Structural and mechanistic insights into Mycothiol disulfide reductase, Mycoredoxin-1 and peroxiredoxin alkyl hydroperoxidase subunit E of Mycobacterium tuberculosis

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NANYANG
TECHNOLOGICAL
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**Structural and mechanistic insights into
Mycothiol disulfide reductase, Mycoredoxin-1 and peroxiredoxin
alkyl hydroperoxidase subunit E of *Mycobacterium tuberculosis***

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Dedication

I dedicate this work to my parents, my brother and my guru Sri Sathya Sai Baba.

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Abbreviations

1D	One-dimensional
2D	Two-dimensional
3D	Three-dimensional
Å	Angström
AhpF	Alkyl hydroperoxide reductase subunit F
AhpC	Alkyl hydroperoxide reductase subunit C
AhpE	Alkyl hydroperoxide reductase subunit E
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
CD	Circular dichroism
CSI	Chemical shift index
DLS	Dynamic light scattering
DSS	2, 2-Dimethyl-2-silapentane-5-sulphonate
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
FADH ₂	Flavin adenine dinucleotide
FPLC	Fast protein liquid chromatography
HSQC	Heteronuclear single quantum correlation
Hz	Hertz
IPTG	Isopropyl-β-D-thiogalactoside
kDa	Kilo-Dalton
LLG	Log likelihood gain
<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
Mtr	Mycothiol disulfide reductase
Mrx-1	Mycoredoxin-1
Mrx-2	Mycoredoxin-2
MSH	Mycothiol (reduced)
MSSM	Mycothiol disulfide (oxidised)
NAC	N-acetyl-L-cysteine

NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
nm	Nanometre
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser effect spectroscopy
NTA	Nitrilotriacetic acid
NTD	N-terminal domain
PAGE	Polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
PCR	Polymerase chain reaction
PDB	Protein Data Bank
ROS	Reactive oxygen species
RNI	Reactive nitrogen intermediate
r.m.s.d.	Root mean square deviation
SAXS	Small angle X-ray scattering
SDS	Sodium dodecyl sulphate
TFE	2,2,2-Trifluoroethanol
TOCSY	Total correlation spectroscopy

Abstract

Mycobacterium tuberculosis (*Mtb*) has the ability to persist within the human host for a long time in a dormant stage and re-emerges when the immune system is compromised. During infection, *Mtb* is exposed to a number of redox stresses inside macrophages, such as reactive oxygen species and reactive nitrogen intermediates, both having the potential to damage a number of cellular components, including lipids, proteins, and DNA [1]. The ability of *Mtb* to persist inside macrophages suggests the presence of elaborated machineries capable of preserving redox homeostasis and countering oxidoreductive stress. *Mtb* is equipped with sophisticated proteins and thiols (mycothiol) responsible for antioxidant defense. Most of these proteins as well as mycothiol are unique to mycobacteria and therefore lacking in the human host, like the mycothiol-dependent system composed of the enzymes mycothione reductase (Mtr), myco-redoxin1 (Mrx-1), and the alkyl hydroperoxidase subunit E (*MtAhpE*), responsible for the reduction of hydrogen peroxide, peroxyntous acid, organic peroxides or S-mycothiolated mixed disulfides, generated by oxidative stress. Clearly, the protein ensemble and mycothiol of the antioxidant defense of *Mtb* differ substantially from that in the mammalian hosts and thus the chances for a selective inhibition of the mycobacterial antioxidant defense system will be high. In the effort to understand the biological mechanisms of the mycobacterial antioxidant defense orchestra, this work aims to provide structural, enzymatic and mechanistic insights into the *Mtb* NADPH dependent Mtr (*MtMtr*), structural details of *Mtb* Mrx-1 (*MtMrx-1*) and *AhpE* (*MtAhpE*) as well as mechanistic insights into the mycothiol/Mrx-1 dependent recycling of peroxiredoxin *MtAhpE*.

In the first part of this thesis, the production of a stable *MtMtr* using a GroEL/ES chaperone-chaperonin system is described. The recombinant *MtMtr* provided a platform for the first low resolution solution structure by small-angle X-ray scattering (SAXS), representing a dimeric enzyme. Substrate induced conformational changes of *MtMtr* in the presence of NADPH and mycothiol were studied in solution by SAXS, demonstrating significant overall changes after NADPH-binding, interpreted by a shift of a dimeric to a tetrameric form of the NADPH-bound *MtMtr*. Genetically engineered mutants of *MtMtr* shed light into the importance of the flexibility of the linker, connecting the catalytic FAD-domain I with the NADPH-domain, as well as the linker, connecting the NADPH- with the FAD-domain II. Furthermore, attempts were made to crystallize

MtMtr, resulting in needle showers. Together, with the linker mutants generated, these may provide a platform for crystallization approaches in the near future.

Together with the generation of recombinant *MtMrx-1*, which was revealed to be monomeric in solution regardless of the redox state and concentration, the ensemble formation of *MtMtr* and *MtMrx-1* was studied by NMR titration. The *MtMtr-MtMrx-1* interaction was characterized by a fast exchange regime and critical residues involved in the protein-protein interaction were identified. In order to shed light into the complete ensemble of the the mycothiol-dependent system, recombinant *MtAhpE* was produced and purified. Its ensemble formation with *MtMrx-1* was analysed by NMR spectroscopy and docking studies, providing insights into the interaction interface and mechanism of action. The interaction model presented revealed, that two molecules of monomeric *MtMrx-1* interact with one dimeric *MtAhpE* with each molecule of *MtMrx-1* reducing one monomer of *MtAhpE*.

The last chapter describes in detail the structural and mechanistic characterisation of *MtAhpE*. The oligomeric state of *MtAhpE* was shown to be a dimer regardless of the redox condition. Interestingly, the formation of a higher molecular weight oligomer was indicated in dynamic light scattering and SAXS studies in the presence of equimolar H₂O₂. Using cysteine-labelling NMR experiments, it was possible to investigate the redox dynamics of the catalytic and peroxidatic cysteine in solution in the presence of various reducing agents. These data enabled to propose a new monothiolic pathway for the reduction of *MtAhpE* by mycothiol in the absence of *MtMrx-1*. Finally, the crystallographic structures of *MtAhpE* in the presence of mycothiol (2.43 Å resolution) and the mycothiol-analogue N-acetylcysteine (2. 2.7 Å resolution) were determined, respectively, to validate the newly proposed pathway in depth.

References:

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Introduction

Introduction

1. Introduction

Tuberculosis (TB) is one of the leading causes of death worldwide claiming approximately 1.7 million lives annually (approximately 1 death every 15 second) (Fig. 1.1). In 2015, as per the World Health Organization (WHO) estimate a total of 10.4 million new TB cases were reported and approximately 1.4 million of those affected died, 1.2 million of whom were HIV-positive”. This is in addition to approximately 2 billion latently affected people (one out of every third person) (1). The synergistic effect of TB- and HIV-infection has further aggravated the epidemics. Lack of knowledge and mismanagement of MDR-TB (multi-drug resistance TB) has resulted in the evolution of extensively drug-resistant strains (XDR-TB; MDR + resistance to a fluoroquinolone and an injectable drug) and super XDR strains (SXDR-TB; resistant to virtually all known anti-TB drugs) (2,3). Therefore, need of the hour is to understand the biology of tuberculosis pathogenesis and development of effective vaccines and rapid detection tools.

Figure 1.1: Global TB incidences 2015. The annual number of incident TB cases relative to population size. The figure was taken from Global Tuberculosis report published by WHO (1).

Mtb, the obligate pathogenic bacteria which causes TB is one of the oldest and most successful human pathogen. *Mtb* is constantly exposed to various oxidative stresses during its cycle of transmission and infection. However the unique ability of *Mtb* to not only endure the redox stress manifested by host but also to synchronize its metabolic pathway accordingly, is central to its survival (4). *Mtb* continuously employs redox and

hypoxia sensor to monitor changes in intracellular redox state and surrounding microenvironments. Using DosS and DosT (senses the changes in O₂, NO and CO levels) as well as WhiB3 and anti-sigma factor RsrA (monitors intracellular redox state), *Mtb* regulates the symphony of its unique metabolic pathway to survive nutrient scarce, oxidative, nitrosative and hypoxic environment inside granulomas and lesions (5).

Mtb employs advanced machinery composed of low molecular weight thiol (such as mycothiol and ergothioneine) and thioredoxin system (composed of thioredoxin, TrxC and TrxB as well as thioredoxin reductase, TrxR) in addition to superoxide dismutase (Sod A and SodC), truncated hemoglobin (trHbN), catalase (KatG), bacterioferritin comigratory protein (Bcp and BcpB) alkyl hydroperoxidase subunit C (AhpC), alkyl hydroperoxidase subunit D (AhpD), and alkyl hydroperoxidase subunit E (AhpE) to neutralize the reactive oxygen species (ROS) and reactive nitrogen intermediate (RNI) generated by the host immune system (Fig. 1.2) (6). This ensures a reductive intracellular environment which is very essential for optimal functioning of its proteins.

Figure 1.2: Anti-oxidant defence system in Mycobacteria. Various components of Mycobacterial defense against ROS and RNI. Mtr is NADPH dependent mycothiol disulfide reductase which recycles MSSM back to MSH. Lpd is lipoamide dehydrogenase and it transfer electrons from NADH to Dihydrolipoamide acetyltransferase (SucB) which in turn transfer electron via AhpD to recycles AhpC. These proteins work in tandem to fight against ROS and RNI inside Mycobacterial cell. Modified according to (4).

Redox balance is also essential for energy metabolism, including glycolysis, the tricarboxylic acid cycle, and oxidative phosphorylation. This ability of mycobacteria to maintain redox homeostasis and metabolic balance is critical for its survival and virulence.

1.2 Oxidative stress in *Mtb*

Oxidative stress in tuberculosis can be endogenous (due to univalent transfer of electrons from electron transport chain to O₂) as well as exogenous (comes into effect due to specialized pathway of host to eliminate the pathogen). During the process of phagocytosis, macrophages produce massive quantities of reactive oxygen species (ROS) and reactive nitrogen intermediate (RNI) upon engulfing *Mtb*. Production of a moderately reactive superoxide radical (O₂^{•−}) is a critical step in the generation of ROI as well as RNI (7). This step is catalyzed by the NADPH-Oxidase (NOX), wherein one electron reduction of O₂ takes place using NADPH as electron donor.

O₂^{•−} can self-react in disproportionation reaction leading to production of hydrogen peroxide. This reaction is catalyzed by the SOD

Hydrogen peroxide (H₂O₂), generated in the above reaction, can react (under physiological condition) with metal ions such as Fe²⁺ and Cu²⁺ to generate the strongest aqueous oxidant (HO[•]) as shown below:

This HO[•] reacts with the cellular machinery instantly and leads to bacterial death. In fact, it has been shown that all the three classes of antibiotics (inhibitor of DNA replication and repair, inhibitor of protein synthesis, and inhibitor of cell-wall turnover stimulate the production of highly deleterious hydroxyl radicals in Gram-negative and Gram-positive bacteria, which ultimately contribute to cell death) (8). It is therefore essential that new anti-TB drugs are designed to generate excess of HO[•] upon interaction with the unique metabolic pathways of *Mtb* (8). Very recently it has been shown that AC2P36-J kills *Mtb* by depleting free thiol pools and promoting the accumulation of reactive oxygen species at acidic pH while showing no macrophage cytotoxicity (9).

H₂O₂ and O₂^{•-} are the most common ROS which mycobacteria faces upon engulfment by macrophages. Most species of mycobacteria such as *Mtb* (10), *M. bovis* BCG (11), *M. microti* (12), *M. leprae* (13) are sensitive to killing by H₂O₂. Similarly, the NO generation pathway is initiated through inducible nitric oxide synthase as shown below:

O₂^{•-} is produced along with NO and rapidly reacts with the latter to form highly reactive peroxynitrite (OONO⁻), which in turn leads to generation of [•]NO₂, NO₂⁻, N₂O₃, S-nitrosothiols and a dinitrosyl-iron complex (14). RNI can effectively kill *Mtb in vitro* at higher concentrations and is bacteriostatic at lower concentration (15). However, it is puzzling, that the presence of NO is imperative for supporting a latent TB infection in the murine model (16). NO inhibits respiration and growth of *Mtb* (17) highlighting its role in TB latency. However, in terms of viability, different species of *Mycobacterium* respond differently when exposed to NO.

1.3 Mechanism of Redox Sensing in *Mycobacteria*

The *Mycobacterium* genome lacks classical redox sensors such as fumarate and nitrate reduction regulatory protein (fnr), hydrogen peroxide-inducible genes activator (oxyR) and redox-sensitive transcriptional activator (soxR) (4,5,18). However, during the course of evolution, Mycobacteria have evolved a complex network of molecular sensors capable of sensing a broad range of redox signals such as oxygen tension, NO, CO, H₂O₂ and NADH/NAD⁺. These molecular sensors include DosRST, a two-component system, as well as WhiB and anti-sigma factor which are described in greater details in following section.

(a) DosRST proteins: DosRST proteins are heme based redox sensors of *Mtb* (Fig.1.3). These were identified along with a regulon of about 50 genes, which are upregulated when *Mtb* cells are exposed to defined hypoxia (19). This paired two component system (2CS) was named as DosRS (20). Exposure to NO also induces a Dos regulon via the DosRS 2CS (17). DosT is a homologue of DosS which specifically phosphorylates DosR. Mutants lacking both DosS and DosT cannot activate the expression of DosR regulated genes (21,22). DosT plays an important role during the earlier phase of hypoxia whereas DosS does the same during the later phase of anaerobiosis (23). Structurally both sensor kinase DosS and DosT possesses two GAF domains (structural motifs present in cyclic GMP (24).

Both DosS and DosT bind heme and have certain biochemical features which allows these proteins to sense the presence of diatomic gases such as O₂, NO and CO and (25,26). Exposure to oxygen can oxidize heme iron of DosS but not DosT, suggesting that DosS acts as a redox sensor, while DosT functions as a hypoxia sensor (26). Recent studies have shown that electron flux via the electron transport chain (ETC) can induce Dos regulon via DosS but not via DosT (27). The DosR regulon helps mycobacteria to maintain high energy levels and balance RH during anaerobiosis as well as resumption of normal growth during normoxia (28). It has been observed that inhibition of DosR binding to target DNA results in reduced survival of bacilli during hypoxia (29).

(b) WhiB3 proteins: *Mtb* harbors seven Wbl proteins, numbered WhiB1 to WhiB7. WhiB3 proteins are iron-sulphur cluster based redox sensor and are used to monitor changes in nutrient levels and intracellular redox state (30). Additionally, they are used in coordinating the cellular metabolism, expression of virulence lipids and cell division. WhiB proteins are small proteins containing four invariant cysteine and a helix turn helix motif and these proteins are conserved throughout actinobacteria. *Mtb* whiB3 mutant strains have been shown to be attenuated for pathophysiology and thus *Mtb*WhiB3 is considered to be the regulator of immunomodulatory virulence factors responsible for pathophysiology (31).

Figure 1.3: Mechanism of redox sensing in *Mtb*. *Mtb* employs DosRST as well as WhiB3 proteins to continuously monitor the change in its microenvironment. Modified according to (32).

WhiB3 possess insulin disulfide reductase activity and could function as glutaredoxin, mycoredoxin or Trx to regulate redox homeostasis (RH) of *Mycobacterium*

(33,34). Thus, *Mycobacteria* use the above system to continuously monitor its microenvironment and to regulate expression of virulence factors and the defense pathway. *Mtb* uses its metabolic flexibility to regulate RH. *Mtb* resides in phagosomes of alveolar macrophages. The phagosomes are believed to be deprived of carbohydrates and amino acids (35,36) and mycobacteria preferentially shift to fatty acids metabolism. This is in agreement with the fact that bacilli recovered from lungs of infected mice uses fatty acids instead of carbohydrates as preferred carbon source (37). It has also been shown that several genes encoding enzymes of the glycolytic pathway are not required for survival of *Mtb in vivo* (38). The *Mtb* genome contains about 100 genes which are part of the β -oxidation pathway (39) and many of these are overexpressed during macrophage infection (40). ATP biosynthesis is a must for mycobacterial survival during hypoxia (41). Thus, the latent state allows *Mtb* to survive with minimal level of ATP during hypoxia.

During normal growth, the ETC of *Mtb* uses the proton-pumping type-I NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II) for influx of electrons. Menaquinones are essential for the transfer of electrons from complex-I and complex-II to cytochrome-c (complex-III) while cytochrome-aa₃ complex is essential for subsequent transfer of electrons to O₂ (42). During hypoxia, *Mtb* uses non-proton-pumping type-II NADH dehydrogenase for electron influx (41) and *cytochrome-bd* instead of *cytochrome-aa₃* for reduction of O₂. Complex-V of ETC, the ATP synthase is expressed both during normal growth and hypoxia and has been the target of anti-TB drug development (43). *Mtb* also uses different terminal electron acceptor other than O₂ such as nitrate and fumarate. It is important to note that while the front-line TB drugs such as Isoniazid and rifampicin can kill greater than 99% of actively growing *Mtb* cells, they fail to eradicate the remaining cells which are persisters with decreased metabolic rate. A recent study (44) has shown that use of cysteine with the drugs prevents formation of persisters and can sterilize the culture. The study attributed this to a shift of menaquinol/menaquinone balance towards the reduced state upon addition of thiols. This in-turn enhances *Mtb* respiration converting persisters to metabolically active cells which are killed by drugs. Another interesting study suggests that simultaneous inhibition of cytochrome *bc₁:aa₃* and the cytochrome *bd* oxidase stops respiration completely and eradicates infection *in vivo* highlighting the importance of the terminal oxidase in respiration in *Mtb* (45).

1.4 Understanding Redox homeostasis and TB pathogenesis

In order to harness the reducing power through catabolism of substrate and to use it for anabolism of cellular components such as lipids, protein and DNA, the maintenance of RH is a prerequisite. Mycobacteria can survive in a highly oxidative environment during the pathogenic cycle and this indicates the presence of an elegant machinery capable of regulating the redox balance and counteracting the oxidoreductive stress (4,5,18). The major redox couples maintaining this balance in *Mtb* are thioredoxins (Trxs), peroxiredoxins (*AhpC* and *AhpE*), NADH/NAD⁺, NADPH/NADP⁺ and thiols such as mycothiol (MSH) and ergothioneine (EGT).

The levels of NAD⁺ (used as cofactor in electron harvesting catabolic processes) and NADPH (used as electron donor in reductive anabolic reactions) are tightly regulated. In *Mtb*, the levels of NADPH and NADP⁺ are kept equimolar about 1:1, while the NADH/NAD⁺ ratio is between 1:3 and 1:10 preferring higher levels of NAD⁺ to aid in electron extraction (46,47). Recent studies have shown that inhibition of respiration by hypoxia (possibly with increase in NO-levels) leads to increase in this ratio due to depletion of NAD⁺ (41). Similar observations have also been made in *Mtb* residing in the mouse lung (47), suggesting that *Mtb* encounters a reductive stress during pathogenesis. It is important to note that a higher NADH/NAD⁺ ratio leads to Isonicotinylhydrazine (INH) resistance (48).

Further highlighting the role of redox homeostasis (RH) in mycobacteria is the fact that the most potent TB drug, isonicotinylhydrazine (INH) kills bacteria by potentially disrupting the redox balance and over-expression of *AhpC* to INH resistance in mycobacteria (49). Therefore, a very fundamental prerequisite to global TB control is deciphering metabolic flexibility which allows mycobacteria to adapt to diverse carbon sources and redox environments it encounters within the host.

1.5 Low molecular weight dependent redox regulation

Low molecular weight (LMW) thiols are produced by all living cells to maintain redox homeostasis. LMW thiols are small compounds with a redox active thiol group that keeps the intracellular environment in a reduced state. In eukaryotes, the major LMW thiols are Glutathione and Coenzyme-A, while in prokaryotes a wide variety of LMW

thiols are described (see Table 1.1). *Mycobacterium* employs LMW thiols to avert oxidative stress and maintains intracellular thiol-disulphide homeostasis (50).

Table 1.1 Distribution of different LMW thiols found across the different life forms.

<u>LMW thiols</u>	<u>Organisms in which LMW thiols are present</u>
Gluathione (GSH)	Eukaryotes and Gram-negative bacteria including <i>Escherichia coli</i> (51)
Trypanothione (T[SH]₂)	Trypanosoma (52)
Gamma glutamyl-cysteine (γGC)	Halobacteria (lactic acid bacteria) (51)
Mycothiol (MSH)	Actinomycetes mainly in <i>Mycobacteria</i> (53)
Cysteine (CySH)	All organisms (51)
Coenzyme A (CoASH)	All organisms (51)
Bacillithiol (BSH)	Fermicutes (51)
Ergothioneine (ESH)	Mycobacteria (54)
Ovothiol (OSH)	Marine invertebrates including <i>Leishmania</i> (51)

Chemically, MSH (Fig. 1.4) comprises a functional cysteine residue, the saccharide inositol and N-glucosamine (55). MSH is present in millimolar concentration and is equivalent of GSH. Mycothiol disulphide reductase (Mtr), a flavoprotein is employed by mycobacteria to maintain MSH in the reduced form. Mtr utilizes NADPH to reduce MSSM to MSH (56). In addition, mycobacteria also produce ergothioneine (EGT), a sulfur-containing histidine derivative with potent antioxidant properties (Fig. 1.4).

Figure 1.4: Redox buffers in Mycobacteria. Two LMW thiols namely MSH (A) and Ergothioneine (B) are major redox buffer in *Mtb*.

Both MSH and EGT are both essential for redox and bioenergetic homeostasis but play a distinct role as shown recently using a transcriptomic analysis (54). In this context,

it is noteworthy that a MSH deficient strain of *M. smegmatis* contains a 20-to 30-fold higher amount of EGT indicating that EGT can compensate for lack of MSH (57). Another interesting fact is that MSH is relatively resistant to auto-oxidation. It has been shown that MSH undergoes copper catalyzed auto-oxidation 30-times slower than free cysteine and seven times slower than GSH (58). This highlights that actinomycetes has a higher ability to cope with oxidative stress due to MSH, making MSH an attractive drug target to disrupt redox balance in *Mtb*.

1.6 Biosynthesis of MSH in Mycobacteria

The MSH biosynthesis pathway has been extensively studied and characterized in Mycobacteria (59),(60). There are five major steps during the biosynthesis of MSH as highlighted in Fig. 1.5. 1L-myo-inositol-1-phosphate (1L-Ins-1-P or Ins-P), the starting material for MSH biosynthesis is produced by inositol phosphate synthase (Ino1) using glucose-6-phosphate (Glc-6-P) as a substrate. The first intermediate [1-O-(2-acetamido-2-deoxy-D-glucopyranosyl)-D-myo-inositol-3-phosphate] or (GlcNAc-Ins-P), in biosynthesis is produced by glycosyltransferase MshA using UDPGlcNAc and Ins-P. The next step, which involves the dephosphorylation of GlcNAc-Ins-P is catalyzed by the MshA2 enzyme and results in the formation of GlcNAc-Ins. The third step involves deacetylation of 1-O-(2-acetamido-2-deoxy-D-glucopyranosyl)-D-myo-inositol also known as GlcNAc-Ins and is carried out in presence of a metalloprotein known as MshB (59). This step results in the formation of 1-O-(2-amino-2-deoxy-D-glucopyranosyl)-D-myo-inositol (GlcN-Ins) which is a primary intermediate found in the mycobacterial extracts (61,62).

Figure 1.5: Biosynthesis of MSH in mycobacteria. There are five major steps during the biosynthesis of MSH. The protein involved during MSH biosynthesis namely MshA, MshB, MshC and MshD has been structurally characterized.

The penultimate step in MSH biosynthesis involves the ligation of a cysteine with GlcN-Ins to produce Cys-GlcN-Ins. This is an ATP-dependent process and is carried out by MshC, a homolog of Cys-tRNA synthetase (63). The final step in MSH biosynthesis is acetylation of amino group of Cys-GlcN-Ins by acetyl-CoA under catalysis by a GCN5 acetyl-transferase, MshD. The MSH pool inside the mycobacterial cell is kept in the reduced form by Mtr which is an NADPH dependent flavoprotein oxidoreductase. While all the enzymes in biosynthesis of MSH has been structurally characterized (64-67), no structural data is available for Mtr.

Similar to the GSH system in Gram-negative bacteria such as *E. coli* and *S. typhimurium*, the MSH system in actinomycetes also keeps the protein thiol in the reduced state. Therefore, like the Glutaredoxin (Grx) which couples the power of glutathione to redox protein, the actinomycetes also contains a small oxidoreductase called as Mycoredoxin (Mrx). The actinomycetes counterpart of the NADPH-dependent glutathione reductase (Gor) which keeps GSH in its reduced state to provide a high GSH/GSSG ratio is Mtr (Fig. 1.6).

Figure 1.6: MSH and GSH dependent protein recycling. Reduction of protein-S-glutathionylations and protein-S-mycothiolations by Grx and Mrx-1 pathways respectively. Mycobacterium employs an MSH/Mrx-1 system to reduce redox active protein such as peroxiredoxin AhpE.

The first enzyme to be characterized to employ MSH/Mrx-1 in the thiol/disulfide redox cascade was arsenate reductase from *C. glutamicum* (CgArsC1 and CgArsC2).

Briefly, Arsenate reacts with MSH to form an arseno-(V)-MSH complex, which is reduced by Mrx1 releasing As-(III) and Mrx1-SS-M that is recycled by the MSH/Mtr/NADPH electron pathway (68). Since then various redox active proteins have been shown to employ the MSH/Mrx-1 dependent redox pathway. These include mycobacterial AhpE (69), mycothiol peroxidase from *C. glutamicum* (70), methionine sulfoxide reductase from *C. diphtheriae* (71) and methionine sulfoxide reductase A from *C. glutamicum* (72).

1.7 MSH/Mrx-1 dependent redox regulation in Mycobacteria

As stated earlier, MSH is one the key redox thiols in actinomycetes including *Mtb*. Very recently a Mrx-1/MSH dependent recycling system has been proposed to be a biologically relevant system for the recycling process of peroxiredoxin AhpE, one of the key enzymes in mycobacterial anti-oxidant defense system (69) (Fig. 1.7).

Figure 1.7 Proposed mechanisms of AhpE reduction in *Mtb*. *Mt*AhpE is oxidized by a peroxide to form *Mt*AhpE-SOH or it may be over-oxidized to *Mt*AhpE-SO₂H leading to inactivation. *Mt*AhpE-SOH is then directly reduced by MSH and this mixed disulphide is then cleaved by *Mt*Mrx-1 (b). Alternatively, *Mt*Mrx-1 forms a mixed disulphide with *Mt*AhpE via the N-terminal cysteine, which is then subsequently cleaved by the C-terminal cysteine (a). Based on (69)

Briefly, the proposed mechanism suggests, that one molecule of MSH forms a mixed disulphide with *Mt*AhpE-SOH followed by a release of water. The mixed disulphide (*Mt*AhpE-SS-M) is cleaved by reduced Mrx-1, leading to the reduction of *Mt*AhpE. This mechanism is called monothiolic mechanism. Alternatively, the same

work also proposed a dithiolic mechanism of *MtAhpE* reduction via *Mrx-1*, wherein a sulphenic derivative of *MtAhpE* is directly reduced by *Mrx-1* in two steps to yield *MtAhpE*-SH. It has been proposed while the MSH pathway might be operational in cytoplasm, the *Mrx-1* pathway might be favored in membranes. MSSM is recycled back to MSH via *Mtr*. The details of various proteins involved in this pathway are described in detail in the next section.

(a) Mycothiol disulfide reductase (Rv2855)

Mtr is a member of the pyridine nucleotide-disulfide reductase superfamily which also includes the glutathione reductase, trypanothione reductase, dihyrolipoamide dehydrogenase and thioredoxin reductase. Like other flavoprotein disulfide reductases, *Mtr* also maintains redox homeostasis allowing proper functioning of various biological processes. Earlier studies have reported that glutathione reductase as well as dihyrolipoamide dehydrogenase exists as mixture of dimers and tetramers in solution (73,74). It has also been shown in case of glutathione reductase from human erythrocytes that the presence of NADPH favors conversion of the dimeric form of enzyme into the less active tetramers (75).

Using transposon mutagenesis in the H37Rv strain Sasseti et. al. has shown that *Mtr* is an essential gene in mycobacteria (76). *Mtr* is a 459-aa long protein which uses FAD and NADPH as co-factors. *MtMtr* has been expressed in *M. smegmatis* and enzymatically characterized (77,78). *MtMtr* contains a disulfide catalytic center. It was reported that the protein exists as a dimer in solution. The reduction of MSSM to MSH follows a bi-bi-ping-pong kinetic mechanism (78). The reported K_{cat} for MSSM is 400 mol per mole of FAD per min. indicating that *MtMtr* is one to two-order slower as compared to other disulfide reductases such as glutathione reductase (78). Since the rate of auto-oxidation for MSH is comparatively lesser to other LMW thiols such as cysteine and GSH, it was proposed that maintenance of MSH inside the mycobacterial cell might still be possible by *MtMtr*. The K_m for MSSM is 73 mM, which is akin to related reductases (78). However, *MtMtr* is specific to MSSM and doesn't show any activity towards oxidised trypanothione and oxidised glutathione (78).

Very recently (79) it was shown that over-expression of *Mtr* in *C. glutamicum* enhances its tolerance against oxidative stress and modulates cellular redox homeostasis by decreasing ROS levels and reducing carbonylation damage. In addition, the study also

showed that over-expression of Mtr resulted in increased activities of several anti-oxidant enzymes such as mycothiol peroxidase (MPx), Mrx-1, Trx-1, and methionine sulfoxide reductase A (MsrA). The importance of MSH and *MtMtr* is highlighted by the fact that MSH is essential for survival during biofilm formation and aldehyde stress in *M. smegmatis* (80) as well as under hypochlorite stress (81). It has been shown that fumarase depletion in mycobacteria results in fumarate accumulation and this in turn leads to succination of thiol (MSH and catalase) resulting in hyper-susceptibility to oxidative stress (82). This further resulted in killing of *Mtb* *in vitro* as well as in chronic phases of infection in a mouse model highlighting the importance of MSH in redox regulation in mycobacteria (82). There have been only few reports of expressing *MtMtr* in *E. coli* for structural and biochemical studies, however the recombinant protein production was not robust (69). Due to this, many research groups have preferentially utilized Mtr from *C. glutamicum* for biochemical studies and no structural data are available for *MtMtr* till date. Since *MtMtr* is one of the important proteins, structural and mechanistic insight will provide novel insights into redox-regulation in mycobacteria.

