CRYSTAL STRUCTURE OF MYCOBACTERIUM TUBERCULOSIS CATALASE-PEROXIDASE*

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RUNNING TITLE: Structure of M. tuberculosis CP
SUMMARY

The *Mycobacterium tuberculosis* catalase-peroxidase is a multi-functional heme-dependent enzyme which activates the core anti-tuberculosis drug, isoniazid. Numerous studies have been undertaken to elucidate the enzyme-dependent mechanism of isoniazid activation, and it is well documented that mutations which reduce activity or inactivate the catalase-peroxidase lead to increased levels of isoniazid resistance in *M. tuberculosis*. Interpretation of the catalytic activities and the effects of mutations upon the action of the enzyme to date have been limited due to the lack of a three-dimensional structure for this enzyme. In order to provide a more accurate model of the three-dimensional structure of the *M. tuberculosis* catalase-peroxidase, we have crystallized the enzyme and now report its crystal structure refined to 2.4-Å resolution. The structure reveals new information about dimer assembly and provides information about the location of residues which may play a role in catalysis including candidates for protein-based radical formation. Modelling and computational studies suggest that the binding site for isoniazid is located near the δ-meso heme edge, rather than in a surface loop structure as currently proposed. The availability of a crystal structure for the *M. tuberculosis* catalase-peroxidase also permits structural and functional effects of mutations implicated in causing elevated levels of isoniazid-resistance in clinical isolates to be interpreted with improved confidence.
INTRODUCTION

Catalase-peroxidases (CPs)\(^1\) are bi-functional, heme-dependent enzymes which exhibit a strong catalatic activity, comparable to that of monofunctional catalases, and a broad spectrum peroxidatic activity. The proposed role of the enzyme is to protect bacteria from toxic molecules including hydroperoxides and hydroxyl radicals that are present in an aerobic environment. CPs are generally homodimers or homotetramers with subunits of about 80 kDa. The single polypeptide chain containing two domains, each of approximately 40 kDa, is proposed to have arisen from a gene duplication event (1). Sequence analysis shows that the N-terminal domain contains a heme-binding motif whilst the C-terminal domain lacks this feature. In spite of the strong catalatic activity displayed by CPs, the sequence shows no homology with catalases. However, both CP domains show highest sequence similarity with yeast cytochrome c peroxidase (CcP) and ascorbate peroxidase (APX) and CPs are therefore classified as belonging to class I of the superfamily of plant, fungal and bacterial peroxidases (2).

The *Mycobacterium tuberculosis* CP has been the subject of numerous studies because of its ability to activate isoniazid (INH), a core compound used to treat tuberculosis. In particular, it has long been observed that INH resistance in tuberculosis-causing mycobacteria has often been correlated with reduced levels of catalase activity (3-5). Subsequently, it was confirmed that the presence of an active CP, encoded by a single gene, *katG*, is sufficient to confer INH sensitivity in *M. tuberculosis*, the organism principally responsible for tuberculosis (6). Studies using *M. tuberculosis* CP obtained either from the organism or in a recombinant form demonstrated that the enzyme is capable of oxidizing INH (7,8); however, the mechanism of oxidation and the precise mode of action of the drug are still subjects for debate. Of the evidence to date, there is some indication that activated INH may inhibit the action of InhA (9) and KasA (10), enzymes which are proposed to be involved in the synthesis of mycolic acids. *In vitro* assays have demonstrated that
inactivation of the InhA enzyme by INH only occurs in the presence of NADH or NAD$^+$ (11), and the crystal structure of InhA containing an INH-NADH adduct present in its active site has been determined (12). Recently published data have also shown that an INH-NADH adduct is an effective inhibitor of InhA (13).

It is established that in vitro activation of INH by M. tuberculosis CP yields acid, amide and aldehyde products (8,14,15). Generation of the amide product has been shown to proceed via the cleavage of a C-N bond (14) which is thought to yield an acyl radical (8,14) that is capable of reacting with NADH or NAD$^+$, for example. Residues and other structural elements which play a key role in the enzyme-mediated activation of INH by M. tuberculosis CP have yet to be definitively assigned. Identification of the binding site for INH in the enzyme is still open to question and is of fundamental importance in understanding both the activation process of the drug and the significance of mutations in the enzyme which may be directly responsible for INH-resistance.

