



Redox Proteins of *Mycobacterium tuberculosis*

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Abstract | Evolution of life took place in a reducing atmosphere. As a reminiscence of this, cytoplasm of all living organisms is reducing in nature. Furthermore most biochemical reactions like energy production etc. are redox in nature requiring either an electron supplier or acceptor. The maintenance of redox state thus becomes extremely crucial for any cell. Cells typically devote considerable resources for the maintenance of their redox state. *M. tuberculosis* successfully evades an array of host generated redox stresses. For this *M. tuberculosis* employs a large number of redox sensors and effectors. In our laboratory we have been studying some of these central players with a hope to be able to gain insights into the pathogen. We shall try to discuss the relevance of redox and our efforts to understand the same in this review.

1 Redox Balance in *Mycobacterium tuberculosis* and Its Importance

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (M.tb), is amongst the leading causes of infection related mortality and morbidity worldwide.¹ Infection typically occurs via the respiratory tract upon inhalation of droplet nuclei containing M.tb. Upon reaching the alveoli the bacteria are phagocytosed by alveolar macrophages and in the initial stages of intracellular existence, M.tb resides within the phagosome. Herein M.tb is exposed to a hostile host environment comprising reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNIs), lysosomal enzymes, and toxic peptides. Moreover, M.tb can persist inside immune-competent individuals for decades in a latent form and may reactivate to cause an active disease when the host is immune-compromised. M.tb elaborates multiple mechanisms to survive in the host environment and has a unique ability to persist for long durations in the host. The sustenance of viability in the presence of severe oxidative stress appears to be of primary importance for successful pathogenesis of M.tb.

The major process for detoxification of oxidative damage to proteins and DNA occurs via the activities of redox enzymes. In our laboratory we have been trying to understand the mechanisms used by M.tb to maintain its cytoplasmic redox status and elucidate the structure-function features of redox enzymes of M.tb (Figure 1). We believe that understanding these mechanisms

can lead to newer avenues for the development of novel therapeutics for both the active and the latent form of infection. Typically redox balance is both sensed and maintained by a set of proteins with a CXXC and TXXC motifs. In this review we discuss the functional and structural features of redox enzymes of M.tb but restrict the discussion to the work performed by our laboratory.

2 CXXC and TXXC Derived Motif Proteins Encoded by the M.tb Genome

Disulphide bonds can alternate with free thiols to play a major role in both the maintenance of proper structure and supply of reducing/oxidizing equivalents. Bacteria thus possess mechanisms that lead to the maintenance of thiol/disulphide homeostasis. Thus, thiol-disulphide-oxidoreductases are found ubiquitously across life with functions ranging from oxidation/reduction/isomerization of disulphide bonds present in proteins to cytoplasmic protein reduction. They have been shown to be critical for protein folding reactions in bacterial periplasm and for electron supply in essential biochemical reactions.² The CXXC motif is amongst the most prevalent motif in proteins and is essential for the activity of thioredoxins (Trx) and thioredoxin like proteins. Moreover, CXXC derived motifs such as TXXC and SXXC motifs containing proteins also have possible redox functions. One such example is the peroxiredoxin family which is a representative member of proteins with the TXXC motif.³ The two amino

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acids present in between the two cysteines in the CXXC motif have been shown to affect the redox potential of the protein and the pKa of the two cysteines, leading to a modulation of its activity.⁴ The *M.tb* genome potentially codes for more than 200 proteins possessing this motif, and these

proteins are distributed across various functional categories (Figure 2). Three-dimensional structures of some of these have been determined, thereby illuminating the structural diversity of their biochemical functions (Figures 3 and 4). In the structures of all these proteins characterized

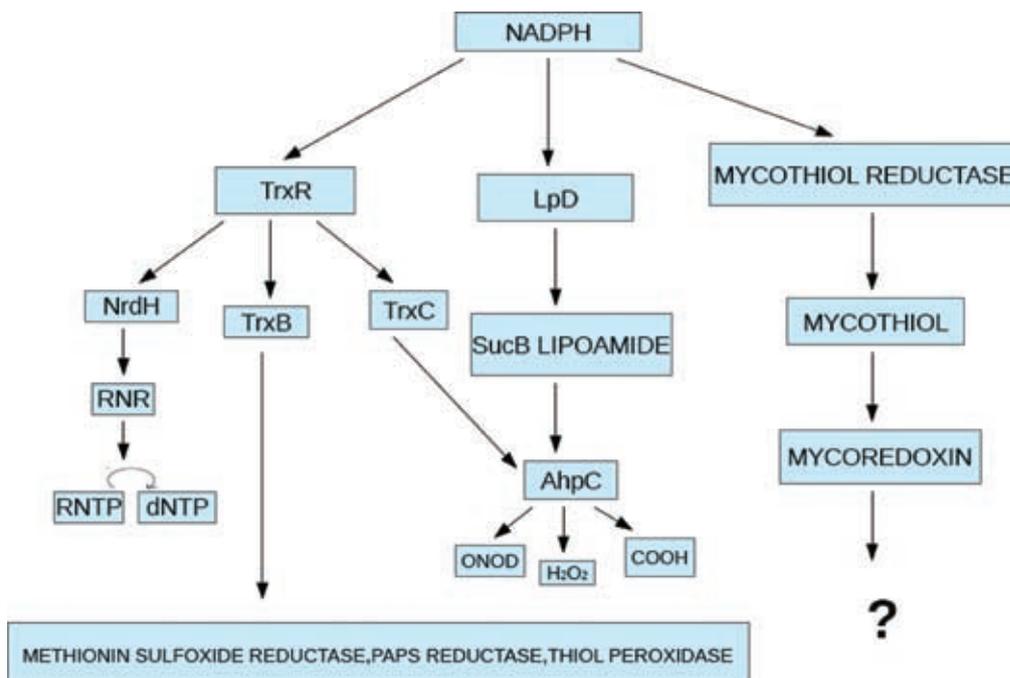


Figure 1: Flow chart representation of the redox pathways in *M.tb*. Arrows indicate the direction of electron flow.