(b) Mycoredoxin (Rv3198.1)

The *Mtb* genome encodes *MtMrx-1*, the actinomycetes homologue of glutaredoxin, which acts as a MSH-dependent reductase of *MtAhpE* (51,83). Genetic studies have shown that *mrx1* deletion mutant in *M. smegmatis* shows increased, but limited sensitivity to H₂O₂, Cu²⁺ and iodoacetamide (IAM). It was speculated that limited sensitivity was due to compensatory upregulation of other anti-oxidant enzymes (68). *MtMrx-1* shares high amino acid sequence similarity with the nucleotide reductase protein namely NrdH-redoxin (Fig. 1.8).

Figure 1.9: Sequence alignment of *MtMrx-1* and related proteins. Structure based sequence alignment of *Mtb* NrdH redoxin, *C. glutamicum* NrdH-redoxin, *E. coli* NrdH-redoxin and *Mtb* Mrx-1. The secondary structure elements of *Mtb* NrdH-redoxin (light blue) and the *Mtb* Mrx-1 (purple) are shown. The CXXC-motif is shown in a red box.

Despite the high sequence similarity, the two proteins are different in function. NrdH-redoxins are small glutaredoxin-like proteins with thioredoxin-like activity, serving as the reducing partners of class Ib ribonucleotide reductase (84). The NMR solution structure of Mrx-1 from *Mtb* was solved recently both under oxidised (PDB ID: 2LQQ) and reduced condition (PDB ID: 2LQO) (83) (Fig. 1.9). The structure consists of a 4-stranded anti-parallel β -sheet surrounded by three α -helices which is typical of structural elements from several oxidoreductases such as thioredoxin, glutaredoxin, NrdH-redoxin and tryparedoxin (84).

Figure 1.9: Structure of Mrx-1 from *Mtb*. NMR solution structure of oxidoreductase *MtMrx-1* (oxidised form; 2LQQ). *MtMrx-1* consists of a 4-stranded anti-parallel β -sheet surrounded by three α -helices. The active site of oxidised Mrx-1 with C14 and C17 forming a disulfide bond. Based on (83)

It has been experimentally shown that MSH forms a mixed disulfide with oxidised Mrx-1, which is cleaved by a second molecule of MSH, releasing reduced Mrx-1 and MSSM (83). The N-terminus of the α 1 helix contains the catalytic center, the CGYC motif. P57, which is directly opposite to the active site, is in *cis* conformation; a feature which is conserved among all proteins with a Trx-fold and is essential for maintenance of the active site conformation. NMR titration studies revealed that T11, W13, V56, T58, T68, N69 and P70 were involved in MSH binding (83). It is interesting to note that very recently a new Mycoredoxin (Rv2466c) termed Mrx-2, was identified in *Mtb* (85). *MtMrx-2* is essential for activation of the TP053 prodrug in a *Mtb H37Rv* cell culture model. It was also demonstrated that unlike Mrx-1, *MtMrx-2* is not very specific, as the (TrxC/TrxR/NADPH) pathway can also regenerate oxidized *MtMrx-2* in addition to the MSH/Mtr/NADPH pathway (85).

(c) Peroxiredoxin AhpE (Rv2238c)

Peroxiredoxins (Prxs) are a ubiquitous family of antioxidant proteins which use redox-active cysteine to reduce peroxide. Among the various classes of Prxs, the subfamily AhpE is unique and least understood. Data mining has indicated that there are only about 25 sequences, mostly belonging to mycobacterial and others closely related bacterial species that can be classified into the AhpE subfamily. Despite this uniqueness, members of this superfamily shares about 30% sequence identity with the Prx-1 subfamily (AhpC subfamily). Although *MtAhpE* is considered as a member of 1-Cys Prxs it shares only 24% sequence identity with human 1-Cys Prx (hORF6) and has been shown to lack the C-terminal domains that functions as dimerization interface in the crystal structure of human 1-Cys Prx (hORF6) (86). Till date, the only two members of this family structurally characterized are *MtAhpE* and *PfAOP* from *P. falciparum*, both of which are anti-oxidant proteins and protects against reactive oxygen species, and share over-all similar secondary structure (Fig. 1.10).

MtAhpE contains only one cysteine namely C45 which is peroxidatic cysteine and lacks the resolving cysteine. *MtAhpE* reacts 10^3 times faster with peroxynitrite (ONOOH) than with H_2O_2 (1.9×10^7 versus $8.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4 and 25 °C) (87,88). *MtAhpE* contains a hydrophobic groove near the dimer interface which allows it to bind to fatty acid hydroperoxide in addition to H_2O_2 . It has been shown that the quaternary structure of *MtAhpE* is controlled by the redox state of the peroxidatic cysteine (87,88).

Figure 1.10. Structure based alignment of MtAhpE with the related structure. Structure based sequence alignment of MtAhpE (SID2), *Plasmodium falciparum* 1-Cys Prxs (PfAOP_1XIY), Human peroxidoxin V (PrxV_1H4O), trypanoxin peroxidase from *Crithidia fasciculata* (TryP_1E2Y). The secondary structure elements of peroxidoxin MtAhpE (orange) and the antioxidants protein from *P. falciparum* (light blue) are shown. All the residues conserved among 1-Cys Prxs are highlighted in blue. The PXXXTXXC-motif, R116 and R139 are shown in a red box. Taken from Kumar et. al. 2016.

Earlier studies have highlighted the overoxidation of *MtAhpE* with equimolar H_2O_2 resulting in the formation of higher molecular weight species, while the excess of H_2O_2 resulted in dimeric species again (88). The crystal structure of *MtAhpE* has been reported both under oxidised (PDB ID:1XVW) and reduced conditions (PDB ID: 1XXU) (86). The crystal structure of *MtAhpE* shows that it forms a tightly associated dimer as well as a ring-like octamer (generated by crystallographic 4-fold symmetry) (Fig. 1.11). In comparison, the crystal structure of the reduced *MtAhpE* (PDB ID 1XXU) was unstable during MD simulation due to unoptimized hydrogen bonding network (89).

Figure 1.11: Oligomeric state of *MtAhpE*. Earlier studies revealed two distinct oligomeric states of *MtAhpE*, AhpE dimer and octameric form (86). The AhpE octamer, formed by operation of the crystallographic 4-fold axis, relating four dimers. Based on PDB ID 1XXU.

It is interesting to note that during the compilation of this work, a study characterizing AhpE in *Thermococcus kodakarensis* was published (90) and it was shown by negative staining electron microscopy that *TkAhpE* adopted an octameric form. It was also shown that like the peroxidase, *TkAhpE* has both structure-dependent holdase chaperone and DNA binding properties. In addition, the structure of *MtAhpE*-WT, F37H and R116A was also reported recently by other groups (91,92).

1.8 Aims of this thesis:

The anti-oxidant defense system of *Mtb* represents an ensemble of proteins which is essential for survival of pathogen. The most important aim of this thesis is to gain structural insights into *MtMtr* and fill the gaps in our understanding of MSH/Mrx-1 dependent redox regulation in *Mtb*. *MtMtr* is the only protein which has been shown to keep MSH in the reduced form and therefore is an attractive drug target. Keeping this in mind, the goals for this part of work will be;

- Cloning and expression of a catalytically active, stable *MtMtr* to an amount suitable for enzymatic and structural studies.
- Solution X-ray scattering (SAXS) studies on *MtMtr* to deduce the oligomeric state and first low-resolution structure.
- Studying substrate induced conformational changes in solution for *MtMtr* in the presence of NADPH and MSSM using SAXS.
- Crystallization of *MtMtr* to obtain diffraction quality crystals.

In spite of several efforts, the interaction between *MtMrx-1* and *MtAhpE* is still to be characterized on a molecular level, in order to understand the redox mechanism. In this regard, the following points will be addressed;

- Cloning, expression, production and purification of *MtAhpE* and *MtMrx-1*.
- SAXS studies on Mrx-1 to investigate possible oligomerization at high concentrations as reported for the related NrdH-redoxin.
- NMR based interaction studies to study the interaction between *MtAhpE* and *MtMrx-1*.
- Cysteine labelled NMR experiments to understand the redox-active center of *MtMrx-1* and changes at the redox center upon titration with *MtAhpE*.

For *MtAhpE*, the mechanistic aspect is still unknown as the substrate bound structure is not available and the biological reductant is still debated. However, recently it has been proposed that *MtMrx-1*/MSH could be the biological reductant of *MtAhpE*.

- Crystallization studies to obtain MSH bound structure of *MtAhpE* to get insights into molecular basis for Mycothiolation.

- SAXS studies on *MtAhpE* to address the redox-linked changes in the quaternary structure. In addition, the oligomeric state of *MtAhpE* is debatable with earlier studies reporting both a dimeric and an octameric form, therefore it is important to study the oligomeric state of *MtAhpE* under different redox conditions as well as in the presence of substrate.
- Cysteine labelled NMR experiments to understand the redox-active center of *MtAhpE* and changes at the redox center upon titration with different reducing agents such as DTT, MSH and NAC.
- Isothermal Titration Calorimetry (ITC) experiments to study nature of interaction between NAC and *MtAhpE*.

Materials and Methods

Materials and Methods

Materials

Materials

2.1 Materials

2.1.1 Chemicals

All the chemicals used for the study were at least of analytical grade. Chemicals were purchased from the following companies:

2.1.1.1 List of Chemicals

Buffers, Media and Salts	Sigma (St. Louis, MO, USA) USB (Sampscott, MA) Calbiochem (Darmstadt, Germany) Fluka (Sigma, Buchs Germany) Roth (Karlsruhe, Germany) Serva (Heidelberg, Germany)
DTT	Hoefer (San Francisco, CA, USA)
Ni ²⁺ -NTA	QIAGEN (Hilden, Germany)
Pefabloc ^{SC}	BIOMOL (Hamburg, Germany)
PMSF	Sigma (St. Louis, MO, USA)
LB Media	BD (Sparks, MD, USA)
Mycothioli	JEMA Biosciences (USA)
N-Acetyl-L-cysteine	Sigma (St. Louis, MO, USA)
Antibiotics	Calbiochem, Sigma and Gibco
IPTG	Fermentas (Vilnius, Lithuania)

2.1.1.2 Electrophoresis reagents

Agarose	Bio-Rad (Hercules, CA, USA)
SDS	Bio-Rad (Hercules, CA, USA)
Glycine	Bio-Rad (Hercules, CA, USA)
APS	Bio-Rad (Hercules, CA, USA)

2.1.1.3 Molecular biology materials

Primers	IDT (Singapore)
Pfu DNA polymerase	Fermentas (Glen Burnie, MD, USA)
<i>NcoI</i> , <i>SacI</i> , <i>BamHI</i>	Fermentas and New England Biolabs
T4 DNA Ligase	Fermentas and NEB

Miniprep Plasmid Kit	Qiagen (Hilden, Germany)
Nucleobond AX mediprep Kit	MN and Co (Düren, Germany)
<i>E coli</i> expression strains	DH5 α , BL21 (DE3), BL21-GroEL/ES
pET-9d1	Grüber lab. (93)

2.1.2 Chromatography

2.1.2.1 Gel filtration Columns

Superdex 75 (10/300) GL	GE Healthcare (Uppsala, Sweden)
Superdex 200-I (10/300) GL	GE Healthcare (Uppsala, Sweden)

2.1.2.2 Instruments and accessories

Akta FPLC	GE Healthcare (Uppsala, Sweden)
Millex Filters (0.45 μ M)	Millipore (Bradford, USA)
Syringe, and accessories	BD Biosciences (New Jersey, USA)

2.1.2.3 Protein concentration, estimation

Centriprep YM10	Millipore (Co-cork, Ireland)
Amicon ulta (3-50kDa)	Millipore (Co-cork, Ireland)

2.1.3 Other instrumentation

PCR Thermocycler:	
Biometra T personal	Biometra (Göttingen, Germany)
Biometra T gradient	Biometra (Göttingen, Germany)
Sonoplus Sonicator	Bandelin (Berlin, Germany)
Micropulser Electroporator	Bio-Rad (Hercules, CA, USA)
Ultraspec 2100 Pro (Spectrophotometer)	Amersham Biosciences (Bucks, UK)
MetalJet-NANOSTAR	Bruker AXS (Karlsruhe, Germany)
NMR (600 and 700 MHz)	Bruker (Karlsruhe, Germany)

2.1.4 Computer software

Vector NTI 10.3.0	Invitrogen (California, United States)
PyMOL v1.5	DeLano Scientific LLC (USA)
Quantity One	Bio-Rad (Hercules, CA, USA)

ATSAS package	EMBL, (Hamburg, Germany)
SPARKY	University of California (USA)
Topspin 1.3	Bruker Biospin (Karlsruhe, Germany)
ScÅtter v2.3a	© Robert P. Rambo (Oxfordshire, UK)
HKL2000 package	Zbyszek and Wladek, 1997 (94)
Refmac5 (CCP4 package)	Murshudov <i>et al.</i> , 1997 (95)
MOLREP	Vagin and Taplyakov, 1997 (96)
PHASER	McCOY <i>et al.</i> , 2007 (97)
Coot	Emsley and Cowtan, 2004 (98)
Ramachandran plot	Ramachandran <i>et al.</i> , 1963 (99)
PROCHECK	Laskowski <i>et al.</i> , 1993 (100)
MolProbity	Davis <i>et al.</i> , 2004 (101)

Methods

Methods

2.2 Methods

2.2.1 Cloning of Mtr (*Rv2855*) from *Mtb*

To amplify the Mtr (*Rv2855*) coding region, oligonucleotide primers incorporating *NcoI* or *BamHI* restriction site (underlined), were designed as tabulated below:

Construct	Oligonucleotide sequence 5'-3'	
MtMtr	forward	GGGTGATGGC <u>CCATGG</u> TCATGGAAACGTACGAC
	reverse	TCGCCG <u>GGATCCT</u> CAACGCAGGCCAAGCAG

For polymerase chain reaction (PCR), *Mtb* genomic DNA was used as template. PCR reaction was set up in a total volume of 50 µl on ice as mentioned below:

Reagents	Amount
Pfu buffer (10x)	5 µl
dNTPs (2 mM)	1.5 µl
Forward primers (100 µM)	0.5 µl
Reverse primers (100 µM)	0.5 µl
Template (genomic DNA)	1 µl
Milli-Q water	40.5 µl
Pfu DNA polymerase	1 µl

All reagents were pipetted into a PCR tube and the reagents were mixed by short vortex. PCR thermocycler was preheated to 99 °C and reaction tubes were placed inside the machine. The following PCR thermocycler program was used for amplification:

Cycle steps	Temperature	Time
Lid	99 °C	
Initial denaturation	96 °C	3 mins
Cycle denaturation	96 °C	30 secs.
Annealing	64 °C	45 secs.
Extension	72 °C	4 mins
Final extension	72 °C	5 mins
End	4 °C	

After amplification, the PCR product was stored at -20 °C, 5 µl of PCR product was applied onto an analytical agarose gel (1.2%) to check its quality. The gel was kept in ethidium bromide solution for 10 mins and observed under UV light. The size of the PCR product was confirmed with the 1 Kb DNA marker as reference. Remaining reaction mixture was applied onto a 1.2% preparative agarose gel.

Figure 2.1: Plasmid map of pET-9d1 *MtMtr* construct showing full map and unique restriction sites used for cloning.

The portions corresponding to the correct PCR product were cut out immediately and purified by gel extraction kit (QIAGEN) as per the manufacturer's protocol. The product was finally eluted in 30 µl of water. 1 µl of gel extracted PCR product was applied onto 1.5% analytical agarose gel for estimating the purity of the sample. Purified DNA was double digested overnight at 37 °C by taking 1 µl each of *NcoI* and *BamHI* enzymes. After overnight digestion, the reaction mixture was purified using a QIAGEN PCR purification kit again. Subsequently, ligation reaction between pET-9d1 vector (Fig. 2.1) (93) and amplified PCR product was set up at room temperature. The reagents used for ligation are as follows:

Reagents	Amount
Ligase buffer (10x)	1 µl
Vector (70 ng/µl)	1 µl
Insert (120 ng/µl)	3 µl
T4 Ligase	1 µl
Milli-Q water	4 µl

The ligation mixture was kept at room temperature for three hours. The reaction was stopped using butanol-precipitation of DNA. 90 µl of Milli-Q water was added to tube to make a final volume of 100 µl. 1 ml of butanol was added into the solution and vortexed thoroughly. Then the mixture was centrifuged at 13,000 x *g* (using Eppendorf Mini-centrifuge) for 10 mins and the supernatant was discarded gently. The pellet was

re-suspended in 150 µl of 70% ethanol and centrifuged again for 5 mins. The supernatant was again removed and the pellet was re-dissolved in 7 µl Milli-Q water.

The ligation mixture was used for the transformation into *Escherichia coli* DH5α cells. Single colonies were picked from the transformation plate on the next day and were inoculated in liquid culture media at 37 °C overnight. The plasmid DNA was isolated using standard protocol (QIAGEN mini-prep kit) and subsequently double-digested and applied onto 1.5% agarose gel, in order to confirm the presence of the ligated insert. DNA sequencing was used for verification of the insert. The verified plasmid was finally transformed into *E. coli* BL21 (DE3) cells for protein production and they were grown on 30 µg/ml kanamycin-containing Luria-Bertani (LB) agar plates. Similar protocols were used to clone *MtAhpE* and *MtMrx-1* from *Mtb*. However technical details of primers used, plasmid map, PCR program, ligation protocols are listed below:

2.2.1.1 Cloning of *MtMtr* C-terminal domain (337-459) from *Mtb*

To amplify the *MtMtr* C-terminal domain (337-459), oligonucleotide primers incorporating *NcoI* or *BamHI* restriction site (underlined), were designed as tabulated below:

Construct		Oligonucleotide sequence 5'-3'
<i>MtMtr</i> C-term	forward	CACCC <u>CCATGG</u> TCATGATCGTCACCGACCACCG ATACGTACCG
	reverse	GCC <u>GGATCC</u> TCAACGCAGGCCAAGCAGCGCGT TTTCCACCAC

The PCR program used for amplification of *MtMtr* C-term from *MtMtr* plasmid as template is tabulated below:

Cycle steps	Temperature	Time
Lid	99 °C	
Initial denaturation	96 °C	3 mins
Cycle denaturation	96 °C	30 secs.
Annealing	68 °C	45 secs.
Extension	72 °C	2 mins
Final extension	72 °C	5 mins
End	4 °C	

The reagents used for ligation of pET9d1 (Fig. 2.2) (93) and double-digested *MtMtr* C-term PCR product are listed below:

Reagent	Amount
Ligase buffer (10x)	1 μ l
Vector (70 ng/ μ l)	1 μ l
Insert (108 ng/ μ l)	3 μ l
T4 Ligase	1 μ l
Milli-Q water	4 μ l

Figure 2.2: Plasmid map of pET-9d1 (93) *MtMtr* C-term construct showing full map and unique restriction sites used for cloning.

2.2.2 Cloning of *MtMrx-1* (Rv3198A) from *Mtb*

To amplify the *MtMrx-1* coding region, oligonucleotide primers incorporating *NcoI* or *BamHI* restriction site (underlined), were designed as tabulated below:

Construct		Oligonucleotide sequence 5'-3'
<i>MtMrx-1</i>	forward	CTAGGTT <u>CCATGGT</u> CATGATCACCGCTGCG
	reverse	GAATGAAGTCC <u>GGATCCT</u> TAACCCGCGATCTTTAC

The PCR program used for amplification of *MtMrx-1* from *Mtb* genomic DNA is tabulated below:

Cycle steps	Temperature	Time
Lid	99 °C	
Initial denaturation	96 °C	3 mins
Cycle denaturation	96 °C	30 secs.
Annealing	64 °C	45 secs.
Extension	72 °C	1 mins
Final extension	72 °C	5 mins
End	4 °C	

30 cycles

The reagents used for ligation of pET9d1 (Fig. 2.3) (93) and double digested *MtMrx-1* PCR product are listed below:

Reagent	Amount
Ligase buffer (10x)	1 μ l
Vector (70 ng/ μ l)	1 μ l
Insert (108 ng/ μ l)	3 μ l
T4 Ligase	1 μ l
Milli-Q water	4 μ l

Figure 2.3: Plasmid map of pET-9d1 (93) *MtMrx-1* construct showing full map and unique restriction sites used for cloning.

2.2.3 Cloning of *MtAhpE* (Rv2238c) from *Mtb*

To amplify the *MtAhpE* coding region, oligonucleotide primers incorporating *NcoI* or *BamHI* restriction site (underlined), were designed as tabulated below:

Construct		Oligonucleotide sequence 5'-3'
<i>MtAhpE</i>	forward	GCGGGCTGGCC <u>CATGG</u> TGATGCTGAACGTC
	reverse	GGCCAAGCACCC <u>GGATC</u> CTTAGGCCGTAAG

The PCR program used for amplification of *AhpE* from *Mtb* genomic DNA is tabulated below:

Cycle steps	Temperature	Time
Lid	99 °C	
Initial denaturation	96 °C	3 mins
Cycle denaturation	96 °C	30 secs.
Annealing	64 °C	45 secs.
Extension	72 °C	2 mins
Final extension	72 °C	5 mins
End	4 °C	

The reagents used for ligation of pET9d1 (Fig. 2.4) (93) and double digested *MtAhpE* PCR product are listed below:

Reagent	Amount
Ligase buffer (10x)	1 μ l
Vector (70 ng/ μ l)	1 μ l
Insert (108 ng/ μ l)	3 μ l
T4 Ligase	1 μ l
Milli-Q water	4 μ l

Figure 2.4: Plasmid map of pET-9d1 (93) *MtAhpE* construct showing full map and unique restriction sites used for cloning.

2.2.4 Cloning of *MtMtr* mutant using site-directed mutagenesis

To generate the desired mutation of Mtr for this study, site-directed mutagenesis method was employed. Site-directed mutagenesis is a method for amplification of the entire plasmid carrying the gene of interest by usage of mutagenic primers. To substitute the desired residues, sets of mutagenic primers were designed. The primer sequences are tabulated below:

Construct	Oligonucleotide sequence 5'-3'	
S144A	forward	GGAG <u>GCCC</u> GGCCGGTGATTCCGCCGGCCATC
S144A	reverse	GCCG <u>GGC</u> TCCGGCGGCTATCACCACTGCTC
D158A/Y159A	forward	CGTC <u>GCCGCC</u> CACACCAGCGATACCGTCATGC G
D158A/Y159A	reverse	GTGTG <u>GGCGGC</u> GACGCCGGACGCGAGGATG
S268A	forward	GGCCGGGTG <u>GCCA</u> ACGCCGACCTGCTGGATGC CGAGCAGGCC
S268A	reverse	GTCGGCGTT <u>GGC</u> CACCCGGCCTGTCGCTACCA ACAGTAGGTC

For the polymerase chain reaction, ~25 ng of plasmid DNA encoding WT Mtr has been used as a template. Total reaction volume was set to 50 μ l and the reaction mixture to were pipetted to PCR tubes on ice for all the steps. Mixture was homogenized by pipetting and centrifugation on a conventional table-top centrifuge (Eppendorf Mini-centrifuge). PCR reaction was set up in a total volume of 50 μ l on ice as mentioned below:

Reagents	Amount
Kappa buffer (5x)	10 µl
Milli-Q water	34 µl
dNTP's (2 mM)	2 µl
Template (plasmid DNA)	1 µl
Kappa HiFi DNA polymerase	1 µl
Forward primers (100 µM)	1 µl
Reverse primers (100 µM)	1 µl

PCR Thermocycler (Biometra T personal) was preheated to 99 °C before placing the PCR tubes on the heating block. The following program was used for the amplification:

Cycle steps	Temperature	Time
Lid	99 °C	
Initial denaturation	95 °C	2 mins
Cycle denaturation	98 °C	20 secs.
Annealing	*	15 secs.
Extension	72 °C	6 mins
Final extension	72 °C	10 mins
End	4 °C	

* as per the primer specifications

To check the quality, 5 µl of each the PCR product was applied on an 1.2% agarose gel. The gel was stained with EtBr solution for 20 mins and observed under UV light upon de-staining.

For each of the constructs, the remaining reaction mixture (45 µl) was used to isolate the amplified plasmid DNA containing the mutation following the next protocol as outlined in Fig. 2.5

Figure 2.5.: Protocol for isolating the mutant plasmid DNA following PCR.

To digest the methylated plasmid DNA that has been used as the template in PCR, restriction with endonuclease *DpnI* has been setup [PCR mixture (30 µl) + CutSmart®

buffer (10x) (6 μ l) + *DpnI* (1 μ l) + MiliQ water (23 μ l)]. The reaction mixture was incubated for 1 hour in water-bath at 37 °C followed by 20 mins of incubation on 80 °C to inactivate the *DpnI* endonuclease.

In order to isolate DNA for subsequent transformation into the electro-competent cells, 40 μ l of MiliQ water followed by 900 μ l of *n*-butanol (HPLC grade) was added to the reaction mixture. After thorough mixing and homogenizing, DNA was precipitated out using the conventional table-top centrifuge (Eppendorf Mini-centrifuge) for 10 mins on 13000 x g. Supernatant was pipetted out and replaced with 500 μ l of 70% ethanol (HPLC grade) to remove the residual butanol. Centrifugation was repeated and subsequently ethanol was removed. The tube containing the pellet with DNA was inverted on a paper inside of the sterile hood in duration of 10 mins to allow residual ethanol to evaporate. Dry DNA pellet was re-dissolved in 7 μ l of MiliQ water on ice in duration of 30 mins.

2.2.5 Competent cell preparation and Electroporation transformation

Electro-competent cells were prepared as per standard protocol (102). Briefly, a single colony of competent cells was inoculated in 50 ml of LB medium and culture was incubated overnight at 37 °C. Two aliquots of 500 ml of prewarmed LB medium in separate 2-liter flasks were inoculate with 25 ml of the overnight bacterial culture. The flasks were incubated at 37 °C with agitation (200 rpm in a rotary shaker). The OD-600 was measured every twenty minutes and at OD-600 of 0.4-0.6, the flasks were rapidly cooled by transferring to the pre-made ice-water bath for 15-30 minutes. The cells were harvested by centrifugation at 1000 x g in ice-cold centrifuge bottles. The cells were resuspended in 500 ml of ice-cold water and the process of resuspension and centrifugation was repeated with 10% glycerol. The supernatant was discarded, and the cell pellet was resuspended in 10 ml ice-cold 10% glycerol. 60 μ l of the suspension was aliquoted to ice-cold Eppendorf's and stored in -80 °C until further use.

Electroporation was done for the transformation of plasmid into electro-competent cells. 50 ng of plasmid DNA was added to 50 μ l of competent cells and incubated on ice for 5–10 mins. Electroporation was done at a constant voltage of 2500 volts (Micropulser Electroporator, Bio-Rad). The cuvette was immediately placed back on ice for one min. Thereafter, 1 ml of LB media [NaCl (5 g/l), Tryptone (10 g/l) and Yeast extract (10 g/l)] was added to the cuvette and the mixture was transferred to microcentrifuge tubes. The

mixture was then incubated at 37 °C for one hour. Finally, appropriate dilutions were prepared before plating the cells on antibiotic selection plates which were incubated at 37 °C for 8-12 hours.

2.2.6 Production and purification of *MtMtr*, *MtMrx-1* and *MtAhpE*

2.2.6.1 Induction test

Reagents: 4 x Lysis buffer
250 mM Tris/HCl, pH 6.8
9.2% SDS
40% Glycerol
0.2% Bromophenol Blue

At least four single colonies were randomly picked up and grown in 10 ml LB media to OD₆₀₀ of 0.6 - 0.7 at 37 °C with shaking of 180 rpm (Infors HT Minitron shaker). The 10-ml culture was then divided into two equal halves of 5 ml each in separate culture tubes. IPTG was added to one set of tubes to reach final concentration of 1 mM to start induction. Another set of tubes was kept as control. The tubes were kept in a shaker-incubator at 37 °C for 3 hours. The concentration of IPTG depends on the characteristics of protein being produced and the conditions were optimized for the respective protein. After 3 hours, the cells were pelleted down and re-suspended with 60 µl of 1 x lysis buffer in the presence of 1 mM DTT. Re-suspended cell mass was heated at 95 °C for 5 mins and 15 µl of each sample was loaded onto a 17% SDS polyacrylamide gel. The expression was more in the induced cultures than uninduced cultures.

2.2.6.2 Solubility test for recombinant *MtMtr*, *MtMrx-1* and *MtAhpE*

Following the successful induction of proteins with appropriate concentrations of IPTG at the optimized temperature and shaking conditions, solubility of the produced proteins was tested in various buffers. 50 ml of culture was grown and induced with IPTG. Cells were pelleted down into five equal fractions of 10 ml each by centrifuging at 10,000 x g for 10 mins. Cells were then re-suspended into five different buffers mentioned below. Freshly prepared protease inhibitors Pefabloc^{SC} in water and 1 mM PMSF (dissolved in 100% isopropanol) were added to a final concentration of 1 mM. Reducing agent such as DTT (1 mM) was used in the preparation.

Buffer 1: 50 mM Tris/HCl, 200 mM NaCl, pH 8.5
Buffer 2: 50 mM Tris/HCl, 200 mM NaCl, pH 7.5
Buffer 3: 25 mM HEPES, 200 mM NaCl, pH 7.5
Buffer 4: 25 mM MES, 200 mM NaCl, pH 7.0
Buffer 5: 50 mM phosphate, 200 mM NaCl, pH 6.5

Resuspension was sonicated at 15% power with KE 76 tip of sonicator (Bandelin Sonoplus) three times for one mins each, with a cooling interval of two mins between each cycle. Supernatant was then separated from the pellet by centrifugation at 10,000 x g and transferred to a fresh tube. Pellets were re-suspended in the respective buffers. All the steps were carried on ice. 25 μ l of pellet and supernatant were loaded onto a 17% SDS gel.

2.2.6.3 Expression of *MtMtr* in *E. coli* BL21 GroEL-ES strain

To ensure proper folding of proteins, chaperones are required as they assist the nascent polypeptide to achieve the right folding pattern to form a stable and well folded protein. The GroEL-ES class of chaperone-chaperonin in *E. coli* aid folding of synthesized proteins on ATP consumption. The GroEL-ES chaperone-chaperonin system forms a cage encapsulating the substrate protein, which might be a partially folded intermediate.

Figure 2.6: GroEL/ES assisted protein folding. GroEL/ES chaperone-chaperonin system forms a cage encapsulating the unfolded or misfolded protein which is then properly folded at expense of ATP molecules.