As a key step towards elucidating the functions of M. tuberculosis CP and, in particular its role in INH activation, we have determined the crystal structure of a recombinant form of M. tuberculosis CP (mtCP) to 2.4-Å resolution. We discuss how mainchain and side chain features relate to the assembly and function of the enzyme. Comparative analyses of sequences and structures of related class I and class III peroxidases predict that the binding site for INH in mtCP will be near the $\delta$-meso edge of the heme, rather than in a surface loop as currently proposed by others (16). Computational analyses of interaction sites for INH, in combination with the availability of an NMR-derived model of a complex between horseradish peroxidase C (HRPC) and INH (17), have enabled us to model the location of the drug within the active site of mtCP. The significance of the identification of this binding site is discussed in the context of the interpretation of radical sites and mutations related to INH resistance in clinical isolates.
EXPERIMENTAL PROCEDURES

Protein Overproduction and Purification

mtCP was purified from Escherichia coli strain UM255 (18) transformed with the pTrc99A derivative, pTBCP, using a modified version of a previously reported protocol (19). To maximise heme incorporation, excess hemin was added to the crude cell extract prior to sonication. Briefly, hemin chloride (Sigma Aldrich, Poole, UK) was dissolved in 1 ml Milli-Q water to a final concentration of 24.5 mM. Addition of 1 µl 10 M NaOH was sufficient to dissolve the hemin chloride yielding a final solution at about pH 6.0. Reconstituted holoenzyme was achieved by adding the hemin solution in a 1 to 25 (v/v) ratio to the cell pellet of 1 l of culture resuspended in 10-15 ml 100 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 6.5) at 4 °C. The suspension was mixed well by vortexing and incubated at 4 °C for 30 min before sonication. Cells were lysed by sonication with 3 x 30 s bursts on power level 8, using a microtip and an XL2020 sonicator (Labcaire Systems, Clevedon, UK). Insoluble material was removed by centrifugation at 10000 g at 4 °C for 50 min. The supernatant was treated with 100 µg/ml DNAse and 10 µg/ml RNAse for 1 h at 4 °C and then recentrifuged as just described.

The supernatant was applied to a DEAE-Sepharose anion exchange column (19). Pooled, active fractions were passed through a Millipore 0.45-µm filter and loaded, at 0.5 ml/min, onto an Amersham Biosciences HiLoad 16/60 Superdex 200 prep grade (120 ml) gel filtration column pre-equilibrated with phosphate buffered saline [PBS: 0.01 M KH$_2$PO$_4$/K$_2$HPO$_4$ buffer, 0.0027 M KCl, 0.137 M NaCl (pH 7.5)] overnight. 1.0-ml fractions were collected, assayed and pooled (19). Pooled, active fractions were then loaded, at 2.0 ml/min, onto an Amersham Biosciences Resource Q (6 ml) anion exchange column pre-equilibrated with 30 ml 100 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 6.5). The column was washed with 12 ml 100 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 6.5) and mtCP was eluted with a 60-ml linear gradient of 0-1.0 M NaCl in 100 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 6.5) at a flow rate of 2.0 ml/min. 1.0-ml
fractions were collected, assayed and pooled. An Amersham Biosciences HiTrap Desalt column was used to remove NaCl and change buffer conditions for crystallization studies. The column was equilibrated with 25 ml 0.1 M sodium acetate (pH 7.0) using a syringe. mtCP was loaded onto the column in 1.5-ml aliquots and eluted with 2 ml 0.1 M sodium acetate (pH 7.0).

Crystallization and Data Collection
Crystals of mtCP were obtained by the vapor-diffusion, hanging-drop method with 2 µl of a 22 mg/ml protein solution and 2 µl of the reservoir solution, which contained 4% (w/v) polyethylene glycol (PEG) 3350 and 0.1 M sodium acetate pH 4.6. After 24 h at 20 °C, red-brown, plate-like crystals were visible. A single crystal was placed in a cryo-buffer containing 20% (v/v) glycerol, 4% PEG 3350 and 0.1 M sodium acetate, pH 4.6 and cooled to 100 K in a nitrogen cryo-stream. Diffraction data were collected on beamline 14.1 at the Synchrotron Radiation Source at Daresbury, UK using an Area Detector System Corporation Quantum-4 CCD detector and a wavelength of 1.488 Å.

Structure Solution, Refinement, and Superpositions
The diffraction data were processed using the program DENZO and scaled with program SCALEPACK (20). A portion of the data set (10%) was flagged for the calculation of $R_{\text{free}}$ and excluded from subsequent refinement. The structure of mtCP was solved by molecular replacement using the crystal structure of the Burkholderia pseudomallei CP (bpCP) (16) as the search model in the program MOLREP (21). Refinement using CNSsolve, Crystallography and NMR System software version 1.1 (22) and model rebuilding with the graphics program O (23) gave a model with a crystallographic $R$-factor for all data of 21.1% and an $R_{\text{free}}$ of 26.8%. Simulated annealing was initially used as well as non-crystallographic restraints. The two molecules of the asymmetric unit were refined as different models using individual $B$ factors. In the final steps, solvent molecules were placed in residue density above 2.5 standard deviations and with at least one hydrogen bond with the refined model.
Water molecules with a $B$ factor higher than 60 Å$^2$ were discarded. Figures were prepared using PyMOL (24). Root mean square (r.m.s.) deviations of superpositions were calculated using ProSup (http://lore.came.sbg.ac.at:8080/CAME/CAME_EXTERN/PROSUP) (25). Coordinates and structure factors have been deposited in the Protein Databank (PDB # 1SJ2)$^2$.

