium dithionite is characteristic of catalase-peroxidases, in contrast to catalase, which is not reducible with sodium dithionite (46). The addition of potassium cyanide (10 mM) to the ferric KatG shifts the Soret band to 425 nm and leads to an additional maximum at 545 nm with a broad shoulder at 575 nm (Fig. 3). The changes in the optical spectrum associated with the binding of a cyanide anion to the catalase-peroxidase are all similar to those observed in the six-coordinate cyanide complexes of horseradish peroxidase (43, 44) and metmyoglobin (45) and are consistent with the conversion of high spin ferric heme to a low spin configuration. This spectrum is distinct from that of the low spin ferric cyanide complex of cytochrome P450_{cama}, which possesses a thiolate proximal ligand (47). The visible absorption spectra of the KatG R463L mutant were identical with wild-type KatG catalase-peroxidase.

| Table I | Summary of the purification of KatG |
|---------|-----------------------------------|
| Step    | Total protein | Total catalase | Specific catalase | Recovery | Purification |
| CFIE    | 2124          | 481            | 226              | 100      | 1            |
| (NH₄)₂SO₄ precipitation | 1161          | 402            | 346              | 83.5     | 1.5          |
| DEAE-Sephacel | 212          | 233            | 1100             | 48.5     | 4.9          |
| S200 Superdex | 77.5         | 142            | 1838             | 29.6     | 8.1          |
| Mono Q  | 17.7          | 42             | 2420             | 8.9      | 10.7         |

*One unit of catalase activity is the amount of enzyme that catalyzes the disproportionation of 1 μmol of H₂O₂/min.
The low temperature EPR spectrum of the ferric form of wild-type KatG is shown in Fig. 4A. The spectrum is complex, exhibiting multiple \( g \) values, which for an enzyme with one heme indicates the presence of a thermal equilibrium of spin configurations and, additionally, the possible ligation of either water or some active site ligand (e.g. a distal histidine) induced by freezing. The spectrum is composed of at least two high spin ferric signals with a rhombic component having resonances at \( g = 6.07, 5.19, \) and \( 1.94, \) and \( g = 5.73, 4.71, \) and \( 1.94, \) and possibly an additional high spin signal with a \( g \) value at 5.50. In addition to the high spin signals, two low spin signals are also present, both of which have their low field feature in the \( g = 3.0 \) region. The spectrum of wild-type KatG in the presence of 200 mM sodium formate (Fig. 4C) shows that the native enzyme has been converted to a single major rhombic, high spin species with \( g = 5.70, 4.73, \) and \( 1.94. \) This type of ferric high spin signal is characteristic of catalase-peroxidases (20, 21, 25) and is similar to the more highly defined rhombic, ferric high spin signals of catalase (46) and horseradish peroxidase (43, 44). The spectrum of the cyanide (30 mM) adduct of wild-type KatG (Fig. 4D) shows a single low spin signal with \( g = 3.04 \) and 2.11. The spectra of these species are typically very temperature-sensitive, with the low field feature of low spin heme usually the sharpest feature in the spectrum. The derivative-shaped crossing and high field down features are usually broad and, therefore, more difficult to observe even at these low temperatures (48). In addition to the major low spin resonance, there is a small residual high spin signal and a small resonance in the \( g = 4.3 \) region attributable to adventitiously bound ferric ions. The presence of this low spin signal is also characteristic of the cyanide adduct of horseradish peroxidase and distinctly different from the ferric low spin signal of the cytochrome P450cam cyanide adduct (49). Furthermore, the presence of either one predominant high spin ferric heme-formate EPR signal or one axial low spin heme-cyanide EPR signal clearly indicates that wild-type KatG contains only one type of heme, consistent with the heme/protein stoichiometry determined above. The EPR spectra of the KatG R463L mutant (Fig. 4B) were virtually identical to that of wild-type KatG.

Catalase and Peroxidase Activity of KatG—KatG possesses high catalase activity (Table II). As with other catalase-peroxidases, the activity shows a significant pH dependence, with a maximum activity at pH 7.0 (Fig. 5). In contrast, the maximum activity of monofunctional catalases plateaus over several pH units (13). The differences in the pH profiles of catalase and peroxidase activity could be a function of either general differences in the reactivities of the different substrates (indicated also by the observation that different peroxidase substrates show different pH profiles) or differences in the pH dependence of the intrinsic enzymic activities. A similar difference in the pH profiles of the catalase and peroxidase activities has been observed for the catalase-peroxidase from Klebsiella pneumoniae (16).

KatG displays saturation kinetics with an apparent \( K_m \) of 5.2 mM for \( \text{H}_2\text{O}_2 \). The observed \( k_{cat}/K_m \) for KatG catalase activity is \( 1.95 \times 10^8 \text{m}^{-1} \text{s}^{-1} \), 1 order of magnitude below that of beef liver catalase (50) and roughly 3 orders of magnitude below the diffusion controlled limit. The specific catalase activity of the recombinant KatG reported here is roughly 6-fold higher than that of “native” KatG purified from M. tuberculosis cell extracts (42) and approximately 150-fold higher than that from M. tuberculosis H37Rv (55). These differences are most likely due to the difficulty of purifying KatG from cell extracts of the natural host as well as significant differences in the purification protocol utilized; for example, these enzymes are known to undergo an “aging” process dependent on how the purified enzymic components are handled.
Enzyme | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$
---|---|---|---
Wild type | $(1.01 \pm 0.15) \times 10^5$ | $5.18 \pm 0.8$ | $(1.95 \pm 0.12) \times 10^6$
R463L | $(1.14 \pm 0.15) \times 10^5$ | $6.16 \pm 0.7$ | $(1.85 \pm 0.1) \times 10^6$

**TABLE II**

Catalase activity of KatG wild type and R463L mutant

**DISCUSSION**

The catalase-peroxidase KatG from *M. tuberculosis* plays a central role in the mechanism of action of the antituberculosis drug isoniazid and, therefore, the development of one type of isoniazid-resistance, all due, ironically, to its natural role as a component of mycobacterial virulence. Previous attempts to purify sufficient amounts of protein for detailed studies from cultures of *M. tuberculosis* were hampered by the slow growth of the bacterium and its contagious nature. Although we and others have already reported preliminary results on the expression of KatG from *M. tuberculosis* in *E. coli* and on some catalytic properties of the enzyme, we present here a more detailed study on the spectroscopic and kinetic properties of recombinant KatG from *M. tuberculosis* and a mutant of KatG frequently found in isoniazid-resistant strains. Overexpression of KatG in *E. coli*, utilizing the expression plasmid pKATII, has allowed the purification of sufficient protein to allow us to carry out the spectroscopic and kinetic studies described here.

Physical characterization of KatG clearly indicates that it is one of a developing class of bacterial enzymes that catalyze both catalase- and peroxidase-like reactions. Optical absorption and EPR spectra are consistent with what has been previously observed for this class of enzymes and suggest that KatG, like horseradish peroxidase and myoglobin, contains a conserved histidine as the proximal ligand. This is supported by sequence alignment of several catalase-peroxidases of this class (including *M. tuberculosis*) and the high resolution crystal structure of cytochrome C peroxidase, which indicates the presence of a conserved histidine on both the distal and proximal side of the active site heme (23). The source of the complexity of the native KatG EPR spectrum is unclear. The addition of the weak field ligand formate, however, converts the complex spectrum to one predominantly high spin feature. Moreover, the addition of a strong field ligand like cyanide leads to an almost complete conversion to a single low spin species. Since conversion to the
either the catalase or peroxidase pathway (Fig. 1). The peroxidase activity of KatG against NAD(P)H thus could ensure efficient detoxification of organic peroxides in the absence of sufficient concentrations of H$_2$O$_2$. This hypothesis will require more detailed studies with a variety of alkyperoxides as substrates.

Another main objective of this study was to characterize the R463L mutant of KatG, a mutation that has been found in roughly 30–50% of all analyzed isoniazid-resistant strains. KatG R463L exhibits identical visible absorption and EPR spectra to wild-type KatG. The KatG R463L mutant also possesses the same catalytic activity with H$_2$O$_2$ as well as a number of other typical peroxidase substrates, including NADH/NADPH. More importantly, both wild-type KatG and the R463L mutant of KatG oxidize isoniazid and mediate the inactivation of InhA with about the same efficiency. These findings are not consistent with previous suggestions that the R463L mutation of KatG might allow KatG to discriminate against isoniazid as a substrate. It should be noted, however, that these results must be interpreted with caution. The in vivo conditions of the mycobacterial cell are unknown and are certainly different from those conditions typical of an in vitro assay. It is conceivable, for example, that KatG R463L could have a higher activity against an as yet unidentified substrate that could be competitive with isoniazid for the activated form of the catalase-peroxidase. It is also possible that the R463L mutant of KatG has nothing to do directly with substrate binding but rather leads to a change in protein-protein interactions within the bacterium that indirectly influences the rate of inactivation of InhA or a additional target sensitive to activated isoniazid (9). Our findings are, however, in agreement with the recent suggestion that the R463L mutation of KatG is a frequent polymorphism and does not affect isoniazid sensitivity. A solid demonstration of the role of the R463L mutant of KatG must await future experiments with isogenic strains of $M. tuberculosis$.

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