Using ATP, the folding is achieved, and the folded protein is released with the disassembly of the GroEL-ES complex and making available the cavity for binding another substrate protein (Fig. 2.6). This system was employed for folding the over expressed *MtMtr*. The cell strain of *E. coli* BL21 GroEL-ES containing pBAD vector was used, which codes for GroEL-ES chaperone-chaperonin and over expresses this to

allow sufficient population of the chaperone to be present to facilitate the folding of the recombinant protein. The promoter present in this vector is induced using 0.5% arabinose. Heat shock competent cells were kindly provided and further prepared as per the following protocol as given by Prof. Oliver Mueller-Cajar's lab (SBS, NTU). Overnight (O/N) culture was set up using the plasmid provided, in Chloramphenicol enriched LB and shaken at 180 rpm at 37 °C. Next morning, this was scaled up to 1 L in Chloramphenicol enriched LB to a starting OD₆₀₀ of 0.1 and allowed to grow at 37 °C. At an OD₆₀₀ of 0.6, the culture was placed on ice for 30 mins and afterwards centrifuged in a Beckman coulter Avanti J-26XP centrifuge at 4,200 rpm for 20 mins at 4 °C. Supernatant LB was decanted and the pellet was re-suspended in previously autoclaved and chilled mixture of 8 ml of 0.1 M CaCl₂ and 15% glycerol. This resuspension was placed on ice for 30 mins and aliquots of 50 µl were drawn into each tube and frozen.

2.2.6.3.1 Transformation using heat shock

A tube of frozen competent cells was equilibrated on ice for 20 mins with the plasmid (pET9d with *MtMtr*). Heat shock was performed by placing this mixture on a water bath set to 42 °C for 90 secs. and immediately returning this to ice for 2 mins. This was re-suspended in 200 µl of LB and allowed to revive for 30 mins at 37 °C in a shaking incubator. The re-suspended cells were plated on Kanamycin-Chloramphenicol LB plates and placed at 37 °C overnight. Single colonies obtained the next day, were picked and checked for expression of the desired protein.

2.2.6.3.2 Large scale production of *MtMtr* in *E. coli* BL21 GroEL-ES cell strain

Overnight inoculum of the transformed plasmid (pET9d with *MtMtr*) in *E. coli* BL21 GroEL-ES was setup in Kanamycin-Chloramphenicol enriched LB at 37 °C. This was used to scale up the large culture next morning, to a starting OD₆₀₀ of 0.1. The liquid culture in 5 L flasks were placed in the shaking incubator at 37 °C and were allowed to grow until the OD₆₀₀ reached 0.5 and were then shifted to an incubator set at 23 °C for equilibration. Arabinose (Final concentration: 0.2%) was added to induce the pBAD vector to express GroEL-ES at 23 °C for 30 mins. This was followed with the addition of IPTG (Final concentration: 1 mM) to induce the pET9d vector to express *MtMtr*. The culture was shake at 180 rpm at 20 °C overnight, after which the cells from the liquid culture were harvested at 8,500 g for 12 mins at 4 °C on Beckman Coulter Avanti J26X centrifuge. The cells were frozen in liquid Nitrogen and stored in -80 °C until further use.

2.2.6.4 Large scale production of *MtMrx-1* and *MtAhpE* in *E. coli* BL21 DE3 cell strain

Overnight inoculum of the transformed plasmid in *E. coli* BL21 DE3 was setup in Kanamycin enriched LB at 37 °C. This was used to scale up the large culture next morning, to a starting OD₆₀₀ of 0.1. To produce *MtMrx-1* and *MtAhpE*, liquid cultures were incubated in Kanamycin (30ug/ml) LB medium at 37 °C with shaking of 180 rpm, until an OD₆₀₀ of 0.6 - 0.7 was achieved. Expression of *MtMrx-1* and *MtAhpE* was obtained by adding of IPTG to a final concentration of 1 mM. Following incubation for another 16-18 hrs at 20 °C, the cells were harvested at 8,500 x g for 12 mins, at 4 °C on Beckman Coulter Avanti J26X centrifuge. The cells were frozen in liquid Nitrogen and stored in -80 °C until further use.

2.2.6.5 Purification of *MtMtr*, *MtMrx-1* and *MtAhpE*

Cells were lysed on ice by sonication using Bandelin Sonuplus with KE76 tip for 3 x 1 mins in the desired buffer. Buffer containing 50 mM HEPES, 250 mM NaCl, pH 7.5, 10% glycerol, 1 mM PMSF and 1mM Pefabloc^{SC} (BIOMOL) was used for *MtMtr* and 50 mM Tris, 250 mM NaCl, pH 7.5, 1 mM PMSF and 1mM Pefabloc^{SC} (BIOMOL) for *MtMrx-1* and *MtAhpE*. Cell debris were separated by centrifugation at 12 000 x g for 35 mins. Supernatant was filtered (0.45 µm; Millipore) and passed over a 2 ml Ni²⁺-NTA resin column to isolate recombinant protein. His-tagged protein was allowed to bind to the matrix for 1.5 h at 4 °C with continuous mixing and eluted with an imidazole-gradient (25 - 400 mM) in respective buffer.

Fractions containing recombinant proteins were identified by SDS-PAGE (103), pooled and concentrated as required using Centricon (30 kDa molecular mass cut off for *MtMtr*, 10 kDa for *MtAhpE* and 3 kDa for *MtMrx-1*) spin concentrators. The concentrated protein samples were further purified by gel filtration chromatography using a Superdex 200-I (10/300) GL column (GE Healthcare) for *MtMtr* and a Superdex 75 (10/300) GL column (GE Healthcare) for *MtMrx-1* and *MtAhpE*.

2.2.7 Dynamic light scattering experiments

Dynamic light scattering (DLS) of *MtAhpE* in 50 mM Tris-HCl pH 7.5, 250 mM NaCl was performed at concentrations of 5 mg/ml. For *MtMtr*, DLS studies were performed in a buffer containing 50 mM HEPES pH 7.5, 250 mM NaCl at concentrations of 1.5 mg/ml. DLS measurements were carried out using a Malvern Zetasizer Nano ZS

instrument in a low-volume quartz batch cuvette (ZEN2112, Malvern Instruments). For measurement, 12 μl of respective protein solution equilibrated with desired buffer solution was used. After 60 secs. equilibration time, the backscattering at 173° was detected for all proteins. Scattering intensities were analyzed using the inbuilt software Zetasizer to calculate the hydrodynamic diameter (D_H), size, and volume distribution. In order to understand the role played by H_2O_2 in modulating the oligomeric state of *MtAhpE*, measurements were made after adding equimolar concentrations of H_2O_2 (incubation time of 1 and 60 mins) as well as five-fold molar excess of H_2O_2 with an incubation time of 60 mins.

2.2.8 SAXS data collection using a home source NANOSTAR

For all the experiments, SAXS datasets were collected by Dr. Malathy Sony Subramanian Manimekalai from Prof. Gerhard Grüber's team and data were processed under her guidance. SAXS data for recombinant proteins were collected using a NANOSTAR SAXS instrument (Bruker AXS, Karlsruhe, Germany) equipped with MetalJet X-ray source (Excillum, Germany), as shown in Fig. 2.7. The X-ray radiation is generated after the impact of electron to a high-speed jet of liquid gallium alloy ($\lambda = 0.13414 \text{ nm}$ with a potential of 70 kV and a current of 2.857 mA), and collimated through Montel optics, followed by two 500 μm scatter-free pinhole system (Incoatec, Germany), to remove any parasitic scattering. A photon counting VÅNTEC-2000 detector (140 mm x 140 mm) (Bruker AXS, Germany) was used at a sample-to-detector distance of 0.67 m covering the range of momentum transfer $0.16 < q < 4 \text{ nm}^{-1}$, where $q = 4\pi \sin(\theta)/\lambda$, in which 2θ is the scattering angle and λ is the X-ray wavelength. The samples were loaded into a vacuum tight quartz capillary and measured at 15 $^\circ\text{C}$ (288 K) temperature, with a sample volume of 40 μl .

SAXS experiments were carried out in a sample volume of 40 μl at four different concentrations each for *MtMtr* in buffer 50 mM HEPES, pH 7.5, 250 mM NaCl, 1 mM DTT and for *MtMrx-1* in buffer containing 50 mM Tris/HCl, pH 7.5, 250 mM NaCl, 1 mM DTT. The data were collected for 30 mins and for each measurement a total of 6 frames at 5 mins intervals were recorded. For *MtMtr* WT and mutants the SAXS data was collected for 10 mins in presence and absence of substrate (NADPH and MSSM) to study substrate induced conformational changes. SAXS measurement for a standard compact globular protein, lysozyme, at a concentration of 5 mg/ml in buffer containing

100 mM acetic acid, pH 4.5 was also done employing the same strategy as above for comparison. For *MtAhpE*, SAXS experiments were carried out at three different concentrations of *MtAhpE* in buffer containing 50 mM Tris/HCl, pH 7.5, 250 mM NaCl, 1 mM DTT for a sample volume of 40 μ l. In case of oxidised *MtAhpE*, no DTT was added to the sample, and no radiation damage of the protein could be detected in the absence of the reducing agent. The data were collected for 10 mins and for each measurement, a total of 10 frames at 1-min intervals were recorded.

Figure 2.7: BRUKER NANOSTAR SAXS instrument. SAXS instrument located in the School of Biological Sciences, Nanyang Technological University. This instrument is equipped with a MetalJet X-ray source, a sample chamber with temperature control holder inside, and a VANTEC-2000 detector, and the beam is collimated with Montel optics and two scatter-free pinholes.

The scattered X-rays, detected by a two-dimensional area detector, were flood-field and spatially corrected. The flood-field correction rectifies the intensity distortions arising due to the non-uniformity in the pixel to pixel sensitivity differences in the detector using a radioactive source (^{55}Fe) supplied by Bruker AXS, Germany. The spatial correction fixes the inherent geometrical pincushion distortion by placing a mask with the regular pattern before the detector and measuring the deviation from regularity in the detected image. The data were converted to one-dimensional scattering as a function of momentum transfer by radial averaging using the built-in SAXS software (Bruker AXS, Germany). The data were normalized by the incident intensity and transmission of the sample using a strongly scattering glassy carbon of known X-ray transmission. The

transmission of X-rays through the sample is determined by an indirect method, in which the integrated counts per unit time for glassy carbon is related to the relative intensity of the direct beam and hence the transmission of the sample. The data were then tested for possible radiation damage by comparing the different data frames and no changes were observed. The scattering of the buffer was subtracted from the scattering of the sample, and all the scattering data were normalized by the concentration.

2.2.8.1 SAXS data analysis: Primary SAXS data processing

The primary SAXS data processing steps were performed using the program package PRIMUS (104,105). To identify the presence of inter-particle interaction between protein molecules with increasing protein concentrations (concentration dependence), the scattering profiles from different protein concentrations were overlapped and compared at low angle region. If all scattering profiles overlapped nicely, it was considered that there is no concentration dependence in the scattering profiles. If the low angle of scattering profiles shifted upward or downward, then there is concentration dependence present in the scattering profiles and the datasets were either extrapolated to infinite dilution or merged together. A non-linear dependence of $\log(I(q))$ vs. q^2 indicates the presence of aggregation (Fig. 2.8).

Figure 2.8: Guinier plot. A non-linear dependence of $\log(I(q))$ vs. q^2 indicates the presence of aggregation. Scattering from aggregated samples strongly influences the entire data set and no further processing should be performed. Aggregation in the sample can be reduced or eliminated by varying buffer conditions, centrifugation, and filtration. The figure was taken from Putnam et. al (106).

Two important parameters, including forward intensity ($I(0)$) and radius of gyration (R_g), were determined from Guinier approximation and pair-distance distribution ($P(r)$) function. The forward intensity ($I(0)$) is the radiation intensity scattered through zero angle ($q = 0$). Simply put, forward intensity ($I(0)$) is intensity at $q=0$ and is proportional

to mass and volume. This parameter cannot be measured and it can only be determined through extrapolation, because the detected intensity cannot be differentiated from the un-scattered intensity that passes through the sample (107,108). $I(0)$ is not dependent on the particle shape and this value can be used to determine the molecular mass of a particle (108). The radius of gyration (R_g) is the root-mean-squared distance of each scatterer from the center of particle. This parameter is shape-dependent, whereby the objects with same volume but with different shapes have different R_g values (107,108). The Guinier approximation (109,110) states that at very low angle ($q < 1/R_g$), the intensity can be represented as:

$$I(q) = I(0)e^{\frac{-q^2 R_g^2}{3}}$$

This means that when the scattering intensity is plotted as $\ln I(q)$ vs q^2 , the intensity exists as a linear function, and the R_g can be calculated from the slope of the curve and $I(0)$ can be derived from the y-intercept of the curve. However, this relationship is only valid for $qR_g < 1.3$ for globular protein. Protein aggregation and inter-particle interaction for all the protein samples were analyzed using the scattering data from the Guinier region (109), where the non-linear behavior at this region indicates the formation of aggregation. During SAXS data analysis, this R_g value is computed using a command-line program AUTORG (111) to speed up the R_g estimation.

To validate these parameters, the R_g and $I(0)$ were also computed based on the pair-distance distribution ($P(r)$) function. This method considers the whole scattering data while computing these values and thus, the computation of both parameters can be more precise (107). The $P(r)$ function describes all the distances between two electrons in a protein particle as a paired-set, and can be computed using the software GNOM (112). This function is derived from the scattering intensity by an inverse Fourier transformation (112,113) and their relationship is represented as:

$$p(r) = \frac{r^2}{2\pi^2} \int q^2 I(q) \frac{\sin qr}{qr} dq$$

Additionally, the maximum dimension (D_{max}) of a particle can also be determined from the $P(r)$ function, where D_{max} is the maximum pair-distance that can be found within the particle.

2.2.8.2 SAXS data analysis: *ab initio* modeling using SAXS

The *ab initio* low-resolution models of the proteins were reconstructed using two programs DAMMIN and DAMMIF (114). DAMMIF generates the *ab initio* models based on the bead modeling method, where the particle is represented as a collection of dummy atoms within a search volume, and each dummy atom can come from the particle or the solvent. A search volume is a sphere that has the diameter equal to the D_{max} determined from $P(r)$ function. The program considers the low angle data in a range of $q < 0.2 \text{ nm}^{-1}$. DAMMIF utilizes the simulated annealing to construct the bead model, and disconnected models are immediately discarded from the simulation, which speed up the simulation time. The calculation of theoretical scattering amplitudes of the models is only performed to the connected models, and a final model that has a lowest discrepancy (χ^2) between the theoretical scattering profile and the experimental data is selected.

To validate the reproducibility of the shape reconstructions, 20 independent *ab initio* reconstructions were performed for each scattering data, and all 20 reconstructed models were used as input for the program suite DAMAVER (115). DAMAVER has a set of programs that align these reconstructed models, select the typical model, and construct an averaged model. The normalized spatial discrepancy (NSD) values are computed between two models, where the two models are similar if the NSD is close to one. Subsequently, a final mean NSD over all model-pairs and their dispersion of NSD are calculated. The reconstructed models which have a high NSD dispersion are then discarded, while the rest of the models are averaged. Beside this, the averaged excluded volume (V_{Ex}) computed from DAMAVER using the DAMMIF models as input, was used to estimate the molecular mass of the protein (116).

2.2.8.3 SAXS data analysis: Protein folding and flexibility assessment

To qualitatively access the globular nature of the particle, a Kratky plot can be generated by transforming the scattering pattern of the particle as $q^2 I(q)$ vs q . For a globular protein with a well-defined and solvent-accessible surface, its Kratky plot will exhibit a typical bell-curve profile with a well-defined maximum. Conversely, for a random chain, the Kratky plot exhibits a plateau or slightly increase at high q region. The Kratky plot cannot distinguish clearly between partially unfolded and fully folded particles since both exhibits a similar profile. To overcome this, a normalized Kratky plot

is generated ($(qR_g)^2(I(q)/I(0))$ vs qR_g), by normalizing the intensity $I(q)$ with the forward intensity $I(0)$, and q with the radius of gyration (R_g) of the particle (108,117-119).

Therefore, the normalized Kratky plot is independent to the particle size and molecular mass, where $I(0)$ is related to the particle molecular mass. This allows one to compare the well-folded globular particle, extended folded particle and fully disordered particle. For the well-folded globular particle, despite their particle sizes, their normalized Kratky plots exhibit a well-defined bell-curve shape, with a maximum $(qR_g)^2(I(q)/I(0))$ value of 1.104 at qR_g value of $\sqrt{3}$ (Fig. 2.9). For the fully disordered particles, their normalized Kratky plots lose the bell-curve shape, and the $(qR_g)^2(I(q)/I(0))$ values increase with the increased qR_g values. For the particles exhibiting the shape in between, their normalized Kratky plots exhibit a profile that lies in between these two extremes.

Figure 2.9: Kratky plot. The Kratky plot identifies unfolded samples. Globular macromolecules follow Porod's law and have bell-shaped curves. Extended molecules, such as unfolded peptides, lack this peak and have a plateau or are slightly increasing in the larger q range. The figure was adopted from Putnam et. al (106).

The theoretical scattering curves from atomic structures were generated and evaluated against experimental scattering curves using CRY SOL (120). Afterwards the theoretical scattering intensity from the high resolution domain structures, together with its DR-chain, are computed using CRY SOL (120) and compared with the experimental scattering intensity, generating the discrepancy called χ^2 value. The χ^2 value is used to analyze the goodness of fit between the experimental and calculated data. A better fit between the experimental and calculated data gives rise to a lower χ value. Therefore, in most of the data fitting and model building programs, the χ value is minimized to generate

a best solution that explains the experimental data. Superimposition between *ab initio* reconstruction and the atomic model was performed using the software SUPCOMB (121). The program represents each input structure as an ensemble of points, then minimizes a normalized spatial discrepancy (NSD) to find the best alignment of two models. NSD is a measure of quantitative similarity between sets of three-dimensional points. The molecular mass was also determined using the volume of correlation, V_c , defined as the ratio of $I(0)$ to its total scattered intensity, is contrast and concentration independent (122).

2.2.8.4 SAXS data analysis: Rigid-body modeling using SAXS

Rigid Body modelling for *MtMtr* was performed using the software CORAL (116) by docking the individual domains of the I-TASSER model against the experimental SAXS data. CORAL modelling involves a simulated annealing protocol that finds the optimal positions and orientations of available high-resolution models of domains and the approximate conformations of the linker portions of polypeptide chain. The rigid-body models are superimposed into the reconstructed low resolution envelopes using the software SUPCOMB (121), where it aligns two models by minimizing the normalized spatial discrepancy (NSD), which is a qualitative measurement of the similarity between the sets of three-dimensional points from two models.

2.2.9. Crystallization of *MtAhpE*-MSH co-crystals

2.2.9.1 Crystallization of native *MtAhpE*

Crystallization of native *MtAhpE* was attempted using vapor diffusion method in the condition originally published (86). The crystallization condition was 1.8 M sodium malonate, 0.1 M sodium acetate, pH 5. The protein concentration used was 2 mg/ml and 5 mg/ml. It was optimized via Hampton research additive screen to decrease the nucleation and promote further crystal growth. The concentration of sodium malonate was also optimized in a range of 1.6 M to 2.2 M, in order to optimize the size of the crystals. Cryoprotectant solution was also optimized by testing different percentages of glycerol in 1.8 M sodium malonate.

2.2.9.2 Crystal diffraction test for native *MtAhpE*

The diffraction of *MtAhpE* crystals were tested in-house at 100 K on a Rigaku RAXIS IV image plate detector with a Rigaku RA-Micro 7 HFM rotating copper anode

generator (Rigaku/MSD). All the diffraction data were indexed, integrated and scaled using the HKL2000 suite program (94).

2.2.9.3 Co-crystallization of *MtAhpE* with MSH and NAC

In order to obtain *MtAhpE*-MSH co-crystals, *in vitro* mycothylation was performed as described in [27], wherein MSSM was first reduced by 5 mM DTT. Meanwhile, the reduced *MtAhpE* (0.3 μ M) was oxidized with an equimolar amount of H₂O₂ and then incubated in the presence of 10 molar excess of reduced MSH. After 30 mins incubation, crystal drops were set up as described earlier for native *MtAhpE* crystals (86). *AhpE*-MSH co-crystals were further optimized and final diffraction quality crystals were obtained in the condition of 1.8 M sodium malonate (pH 5.0), 0.1 M sodium acetate (pH 4.5) and 5% (v/v) glycerol. In case of NAC, the oxidized *MtAhpE* protein was directly incubated with 5 molar excess of NAC and crystal drops were set in the same condition. The crystals were flash frozen in liquid nitrogen at 100 K in a crystallization buffer containing 25% (v/v) glycerol.

2.2.9.4. Synchrotron data collection and processing of X-ray diffraction data

The synchrotron datasets were collected under the guidance of Dr. Asha Balakrishna from Prof. Gerhard Grüber's team. X-ray diffraction data of *MtAhpE* crystals were collected at the synchrotron beamline 13B1 (National Synchrotron Radiation Research Centre, Taiwan) using the Q315 detector, as a series of 0.5 oscillation images, covering a crystal rotation range of 200° on the cryo-cooled crystals at 100 K. The dataset was indexed, integrated, and scaled using HKL2000 suite. The Matthews coefficient (123) confirmed the presence of four molecules in the asymmetric unit with a corresponding solvent of 53%.

The structure was solved by molecular replacement using the model of *MtAhpE* (PDB ID: 1XXU; [5]). Manual corrections of the model were done with COOT (98) and refinement was carried out using REFMAC (95) of CCP4 suite. A check on the stereochemical quality of the final model was assessed using PROCHECK (124), identifying that 90.5% of all the residues were within the core regions of the Ramachandran plot and 9.5% were within the allowed regions. Molprobit analysis (101,125) indicated that the overall geometry of the final model ranked in the 100th percentile (Molprobit score of 1.26). The clash score for all atoms was 2.65 corresponding to a 100th percentile ranking of structures of comparable resolution. Omit

difference maps were used to remove model bias. Refined omit maps were calculated by omitting from the model a region of 8 Å around amino acid C45 and refining the model. The active cysteine (C45-SH) was modelled as cysteine sulfenic acid (C45-SOH) in two of the chains, to accommodate for the extra density connected to the sulphur atom of C45-SH. All the figures were drawn using PyMOL (126,127). The atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB ID: 5ID2).

2.2.9.5 Crystallization studies on *MtMtr*

Crystallization was carried out with the help of Dr. Asha Balakrishnan from Prof. Gerhard Grüber's team. Various commercially available crystallization kits were used as listed in the following table 2.1. These kits contain several previously made conditions of buffer with additives, precipitants and cryoprotectants which have been tried and tested to produce crystals. Hanging drops were set up by mixing 1 µl of the purified *MtMtr* in buffer 50 mM HEPES, pH 7.5, 250 mM NaCl and 10% glycerol with 1 µl of the reservoir solution and equilibrated against 200 µl reservoir solution.

Table 2.1 Various crystallization screens used for *MtMtr*

• Hampton Crystal Screen I and II
• Wizard I, II, III and IV
• Precipitant Synergy I and II
• PEG/ION I and II
• MORPHEUS-I and II
• INDEX-I and II
• CRYO I and II
• Grid Screen Ammonium sulphate
• Grid Screen MPD
• Grid Screen PEG 6000

2.2.10 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is one of the principal techniques used to study the structural and functional aspects of macromolecules such as proteins. The technique enables to determine the solution structure of proteins and to study protein-ligand and protein-protein interactions. As hydrogen is one of the most receptive and abundant NMR active nuclei besides other isotopes such as ^{15}N , ^{13}C , ^{31}P etc., it can be observed in magnetic

resonance. Any nuclei having mass number, which exhibits net intrinsic magnetic momentum and angular momentum, can be defined as NMR active. All the NMR experiments were collected on Bruker Avance 600/700 MHz machines at SBS NMR core facility.

2.2.10.1 ^{15}N single and ^{13}C ^{15}N double labeling of proteins

Reagents: Minimal Media (M9)
42 mM Na_2NPO_4
22 mM KH_2PO_4
8.5 mM NaCl
1 g/l $^{15}\text{NH}_4\text{Cl}$
0.1 mM CaCl_2
2 mM MgSO_4
10 g D-Glucose
30 μM FeCl_3
5 ng/l Thiamine
Antibiotics *

* specific to respective constructs

For NMR experiments, either single (^{15}N) or double (^{13}C - ^{15}N) labelled recombinant proteins were produced in *E. coli* BL21 (DE3) cells and purified according to the established protocols described earlier. The clones were grown in M9 minimal media to yield labelled protein. 30 ml of overnight culture was pelleted down at low centrifugal force at room temperature and washed with minimal media and then transferred to large volume of minimal media with OD_{600} of 0.1. The cultures were grown in incubator shaker at 37 °C till OD_{600} of 0.6 - 0.7 was achieved. The protein production was induced with IPTG to final concentration of 1 mM overnight at 20 °C. Cells were harvested and frozen in liquid nitrogen and stored at -80 °C till purification.

2.2.10.2 Production and purification of (3- ^{13}C)-Cys-labelled *MtAhpE* and *MtMrx-1* and ^{15}N -Arg-labelled *MtAhpE*

E. coli BL21 (DE3) transformed cells were plated on LB-agar, from which a single colony was selected to prepare a 15 ml LB seed culture. After overnight growth, the culture was centrifuged at 3,000 x g for 5 mins. at 4 °C to pellet out the cells. The cells were washed and subsequently suspended in 1 liter M9-minimal media supplemented

with MgSO_4 , CaCl_2 , thiamine, FeCl_3 , trace elements and 19 unlabeled amino acids (128) with the exception of ($3\text{-}^{13}\text{C}$)- L-cysteine or $^{15}\text{N}_4$ L-Arginine as needed. Starting with an OD_{600} of 0.08, the cells were allowed to grow to an OD_{600} of 0.6 at 37 °C at which point the culture was induced with IPTG to a final concentration of 1 mM. In addition, 25 mg of ($3\text{-}^{13}\text{C}$)-L-Cysteine or $^{15}\text{N}_4$ L-Arginine, were added as required and the culture was allowed to grow overnight at 20 °C. The cells were harvested by centrifugation at 8,500 x g for 15 mins., frozen in liquid nitrogen and stored at -80 °C till purification.

2.2.10.3 1D- ^1H and multi-dimensional (2D, 3D) ^{13}C - ^{15}N NMR spectroscopy

The NMR experiments were performed under the guidance of Dr. Wilson Nartey and Dr. Shin Joon. 1D NMR spectra of various proteins were collected at temperatures ranging from 283 K to 308 K on core facility Avance 600 MHz instruments (Bruker, Billerica, MA). Un-labelled or labelled protein sample in phosphate buffer, pH 6.8 was used in presence of 10% D_2O (v/v) to record the spectrum. For 2D experiments like HSQC (Hetero nuclear Single Quantum Coherence), ^{15}N labelled proteins in 90% H_2O and 10% D_2O in 50 mM phosphate buffer (pH 6.8) were used to collect data. Different parameters including temperature, buffer, and protein concentration were optimized before making final measurements. Pulse was also calibrated accordingly. ^{15}N - ^{13}C labelled samples were utilized to collect 3D spectra such as CBCA(CO)NH, HNCACB in 10% (v/v) D_2O lock signal. Baseline corrections were applied wherever necessary. The proton chemical shift was referenced to the methyl signal of DSS (2, 2-dimethyl-2-silapentane-5-sulphonate (Cambridge Isotope Laboratories)) as an external reference to 0 ppm.

2.2.10.4 ^1H - ^{13}C and ^1H - ^{15}N HSQC-NMR spectroscopy of *MtAhpE*

The NMR experiments were performed under the guidance of Dr. Wilson Nartey. Purified ^{13}C -Cysteine labelled and ^{15}N -Arginine labelled *MtAhpE* was prepared for NMR spectroscopy experiments by buffer-exchanging into phosphate buffer (50 mM phosphate, 100 mM NaCl, pH 6.8). 10% D_2O was added to and mixed with 180 μl of the sample. Indirect referencing using 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was used to calibrate the ^{13}C chemical shifts. The sample was centrifuged and transferred into a Shegami tube. All measurements were acquired on a 700-MHz NMR spectrometer with 4 RF channels (Bruker Avance, Bremen, Germany) equipped with 5 mm z-gradient TXI (Triple Resonance Probes) cryoprobe, one 4 mm HR-MAS probe, one 5 mm z-gradient

PATXI probe. ^1H - ^{13}C HSQC was acquired with 96 scans using the pulse-program *hsqcetf3gpsi2* (129,130). In this experiment, *MtAhpE* data were collected in its oxidized state and in the presence of either 10 molar excess of hydrogen peroxide (H_2O_2), *tris*(2-carboxyethyl) phosphine (TCEP), DTT, reduced MSH or reduced MSH plus H_2O_2 . MSH was purchased from JEMA Biosciences, San Diego, CA, USA. MSSM was reduced as per the protocol provided by the supplier. Accordingly, MSSM was first dissolved in 50 mM Tris/HCl, pH 8.0. DTT was then added to a concentration of 20 mM. Afterwards, the solution was incubated at room temperature for 20 mins. The NMR sample was incubated for 30 mins at RT with the respective reducing agent before data collection. In the case of ^{15}N -Arginine-labelled titration, a five-molar excess of MSH was used. The MSH analogue, NAC, a low molecular weight thiol that can be formed from MSH enzymatic degradation in *Mtb* was also used to study the reduction of *MtAhpE*. In order to investigate the slow oxidation of C45-SH to C45-SOH and subsequently to C45-SO₂H and C45-SO₃H, a set of NMR experiments (12 hours) were done with ^1H - ^{13}C -labelled *MtAhpE*. In order to confirm whether *MtMrx-1* forms a mixed disulphide with *MtAhpE* as previously shown by mass spectrometry (69), a three-molar excess of *MtMrx-1* was incubated with *MtAhpE* and the state of C45 was observed using NMR spectroscopy.

These experiments were designed based on the knowledge that a chemical shift in the ^{13}C -dimension of a cysteine can be used to characterize its redox state. In brief, for a cysteine, the H β -chemical shifts are in the range of 2.5 to 3.8 ppm and C β -chemical shifts are in the range of 25.0 to 60.0 ppm based on the redox state of the cysteine. Accordingly, peaks of a ^1H - ^{13}C -HSQC spectrum can be used to unambiguously assign the correct redox state of a cysteine residue. If the C β -shift is less than 32.0 ppm or greater than 35.0 ppm, the redox state is assigned to be reduced or oxidized, respectively. The presence of other cysteine derivatives can also be obtained by virtue of their chemical shifts. Crane *et al.* (131) have systematically reported chemical shifts for various redox states of a cysteine (C-SH = 29.5 ppm, C-S-OH = 39.6 ppm, C-SS-Cys = 38.7 ppm, C-S(=O)OH = 58.8 ppm, and C-S(=O)₂OH = 56.0 ppm).

