CHAPTER 2

Characterization of the *Mycobacterium tuberculosis* H37Rv Alkylhydroperoxidase (AhpC) Points to the Importance of Ionic Interactions in Oligomerization and Activity

Running title: Biochemical Characterization of *M. tuberculosis*AhpC

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2.1. Introduction

Isoniazid has been one of the frontline drugs used in tuberculosis control for more than three decades. Resistance to isoniazid develops primarily due to point mutations in the \textit{katG} locus, encoding the catalase peroxidase enzyme \cite{1}. The loss of \textit{katG} function in isoniazid strains therefore renders \textit{Mycobacterium tuberculosis} susceptible to the toxic peroxide radicals \cite{2}. Under these conditions, overexpression of another gene, \textit{ahpC}, encoding an alkyl hydroperoxide reductase, has been frequently found to occur \cite{3}. We have attempted to characterize the AhpC enzyme biochemically, and report its functional properties in this communication.

The mycobacterial AhpC is a unique enzyme in many ways compared to its well-characterized homologues in enteric bacteria. For example, the \textit{M. tuberculosis} enzyme possesses three cysteine residues, as compared to two of \textit{Salmonella typhimurium} and \textit{Escherichia coli} enzymes \cite{4, 5}. Very recently it has been shown that all the three cysteines residues are important for \textit{M. tuberculosis} AhpC activity \cite{6}. Another important difference between the \textit{M. tuberculosis} AhpC and that derived from other closely related species, is that the electron donor partner of AhpC, the FAD/NADPH binding subunit AhpF, is absent in \textit{M. tuberculosis} unlike in \textit{E. coli} and \textit{S. typhimurium}. We show in this manuscript that the mycobacterial AhpC can use DTT as an electron transfer partner \textit{in vitro}, and rapidly oxidize DTT in the presence of the substrate. Furthermore, we show that unlike its dimeric homologues from enteric bacteria, the mycobacterial AhpC is decameric in nature.

2.2. Experimental Procedures:

2.2.1. \textit{M. tuberculosis} \textit{ahpC} gene cloning and protein purification:

The 588 bp long gene encoding \textit{M. tuberculosis} AhpC was PCR amplified using the genomic DNA of \textit{M. tuberculosis H37Rv} and overexpressed in the pET23A (+)/BL21 (DE3) expression system. The overexpressed 6-Histidine tagged protein,
named as AhpCHis6, was purified by Ni-NTA affinity chromatography. Briefly, cells harboring pET23A along with AhpCHis6 insert were grown till they reached an OD\textsubscript{600nm} of 0.6, and then induced with 0.6 mM IPTG. The lysed cells were centrifuged at 30,000 g for 45 minutes and the supernatant was loaded onto the Ni-NTA column. The column was washed with 50mM tris-HCl buffer pH 7.5 containing 200mM NaCl and 6% glycerol. The protein was eluted with the same buffer supplemented with 200mM imidazole. The purity of the protein was checked on SDS-PAGE where it appeared as a single band (unpublished data).

2.2.2. Size-exclusion chromatography:

Size exclusion chromatography was performed at room temperature using either FPLC or SMART systems (Pharmacia Amersham) equipped with superdex-200 HR 10/30 or superdex-200 PC 3.2/30 columns respectively. Calibration of the columns was performed using molecular weight standards for gel filtration chromatography supplied by Sigma. Briefly, for calibration of both the columns, thyroglobulin (669 kDa), apoferritin (443 kDa) β amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa) and carbonic anhydrase (29 kDa) were used as standard markers at recommended concentrations. Standard proteins were dissolved into equilibration buffer, i.e. 50mM tris-HCl pH 7.5 buffer containing desired concentration of NaCl. The column was equilibrated and then each protein was applied to the column to determine elution volume ($V_e$) of the protein. The void volume ($V_o$) of the column was determined by running the blue dextran on the column. The calibration curve was plotted as $V_e/V_o$ versus logarithmic molecular weight. No difference in the elution volume of the standard proteins as well as in their calibration curve was observed when the column calibration was done at different concentrations of NaCl, i.e. 150mM, 300mM, 1.5M or without NaCl.