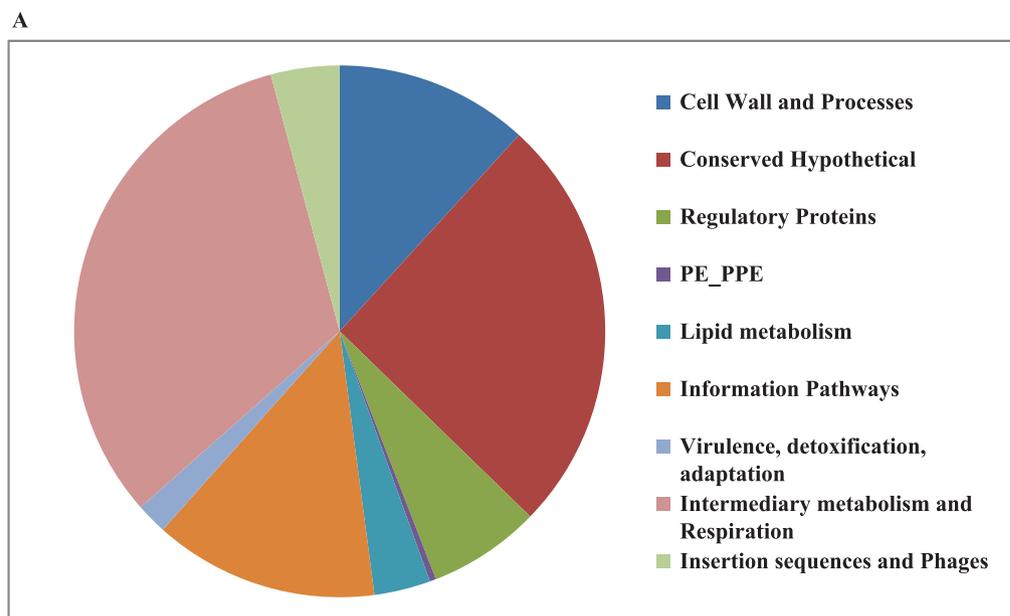


Figure 2: (Continued).

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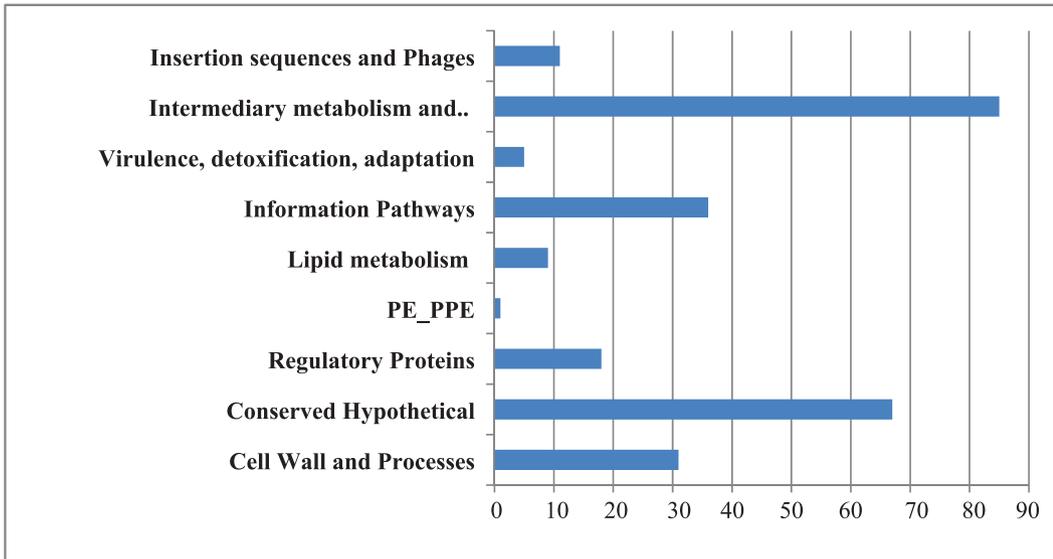


Figure 2: Distribution of CXXC proteins in M.tb. The entire proteome of M.tb was downloaded from the Tuberculist server. A perl script was run locally to find proteins with CXXC motif. These proteins were then also categorized according to functional categories as described in Tuberculist. Shown is the distribution of CXXC proteins across functional categories (A) Pie chart (B) Bar graph with X axis representing the number of such proteins.