2.2.10.5 NMR data collection and processing

All NMR experiments were performed at 20 °C on 700 MHz NMR spectrometer (Bruker Avance) equipped with 5 mm triple-resonance ($^1\text{H}/^{15}\text{N}/^{13}\text{C}$) single axis gradient probes and a cryoprobe. All the protein samples were prepared in respective buffer and

10 % D₂O. D₂O was added for the locking of the sample. Appropriate pulse calibrations and other parameters including temperature, buffer and protein concentration were optimized before making final measurements. To assign the backbone of *MtMrx-1*, The resonance assignments were achieved from conventional 2D and 3D heteronuclear NMR experimental data, collected using 1.0 mM of uniformly ¹³C/¹⁵N-labeled sample. ¹H-¹⁵N HSQC, HNCACB, CBCA(CO)NH, HNCA and HN(CO)CA experiments were performed in order to obtain the backbone resonance assignment. In all practical cases, 3D triple resonance spectra were recorded in non-uniform sampling (NUS) (132) of the indirect dimension as 20-25% sampling rates and reconstructed using compressed sensing (CS) scheme using MDDNMR software (133,134). All data were processed with NMR-Pipe and NMRDraw (135) and spectra were analyzed using SPARKY (136).

In order to investigate the molecular interaction between *MtMtr* and *MtMrx-1*, reduced ¹⁵N labelled *MtMrx-1* was prepared as described above. Unlabelled *MtMtr* was titrated stepwise into a 0.1 mM reduced ¹⁵N labelled *MtMrx-1*. ¹H-¹⁵N HSQC spectra were recorded at molar ratios of 1(*MtMrx-1*):1(*MtMtr*), 1:2 and 1:3.5, respectively. To study the interaction between *MtAhpE* and *MtMrx-1*, titration of 0.25 mM of labelled *MtMrx-1* with oxidized *MtAhpE* was carried out to achieve incremental molar equivalents of 0.5, 1, 2, and 4.

2.2.10.6 Resonance assignment

In the initial stage, protein molecules must be assigned in order to investigate NMR data for analysis of any kind of interaction or dynamic studies. Scalar couplings, via dipolar coupling and via chemical shift are useful information for sequence specific assignment of protein backbone. Depending on the type of sample whether it is a unlabeled or isotopically labelled protein (137), different strategies are employed to assign the resonance. In NMR, a 1D spectrum is useful to investigate dispersion of resonance line of protein and also it gives an idea about the folding of the protein. But by doing 1D experiment it is impossible to resolve all resonance lines in a spectrum for protein. Assignments can be done using the sequential assignment approach based on Homonuclear 2D Correlation Spectroscopy (COSY) or Total Correlation Spectroscopy (TOCSY) (138) and 2D Nuclear Overhauser Effect Spectroscopy (NOESY). The TOCSY experiment is basically used to identify the resonance position within each amino acid spin system and the NOESY experiment is used for the sequential assignment

of the spin systems present in the amino acids. For a protein/peptide NMR spectrum, amide hydrogens resonances are generally the best dispersed and observed between 6-12 ppm. The side chain resonance assignments are generally best done by examining the peaks in the backbone amide regions. This process is known as the sequential assignment strategy developed by Wüthrich *et al* (139).

For larger molecules (>10 kDa) the sequential assignment strategy by using above assignment approach is not useful, because overlaps of the NMR signals make the assignment complicated. For that it requires the addition of a third dimension by the replacement of naturally abundant isotope ^{14}N with ^{15}N and ^{12}C by ^{13}C . Hence, assignment is based on triple resonance experiments allowing further decrease in the overlap of NMR signals. There are six triple resonance experiments commonly used in chemical shift assignments of the backbone atoms of the protein: HNCA, HN(CO)CA, HNCACB, CBCA(CO)HN, HNCO and HN(CA)CO.

In HNCA experiment, the magnetization is passed from ^1H to ^{15}N and then via the N- $\text{C}\alpha$ J-coupling to the $^{13}\text{C}\alpha$ and then back again to ^{15}N and ^1H hydrogen for detection. The chemical shift is evolved for $^1\text{H}^{\text{N}}$ as well as the $^{15}\text{N}^{\text{H}}$ and $^{13}\text{C}\alpha$, resulting in a 3-dimensional spectrum (140).

Figure 2.10 Magnetization transfer scheme for 3D NMR HNCA experiments for backbone assignment showing the magnetization transfer through NMR active nuclei. The magnetization is passed from ^1H to ^{15}N and then via the N- $\text{C}\alpha$ J-coupling to the $^{13}\text{C}\alpha$ and then back again to ^{15}N and ^1H hydrogen for detection. The figure is based on (140).

Since the amide nitrogen is coupled both to the $C\alpha$ of its own residue and that of the preceding residue, both these transfers occur and peaks for both $C\alpha$ s are visible in the spectrum. However, the coupling to the directly bonded $C\alpha$ is stronger and thus these peaks will appear with greater intensity in the spectra (140)

In HN(CO)CA, the magnetisation is passed from 1H to ^{15}N and then to ^{13}CO . From here it is transferred to $^{13}C\alpha$ and the chemical shift is evolved. The magnetisation is then transferred back via ^{13}CO to ^{15}N and 1H for detection. The chemical shift is only evolved for the $^1H^N$, the $^{15}N^H$ and the $^{13}C\alpha$, but not for the ^{13}CO . This results in a spectrum which is like the HNCA, but which is selective for the $C\alpha$ of the preceding residue (140).

Figure 2.11 Magnetization transfer scheme for 3D NMR HN(CO)CA experiments for backbone assignment showing the magnetization transfer through NMR active nuclei. The magnetization is passed from 1H to ^{15}N and then to ^{13}CO . From here it is transferred to $^{13}C\alpha$ and the chemical shift is evolved. The magnetization is then transferred back via ^{13}CO to ^{15}N and 1H for detection. The figure is based on (140).

In HNCACB, the magnetization is transferred from $^1H\alpha$ and $^1H\beta$ to $^{13}C\alpha$ and $^{13}C\beta$, respectively, and then from $^{13}C\beta$ to $^{13}C\alpha$. From here it is transferred first to $^{15}N^H$ and then to $^1H^N$ for detection. Transfer from $C\alpha_{i-1}$ can occur both to $^{15}N_{i-1}$ and $^{15}N_i$, or viewed the other way, magnetization is transferred to $^{15}N_i$ from both $^{13}C\alpha_i$ and $^{13}C\alpha_{i-1}$. Thus, for each NH group there are two $C\alpha$ and $C\beta$ peaks visible. The chemical shift is evolved simultaneously on $^{13}C\alpha$ and $^{13}C\beta$, so these appear in one dimension. The chemical shifts evolved in the other two dimensions are $^{15}N^H$ and $^1H^N$ (140).

Figure 2.12 Magnetization transfer scheme for 3D NMR HNCACB experiments for backbone assignment showing the magnetization transfer through NMR active nuclei. The magnetization is transferred from $^1\text{H}\alpha$ and $^1\text{H}\beta$ to ^{13}Ca and $^{13}\text{C}\beta$, respectively, and then from $^{13}\text{C}\beta$ to ^{13}Ca . From here it is transferred first to $^{15}\text{N}^{\text{H}}$ and then to $^1\text{H}^{\text{N}}$ for detection. The figure is based on (140).

In CBCA(CO)HN, the magnetization is transferred from $^1\text{H}\alpha$ and $^1\text{H}\beta$ to ^{13}Ca and $^{13}\text{C}\beta$, respectively, and then from $^{13}\text{C}\beta$ to ^{13}Ca . From here it is transferred first to ^{13}CO , then to $^{15}\text{N}^{\text{H}}$ and then to $^1\text{H}^{\text{N}}$ for detection. The chemical shift is evolved simultaneously on ^{13}Ca and $^{13}\text{C}\beta$, so these appear in one dimension. The chemical shifts evolved in the other two dimensions are $^{15}\text{N}^{\text{H}}$ and $^1\text{H}^{\text{N}}$. The chemical shift is not evolved on ^{13}CO (140).

Figure 2.13 Magnetization transfer scheme for 3D NMR CBCA(CO)HN experiments for backbone assignment showing the magnetization transfer through NMR active nuclei. The magnetization is transferred from $^1\text{H}\alpha$ and $^1\text{H}\beta$ to ^{13}Ca and $^{13}\text{C}\beta$, respectively, and then from $^{13}\text{C}\beta$ to ^{13}Ca . From here it is transferred first to ^{13}CO , then to $^{15}\text{N}^{\text{H}}$ and then to $^1\text{H}^{\text{N}}$ for detection. The figure is based on (140).

In HNCO, magnetization is passed from ^1H to ^{15}N and then selectively to the carbonyl ^{13}C via the $^{15}\text{N}^{\text{H}}\text{--}^{13}\text{CO}$ J-coupling. Magnetization is then passed back via ^{15}N

to ^1H for detection. The chemical shift is evolved on all three nuclei resulting in a three-dimensional spectrum (140).

Figure 2.14 Magnetization transfer scheme for 3D NMR HNCO experiments for backbone assignment showing the magnetization transfer through NMR active nuclei. The magnetization is passed from ^1H to ^{15}N and then selectively to the carbonyl ^{13}C via the ^{15}N - ^{13}C J-coupling. Magnetization is then passed back via ^{15}N to ^1H for detection. The figure is based on (140).

Finally, in HN(CA)CO the magnetization is transferred from ^1H to ^{15}N and then via the N- $\text{C}\alpha$ J-coupling to the $^{13}\text{C}\alpha$. From there it is transferred to the ^{13}CO via the $^{13}\text{C}\alpha$ - ^{13}CO J-coupling. For detection, the magnetization is transferred back the same way: from ^{13}CO to $^{13}\text{C}\alpha$, ^{15}N and finally ^1H .

Figure 2.15 Magnetization transfer scheme for 3D NMR HN(CA)CO experiments for backbone assignment showing the magnetization transfer through NMR active nuclei. the magnetization is transferred from ^1H to ^{15}N and then via the N- $\text{C}\alpha$ J-coupling to the $^{13}\text{C}\alpha$. From there it is transferred to the ^{13}CO via the $^{13}\text{C}\alpha$ - ^{13}CO J-coupling. For detection, the magnetization is transferred back the same way: from ^{13}CO to $^{13}\text{C}\alpha$, ^{15}N and finally ^1H . The chemical shift is only evolved on ^1H , ^{15}N and ^{13}CO and not on the $^{13}\text{C}\alpha$. The figure is based on (140).

The chemical shift is only evolved on ^1H , ^{15}N and ^{13}CO and not on the $^{13}\text{C}\alpha$. The result is a three-dimensional spectrum. Because the amide nitrogen is coupled both to the $\text{C}\alpha$ of its own residue and that of the preceding residue, both these transfers occur and transfer to both ^{13}CO nuclei occurs. Thus, for each NH group, two carbonyl groups are observed in the spectrum. But because the coupling between N_i and $\text{C}\alpha_i$ is stronger than that between N_i and $\text{C}\alpha_{i-1}$, the $\text{H}_i\text{-N}_i\text{-CO}_i$ peak generally ends up being more intense than the $\text{H}_i\text{-N}_i\text{-CO}_{i-1}$ peak (140).

2.2.11 HADDOCK modelling of *MtAhpE-Mrx-1* ensemble.

The docking studies were performed under the guidance of Dr. Malathy Sony Subramanian Manimekalai from Prof. Gerhard Grüber's team. Experimentally identified binding interfacial residues were used to perform docking studies and to model the *MtAhpE-Mrx-1* ensemble using the HADDOCK webserver (141,142). The coordinates for *MtMrx-1* were fetched from the Protein Data Bank (PDB ID: 2LQQ; (83)). Surface-exposed residues experiencing significant perturbation (CSPs and Intensity loss above 1 δ) during titration were used as restraints. Since HADDOCK requires a set of ambiguous interaction restraints, residues directly interacting at the interface need to be specified as "active" residues and neighbouring residues as "passive". The experimentally obtained interacting residues were set as active. In the case of *MtAhpE*, a dimer comprising chain *A* and *B* of coordinates from the PDB database (PDB ID: 5ID2; (143)) was renumbered to treat each monomer as a unique entity. Briefly, the submitted subunits were placed in space with an approximate distance of 25 Å and a complex was formed through rigid body energy minimization, which resulted in an initial 1,000 structures. The top 200 lowest energy models from the above minimization were subjected to a simulated annealing in torsion angle space procedure and subsequent flexible refinement in explicit solvents. Models, which fulfilled the interfaced ligand r.m.s.d cut-off 5 Å, were clustered into seven groups. The best model in the highest scoring cluster was selected for analysis. The model was visualized using PyMOL (126,127).

2.2.11.1 HADDOCK modelling of *MtAhpE-MSH* complex

The docking studies were performed by Dr. Malathy Sony Subramanian Manimekalai from Prof. Gerhard Grüber's team. The presented crystallographic structures of *MtAhpE-SOH* (chain *D*) and *MtAhpE-SH* (chains *B* and *C*) clearly indicated the influence of R116 and $\beta 9/\alpha 5$ loop on the active center (C45) and its role in the

coordination of peroxidase activity. Therefore, both the oxidized and reduced (chain C) forms of *MtAhpE* were used for docking studies. The three-dimensional structure of MSH was retrieved from the ZINC-database (144) and docked at the defined active site using HADDOCK (High Ambiguity Driven DOCKing) (141,142). The channel around residues C45, T42, and R116 were used to define the active site region for performing the molecular docking studies. Since HADDOCK requires a set of ambiguous interaction restraints, the residues C45, T42 and R116 are specified as “active” residues and the neighbouring residues as “passive”. Similar docking studies were also performed for the bromide-soaked reduced structure (PDB ID: 1XXU (86)) and the previously reported oxidized structure (PDB ID: 1XVW (86)).

2.2.12 Isothermal titration calorimetry

ITC was used to study the macromolecular interactions in the solution. It permits determination of binding affinities and also the binding constant of protein-protein interaction (145). Thermodynamic parameters such as entropic and enthalpic components of such binding reactions can be resolved using ITC. Interaction of NAC with *MtAhpE* was monitored by ITC. The NAC was then loaded in the syringe and titrated in 1.5 µl injections against variable concentration of protein placed in the sample cell in a MicroCal iTC200 microcalorimeter (Microcal, Northampton, UK). All experiments were performed under identical conditions at 25 °C. The binding curve was determined by least squares method and the binding isotherm was fitted using Origin v7.0 (Microcal) assuming a single-site binding model.

2.2.13 Measurement of NADPH-dependent disulfide reductase activity

NADPH-dependent disulfide reductase activity was monitored at 340 nm following the decrease in NADPH absorbance using a stopped flow spectrophotometer Applied Photophysics SX20. The assay was carried out at 25 °C containing a 50-mM phosphate buffer at pH 7.0, 100 mM ammonium sulfate and 0.5 mM EDTA. One syringe arm contained a final concentration of 200 µM of NADH, and the other arm contained a final concentration of 0.5 µM *MtMtr* with increasing amounts of MSSM (10 µM, 20 µM, 40 µM and 50 µM, respectively).

Results

3. Results

Mtr/Mrx-1/MSH dependent recycling of redox-active proteins in *Mycobacteria*

Mycobacteria employ a versatile machinery of the MSH-dependent system, containing the proteins Mtr, Mrx-1 and AhpE. The NADPH dependent Mtr regulates the balance of oxidized–reduced MSH, to ensure a reductive intracellular environment for optimal functioning of its proteins even upon exposure to oxidative stress as well as recycling of various redox-active proteins such as AhpE via the Mrx-1/MSH pathway (Fig. 3.1).

Figure 3.1: Electron transfer in the Mrx-1/MSH-dependent recycling of *MtAhpE*. An electron is transferred from NADPH to MSH via Mtr which recycles MSSM to MSH. *MtAhpE*-SH is oxidized by a peroxide to form a sulfenic acid. The latter is then directly reduced by MSH through an intermediate disulfide formation, followed by reduction by *MtMrx1* or directly by two molecules of MSH. While both *MtMrx-1* and *MtAhpE* have been structurally characterized, there is no structural information available on *MtMtr*. Figure is adapted from (146)

For clear understanding, the major findings of this work are presented in form of three chapters. In chapter 3, the cloning, expression, and purification of *MtMtr* has been presented, wherein details of use of the chaperone GroEL/ES to aid in solubility of recombinant *MtMtr* is described. This is followed by the determination of the first low-resolution SAXS structure of Mtr and crystallization attempts to unravel the high-resolution structure. Chapter 4 describes cloning, expression, and purification of *MtMrx-1* and new insights obtained upon NMR titration of (*MtAhpE* and *MtMrx-1*) and (*MtMrx-1* and Mtr). The chapter also describes modelling of the *MtAhpE*-*MtMrx-1* complex

based on NMR restraints using HADDOCK. Finally, chapter 5 presents the cloning, expression, purification and crystallization of *MtAhpE*. The SAXS structure of *MtAhpE* has been determined to confirm the oligomeric state during redox modulation. This is followed by NMR experiments which allowed to unravel the new pathway for recycling of *MtAhpE* in the absence of *MtMrx-1*.

Structural and functional insights into the Mtr from *Mtb*

3.1 Introduction

In past, *MtMtr* has been successfully expressed in *M. smegmatis* and enzymatically characterised (77,78). It has been shown that *MtMtr* is highly specific for MSSM as substrate and lacks activity towards the analogous glutathione or trypanothione. So far, no structural data are available and earlier attempts to recombinantly express *MtMtr* in *E. coli* resulted in an unstable protein (69).

In this chapter, cloning, expression, production and purification of *MtMtr* from *E. coli* cells are described. 1D NMR experiments were performed to judge proper protein folding and NADPH consumption assays verified that the protein was enzymatically active. This is followed by SAXS experiments which enabled us to deduce low-resolution structure of *MtMtr* and together with DLS studies the oligomeric state of *MtMtr* in solution. Next, SAXS experiments in the presence of substrate are described. Finally, attempts made to obtain crystals are presented.

3.2 I-TASSER modelling of *Mtr* from *Mtb*

In recent years, the 3D structure modelling program have become very reliable. It is therefore imperative that these tools are utilised to obtain preliminary structural information. The sequence of *MtMtr* was submitted to the I-TASSER webserver (147) to model the 3D structure as there was no structural data available for this protein. The topmost template identified by I-TASSER to model *MtMtr* was the structure of the Dihydrolipoamide dehydrogenase from *Thermus thermophilus* (PDB ID: 2YQU), which shares a sequence similarity of 28% percent with *MtMtr*. The latter is proposed to be made up by a Pyridine nucleotide-disulphide oxidoreductase domain (residues 1-320), connected by a linker sequence to the so-called Pyridine nucleotide-disulphide oxidoreductase, dimerization domain (residues 345-459).

In addition, the crystal structure of the Human glutathione reductase (PDB ID: 3DJG) was used to generate a dimer model of *MtMtr* for fitting into SAXS bead model. The sequences of *MtMtr*, dihydrolipoamide dehydrogenase and Human glutathione reductase was aligned using Clustal Omega (148) using Jalview software (149) and is depicted in Fig. 3.2. It can be seen that the overall secondary structure is conserved.

Figure 3.2 Sequence alignment of *MtMtr*. Structure based sequence alignment of *MtMtr*, *H. sapiens* Glutathione reductase and *T. thermophilus* Dihydrolipoamide dehydrogenase. The secondary structure elements of *MtMtr* I-TASSER prediction (top) and *T. thermophilus* Dihydrolipoamide dehydrogenase (bottom) are shown. The secondary structural elements β -sheet (green) and α -helix (blue) are depicted and were calculated based on an I-TASSER model for the *MtMtr* and the crystal structure of the Dihydrolipoamide dehydrogenase.

3.3 Cloning, production, and purification of *MtMtr*

The *Mtr* coding region was amplified using *Mtb* genomic DNA as template. PCR amplification resulted in a clear band corresponding to 1380 bp as shown in Fig. 3.3. After ligation and transformation, the presence of an insert was verified using control digestion using *Bam*HI and *Nco*I. The insert sequence was verified using sequencing. The successfully cloned constructs were transformed into *E. coli* BL21 (DE3) for overproduction. The production was confirmed using induction test (Fig. 3.4-A).

Figure 3.3. PCR amplification of the *MtMtr* gene from *Mtb* genomic DNA using PCR. DNA sample of 5 μ l was loaded on an 1.2% agarose gel.

The SDS-PAGE of recombinant protein revealed a prominent band of about 50 kDa. A solubility test performed on one of the positive colonies of the induction test revealed that the recombinant protein was sparingly soluble when cells were grown at 20 °C. Based on the solubility test, 50 mM HEPES, 250 mM NaCl, pH 7.5 was selected as appropriate buffer for purification of the recombinant protein (Fig. 3.4-B).

Figure 3.4 A-B: Induction and solubility test of the *MtMtr*. (A) Induction test SDS gel showing induced (+ve) and uninduced (-ve) samples. Induction was done with 1 mM IPTG at 37 °C for 3.5 hrs. A prominent band is seen around 50 kDa which corresponds to the recombinant protein *MtMtr*. (B) Solubility test of *MtMtr*: 17% SDS gel showing the protein in pellet (P) and supernatant (SN) in various buffers (1-5) namely Buffer 1: 50 mM Tris/HCl, 200 mM NaCl, pH 8.5; Buffer 2: 50 mM Tris/HCl, 200 mM NaCl, pH 7.5; Buffer 3: 25 mM HEPES, 200 mM NaCl, pH 7.5; Buffer 4: 25 mM MES, 200 mM NaCl, pH 7.0; Buffer 5: 50 mM phosphate, 200 mM NaCl, pH 6.5. Although the major portion of the recombinant protein is seen in the pellet, a very small portion of the *MtMtr* is soluble.

Due to sparingly soluble nature of the protein, it was decided to co-express chaperones along with recombinant *MtMtr* to increase its solubility. Therefore, a cell strain of *E. coli* BL21 GroEL-ES containing a pBAD vector, which codes for GroEL-ES, was used. To express the recombinant protein, liquid cultures were grown in a shaker incubator in LB medium, containing kanamycin (30 µg/ml), for about 4 h at 37 °C until an OD600 of 0.6–0.7 was reached. The temperature was then lowered to 24 °C and arabinose (1 mg/ml) was used to induce the expression of GroEL/ES for 45 mins.

To induce the production of *MtMtr*, cultures were supplemented with IPTG to a final concentration of 1 mM, followed by overnight incubation at 20 °C. In addition, the liquid culture media for cells expressing *MtMtr* contained chloramphenicol (35 µg/ml) for maintenance of the pBAD plasmid vector. The use of *E. coli* BL21 GroEL-ES resulted in a 20-fold increase in solubility of recombinant *MtMtr*.

Figure 3.5 A-C: Purification of the *MtMtr* (A) Ni^{2+} -NTA purification profile showing the desired bands of *MtMtr*. Fractions from 100 mM to 250 mM imidazole were pooled and used for further purification. Lane L represents a protein marker. The gel was stained with Coomassie Brilliant Blue G250 (B) SuperdexTM S200 purification profile of *MtMtr*, insert in Fig. B shows the SDS-PAGE gel after SuperdexTM S200 purification. Lane 1 and 2 represents a protein marker and purified *MtMtr* after size exclusion chromatography, respectively. (C) SDS-PAGE gel showing purified *MtMtr* in presence and absence of DTT.

Since the recombinant protein was cloned with a 6-His-tag at the N-terminus, Ni^{2+} -affinity chromatography was used as a first step of purification. Recombinant *MtMtr* was separated from the other impurities using an imidazole gradient (15-400 mM) in buffer consisting of 50 mM HEPES, 250 mM NaCl, pH 7.5, 1 mM PMSF. Clear bands were identified in the fractions 75-300 mM on an SDS-gel (Fig. 3.5-A).

The 100 to 250 mM of imidazole fractions were pooled, concentrated and then passed through a size exclusion Superdex 200-I (10/300) GL column (GE Healthcare), to get pure and homogeneous protein (Fig. 3.5-B). *MtMtr* eluted at 14.5 ml, indicating the presence of dimeric species. SDS-PAGE gel was performed to analyze the purity of the isolated protein (Fig. 3.5-B inset). Protein concentrations were determined by a NanoDrop machine (BioSpec-nano, Shimadzu Scientific Instruments) to perform subsequent experiments. With the use of GroEL/ES to aid in protein folding, about 1 ml of 10 mg/ml protein was obtained from 3 gm of cells. In addition, samples of purified *MtMtr* with and without DTT were applied onto 17% SDS gels to check for possible oligomeric states. The samples with DTT were heated at 95 °C for 5 mins. before loading onto the gel. As shown in Fig. 3.5-C, in the samples without DTT an additional band was observed indicating a possible higher oligomer. To confirm the results obtained from SEC as shown above as well as to get insight into hydrodynamic radius, DLS experiment of *MtMtr* in 50 mM HEPES pH 7.5, 250 mM NaCl was performed. Experiments were performed at concentrations of 1 mg/ml using a Malvern Zetasizer Nano ZS instrument. As shown in Fig. 3.6-A, the DLS profile of *MtMtr* revealed a dimeric species with an estimated molecular weight of 137 ± 23.8 kDa.

Figure 3.6 A-B: Dynamic light scattering studies on *MtMtr*. (A) DLS-plot of *MtMtr* indicating a dimeric species with molecular weight estimate of 137 ± 23.8 kDa. The hydrodynamic radius was determined to be 5.033 ± 1.2 nm. (B) DLS correlation function for *MtMtr* in 50 mM HEPES pH 7.5, 250 mM NaCl.

The data indicate the presence of a dimeric species in solution. The hydrodynamic radius was determined to be 5.033 ± 1.2 nm. The correlation function is shown in Fig. 3.6-B. In order to confirm the proper folding of *MtMtr*, 1D-NMR was used and NADPH oxidation was monitored at 340 nm to confirm that the *MtMtr* is enzymatically active.

3.4 1D NMR indicates that recombinant *MtMtr* is well folded in solution

The concept of using 1-D NMR to estimate protein folding is based on the idea that if a protein is well folded, the peaks are sharp and narrow and cover a large range of chemical shifts. On the other hand, if the protein is unfolded or partially folded, the peaks are usually broader and not as widely dispersed. An 1D NMR spectrum was acquired for the purified *MtMtr* in 50 mM phosphate, 50 mM NaCl, pH 6.8 as shown in Fig. 3.7. The 1D NMR spectrum indicates a well folded protein with well-dispersed and distinct characteristic peaks in the amide region (6 to 10 ppm), α -region (3.5 to 5 ppm), and uphill shifted methyl protons region (-0.5 to 1.0 ppm).

Figure 3.7: 1D NMR spectrum of *MtMtr*. One dimensional NMR spectrum of *MtMtr* recorded at 298 K in 50 mM phosphate buffer, pH 6.8, 50 mM NaCl and 5 mM EDTA on Bruker Avance 700 MHz machine.

3.5 NADPH dependent assay indicates that *MtMtr* is enzymatically active

Since Mtr utilizes NADPH to convert MSSM to MSH, a NADPH dependent assay was performed to measure, whether the purified protein is active. In this method, the decrease in NADPH absorbance was monitored spectrophotometrically at 340 nm. Due to the ability of NADPH to differentially absorb at this wavelength, the transfer of electrons between NADPH via FAD and the cysteine catalytic centres of Mtr results in

the decrease of NADPH absorbance at 340 nm in presence of the substrate MSSM. The reaction mixture contained 0.5 μM *MtMtr*, 200 μM NADPH in a buffer containing 50 mM phosphate pH 7.0, 100 mM ammonium sulfate, 0.5 mM EDTA and increasing amounts of substrate MSSM. The assay was carried out using a stopped-flow spectrophotometer in the presence of varying amounts of MSSM (10 μM , 20 μM , 40 μM and 50 μM). As shown in Fig. 3.8, saturation of the reaction was obtained at around 45 secs. At 50 μM MSSM, the rate of reaction increased drastically, because this concentration of substrate is probably close to the K_m and there ideally half of the maximum velocity can be attained. In fact, for Mtr expressed in *M. smegmatis*, the reported K_m for MSSM is $70 \pm 30 \mu\text{M}$ (78). The results presented clearly indicate that recombinant *MtMtr* is enzymatically active. In addition, one cannot rule out the phenomenon of cooperativity as NADPH promotes tetramerization of Mtr, wherein the NADPH domain from two dimers come in close proximity inside the tetramer.

Figure 3.8: NADPH dependent activity assay for *MtMtr*. The decrease in NADPH absorbance was monitored at 340 nm and the experiment was performed using a stopped-flow spectrophotometer Applied Photophysics SX20. The assay was carried out at 25 °C in a solution, containing 50 mM phosphate buffer at pH 7.0, 100 mM ammonium sulfate and 0.5 mM EDTA. In this assay 0.5 μM *MtMtr*, 200 μM NADPH, and increasing amount of MSSM were used.

3.6 SAXS studies on *MtMtr*

SAXS experiments of *MtMtr* were carried out in order to determine the solution shape as well as unambiguously assign the oligomeric state of *MtMtr* in solution. SAXS experiments were performed. The experiments were carried out using freshly prepared *MtMtr* at concentrations of 1.2, 2.1, 3.0, and 4.5 mg/ml (Fig. 3.9-A). The Guinier plots at low angles for the different concentrations appeared linear and revealed good data

quality with no indication of protein aggregation (Fig. 3.9-B). From the Guinier approximation a radius of gyration of 34.57 ± 0.72 Å was derived which was consistent for the four concentrations measured (Table 3.1). The R_g -values (34.37 ± 0.23 Å) extracted from the distance distribution function agreed to the one derived from the Guinier region.

Figure 3.9 A-B: Solution X-ray scattering studies of *MtMtr*. (A) SAXS-patterns of *MtMtr* in 50 mM HEPES, pH 7.5 and 250 mM NaCl and 1 mM DTT. The black, red, blue, and green curves represent the scattering pattern at 1.2, 2.1, 3.0, and 4.5 mg/ml of *MtMtr*, respectively. (inset) The Guinier plots at low angles are linear, indicating no aggregation.

The maximum particle dimension, D_{max} , was determined to be 109 ± 3 Å (Fig. 3.10-A). The normalized Kratky plot of *MtMtr* is shifted from a well-defined bell curve profile of lysozyme (Fig. 3.10-B), indicating that *MtMtr* might exhibit a slightly flexible conformation in solution (150).

Figure 3.10 A-B: SAXS data analysis of *MtMtr*: P(r) function and Kratky plot (A) Distance distribution functions of *MtMtr* at 3.0 mg/ml concentration. (B) Normalized Kratky plot of *MtMtr* (•; black) and the compact globular lysozyme (•; blue) with a peak (---; grey), representing the theoretical peak and assuming an ideal Guinier region of a globular particle.

The *ab initio* low resolution shape reconstructed for *MtMtr* using DAMMIF had normalized spatial discrepancy (NSD) values of 0.79 ± 0.03 and the average structure is shown in Fig. 3.11. The molecular mass determined by the Porod-hydrated volume, excluded volume and volume of correlation is approximately 92 ± 10 kDa, confirming the presence of a dimeric *MtMtr* in solution.

Figure 3.11 SAXS data analysis of *MtMtr*: DAMMIF model. Superimposition of the DAMMIF model to the I-TASSER model. The three domains, FAD-binding, NADPH-binding and interface domains are coloured as yellow, brown, and blue, respectively, for one subunit and the other subunit is shown in lighter shaded colours.

Since no high-resolution structure of *MtMtr* is available a homology model was generated employing the I-TASSER program as described earlier. A dimer model was then produced based on the crystal structure of human glutathione reductase. Like the homologous human glutathione reductase structure, *MtMtr* also has three domains viz, FAD-binding, NADPH-binding, and interface domains, wherein the FAD-binding and interface domains take part in the dimer formation. The dimer model of *MtMtr* fits with a discrepancy of $\chi^2 = 0.70$ to the experimental data (Fig. 3.12) and superimpose to the solution shape with an NSD of 2.2. The dimer model did not fill the entire solution shape leaving a volume of density unoccupied and showed that one of the NADPH-binding domain of the dimer did not fit in to the shape (Fig. 3.11), which is also confirmed by the relative less fitting of the model at lower q-value ($q < 1.5 \text{ \AA}^{-1}$). As indicated by the normalized Kratky plot, *MtMtr* might be flexible. It was speculated that the NADPH-binding domain might have a different conformation in solution since FAD-binding and interface domains participates in the dimer formation. Rigid-body modelling using

CORAL (116) was carried out which exhibited two conformations for the NADPH-binding domain. One subunit of the dimer model has a closed conformation similar to the I-TASSER model and the other subunit has an extended conformation (Fig. 3.13).

Figure 3.12: SAXS data analysis of *MtMtr*: CRY SOL fit analysis. The fit of the theoretical scattering curve calculated using CRY SOL for the DAMMIF model (—; red) and the CORAL model (—; green) of *MtMtr* with the experimental scattering data (○; black) of *MtMtr*.

This CORAL model fits better in the lower q -value ($q < 1.5 \text{ \AA}^{-1}$) to the experimental data and has a χ^2 value of 0.60. The CORAL model superimposes well into the solution shape with an NSD of 1.8 and the extended NADPH-binding domain fits into the previously unoccupied density of the shape indicating that the NADPH-binding domain is flexible in solution and might exist in different conformation (Fig. 3.13).

Figure 3.13: Solution shape of *MtMtr* deduced using CORAL. The CORAL model of *MtMtr* (ribbon representation) is superimposed into the solution shape. The three domains, FAD-binding, NADPH-binding and interface domains are coloured as yellow, brown, and blue, respectively, for one subunit and the other subunit is shown in lighter shaded colours.

Table 3.1: SAXS-data collection and scattering derived parameters of the reduced *MtMtr*

Data collection parameters	
Instrument (source & detector)	Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VÅNTEC-2000 detector
Beam geometry	100 μm slit
Wavelength ($\text{\AA}$)	0.134
q -range ($\text{\AA}^{-1}$)	0.16 – 4
Exposure time (minutes)	30 (5 frames x 6 minutes)
Temperature (K)	288.15
Protein sample	<i>MtMtr</i>
Concentration range (mg ml^{-1})	1.2-4.5
Structural parameters[#]	
$I(0)$ (cm^{-1}) [from $P(r)$]	83.14 ± 0.67
R_g ($\text{\AA}$) [from $P(r)$]	34.37 ± 0.23
$I(0)$ (cm^{-1}) (from Guinier)	84.31 ± 1.35
R_g ($\text{\AA}$) (from Guinier)	34.57 ± 0.72
D_{max} ($\text{\AA}$)	109 ± 3
Porod volume estimate ($\text{\AA}^3$)	149580
Dry volume from sequence ($\text{\AA}^3$) ‡ (monomeric)	60242
Molecular mass determination	
Calculated monomeric <i>MM</i> (kDa) [from sequence] *	49.8
<i>MM</i> from Porod invariant ($V_p/1.6$) (kDa)	94 ± 10
<i>MM</i> from excluded volume ($V_{ex}/2$) (kDa)	92 ± 10
<i>MM</i> from volume of correlation, V_c (kDa)	89 ± 10
Software employed	
Primary data reduction	BRUKER SAS
Data processing	PRIMUS
<i>Ab initio</i> analysis	DAMMIF
Validation and averaging	DAMAVAR
Computation of model intensities	CRY SOL
Rigid-body modelling	CORAL
3D graphics representations	PyMOL

reported for highest concentration

‡ <http://www.basic.northwestern.edu/biotools/proteincalc.html>* http://web.expasy.org/compute_pi/

3.7 Substrate induced conformational changes in *MtMtr*

Since asymmetry was observed in the NADPH binding domain of the *MtMtr* dimer in solution, SAXS experiments were carried out in the presence of substrates. As the kinetics of MSSM reduction is fast, the substrates NADPH and MSSM were individually incubated with *MtMtr* in the ratio of 1:5 for 30 mins and SAXS data were collected for 10 mins (10 frames X 1 mins each) with 3.5 mg/ml of fresh *MtMtr* in the absence of any reducing agent, and in the presence of NADPH and MSSM (Fig. 3.14-A).

The Guinier plots at low angles for the different concentrations appeared linear and revealed reliable data quality with no indication of protein aggregation (Fig. 3.14-B). From the Guinier approximation, a radius of gyration (R_g) of 35.54 ± 0.97 Å for oxidized, 35.92 ± 0.96 Å in the presence of MSSM and 47.35 ± 1.71 Å in the presence of NADPH were derived. The R_g -value of oxidized *MtMtr* and in the presence of MSSM are consistent to the reduced *MtMtr* form, however in presence of NADPH, *MtMtr* had larger R_g -value. The R_g -values extracted from the distance distribution function agreed to the one derived from the Guinier region.

Figure 3.14.A-B: SAXS pattern of *MtMtr* in the presence of substrates. (A) SAXS pattern of *MtMtr* in the presence of substrates in a buffer containing 50 mM HEPES, pH 7.5 and 250 mM NaCl. (B) The Guinier plots for the respective concentrations at low angles are linear, indicating no aggregation. The black, red and blue curves represent the scattering pattern for oxidised *MtMtr* and *MtMtr* incubated with MSSM and NADPH, respectively.

The maximum particle dimension, D_{max} , was determined was also higher in the presence of NADPH (150.05 ± 2 Å) (Fig. 3.15-A). The scattering data statistics are given in (Table 3.2). The normalized Kratky plot of *MtMtr* in presence of NADPH has a larger shift when compared to the oxidized or in the presence of MSSM (Fig. 3.15-B),

indicating that *MtMtr* might have taken a more extended form or a flexible form or a higher oligomeric state in solution.

Figure 3.15 A-B: SAXS-data analysis of *MtMtr* in the presence of substrates: P(r) function and Kratky plot (A) Distance distribution functions at 3.5 mg/ml concentration. The *black*, *red* and *blue* curves represent the oxidized *MtMtr* and *MtMtr* incubated with MSSM and NADPH, respectively (B) Normalized Kratky plot of oxidized *MtMtr* (●; *black*), *MtMtr* incubated with MSSM (●; *red*) and *MtMtr* incubated with NADPH (●; *blue*) and the compact globular lysozyme (●; *cyan*) with a peak (---; *grey*), representing the theoretical peak and assuming an ideal Guinier region of a globular particle.

The CORAL model of the reduced *MtMtr* had a good fit to the experimental data with χ^2 -value of 0.442 and 0.495 for the oxidized and MSSM bound form respectively, However, it had a poor fitting to the NADPH bound form ($\chi^2 = 1.372$) (Fig. 3.16).

Figure 3.16: SAXS-data analysis of *MtMtr* in the presence of substrates: CRYSOLO fit analysis. The fit of the theoretical scattering curve calculated using CRYSOLO for the CORAL model (—) of the reduced *MtMtr*. The *black*, *red* and *blue* curves represent the oxidized *MtMtr* and *MtMtr* incubated with MSSM and NADPH, respectively

Table 3.2: SAXS-data collection and scattering derived parameters of *MtMtr*-WT in the presence of substrates.**Data collection parameters**

Instrument (source & detector)	Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VÅNTEC-2000 detector		
Beam geometry	100 μm slit		
Wavelength ($\text{\AA}$)	1.34		
q -range ($\text{\AA}^{-1}$)	0.016 – 0.4		
Exposure time (minutes)	10 (10 frames x 1 minutes)		
Temperature (K)	288.15		

Protein sample

	<i>MtMtr</i> -WT	<i>MtMtr</i> -WT + MSSM	<i>MtMtr</i> -WT + NADPH
Concentration range (mg ml^{-1})	3.6	3.6	3.6

Structural parameters

I(0) (arbitrary units) [153]	15.74 ± 0.18	16.90 ± 0.16	27.66 ± 0.35
R_g ($\text{\AA}$) [153]	35.41 ± 0.35	36.10 ± 0.25	47.15 ± 0.50
I(0) (arbitrary units) (from Guinier)	15.98 ± 0.31	17.09 ± 0.33	28.51 ± 0.80
R_g ($\text{\AA}$) (from Guinier)	35.54 ± 0.97	35.92 ± 0.96	47.35 ± 1.71
D_{max} ($\text{\AA}$)	110.97	110.86	150.05
Porod volume estimate ($\text{\AA}^3$)	154668	151735	211602
Dry volume from sequence ($\text{\AA}^3$) ‡ (monomeric)	60242	60242	60242

Molecular mass (Mm) determination

Calculated monomeric <i>Mm</i> (kDa) [from sequence] *	49.8	49.8	49.8
<i>Mm</i> from Porod invariant ($V_p/1.6$) (kDa)	96.6	94.8	132.2
<i>Mm</i> from excluded volume ($V_{ex}/2$) (kDa)	94.1	91.1	139.4

Software employed

Primary data reduction	BRUKER SAS
Data processing	PRIMUS
<i>Ab initio</i> analysis	DAMMIF
Validation and averaging	DAMAVAR
Computation of model intensities	CRY SOL
3D graphics representations	PyMOL

‡ <http://www.basic.northwestern.edu/biotools/proteincalc.html>* http://web.expasy.org/compute_pi/

3.8 Design of *MtMtr* mutation to probe conformational changes in solution

The results presented above indicates *MtMtr* exhibits NADPH induced conformational changes in solution. Looking at the topological diagram of *MtMtr* (Fig. 3.17-A), one can rationalize that this could be due to the flexibility of linker between the FAD domain and NADPH domain. To confirm this observation and to gain further insights, two single and one double mutations were planned in the linker region based on substrate bound structure of glutathione reductase and I-TASSER model of *MtMtr* which could possibly abolish the interaction between linker and NADPH domain of *MtMtr*. S268 and S144 makes polar contact with D305. D158 makes contact with Q240 and L259 and Y159 with Q240, G238 and L260. All the four residues S268, S144, D158 and Y159 were mutated to alanine (Fig. 3.17-B and C).

Figure 3.17 A-C: Topological model of *MtMtr* and planned mutations. (A) Topological model of Mtr generated using PDBsum using I-TASSER model of *MtMtr*. The linker region (green), NADPH domain (brown), FAD domain (Yellow) and dimerization domain (blue) are differently coloured. The NADPH domain is sandwiched between two parts of FAD domain. (B) Planned mutation to further probe the effect of substrate binding. (C) S268 and S144 makes polar contact with D305. D158 makes contact with Q240 and L259 and Y159 with Q240, G238 and L260. All the four residues S268, S144, D158 and Y159 were mutated to alanine.

Mutagenic primers used for PCR are mentioned in the materials and method (section 2.2.4). The PCR product is shown in Fig. 3.18-A. After confirming the presence of mutations by DNA-sequencing, the plasmid DNA was transformed into *E. coli* B121-GroEL/ES strain for protein expression followed by an induction test (Fig. 3.18-B) and large-scale purification.

Figure 3.18 A-B: Site directed mutagenesis and Induction test. (A) PCR amplification product of Mtr mutants S268A (lane-1), S144A (lane-2), D158A-Y159A (lane-3) and 1 kb DNA ladder (lane-M). 5 μ l of DNA sample was loaded on an 1.2% agarose gel. (B) Induction test SDS gel showing induced (+ve) and uninduced (-ve) samples. Induction was done with 1 mM IPTG at 37 $^{\circ}$ C for 3.5 hrs. A prominent band is seen around 50 kDa which corresponds to the recombinant protein *MtMtr* mutants.

The mutants were purified using the same protocol as wild type (WT) but without DTT (described in detail in section 3.3). All the mutants eluted at the same volume as WT *MtMtr* in SEC (Fig. 3.19 A-D) and the purity of recombinant protein was evaluated using SDS-PAGE (Fig. 3.19 inset). WT *MtMtr* and the mutants were used for SAXS studies.

Figure 3.19 A-D: Purification of the *MtMtr* mutants. Superdex™ S200I purification profile of *MtMtr* mutants S144A (A), S268A (B), D158A-Y159A (C). SDS-PAGE gel after Superdex™ S200 purification. lane M stands for a protein marker and purified WT *MtMtr* and mutants after SEC are shown as labelled. Comparison of Superdex™ S200I purification profile of mutants with that of WT *MtMtr*.

3.9 SAXS experiments of *MtMtr* mutants in the presence of substrate

SAXS data were collected for 10 min (10 frames x 1 min. each) with 3.5 mg/ml of freshly prepared *MtMtr* proteins (WT, S268A, S144A, DY_AA) under three conditions: (a) substrate-free, in the presence of (b) NADPH or (c) MSSM (Fig. 3.20 A-F). The Guinier plots at low angles for the different concentrations appeared linear and revealed reliable data quality with no indication of protein aggregation (Fig. 3.20). From the

Guinier approximation, the R_g values of the Mtr mutants under the three conditions mentioned above were derived.

Figure 3.20 A-F: SAXS pattern of *MtMtr* mutants in the presence of substrates. SAXS patterns of *MtMtr*-S144A (A), *MtMtr*-S268A (C) and *MtMtr*-D158A-Y159A (E) in the presence of substrates in a buffer containing 50 mM HEPES, pH 7.5 and 250 mM NaCl. The Guinier plots for *MtMtr*-S144A (B), *MtMtr*-S268A (D) and *MtMtr*-D158A-Y159A (F) at low angles are linear, indicating no aggregation. The black, red and blue curves represent the scattering pattern for oxidised *MtMtr* and *MtMtr* incubated with MSSM and NADPH, respectively.

The R_g -values extracted from the distance distribution function agreed to the one derived from the Guinier region. The scattering parameters are presented in the tables 3.4, 3.5 and 3.6 for the mutants S144A, S268A and D158A-Y159A, respectively. The R_g -value of the substrate-free *MtMtr* mutants was comparable to that of the substrate-free WT-*MtMtr* (WT: 35.54 ± 0.97 Å, S268A: 36.03 ± 0.98 Å, S144A: 33.90 ± 0.81 Å, and D158A-Y159A: 35.94 ± 0.98 Å). This indicates that the mutation didn't affect the overall shape of the molecule as compared to the WT enzyme. This was validated by the D_{max} values of the *MtMtr* mutants which were in good agreement with that of WT-*MtMtr* (WT: 110.97 ± 10 Å, S268A: 110.48 ± 10 Å, S144A: 110.02 ± 10 Å and D158A-Y159A: 112.77 ± 10 Å) (Fig 3.21-A, C and E). The SAXS data indicated that MSSM did not alter the R_g -values (WT: 35.92 ± 0.96 Å, S268A: 35.88 ± 1.53 Å, S144A: 34.90 ± 0.87 Å and D158A-Y159A: 34.48 ± 0.82 Å) and D_{max} values (WT: 110.86 ± 10 Å, S268A: 109.37 ± 10 Å, S144A: 107.51 ± 10 Å and D158A-Y159A: 111.03 ± 10 Å) significantly. Rather these values were comparable to the substrate-free form of the respective mutants (Fig 3.21-A, C and E).

However, in the presence of NADPH, an interesting observation was made. The R_g -value and the D_{max} did not increase significantly for the *MtMtr* mutants, S144A (R_g -value 34.61 ± 0.88 Å; D_{max} 110 ± 10 Å) and D158A-Y159A (R_g -value 35.77 ± 0.96 Å; D_{max} 110 ± 10 Å). This indicated that the mutants did not undergo substrate-induced conformational changes in solution as observed for the WT. The *MtMtr* mutant S268A however, showed an increased R_g -value (39.33 ± 1.20 Å) in the presence of NADPH as compared to the substrate-free form. Consistent with the R_g -value, the D_{max} was also higher in the presence of NADPH (125 ± 12 Å) for the S268A mutant (Fig 3.21-C).

The normalized Kratky plot of the *MtMtr* mutants in the presence of substrate was generated (Fig. 3.21 B, D and F). The plots did not show any increase in conformational flexibility for all the mutants in the substrate-free form and upon incubation with MSSM. It is interesting to note that, consistent with the increase in the R_g -value and D_{max} , the normalized Kratky plot for the *MtMtr* mutant S268A showed a slightly higher conformational flexibility in the presence of NADPH, as compared to the substrate-free form. The results presented above indicate that the mutant S268A has the R_g - and D_{max} values in between WT and S144A or D158A-Y159A. This indicates that while S144A and D158A-Y159A could completely abolish the NADPH-induced conformational

changes, the S268A mutation has intermediate effect and residual increase in R_g and D_{max} was still seen in solution.

Figure 3.21 A-F: SAXS-data analysis of *MtMtr* mutants in the presence of substrates: P(r) function and Kratky plot. Distance distribution functions for *MtMtr*-S144A (A), *MtMtr*-S268A (C) and *MtMtr*-D158A-Y159A (E). The black, red and blue curves represent the oxidized *MtMtr* and *MtMtr* incubated with MSSM and NADPH, respectively. Normalized Kratky plot for *MtMtr*-S144A (B), *MtMtr*-S268A (D) and *MtMtr*-D158A-Y159A (F). The plot for oxidized *MtMtr* (•; black), *MtMtr* incubated with MSSM (•; red) and *MtMtr* incubated with NADPH (•; blue) and the compact globular lysozyme (•; cyan) with a peak (---; grey), representing the theoretical peak and assuming an ideal Guinier region of a globular particle.

Table 3.3: SAXS-data collection and scattering derived parameters of *MtMtr-S144A* in the presence of substrates.**Data collection parameters**

Instrument (source & detector)	Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VÅNTEC-2000 detector		
Beam geometry	100 μm slit		
Wavelength ($\text{\AA}$)	1.34		
q -range ($\text{\AA}^{-1}$)	0.016 – 0.4		
Exposure time (min)	10 (10 frames x 1 min)		
Temperature (K)	288.15		
Protein sample	<i>MtMtr-S144A</i>	<i>MtMtr-S144A</i> + MSSM	<i>MtMtr-S144A</i> + NADPH
Concentration range (mg ml^{-1})	3.9	3.9	3.9

Structural parameters

$I(0)$ (arbitrary units) [153]	15.03 ± 0.19	16.68 ± 0.15	15.51 ± 0.18
R_g ($\text{\AA}$) [153]	34.66 ± 0.40	35.60 ± 0.26	35.39 ± 0.39
$I(0)$ (arbitrary units) (from Guinier)	14.94 ± 0.25	16.75 ± 0.29	15.43 ± 0.27
R_g ($\text{\AA}$) (from Guinier)	33.90 ± 0.81	34.90 ± 0.87	34.61 ± 0.88
D_{max} ($\text{\AA}$)	110.02	107.51	109.89
Porod volume estimate ($\text{\AA}^3$)	156609	152517	164542
Dry volume from sequence ($\text{\AA}^3$) ‡ (monomeric)	60242	60242	60242

Molecular mass determination

Calculated monomeric Mm (kDa) [from sequence] *	49.8	49.8	49.8
Mm from Porod invariant ($V_p/1.6$) (kDa)	97.8	95.3	102.8
Mm from excluded volume ($V_{ex}/2$) (kDa)	95.0	90.0	98.2

Software employed

Primary data reduction	BRUKER SAS
Data processing	PRIMUS
<i>Ab initio</i> analysis	DAMMIF
Validation and averaging	DAMAVR
Computation of model intensities	CRY SOL
3D graphics representations	PyMOL

‡ <http://www.basic.northwestern.edu/biotools/proteincalc.html>* http://web.expasy.org/compute_pi/

Table 3.4: SAXS-data collection and scattering derived parameters of *MtMtr-S268A* in the presence of substrates.**Data collection parameters**

Instrument (source & detector)	Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VÅNTEC-2000 detector		
Beam geometry	100 μm slit		
Wavelength ($\text{\AA}$)	1.34		
q -range ($\text{\AA}^{-1}$)	0.016 – 0.4		
Exposure time (min)	10 (10 frames x 1 min)		
Temperature (K)	288.15		
Protein sample	<i>MtMtr-S268A</i>	<i>MtMtr-S268A</i> + MSSM	<i>MtMtr-S268A</i> + NADPH
Concentration range (mg ml^{-1})	3.9	3.9	3.9

Structural parameters

$I(0)$ (arbitrary units) [153]	15.28 ± 0.18	17.09 ± 0.16	16.74 ± 0.17
R_g ($\text{\AA}$) [153]	36.48 ± 0.33	37.10 ± 0.23	39.27 ± 0.37
$I(0)$ (arbitrary units) (from Guinier)	15.38 ± 0.30	17.15 ± 0.43	17.00 ± 0.39
R_g ($\text{\AA}$) (from Guinier)	36.03 ± 0.98	35.88 ± 1.53	39.33 ± 1.20
D_{max} ($\text{\AA}$)	110.48	109.37	125.12
Porod volume estimate ($\text{\AA}^3$)	166370	166936	176627
Dry volume from sequence ($\text{\AA}^3$) ‡ (monomeric)	60242	60242	60242

Molecular mass determination

Calculated monomeric M_m (kDa) [from sequence] *	49.8	49.8	49.8
M_m from Porod invariant ($V_p/1.6$) (kDa)	103.9	104.3	110.3
M_m from excluded volume ($V_{\text{ex}}/2$) (kDa)	98.2	96.8	111.7

Software employed

Primary data reduction	BRUKER SAS
Data processing	PRIMUS
<i>Ab initio</i> analysis	DAMMIF
Validation and averaging	DAMAVR
Computation of model intensities	CRY SOL
3D graphics representations	PyMOL

‡ <http://www.basic.northwestern.edu/biotools/proteincalc.html>* http://web.expasy.org/compute_pi/

Table 3.5: SAXS-data collection and scattering derived parameters of *MtMtr*-D158A-Y159A in the presence of substrates.**Data collection parameters**

Instrument (source & detector)	Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VÅNTEC-2000 detector		
Beam geometry	100 µm slit		
Wavelength (Å)	1.34		
q -range (Å ⁻¹)	0.016 – 0.4		
Exposure time (min)	10 (10 frames x 1 min)		
Temperature (K)	288.15		
Protein sample	<i>MtMtr</i> -D158A -Y159A	<i>MtMtr</i> -D158A -Y159A + MSSM	<i>MtMtr</i> -D158A -Y159A + NADPH
Concentration range (mg ml ⁻¹)	3.6	3.6	3.6

Structural parameters

I(0) (arbitrary units) [153]	16.41 ± 0.18	18.01 ± 0.17	16.21 ± 0.16
R_g (Å) [153]	35.78 ± 0.37	35.69 ± 0.31	35.83 ± 0.32
I(0) (arbitrary units) (from Guinier)	16.64 ± 0.32	17.79 ± 0.30	16.39 ± 0.31
R_g (Å) (from Guinier)	35.94 ± 0.98	34.48 ± 0.82	35.77 ± 0.96
D_{max} (Å)	112.77	111.03	110.27
Porod volume estimate (Å ³)	155977	155085	159131
Dry volume from sequence (Å ³) ‡ (monomeric)	60242	60242	60242

Molecular mass determination

Calculated monomeric M_m (kDa) [from sequence] *	49.8	49.8	49.8
M_m from Porod invariant ($V_p/1.6$) (kDa)	97.4	96.9	99.4
M_m from excluded volume ($V_{ex}/2$) (kDa)	97.0	93.3	95.7

Software employed

Primary data reduction	BRUKER SAS
Data processing	PRIMUS
<i>Ab initio</i> analysis	DAMMIF
Validation and averaging	DAMAVR
Computation of model intensities	CRYSOL
3D graphics representations	PyMOL

‡ <http://www.basic.northwestern.edu/biotools/proteincalc.html>* http://web.expasy.org/compute_pi/

As stated earlier, one of the possible explanation for increase in R_g -value as well as D_{max} could be due to presence of different oligomeric species in solution. So therefore, the scattering curves of WT-*MtMtr* under different condition were analysed for the presence of mixture of dimer and tetramer using OLIGOMER program from ATSAS suite (116). A tetramer-model generated using PDB ID: 2F5Z was used in addition to the I-TASSER dimer model. Briefly, OLIGOMER fits an experimental scattering curve from a multi-component mixture of proteins to find the volume fractions of each component in the mixture. The analysis was carried out by Dr. Malathy Sony Subramanian Manimekalai from Prof. Gerhard Grüber's team. As shown in figure 3.22-A, the mixture of dimer-tetramer fits the experimental data of WT-*MtMtr* in the presence of NADPH with lower χ^2 value (0.53) as compared to CORAL model of the reduced *MtMtr* (χ^2 value = 1.372) shown earlier in figure 3.16.

These results agree with earlier studies using analytical ultra-centrifugation which reported that glutathione reductase as well as dihyrolipoamide dehydrogenase exists as mixture of dimer and tetramer in solution (73,74). It is very surprising that while the WT-*MtMtr* in substrate-free form has predominant population of dimer, in the presence of NADPH the oligomer equilibrium was completely shifted towards tetramer (Fig 3.23). This is in agreement with an earlier study (75) which reported that the presence of NADPH favors conversion of dimer of glutathione reductase into less active tetramers.

Figure 3.23: Volume fraction of dimeric and tetrameric WT-*MtMtr*. Percentage volume fraction of dimeric and tetrameric oligomer of WT-*MtMtr* in solution in substrate-free form and in presence of NADPH. The data indicates clear shift in oligomer-equilibrium towards tetramer upon incubation with NADPH. The analysis was carried out using OLIGOMER software (105).

In order to evaluate whether, the various mutations generated could affect the dimer-tetramer equilibrium, the OLIGOMER analysis was carried out for the various mutants of *MtMtr* under different condition mentioned earlier and the volume fraction of dimer-tetramer is reported in table 3.6. The results presented reflects that in the presence of NADPH, mutant S268A has the tetrameric fraction in between WT and S144A or D158A-Y159A. This indicates that while S144A and D158A-Y159A could completely abolish the NADPH-induced oligomeric changes, the S268A mutation has intermediate effect and residual shift in oligomeric equilibrium was still seen in solution. It is also

clearly evident that mutant S144A has higher volume fraction of dimer as compared to tetramer and may be a good candidate for the crystallisation studies. In addition, the presence of NADPH as an additive in crystallisation may be helpful.

Table 3.6: Percentage volume fraction of dimer and tetramer for WT-*MtMtr* and *MtMtr*-mutants under different conditions.

	Dimer (%)	Tetramer (%)	OLIGOMER fit (χ^2 value)
WT- <i>MtMtr</i>	77.8 $\pm$ 2.1	22.1 $\pm$ 1.7	0.45
WT- <i>MtMtr</i> + MSSM	75.8 $\pm$ 2.0	24.1 $\pm$ 1.7	0.47
WT- <i>MtMtr</i> + NADPH	24.1 $\pm$ 2.0	75.9 $\pm$ 1.7	0.53
S144A- <i>MtMtr</i>	84.0 $\pm$ 2.0	16.0 $\pm$ 1.6	0.48
S144A- <i>MtMtr</i> + MSSM	77.3 $\pm$ 1.9	22.6 $\pm$ 1.6	0.52
S144A- <i>MtMtr</i> + NADPH	74.9 $\pm$ 2.0	25.0 $\pm$ 1.7	0.48
S268A- <i>MtMtr</i>	69.6 $\pm$ 2.1	30.3 $\pm$ 1.8	0.46
S268A- <i>MtMtr</i> + MSSM	60.2 $\pm$ 2.0	39.7 $\pm$ 1.7	0.47
S268A- <i>MtMtr</i> + NADPH	57.3 $\pm$ 2.1	42.7 $\pm$ 1.8	0.45
D158A-Y159A- <i>MtMtr</i>	79.8 $\pm$ 2.0	20.1 $\pm$ 1.7	0.48
D158A-Y159A- <i>MtMtr</i> + MSSM	77.0 $\pm$ 1.9	23.0 $\pm$ 1.6	0.46
D158A-Y159A- <i>MtMtr</i> + NADPH	77.8 $\pm$ 2.1	22.2 $\pm$ 1.7	0.45

Since crystallization studies described in next section were carried out much before the above results were obtained, all the crystallization was attempted using WT-*MtMtr*. Nevertheless, the above results are of importance and provide insights into substrate induced shift in oligomer equilibrium and must be kept in mind during future crystallisation studies.

3.10 Crystallization studies of *MtMtr*

In order to determine a high-resolution structure of the *MtMtr*, crystallization was attempted using hanging drop vapor diffusion method at 18 °C. Initially a range of protein concentrations was used ranging from 5-20 mg/ml. The concentration of protein is a key variable as samples too concentrated results in amorphous precipitate, while samples too dilute leads to clear drops. The overall approach is described below:

Figure 3.24. Flowchart showing various steps tried to crystallize *MtMtr*. Few conditions resulted either in phase separation (Cryo-I #38 and Cryo-I #47) or granular aggregates (MembFac #34 and PEG/Ion 2 #28) or crystalline precipitate (Wizard-II #17).

The appropriate protein concentration for crystallization can be judged by using Hampton PCT Pre-Crystallization Test. Based on condition 4 (0.1 M Tris hydrochloride pH 8.5, 2.0 M Ammonium sulfate) and 6 (0.1 M Tris hydrochloride pH 8.5, 0.2 M Magnesium chloride hexahydrate, 30% Polyethylene glycol 4,000) of Hampton Crystal Screen I, two different concentrations of 9 mg/ml and 15 mg/ml were chosen for screening commercially available crystallization screens. Initially the buffer containing 50mM Tris, pH 7.5, 250 NaCl and 50mM HEPES, pH 7.5, 250 NaCl was used for screening and no hits were observed. Most of condition resulted in precipitation. Thereafter to increase the solubility, 10% glycerol was included in buffer. During this time, few condition resulted either in phase separation (Cryo-I #38 and Cryo-I #47) or granular aggregates (MembFac #34 and PEG/Ion 2 #28) or crystalline precipitate (Wizard-II #17) (Fig. 3.24). Initially attempts were made to reproduce these conditions both in commercial solution as well as self-made solution. While most of these conditions didn't result in significant improvement, condition from MembFac #34 (0.1 M Ammonium sulfate, 0.1 M HEPES sodium pH 7.5, 10% w/v Polyethylene glycol) appeared promising and very small needle showers were observed (Fig. 3.25-A). Optimizations of this conditions was done by varying the precipitant and salt concentration, changing buffer or pH in self-made grid screens.