The columns were equilibrated with at least 3-bed volumes of the elution buffer prior to each run. A typical flow rate of 0.5 ml/min or 100μl/min was maintained for the FPLC or the SMART systems respectively. Absorbance at 280 nm was measured to monitor elution of the protein from the column.
concentration of AhpCHis6 in tris-HCl pH 7.5 was used for all the gel filtration experiments. To study the effect of NaCl concentration on oligomerization, the protein was incubated at various concentrations of NaCl, and the column was equilibrated with the desired concentration of NaCl before loading the protein on each column.

2.2.3. Crosslinking of AhpCHis6:

Chemical crosslinking of AhpCHis6 was carried out according to the well-established protocols [7, 8]. 0.5 mg/ml AhpCHis6 in Triethanolamine (TEA) buffer pH 9.0, was incubated with dimethyl pimelimidate (DMP) for 10 minutes at 25°C. To terminate crosslinking, the protein was precipitated with 20% Trichloroacetic acid (TCA). The precipitate was then redissolved in SDS and loaded onto 10% SDS-PAGE.

2.2.4. DTNB Assay:

Free -SH groups in AhpCHis6 were determined spectrophotometrically at 412nm using DTNB (5,5'-dithiobis-2-nitrobenzoic acid) [9]. 1.2 μM AhpC was incubated at 25°C with 0.8mM DTNB in 50mM potassium phosphate buffer (KPi) pH 7.5. Stoichiometry of the reaction was calculated by using the extinction coefficient of 14,150 M⁻¹cm⁻¹ for thionitrobenzoate (TNB⁻²) anion in the absence of SDS and a value of 13,600M⁻¹cm⁻¹ in the presence of 5% SDS. In another experiment, the enzyme was incubated with 5% SDS for 1 hour at room temperature in the presence of 50mM dithiothreitol (DTT). Subsequently the excess of DTT was removed by 20% TCA precipitation. The protein precipitate was washed twice with 20% TCA and redissolved in 6M Urea-KPi buffer pH 7.5. Each assay was repeated for 3 times.

2.2.5. Activity Assay by Ferrithiocyanate method:

Enzyme activity was monitored by measuring removal of peroxide substrate, t-butyl hydroperoxide, from reaction mixture. Peroxides form a complex with
ferrithiocyanate with an absorption maximum of 480 nm [10,11]. 44 μM AhpCHis6 protein in Kpi buffer pH 7.0 containing 1mM EDTA and 300mM NaCl, was incubated at 37°C with 2mM tertiary butyl-hydroperoxide in a 1ml-reaction mixture. The reaction was initiated by the addition of 10mM DTT and was terminated by adding 20% TCA. After the removal of precipitated protein by centrifugation, 0.2 ml of 10mM ferrous ammonium sulfate and subsequently 0.1 ml of 2.5M potassium thiocyanate were added to a 1.0 ml aliquot of the supernatant. Absorption of the red ferrithiocyanate complex was measured at 480nm spectrophotometerically and was compared to t-butyl hydroperoxide standards. Km and V_max for AhpCHis6 was calculated by following the steady state kinetics, in which the substrate concentration (t-butyl hydroperoxide) was varied and the removal of the substrate was measured by thiocyanate method.

2.2.6. Homology modelling:

Sequence homology of *M. tuberculosis* AhpC was searched using BLAST for similar three-dimensional structures in the protein databank [12]. Sequence alignment of *M. tuberculosis* AhpC with 1-cysteine and 2-cysteine peroxiredoxins (Prxs) was carried out by CLUSTALW [13]. Coordinates of 2-cys Prx family, Heme binding protein 23kDa (HBP23; pdb ID: 1qq2) were used as the template for three dimensional model constructions. One major insertion of 11 residues in the *M. tuberculosis* AhpC with respect to the HBP23 was modeled as an antiparallel β-hairpin in continuation with the preceding β-sheet. All other residues which were different between the two proteins were appropriately mutated on a Silicon Graphics workstation using the program "O" [14]. After performing the mutations, best side chain conformations were chosen to avoid short contacts. The resulting model was then energy minimized using distance dependent dielectric constant and other standard parameters in the program DISCOVER.
2.2.7. Fourth Derivative Spectra:

Absorption spectra and their 4\textsuperscript{th} derivative were monitored on a Shimadzu UV 1601A spectrophotometer using derivative interval of 2.0nm. The enzyme was used in a concentration of 2mg/ml in 50mM tris-HCl buffer pH 7.5. To reduce the protein, DTT was added to a final concentration of 10mM. Spectra were recorded in a range of 190 to 300 nm.