Superpositions of the $mt$CP crystal structure with crystal structures of CcP (26), recombinant soybean ascorbate peroxidase (rsAPX) (27), and HRPC (28) were carried out using Swiss PDB Viewer (29) and were based upon Cα coordinates in all these structures. INH was docked into the active site of $mt$CP based upon a superposition of the $mt$CP crystal structure with the HRPC-INH NMR-derived model (17) using all atoms of the heme moiety, the conserved distal His and Arg residues and the conserved proximal His and Asp residues.

**GRID calculations**

GRID, a computational program by P. J. Goodford (30), was designed to detect energetically favorable binding sites on proteins, structurally determined by X-ray crystallography or NMR, with the view that this information could be used in the rational design of drugs. In GRID, the target molecule is the species being evaluated and the probe molecule is a small biologically active species such as water, amine nitrogen, carboxy oxygen, or hydroxyl. GRID calculates the interaction energies between the probe molecule and the target molecule accounting for both the shape and energy of the two species. Analysis of GRID data yields three-dimensional maps which can be contoured to identify the location of energetically favorable interaction sites.

GRID calculations (30) were performed on atomic coordinate files of the crystal structures of $mt$CP, $bp$CP (16), *Haloarcula marismortui* CP (hmCP) (31) and HRPC (28). All remarks and water molecules were removed from the PDB files prior to processing. Missing residues were added in idealized positions based on the position of surrounding residues by Swiss
PDB Viewer (29) and the structures were minimized using CNSsolve (22) version 1.1, at one refinement cycle of two hundred minimization steps.

The atomic coordinate files were prepared for use in GRID using the program GRIN. The total charge of each structure was determined in GRIN and appropriate counterions (Na⁺ or Cl⁻) were placed near charged residues with care taken to avoid the channel region near the heme. Four GRID probes, NH, NH₂, carbonyl oxygen and aromatic neutral amide, were selected to model the functional groups of INH. The move directive in GRID was set to -1 to allow the counterions to move in response to the probe. All GRID calculations were performed on the charge balanced protein coordinate files at a resolution of 0.5 Å. The output data generated by GRID were read into MSI Insight II (Accelrys, Cambridge, UK) software version 2000 for visualization of the energy maps. The GRID maps were converted into ASCII files using MSI Insight II and read into DS ViewerPro (Accelrys, Cambridge, UK) for visualisation and figure preparation.
RESULTS AND DISCUSSION

Fold and assembly of the M. tuberculosis CP

The mtCP crystal structure was determined to a resolution of 2.4 Å, with $R_{\text{cryst}}$ and $R_{\text{free}}$ values of 21.1% and 26.8 %, respectively. Full data collection and refinement statistics are summarized in Table 1. The mtCP crystal structure shows that the enzyme assembles as a functional homodimer. The electron density map of each monomer includes residues 24-740 of the polypeptide chain, one heme $b$ moiety and three glycerol molecules. In addition 703 water molecules associated with the homodimer were also identified and refined. Each monomer (Fig. 1) is composed of two domains which are mainly $\alpha$-helical and display a common core structure shared by members of the bacterial and plant peroxidase families including CcP (26), APX (27,32) and HRPC (28). The N-terminal domain of mtCP contains the active site of the enzyme which includes the heme $b$ prosthetic group. The fold of the C-terminal domain of mtCP is similar to that of the N-terminal domain, consistent with the proposal that the enzyme arose from a gene duplication event (1). No heme is observed in this domain which is also consistent with the observation that the C-terminal domain lacks signature sequences indicative of a heme-binding site (2).

The crystal structure of mtCP shows that the enzyme adopts a similar fold to those reported for the crystal structures of hmCP (31) and bpCP (16). mtCP shares 55% identity and 69% similarity with hmCP, and 66% identity and 77% similarity with bpCP. C$\alpha$ backbone superpositions show r.m.s. deviations of 1.18 Å and 0.82 Å between mtCP with hmCP or bpCP, respectively. Active site residues are conserved and align well in all three structures, and there are few structural differences in the three-dimensional arrangement of the polypeptide chains. The most significant difference is at the N-terminus of mtCP, although electron density is lacking for the first 23 residues, extra residues are visible (24-30, highlighted in Fig. 1) in comparison with the hmCP (31) and bpCP (16) crystal structures.
(seven more than bpCP and six more than hmCP). The implications of this extra visible region in homodimer assembly are discussed below. Fig. 1 also highlights a surface loop region (residues 278-312 in mtCP), which is present in similar conformations in all three CP crystal structures. In bpCP this loop region has been proposed to form a substrate binding site where INH could interact with the enzyme (16) although, as discussed below, data presented here and in Pierattelli et al. (17) suggest that this loop may not be the principal binding site of the drug.