Figure 3.25 A-F: Optimization of initial hits observed during *MtMtr* crystallization. Crystallization buffer conditions used are (A) MembFac #34, (B) MembFac #34* (1.5 M Ammonium sulfate, 0.1 M HEPES sodium pH 7.5, 10% w/v Polyethylene glycol), (C) MembFac #34* (1.0 M Ammonium sulfate, 0.1 M HEPES sodium pH 7.5, 10% w/v Polyethylene glycol), (D) MembFac #34 additive 12 (0.1 M Nickel(II) chloride hexahydrate), (E) MembFac #34 additive 89 (40% v/v Formamide) and (F) MembFac #34 (different drop) respectively. These hits appeared after 7 days. All crystallization was carried out at 18 °C.

The small granules that were observed under the first condition MembFac #34 (0.1 M Ammonium sulfate, 0.1 M HEPES sodium pH 7.5, 10% w/v Polyethylene glycol) was optimized by grid screen and resulted in bigger granules (Fig. 3.25-B) and micro crystalline (Fig. 3.25-C) precipitate depending on concentration of ammonium sulfate used. However even after multiple rounds of optimization, no further improvement was observed. Thereafter, additional crystallisation trials were carried out using Additive Screen™, (Fig. 3.25-D, E and F). Attempts were also made by micro- and macro-seeding, temperature variation at various steps but no significant improvement in crystallization was observed.

In addition, attempts were made to clone and express the C-terminal dimerization domain (residues 337-459) alone for structural studies. While it was possible to get the protein cloned and expressed (Fig. 3.26-A), the level of solubility was negligible (Fig. 3.26-B) and therefore this was not pursued further.

Figure 3.26 A-B: Induction and solubility test of *MtMtr* C-term domain. (A) Induction test SDS gel showing induced (+ve) and uninduced (-ve) samples. Induction was done with 1 mM IPTG at 37 °C for 3.5 hrs. A prominent band is seen around ~13 KDa which corresponds to the recombinant protein *MtMtr*. (B) Solubility test of *MtMtr* C-term domain: 17% SDS gel showing the protein in Pellet (P) and supernatant (SN) in various buffers (1-5) namely Buffer 1: 50 mM Tris/HCl, 200 mM NaCl, pH 8.5; Buffer 2: 50 mM Tris/HCl, 200 mM NaCl, pH 7.5; Buffer 3: 25 mM HEPES, 200 mM NaCl, pH 7.5; Buffer 4: 25 mM MES, 200 mM NaCl, pH 7.0; Buffer 5: 50 mM phosphate, 200 mM NaCl, pH 6.5. The major portion of the recombinant protein is seen in the pellet indicating the protein is insoluble under the condition used.

Mechanistic insights into interaction between peroxiredoxin AhpE and Mrx-1 from *Mtb*

4.1 Introduction to oxidoreductase *MtMrx-1*

Recent studies using genetic approach have shown that *MtMrx-1* deletion strains are more susceptible to oxidative stress (83). *MtMrx-1* acts as oxido-reductase, linking the MSH electron transfer pathway to a redox-active protein such as a 1-Cys peroxiredoxin, *MtAhpE*, in mycobacterium and mycothiol peroxidase (Mpx) in *Corynebacterium glutamicum* (70,151). The reduced *MtAhpE* (*MtAhpE*-SH) scavenges peroxides to become converted to *MtAhpE*-SOH. To provide continuous availability of *MtAhpE*-SH, *MtAhpE*-SOH needs to be reduced and recycled. Mrx-1 functions by reducing the oxidised cysteine residue (Cys-SOH) in case of *MtAhpE* as well as the protein-MSH mixed disulphide as described for mycothiol peroxidase (151,152).

The atomic details of this *MtAhpE*-*MtMrx-1* and *MtMtr*-*MtMrx-1* interaction are described in this chapter. This chapter begins with cloning, production, and purification of *MtMrx-1* followed by SAXS experiments to confirm the oligomeric state of *MtMrx-1* in solution. NMR spectroscopy experiments were performed to assign the HSQC spectrum of *MtMrx-1*. This is followed by NMR titration experiments between *MtAhpE* and *MtMrx-1* and cysteine-labeled NMR experiments performed to study the state of the catalytic cysteine of *MtMrx-1*. In addition, details are provided about the NMR restraints based molecular docking calculations. Finally, the NMR titration experiments between *MtMrx-1* and *MtMtr* are described.

4.2 Cloning, production, and purification of *MtMrx-1*

The *MtMrx-1* coding region was amplified using *Mtb* genomic DNA as template. PCR amplification resulted in a clear band corresponding to about 255 bp as shown in Fig. 4.1. The PCR product was gel extracted, digested using *Bam*HI and *Nco*I restriction enzymes and was successfully ligated into the pET9d-1 vector and transformed into DH5 α cells using electroporation. The presence of the insert was verified using control digestion using the enzymes *Bam*HI and *Nco*I. The correct clones were sent for sequencing and the insert sequence were verified. One of the successfully cloned constructs was transformed into *E. coli* BL21 (DE3) for overproduction.

Figure 4.1: PCR amplification of *MtMrx-1*. PCR amplification of Mrx-1 gene from *Mtb* genomic DNA using PCR. DNA sample of 5 μ l was loaded on an 1% agarose gel.

The production was confirmed using induction test (Fig. 4.2). The SDS-PAGE of recombinant protein revealed a prominent band of about ~10 kDa. A solubility test performed with one of the positive colonies of the induction test revealed that a small fraction of recombinant protein was found to be soluble when cells were grown at 20 °C. Based on the solubility test, 50 mM Tris, 250 mM NaCl, pH 7.5 was selected as appropriate buffer for purification of the protein. Since *MtMrx-1* was cloned with a 6-His-tag at the N-terminus, Ni^{2+} -affinity chromatography was used as first step of purification. The recombinant *MtMrx-1* was separated from the other impurities using an imidazole gradient (15-400 mM) in buffer consisting of 50 mM Tris, 250 mM NaCl, pH 7.5.

Figure 4.2: Induction test of *MtMrx-1*. SDS gel showing induced (+ve) and uninduced (-ve) samples. Induction was done with 1 mM IPTG at 37 °C for 3 hrs. A prominent band is seen around 10 KDa, which corresponds to recombinant *MtMrx-1*.

Clear bands were identified in the fractions 75-300 mM on an SDS-gel (Fig. 4.3-A) which was stained with Coomassie Brilliant Blue G250. Since pure protein was found at 75 to 250 mM imidazole concentrations, these fractions were pooled together and concentrated. The concentrated fraction was then passed through a size exclusion Superdex 75 (10/300) GL column (GE Healthcare), to get pure and homogeneous protein (Fig. 4.3-B). The protein eluted at 15.2 ml on a S-75 column, corresponding to a monomeric species of around 10 kDa. The SDS-gel was carried out to analyze the purity of the isolated protein (Fig. 4.3-B).

Figure 4.3 A-B: Purification of the *MtMrx-1* from *Mtb* (A) Ni^{2+} -NTA purification profile showing the desired bands of *MtMrx-1*. Fractions from 75 mM to 250 mM imidazole were pooled and used for further purification. (B) SuperdexTM S75 purification profile of *MtMrx-1*. Insert in Figure B shows the SDS gel after SuperdexTM S75 purification. In Figure B, lane 1 and 2 represent a protein marker and profile of protein after size exclusion chromatography, respectively.

4.3 SAXS studies on *MtMrx-1*

Recent studies have shown that NrdH-redoxin of *Mtb* is a monomer in solution and forms a non-swapped dimer at high protein concentrations (84). To confirm the monomeric state of *MtMrx-1* in solution and to investigate a possible concentration dependent effect on oligomerization of *MtMrx-1*, SAXS-patterns of *MtMrx-1* were recorded at four concentrations (1.5, 2.4, 3.5 and 6.0 mg/ml). SAXS pattern at different concentrations showed comparable final composite scattering curves (Fig. 4.4-A). No radiation damage was detected over the data collection time. Guinier plots at low angles were linear and revealed good data quality with no indication of protein aggregation (Fig. 4.4-B). The R_g values from the Guinier approximation was consistent for the four concentrations measured with a value of 16.73 ± 0.17 Å (Table 4.1). The R_g -values (16.99 ± 0.50 Å) extracted from the $P(r)$ function agreed to the one derived from the Guinier region.

Figure 4.4 A-B: SAXS pattern of *MtMrx-1*. (A) SAXS patterns of *MtMrx-1* in 50 mM Tris/HCl, pH 7.5 and 250 mM NaCl and 1 mM DTT. The black, red, blue and pink curves represent the scattering pattern at 1.5, 2.4, 3.5 and 6.0 mg/ml of *MtMrx-1*, respectively. (B) The Guinier plots for the respective concentrations at low angles are linear, indicating no aggregation.

The distance distribution functions determined were similarly shaped for all the concentrations and therefore the highest concentration data set was used for further analysis (Fig. 4.5-A) and the D_{max} was determined to be 52 ± 2 Å. The normalized Kratky plot ($(qR_g)^2 (I(q)/I(0))$ vs. qR_g) of *MtMrx-1* shifted from a well-defined bell curve profile (Fig. 4.5-B). The peak is deviated to slightly higher values and the profile is broadened, indicating that *MtMrx-1* exhibits a slightly flexible conformation in solution (150).

Figure 4.5 A-B: SAXS-data analysis of *MtMrx-1*: P(r) function and Kratky plot. (A) Distance distribution functions of *MtMrx-1*. The maximum particle dimension was calculated to be 52 ± 2 Å. (B) Normalized Kratky plot of *MtMrx-1* (●; blue) and the compact globular lysozyme (●; red) with a peak (---; grey), representing the theoretical peak and assuming an ideal Guinier region of a globular particle.

The *ab initio* reconstruction of the gross shape of reduced *MtMrx-1* had a good fit to the experimental data in the entire scattering range with a discrepancy of $\chi^2 = 0.99$. Twenty independent DAMMIF reconstructions produced similar envelopes with NSD value of 0.984 ± 0.08 and the average structure is shown in Fig. 4.6. The NMR structure of *MtMrx-1* fitted very nicely in SAXS envelope, indicating good data quality.

Figure 4.6: *ab initio* reconstruction of *MtMrx-1*. Average bead model of *MtMrx-1* at a concentration of 6.0 mg/ml. The model clearly indicates the monomeric form of *MtMrx-1* in solution. The NMR structure of *MtMrx-1* (shown in the ribbon) is well embedded into the *ab initio* model. The *ab initio* model was fitted onto the NMR structure (PDB ID 2LQQ; red) using SUPCOMB(121).

Table 4.1: SAXS-data collection and scattering derived parameters of the *MtMrx-1*

Data collection parameters	
Instrument (source & detector)	Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VÅNTEC-2000 detector
Beam geometry	100 μm slit
Wavelength ($\text{\AA}$)	0.134
q -range ($\text{\AA}^{-1}$)	0.16 – 4
Exposure time (min)	30 (5 frames x 6 min)
Temperature (K)	288.15
Protein sample	<i>MtMrx-1</i>
Concentration range (mg ml^{-1})	1.5-6.0
Structural parameters[#]	
$I(0)$ (cm^{-1}) [from $P(r)$]	31.99 ± 0.35
R_g ($\text{\AA}$) [from $P(r)$]	16.99 ± 0.50
$I(0)$ (cm^{-1}) (from Guinier)	32.16 ± 0.20
R_g ($\text{\AA}$) (from Guinier)	16.73 ± 0.17
D_{max} ($\text{\AA}$)	52 ± 2
Porod volume estimate ($\text{\AA}^3$)	13722
Dry volume from sequence ($\text{\AA}^3$) $\ddagger$ (monomeric)	12237
Molecular mass determination	
Calculated monomeric MM (kDa) [from sequence] *	10.1
MM from Porod invariant ($V_p/1.6$) (kDa)	8.5 ± 2
MM from excluded volume ($V_{ex}/2$) (kDa)	8.7 ± 2
MM from volume of correlation, V_c (kDa)	9.0 ± 2
Software employed	
Primary data reduction	BRUKER SAS
Data processing	PRIMUS
<i>Ab initio</i> analysis	DAMMIF
Computation of model intensities	CRY SOL
Validation and averaging	DAM AVER
3D graphics representations	PyMOL

[#] reported for highest concentration

$\ddagger$ <http://www.basic.northwestern.edu/biotools/proteincalc.html>

* http://web.expasy.org/compute_pi/

The molecular mass as determined by the Porod-hydrated volume, excluded volume and volume of correlation is approximately 9 ± 2 kDa, confirming the presence of a monomeric *MtMrx-1* in solution. The theoretical scattering curve for the monomer from the NMR structure (PDB ID: 2LQQ; (83)) was computed using CRY SOL (120) and fitted nicely with the experimental curve with the χ^2 -value of 0.67 (Fig. 4.7), underlining the monomeric nature of the protein in solution.

Figure 4.7: SAXS-data analysis of *MtMrx-1*: CRY SOL fit analysis. The fit of the theoretical scattering curve (—; *black*) calculated using CRY SOL for the NMR structure of *MtMrx-1* (PDB ID: 2LQQ) with the experimental scattering data (○; *pink*) of *MtMrx-1*.

4.4 NMR spectroscopy experiments of *MtMrx-1*

To study the interaction between *MtAhpE* and *MtMrx-1*, NMR spectroscopy experiments were performed. Initially, 3D experiments were performed to assign the HSQC spectrum of *MtMrx-1*. This was followed by NMR titration experiments between *MtAhpE* and *MtMrx-1* to map the interacting residues and finally cysteine-labeled NMR experiments to study the state of catalytic cysteine of *MtMrx-1*.

Firstly, a 1D NMR spectrum of the purified *MtMrx-1* was acquired in 50 mM phosphate, 50 mM NaCl, pH 6.8. The 1D spectrum which indicated a well folded protein with well-dispersed and distinct characteristic peaks in the amide region (6 to 10 ppm), α -region (3.5 to 5 ppm), and uphill shifted methyl protons region (-0.5 to 1.0 ppm) and is shown in Figure 4.8.

Figure 4.8: 1D NMR spectrum of *MtMrx-1*. 1D NMR spectrum of *MtMrx-1* recorded at 298 K in 50 mM phosphate buffer, pH 6.8, 50 mM NaCl on Bruker Avance 700 MHz machine. 1D spectrum clearly indicates the protein is well folded.

A well-dispersed ^1H - ^{15}N HSQC-spectrum of *MtMrx-1* was observed. This indicates that the protein is properly folded and that the protein is very likely monodispersed in solution. Around 100 peaks were identified on the HSQC spectrum which indicated, that it was possible to observe all non-proline peaks on the spectrum. The HSQC spectrum is shown in the Fig. 4.9.

Figure 4.9: ^1H - ^{15}N HSQC-spectrum of *MtMrx-1*. The spectrum was recorded in 50 mM phosphate, 50 mM NaCl buffer, pH 6.8 on a Bruker Avance 700 MHz machine at 298 K.

Sequential backbone assignment was carried out using a non-uniform sampling (NUS) strategy. For assignment purpose, four sets of 3D-data were acquired namely NUS_HNCACB, NUS_HNCOCA, NUS_CBCACONH, and NUS_HNCA (Figure 4.10).

Figure 4.10: Sequential assignment of the backbone resonances of *MtMrx-1*. Representative strips are shown from a Non-Uniform Sampling (NUS) 3D HNCACB and 3D CBCA(CO)NH. Sequential assignment indicated by arrows is shown for residues K32-I36. Data were acquired at 298 K using a 700 MHz machine, equipped with a triple resonance cryoprobe.

This dataset enabled backbone assignment of all 84 amino acids of *MtMrx-1* as shown in the Fig. 4.11.

Figure 4.11: Assigned ^1H - ^{15}N HSQC-spectrum of *MtMrx-1*. The peaks corresponding to all the 84 amino acids were assigned and labelled. The spectrum was recorded in 50 mM phosphate, 50 mM NaCl buffer, pH 6.8 on a Bruker Avance 700 MHz machine at 298 K.

After the assignment of C α - and C β -chemical shifts, the secondary structure prediction of *MtMrx-1* was carried out by subtracting C α - and C β -chemical shifts from their random coil values ($\Delta C\alpha$ - $\Delta C\beta$). The predicted structure is in agreement with the published *MtMrx-1* NMR structure [7] and reveals three α -helices and four β -strands as shown in Fig. 4.12.

Figure 4.12: NMR spectroscopic characterization of *MtMrx-1*. The secondary structure elements of the NMR structure of *MtMrx-1* [5] are represented above chemical shift index plot. The scores were derived from $\Delta C^\alpha - \Delta C^\beta$ based on backbone assignment of *MtMrx-1*. A continuous stretch of positive and negative chemical shift index values indicates presence of α -helical and β -strand secondary structure, respectively.

The well dispersed and resolved ^1H - ^{15}N HSQC-spectrum of *MtMrx*-1 enabled us to perform NMR-titration experiments with *MtAhpE*. The ^1H - ^{15}N -HSQC obtained for the labelled *MtMrx*-1 alone, overlaid with those of increasing amounts of *MtAhpE*, exhibit characteristic peak shifts, which indicate transient interactions (Fig. 4.13-A).

Starting from a molar ratio of 0.5 of *MtAhpE* where no change was observed, the titration was carried out in ratios of 1:1, 1:2 and 1:4 wherein further shifting of the peaks were observed. The above-mentioned observation is in agreement with a transient complex formation [44]. In this situation, the chemical shifts change continuously while the binding partner *MtAhpE* is added. Essentially, the interaction is weak and transient such, that the ensemble exists only for the part of time it takes to record the NMR-data. Therefore, the observed chemical shift is a weighted average of the chemical shifts for the protein in its free state and in the complex. The shift progressively occurs until saturation is reached. The key residues undergoing significant chemical shift perturbation included Y16 and C17 of the CGYC-motif of *MtMrx-1* and E37, H38, N39 and R40 in the region between $\beta 2$ and the tip of helix $\alpha 2$ (Fig. 4.13-B). To identify the residues involved in the *MtAhpE-MtMrx-1* interaction, chemical shift differences for the nitrogen and proton dimension were first calculated separately.

The combined chemical shift difference was calculated using the equation; combined shift difference = $[(\Delta^1\text{H})^2 + (\Delta^{15}\text{N}/6.51)^2]^{0.5}$. To obtain significant shifting peaks, residues with shifts higher than the average of all combined shifts plus two standard deviations were used to delineate the interacting residues (Fig. 4.14 A). All residues, which showed significant chemical shift perturbation, were mapped on the NMR structure of *MtMrx-1* (Fig. 4.14-B).

Figure 4.14 A-B: NMR analysis of *MtMrx-1* and *MtAhpE* interaction. (A) Combined ^1H and ^{15}N chemical shift difference between the ^1H - ^{15}N HSQC resonances of the *MtMrx-1* alone and in the presence of four molar excess of *MtAhpE*. CSP above the horizontal line are significant. (B) Surface representation of *MtMrx-1* displaying residues undergoing significant chemical shift perturbation (red). The cysteine catalytic site of the *MtMrx-1* is coloured yellow.

The surface exposed residues formed a well-defined patch around the active site residue CXXC-motif. E44 and S12 were also mapped onto the structure, since these residues show significant chemical shift perturbation and are near the active residues. The distribution of the affected residues delineates an interface for the *MtAhpE-MtMrx-1* interaction.

4.6 HADDOCK modelling of *MtAhpE-MtMrx-1* complex

The obtained NMR restraints (S12, Y16, C17, E37, H38, N39, R40 and E44) for *MtMrx-1*, together with the active site C45 of *MtAhpE*, were then used to drive an HADDOCK-docking simulation. The top scoring HADDOCK model from the highest scoring cluster was selected (HADDOCK Score -60.4 +/- 6.8; Restraint violation energy: 54.0 +/- 20.4 Kcal/mol; Buried surface area: 1545.6 +/- 111.4 Å²; Z-score: -1.2). On a closer look, *MtAhpE* with its C45 at the dimer interface forms a positively charged cavity and amino acids undergoing significant chemical shift perturbation in *MtMrx-1* form a part of a negatively charged surface, allowing electrostatic interactions between the two charged surfaces (Fig. 4.15 A-B).

Figure 4.15 A-C: HADDOCK modelling of *MtAhpE* and *MtMrx-1* complex. A model of electrostatic surface of (A) *MtAhpE* and (B) *MtMrx-1* showing the active site cysteine. (C) Electrostatic surface representation of the docked complex.

The resulting *MtMrx-1-MtAhpE* complex model (Fig. 4.15-C) indicates the spatial proximity of the two catalytic sites; namely C14 of *MtMrx-1* and C45 of *MtAhpE*. Since *MtAhpE* is a dimer in solution, two molecules of *MtMrx-1* are required for reduction of one molecule of *MtAhpE*. An *MtMrx-1* monomer docks into the active site of *MtAhpE*

at the dimer interface and therefore, residues from both monomers come in close contact with the residues of *MtMrx-1* as shown in Fig. 4.16. The complex model reveals that the CGYC-motif of *MtMrx-1* comes near C45 of *MtAhpE* with Y16 forming a hydrogen bond with Q134 of *MtAhpE*. H38 of *MtMrx-1* forms a hydrogen bond with G43 of *MtAhpE*. The complex model reveals a total of seven hydrogen bond contacts between *MtMrx-1* and *MtAhpE* (Fig. 4.16).

Figure 4.16: NMR restraint based docking of *MtMrx-1* and *MtAhpE*. The interaction profile of *MtAhpE*-*MtMrx-1* ensemble. Residues from both monomers of *MtAhpE* come in close contact with the residues of *MtMrx-1*.

This interaction allows residue C14 of *MtMrx-1* to form a disulphide bond with C45 of *MtAhpE* with the release of a water molecule. Subsequently, C17 of *MtMrx-1* attacks to break the mixed disulphide and an intramolecular disulphide bond can be formed between C17 and C14 of *MtMrx-1*. The observed epitope of this interaction provides an opportunity to develop inhibitors of protein-protein interaction to disrupt the recycling of *MtAhpE*.

4.7 NMR provides insights into the catalytic sites of *MtMrx-1*

One of the two proposed reducing systems of *MtAhpE* is the MSH/*MtMrx1* pathway, in which mycoredoxin is predicted to form a *MtAhpE*-SS-Mrx complex, which serves as a substrate for the reduced mycoredoxin (*MtMrx1*-(S)₂), leading to the reduced *MtAhpE*-SH. To examine the mechanism in depth as well as the conditions required for

the formation of this mixed disulphide, NMR studies using ^{13}C -labeled cysteines of *MtMrx-1* were carried out. The ^1H - ^{13}C -HSQC spectrum of the oxidized ^{13}C -labeled *MtMrx-1* alone revealed three major ^{13}C -chemical shift peaks at 28.4 ppm, 39.6 ppm and 59 ppm (Fig. 4.17-A), reflecting the presence of a reduced- (*MtMrx-1*-SH), sulphenic- (*MtMrx-1*-SOH) and sulphinic form (*MtMrx-1*-SO₂H) in solution, respectively. To delineate the reduced states of *MtMrx-1*, the reducing agent DTT has been employed, causing a reduced doublet peak at 26 ppm (Fig. 4.17-B).

Figure 4.17 A-B: ^{13}C -Cys labelled HSQC-spectrum of *MtMrx-1* under different conditions. (A) Oxidized *MtMrx-1*, (B) *MtMrx-1* reduced with 5 molar excess of DTT. The peaks corresponding to -SH (purple), -SOH (pink), -SO₂H (dark green) are shown and indicates different redox states of cysteines under different conditions.

Furthermore, the ability of MSH to reduce *MtMrx-1* was examined by the addition of molar excess of MSH (Fig. 4.18-A).

Figure 4.18 A-B: ^{13}C -Cys labelled HSQC-spectrum of *MtMrx-1* under different conditions. (A) *MtMrx-1* in the presence of MSH, (B) *MtMrx-1* in the presence of 3 molar excess of *MtAhpE*. The peaks corresponding to $-\text{SH}$ (purple), $-\text{SOH}$ (pink), $-\text{SO}_2\text{H}$ (dark green) are shown and indicate different redox states of cysteines under different conditions.

In order to confirm whether *MtMrx-1* forms a mixed disulphide with *MtAhpE* as previously shown by mass spectrometry [6], a three-molar excess of *MtMrx-1* was incubated with cysteine-labeled *MtAhpE* and the state of C45 was observed using NMR spectroscopy. The NMR spectrum of the *MtAhpE-Mrx-1* mixture (1:3) was collected and a peak at $^{13}\text{C}=42$ ppm, which corresponds to an *AhpE-SS-Mrx-1* disulphide, was observed (Fig. 4.19). This result confirms an intermediate state of the *MtMrx-1*-dependent reduction of *MtAhpE* in solution.

Figure 4.19: ^{13}C -Cys labelled HSQC-spectrum of *MtAhpE* in the presence of *MtMrx-1*. Oxidised *MtAhpE* in the presence of 3 molar excess of *MtMrx-1*. The peaks corresponding to $-\text{SH}$ (blue), $-\text{SOH}$ (red), $-\text{SO}_2\text{H}$ (green) are shown and indicates different redox states of C_P under different conditions.

4.8 NMR provides insights into the interaction between *MtMrx-1* and *MtMtr*

The experiment was performed with help of Dr. Shin Joon from Prof. Gerhard Grüber's team and data were analysed by Dr. Shin Joon. The highly resolved and dispersed NMR-spectrum of *MtMrx-1* enabled titration experiments to be performed at three different molar ratios of *MtMrx-1* to *MtMtr* (1:1, 1:2 and 1:3.5). During titration experiments, separated ^{15}N and HN-resonances were observed for some residues, and relative peak intensities changed by increasing molar ratios of *MtMtr* (Fig. 4.20). A plot

of relative change of intensities for separated cross peaks after addition of *MtMtr* at molar ratio 1:3.5 (*MtMrx*-1: *MtMtr*) is shown in Fig. 4.21. Since no line broadening effects were observed after addition of *MtMtr* to *MtMrx*-1, the relative change of intensities was calculated based on the peak height of each of the separated peaks.

Figure 4.20: ^{15}N labelled HSQC-spectrum of *MtMrx*-1 upon titration with *MtMtr*. The overlaid ^1H - ^{15}N -HSQC spectra of *MtMrx*-1 without (red) and with *MtMtr* (molar ratio 1:3.5); the spectra show an intensity change in some separated cross-peaks on *MtMtr* addition as indicated by arrows.

Based on previously reported chemical shift information in the BMRB database of the reduced and oxidized *MtMrx*-1 [accession code: 18322 (reduced) and 18325 (oxidized)], the analysis identified that the chemical shifts of separated additional resonances correspond to those of the oxidized form of *MtMrx*-1. This is also supported by changes of the collected $^{13}\text{C}\alpha/^{13}\text{C}\beta$ chemical shift data of residues C14 and C17 corresponding to oxidized *MtMrx*-1. In cases of the *MtMrx*-1 amino acids T11, S12, W13($\text{NH}\epsilon$), C14 and C17, we observed two separated cross peaks in the HSQC spectra.

One peak with higher intensity was identified as a reduced form, while another one was identified as an oxidized form (Fig. 4.20).

Based on the intensity ratio between the reduced and oxidized form, we can deduce the population of the reduced to oxidized form of *MtMrx*-1 as 90% (reduced) to 10% (oxidized). During titration, the cross-peak intensity of the reduced form decreased and the intensity of the oxidized form gradually increased and an average population of oxidized *MtMrx*-1 was deduced as ~50% (Fig. 4.21). These observations demonstrate a molecular interaction between *MtMtr* and -*Mrx*-1, including a conformational transition from a reduced to an oxidized form of *MtMrx*-1 caused by molecular interactions of both enzymes.

Figure 4.21. NMR analysis of *MtMrx*-1 interaction with *MtMtr* Plot of relative change of intensity ratios in separated resonances in HSQC spectra. Intensity ratios between oxidized and reduced resonances of *MtMrx*-1 of without (RI_o) and with *MtMtr* (RI_i) was calculated based on the peak heights. Relative change of intensity ratio was determined by RI_o/RI_i .

No significant chemical shift perturbations of the reduced and oxidized form or intermediate resonances were observed during titration, which means that the molecular interaction between both enzymes is weak and that a kind of fast exchange process occurs. Therefore, an exact binding epitope of *MtMrx*-1 to *MtMtr* cannot be assigned. However, one can deduce that the residues L6-C17, V34-G47, V59, K60 and T68-L79 might be directly or indirectly involved in protein-protein interaction based on the relative change of peak intensity ratio for each separated peak.

Structural and mechanistic insights into peroxiredoxin AhpE from *Mtb*

5.1 Introduction to peroxiredoxin *MtAhpE*

MtAhpE has been studied extensively in recent years as a model system to understand 1-Cys peroxiredoxin (69,86-88,91,153). In general, *MtAhpE*-SH catalyzes the reduction of H_2O_2 to water and in turn gets converted to *MtAhpE*-SOH. For a long time, the pathway which recycles the *MtAhpE*-SOH was not known. Recently, the MSH/Mrx-1 dependent system has been proposed to recycle the *MtAhpE*-SOH in mycobacterial species (69). It has been proposed while the MSH pathway might be operational in cytoplasm, the Mrx-1 pathway might be favored in membranes. Both these pathways require Mrx-1 for continuous recycling of oxidised *MtAhpE*-SOH. However, a detailed mechanism of the recycling process remains unclear.

This chapter describes cloning, production, and purification of *MtAhpE* is described in detail, followed by DLS and SAXS experiments to confirm the oligomeric state of *MtAhpE* in solution and changes in its quaternary structure in presence of substrate. This is followed by NMR studies carried out to understand the interaction of MSH with *MtAhpE* and to study the redox state of peroxidatic cysteine (Cp). The chapter concludes with crystallization studies which was attempted to achieve an AhpE-MSH complex.

5.2 Cloning, production, and purification of *MtAhpE*

The *MtAhpE* coding region was amplified using *Mtb* genomic DNA as template. PCR resulted in a clear band corresponding to 462 bp as shown in Fig. 5.1. The PCR product was gel extracted, digested using *Bam*HI and *Nco*I, ligated into pET9d-1 vector, and transformed into DH5 α cells using electroporation. The presence of the insert was confirmed using control digestion using *Bam*HI and *Nco*I. The insert sequence was verified using sequencing. The successfully cloned constructs were transformed into *E. coli* BL21 (DE3) for overproduction. The production was confirmed using induction test (Fig. 5.2-A).

Figure 5.1 PCR amplification of *MtAhpE*. PCR of *MtAhpE* gene from *Mtb* genomic DNA using PCR. DNA sample of 5 μ l was loaded on an 1% agarose gel.

The SDS-PAGE of recombinant protein revealed a prominent band of about 16 kDa. Based on the solubility test, 50 mM Tris, 250 mM NaCl, pH 7.5 was selected as appropriate buffer for purification of recombinant *MtAhpE*.

Figure 5.2. A-B: Induction and solubility test for recombinant *MtAhpE*. (A) Induction test of *MtAhpE*: SDS gel showing induced (+ve) and uninduced (-ve) samples. Induction was done with 1 mM IPTG at 37 °C for 3 hrs. A prominent band is seen around 15 kDa which corresponds to recombinant *MtAhpE*. (B) Solubility test of *MtAhpE*: 17% SDS gel showing the protein seen in pellet (P) and supernatant (SN) in various buffers (1-5) as described earlier. Although the major portion of recombinant *MtAhpE* is seen in the pellet, some portion of *MtAhpE* is soluble.

Since the recombinant protein was cloned with 6-His-tag at the N-terminus of *MtAhpE*, Ni²⁺-affinity chromatography was used as a first step of purification. *MtAhpE* was separated from the other impurities using an imidazole gradient (15-400 mM) in buffer consisting of 50 mM Tris, 250 mM NaCl, pH 7.5, 1 mM PMSF. Clear bands were identified in the fractions 75-300 mM on an SDS-gel (Fig. 5.3-A).

Figure 5.3. A-B: Purification of *MtAhpE* (A) Ni²⁺-NTA purification profile showing the desired bands of *MtAhpE*. Fractions from 75 mM to 300 mM imidazole were pooled and used for further purification. (B) Superdex™ S75 purification profile of *MtAhpE*, insert in figure B shows the SDS-PAGE gel after Superdex™ S75 purification. The grey color shows peak fractions which were pooled and used for subsequent experiments. In Figure B lane, 1 and 2 represent a protein marker and profile of protein after size exclusion chromatography, respectively.

Since pure protein was found at 75 to 300 mM imidazole concentrations, these fractions were pooled together and concentrated, and then passed through a size exclusion Superdex 75 (10/300) GL column (GE Healthcare), to get pure and homogeneous protein (Fig. 5.3-B). The protein eluted at 11.5 ml on a S-75 column, corresponding to a dimeric species of around 35 kDa. SDS-PAGE was performed to analyze the purity of the isolated proteins (Fig. 5.3-B).

5.3 DLS studies indicates substrate-induced conformational changes in *MtAhpE*

To confirm the results obtained by SEC above as well as to investigate changes in the oligomeric state in the presence of substrate, DLS of *MtAhpE* was performed in buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl. These experiments were carried out at concentrations of 5 mg/ml using a Malvern Zetasizer Nano ZS instrument. To understand the role played by H₂O₂, measurements were made after adding equimolar concentrations of H₂O₂ (incubation time of 1 and 60 min) as well as five-fold molar excess of H₂O₂ with an incubation time of 60 min. As shown in Fig. 5.4, the DLS profile of native *MtAhpE* revealed a dimeric species with an estimated molecular weight of 37 kDa, confirming the SEC results described above. The DLS profile of sample incubated with equimolar H₂O₂ revealed the formation of a higher molecular weight species of about 72 kDa with slower diffusion properties, which was independent of the incubation time used (1 or 60 min; black dashed and solid line respectively). In comparison, the profile in the presence of an excess of five-fold molar excess H₂O₂ (60 min incubation) was similar to the one of the native *MtAhpE*, indicating the formation of a dimeric enzyme (blue line).

Figure 5.4. Dynamic light scattering studies on *MtAhpE*. DLS-plot of native *MtAhpE* (red) indicating a dimeric species, and *MtAhpE* in the presence of equimolar H₂O₂ (black dashed line; incubation time of 1 min and black solid line; incubation time of 60 min) indicating presence of higher molecular weight oligomer. However, in presence of an excess of H₂O₂ (60 min incubation; blue) plot was similar to the native *MtAhpE*, reflecting the formation of a dimeric enzyme.

These results agree with earlier studies by Hugo et al. (88) reporting that the addition of equimolar concentrations of H_2O_2 led to an increase of higher oligomers of *MtAhpE* in a time-dependent manner (1 to 60 min), whereby the addition of excess H_2O_2 resulted again in a dimeric form.

5.4 SAXS studies on *MtAhpE*

The quaternary structure of *MtAhpE* in solution is proposed to be regulated by the redox state, existing as a dimer in the reduced state and slowly forming high molecular weight aggregates upon oxidation in the presence of equimolar peroxide. To gain insight into the solution behavior under different redox states and to confirm the oligomeric state of *MtAhpE*, SAXS experiments were carried out at different concentrations under different redox environment.

The SAXS scattering pattern were measured at three different protein concentrations (2.3, 3.4 and 5 mg/ml) in a buffer containing 50 mM Tris/HCl, pH 7.5, 250 mM NaCl and 1 mM DTT for the reduced protein as shown in Fig. 5.5-A. For the oxidized protein, separate purification was made in buffer containing 50 mM Tris/HCl, pH 7.5, 250 mM NaCl without DTT and three different concentrations (2.6, 4.8 and 5.2 mg/ml) were used for measuring the scattering pattern. The final composite scattering curve for reduced *MtAhpE* is shown in Fig. 5.5-A and that for oxidized *MtAhpE* is shown in Fig. 5.6-A. No radiation damage and significant concentration effect was detected over the data collection time.

Figure 5.5.A-B: SAXS pattern of reduced *MtAhpE*. (A) SAXS pattern of *MtAhpE* in 50 mM Tris/HCl, pH 7.5, 250 mM NaCl. The red, blue and the pink curve represents the scattering pattern at 2.3, 3.4 and 5.0 mg/ml, respectively. (B) The Guinier plots at low angles are linear, indicating no aggregation.

Inspection of the Guinier plots revealed good data quality and no protein aggregation under different redox states as shown in Fig. 5.5-B and 5.6-B for reduced and oxidized *MtAhpE*, respectively.

Figure 5.6.A-B: SAXS pattern of oxidized *MtAhpE*. (A) SAXS pattern of *MtAhpE* in 50 mM Tris/HCl, pH 7.5, 250 mM NaCl. The blue, red and the green curve represents the scattering pattern at 2.6, 4.8 and 5.2 mg/ml, respectively. (B) The Guinier plots at low angles are linear, indicating no aggregation.

After confirming the data quality, data for both the reduced and oxidized enzyme were processed using ATSAS 2.7.2 package and were compared to understand the changes upon redox modulation. For the reduced enzyme, R_g values from the Guinier approximation was consistent for the three concentrations (2.3, 3.4 and 5 mg/ml) measured with a value of 24.75 ± 0.18 Å (Table 5.1). The R_g -values (24.65 ± 0.50 Å) extracted from the $P(r)$ function agreed to the one derived from the Guinier region.

Figure 5.7.A-B: Solution X-ray scattering studies of *MtAhpE*: $P(r)$ function and Kratky plot. (A) Distance distribution functions of reduced- (5.0 mg/ml; red) and oxidized *MtAhpE* (5.2 mg/ml; blue). The difference in the range of 40 to 60 Å for the oxidized and reduced *MtAhpE* is indicated by an arrow. (B) Normalized Kratky plot of oxidised- (●; blue), reduced *MtAhpE* (●; red), and the compact globular lysozyme (●; grey) with a peak (---; grey), representing the theoretical peak and assuming an ideal Guinier region of a globular particle. When compared to the reduced *MtAhpE*, the profile of the oxidized protein is slightly deviated. This, as well as small shifts in the peak of the oxidized form, is shown by arrows.

The distance distribution functions determined were similarly shaped for both reduced and oxidised *MtAhpE* (Fig. 5.7-A) and the D_{max} was determined to be 76 ± 3 Å. The right-skewed distribution indicates that the protein has an elongated shape. The normalized Kratky plot of reduced *MtAhpE* shifted from a well-defined bell curve profile (Fig. 5.7-B). The peak is deviated to slightly higher values and the profile is broadened, indicating that reduced *MtAhpE* exhibits an extended conformation and might be less rigid in solution.

The determined R_g - (24.12 ± 0.40 Å) and D_{max} values (76 ± 4 Å) as well as the normalized Kratky plot of the oxidised *MtAhpE* were similar to the ones of the reduced enzyme. This data indicates that the dimeric state of *MtAhpE* in solution is independent to the oxidation state. However, the comparison of the distance distribution functions of the oxidised and reduced *MtAhpE* revealed differences in the range of 40 to 60 Å, indicating some alteration inside the dimer molecule due to redox modulation. This is also confirmed by the comparison of the normalized Kratky plots (Fig. 5.7-B). The profile for the oxidised *MtAhpE* is slightly deviated outward and a small shift in the peak was also observed, reflecting a slight conformational change of the oxidised form.

Figure 5.8: The *ab initio* DAMMIN reconstruction of *MtAhpE*. A DAMFILT bead model of *MtAhpE* at a concentration of 5 mg/ml. The model clearly indicates that *MtAhpE* is a dimer in solution. The crystal structure of *MtAhpE* (shown in ribbon) is well embedded into the *ab initio* model. The *ab initio* model was fitted onto the crystal structure of *MtAhpE* (PDB ID:5ID2) using SUPCOMB.

The *ab initio* shape for *MtAhpE* was determined by DAMMIN using default parameters (P1 symmetry). Twenty independent DAMMIN calculations were performed and the DAMAVER program was used to produce the final average structure of 20 independent reconstructions. A DAMFILT model is shown in (Fig. 5.8) with a NSD value of 0.586 ± 0.014 . The *ab initio* model was fitted onto the crystal structure (PDB ID: 5ID2) using SUPCOMB, where the crystal structure of the dimeric *MtAhpE* is well embedded into the *ab initio* model.

The theoretical scattering curves of the crystallographic dimer of *MtAhpE* (PDB ID: 5ID2) was calculated using CRY SOL and was fitted with the experimental scattering data. The corresponding fit with the experimental data led to a χ^2 value of 0.169 (Fig. 5.9). The theoretical pattern of a dimer fits very well with the experimental data and this indicates that only a dimeric species of *MtAhpE* is present in solution.

Figure 5.9: Fitting of experimental data using CRY SOL. The fitting curve calculated using CRY SOL from the dimeric crystal structure of *MtAhpE* (PDB ID: 5ID2; black) with the experimental data (pink) of *MtAhpE* recorded at a concentration of 5 mg/ml.

Table 5.1: SAXS-data collection and scattering derived parameters of the reduced- and oxidized *MtAhpE*

Data collection parameters		
Instrument (source & detector)	Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VÅNTEC-2000 detector	
Beam geometry	100 μm slit	
Wavelength ($\text{\AA}$)	0.134	
q -range ($\text{\AA}^{-1}$)	0.16 – 4	
Exposure time (min)	10 (10 frames x 1 min)	
Temperature (K)	288.15	
Protein sample	reduced <i>MtAhpE</i>	oxidized <i>MtAhpE</i>
Concentration range (mg ml^{-1})	2.3 – 5.0	2.6 – 5.2
Structural parameters[#]		
$I(0)$ (cm^{-1}) [from $P(r)$]	12.51 ± 0.18	10.65 ± 0.10
R_g ($\text{\AA}$) [from $P(r)$]	24.65 ± 0.50	24.07 ± 0.17
$I(0)$ (cm^{-1}) (from Guinier)	12.44 ± 0.10	10.72 ± 0.11
R_g ($\text{\AA}$) (from Guinier)	24.75 ± 0.18	24.12 ± 0.40
D_{max} ($\text{\AA}$)	76 ± 3	76 ± 4
Porod volume estimate ($\text{\AA}^3$)	51 013	40 342
Dry volume from sequence ($\text{\AA}^3$) ‡ (monomeric)	20 350	20 350
Molecular mass determination		
Calculated monomeric MM (kDa) [from sequence] *	17	17
MM from Porod invariant ($V_p/1.6$) (kDa)	30 ± 2	25 ± 3
MM from excluded volume ($V_{\text{ex}}/2$) (kDa)	30 ± 3	28 ± 3
MM from volume of correlation, V_c (kDa)	37 ± 3	33 ± 3
Software employed		
Primary data reduction	BRUKER SAS	
Data processing	PRIMUS	
<i>Ab initio</i> analysis	DAMMIN	
Validation and averaging	DAMAVAR	
Computation of model intensities	CRY SOL	
3D graphics representations	PyMOL	

‡ <http://www.basic.northwestern.edu/biotools/proteincalc.html>* http://web.expasy.org/compute_pi/

5.5 SAXS experiments of *MtAhpE* in the presence of substrate

In order to confirm whether the addition of equimolar peroxide resulted in a higher molecular weight form as observed in the DLS studies as shown in section 5.3, SAXS experiments were carried out at a concentration of 5 mg/ml in presence of equimolar concentration of peroxide as well as excess of peroxide. The SAXS data demonstrated that the R_g - (23.56 ± 0.44 Å) and D_{max} values (76 ± 5 Å) did not alter after addition of 5 molar excess of H_2O_2 , while the R_g - (27.71 ± 0.42 Å) and D_{max} values (98 ± 7 Å) increased in the presence of equimolar concentrations of H_2O_2 as shown in Fig. 5.10. This clearly indicates that the addition of equimolar peroxide results in a higher molecular weight form.

Figure 5.10 A-B: SAXS experiment of *MtAhpE* in the presence of substrate. (A) SAXS patterns of *MtAhpE* (5.0 mg/ml) after incubation of either equimolar concentration or excess of H_2O_2 for 30 min. (B) Distance distribution functions of *MtAhpE* derived from the respective scattering curves.

In summary, the SAXS experiments clearly indicate that both the oxidised and reduced *MtAhpE* are dimeric in solution and that in the presence of equimolar substrate the quaternary structure of *MtAhpE* shifts from a dimer to a higher molecular weight structure. These results are in agreement with the DLS results described in section 5.3.

5.6 NMR provides novel insights into the recycling of *MtAhpE*

Recent studies proposed a MSH/Mrx-1 dependent pathway to reduce *MtAhpE*. Briefly, using mass spectrometry, MSH was shown to form a mixed disulphide (*MtAhpE*-SS-MSH) complex, which serves as a substrate for the reduced mycoredoxin ($Mrx1-(SH)_2$), which cleaves this mixed disulphide leading to the formation of *MtAhpE*-SH. In order to confirm and investigate this in detail, NMR experiments were carried out.

Firstly, a 1D NMR spectrum was acquired for purified *MtAhpE* in 50 mM phosphate, 50 mM NaCl, pH 6.8. The 1D spectrum (Fig. 5.11) indicated a well folded protein with well-dispersed and distinct characteristic peaks in the amide region (6 to 10 ppm), α -region (3.5 to 5 ppm), and uphill shifted methyl protons region (-0.5 to 1.0 ppm) (Fig. 5.11).

Figure 5.11: 1D NMR spectrum of *MtAhpE*. 1D NMR spectrum of *MtAhpE* recorded at 298 K in 50 mM phosphate buffer, pH 6.8, 50 mM NaCl and 5 mM EDTA on a Bruker Avance 700 MHz machine.

Since *MtAhpE* contains only the cysteine, C45 which is the peroxidatic cysteine, this residue was specifically observed using ^{13}C -labeled cysteine. 2D NMR (^1H - ^{13}C -HSQC) was then used to understand the redox state of peroxidatic cysteine. All the experiments were carried at 298 K. To understand the conditions required for the formation of this mixed disulphide, NMR studies were carried out using the selectively ^{13}C -labeled cysteine of *MtAhpE* under different redox states. The ^1H - ^{13}C -HSQC spectrum of the oxidized ^{13}C -labeled *MtAhpE* alone revealed three major ^{13}C -chemical shift peaks at 28.4 ppm, 39.6 ppm and 59 ppm as shown in Fig. 5.12-A. These peaks indicate the presence of a reduced- (AhpE-SH), sulphenic- (AhpE-SOH) and sulphinic- (AhpE-SO₂H) form in solution, respectively. Using this spectrum as a control, a 10-molar excess of peroxide was added to study changes in the redox states. This resulted in a

single peak at 59 ppm (Fig. 5.12-B) representing a highly over oxidized *MtAhpE*-SO₂H. These results agreed with previous experiment in which excess of peroxide led to the irreversible formation of the sulphinic derivative of *MtAhpE* (87).

Figure 5.12.A-B: ¹³C-Cys labelled HSQC-spectrum of *MtAhpE* under different redox conditions. (A) Oxidized *MtAhpE*, (B) *MtAhpE* in presence of excess of H₂O₂. The peaks corresponding to –SH (blue), –SOH (red), –SO₂H (green) are shown and indicate different redox states of C_P under different conditions.

To delineate the reduced states of *MtAhpE*, the two reducing agents TCEP (*tris*(2-carboxyethyl) phosphine) and DTT were used in 5 molar excess of *MtAhpE*. Samples were incubated for 30 min prior to data collection. The reduction with TCEP and DTT resulted in two peaks at 30 ppm (Fig. 5.13-A) and 26 ppm (Fig. 5.13-B), respectively.

Figure 5.13. A-B: ¹³C-Cys labelled HSQC-spectrum of *MtAhpE* in the presence of different reducing agents. (A) *MtAhpE* in the presence of 5 molar excess of TCEP, (B) *MtAhpE* in the presence of 5 molar excess of DTT. The peaks corresponding to –SH (blue), –SOH (red), –SO₂H (green) are shown and indicate different redox states of C_P under different conditions.

The differences in reduced chemical shift positions represent the differences in the mechanism of reduction by each of these reducing agents. Finally, the ability of MSH to interact with C45 of *MtAhpE* was examined by the addition of 5-molar excess of MSH (Fig. 5.14-A) or MSH + H₂O₂ (Fig. 5.14-B). Interestingly, in both cases, two additional reduced peaks could be observed, which were characteristic to the presence of MSH. The above data implicated for the first time a possible by-pass mechanism for *MtAhpE* reduction, where MSH can first form an adduct with *MtAhpE*, followed by a reduction mediated by a second molecule of MSH.

Figure 5.14.A-B: ¹³C-Cys labelled HSQC-spectrum of *MtAhpE* in the presence of MSH and MSH plus H₂O₂. (A) *MtAhpE* in the presence of 5-molar excess MSH (B) *MtAhpE* in the presence of 5 molar excess of MSH plus H₂O₂. The peaks corresponding to –SH (blue), –SOH (red), –SO₂H (green) are shown and indicates different redox states of C_P under different conditions. The peak for –SS– is shown in orange color and corresponds to probable AhpE-SS-M complex.

Interestingly, by comparing the two spectra, an additional oxidized peak was observed at ¹³C=42 ppm in the presence of H₂O₂, corresponding to a possible disulphide adduct (–SS–) (Fig. 5.14-B). This confirms previous mass spectrometry analysis, where an AhpE-SS-M mixed disulphide was observed in the presence of peroxide [6]. The fact that a reduced state and not a mixed disulphide was observed when MSH alone was added indicated a MSH-dependent mechanism of *MtAhpE* reduction, whose kinetics may be too fast to be observed by conventional HSQC.

To understand whether the MSH-analogue NAC could also reduce *MtAhpE*, the experiment was performed using 5 molar excess of NAC. It was interesting to note that the reduced peaks are similar to that observed in the case of MSH (Fig. 5.15-A). This

confirms that NAC works similarly as MSH and provides an alternative reducing agent to study *MtAhpE*. Most importantly like MSH, NAC could also directly reduce *MtAhpE*-SOH to *MtAhpE*-SH without the requirement of Mrx-1.

It was also observed that *MtAhpE* is slowly over-oxidized in the absence of a reducing agent and peaks corresponding to the sulphinic- (*AhpE*-SO₂H) and sulphonic form (*AhpE*-SO₃H) in solution were observed when the NMR experiment was carried out over a longer duration of 12 hrs. (Fig. 5.15-B). This is the first report of *MtAhpE* with peroxidatic cysteine in the sulphinic acid form and may represent an irreversible inactivated form, which may not be biologically relevant due to continuous recycling of *MtAhpE*-SOH to *MtAhpE*-SH.

Figure 5.15.A-B: ¹³C-Cys labelled HSQC-spectrum of *MtAhpE* in the presence of NAC and in absence of any reducing agent. (A) *MtAhpE* in the presence of 5 molar excess of NAC, (B) *MtAhpE* in the absence of any reducing agent. The peaks corresponding to -SH (blue), -SOH (red), -SO₂H (green) are shown and indicates different redox states of C_P under different conditions.

In summary, a new pathway for recycling of *MtAhpE* was discovered which involves direct reduction by MSH in the absence of *MtMrx*-1, which along with the other two pathways recently proposed are described schematically in Fig. 5.16. The above results also enabled us to understand that it might be possible to trap *MtAhpE*-SS-M mixed disulphide adduct in presence of MSH and H₂O₂ and therefore, an attempt was made to crystallize the complex as described in the next section.

Figure 5.16: The proposed mechanism of a Mrx1-independent reduction of MtAhpE. (I) The newly proposed mechanism of MtAhpE reduction, which is entirely independent on Mrx-1. In the first step, an MSH molecule forms an adduct with MtAhpE. The S-Mythiolated adduct is then attacked by a second molecule of MSH, leading to the release of MSSM and the generation of reduced MtAhpE in a process akin to the 2-step reduction of Mrx1 [46]. (II) One of the accepted mechanisms of MtAhpE reduction suggests, that one molecule of MSH forms a mixed disulphide with MtAhpE-SO₃H followed by a release of water. The mixed disulphide is attacked by reduced Mrx-1, leading to the reduction of MtAhpE. (III) Another mechanism of MtAhpE reduction via Mrx-1 proposes that a sulphenic derivative of MtAhpE is directly reduced by Mrx-1 in two steps to yield MtAhpE-SH.

5.7 Crystallization of *MtAhpE* and *MtAhpE*-MSH co-crystals

Initially *MtAhpE* crystals were obtained under the condition already published (86) (1.8 M sodium malonate pH 5.0 mixed with 0.1 M sodium acetate pH 4.5). Both hanging drop and sitting drop method of vapour diffusion were tried. The concentration of *MtAhpE* used for crystallization was 2 mg/ml, 5 mg/ml and 9 mg/ml. The crystals were obtained after 24 hrs, however they were of very small size (Fig. 5.17-A). In order to increase the size of the crystals, crystallization was optimized using 5-15% glycerol as additive which solubilize the protein further and prevents excessive nucleation. Crystals of bigger size (0.05 x 0.05 x 0.2 mm) were obtained (Fig. 5.17-B) and used for diffraction test. Bigger single crystals (Fig. 5.17-C) were tested in-house at 100 K on a Rigaku RAXIS IV image plate detector with a Rigaku RA-Micro 7 HFM rotating copper anode generator. These crystals showed diffraction spots going up to 4 Å (Fig. 5.17-D).

Figure 5.17. A-D: Crystallization of *MtAhpE*. Selected pictures of native *MtAhpE* crystals and diffraction pattern. Crystal drops were set up in the condition 1.8 M sodium malonate pH 5, 0.1 M sodium acetate pH 4.5. (A) Small crystals obtained initially, (B) Larger crystals of *MtAhpE* obtained after adding 10% glycerol, (C) Single crystal used for diffraction test and (D) Diffraction pattern, respectively.

After diffraction test of *MtAhpE* crystals, same condition (1.8 M sodium malonate pH 5.0 mixed with 0.1 M sodium acetate pH 4.5) was then used for co-crystallization of *MtAhpE* with different concentration of MSH in presence of H_2O_2 .

Figure 5.18.A-B: Crystallization of *MtAhpE*-MSH crystals. (A-B) Selected pictures of *MtAhpE*-MSH crystals. Crystal drops were set up in the condition 1.8 M sodium malonate, 0.1 M sodium acetate pH 5, 10% glycerol in the presence of MSH and H_2O_2 .

Crystals of *MtAhpE* grown in presence of MSH and H_2O_2 (tetragonal space group $P4_2$) were obtained as shown above in Fig. 5.18.A-B and the diffraction data were collected with the help of Dr. Asha Balakrishna from Prof. Gerhard Grüber's team at NSRRC, Taiwan. The structure was solved together with Dr. Asha Balakrishna. The crystallographic structure was refined to 2.43 Å resolution with a final R-factor and R-free (calculated with 5% of reflections that were not included in the refinement) of 0.17 and 0.21, respectively (Table 5.2).

The asymmetric unit consists of four molecules comprising of two dimers (AB and CD, respectively) with a solvent content of 53.33%, 218 water molecules, two molecules of glycerol and one molecule of acetate ion in the final structure (Fig. 5.19). Although MSH was not seen bound to AhpE in the final structure, several differences were clearly evident in the active site as well as the C-terminal loop position in different monomers of the asymmetric unit. For a clear understanding, chain A and D are here after called oxidized (*MtAhpE*-SOH) conformation, and chain B and C, are called reduced conformation (*MtAhpE*-SH) which occurred due to MSH reduction. The r.m.s.d. between chain B and chain C was 0.168 Å, whereas for chain C and chain D the determined r.m.s.d. value was 0.75 Å for 153 Cα atoms.

Figure 5.19: Crystal structure of *MtAhpE* in an asymmetric unit. The chains are coloured A; yellow, B; orange, C; red and D; blue, respectively. In chain A and D, the C_P is in the oxidized state, where as in the chain B and C, it is determined to be in the reduced state. The *inset* shows the electron density omit map contoured at 2 sigma level for C_P (chain D, blue).

As revealed by the recently determined *MtAhpE* crystal structure [5], *MtAhpE* contains nine β -strands, β 1- β 9, and five α -helices, α 1- α 5, with its characteristic C_p at position 45 (C45) (Fig. 5.20). C45 is located at helix 2, inside the atypical PXXXTXXC-motif of the Prx-active site and surrounded by a trio of the conserved residues P38 and T42 of the $_{38}\text{PXXXTXXC}_{45}$ motif and R116 present in-between β 7 and β 8 of *MtAhpE*.

Figure 5.20: Crystal structure of *MtAhpE*. Cartoon representation of one monomer of chain B of *MtAhpE*. *MtAhpE* contains nine β -strands, β 1- β 9, and five α -helices, α 1- α 5, with its characteristic C_p at position 45 (C45). The C_p, as well as the active site R116 is shown as sticks. The C-terminal loop $_{131}\text{EMKQPGEVRD}_{140}$ in-between β 9 and α 5 is colored red.

Table 5.2: Data collection, refinement, and processing statistics of the *MtAhpE* structure [PDB ID :5ID2]**Data collection statistics:**

Wavelength	1.00
Space group	P4 ₂
Unit cell parameter (Å)	$a = b = 146.91, c = 33.62$
	$\alpha = \beta = \gamma = 90 (^{\circ})$
Resolution range (Å)	30-2.43
Solvent content (%)	53.34
Total number of reflections	111 827
Number of unique reflections	27 862
I/ σ	14.5 (2.9)
Completeness (%)	99.6 (97.3)
R-merge (%)	6.7 (27.6)
Redundancy	4.0 (3.9)

The values in the parentheses is for the last resolution shell (2.52 - 2.43 Å)

Refinement statistics:

R-factor (%)	17.6
R-free (%)	21.6
Number of amino acid residues	623
Ramachandran statistics	
Most favored (%)	90.3
Additionally allowed (%)	7
Generously (%)	0
Disallowed (%)	0
R.M.S. Deviations	
Bond lengths (Å)	0.006
Bond angles ($^{\circ}$)	1.05
Mean atomic B-values	
Overall	27.4
Wilson B-Factor	29.01
Clash score	3.33
Molprobit score	1.31

The position of amino acid T42 in the $_{38}\text{PXXXTXXC}_{45}$ motif enables C45 to interact with the substrate and P38 provides a hydrophobic surface, shielding the reactive cysteine sulfenic acid (-SOH) intermediate from further oxidation by peroxides and peroxynitrite. An active site arginine (R116), which would lower the pK_a of the peroxidatic cysteine by stabilizing its thiolate form, is conserved among all Prxs [40]. R116 is in close proximity to C45 and stabilizes the Cp thiolate via a hydrogen bond with the backbone amide hydrogen at a distance of 3.07 Å (Fig. 5.21).

Figure 5.21: Electron density map (2FoFc) of the 131EMKQPGEVRD140 C-terminal loop. *MtAhpE* (chain B; orange) with the cysteine in the MSH-reduced conformation. The electron density map (2FoFc) of the $_{131}\text{EMKQPGEVRD}_{140}$ -loop is contoured at 1 sigma level and the interacting conserved residues “RER” are shown in stick representation.

The active site formation becomes stabilized by the hydrogen bond interaction of residue E48 of helix α_2 , and R139 of the C-terminal loop in-between β_9 and α_5 (Fig. 5.22). Among 1-Cys Prxs, a conserved histidine precedes the PXXXTXXC-motif. However, in case of *MtAhpE*, the histidine residue is replaced by amino acid F37. The relevance of amino acids R116, P38, E48 and T42 is in line with recent MD simulations of *MtAhpE* in the presence of H_2O_2 , which described the importance of hydrogen bonding interactions and the inter-molecular network formed by these residues [41].

Figure 5.22: Superimposition of the *MtAhpE*-SH (chain *C*, red) and *MtAhpE*-SOH (chain *D*, blue). The hydrogen bond network, which stabilizes the CP, is shown in the inset and the interactions within 5 Å are shown in dotted lines. All the residues involved in the hydrogen bonding are presented as sticks and are labelled.

Fig 5.23 A-B reveal that these critical residues form a similar molecular network in the presented crystallographic structure in chain *C* (*MtAhpE*-SH) and chain *D* (*MtAhpE*-SOH). Furthermore, the catalytic C45 containing helix $\alpha 2$ is stabilized by a hydrogen bond network between amino acid Q46 and D50 of helix $\alpha 2$ and W80 and S84 of helix $\alpha 3$.

Figure 5.23 A-B: Hydrogen bonding interactions and the inter-molecular network formed by key residues in *MtAhpE*. The molecular network and hydrogen bonding of key residues in the active site in *MtAhpE*-SH (chain *C*, red) and *MtAhpE*-SOH (chain *D*, blue). All the residues involved are presented as sticks and are labelled.