2.3. Results:

2.3.1. Activity of purified AhpCHis6:

The physiological electron donor of mycobacterial AhpC is unknown at present. We chose DTT for performing activity assay of the recombinant AhpCHis6, since DTT is capable of transferring electrons to peroxide during catalysis in mammalian thiol peroxidases [15]. We found that DTT was rapidly oxidized when both the enzyme and the substrate were present in the reaction mixture. On the other hand, DTT could not be oxidized when either the enzyme or the substrate, or both of them were omitted from the reaction mixture. Similarly, when AhpC was replaced with BSA for monitoring the desired activity, DTT was not oxidized (Figure 2.1)

To confirm the enzymatic activity, thiocyanate assay [11] was performed to measure decrease in substrate concentration as described in experimental procedures. Upon incubation of the enzyme with t-butyl hydroperoxide and DTT, a continuous decrease in the amount of t-butyl hydroperoxide was observed as the reaction proceeded. In contrast, control reactions without the enzyme, or with BSA in place of AhpCHis6, showed a constant absorbance at 480 nm. (Figure 2.2A). These results therefore indicated that the recombinant AhpCHis6 was fully functional and was capable of catalyzing the reduction of t-butyl hydroperoxide using DTT as an electron transfer partner.

On probing the effect of NaCl concentration on enzyme activity, we found that the enzyme was most active at 300mM NaCl concentration. The activity of the enzyme gradually increased with the concentration of NaCl, and was optimal at
Figure 2.1: AhpCHis6 catalyzed oxidation of DTT.

Solid line and filled squares indicate the reaction in the presence of AhpCHis6, hollow rectangles and dotted line represent oxidation in the absence of the enzyme, and hollow triangles and dashed line represent oxidation in the absence of CHP. DTT thus can be oxidized only in presence of the enzyme and the substrate CHP.
Figure 2.2: Thiocyanate assay and NaCl dependent activity of AhpCHis6

(a) Removal of peroxides by AhpCHis6 measured by thiocyanate method. Solid line indicates the progress of reaction in presence of the enzyme. Dashed line indicates the control reactions.

(b) Enzyme activity of AhpCHis6 measured by the oxidation of DTT as a function of NaCl concentration. The enzyme is most active in the presence of 300mM NaCl and beyond this concentration loses its activity rapidly.
300mM concentration of NaCl. On increasing the concentration of NaCl further, there was a significant decrease in the DTT oxidation (Figure 2.2B). The loss of activity in higher concentration of NaCl, particularly at 1.5M NaCl, is indicative of important role of ionic interactions during catalysis. Inspection of the 3D model showed the presence of a chloride ion-binding site next to the active site cysteine residues. This region although partially solvent shielded is dominated by the presence of charged side chain residues Glu 64, Arg 133 and Arg 156. We thus believe that activity of the enzyme may be lost at higher salt concentrations due to the screening effect on electrostatic interactions.

To confirm the NaCl dependent activity of AhpCHis6, we calculated the Km and $V_{\text{max}}$ kinetics parameters in the presence of 100mM and 300mM NaCl. In accordance with the increased activity of the enzyme, we observed that the Km of AhpCHis6 in 300mM NaCl was 35mM, compared to 8mM in the presence of 100mM NaCl. However, not only Km was affected, but $V_{\text{max}}$ also doubled in 300mM NaCl (data not shown). Thus, the enhanced activity of the enzyme at 300mM NaCl ionic strength cannot be explained based solely on the reduced substrate affinity. Nonetheless, the salt concentration dependence suggests an important role of ionic interactions in catalytic activity of the enzyme.