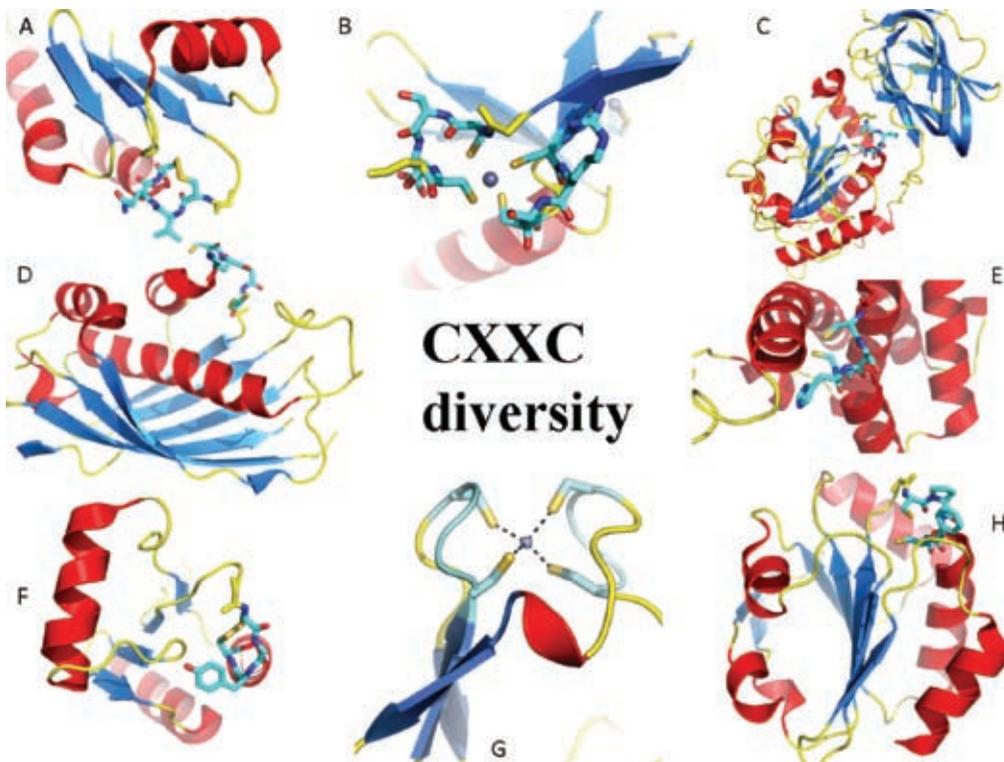


Figure 3: The diversity of CXXC motif is depicted by representative images of M.tb CXXC containing proteins. The cartoon is coloured by secondary structure with red showing helix, yellow showing loops and blue showing α -helix. The CXXC motif is highlighted, coloured by atom and represented as sticks. The individual structures shown are (A) NrdH, pdb id: 4HS1, (B) FurB, pdb id: 2O03, (C) DipZ, pdb id: 2HYX, (D) FcoT, pdb id: 2PFC, (E) AhpD, pdb id: 1KNC, (F) Mycoredoxin, pdb id: 2LQQ, (G) Rubedoxin C with Zinc atom shown as sphere, pdb id: 2KN9, (H) TrxC, pdb id: 1LU4.

