Mechanistic insights into Escherichia coli alkyl hydroperoxide reductase complex formation

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**Mechanistic insights into *Escherichia coli* Alkyl
hydroperoxide Reductase complex formation**

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Dedicated to my mum

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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
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>
EM	Electron microscopy
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

Abbreviations

x

LLG	Log likelihood gain
NADH	Nicotinamide adenine dinucleotide
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
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PPM	Parts per million
r.m.s.d.	Root mean square deviation
SDS	Sodium dodecyl sulphate
TFE	2,2,2-Trifluoroethanol
TOCSY	Total correlation spectroscopy
Tris	Tris-(hydroxymethyl) aminomethane

Abstract

Abstract

Oxidative stress, an imbalance between the amount of Reactive Oxygen Species (ROS) and antioxidant defences, is detrimental to the survival of both benign and pathogenic bacteria [1]. To keep ROS in balance bacteria have evolved various antioxidant enzymes, among them the alkyl hydroperoxide reductase (AhpR), consisting of 21 kDa AhpC and 57 kDa AhpF, which serve as a primary hydroperoxide scavenger that reduces hydroperoxide to water and its corresponding alcohol, and preventing thereby damages like protein oxidation, lipid peroxidation and DNA damage [2-5]. AhpC and -F represent one of the most efficient enzyme systems responsible for redox homeostasis in bacteria [6]. To do that, the *Escherichia coli* AhpC (*EcAhpC*) directly donates electrons to peroxides reducing them to less harmful products. The oxidized AhpC is reduced by AhpF via the disulphide center (-CxxC-) in its N-terminal domain (NTD), with a catalytic turnover rate measured to be $4.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ in *Salmonella typhimurium* [7]. By employing biophysical approaches, this thesis set out to understand the mechanistic basis for this efficiency. The C-terminal tail of AhpC has been implicated as a hydroperoxide sensor in eukaryotes but not in most bacteria [8]. By generating C-terminally truncated mutants, *EcAhpC*₁₋₁₇₇, *EcAhpC*₁₋₁₈₂, *EcAhpC*₁₋₁₈₅, together with site-specific mutations *EcAhpC*_{K186A} and *EcAhpC*_{I187G}, the critical role of the C-terminus in oligomerization under reducing conditions has been examined. Since oligomerization has been associated with catalytic efficiency [7], NADH-dependent assays have been used to associate the C-terminal mutations to catalytic efficiency. In an attempt to gain structural insight into the C-terminal tail, the *EcAhpC*₁₋₁₈₂ crystal structure has been solved in its oxidised state that revealed five molecules in an asymmetric unit. The ring shape decamer conformation was generated by applying a symmetry calculation with the asymmetric unit cell. The absence of the C-terminal tail in the crystal structure indicates a flexibility of this region.

Furthermore, the first interaction studies between the *EcAhpC* and *EcAhpF* have been performed. By generating constructs of the N- and C-terminal domains of *EcAhpF*, it has been confirmed that *EcAhpF* interacts entropically via its NTD with *EcAhpC*. The affinity for the *EcAhpF*_{NTD}-*EcAhpC* interaction was calculated to be 3.2 μM . The effect of the redox-state of *EcAhpC* has been found to be important to this interaction as only the oxidized AhpC interacts with the NTD. In comparison, reduction abolished the interaction altogether. Importantly, by using NMR spectroscopy, it has been revealed that the interaction of the full-length *EcAhpC* and

the NTD is long-lived, whereas the C-terminal free AhpC (*EcAhpC*₁₋₁₇₂) interacts very dynamically with the NTD. This dynamic nature of the *EcAhpC*₁₋₁₇₂-*EcAhpF*₁₋₂₁₂ interaction enabled the observation of chemical shift perturbations of residues of the isotopically labelled NTD. Information obtained have been used to drive a HADDOCK docking of the complex and subsequently a binding epitope of the C-terminal tail of *EcAhpC* which it is proposed to wrap around the backside of *EcAhpF*_{NTD}. Taken together, this thesis has revealed a novel binding mechanism, where the C-terminus of AhpC wraps around the NTD to slow the dissociation rate upon complex formation for an efficient electron transfer process to occur, and a release mechanism mediated by a well-described conformational change of the C-terminus of *EcAhpC* upon reduction.

In addition, by employing a high ionic buffer system, solution structures of the full-length oxidized *EcAhpC* have been solved using small angle x-ray scattering (SAXS) throwing light on the flexibility of the C-terminus and its importance to the *EcAhpC*-*EcAhpF* complex formation. In addition, the orientation of the full-length proteins in the complex in solution agrees very well with models obtained by NMR.

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1. Introduction

1.1 Reactive Oxygen Species (ROS) and their effects on life

Over 2.7 billion years ago, during the archaean period, when the earth was still in the process of cooling and conditions were still extreme, most living things existing were anaerobes since the atmosphere was devoid of oxygen [9, 10]. As the atmosphere became gradually saturated by oxygen from early oxygenic photosynthetic organisms, many existing anaerobes transitioned from anaerobic metabolism to aerobic metabolism and were faced with a dangerous assortment of reactive oxygen species (ROS), a by-product of oxygen metabolism [10]. In order to survive these assortments, early aerobic organisms evolved mechanisms to contain the reactive oxygen species and these have been passed on through all the domains of life.

ROS are generated as a by-product of oxygen metabolism. In an oxygen-rich environment, there is an excess of molecular oxygen available for aerobic organisms. This is mainly due to the fact that dioxygen is non-polar and hence easily diffuses through the membranes of cells at a rate faster than the rate at which the cells can utilize it [11]. The paramagnetic nature of oxygen and the presence of unpaired electrons in the outer shell dioxygen molecules serve as an impediment to most chemical reactions [12]. This, however, allows the univalent addition of electrons to produce reactive intermediates such as superoxides (O_2^-), peroxide (H_2O_2) and hydroxyl radicals ($HO^\cdot$) (Figure 1.1). The source of electrons for this step-wise reduction of oxygen is usually paramagnetic transition metals, which in biological systems are cofactors for flavins, respiratory quinines and metal centres [10]. Most of these enzymatic systems are known to be electron carriers in the respiratory chain, hence the detection of traces of O_2^- and H_2O_2 in inverted bacterial membranes and sub mitochondrial particles [10, 13]. In the respiratory chain, up to 2% of electrons that are to be shuttled along the chain for useful metabolic purposes are lost to the environment where they are used by available oxygen molecules. The main determining factor is the half-life of the electron

Figure 1.1: Generation of reactive oxygen species (ROS) from the step-wise addition of electrons to molecular dioxygen. The step-wise addition of electrons is made possible because of the fact that molecular oxygen has parallel unpaired electrons in the discrete orbital ensuring that electrons can be accepted one at a time [12].

centres; those that are slow in transferring the electrons to the next centre in the chain are likely to lose it to the environment [9, 14, 15]. It has been shown that there are other sources of electrons besides respiratory proteins. In an experiment where bacteria mutant lacking respiratory enzymes were generated, they were still able to produce superoxides and peroxides at substantial rates. It was then suggested that other non-respiratory flavoproteins like glutamate synthase, lipoamide dehydrogenase, and glutathione reductase may be contributing to the electron pool [16, 17, 18-20]. Peroxisomes also act as a critical source of electrons for molecular oxygen reduction. Thus during the lipid degradation, electrons can be transferred from cofactors such as FADH_2 to oxygen generating superoxides and subsequently peroxides. In plants, the electron sources can come from the photosynthetic process [21]. Besides the endogenous sources of ROS discussed above (Figure 1.2), ROS can be generated externally. UV radiation and ionizing radiation such as gamma rays can break down peroxides and water to generate the highly reactive hydroxyl radicals, a process known as radiolysis [22, 23]. Chemicals that react to form peroxides, promote superoxide formation, release ions and can be metabolized to radicals are sources of exogenous ROS [24]. Lactic acid bacteria are a prominent source of H_2O_2 and use it as a chemical arsenal against other bacteria [25]. Higher organism employs H_2O_2 to kill bacteria trapped in macrophages by phagocytosis [26]. The physiological concentration of ROS is not only tolerated but is also useful in cell signalling and homeostasis. It is central in inducing apoptosis in cells and regulates the expression of genes and activating cell signalling cascades. However, it cannot be underestimated the damage ROS can do to the cell. The ability of cells to keep the levels of ROS within physiological range is critical to prevent “oxidative stress” [1]. Lipid peroxidation is highly increased in the presence of ROS [27]. The initial attack by a single hydroxyl radical on a polyunsaturated fatty acid of the membrane phospholipids can lead to the generation of lipid-based radicals which are capable of propagating devastating chain reactions. An example of such radicals is malondialdehyde (MDA) a well-studied radical known to damage cell membranes [4]. In addition to the formation of lipid radicals, lipid peroxidation leads to the formation of lipid hydroperoxides, species that can be broken down into alkoxy radicals, alcohols, lipid epoxides and alkanes through a reaction catalysed by iron (II) [28]. The overall effect of lipid peroxidation is the increase in membrane permeability and fluidity mediated by the breakdown of fatty acid chains. Proteins are also a major target for ROS attack through direct and indirect

modification. By nitrosylating, carbonylating, glutathionylating and stimulating disulphide bond formation in proteins, ROS can directly modulate the activity of proteins. Thus by changing the surface charges of the protein and increasing its susceptibility to proteolysis [29]. Among all the amino acids, the most vulnerable to ROS modification are the sulphur-containing amino acids and the thiol groups. Thus, an ROS can remove a proton from two different cysteine moieties to form thiyl radicals which can subsequently come together to form a non-physiological disulphide bond [28]. Superoxides are known to irreversibly inactivate enzymes by oxidizing their iron-sulphur centres [30]. Indirect protein modification occurs through the conjugation of the by-products of lipid peroxidation with proteins [31]. Finally, ROS can substantially damage DNA of all origins (chloroplast, nuclear and mitochondrial) [32]. Due to the critical role that DNA plays in the synthesis of proteins and other cell processes, any oxidative damage to the DNA has a cascade effect on the physiology of the cells [29]. The above described devastating effect of ROS implies that cells should be able to maintain physiological concentrations of ROS at all times.

Figure 1.2: Exogenous and endogenous sources of hydrogen peroxide for *E. coli* cells. Exogenous sources of hydrogen peroxide include H_2O_2 -excreting microbes such as lactic acid bacteria, the NADPH oxidase responses of plants and macrophages and photochemically driven redox reactions. The endogenous source of hydrogen peroxide is the incomplete reduction of oxygen in the respiratory chain. OxyR proteins in *E. coli* are the transcription factor for *ahpC* and *ahpF* genes in response to hydrogen peroxide and is sensitive to the H_2O_2 level of 0.1 μM . This figure has been adopted with modification from Mishra and Imlay, 2012 [1].

1.2 Detoxification of Reactive Oxygen Species by Scavenger enzymes

In *E. coli*, there are numerous enzyme systems that are responsible for controlling ROS (Figure 1.3). Superoxide dismutase (SOD) is responsible for neutralizing superoxides by converting two molecules of superoxide into hydrogen peroxide and water [10]. The inability of superoxides to traverse cell membranes has led to the evolution of different types of SOD each residing in a specific cellular compartment [33, 34]. All the various SODs contain metal centers which facilitate their reaction with O_2^- . They are under the regulation of the superoxide response (*SoxRS*) system.