### 2.3.2. Oligomeric assembly of recombinant AhpCHis6:

In order to determine if the loss of activity at higher NaCl concentrations is attributed to the loss of quaternary structure of the enzyme, we performed size exclusion chromatography. Results of the experiments in presence of 250mM NaCl, quite unexpectedly, showed that the molecule migrated as a mass of 253 kDa on analytical gel filtration column, unlike its dimeric homologues from other bacteria. The homogeneity of this peak suggested that AhpCHis6 might form a 10-12 mer in solution (Figure 2.3A). Although we could not perform similar experiment in absence of NaCl due to the instability of the protein, we believe that the protein would possess the same oligomeric structure in absence of salt also.

The activity of AhpCHis6, as mentioned earlier, reduced considerably at high concentrations of NaCl. Size exclusion chromatography showed that in the presence
Figure 2.3: Gel filtration chromatograms of AhpCHis6.

(a) The elution profile of the purified AhpCHis6 on superdex-200 HR 3.2/10 column. The column was pre-equilibrated in 50mM Tris-HCl buffer of pH 7.5 containing 250 mM NaCl before loading the protein. Comparison with standard molecular weight markers showed that the elution volume corresponded to a 10-12 mer species.

(b) The elution profile of AhpCHis6 in 1.5 M NaCl. The experimental conditions are identical as in (a), but for the high concentration of the salt (1.5M NaCl). The protein clearly eluted as a homogenous dimer in this experiment.

(c) The elution profile of AhpCHis6 in 1.5M NaCl and 10mM DTT. With the addition of DTT, the monomeric species of the enzyme starts becoming prominent, in addition to the dimer.
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of 1.5M NaCl, the decamer disintegrated into a dimer (Figure 2.3B). The dimer, obtained in 1.5M NaCl, further dissociated into monomers in presence of 10mM DTT (Figure 2.3C). Dissociation of dimers into monomers in presence of DTT suggested presence of an intersubunit disulfide linkage in the protein. These results thus indicated that the oligomeric assembly of AhpCHis6 might be stabilized through ionic interactions. Since the activity of AhpCHis6 is attributed to decameric quaternary structure, it seems that in cellular environment the protein maintains its decameric assembly.

To further investigate the pattern of AhpCHis6 oligomerization, we crosslinked the AhpCHis6 with ε-amine specific crosslinker, DMP, followed by SDS-PAGE of the crosslinked species. Five major species were observed and the molecular weight values corresponded to those calculated for the monomer, dimer, trimer, tetramer, and pentamer (Figure 2.4). As such therefore the data indicate that the fundamental unit of quaternary structure is a 'pentamer'. Furthermore, since analytical gel filtration data show that AhpCHis6 is a 10-12 mer, the likelihood is that AhpCHis6 has a paired pentameric subunit arrangement.

2.3.3. Role of Cysteines and ionic interactions in the oligomerization of AhpCHis6:

The purified AhpcHis6 appears as a single band of molecular weight 25 kDa on SDS-PAGE when reduced with β-mercaptoethanol. However, under non-reducing conditions several high molecular weight bands were also observed. The most prominent of these bands corresponded to a molecular weight of ~45 kDa, suggesting the presence of a dimeric species. The absence of a dimer in reducing conditions and its presence under non-reducing conditions suggests an intersubunit disulfide bond. Thus, monomers within a dimer are likely to be held together by disulfide linkages.

The amino acid sequence of mycobacterial AhpC contains a total of three Cys residues. To confirm if the cysteines, by some means, were responsible for the multimeric aggregation of the protein, a DTNB assay was carried out. The total numbers of free sulphhydryl groups in AhpCHis6 estimated from DTNB analysis under various treatments are listed in Table 2.1. These results clearly showed that there was
Figure 2.4: 10% SDS-PAGE scans showing crosslinked subunits of AhpCHis6.

Molecular weights of the bands are as follows: band 1, 25kDa; band 2, 43kDa; band 3, 68 kDa; band 4, 98 kDa; band 5, 120 kDa. These molecular weights respectively correspond to the monomer and cross-linked dimer, trimer, tetramer and pentamer respectively of AhpCHis6. Beyond the pentamer, other species are notably absent, suggesting that the basic unit of quaternary structure is a pentamer.
Table 2.1: DTNB titration of AhpCHis6:

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>µmol RSH/µmol subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native AhpC</td>
<td>0.76 ± 0.10</td>
</tr>
<tr>
<td>AhpC + 5%SDS</td>
<td>0.68 ± 0.01</td>
</tr>
<tr>
<td>AhpC + 50mM DTT + 5%SDS</td>
<td>2.46 ± 0.17</td>
</tr>
</tbody>
</table>
only one free sulphhydryl group per subunit of purified recombinant AhpCHis6. On the other hand, treatment of AhpCHis6 with 10mM DTT along with 5% SDS resulted in all the three cysteines in their reduced forms. Thus, the protein possessed only one disulfide bond per monomer in its oxidized state. This disulfide bond as we have shown earlier, crosslinked the two monomers in a dimer. Consequently the third cysteine was not involved in any disulfide linkage ruling out possible oligomerization arising out of disulfide bond.

2.3.4. Conformational changes in recombinant AhpCHis6:

UV Absorption 4th derivative spectra of the oxidized and reduced protein are shown in Figure 5. These spectra showed significant differences between the oxidized and the reduced states of the protein. The 4th derivative spectra of the oxidized protein revealed a peak at 290nm, corresponding to tryptophan residues. This peak was absent in AhpCHis6 protein reduced with 10mM DTT (Figures 2.5A and 2.5B). The derivative spectra therefore indicated that tryptophan residues were exposed to the solvent in the oxidized form of the protein but were solvent shielded in the reduced AhpCHis6. A conformational change is thus likely to occur during oxidation-reduction of the protein.

Three-dimensional model of the protein generated using HBP23 coordinates [16] was inspected to obtain insights into possible conformational changes. The model showed that Cys 61 of one subunit is crosslinked with Cys 174 of another (Figure 2.6). The model also showed two partially solvent exposed tryptophan residues, 47 and 96, in the vicinity of the active site. These two tryptophan residues are likely to be affected most by conformational changes related to oxidation-reduction of the protein, due to their proximity to the loops containing all the three important cysteines residues. Thus, movement of the loops containing Cys 61, Cys 174 and Cys 176, is likely to lead to burial of Trp 47 and Trp 96, which are otherwise solvent exposed in the oxidized protein.
Figure 2.5: 4th Derivative absorption spectra of AhpCHis6:

(a) Reduced AhpCHis6 and (b) Oxidized AhpCHis6

The change due to tryptophan residues is indicated in (b) by an arrow, showing clearly a change in the environment of tryptophan residues in the two states. This change presumably takes place in the neighbourhood of Trp 47 and 96 as discussed in the text.
Figure 2.6: Ribbon diagram of the model constructed using 2-cysteine Prx coordinates.

The three catalytically important cysteines are shown. Importance of charged residues is highlighted by the presence of Glu 64, Arg 133 and Arg 156 next to the active site. Two-tryptophan residues 47 and 96, which we believe give rise to difference in the absorption spectra, are also shown. The figure was produced using MOLSCRIPT [29].
2.4 Discussion:

*M. tuberculosis* lack the classical oxidative stress responses, with the *oxyR* regulator being absent from the genome of *M. tuberculosis* [17, 18]. In the absence of *OxyR* regulated defense, the catalase peroxidase enzyme encoded by the *katG* gene has been found to be the only peroxide inducible gene in *M. tuberculosis*. The isoniazid resistant strains of *M. tuberculosis* are therefore especially susceptible to oxidative damage due to the non-functional catalase peroxidase. In the absence of a functional *oxyR* response and the malfunctioning *katG*, the tuberculosis bacilli are therefore faced with an unusual situation of survival within the host macrophages without an effective oxidative stress response. In these strains of *M. tuberculosis*, the *ahpC* gene has been shown to be frequently overexpressed [19]. AhpC, has also been shown to be vital for mycobacterial survival within macrophages [20], and therefore forms an attractive drug target. We have undertaken work to biochemically characterize this important enzyme.

Eubacteria such as *E. coli*, *S. typhimurium* and *Bacillus subtilis* possess AhpF protein as electron donor partner of AhpC [4, 21, 22]. While in yeast and mammalian systems, the thioredoxin/thioredoxin reductase system substitutes AhpF [23, 15]. It has also been shown that NADH oxidase can also reduce AhpC in *Amphibacillus xylanus* [24]. Hence, it appears that there is no common electron transfer partner of AhpC. All these partners known till date share only one common characteristics, i.e. they are FADH/NAD(P)H binding proteins. In the absence of AhpF protein, hence, *M. tuberculosis* may use thioredoxin or NADH oxidase as an electron dononating partner during catalysis.