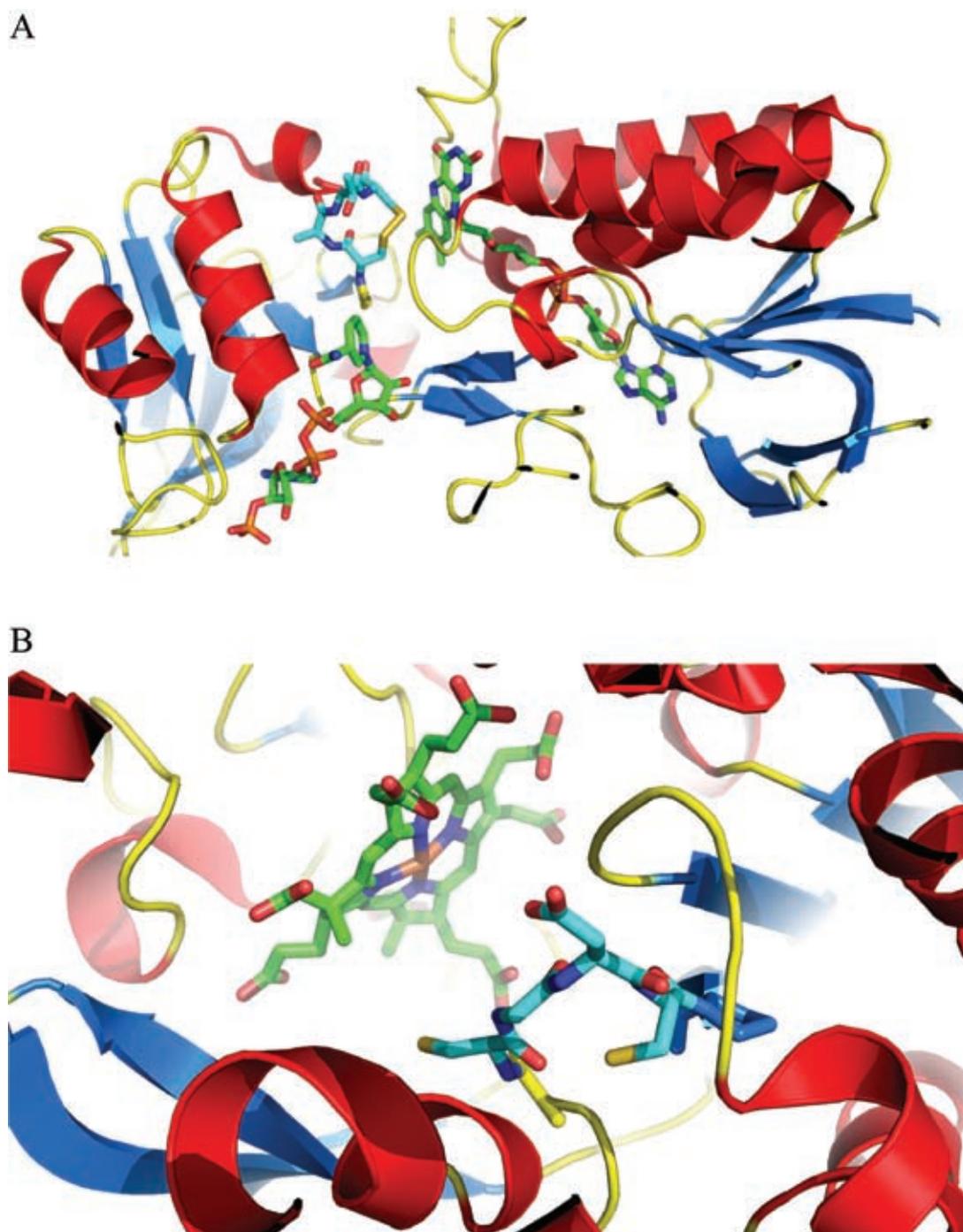


Figure 4: CXXC motif in proximity to cofactors. (A) NADPH and FAD in TrxR, pdb id: 2A87 (B) heme ring in NirA, pdb id: IZJ9.

so far, first Cys and the second Cys in the motif are always N-cap and N3 position of an α -helix (Figure 5). This structural arrangement brings other residues in close proximity, and plays an important role in the maintenance of the pKa of the active site Cys-residues.

3 The Peroxiredoxin Family of Proteins

Alkylhydroperoxidase (AhpC) and Thiol peroxidase (Tpx) are important *M.tb* redox proteins belonging to the peroxiredoxin family of proteins. *M.tb* Tpx can be reduced by two of the three potential thioredoxins, TrxB and TrxC, encoded

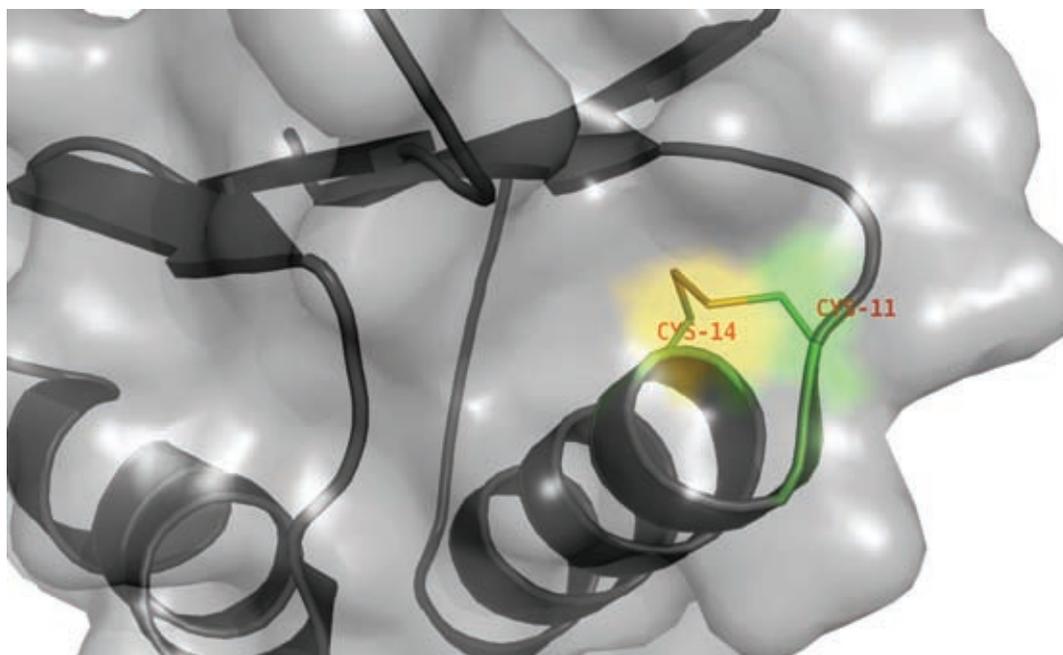


Figure 5: The CXXC motif is typically located at the beginning of an α -helix, with the first cysteine surface exposed. Since the pKa of the first Cysteine is less than 7 in the reduced form under physiological conditions, this cysteine exists as thiolate ion. Representative image of M.tb NrdH in cartoon representation with surface, shown in sticks is the disulphide bond of the CXXC motif.

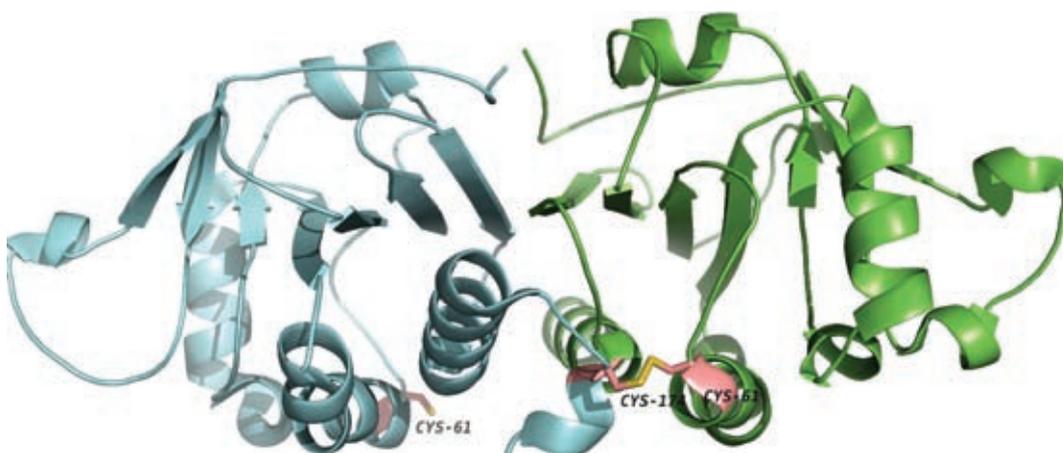


Figure 6: Cartoon representation of M.tb AhpC with cysteine residues shown as sticks and coloured by atom. The two molecules shown here form an intra-molecular dimer as indicated, pdb id: 2BMX.