The observation of a homodimer in the crystal structure of mtCP (Fig. 2A) agrees well with gel filtration (19,33) and small-angle X-ray scattering studies (34) which demonstrate that this enzyme is a dimer in solution. Inspection of the crystal structure of mtCP suggests that the most important inter-domain interactions are between the amino- and carboxy-terminal domains of the two monomers which form the functional homodimer. The lack of electron density for the first 23 residues of mtCP suggests that there is conformational flexibility at the N-terminus. However, there also appear to be significant inter-domain interactions between N-terminal residues of mtCP as was previously proposed using the yeast two-hybrid assay (35) and as was observed in the bpCP crystal structure (16). The N-terminal residues appear to “hook” around each other in a manner which may further stabilize the formation of the dimer, shown schematically in Fig. 2B, a detail not previously observed in the hmCP (31) or bpCP (16) crystal structures. This stabilization appears to be mediated by the presence of stacking interactions between Tyr28 and Tyr197, and Trp38 and Trp204 from opposite monomers (Fig. 2C). This long N-terminal extension appears unique to the CPs and is not present in other members of the class I peroxidase family e.g. CcP (26) and APX (27,32) (Fig. 3A). Hence, although inter-domain contacts between monomers of mtCP appear to be important in mediating homodimer formation, it is also interesting to note this “hook” motif may also play a significant role in dimer assembly.
Active site of \textit{M. tuberculosis} CP

The architecture of the heme-containing active site of \textit{mt}CP (Fig. 4) is similar to those of \textit{hm}CP and \textit{bp}CP, and closely resembles other class I peroxidases. The heme protoporphyrin IX moiety embedded within the active site of \textit{mt}CP is fully occupied and unmodified. The level of heme incorporation in CPs has been shown to vary from sub-stoichiometric to stoichiometric values (e.g. \textit{(19,33,36)}). In the case of \textit{mt}CP, occupancy of heme was raised from 0.5 heme/dimer \textit{(19)} to 2 hemes/dimer by incubating \textit{E. coli} cells containing overproduced enzyme with a suspension of hemin just prior to sonication. This improved occupancy of heme may indeed have reduced heterogeneity in preparation of this protein thus enabling successful crystallization of the enzyme.

Surrounding the heme within \textit{mt}CP are a number of structural elements which typify CP enzymes of the class I peroxidase family. As shown in Fig. 4A, the constellation of the six conserved key active site residues, Arg104, Trp107 and His108 in the distal pocket and His270, Trp321 and Asp381 in the proximal pocket, is present in a near identical arrangement to that observed in both the \textit{hm}CP and \textit{bp}CP crystal structures, as well as in the monofunctional class I peroxidases such as APX \textit{(27,32)} and CcP \textit{(26)}. In addition, electron density clearly describes covalent linkages between C$_{\eta 2}$ of Trp107 and C$_{\epsilon 1}$ of Tyr229 and between C$_{\epsilon 2}$ of Tyr229 and S$_{\delta}$ of Met255 (Fig. 4B). That this unusual adduct has now been observed in three CP structures would suggest that it is a characteristic common to all CPs. Although initial attempts to confirm the presence of this adduct in solution by mass spectrometry were unsuccessful for \textit{hm}CP \textit{(31)}, subsequent mass spectrometric analysis has shown the adduct to be present in both \textit{bp}CP \textit{(37)} and the \textit{Synechocystis} CP \textit{(38)}. Numerous site-directed mutagenesis studies of the class I monofunctional peroxidases, APX and CcP, have established that the six conserved residues in the active site play varying roles in the peroxidatic activity of these enzymes (e.g. reviewed in \textit{(39-44)}). Notably, these enzymes do not display significant levels of catalatic...
activity. In CPs, the most dramatic effects upon activity are observed for mutations involving the covalently modified distal site tryptophan residue, Trp107 in mtCP (Fig. 4A), which severely impair the catalatic activity with, in general, limited effects upon the peroxidatic activity of the enzyme (45,46). Mutation of mtCP Tyr229, or its equivalent in the Synechocystis CP, to phenylalanine also results in the loss of catalatic activity (47,48). Taken together these data further support the role of this adduct in maintaining the catalatic activity of the enzyme.

Four well-ordered water molecules can also be identified above the heme, within the distal pocket of the mtCP crystal structure (Fig. 4). Wat7, Wat235 and Wat427 occupy very similar positions to those observed for water molecules in the bpCP (16) and hmCP (31) crystal structures. mtCP also contains an additional water molecule, Wat352, located near the adduct and hydrogen-bonded to Wat427. In the hmCP crystal structure (31) a similar water molecule, Wat376, is also found near the adduct. In the bpCP crystal structure (16), the position of this water is occupied by a postulated perhydroxy modification on the vinyl group of the porphyrin ring of the heme. Notably, a water molecule is observed ligated to the heme iron at the sixth coordination position in the distal pocket of bpCP (16) and in one of the monomers of hmCP (31). In the mtCP crystal structure, however, no equivalent water molecule is observed and the heme appears only in a 5-coordinate state (Fig. 4), which is consistent with a number of spectroscopic studies of the enzyme (36,49,50).

Spectroscopic correlation: sites of radical formation in M. tuberculosis CP

In heme-containing peroxidases, a common catalytic cycle is observed which involves reaction of the ferric iron with hydrogen peroxide resulting in the formation of an oxyferryl species known as Compound I (51,52). In most peroxidases, Compound I is characterized by an Fe(IV)=O heme and a porphyrin π-cation radical (reviewed by (53)). The most notable exception is CcP where the radical is not localised on the porphyrin ring but is instead on the
proximal Trp191 residue (54-56), equivalent to Trp321 in mtCP (Fig. 4). The Compound I spectrum for CP based on studies of alkyl peroxide-mediated oxidation of CPs is similar to that for other peroxidases and therefore was initially attributed to a protoporphyrin radical species (57,58). Although initial EPR studies drew similar conclusions (36), recent studies have suggested that protein-based radicals may indeed be generated involving tyrosine and/or tryptophan residues (47,59-61). mtCP contains 21 tyrosine residues and 24 tryptophan residues which are scattered throughout the structure of the enzyme as shown in Fig. 5. The most recent evidence for the presence of a protein-based radical in mtCP has been the identification of a tyrosyl radical (47,59) proposed to occur at Tyr353 based upon EPR, spin trapping and mutational studies (60). As shown in Fig. 5, Tyr353 is located approximately 15 Å from the heme Fe, on the surface of the structure and now seems an unlikely candidate when considered in the context of the crystal structure. The side chain of Tyr353 points away from the surface of the protein, making it perhaps a likely target for radical trapping; however, given the number of tyrosines in the structure, the presence of a tyrosyl radical is still possible but could perhaps involve other tyrosine residues which are less solvent exposed and/or are located nearer to the heme (Fig. 5). EPR studies of a Synechocystis CP enzyme have also demonstrated the presence of a tyrosyl radical and a tryptophanyl radical in addition to a porphyrin-based radical (61). Although no residue has yet been assigned to the tyrosyl radical, Trp106 in this enzyme has been proposed as the possible site for the tryptophanyl radical. In mtCP the equivalent residue is Trp91, shown in Fig. 5. This residue is located even further away from the heme Fe, approximately 19 Å, compared to Tyr353, and again seems an unlikely candidate for radical formation in the M. tuberculosis enzyme. Indeed, the importance of this radical species may be specific to the Synechocystis CP as no tryptophanyl radical has yet been reported in the M. tuberculosis enzyme although, as shown in Fig. 5, there are numerous potential sites for radical formation of this type including the covalent adduct between Trp107, Tyr229 and Met255.

An alternative binding site for INH in M. tuberculosis CP
The location of a binding site for aromatic compounds in CPs has been postulated to be in the conserved surface loop, highlighted in Fig. 1, based upon the presence of a peak of electron density observed within this loop region in the \textit{bpCP} crystal structure (16). The authors speculate that this density might be occupied by a pyridine-like metabolite, although no biochemical data are yet published to support this hypothesis. In \textit{mtCP}, a small peak of electron density is also observed in the same site (Fig. 6A) but it appears too small to accommodate a pyridine-like metabolite and could in fact represent poorly ordered solvent or a small hydrophobic molecule. The identification of an aromatic compound binding site in a surface loop is unusual. Crystal structures of monofunctional peroxidases from all three classes (APX with salicylhydroxamic acid (SHA) (62); \textit{Arthromyces ramosus} peroxidase with SHA (63); HRPC with benzhydroxamic acid (BHA) (64)) have shown a common binding site for aromatic compounds located near the $\delta$-meso edge of the heme. Within the class I peroxidase family this loop appears only to be observed as a conserved feature in the CPs and, as shown in the sequence alignment in Fig. 3A, it is an insertion between the conserved proximal histidine and tryptophan residues. Superpositions of the crystal structures of \textit{mtCP} with the class I peroxidases, APX and CcP (Fig. 3B) clearly show how the loop is distinguished from the core structure of all these enzymes and localized near the distal side of the heme pocket. Comparison of the \textit{mtCP} crystal structure with the structure of the class III peroxidase HRPC (Fig. 3C) shows that this loop also adopts a unique structure which differs significantly from an insertion within HRPC at the same position which adopts an $\alpha$-helical structure localised nearer to the proximal side of the heme.

Fig. 6B shows an alternative binding mode for INH near the $\delta$-meso heme edge (INH1) in comparison with the site proposed (INH2) in the \textit{bpCP} crystal structure (16). The position of INH1 is based upon superposition of the \textit{mtCP} crystal structure with an NMR-derived HRPC-INH complex (17) which shows INH to be bound near the $\delta$-meso heme edge in a similar position to BHA in the HRPC-BHA crystal structure (64). The location of INH1 (Fig. 6B) also agrees well with NMR studies using $^{15}$N-labelled INH which reported a separation distance of
3.8 ± 0.8 Å between the amide nitrogen of INH and the heme iron of *mt*CP (65). Fig. 7A shows the energy map of complementary GRID analysis undertaken on *mt*CP using GRID probes selected to represent the hydrazinyl, carbonyl and aromatic moieties in the INH molecule. The GRID map aligns well with the position of INH1 (Fig. 6B) and identifies residues Arg104, Trp107 and His108, located in the distal pocket of *mt*CP (Fig. 4A), to be involved in favorable energetic interactions. In addition, GRID also identified Asp137 as having energetically favorable interactions with the selected probes. This residue has the potential to make hydrogen-bonding interactions between its carboxylic acid side chain and the pyridinyl N1 moiety of INH. In HRPC, residues Arg38, Phe41, and His42 are predicted to be key residues involved in INH binding (Fig. 7B) and were located by GRID calculations as energetically favorable toward the NH, carbonyl oxygen and aromatic neutral amide probes. The NH$_2$ probe also formed favorable interactions with residues Pro139 and Leu138 in HRPC. The location of favorable interactions in this GRID map of HRPC overlaps well with the position of INH in the NMR-derived HRPC-INH complex (17). GRID maps derived for *bp*CP (16) and *hm*CP (31) also gave very similar results, predicting that the primary site of INH/aromatic compound interaction is in the distal pocket of these enzymes. In all three CP crystal structures no significant energetic interactions were observed near the surface loop (Fig. 6A). The absence of this loop in the monofunctional peroxidases HRPC, APX and CcP, which have all been shown to turnover INH (17,66-68) further supports the proposal that INH1 (Fig. 6B) is located in the primary binding site for INH, and that this *mt*CP-INH1 model is representative of the complex formed during oxidation of the drug (see also (17)).

*Role of mutations in M. tuberculosis CP associated with INH resistance*

Resistance to INH is a continuing problem in the development of effective therapeutic regimes designed to eliminate infections arising from *M. tuberculosis* (the principle cause of tuberculosis in humans). Classical resistance to INH arises from deletions or point mutations in *katG* which encodes *mt*CP (e.g. (6,69-71), although in some cases, INH resistance can arise in genes encoding enzymes such as InhA and KasA required for
production of mycolic acids which are essential to the survival of the organism (reviewed in (72)). Using the mtCP crystal structure the effects of a number of point mutations located in the proposed INH binding site in the distal pocket can now be rationalized to some extent (Fig. 8).

For example, mutations affecting Ser315 are amongst the most commonly occurring (69,73-75) resulting in up to a 200-fold increase in the Minimum Inhibitory Concentration (MIC) for the drug (76). Ser315 has been reported to be mutated to asparagine, isoleucine, arginine and glycine although the most frequently occurring mutation is to threonine. As a result, a number of in vitro studies have been undertaken to understand the origins of resistance using the S315T mutant as a model (50,77-86). In the absence of a structure for mtCP it was recently postulated that Ser315 forms hydrogen bonds to one of the heme propionate groups and that mutation to threonine would therefore modify the heme pocket altering INH binding (16,50). Based upon the proposed binding site for INH in the bpCP crystal structure (INH2 in Fig. 6B), the S315T mutation was predicted to alter the binding site for the hydrazinyl moiety of INH and/or affect the transfer of electrons to the heme. As shown in Fig. 8, Ser315 is located at the periphery of the INH binding pocket at the bottom of the substrate access channel. Mutation to threonine in this model of the mtCP-INH complex could reduce affinity of the enzyme for the drug by increasing the steric bulk at this position and reducing but perhaps not fully blocking access to this substrate binding site. This is consistent with recently reported Raman data which drew a similar conclusion based upon the observation of new vibrational modes in the mutant compared to the wild-type enzyme which were attributed to conformational changes in the heme pocket (50). Ser315 is not predicted to play a significant role in the proposed mechanism for the enzyme-catalyzed activation of the drug (see (17)). This is consistent with the observation that the S315T mutant displays a reduced affinity for INH (78) but is able to oxidize INH with a rate equivalent to the wild-type enzyme (80). Furthermore, with the exception of glycine, the other mutations at this position would also increase steric bulk and further limit access to the
binding site. Taking the mutation to glycine into account, larger conformational changes may also occur which could reduce the affinity and potentially alter the orientation of INH in the active site of the enzyme thus reducing the enzyme’s ability to efficiently activate the drug. Interestingly, recent comparative studies of superoxide reactivity of the wild-type and S315T enzymes indicate INH affinity may not be greatly altered but that the mutant is unable to form an oxyferrous intermediate capable of oxidizing INH (86). The authors suggest that the effect of the S315T mutation upon superoxide-dependent activation of INH may be more related to localized changes in hydrogen bonding patterns or redox potentials. Thus, considering the various proposals regarding the activities of S315T, a crystallographic determination of this mutant in the future may help to clarify the origins of its role in resistance.

