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Purification and Characterization of Recombinant Catalase-Peroxidase, Which Confers Isoniazid Sensitivity in *Mycobacterium tuberculosis**

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The *Mycobacterium tuberculosis katG* gene encodes a dual-function enzyme called catalase-peroxidase, which confers sensitivity in *M. tuberculosis* to isonicotinic acid hydrazide. We have constructed a system for the high level expression of a recombinant form of this enzyme by amplifying the *katG* gene from the pYZ56 construct (1) and subcloning into a vector suitable for expression in *Escherichia coli*. The resulting plasmid, pTBCP, produced the catalase-peroxidase in large quantities, corresponding to 30% of total cell protein. The enzyme has been purified to homogeneity and appears to be a dimer in the native form. Using either hydrogen peroxide or *t*-butyl hydroperoxide and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as substrates, k_{cat} and K_m values have been obtained for both catalatic and peroxidatic activities, respectively. The availability of significant quantities of an active, folded, recombinant form of *M. tuberculosis* catalase-peroxidase should thus facilitate future studies of its role in drug activation and antibiotic resistance.

In the middle of the 18th century, tuberculosis was probably the leading cause of death world-wide. Improvements in health, hygiene, and general living conditions resulted in a steady decline in mortality. By 1952, when the anti-tuberculosis drug isonicotinic acid hydrazide (isoniazid, INH)[1] was discovered, a further decline in mortality had taken place, and it was generally believed that the disease would eventually disappear in the Western world. Despite this optimism, the migration of populations and the spread of immunodeficiency resulting from human immunodeficiency virus infection has resulted in an accelerating rise in the incidence of tuberculosis since 1985 (2). This trend of increasing incidence of infection has also been accompanied by an increase in frequency of multi-drug-resistant strains; in the case of the immunodeficient patient, these may prove fatal within months (3–6). In 1993, the World Health Organization declared tuberculosis a global emergency. WHO figures from 1995 indicate that one third of the world's population is already infected with tuberculosis-causing bacillus and that more than 50 million people may already be infected with drug-resistant strains. The primary cause of multi-drug-resistant strains is poorly managed tuberculosis control programs. These facts emphasize the need for alternative treatment strategies to combat the resurgence of tuberculosis, which in turn, given the complexity of the disease, will demand a better understanding of the origins of drug resistance as well as the mechanisms of action of existing drugs such as INH.

It has long been observed that INH resistance in mycobacteria has been often correlated with reduced levels of catalase activity (7, 8). It has been confirmed that the presence of active catalase-peroxidase (CP), encoded by a single gene *katG*, is sufficient for INH sensitivity in *Mycobacterium tuberculosis* (1). This protein belongs to a family of bifunctional heme-dependent enzymes, showing both catalase and peroxidase activities, known as hydroperoxidase I. Point mutations or deletions in *M. tuberculosis katG* have been found in clinical isolates with increased levels of INH resistance (9, 10). The requirement for a *katG* gene product has been further supported by the observation that transformation of a plasmid harboring this gene into INH-resistant *M. tuberculosis* strains can restore INH sensitivity (11).

It appears that both the presence of a functional CP and point mutations affecting *oxyR*, a central regulator of peroxide stress response tightly linked to the *ahpC* gene, encoding alkyl hydroperoxidase, are required for INH sensitivity in *M. tuberculosis* (12–14); however, the mechanism of action of INH in the bacterium has not yet been completely defined. It has been shown that INH is capable of being oxidized by the *katG* gene product (15), although the reactive intermediates responsible for the cellular effects of INH have yet to be identified. Some evidence indicates that INH may act as an anti-metabolite of NAD and pyridoxal phosphate, both of which are important co-enzymes (16–18). It has also been suggested that INH interferes with the synthesis of the long chain α-branched, β-hydroxy fatty acids (mycolic acids), which are present in mycobacterial cell walls (19–22). Recently, mutations in another *M. tuberculosis* gene, *inhA*, have been shown to also confer resistance to INH and ethionamide, a related anti-tuberculosis drug, in strains with normal CP activity (23). The *inhA* gene encodes an NADH-dependent enzyme that may participate in the mycolic acid synthesis. Furthermore, enzymological studies have suggested that a *katG*-activated form of isoniazid may be exerting its effects through an interaction with the *inhA* gene product, possibly involving NADH (24–26). A recent study has

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[1] The abbreviations used are: INH, isonicotinic acid hydrazide (isoniazid); ABTS, 2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid); CP, catalase-peroxidase; *mt*CP and KatG, recombinant *M. tuberculosis* catalase-peroxidase expressed in *E. coli*; *t*-BuOOH, *t*-butyl hydroperoxide; IPTG, isopropyl-1-thio-β-D-galactopyranoside; IEF, isoelectric focusing; ICP-AES, induced coupled plasma-atomic emission spectroscopy.

indeed isolated a radiolabeled INH-*inhA* gene product adduct, although the exact nature of the interaction has yet to be resolved (27).