by M.tb, while AhpC can accept electrons only from TrxC.⁵

In M.tb, the thioredoxins (Trx) and thioredoxin reductase (TrxR) are thought to be involved in mediating peroxide, hydroperoxide and dinitrobenzene detoxification,⁵ though M.tb does not appear to possess the ortholog of the classical oxidative stress response regulator, OxyR present in several gram negative bacteria.⁶ Majority of the

oxidative radical scavenging is performed by the catalase peroxidase encoded by the *katG* gene. Our interest in AhpC started with the prior observation that it is overexpressed in isoniazid resistant strains of M.tb.⁷ In these strains the *katG* gene (encoding a catalase peroxidase) is inactive, rendering M.tb resistant to the activity of the prodrug isoniazid. We speculate that the overexpression of *ahpC* in the aforementioned isoniazid resistant strains may

constitute an adaptive response to compensate for the lack of KatG though convincing evidence for this notion is lacking.

The alkyl hydroperoxidase (AhpC) of *M.tb* is unique in many ways compared with its well characterized ortholog from *E. coli*. AhpC has also been shown to be vital for mycobacterial survival within macrophages,²⁰ and thus seemed an attractive drug target. We characterized AhpC biochemically for its functional and oligomerization properties⁸ to find that AhpC is a decameric protein,⁹ a pentamer of dimers held together by ionic interactions. Dimerization of individual subunits takes place through an inter-subunit disulphide linkage. The ionic interactions play a significant role in its activity. Furthermore, using UV absorption spectroscopy, we proposed that significant conformational changes take place during oxidation and reduction of the inter-subunit disulphide linkage.⁸ Such biochemical evidence were later corroborated in the crystal structure of AhpC (Figure 6).¹⁰

4 Thioredoxin System

4.1 The thioredoxin pathway

Thioredoxins represent a ubiquitously present class of proteins characterised by the Thioredoxin fold.¹¹ Most subclasses of thioredoxins function as general disulfide reductases, i.e. they have a broad spectrum of reducing targets. In some other cases they have a very specific target. Prominent examples of electron supply by thioredoxin class of proteins are during deoxyribonucleotide synthesis, sulphur assimilation, detoxification of ROI and RNI, repair of proteins, and redox regulation.¹² While maintaining its substrates in the reduced form, thioredoxin gets oxidized, which in turn is reduced by the FAD-containing enzyme TrxR, which extracts electrons from NADPH.¹³

The mechanism for the CXXC proteins in thioredoxins was first described for *E. coli* TrxA.¹⁴ Within the CXXC motif the two cysteines possess different pKas. In the case of *E. coli* TrxA pKa of the first cysteine is 6.7 (meaning that at physiological pH the -SH exists as S⁻) and that of the second cysteine is >9.¹⁴ The maintenance of pKa of the two Cys-residues is crucial for the electron transport between thioredoxin and its substrates, and between Trx and TrxR (Figure 5).

4.2 Structural and biochemical studies of thioredoxins and thioredoxin reductase reveal novel features

The genome of *M.tb* has the potential to code for three thioredoxins (TrxA, TrxB, and TrxC) and one

thioredoxin reductase. However, *M.tb* and several other bacteria do not code for any proteins of the glutathione reductase system⁶ making thioredoxins of great interest, since inhibition of their activity would potentially be lethal to the bacteria.

The three thioredoxins were analysed for their functional activity to reduce a model substrate, insulin, in the presence of DTT and were found to act as efficient reductants of insulin.¹⁵ The redox potential for TrxA, TrxB and TrxC was found to be -248, -262 and -269 mV respectively. When DTT was replaced by TrxR and NADPH, only TrxB and TrxC were found to be electron acceptors, clearly indicating that *M.tb* TrxA cannot accept electrons from *M.tb* TrxR. Furthermore, it was observed that in the H37Rv strain of *M.tb*, robust expression of both *trxB* and *trxC* could be detected both in the presence and absence of oxidative stress whereas that for *trxA* was not detected, suggesting that expression of *trxA* may be rendered cryptic.¹⁵ Functional genetic studies in *E. coli* also demonstrate that TrxB and TrxC can and TrxA cannot compensate for the lack of thioredoxins in *E. coli*.

4.3 Structural studies on *M.tb* TrxR

The crystal structure of *M.tb* TrxR (Figure 4a) consists of a FAD-binding domain (residues 10–125 and 251–318) and a NADPH-binding domain (residues 126–250).¹⁶ The structure resembles *E. coli* TrxR closely with both the domains having a similar fold (RMS deviation of approximately 1.7 Å). The structure also showed evidence for bound NADPH with Arg186, Arg187 and Arg191 interacting with 2'-phosphate of NADPH. Furthermore, the NADPH molecule had a high temperature factor indicating disorder in its binding. The FAD-binding domain formed most of the inter-subunit interactions, which may be the reason for high flexibility of the NADPH domain allowing for large conformational changes during the redox cycle. Comparison of this structure with *E. coli* TrxR shows that the relative positions of the domains in *M.tb* TrxR are rotated by approximately 11° compared to *E. coli* TrxR structure.

TrxR can have two possible conformations, i.e. oxidised (F_O) or reduced (F_R) and the TrxR molecule is speculated to alternate between the two conformations. The crystal structure solved by us was of the F_O conformation. Large-scale conformational changes required for F_R/F_O transition were probed using normal mode analyses (NMA). NMA revealed that in this case three dominating modes could explain most of the conformational changes needed to switch in between the two forms. The largest contributor was the 8th mode contributing about 35% of the required motions.

Examination of motions of this mode showed that rotation of the NADPH domain leads to surface exposure of the active site.

Furthermore, it was seen that the F_O to F_R transition involved anti-correlated motions between the NADPH and FAD domain, which leads to the formation of a salt bridge between the side chains of Arg130 and of Glu48 concomitant with hydrogen-bond breakage between main-chain carbonyl of Ile243 with guanidine group of Arg117. Moreover, the hydrogen bond between Ser138 and Asn51 was broken while that between Ser138 and Asn294 was seen. Concurrently the hinge region, which is a small β -sheet, remains stable to small perturbations enabling motion between the two domains. Overall it appeared that complex inter-domain conformational changes are needed for a change in conformation from the F_R to the F_O form and vice versa, the changes being important for successful electron transfer between Trx and TrxR (Figure 7).

4.4 NrdH redoxins

Ribonucleotide reductases (RNR) are essential enzymes that catalyse the reduction of ribonucleotides to deoxyribonucleotides, a reaction essential for the synthesis of deoxyribonucleotides (dNTPs) and thus essential for all life forms.¹⁷ There are three major classes of RNRs, namely classes I, II, and III, a classification based on their cofactor requirement and free radical mechanism. All RNRs function by using free radicals.

Class I RNRs are subdivided into class Ia and class Ib. The latter are found only in eubacteria and some bacteriophages, while the canonical class I

RNRs (class Ia) are found in almost all eukaryotes, in most eubacterial phyla, few archaea and dsDNA viruses.¹⁷ Class Ib RNRs possess a distinct operonic structure also encoding for a flavodoxin protein encoded by the *nrdI* gene, and in most cases an *nrdH* gene coding for a redoxin specific for reduction of the class Ib RNR.

M.tb codes for a class Ib ribonucleotidereductase (RNR), encoded by the *nrdE* and *nrdF2* genes,¹⁸ along with another putative class II RNR, encoded by *nrdZ*. The *nrdF2* gene is essential for in vitro growth. NrdZ, cannot substitute for the class Ib enzyme, NrdEF2, and furthermore, an *nrdZ* knock out can successfully survive under in-vitro growth conditions.¹⁹ *nrdZ* is induced under microaerophilic conditions, while the expression of other genes is reduced upon oxygen deprivation. Nevertheless a $\Delta nrdZ$ mutant can survive hypoxia in vitro. Also, the $\Delta nrdZ$ mutant survives equally well as the wild type in B6D2/ F_1 mouse infection model.¹⁹

Class I RNRs consist of a large α -subunit bearing the active site and the allosteric binding site and a small β -subunit which harbours a stable tyrosyl radical. Class I RNRs form an $\alpha_2\beta_2$ type holo-enzyme complex, which is the active form. Furthermore for the *E. coli* class Ia enzyme, the $\alpha_4\beta_4$ arrangement is also possible as an allosterically inhibited form and may constitute a mechanism of activity regulation.²⁰

Functionally, NrdH functions like both thioredoxins and glutaredoxins, in the sense that both glutaredoxins and thioredoxins can supply electrons to class Ia RNR while NrdH seems to substitute for this function for class Ib. NrdH redoxins

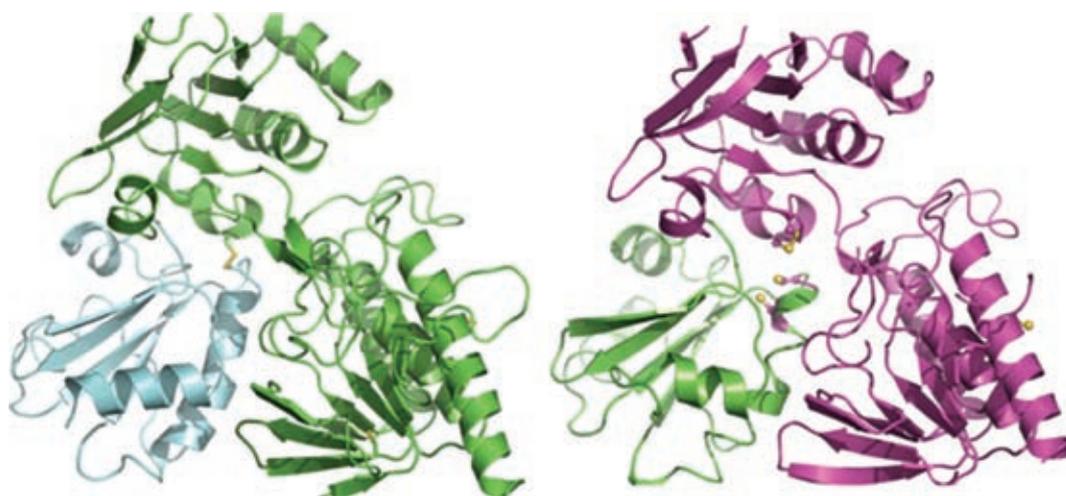


Figure 7: Proposed mode of Trx-TrxR interaction. Left Panel shows cartoon representation of the *E. coli* Trx-TrxR structure with the intra-molecular disulphide shown in sticks, pdb id 1F6M; chain A and chain C. Right panel shows the proposed mode of *M.tb* TrxR-TrxC interaction by superposing TrxC (pdb id: 1LU4) and TrxR (pdb id: 2A87) over their respective counterparts in 1F6M.