On the basis of our results of DTT oxidation we found that DTT can transfer electron to substrate via AhpC, which suggests that, the mycobacterial AhpC may use small size molecule as an electron transfer partner. Hence, we hypothesize that the possible electron donor of mycobacterial AhpC could be either thioredoxins or mycothiol, a major low molecular weight thiol, analogous to glutathione. Our speculations are also supported by the findings that genes responsible for mycothiol
synthesis can be induced by oxidative stress [25]. These speculations need further experimental evidences, which are under progress in our laboratory.

Interestingly, we found that the activity of the alkylhydroperoxidase enzyme was dependent on salt concentration. Our findings that the enzyme was most active at 300mM NaCl are surprising since the physiological concentration of salts in cellular environment is far less than 300mM. The 3D model of mycobacterial AhpC indicated the presence of a chloride ion-binding site in AhpC. Earlier it has been reported that 2-Cys peroxiredoxin of human bind to chloride ions to maintain the active site structure [16], which confirm our results. Occurrence of several charged side chains in the proximity of catalytic cysteine residues suggests that these side chains may be crucial for the maintenance of the active site configuration. All the charged side chains in the active site are conserved in evolution and thus support our observation that ionic interactions may play a crucial role in the activity of mycobacterial AhpC.

To probe if ionic interactions were important for subunit association, and if the loss of activity was a consequence of disintegration of subunit structure, we carried out gel filtration experiments. While we were expecting a dimeric nature of the enzyme similar to its homologues from other bacteria, the mycobacterial enzyme showed interestingly that it is decameric in nature. Decamerization has previously been reported for AhpC from \textit{A. xylanus} [26] and human TPx-B [27]. There is no report available yet on the physiological state of mycobacterial AhpC oligomerization and its \textit{in vivo} role in oxidative stress.

Very recently oligomeric nature of mycobacterial AhpC has also been reported by Hillas \textit{et al.} [6]. Although the previous report on mycobacterial AhpC is not certain on the number of subunits in the oligomer, we have shown conclusively here that enzyme is a decamer, through a combination of chemical cross-linking studies, and precise analytical gel-filtration experiments. Further, the decamer of AhpCHis6 could be dissociated at high salt concentrations, making dimer a dominant species. The dimers could be dissociated into monomers only in reducing conditions, implying presence of an intersubunit disulfide linkage.

The intersubunit disulfide has been observed in the structure of 2-Cys peroxiredoxin, but has been suggested to be formed only during the catalytic cycle
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[16, 28]. The equivalent cysteine residues in \textit{M. tuberculosis} would be Cys 61 and Cys 174 derived from two different subunits. Interestingly, the mycobacterial AhpC contains a cysteine residue at the 176\textsuperscript{th} position in the sequence, very close to the intersubunit disulfide as observed in the three dimensional model. The third cysteine residue could be successfully modelled in the three dimensional structure, contrary to the suggestion of Hillas \textit{et al.} [6]. All the three cysteines residues have very recently been shown to be crucial for the activity of the enzyme by site directed mutagenesis studies [6]. This gives rise to an interesting speculation that Cys 174 and Cys 176 may be involved in a disulfide link, reduction of which leads to the formation of the intersubunit Cys 61-Cys174 disulfide.

In our 3D model of AhpCHis6, we found that Trp47 and Trp96 are exposed to solvent in oxidized state of the enzyme. This observation is consistent to our UV absorption spectra studies. During oxidation and reduction of AhpCHis6, there is rearrangement of Trp 47 and 96. Such type of rearrangement arises due to the movement of loops containing Cys 61, 174 and 176, which lead to burial of Trp 47 and 96 in the reduced state of AhpCHis6. Such types of conformational changes have also been observed for human TPx-B and other bacterial AhpC [27, 28].

Biochemical characterization of \textit{M. tuberculosis} AhpC thus shows that ionic interactions play an important role in its function as well as oligomerization. These results should lead to initiation of rational drug design work using this enzyme as a potential target.
2.5. References:


