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Structural and Functional Studies on Mycobacterium Tuberculosis Alkylhydroperoxidase C (Ahpc).

With about one-third of the world's population infected and nearly three million deaths annually, *Mycobacterium tuberculosis* is arguably the world's most successful human pathogen. Over millions of years, *M. tuberculosis* has been surviving and multiplying in the human macrophages, which is the host's main weapon to kill invading organisms. The macrophages generate a series of toxic material including ROS and RNIs, which cause oxidative damage to the proteins, lipids and nucleic acids. Hence, it is obvious that the pathogens surviving within the macrophages must have a strong defense mechanism against the oxidative and nitrosative stress. This line of thought motivated the present studies on alkylhydroperoxidase C/ alkylhydroperoxide reductase C (AhpC), an important component of oxidative stress defenses in *M. tuberculosis*. The current study illustrates the structural and biochemical aspects of *M. tuberculosis* AhpC.

Oxidative damage is an unavoidable consequence of the aerobic lifestyle and is caused by ROS and hydroxyl radicals. Similar to the oxidative stress, nitrosative stress is also known to damage the cellular components. Chapter 1 describes the mechanism of oxidative damage to proteins, lipids and nucleic acids and the adaptive responses of prokaryotes in defense against this damage. This chapter also deals with the current scenario of *M. tuberculosis* defense mechanisms. Several unique characteristics of the *M. tuberculosis* defense mechanisms against the oxidative stress, particularly the lack of OxyR inducible responses and its link to the sensitivity to isoniazid drug have been reviewed in Chapter 1. Mutations in the *katG* gene locus generates INH resistant *M. tuberculosis* strains, harboring an additional mutation in the promoter of *ahpC* gene, which results in the overexpression of AhpC protein. The details of proposed significance of AhpC overexpression in INH resistant strains of *M. tuberculosis* have been elaborated in section 1.5.

Section 1.6 describes the concept of peroxiredoxin, a cysteine based peroxidase. The 3-dimensional structural information of some of the Prx members namely hORF6, HBP23, TPx-B, TryP, PrxV and Salmonella AhpC have also been reviewed in this section. Section 1.7 of Chapter 1 includes the rationale behind the

present studies on *M. tuberculosis* AhpC. It also comprises a brief introduction to the project undertaken.

The biochemical characterization of AhpC indicated that AhpC of *M. tuberculosis* is unique in many ways compared to its well-characterized homologues from enteric bacteria. Chapter 2 illustrates these preliminary biochemical characterizations of *M. tuberculosis* AhpC protein. In this Chapter, it has been shown that AhpC is a decameric protein, composed of five identical dimers held together by ionic interactions. Dimerization of individual subunits takes place through an intersubunit disulfide linkage. The ionic interactions play a significant role in enzymatic activity of the AhpC protein. The UV absorption spectrum and the 3D model of AhpC suggested that interesting conformational changes might take place during the oxidation and reduction of the intersubunit disulfide linkage. Based on the analysis of the active site from the generated model it has been proposed that in the absence of the partner AhpF subunit in *M. tuberculosis*, the mycobacterial AhpC might use small molecule such as mycothiol, for completing its enzymatic cycle. The results included in Chapter 2 have been published. (Chauhan, R and Mande, S. C. 2001. Characterization of the *Mycobacterium tuberculosis* H37Rv Alkylhydroperoxidase (AhpC) points to the importance of ionic interactions in oligomerization and activity. *Biochemical Journal* 354, 209-215).