share high sequence similarity to glutaredoxins (Figure 8) but are paradoxically thioredoxin-like in their function. They behave like thioredoxins in terms of capacity to accept electrons from thioredoxin reductase. NrdH redoxins possess a thioredoxin fold and contain an active site motif CXXC, features that are common to thioredoxins and glutaredoxins.

Based on phylogeny of NrdH, Grx and Trx we suggest that NrdH or NrdH-like proteins might be evolutionary precursors of both Thioredoxins and Glutaredoxins. NrdH redoxins lack a glutathione-binding site common to glutaredoxin,²¹ and are characterised by the presence of two sequence motifs (1) the active site CVQC, (2) WSGFRP motif.

Prior structure of *C. ammoniagenes* NrdH showed a domain-swapped dimer which was formed by an inter-chain two-stranded anti-parallel β -sheets with dimer interface stabilised by electrostatic interactions. This was the first example of domain swapping in the thioredoxin family.²² It was also postulated that domain-swapping in dimers may be an in vivo phenomenon and constitute a mechanism for activity regulation.²²

4.5 Ultra high resolution structure of NrdH

We were successful in solving the structure of *M.tb* NrdH at 0.87 Å resolution.²³ The structure was

solved using molecular replacement, and showed clear electron density starting from the second residue onwards. Also visible were the six histidine residues at the C-terminus arising from the affinity tag. It also appeared that this C-terminus tag interacted with the neighbouring molecule promoting crystal formation. Further, based on the structures of NrdH and TrxR, we were able to model the interaction between the two proteins (Figure 9). The active site cysteines were disulphide bonded and the structure represented the oxidised state of the molecule (Figure 10A). We could successfully rule out any possibility of domain swapping with extremely clear electron density (Figure 10B) of the hinge region which helped us in postulating that the *C. ammoniagenes* domain swapped NrdH structure could well have been a crystallization artefact. Using size exclusion chromatography and Dynamic Light Scattering we showed that in solution *M.tb* NrdH existed either as a dimer (in low salt 10 mM Tris) or as a monomer (in 10 mMTris, 1M NaCl).

The ultra-high resolution structure allowed us to model several side chains in alternate conformations along with occupancy refinement. Interestingly Thr7 was present in two alternate conformations with occupancies of 0.4 and 0.6 respectively. In the former form, the hydroxyl group of this threonine faces the redox active disulphide leaving little space for the thiol group

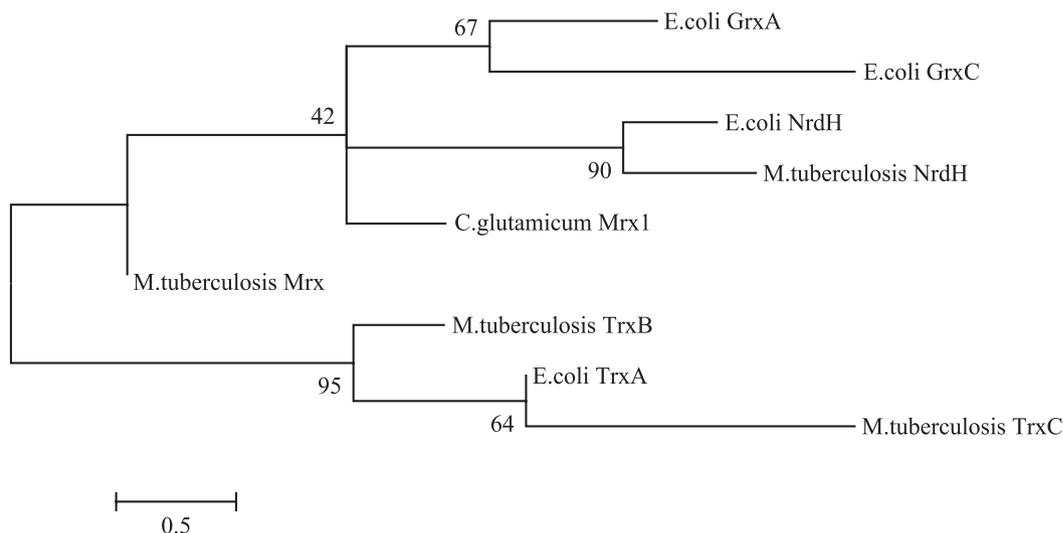


Figure 8: Phylogenetic Tree of NrdH, thioredoxins and glutaredoxin-like proteins using the maximum likelihood method. This was based on the JTT matrix-based model and was conducted using MEGA5. Shown is the bootstrap consensus tree inferred from 500 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. Initial tree was obtained by maximum parsimony method. The tree is drawn to scale; branch lengths represent number of substitutions per site. Gaps and missing data were not used.

of Cys14 to exist in the reduced form. In the latter form a small cavity is seen to exist next to Cys14, which might be required to accommodate the sulfhydryl group of the reduced Cys when it moves away from its oxidized position. Based on

this observation we proposed that in the oxidized state of NrdH, as captured by this crystal structure, Thr7 can exist in either of the two forms although in the reduced state only one of the forms would be allowed.



Figure 9: Proposed mode of NrdH-TrxR interaction. Left Panel shows cartoon representation of the M.tbNrdH (yellow) (pdb id: 4HS1) superposed over the thioredoxin counterpart in *E. coli* Trx-TrxR structure and M.tb TrxR (magenta) modelled as for figure 6. Right panel shows the C α trace of NrdH and Trx in the proposed interaction modes of M.tb TrxR-TrxC/NrdH.

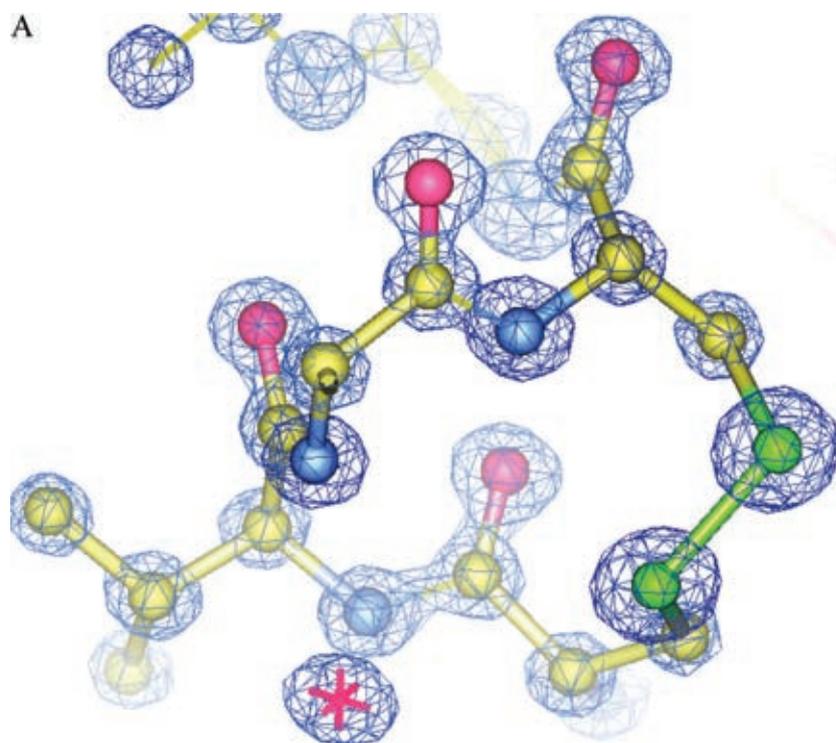


Figure 10: (Continued).

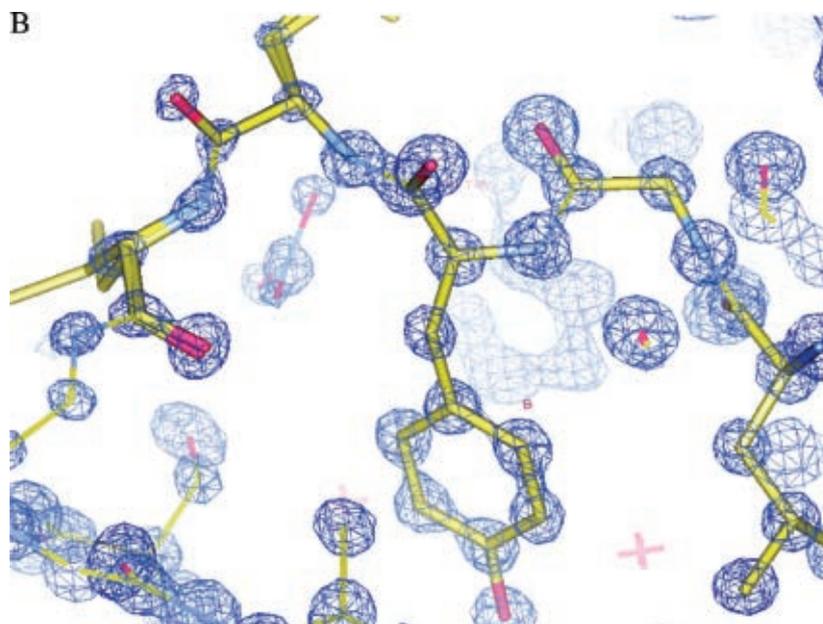


Figure 10: Electron density map of (A) the active site region of *M.tb* NrdH, (B) The region corresponding to domain swap in *C. ammoniagenes* structure. Electron density map was calculated using Shelxl and displayed using coot in the default colouring scheme, contoured at 3σ with ball and stick representation from 11th cysteine to 14th.

5 Concluding Remarks

In the host, *M.tb* suffers from stresses like redox imbalance and nutrition deprivation and to cope with these it possesses a considerable degree of metabolic versatility that allows it to survive in the hostile environment inside macrophages. This is done by utilizing a myriad of proteins spanning all forms of metabolic activity. Regarding the ability to cope with redox stress *M.tb* is equipped with several protective enzymes and specialized biochemical pathways that maintain its cytoplasmic redox homeostasis. The elucidation of the mechanisms by which *M.tb* evades these stresses will open new avenues of therapeutic intervention.

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Shekhar C. Mande obtained his PhD in Molecular Biophysics from the Indian Institute of Science in 1991. He has been working in the field of M.tb structural biology since 1996. His current research interests include obtaining insights into biochemical functions of proteins using 3D structural knowledge and large scale network analysis of protein: protein interaction networks.