Based upon the availability of the mtCP crystal structure, certain residues in its active site have been postulated to be involved in enzyme-catalyzed activation of INH (17) but only the distal His108 residue has been a site for mutations conferring increased resistance to the drug (Fig. 8). This residue is proposed to be involved in the binding and enzyme-catalyzed activation of INH via interactions with the hydrazinyl moiety of the drug (17). In monofunctional peroxidases, this distal histidine has been shown to be particularly important in the formation of the oxyferryl Compound I (reviewed by (53)). In CcP, mutation of this residue, His52, to leucine results in a $10^5$ reduction of activity (87). In CPs, mutation of this residue results in a substantial reduction in catalatic and peroxidatic activities (45,46). With regard to INH resistance, His108 in mtCP has been reported to be mutated to glutamic acid and glutamine (e.g. (70,71)). These mutations may reduce the affinity of the enzyme for INH but glutamic acid/glutamine do have hydrogen bond donor and acceptor groups which could allow INH to bind in the distal pocket based upon the model presented here. However, neither of these residues would be predicted to shuttle protons in the same manner as His108 in the enzyme-catalyzed activation pathway (17). In addition, the A110V mutant associated with increased levels of INH resistance (88) may in fact, be exerting its effects by
altering the local conformation of His108 which could in turn alter its ability to bind and/or activate INH.

Based upon the mtCP structure, Asp137 has also been identified to play a key role in the binding and activation of INH as this residue appears to be a CP-specific proton donor in the enzyme-catalyzed activation pathway (discussed in (17)). No mutants associated with INH resistance have been reported to involve Asp137 although a number have been observed nearby including N138S, A139P, S140N or D142A (6,69,70,73,88) (Fig. 8). These mutants might be exerting their effects through local conformational changes which could alter the orientation of the Asp137 side chain, making it less effective in binding INH and/or catalyzing the turnover of this drug. It is interesting to note that in the catalatic reaction carried out by the enzyme, Asp137 is also postulated to act as a proton donor (89). Mutations at this position would therefore have the potential to drastically alter the enzyme’s ability to function as a catalase. It is therefore possible that slight repositioning of the side chain of Asp137 through mutations nearby may be sufficient to significantly reduce INH binding and/or activation whilst retaining sufficient levels of catalase activity in vivo.

In summary, we have presented the crystal structure of CP from M. tuberculosis which reveals new information about the assembly of class I peroxidases and provides a structural framework to understand further the multi-functional properties of the enzyme. We have also predicted a new primary binding site for INH in mtCP. The model of this complex identifies residues which may play key roles in interactions with the drug and can be used to rationalise the effects of a number of mutants within the enzyme which confer elevated levels of resistance to INH. This work should therefore stimulate more rationally designed structure-function studies aimed at elucidating the catalytic activities of this enzyme including INH activation.
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FOOTNOTES

1 The abbreviations used are: CP, catalase-peroxidase; CcP, cytochrome c peroxidase; APX, ascorbate peroxidase; INH, isonicotinic acid hydrazide (isoniazid); mtCP, Mycobacterium tuberculosis catalase-peroxidase; HRPC, horseradish peroxidase C; PBS, phosphate buffered saline; PEG, polyethylene glycol; bpCP, Burkholderia pseudomallei catalase-peroxidase; r.m.s., root mean square; rsAPX, recombinant soybean ascorbate peroxidase; hmCP, Haloarcula marismortui catalase-peroxidase; SHA, salicylhydroxamic acid; BHA, benzhydroxamic acid; MIC, minimum inhibitory concentration; sAPX, soybean ascorbate peroxidase; pAPX, pea ascorbate peroxidase.

2 The coordinates and structure factors for mtCP are available in the Research Collaboratory for Structural Bioinformatics Protein Databank (http://www.pdb.org) under PDB # 1SJ2.
FIGURE LEGENDS

Figure 1: mtCP monomer. N-terminal domain is in light pink, C-terminal domain is in dark pink. The heme is in gray. N-terminal residues 24-30 are highlighted in green. Residues 278-312 are highlighted in red.

Figure 2: Structure of mtCP. (A) Global structure of the homodimer. Subunit 1 is in pink (N-terminal domain is in light pink, C-terminal domain is in dark pink), subunit 2 is in blue (N-terminal domain is in light blue, C-terminal domain is in dark blue). The heme is in gray. (B) Schematic representation of the homodimer. N-terminal residues of each monomer subunit form interlocking “hooks” via hydrophobic interactions including residues Y28 and Y197, W38 and W204 as shown in (C). A black ellipse represents the axis of two-fold symmetry.

Figure 3: Comparison of mtCP with monofunctional peroxidases. (A) Structure-based sequence alignment of the N-terminal domain of mtCP with CcP, soybean ascorbate peroxidase (sAPX), pea ascorbate peroxidase (pAPX) and HRPC. Residues forming a large insertion in the mtCP structure (residue 278-312) are highlighted in red and residues forming a large insertion in HRPC (residue 177-215) are in dark blue. Six key active site residues are shown in green, the mtCP active site adduct is shown in yellow and residues proposed to be important for INH binding are shown in light blue (17). (B) Structural superposition of mtCP (pink) with CcP (lime) and sAPX (orange). (C) Structural superposition of mtCP (pink) with HRPC (dark green). The extra loop found in mtCP is in red, the extra helices in HRPC are in blue.

Figure 4: Heme environment of mtCP. (A) On the distal side, residues R104, W107 and H108 are shown as well as four water molecules (labelled with red w). Note also that W107 forms an adduct with Y229 and M255. On the proximal side, H270, W321 and D381 are shown. Hydrogen bonds are shown with dotted lines. (B) Simulated annealing composite
Fo-Fc omit electron density map contoured at 2\(\sigma\) (in marine) with the model of this region superimposed. Protein carbon atoms are depicted in pink, oxygen atoms in red, nitrogen atoms in blue, sulfur atoms in orange, heme carbons atoms in gray, oxygen atoms in magenta and nitrogen atoms in slate.

**Figure 5: Tryptophan and tyrosine residues in N-terminal domain of mtCP crystal structure.** Tryptophan residues are shown in blue and tyrosine residues in pink. Two residues proposed to be the site of the Compound I radical are highlighted in green (60,61). The C\(\alpha\) trace of mtCP is shown in pink.

**Figure 6: Potential binding sites for INH in mtCP.** (A) mtCP Fo-Fc electronic density contoured at 3\(\sigma\) in the region where INH is proposed to bind (16). Residues surrounding this region are labelled. (B) Two potential binding sites for INH (green). INH docked into mtCP based on HRPC-INH model (17) (INH1). INH docked into mtCP based on bpCP undefined electron density (16) (INH2). Protein carbon atoms are depicted in pink, oxygen atoms in red, nitrogen atoms in blue, heme carbons atoms in gray, oxygen atoms in magenta and nitrogen atoms in slate. Water molecules are labelled with red w.

**Figure 7: GRID maps and diagrams.** Energetically favorable binding sites for the NH (green), NH\(_2\) (blue), carbonyl oxygen (red) and aromatic neutral amide (purple) probes in (A) mtCP (pink) and (B) HRPC (dark green) are shown. GRID map contour levels are set at -8, -9, -12 and -8.5 kcal/mol for the NH, NH\(_2\), carbonyl and aromatic neutral amide probes, respectively. In all panels the heme carbon and iron atoms are in gray, whilst the oxygen atoms are depicted in purple and the nitrogen atoms are shown in cyan. Important amino acid residues are indicated as wire figures. This figure was produced using DS ViewerPro 5.0 (Accelrys, Cambridge, UK).
Figure 8: Positions of clinical mutations close to INH binding site in *mtCP*. Residues which have been found to be mutated in clinical isolates are shown in green. Residues R104, W107, D137, Y229, M255, H270, W321 and D381 are shown in pink to delineate the active site. INH is shown in green and the heme in gray. A109 and L141 are also shown in pink for clarity.
### TABLE 1

Data collection and refinement statistics

| Data collection       |       |
|-----------------------|-------|
| Wavelength (Å)        | 1.488 |
| Space group           | $P_{4_2}2_1$ |
| Unit cell (Å)         |       |
| $a = b$               | 150.3 |
| $c$                   | 154.3 |
| Resolution (Å)        | 30 – 2.4 |
| Observed reflections  | 1932986 |
| Unique reflections    | 61822 |
| Completeness (%)      | 90.4 (89.1)$^a$ |
| $I/\sigma(I)$         | 11.2 (4.2) |
| $R_{sym}$ (%)         | 15.9 (39.1) |

| Refinement statistics |
|-----------------------|
| $R_{cryst}$ (%)       | 21.1 |
| $R_{free}$ (%)        | 26.8 |

| Residues              |       |
|                       | 24 – 740 |
| Number of water molecules | 703 |
| R.m.s. deviation      |       |
| bond lengths (Å)      | 0.0062 |
| bond angles (°)       | 1.26  |

| Average $B$ factors (Å$^2$)       |     |
|-----------------------------------|-----|
| main chain                        | 38.4 |
| side-chain                        | 38.6 |
| heme                              | 30.7 |
| water molecules                   | 38.6 |
| glycerol                          | 42.3 |

| Ramachandran statistics$^e$ (%) |
|----------------------------------|
| most favored regions             | 88.2 |
| additional allowed regions       | 11.3 |
| generously allowed regions       | 0.5  |

$^a$ Numbers in parenthesis represent values in the highest resolution shell (last of 10 shells)

$^b$ $R_{sym} = \Sigma_i |I(h,i) - <I(h)>| / \Sigma_i |I(h,i)|$ where $I(h,i)$ is the intensity value of the $i$th measurement of $h$ and $<I(h)>$ is the corresponding mean value of $I(h)$ for all $i$ measurements.

$^c$ $R_{cryst} = \Sigma ||F_{obs}|-|F_{calc}||/\Sigma |F_{obs}|$, where $|F_{obs}|$ and $|F_{calc}|$ are the observed and calculated structure factor amplitudes respectively.

$^d$ $R_{free}$ is the same as $R_{cryst}$ but calculated with 10% subset of all reflections that was never used in crystallographic refinement.

$^e$ As evaluated by PROCHECK (90).
Figure 7

A

B

H108
R104
D137
W107
H42
G69
R38
F41
Crystal structure of Mycobacterium tuberculosis catalase-peroxidase
Thomas Bertrand, Nigel A. J. Eady, Jamie N. Jones, Judit M. Nagy, Brigitte
Jamart-Gregoire, Emma Lloyd Raven and Katherine A. Brown

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