Elucidation of the nature of the intermediates associated with INH activation and the interactions of these intermediates with other cellular targets should be facilitated by the availability of substantial quantities of pure, native *M. tuberculosis* CP. Other CP enzymes have been successfully purified from a variety of sources. They are generally multimers composed of identical subunits approximately 80 kDa in size. The CP enzymes from *Escherichia coli* (28, 29), from *Mycobacterium smegmatis* (30) and the photosynthetic bacterium *Rhodopseudomonas capsulata* (31, 32) are tetrameric, whereas those from *Bacillus stearothermophilus* (33) and *Comamonas compransoris* (34) are dimeric, and the CP from *Halobacterium halobium* is monomeric (35). Although purification of native *M. tuberculosis* CP has in fact been achieved from the bacterium itself (36, 37), the slow growth of this organism, lack of a good expression system, and concerns associated with handling large quantities of *M. tuberculosis* have motivated us to develop a means for the production of this protein by an alternative route.

In this article, we report on the design and construction of a system for the high level expression of a recombinant, native form of *M. tuberculosis* CP in *E. coli* and compare our results to another recently developed *E. coli* expression system for the enzyme (38). We also describe the purification and the initial characterization of catalatic and peroxidatic activities for this recombinant form of *M. tuberculosis* CP.

EXPERIMENTAL PROCEDURES

Materials

Oligonucleotides were synthesized on a Perkin-Elmer ABI 392 DNA synthesizer (Warrington, United Kingdom (UK)). Media reagents, KCN, K_2HPO_4, and KH_2PO_4 were obtained from Merck Ltd. (Lutterworth, UK). Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was from Genesys (London, UK). Precast isoelectric focusing (IEF), native and sodium dodecyl sulfate (SDS) polyacrylamide gels, protein molecular weight standards, and Coomassie Brilliant Blue were from Pharmacia Biotech (St. Albans, UK). Restriction enzymes were purchased from New England Biolabs (UK) Ltd. (Hitchin, UK). Polymerase chain reaction (PCR) reagents, including *Pfu* polymerase, were obtained from Stratagene Ltd. (Cambridge, UK). All other chemicals mentioned in the paper were from Sigma-Aldrich Co. Ltd. (Poole, UK).

Expression Plasmid Construction

Plasmid pYZ56 (1) is a pUC19 derivative encoding the *M. tuberculosis* CP (*katG* gene product) with an N-terminal 40-amino acid fusion containing a portion of the *E. coli lacZ* gene and some additional *M. tuberculosis* genomic DNA sequence upstream of the GTG start codon of the *katG*. Purified plasmid was prepared from overnight cultures of *E. coli* UM255 (*pro leu rpsL hsdM hsdR endI lacY katG, katE*::Tn*10 recA*; Ref. 39) transformed with pYZ56 using Wizard Minipreps DNA purification system (Promega Ltd., Southampton, UK). This plasmid was then used as a template in the amplification of the *M. tuberculosis katG* gene in a PCR using two synthetic oligonucleotide primers. Primer 1 (5′-AGTGAGGAATTCGTGCCCGAGCAACAC-3′) incorporates a unique *Eco*RI site, which introduces three additional amino acids upstream of the mycobacterial GTG start codon (translated as Val). The expressed protein is thus expected to have the N-terminal sequence Met-Glu-Phe-Val. Primer 2 (5′-CAGGAAGCTTCAACCCGAATCAGCGCACGTC-3′) introduces a unique *Hin*dIII site downstream of the TGA stop codon. Fifty cycles (95 °C, 1 min; 52 °C, 3 min; 72 °C, 3 min), linked to a final cycle of 72 °C for 10 min, of PCR using the Stratgene *Pfu* DNA polymerase kit were used to generate a 2218-base pair fragment encoding the *M. tuberculosis* CP. The fragment was gel-purified, digested with *Eco*RI and *Hin*dIII, and ligated into the *Eco*RI and *Hin*dIII sites of the IPTG-inducible expression vector p*Trc*99A (Pharmacia Biotech) to produce the plasmid pTBCP (Fig. 1). Initial clones were obtained by transformation of the *katG*-positive *E. coli* strain XL1-Blue MRF′ (Stratagene Ltd., Cambridge, UK) using a Gene Pulser II Apparatus (Bio-Rad Laboratories Ltd., York, UK).

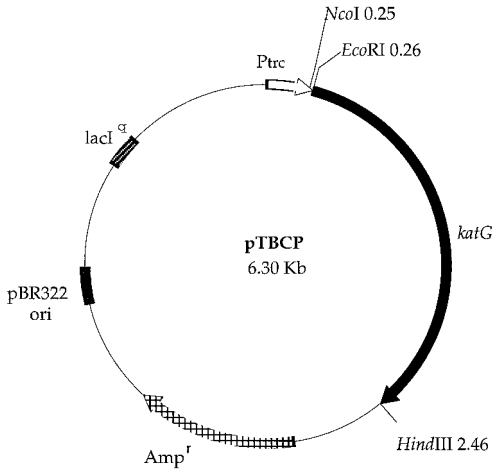

FIG. 1. ***E. coli* expression plasmid for the *M. tuberculosis katG* gene.** Plasmid pTBCP was constructed by ligation of a PCR-generated fragment from pYZ56, encoding *mt*CP, into the *Eco*RI and *Hin*dIII sites of p*Trc*99A (47) as described under "Experimental Procedures." The translated gene product, *mt*CP, is produced using the start codon contained in the *Nco*I restriction site, immediately upstream of *Eco*RI cloning site, and thus contains three additional residues (Met-Glu-Phe) at its N terminus.