Studies described in Chapter 2 are further extended to understand the mechanism of action of *M. tuberculosis* AhpC and constitute Chapter 3 of the present dissertation. In these studies, It has been demonstrated that AhpC is capable of acting as a general anti-oxidant by protecting a range of substrates including supercoiled DNA. Active site Cys to Ala mutants shows that all the three cysteine residues are important for the activity. Cys-61 plays a central role in the activity and Cys-174 also appears to be crucial. Interestingly, C174A mutant is inactive, but the double mutant C174/176A shows significant revertant activity. Kinetic parameters indicate that C176A mutant is active, although much less efficiently. Based on the results described in Chapter 3, it has been suggested that *M. tuberculosis* AhpC belongs to a novel peroxiredoxin family and might follow a unique disulfide relay reaction mechanism which has been discussed in detail in this Chapter. This chapter is also a

reproduction of the published work (Chauhan, R. and Mande, S. C. 2002. Site directed mutagenesis reveals a novel catalytic mechanism of *Mycobacterium tuberculosis* Alkylhydroperoxidase C. *Biochemical Journal* 367, 255-261).

Chapter 4 of this study includes experimental details of *M. tuberculosis* AhpC cloning, overexpression and purification of the protein. The methodology followed for AhpC crystallization is also included in this chapter. The chapter describes the failure of AhpC crystallization, which directed the study to initiate site directed mutagenesis work to obtain suitable mutants for crystallization experiments. Furthermore, the generated mutants have been studied to test their solubility, enzyme activity and oligomerization behavior. Two mutants are found favorable for crystallization experiments namely, R116L and AhpCdel1. Both the mutants show the enzyme activity equivalent to the wildtype AhpC. However, the quaternary state characterization shows that the mutant, R116L is dimeric and AhpCdel1 is decameric under reducing environment. The rationale of mutagenesis to obtain suitable protein for crystallization experiments and the results of preliminary characterization and crystallization trials of these mutants are discussed in detail in Chapter 4.

Since suitable crystals are not available for X-ray diffraction studies of the AhpC enzyme, an alternative approach has been taken to gain insight into the 3D structure of *M. tuberculosis* AhpC. Homology model of AhpC was built using available 3D structures of eukaryotic peroxiredoxins as the templates. The generated model is further validated using PROCHECK and 3D-profile programs. The model of *M. tuberculosis* AhpC reveals several interesting features of the AhpC enzyme. The eleven amino acid long loop located at the N-terminal of the protein sequence has been found near the dimer-dimer interface. The generated model has further helped in designing the experiments to obtain suitable mutants for crystallizations. The generation of the above model and its structural details are explained in the last section of Chapter 4.

The physiological redox partners of *M. tuberculosis* AhpC are not yet known. The generated model of AhpC suggests that small molecules like mycothiol can act as redox partners of *M. tuberculosis* AhpC. To test this hypothesis, *in vitro* activity of *M. tuberculosis* AhpC has been tested using the various reducing agents as electron-

transferring components. It has been observed that mycothiol, β-mercaptoethanol and glutathione cannot assist AhpC activity. The preliminary results of these experiments are included in Appendix A of this thesis. However, the possibilities of all the three thioredoxins of *M. tuberculosis* acting as redox partners have not yet been attempted in the present study due to the unavailability of the purified thioredoxins and thioredoxin reductase proteins of *M. tuberculosis*. Current laboratory efforts are being directed to clone and purify all the three thioredoxins and single thioredoxin reductase using Ni-NTA affinity chromatography method. On the availability of these purified proteins, their possibility to act as redox partners of *M. tuberculosis* AhpC can be tested.

All these studies undertaken have significantly contributed in the understanding of *M. tuberculosis AhpC* as a peroxiredoxin enzyme. These studies have also demonstrated that *M. tuberculosis* AhpC has a distinct reaction mechanism in comparison to the known eukaryotic peroxiredoxins. Hence, this information can further be utilized in initiating structure based drug design work using *M. tuberculosis* AhpC as a drug target. These studies have also established that the oligomerization and redox catalytic cycle of *M. tuberculosis* AhpC is linked via conformational changes in the protein. Although the crystallization work on AhpC has not yet been successful, it is hoped that studies on the recently generated mutants, R116L and AhpCdel1 might provide some clues on the link between the redox reaction mechanism and the oligomerization of *M.tuberclosis* AhpC.