In comparison, except for the hydrogen bond with S84 these hydrogen bonds were missing in the previously solved bromide-soaked reduced (PDB ID: 1XXU [5]) and the oxidized structure of *MtAhpE* (PDB ID: 1XVW [5]), as the side chain of Q46 was not flipped in both structures as shown in Fig. 5.24 A-B. Analysis of the electron density map of the bromide-soaked crystal structure (1XVW [5]) reveals that the alternate conformation of Q46 was wrongly assigned, as confirmed recently by Wouter et al [42].

Figure 5.24. A-B: Comparison of earlier solved structure of *MtAhpE* with presented structure. Superimposition of the (A) *MtAhpE*-SH (chain B, orange) and 1XXU structure (chain B, light grey) and (B) *MtAhpE*-SOH (chain D, blue) and 1XVW structure (chain A, wheat). The r.m.s.d between *MtAhpE*-SH (chain B) and 1XXU is 0.518 Å, whereas between *MtAhpE*-SOH (chain A) and 1XVW is 0.628 Å for 153 Cα atoms. The hydrogen bond network between Q46 and the neighboring residues D50, W80 and S84 in *MtAhpE*-SH and *MtAhpE*-SOH are shown, whereas in the respective chains of structure 1XXU and 1XVW only the hydrogen bond between Q46 and S84 is present. For clarity, only the α2 and α3 regions are shown. All the residues involved in the hydrogen bonding are presented as sticks and are labelled.

5.7.1 Mechanistic insights into the active site of *MtAhpE*

Although no MSH was bound in the final structure of the MSH co-crystallized *MtAhpE*, C45 in chain *B* and *C* were reduced, mimicking the reduced state of Cp in the catalytic cycle of *MtAhpE*. A comparison of the oxidised conformation of chain *D*, in which the side chain of R116 was facing outwards, highlighting that side-chain of R116 of the reduced conformation moved inwards and comes in proximity to Cp (Fig. 5.25). In parallel, the C-terminal loop ($_{131}\text{EMKQPGEVRD}_{140}$) in the reduced conformation moves inwards by about 6 Å, coming closer to the active cysteine. This inward movement of the $_{131}\text{EMKQPGEVRD}_{140}$ -loop as well as the flip in the side chain of R116 partially closes the active site pocket (Fig. 5.25). The outward movement of R116 in the oxidised chain causes a distance of 8.4 Å between the amide hydrogen and C45-SOH. This together with the movement of the $_{131}\text{EMKQPGEVRD}_{140}$ -loop forms a channel to allow the reductant/substrate to enter and to come in proximity to the reactive C45. In addition, earlier studies demonstrated a *pKa* value of 5.2 and 6.6 for the thiolate- and sulphenic acid form of *MtAhpE*, respectively [7]. This data would be consistent with the outward movement of R116 relative to C45, resulting in an increased *pKa* of the sulphenic form.

Figure 5.25: Surface potential of *MtAhpE* in reduced and oxidized state. The surface potential of *MtAhpE*-SH and *MtAhpE*-SOH are shown in the cartoon. The electrostatic potential surface was calculated using APBS and mapped at contouring levels from -3kT (*blue*) to 3 kT (*red*). C45 and R116 of the respective chains are shown in stick representation.

Among the many side chains that displayed alternate conformations in the structure, R116 in chain *A* presents an interesting behavior as it is present in two alternate

conformations with occupancies of 0.4 (conformation *A*) and 0.6 (conformation *B*). In the so-called *A*-conformation, the backbone amide hydrogen comes closer to the C45–SOH (2.65 Å), whereas in the *B*-conformation the side chain is directed away and the amide nitrogen is at a distance of 8.29 Å from C45–SOH (Fig. 5.26). Since the R116 is a key residue which plays an important role in redox modulation, the alternate conformation represents a transition state which might have been trapped due to crystallization.

Figure 5.26: Alternate configuration of R116 as observed in chain A. The active site of *MtAhpE*-SOH (chain A), in which C_P is oxidized to sulfenic acid (C45-SOH), and residues E48, R116 and R139 are shown in sticks. Electron density maps (2FoFc) showing the C45-SOH- and the alternate conformations of R116. The map is contoured at 2 sigma level.

This observation raised the question whether residue C45, which is in the oxidized state in chain *A* and *D*, was caused by radiation damage? Therefore, the density maps at different stages of data collection were compared. It was observed that C45 in all the four chains (*A-D*) were in the reduced state for the data of the initial 25 frames and that the R116 side chain was in close proximity to C45 (Fig. 5.27). Subsequently, in the later frames, the side chain of R116 was shown to flip and a positive density was observed near C45, which was later refined to its oxidized state in chain *A* and *D*. These data confirmed that MSH reduced C45 in all four chains primarily, as revealed by the NMR results presented, and that the oxidized state observed in chains *A* and *D* was probably due to radiation damage during data collection.

Figure 5.27: 2FoFc map of the active site of *MtAhpE* for initial 25 frames. The 2FoFc map of the active site of *MtAhpE* for all the chains for the first 25 frames (A; yellow, B; orange, C; red, D; blue). Only the residues CP and R116 of all the chains are shown as sticks for clarity.

5.8 NMR confirms important role played by R116 and R139

To confirm the contribution of R116 and R139 in MSH-binding, NMR experiments were performed with selectively labelled arginine-*MtAhpE* in the presence and absence of MSH. As presented in Fig. 5.28, eight out of nine arginines of *MtAhpE* were observed in the NMR-spectrum of ^{15}N -Arginine labelled *MtAhpE*. After addition of MSH (five molar excess) two arginine peaks, probably R116 and R139, showed a clear chemical shift (Fig. 5.28; *inset*), which is in an agreement with the observed conformational changes of these arginines observed in the reduced structure of *MtAhpE*.

Figure 5.28: NMR confirms role played by R-116 and R-139. ^{15}N -Arginine labelled HSQC-spectrum of *MtAhpE* upon titration with MSH. Eight out of nine arginines could be mapped onto the oxidized HSQC-spectrum of *MtAhpE*. Two of these peaks were seen to shift upon addition of five molar excess of MSH, which corroborates with residues R116 and R139 seen to be moving in the crystallographic structure.

5.9 ITC confirms MSH analogue NAC indeed binds to *MtAhpE*

In order to confirm that NAC indeed binds *MtAhpE* as observed during NMR experiments, ITC experiments were carried out using a ITC₂₀₀ microcalorimeter instrument (MicroCal, Northampton, UK), to study the binding of *MtAhpE* with NAC. Both protein and ligand samples were prepared in identical buffer composed of 50 mM Tris-HCl, 250 mM NaCl (pH 7.5). As shown in Figure 5.29, titration of *MtAhpE* with NAC resulted in an overall negative heat, indicating an exothermic reaction. The data were fitted with single site binding model and binding affinity K_D was estimated in μM range. As clearly evident from ITC signature plot, the current reaction is enthalpy driven and mainly stabilised by hydrogen bonding interaction. The K_D value of $13.9 \mu\text{M}$ indicates moderate to strong binding. Keeping this result in mind, an attempt was made to crystallize *MtAhpE* in the presence of NAC as described in next section.

Figure 5.29: ITC interaction profiles for *MtAhpE* titration with NAC. **(A)** The upper panel shows the injection after baseline correction and the lower panel show the heat release for each injection. The solid lines reveal the fitting of the data to a function based on a one-site binding model. **(B)** Binding signature of the interaction of *MtAhpE* with NAC. The free energy of the reaction (ΔG) is a function of both the enthalpic contribution (ΔH) and entropic contribution ($-T\Delta S$). It is definitive that this reaction is mainly enthalpy driven.

5.10 Confirming the newly proposed by-pass mechanism: Crystallization studies on *MtAhpE* in presence of NAC

In order to confirm the reduction of *MtAhpE* by NAC, crystallization was attempted in the condition described earlier for the apo-crystals (1.8M sodium malonate pH 5.0 mixed with 0.1M sodium acetate pH 4.5). For crystallization, *MtAhpE* was purified without DTT. The diffraction data was collected at NSRRC, Taiwan by Dr. Neelagandan Kamariah from the Prof. Gerhard Grüber's group and structure was solved using molecular replacement. The structure was solved at 2.7 Å in I4 space group and it was observed that in both the chain, the peroxidatic cysteine (C45) was in reduced state (-SH), confirming that NAC has reduced the oxidised *MtAhpE*-SOH (Fig. 5.30 A-B).

Figure 5.30 A-B: Structure of *MtAhpE* crystallized in the presence of NAC. The snapshot of the electron density map of *MtAhpE*-NAC reveals that C45 is in the reduced state in both (A) chain A (light yellow) and (B) chain B (forest green). The structure was solved at 2.7 Å and in both the chains, R116 comes close to C45 as observed in case of the reduced structure.

Discussion

Discussion

MSH is a glutathione homologue present in few actinomycetes such *Mycobacteria* and *C. glutamicum* (154,155). It is one of the dominant thiols in mycobacteria with the cellular concentration ranging from 3-5 mM in *Mtb* and 6 mM in *M. smegmatis* (156). Previous studies have shown that mutants deficient in MSH, exhibit increased sensitivity to various toxins and antibiotics, indicating that MSH is involved in detoxification (50). It was also shown that MSH and S-nitrosomycothiols reductase are required for survival under aldehyde stress and biofilm formation in *M. smegmatis* (80). Therefore, the enzymes involved in the biosynthesis of MSH and recycling of MSSM to MSH (NADPH-dependent Mtr) are indispensable for redox regulation in *Mtb* and have triggered much interest as drug targets [51]. This work deals with the structural and mechanistic studies on the protein ensemble of *MtMtr*, *MtMrx-1* and *MtAhpE* using the NADPH provided electron for the recycling of mycothiol, one of the major molecules for redox homeostasis of mycobacteria.

6.1 *MtMtr*, a dimeric enzyme with an extended shape in solution

In this work a comprehensive protocol for the high-level production and purification of stable recombinant *MtMtr* protein in *E. coli* using GroEL-ES chaperone-chaperonin system was established. The purified protein was enzymatically active as confirmed by the NADPH-consumption assay in the presence of MSSM as described in section 3.5. The protein was shown to be well-folded by 1D-NMR spectroscopy studies as shown in section 3.4. As a first step toward structural characterization *MtMtr* it was necessary to determine the oligomeric state of the recombinant protein as oligomerization plays an important role in modulating activity of redox active proteins (157).

In the presented work, the complementary approaches of SEC (section 3.3), DLS (3.3) and SAXS (3.6) demonstrated unambiguously that *MtMtr* forms a homodimer in the solution which is in agreement with previous studies (78). Like glutathione reductase, the *MtMtr* contains three domains namely the NADPH-binding-, the FAD-binding-, and the dimerization-domain; of which the latter two participate in substrate-binding and dimer formation, respectively. The structural model of *MtMtr* was superimposed onto the SAXS solution shape as shown in Figure 3.11. The superimposition revealed a region of unoccupied density. In addition, one NADPH-binding domain of the dimer model was protruding out of the solution shape. It is possible that the poor fitting was caused due to domain movement and the inter-domain flexibility arising due to linker between the

FAD- and NADPH-binding domains. In recent years, with advancement in software programs, it is now possible to model flexible-systems using a SAXS-based method called CORAL (116), which was used for rigid body modelling of *MtMtr*, and allowing free rotation of the NADPH-binding domain. This resulted in an extended conformation of *MtMtr* which fitted better with the experimental scattering curve. This model showed that *MtMtr* is flexible with the two NADPH-binding domains oriented in an asymmetric conformation.

Since asymmetry was observed for the NADPH domain, the question was addressed, whether substrate binding could affect the conformational dynamics of *MtMtr* in solution. Therefore, SAXS experiments were performed in the presence of NADPH or MSSM. An increment in the R_g and D_{max} values was observed in the presence of NADPH, indicating substrate-induced conformational changes in solution. To further investigate this result, mutations of *MtMtr* (S144A, S268A and double mutant D158A-Y159A), which could possibly abolish the interaction between the linker and NADPH domain, were generated and examined using SAXS. The R_g -values of the S144A and D158A-Y159A mutants in the substrate-free form and in the presence of MSSM or NADPH were comparable. However, in case of S268A, the incubation with NADPH resulted in an increment in the R_g and D_{max} values as compared to the substrate-free form. This allowed us to conclude that the mutations S144A or D158A-Y159A abolish substrate-induced conformational changes in solution.

Previous studies have reported that the related glutathione reductase as well as dihyrolipoamide dehydrogenase exists as a mixture of dimer and tetramer in solution (73,74). Keeping this in mind, OLIGOMER analysis was carried out for scattering data to investigate possible presence of mixture populations in solution. In case of WT-*MtMtr*, it was observed that the substrate-free form occurs predominantly as a dimer in solution (Tab. 3.1, Fig. 6.1). In comparison, binding of the substrate NADPH alters the oligomer equilibrium to a tetrameric form of a WT-*MtMtr*-NADPH-bound complex. These result agrees with earlier studies of the related glutathione reductase, reporting that the presence of NADPH favors conversion of the dimeric glutathione reductase into less active tetramers (75). Another interesting observation of the presented data is, that the S144A mutant of *MtMtr* has the highest fraction of dimer population and therefore represents a good candidate for future crystallisation attempts. Studies on glutathione

reductase have shown that dynamics of dimer-tetramer equilibrium is also affected by pH as well as temperature (158). Therefore, such studies on dimer-tetramer equilibrium of *MtMtr* is important to understand the protein dynamics and behaviour in solution and will be a matter of future investigation, including the aspect of its biological significance. In general, this is of great relevance for proteins involved in redox homeostasis as shown for the dimer-dodecamer equilibrium of the alkyl-hydroperoxidase reductase subunit C of *Mtb* (159) and the dimer-decamer formation of prokaryotic AhpC, whose equilibrium is redox modulated (6,160,161).

Figure 6.1: Volume fraction of dimeric and tetrameric WT-*MtMtr*. Percentage volume fraction of dimeric and tetrameric oligomer of WT-*MtMtr* in solution in substrate-free form and in the presence of NADPH. The data indicate a clear shift in oligomer-equilibrium towards a tetramer upon incubation with NADPH. The analysis was carried out using OLIGOMER (105).

The attempts to obtain a high-resolution structure of *MtMtr* by protein crystallization resulted in promising conditions, but none of these provided a single, well diffracting crystal. One reason which can be attributed to this is the presence of intrinsic flexibility in *MtMtr*. Therefore, an attempt was made to generate the C-terminal dimerization domain of *MtMtr*. However, the recombinant protein was insoluble even when expressed in the BL21-GroEL/ES strain. In this regard, it is worthwhile to mention that the tetrameric-form of WT *MtMtr* with a molecular weight of 200 kDa makes it

amenable to EM studies. As highlighted earlier, the *MtMtr* mutant S144A with its high dimeric population might be a suitable candidate for crystallization trials in the future.

6.2 Interaction between *MtMtr* and *MtMrx-1* is a fast-exchange process

The high production and stability of the recombinant *MtMtr* enabled to investigate the interaction between *MtMrx-1* and *MtMtr*. NMR titration experiments revealed that there was molecular interaction between *MtMtr* and *MtMrx-1* as well as the conformational transition from a reduced to an oxidized form of *MtMrx-1* caused by the interaction with *MtMtr*. This is the first report on interaction between *MtMtr* and *MtMrx-1*. Although the exact binding epitope on *MtMrx-1* can't be mapped as the NMR data indicated that conformational transition of *MtMrx-1* is a kind of fast exchange process between two forms of *MtMrx-1*, the conserved active CGYC (L6-C17) motif of *MtMrx-1* as well as the regions V34-G47, V59-K62 and T68-L79 could be identified to be directly or indirectly involved in the interaction with *MtMtr*. With the availability of new insights about the dimer-tetramer equilibrium, it is still to be investigated whether one form or another of *MtMtr* interacts differently with monomeric *MtMrx-1* and how this affects the ensemble formation in solution.

6.3 *MtMrx-1* interacts transiently with *MtAhpE* during catalytic cycle

Besides its interaction with *MtMtr*, the MSH-dependent oxido-reductase *MtMrx-1* plays a significant role in the reduction of *MtAhpE*-SOH. In this thesis, attempts were made to unravel the mechanistic features of *MtAhpE*-*MtMrx-1* interaction. The SAXS experiments confirm *MtMrx-1* to be monomeric irrespective of the concentrations used. This is unlike the related NrdH-redoxin, which dimerizes at higher concentrations (84). The monomeric nature of *MtMrx-1* becomes essential during the interaction with the dimeric *MtAhpE*, where two monomeric *MtMrx-1* interact with *MtAhpE* without any steric hindrances as shown in Figure 6.2. The NMR-titration data between *MtMrx-1* and *MtAhpE* presented in section 4.5 extend the insight about the strength of the interaction as well as the binding epitope. The results demonstrate that the *MtMrx-1* and *MtAhpE* interaction is weak and transient with the involvement of the active site residues and a surface exposed additional region between $\beta 2$ and the tip of helix $\alpha 2$ of *MtMrx-1*. A complex model was generated based on the restrained obtained from NMR-titration experiments.

The docked model demonstrates that *MtMrx-1* interacts at the dimer interface of *MtAhpE*, with both subunits contributing to the complex formation. The three most important residues (Y16, E37 and H38) that show the highest perturbation in *MtMrx-1* are involved in hydrogen bonding interaction with *MtAhpE*. Several other interactions of *MtMrx-1* with both subunits of the *MtAhpE* dimer were identified and might hint at the binding epitope. Based on this complex model, the speculation can be made that these interactions aid in the formation of an intermolecular disulphide bond between *MtAhpE* and *MtMrx-1* allowing reduction of the former by the latter. It is noteworthy, that the overall pattern of binding of *MtAhpE* and *MtMrx-1* is similar to a complex of homolog yeast alkyl hydroperoxide reductase (Ahp1) and thioredoxin (trx2) (162). Finally, the presented complex model may pave the way for the development of chemical agents or peptide-mimetics that can be used to block this essential interaction.

Figure 6.2: Mechanism of a *MtMrx1*-dependent reduction of *MtAhpE*. One of the accepted mechanisms of *MtAhpE* reduction via *MtMrx1* in which a sulphenic derivative of *MtAhpE* is directly reduced by *MtMrx1* in two steps to yield *MtAhpE*-SH. Based on the current study a structural model is shown, to explain the catalytic cycle of dimeric *MtAhpE* and subsequent recycling by *MtMrx-1*.

6.4 *MtAhpE* is a dimer in solution regardless of the redox state

Structural insights into *MtAhpE* are fundamental to understand the intra-molecular electron transfer mechanism(s) during the reduction process of this key enzyme in antioxidant defence via MSH. The crystallographic dimers of *MtAhpE* reflect the dimeric

form of *MtAhpE* in solution as demonstrated by the DLS-data, the determined Porod-hydrated- and excluded volume, the solution shape of *MtAhpE* as well as the good fit of the theoretical scattering curve of the crystallographic dimer of this enzyme. Furthermore, the comparison of the oxidised and reduced enzyme showed similar R_g - and D_{max} values, indicating that *MtAhpE* in its final oxidized- and reduced state is dimeric in solution as shown recently (87). It is noteworthy that the DLS studies indicate, that an equimolar concentration of peroxide leads to an immediate formation of higher molecular weight oligomer of *MtAhpE*, whereas excess amount of peroxides results in a dimeric state of the enzyme. During an outburst of ROS, at which the amount of peroxide will be high, *MtAhpE* will be dimeric and will undergo maximum catalysis. It is possible that this higher molecular structure might correspond to an octameric form of the protein as shown recently for *TkAhpE* using negative staining electron microscopy (90).

In addition, by characterizing the different redox populations of the catalytic C45 by solution NMR, the ability of MSH to reduce *MtAhpE*-SOH without the need of *MtMrx-1* was presented. This new pathway proposed was validated using NAC which reduced the *MtAhpE* in absence of *MtMrx-1*. Furthermore, crystallization of oxidised *MtAhpE* in the presence of MSH or NAC was carried out and it was observed that Cp was in the reduced state in the crystal structure. It still remains to be investigated which of the various pathways is predominant for *MtAhpE* reduction. A recent study proposes that since the cellular concentration of MSH is in the millimolar range, the *Mrx-1* cellular concentration should be at least 50 μ M to be a relevant competitor (154).

6.5 *MtAhpE* exhibits slight conformational changes during redox modulation

As highlighted recently by Zeida *et al.* (153), a hydrophobic patch is formed at the interface of the *MtAhpE* dimer by the residues L39, A40, F41, V71, P73, T76, I79, F94, H97, F107, Q110, A111, and G112, enabling long fatty acid hydroperoxides to bind and to locate the reactive group of the peroxide substrate nearby the peroxidative cysteine. As revealed by the normalized Kratky plot, *MtAhpE* exhibits a profile, indicating less rigidity in solution when compared to a compact enzyme like lysozyme. Therefore, a slight movement around the dimer interface could provide the alterations needed for substrate-binding, transition-state, reduction of the hydroperoxide and product release.

In context of the substrate MSH, the comparison of the oxidised- and MSH-reduced structure demonstrates that inward movements of the $^{131}\text{EMKQPGEVRD}_{140}$ -loop (Fig.

6.3), including R139, as well as the side-chain of amino acid R116, occur in the reduced conformation, which partially closes the active site pocket. Similar movements were observed in a recently described bromide-soaked reduced structure of *MtAhpE* [5]. In comparison, the outward movement of R116 in the oxidised chain causes a distance of 8.4 Å between the amide hydrogen and C45-SOH, which together with the movement of the ${}_{131}\text{EMKQPGEVRD}_{140}$ -loop generates a channel for MSH to enter and to come in close distance to the reactive C45. Importantly, the movements of the two arginine residues were confirmed by the chemical shifts of ${}^{15}\text{N}$ -Arginine labelled *MtAhpE*. To address the question whether binding of one MSH to the dimeric *MtAhpE* may cause a co-operative effect on the binding of another MSH to the second catalytic site of the dimer, molecular docking studies were carried out.

Figure 6.3: Conformational alterations of *MtAhpE*. Model of the oxidised (*blue*) and reduced (*red*) *MtAhpE* dimers achieved by superimposition of the oxidized chain to reduced chain and *vice versa*. The flipping in the ${}_{131}\text{EMKQPGEVRD}_{140}$ -loop between the oxidized and reduced structure is shown by arrows. In the reduced dimer, the ${}_{131}\text{EMKQPGEVRD}_{140}$ -loop moves inward, while in the oxidized chain it moves outward.

6.6 Molecular docking provides insights into binding of MSH

MSH in the oxidized structures (chain *D*) occupies the open channel created by R116 and the ${}_{131}\text{EMKQPGEVRD}_{140}$ -loop, and interacts with amino acids C45, T42 and R116 (Fig. 6.4-A). In this regard, the acetyl group of MSH interacts with R116, the S-

atom of the cysteine moiety interacts with residue T42 and the glucosamine ring of MSH is closer to the $\alpha 4$ - $\beta 6$ -loop, being stabilized by the hydrogen bonding interaction with residue N108 and Q110 in the oxidized chain *D* (Fig. 6.4-B). The inositol ring faces the solvent and is mostly held in place by hydrophobic interactions of P38, L39, A111 and I113. The residues I44 and P135 also render hydrophobic interaction to the MSH binding.

Figure 6.4 A-B: Docking model of *MtAhpE*-SOH with MSH. (A) A model of docked MSH (MSH; shown as sticks in *green*) with the *MtAhpE*-SOH structure (*light blue*). The interacting residues are shown in *blue* in stick representation. (B) The interaction profile of MSH-*MtAhpE* in the oxidized chain is shown.

In the reduced structure (chain *C*), MSH is moved closer to the $\alpha 3$ - $\beta 2$ -loop because of the closing of the channel (Fig. 6.5-A). The cysteine group of MSH interacts with C45 and T42 and the glucosamine ring has hydrogen bonding interaction with G44. The non-polar residues P38, L39, I44, I113 and G136 along with R116 provide hydrophobic interaction to stabilize the binding (Fig. 6.5-B).

Figure 6.5 A-B: HADDOCK docking studies on *MtAhpE*. (A) Superimposition of the oxidized chain (*light blue*; chain *D*) and reduced chain (*salmon*; chain *C*) of *MtAhpE* along with the docked MSH (stick). The $^{131}\text{EMKQPGEVRD}_{140}$ -loop is indicated and shown in *magenta*. Because of the R116 flip and the $^{131}\text{EMKQPGEVRD}_{140}$ -loop movement, MSH occupies different positions in the binding region. (B) A schematic 2D representation of the MSH-*MtAhpE* interactions for reduced chain *C*.

Comparatively the oxidized structures (chain *D*) make stronger interactions with MSH as evidenced by the interaction profiles, suggesting that the opening of the channel might be necessary for binding of MSH.

In summary, the *Mtb* NADPH dependent *MtMtr*, -*Mrx*-1, and -*AhpE* were cloned, expressed and structurally characterized. The first low resolution structure of *MtMtr* was deduced and the effect of substrate-binding induced conformational dynamics in solution were investigated. The shift in oligomer equilibrium to a tetrameric state of *MtMtr* is a new finding. In addition, it was shown that *MtMtr* might interact with *MtMrx*-1 leading to oxidation of *MtMrx*-1. Besides the interaction with *MtMtr*, the MSH-dependent enzyme *MtMrx*-1 plays a further central role in the reduction of *MtAhpE* (Fig.-6.6). The interaction between *MtAhpE* and *MtMrx*-1 was mapped using NMR spectroscopy and molecular docking was employed to generate a model of complex.

In addition to this, a new pathway for *MtAhpE* reduction was discovered. In this pathway, one molecule of MSH forms an adduct with *MtAhpE* (*MtAhpE*-SSM), followed by a second molecule of MSH, which releases *MtAhpE*-SH and MSSM in a typical monothiolic mechanism. This pathway in addition to earlier existing pathway, wherein two molecules of *MtMrx*-1 bind to an *MtAhpE* dimer with each molecule reducing one *MtAhpE* monomer, presents a more complete picture of the *MtAhpE* recycling pathways (Fig. 6.6). Since many redox active proteins have been proposed to be recycled via the *Mrx*-1/MSH dependent pathway, the results presented here not only allow to gain a deeper understanding on the recycling of *MtAhpE* but also of various redox proteins in general.

Figure 6.6. The interplay of three key enzymes in MSH/Mrx-1 dependent redox regulation. After oxidation of NADPH by the dimeric *Mtr* (left) the enzyme recycles MSSM to MSH. So far, a mechanism was described in which *MtrMtx1* reduces *MtrAhpE* via a sulphenic derivative of *MtrAhpE*, which becomes reduced by *MtrMtx1* (brown) in two steps to yield *MtrAhpE-SH* (sky blue). Based on the current study a structural model is shown, to explain the catalytic cycle of dimeric *MtrAhpE* and subsequent recycling by *MtrMtx-1*. In addition, a new pathway wherein two molecules of MSH directly reduce *MtrAhpE* has been proposed in this work. In addition, it was shown using SAXS studies in presence of substrate that upon incubation with NADPH there is shift in oligomer equilibrium of *MtrMtr* towards tetramer form.

Conclusions

Conclusions

Conclusions

The MSH/Mrx-1 dependent system for redox regulation is central to mycobacterial defense against oxidative stress it faces during the cycle of infection-transmission and is an attractive drug target. By employing a GroEL/ES chaperone-chaperonin system, stable and well-folded *MtMtr* could be obtained which was then used to get first low-resolution structure. In addition, it was observed that there is shift in oligomer equilibrium towards tetramer upon incubation with NADPH. Using NMR spectroscopy, it was empirically proven that interaction of *MtMtr* with *MtMrx-1* leads to oxidation of the latter. In addition, crystallization of *MtMtr* was tried but single crystals were not obtained.

The oligomeric state of *MtMrx-1* and *MtAhpE* was confirmed as monomer and dimer, respectively. In case of *MtAhpE*, substrate-induced changes in the quaternary structure were studied wherein higher molecular species were observed in the presence of equimolar H₂O₂. The interaction between *MtAhpE* and *MtMrx-1* has been mapped and molecular docking was employed to describe the *MtAhpE-MtMrx-1* ensemble formation.

Using cysteine-labeled NMR spectroscopy, the changes in the redox state of the catalytic Cp of *MtAhpE* was observed in the presence of various reducing agents such as MSH, DTT and NAC. A new pathway for reduction of *MtAhpE*-SOH by MSH in the absence of *MtMrx-1* was proposed. This was further validated by obtaining crystal structures of *MtAhpE* in presence of MSH and NAC.

In summary, this work provides new structural and mechanistic insights in MSH/Mrx-1 dependent redox modulation in mycobacteria and will be of significant importance for future drug discovery studies.

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Author's publication related to the project

- **Kumar, A.**, A.M. Balakrishna, W. Narthey, M.S.S. Manimekalai, and G. Grüber, Redox chemistry of Mycobacterium tuberculosis alkylhydroperoxide reductase E (AhpE): Structural and mechanistic insight into a mycoredoxin-1 independent reductive pathway of AhpE via mycothiol. *Free Radic Biol Med*, **2016**. 97: p. 588-601. [IF: 5.886]
- **Kumar, A.**, W. Narthey, J. Shin, M.S.S. Manimekalai, and G. Grüber, Structural and mechanistic insights into Mycothiol Disulphide Reductase and the Mycoredoxin-1-alkylhydroperoxide reductase E assembly of Mycobacterium tuberculosis. *Biochim Biophys Acta*, **2017**. [IF: 4.826]
- Wong, C.F., J. Shin, M.S. Subramanian Manimekalai, W.G. Saw, Z. Yin, S. Bhushan, **A. Kumar**, P. Ragunathan, and G. Grüber, AhpC of the mycobacterial antioxidant defense system and its interaction with its reducing partner Thioredoxin-C. *Sci Rep*, **2017**. 7(1): p. 5159. [IF: 4.847]
- **Kumar, A.**, M.S.S. Manimekalai, and G. Grüber, Substrate-induced structural alterations of Mycobacterial mycothiol disulphide reductase and critical residues involved. **2017**. FEBS letters (submitted) [FEBSL-17-1342]

Posters and Abstracts

- **Kumar, A.**, A.M. Balakrishna, W. Narthey, M.S.S. Manimekalai, and G. Grüber. Structural and mechanistic insight into 1-Cys peroxiredoxin AhpE from Mycobacterium tuberculosis. in EMBO Conference "Tuberculosis 2016: Interdisciplinary research on tuberculosis and pathogenic mycobacteria". 2016. Institute Pasteur, Paris, France.

Awards

- **EMBO Travel Award 2016** for EMBO Tuberculosis Conference "**Tuberculosis 2016: Interdisciplinary research on tuberculosis and pathogenic mycobacteria**" held at Institute Pasteur, Paris, France between 19-23, September 2016.
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