Screening for IPTG-induced Overexpression

The screening was done in two stages. In the first stage, 10-ml cultures of *E. coli* strain XL1-Blue (pTBCP) arising from single colony inoculations were grown in LB medium containing 100 μg/ml ampicillin overnight. The following day, 1 ml of each culture was inoculated into 100 ml of the same medium and grown at 37 °C with shaking. When an A_{550} of 0.2 was reached, IPTG was added at a concentration of 0.2 mg/ml and the culture was grown overnight at 37 °C with shaking. A 10-ml aliquot from each culture was removed, and cells were pelleted by centrifugation at 3400 × *g*, 4 °C for 20 min. Each pellet was resuspended in 1 ml of 100 mM K_2HPO_4/KH_2PO_4 phosphate buffer (pH 6.0) and sonicated using three 10-s bursts at full power with an XL 2020 sonicator (Labcaire Systems Ltd., Avon, UK). Insoluble material was removed by centrifugation at 20,000 × *g*, 4 °C for 20 min. Insoluble material and supernatants were assessed for the presence of overproduced, active CP as an intensely staining band on SDS-polyacrylamide gels stained with Coomassie Brilliant Blue and on native gradient polyacrylamide gels stained for catalase and peroxidase activities (described in the following section). In the second stage, *E. coli* strain UM255 was transformed with pTBCP that had been purified from an *E. coli* XL1-Blue (pTBCP) culture, which had demonstrated the presence of soluble, active, recombinant *M. tuberculosis* CP (now designated *mt*CP) in stage 1. Single colonies of UM255(pTBCP) were inoculated into 10-ml aliquots of 2 × YT medium containing 100 μg/ml ampicillin and 10 μg/ml tetracycline and grown overnight at 37 °C with shaking. The following day, 5 ml of each culture was inoculated into 500 ml of the same medium and grown at 37 °C with shaking. When an A_{550} of 0.2 was reached, IPTG was added at a concentration of 0.2 mg/ml and a time-course analysis of overexpression (shown under "Results") was assessed using the same polyacrylamide gels described in the following section. Glycerol stocks were prepared of positive clones.

DNA Sequence Analysis

The PCR-generated fragment encoding *mt*CP, used for the construction of pTBCP was sequenced on a ALF II DNA workstation (Pharmacia Biotech).

Gel Electrophoresis and Staining

Protein samples obtained from expression and purification studies were analyzed using precast 10–15% polyacrylamide gradient or IEF Phast Gels in conjunction with a Phast electrophoresis system (Pharmacia Biotech). SDS and native polyacrylamide gels were stained with Coomassie Brilliant Blue using the protocol supplied for the developer unit of the Pharmacia Phast System. Native and IEF-polyacrylamide gels were stained for either catalase or peroxidase activity. Catalase activity was visualized after a brief incubation in 0.3% hydrogen peroxide with a mixture of potassium ferrocyanide and ferric chloride using a published protocol (40, 41). Peroxidase activity was visualized with

3,3′-diaminobenzidine tetrachloride using methods described elsewhere (41, 42).

Purification of mtCP (Table I)

Crude Cell Extract (Step i)—Four liters of 2 × YT medium containing 100 μg/ml ampicillin and 10 μg/ml tetracycline were inoculated with 100 ml of an overnight culture of *E. coli* strain UM255(pTBCP) that had been grown in the same medium. Cells were grown at 37 °C with shaking until a cell density corresponding to an A_{550} of 0.2 was reached. IPTG was then added to a concentration of 0.2 mg/ml, and growth was allowed to continue for another 12 h under the same conditions. Cells were harvested by centrifugation at 6000 × *g*, 4 °C for 1 h. Cell pellets were routinely stored frozen at −20 °C at this point. Frozen cells were then thawed and resuspended in 10–15 ml of 100 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.0) containing 0.5 mM EDTA (buffer A). Cells were lysed by sonication with three 30-s bursts at full power. Insoluble material was removed by centrifugation at 9000 × *g*, 4 °C for 1 h. The resulting supernatant was treated with 100 μg/ml of DNase and RNase for 1 h at 4 °C and then recentrifuged as just described.

DEAE-Sepharose Anion-exchange Chromatography (Step ii)—A Pharmacia XK-16/40 column containing 25 ml of DEAE-Sepharose (Fast Flow) attached to a Pharmacia FPLC system was equilibrated with buffer A. The final supernatant from step i was passed through a Millipore 0.45-μm filter and loaded onto the column with a flow rate of 0.5 ml/min. The column was washed with 90 ml of buffer A, and *mt*CP was eluted with a 100-ml linear gradient of 0–1.0 M NaCl in buffer A (0.5 ml/min). Two-ml fractions were collected and assayed for peroxidase and catalase activities using the methods described under "Kinetic Characterization." Active fractions were assessed for purity using SDS-polyacrylamide gel electrophoresis, pooled, and then dialyzed against 10 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.0) containing 0.5 mM EDTA (buffer B) at 4 °C overnight.

Mono Q Anion-exchange Chromatography (Step iii)—A Pharmacia Mono Q HR 5/5 column (1 ml) attached to a Pharmacia FPLC system was equilibrated with buffer B. The dialyzed pool from step ii was briefly centrifuged, passed through a Millipore 0.45-μm filter, and loaded onto the column at a flow rate of 1 ml/min. The column was washed with 10 ml of buffer B and eluted with a 30-ml linear gradient of 0–1.0 M NaCl in buffer B (1 ml/min). Fractions (1 ml) were collected, and assayed and pooled as in step ii.

Superdex 200 Gel Filtration Chromatography (Step iv)—A Pharmacia Superdex 200 HR 10/30 (24 ml) gel filtration column was equilibrated with phosphate-buffered saline overnight. The pooled fractions from step iii were passed through a Millipore 0.45-μm filter and loaded onto the column with a flow rate of 0.2 ml/min. Fractions (1 ml) were collected, and assayed and pooled for *mt*CP as described in step ii.

Purification of the Catalase-Peroxidase Fusion Protein from pYZ56 (Table II)

Four liters of 2 × YT medium containing 100 μg/ml ampicillin and 10 μg/ml tetracycline were inoculated with 200 ml of an overnight culture of *E. coli* strain UM255(pYZ56) that had been grown in the same media. Cells were grown at 37 °C, with shaking, overnight. Cells were harvested by centrifugation at 6000 × *g*, 4 °C for 1 h. Cell pellets were routinely stored frozen at −20 °C at this point. Frozen cells were then thawed and resuspended in 15 ml of 100 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.0) containing 0.5 mM EDTA (buffer A). Cells were lysed by sonication with three 30-s bursts at full power. Insoluble material was removed by centrifugation at 9000 × *g*, 4 °C for 1 h. The resulting supernatant was treated with 100 μg/ml DNase and RNase for 1 h at 4 °C and then recentrifuged as described previously. All subsequent chromatographic steps for purification of the fusion form of the CP were carried out as described earlier in steps ii–iv of the *mt*CP purification.

Protein Analysis

Protein concentrations were determined as described by Bradford (43) using the Bio-Rad protein assay reagent. N-terminal amino acid sequencing was performed on the Mono Q-purified *mt*CP after gel electrophoresis and transfer to ProBlott PVDF membrane (Applied Biosystems, Foster City, CA). Samples were sequenced according to the manufacturer's instructions with a ABI 477A Protein Sequencer (Perkin-Elmer, Warrington, UK). The final purified *mt*CP was also analyzed by capillary electrophoresis on an ISCO model 3850 capillary electropherograph to confirm the purity of the final material. Induced coupled plasma-atomic emission spectroscopy (ICP-AES; Fisons ARL 3580B) was used to determine S, Fe, and Mn content of purified *mt*CP.

Potassium Cyanide Binding

A 0.1 mM solution of KCN in 10 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5) was prepared in a fume hood by dilution of a 5% (w/v) stock solution of KCN. Precautions were taken to avoid contact with the poison, and a cyanide antidote was also kept available. At the conclusion of these experiments, all cyanide-containing solutions were destroyed by the addition of sodium hypochlorite. The binding of cyanide to *mt*CP was followed spectrophotometrically by increasing the KCN concentration in a 1 μM enzyme solution by 10 μM increments. The potassium cyanide and enzyme mixture was incubated at 25 °C for at least 10 min prior to measuring the absorbance spectra. The absorbance change was recorded between 390 nm and 450 nm.

Kinetic Characterization

Spectrophotometric measurements were made on a Perkin-Elmer Lambda 3 UV-visible spectrophotometer interfaced to a personal computer.

Catalase Assay—Enzyme assay solutions (1.0 ml) were made up in MilliQ water containing 10 mM K_2HPO_4/KH_2PO_4 (pH 7.5) and H_2O_2 (25 mM). After equilibration at 25 °C for 5 min, *mt*CP was added and the degradation of H_2O_2 was monitored spectrophotometrically at 240 nm (ϵ_{240} = 43.6 M^{-1} cm^{-1}) as described previously (44). One unit of catalase activity catalyzes the decomposition of 1 μmol of H_2O_2/min at 25 °C.

For determination of steady-state parameters, rate measurements were made as described above except that the amount of *mt*CP was increased to 20 units as defined above. A 5-s delay was allowed after initiation of the reaction. The initial rate was then determined by least-squares fitting of the first 60% of the progress curve (between 0 and 100 s, depending upon the initial concentration of H_2O_2) to a straight line. The apparent K_m for H_2O_2 was determined by varying the H_2O_2 concentration between 5 mM and 100 mM and fitting the experimental rates to the Michaelis-Menten equation by nonlinear regression using the data analysis package Igor (Wave Metrics).

Peroxidase Assay—Enzyme assay solutions (1.0 ml) were made up in MilliQ water containing 10 mM K_2HPO_4/KH_2PO_4 (pH 7.5) containing *m*-chloroperoxybenzoic acid (0.5 mM) and 2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; 4 mM). After equilibration at 25 °C for 5 min, *mt*CP was added and the oxidation of ABTS was monitored spectrophotometrically at 405 nm (ϵ_{405} = 18.6 mM^{-1} cm^{-1}) as described previously (45). One unit of peroxidase activity catalyzes the oxidation of 1 μmol of ABTS/min at 25 °C.

Steady-state parameters were determined using 20 units of *mt*CP as described in the procedure above with the exception that *m*-chloroperoxybenzoic acid was replaced with *t*-butyl hydroperoxide (*t*-BuOOH). The K_m for ABTS was determined by varying the ABTS concentration between 0.2 mM and 80 mM while maintaining a constant concentration of *t*-BuOOH of 23 mM. The K_m for *t*-BuOOH was determined by holding the ABTS concentration constant at 80 mM while varying the *t*-BuOOH concentration between 2.9 mM and 115 mM. Initial rates were determined analogously to that described for the catalase assay above. The K_m values for ABTS and *t*-BuOOH were estimated by fitting the appropriate experimental rates to the Michaelis-Menten equation by nonlinear regression using the data analysis package Igor (Wave Metrics).

RESULTS

DNA Analysis—DNA sequence analysis of pTBCP revealed no sequence deviations in the *M. tuberculosis katG* gene when compared with the published entry in GenBank (GBM21516).

E. coli Expression of mtCP—The levels and time course of IPTG-induced expression of *mt*CP were analyzed by SDS-reducing PAGE of cell lysates of *E. coli* UM255 transformed with pTBCP. A prominent new protein band having an apparent molecular mass of 80 kDa appeared 1 h after induction and increased to a maximum level within 10–12 h (Fig. 2). Analysis of these lysates using native polyacrylamide gels showed the presence of a diffuse band demonstrating significant levels of both catalatic and peroxidatic activities when appropriately stained (Fig. 3). To further characterize the *mt*CP and compare its properties with the fusion form of this protein produced by the pYZ56 construct, both the native and fusion proteins were purified from *E. coli* UM255 transformed cells.

Purification of M. tuberculosis Catalase-Peroxidases—The purification of recombinant *mt*CP, expressed in *E. coli*, was accomplished by a four-step protocol (Table I) and yielded 51

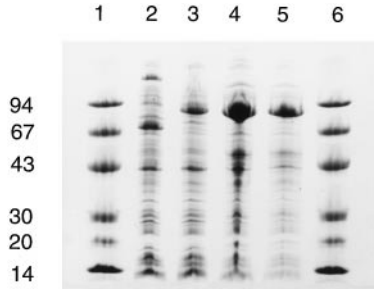

FIG. 2. **Expression time course for *mt*CP from *E. coli* as followed by SDS-polyacrylamide electrophoresis (10–15%) of whole cell extracts.** *Lanes 1* and *6*, marker proteins. *Lanes 2–5*, 2, 4, 8, and 12 h after induction, respectively. Molecular weight standards used were phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400).

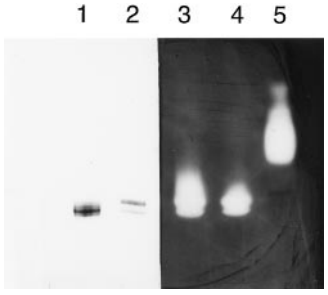

FIG. 3. **Demonstration of enzymic activities of *mt*CP by polyacrylamide gel electrophoresis (10–15%).** Gels were developed and stained as described under "Experimental Procedures." *Lanes 1* and *2*, peroxidase activity stains of crude extract of UM255(pTBCP) and purified *mt*CP, respectively. *Lanes 3–5*, catalase activity stains of crude extract UM255(pTBCP), purified *mt*CP, and bovine liver catalase (as a control), respectively.

mg of protein from 4 liters of bacterial culture. Most contaminating proteins were removed in the first chromatographic step using a DEAE-Sepharose (fast flow) column (Fig. 4, *lane 3*). The *mt*CP bound to the subsequent Mono Q column eluted at 0.35 M NaCl and showed an 50% increase in specific activity (Fig. 4, *lane 4*). Fractionation using a Superdex 200 gel filtration column yielded the final preparation at greater than 98% purity (Fig. 4, *lane 5*) with an estimated molecular mass of 160 kDa, suggesting that *mt*CP is a homodimer in the native form. Native and IEF-polyacrylamide gel electrophoresis indicated the presence of two closely migrating bands, although the purified *mt*CP migrated as a single band of approximately 80 kDa using SDS-reducing PAGE (Fig. 4, *lane 4*). These multiple bands stained positively for both catalatic and peroxidatic activities (Fig. 3, *lanes 2* and *4*) with estimated molecular mass values of 160 kDa and pI values of 3.7 and 3.75.

The purification summary of the recombinant *mt*CP containing a 40-amino acid N-terminal fusion produced using the pYZ56 expressed in *E. coli* construct is shown in Table II for comparison. Using a similar four-step protocol, 4 liters of bacterial culture yielded 12 mg of pure protein. The fractionation step using a Superdex 200 gel filtration column yielded the final preparation at greater than 95% purity, with an estimated molecular mass of 170 kDa, suggesting that the presence of the fusion peptide does not inhibit the formation of a homodimer form of this enzyme. However, total yield of protein and, in particular, specific activities for both catalatic and peroxidatic activities are substantially reduced for the fusion protein compared with native *mt*CP. In addition, native and IEF-polyacrylamide gel electrophoresis indicated the presence of three closely migrating bands, which stained positively for both catalatic and peroxidatic activities (data not shown), possibly indicative of additional structural variations arising from the presence of the fusion peptide.

Protein Analysis—The N-terminal sequence for the first 5 amino acids confirms the presence of the new N-terminal sequence Met-Glu-Phe, predicted by the cloned insert, followed by Val and Pro corresponding to the expected N-terminal sequence from its published DNA sequence (46). The results of capillary zone electrophoresis clearly showed that the purified sample contained one species of protein. ICP-AES analysis of purified *mt*CP indicated approximately 0.5 heme/dimer. No manganese could be detected in the sample. The R_Z value (A_{405}/A_{280}) of pure *mt*CP was 0.21 in 10 mM potassium phosphate, pH 6.0 at 25 °C. The extinction coefficient was calculated by comparing the S content determined by ICP-AES and the absorbance at 280 nm. It was found to be $1.5 \pm 0.1 \times 10^5$ M^{-1} cm^{-1}.

Potassium Cyanide Binding—The absorption spectra of *mt*CP and, as a complex with KCN, are shown in Fig. 5. The absorption spectrum for the unligated *mt*CP is essentially indistinguishable from that obtained by Gayathry-Devi *et al.* (37) for the native enzyme purified from *M. tuberculosis*. Addition of KCN to the *mt*CP resulted in a shift of the Soret band from 405 to 422 nm. Titration of *mt*CP with increasing amounts of KCN was followed spectrophotometrically and the data fitted to a single site binding isotherm by nonlinear regression using Igor (Wave Metrics). The affinity for cyanide was found to be 6 μM.

Kinetic Characterization—Assays performed for the two activities of *mt*CP produced distinct sets of kinetic parameters. For the catalatic reaction, the apparent K_m for hydrogen peroxide degradation is 30 ± 7 mM with k_{cat} 2300 ± 190 s^{-1}. In comparison, for the peroxidatic reaction using *t*-BuOOH, the apparent K_m for ABTS oxidation is 0.96 ± 0.38 mM, with k_{cat} 4.5 ± 0.35 s^{-1}. These data and data from other sources are summarized in Table III and compared under "Discussion."

DISCUSSION

Expression of Recombinant M. tuberculosis Catalase-Peroxidase in E. coli—This paper describes the design and construction of a system for the high level production of *M. tuberculosis* CP (*mt*CP) in *E. coli* and demonstrates its utility for studying the functional properties of this enzyme. In general, expression of mycobacterial proteins in *E. coli* hosts, when achieved, offers several advantages. In the case of *mt*CP, production of substantial quantities of soluble, active material eliminates the need to use large scale preparations of virulent *M. tuberculosis* (37). The use of the *E. coli* host strain UM255, which lacks endogenous catalatic and peroxidatic activities (*pro leu rpsL hsdM hsdR endI lacY katG, katE*::Tn*10 recA*; Ref. 39), eliminates the possibility of background contamination, which can affect both protein isolation and activity assessments.

In these studies, we have constructed a new mature *mt*CP that has just three residues, Met-Glu-Phe, prior to the start codon. Construction of the pTBCP plasmid required the addition of these residues to create an *Eco*RI restriction site without substantially altering the sequence of the N-terminal residues of the *katG*-encoded gene product. PCR amplification of the *katG* gene from pYZ56 with the appropriately placed *Eco*RI (5′) and *Hin*dIII (3′) restriction sites allowed insertion of the *katG* gene into the p*Trc*99A plasmid to take advantage of the highly efficient IPTG-inducible *trc* promoter (47).

It is important to note that the *mt*CP was expressed in *E. coli* as a mature protein, only slightly modified at the N terminus by the addition of a tripeptide and not as a fusion protein. Although no crystal structure exists of any CP-type enzyme, it is not unreasonable to assume that the introduction of three amino acids at the N terminus would have substantially less structural and functional perturbation compared with the 40-

TABLE I
Purification of mtCP from 4 liters of culture

Step	Volume	Concentration	Total amount	Specific activity		Total units		Yield		Purification factor	
				Cat[a]	Per[b]	Cat	Per	Cat	Per	Cat	Per
	ml	*mg/ml*	*mg*	*units/mg*		*units*		*%*		*-fold*	
Crude extract	10	191	1910	431	102	823,000	195,000	100	100	1	1
DEAE-Sepharose	12	92.0	1104	637	148	703,000	163,000	85	84	2	1
Mono Q	4.0	61.0	244	1100	312	268,000	76,100	33	39	3	3
Superdex 200	3.0	17.0	51	2112	504	108,000	25,700	13	13	5	5

[a] Cat, catalase activity; 1 unit of catalase activity catalyzes the decomposition of 1 μmol of H_2O_2/min at 25 °C.
[b] Per, peroxidase activity; 1 unit of peroxidase activity catalyzes the oxidation of 1 μmol of ABTS/min at 25 °C.

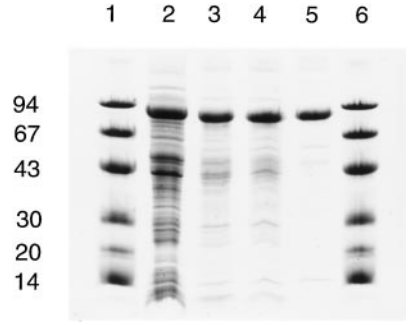

FIG. 4. **Purification of *mt*CP as monitored by SDS-polyacrylamide gel electrophoresis (10–15%).** *Lanes 1* and *6*, marker proteins (described in Fig. 2). *Lane 2*, crude extract of UM255(pTBCP) 12 h after induction by IPTG. *Lane 3*, pooled active fractions after DEAE Sepharose chromatography. *Lane 4*, pooled active fractions after Superdex 200 gel filtration. *Lane 5*, *mt*CP after Mono Q chromatography.

amino acid fusion peptide present in the protein expressed from the pYZ56 construct. Comparison of the relative specific activities of these two enzymes did indeed indicate a lower specific activity for the protein expressed using the latter system, as well as additional heterogeneity in IEF-polyacrylamide gel analysis. Additionally, a second *E. coli* expression system for the *M. tuberculosis* CP was reported earlier this year (38). Using a β-indol acrylic acid-controlled expression vector and a four-step purification protocol yielded 3 mg of highly pure KatG/liter of cell culture. The system described here appears to be a significant improvement in both ease of preparation (three-step protocol) and final yield (12.8 mg/liter of cell culture). Notably, the specific catalatic activities of both preparations are comparable: 2420 units/mg (38) compared with 2112 units/mg presented here. Although the precise yield differences is difficult to assess, it is possible that the IPTG-inducible pTBCP construct and the three-amino acid N-terminal peptide present on *mt*CP may be responsible for enhanced expression of the enzyme in these studies. Therefore, we conclude that the pTBCP construct described here represents a substantial improvement in both the quantity of material that can be produced in *E. coli*, as well as the quality of the protein, which one would expect to be more representative of the true wild-type protein from *M. tuberculosis*.

Functional Properties of mtCP—The current interest in defining the functional properties of the CP from *M. tuberculosis* stems principally from its role in the activation of the antituberculosis drug isoniazid (INH). The action of INH on *M. tuberculosis* has been the subject of a wide range of biochemical and microbiological studies (reviewed in detail in Refs. 8, 48, and 49). Despite the expanding effort to identify INH-resistant strains and devise new clinically-based treatment regimes, characterization of the *M. tuberculosis* CP has been limited to a few mechanistic studies focused upon the isoniazid-activation pathway (13, 23–25). Identification of the true intermediates associated with isoniazid activation, establishment of defined kinetic protocol for assessing activities, and extension of the structural characterization of this enzyme all demand substantial quantities of pure protein, which can be obtained from the expression system described here. In this first instance, this material has provided us with the opportunity to initiate a more detailed characterization of the general functional properties of this enzyme.

The *mt*CP is somewhat distinct in its structural organization. Like many other bacterial CPs, the *mt*CP appears to be composed of identical subunits approximately 80 kDa in size. However, unlike the *M. smegmatis* enzyme, which is tetrameric (30), the *mt*CP appears to be functional as a homodimer. This observation agrees with the result of Winder and Collins (50), although Gayathry-Devi *et al.* (37) have reported observing a trimeric form of this protein. It also binds heme with a likely stoichiometry of 1 heme/dimer, the same ratio as obtained for KatG (38).

The potassium cyanide binding studies revealed a decreasing absorbance at 405 nm and a corresponding increasing absorbance at 422 nm, suggesting that *mt*CP is converting between free (high spin) and cyanide-bound (low spin) states. The affinity for cyanide was comparable to other peroxidases.

A detailed steady-state protocol for assaying both catalatic and peroxidatic activities has been developed. The classic activity assays for catalase and peroxidase involve the decomposition of hydrogen peroxide, since hydrogen peroxide is substrate of both catalases and peroxidases. However, in dual-function enzymes measuring both activities with the same oxidizing substrate cannot give true peroxidatic activity values owing to the competing catalatic reaction. We found that catalase activity could be followed by measuring the decay of hydrogen peroxide at 240 nm, as observed previously (44). Peroxidase activity was measured by using the alternative peroxidase substrates, *m*-chloroperoxybenzoic acid or *t*-BuOOH, as oxidants. The reaction was then followed by measuring the oxidation rate of ABTS spectrophotometrically at 405 nm. As can be seen from Table III, the catalytic efficiency of the *mt*CP for catalatic activity is approximately 1 order of magnitude below the recombinant KatG form (38) and the native *M. smegmatis* CP (30). The catalytic efficiency for peroxidase activity is also 1 order of magnitude below the native *M. smegmatis* CP (30). It is interesting to note that the differences between *mt*CP and *M. smegmatis* CP are principally due to an elevation of substrate-dependent K_m values. However, the kinetic parameters for the *mt*CP catalatic activity are both reduced compared with the KatG form. The *E. coli* CP, a tetrameric enzyme like the *M. smegmatis* CP, demonstrates similar elevated catalatic activity, although such activity does not approach classic mammalian catalases such as horse liver, which are dominated by large k_{cat} values. A somewhat analogous comparison can be made for ABTS oxidation by the dual-function mycobacterial CP enzymes and the well studied horseradish peroxidase, although the differences (less than 1 order of magnitude in k_{cat}/K_m) are not as pronounced.

In the context of these studies, comparison of rate constants

TABLE II
Purification of mtCP with the 40-amino acid fusion from 4 liters of culture

Step	Volume	Concentration	Total amount	Specific activity		Total activity		Yield		Purification factor	
				Cat[a]	Per[b]	Cat	Per	Cat	Per	Cat	Per
	ml	*mg/ml*	*mg*	*units/mg*		*units*		*%*		*-fold*	
Crude extract	6.5	69	445	241	16	108,000	7080	100	100	1	1
DEAE-Sepharose	9.0	18	162	490	20	79,300	3260	74	46	2	1
Mono Q	5.0	17	86.5	814	31	70,500	2690	65	38	3	2
Superdex 200	2.0	6.0	12.0	1140	63	13,600	754	13	11	5	4

[a] Cat, catalase activity; 1 unit of catalase activity catalyzes the decomposition of 1 μmol of H_2O_2/min at 25 °C.
[b] Per, peroxidase activity; 1 unit of peroxidase activity catalyzes the oxidation of 1 μmol of ABTS/min at 25 °C.

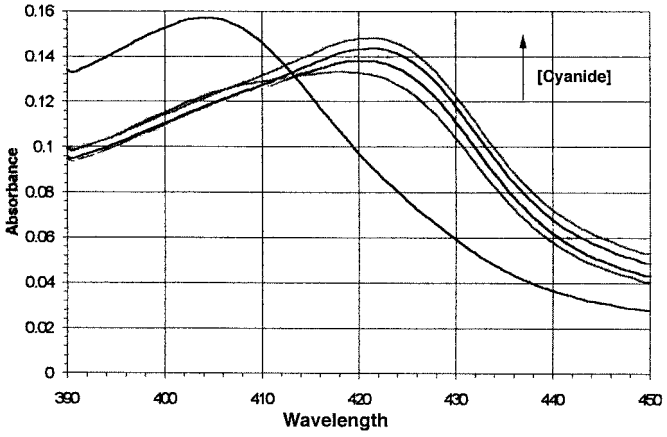

FIG. 5. **Spectrophotometric analysis of cyanide binding.** The cyanide concentration was increased from 0 to 50 μM by 10 μM increments. The enzyme concentration was 1 μM.

might suggest that *mt*CP functions predominantly as a catalase, as noted previously for the *E. coli* enzyme (28). Interestingly, sequence homologies with other non-CP catalases are poor and no classic catalase-heme binding site can be identified. In contrast, homology with cytochrome *c* peroxidase has already been noted (51), with the highly conserved residues in the peroxidase heme-binding site conserved in the N-terminal domain of *mt*CP. Rapid formation of compound I, the reactive oxyferryl enzyme, should proceed for either catalatic or peroxidatic reaction schemes. However, effective peroxidatic activity could be generated, driven by high substrate affinities, as demonstrated for ABTS *in vitro* (K_m = 0.96 mM, surpassing horseradish peroxidase), and possibly for INH *in vivo*. The use of a single peroxidatic-type heme to carry out both catalatic and peroxidatic activities, however, remains an open question.

The proposed role of CP is to protect the bacteria from toxic molecules including hydrogen peroxide and hydroxyl radicals that are present in an aerobic environment. Recent interest in these enzymes stems from the discovery that *M. tuberculosis* CP activates the anti-tuberculosis drug INH, although the structure of the activated INH has remained elusive. Recent reports have also suggested that the *M. smegmatis* CP oxidises INH via an oxidase-type reaction, utilizing hydrazine to produce an oxyferrous enzyme (52). Alternatively, it may behave as a manganese-dependent peroxidase, generating Mn(III) as the proximal oxidant (53). The absence of manganese in the purified *mt*CP does not discount the latter activity, as Mn(II) would be relatively labile. The availability of substantial quantities of pure *mt*CP will facilitate studies to verify the presence of an analogous oxidase activity in the *M. tuberculosis* enzyme, as well as the identification and isolation of chemical species relevant to its reaction mechanisms. Utilization of pure *mt*CP in structure-based studies is currently under way and also offers the possibility of elucidating the origin and relative importance of the activities of this enzyme.

TABLE III
Kinetic parameters for selected enzymes with catalatic and/or peroxidatic activities

Enzyme	Substrate	K_m	k_{cat}	k_{cat}/K_m
		mM	s^{-1}	$M^{-1}s^{-1}$
Catalatic activity				
*mt*CP[a]	H_2O_2	30	2.3×10^3	7.7×10^4
KatG (38)	H_2O_2	5.2	1.0×10^4	1.9×10^6
M. smegmatis CP (30)	H_2O_2	1.4	2.4×10^3	1.7×10^6
E. coli CP (28)	H_2O_2	3.9	1.6×10^4	4.1×10^6
Horse liver catalase	H_2O_2	1100	3.8×10^7	3.5×10^7
Peroxidatic activity				
*mt*CP[a]	ABTS	0.96	4.5	4.7×10^3
M. smegmatis CP (30)	ABTS	0.11	3.7	3.4×10^4
HRP (54)	ABTS	3.7[b]	670[c]	1.8×10^5
*mt*CP[a]	*t*-BuOOH	93	10	1.1×10^2

[a] Recombinant *M. tuberculosis* CP expressed in *E. coli* UM255 prepared in this study.
[b] Ref. 45.
[c] Boehringer Mannheim Biochemica Catalogue.

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Purification and Characterization of Recombinant Catalase-Peroxidase, Which Confers Isoniazid Sensitivity in *Mycobacterium tuberculosis*

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