Figure 1.3: Schematic representation of the network of antioxidant enzymes in *E. coli*. The popularly known thioredoxin antioxidant system in bacteria consist of the Trx1 and Trx2, which is reduced by the dedicated reductase TrxR, with the later receiving electrons from NADPH. Once reduced, Trx can donate electrons to the three thiol peroxidases; BCP, Tpx and AhpC together with protein repair molecules like Msr and other molecules like RNR, which is essential for DNA synthesis, and PAPS. Grx, although not as ubiquitous as Trx essentially possess similar functionalities. The dedicated peroxide quencher, AhpC is reduced by AhpF which receives electrons from NADH. The transcription factor OxyR regulates the expression of the antioxidant enzyme AhpC, catalase, GR, Trx2 and Grx1. This figure has been adapted from Lu and Holmgren, 2014 [35].

The regulation of H_2O_2 is not as straight forward as that of O_2^- since more than one set of enzyme systems is responsible for its neutralization [10]. The fact that bacteria live in varying environments has ensured that they are able to mount different antioxidant strategies to combat oxidative stress [1]. Most of these antioxidant systems can be classified as thiol-dependent peroxidases or thioredoxin (Trx)- and glutathione (GSH)-dependent systems. The latter is abundant in various bacteria. This is seen in the ubiquitous nature of thioredoxin in bacteria. However, GSH is limited in some bacteria [35]. In *E. coli*, thioredoxin molecules mainly Trx1 and Trx2 are encoded by

trxA and *trxC*, the two differing in structural and regulatory features. The dedicated reductase for Trx is a flavoenzyme known as thioredoxin reductase (TrxR) and is encoded by *trxB* and ensures that thioredoxin is kept in a reduced state for downstream activities (Figure 1.3). The electron for the reduction of Trx by TrxR is provided by NADPH (Figure 1.3). Trx functions basically as thiol disulphide reductase and also commonly found as sub domains in other proteins where it acts as functional complexes [36]. Thioredoxin is able to donate hydrogen to ribonucleotide reductase (RNR) thereby plays a critical role in DNA synthesis [37]. It is implicated in sulfur assimilation by donating hydrogen to intermediates, thus phosphoadenosine-phosphosulphate (PAPS), in the reduction of sulphate to sulphite [38]. It also donates hydrogen to methionine sulfoxide reductase (Msr), an enzyme critical for repairing oxidized sulphur amino acids in protein [39]. Thus when methionine in proteins is oxidized to methionine sulfoxide (Met-O) under both physiological and oxidative stress, Msr reverses the process reducing Met-O, the electrons for this process are provided by Trx1. Thiol-dependent peroxidases like the bacterioferritin comigratory protein (BCP), AhpC and thiol peroxidase (Tpx) interacts with Trx, with the later acting as a reductase [1]. *E. coli* Trx1 is known to specifically reduce the homodimeric Tpx from its intrasubunit disulphide to free thiols [40]. The catalytic efficiency for the Michaelis complex formed between them has been calculated to be $3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, whereas monomeric BCP catalyses hydrogen peroxide together with Trx1 with apparent V_{max}/K_m of $1.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ [40]. Moreover, it has been found that other reductants like the glutaredoxins (Grx1 and Grx3) and Trx2 can also supply electrons to BCP [35].

Among the various enzymes, the three most studied are the catalase enzymes (KatE and KatG) and the alkyl hydroperoxide reductase system (Ahp) [41]. The AhpR system consists of two proteins; AhpF and AhpC, a two-component system that obtains its electrons from NADH. Both the AhpR and catalase are under OxyR-regulation. Under physiological condition or during normal aerobiosis, when the concentration of H_2O_2 is approximately 50 nM, the OxyR is virtually inactive [10]. But when the concentration of H_2O_2 increases dramatically as a result of externally generated H_2O_2 entering the cells, the OxyR becomes activated through the interaction of H_2O_2 with the two conserved cysteines [42-44]. This subsequently results in the formation of a disulphide bond which essentially constrains the OxyR into a certain conformation, hence triggering it to act as a positive transcription factor for *catalase*

and the *AhpR* genes (Figure 1.2) [10]. During this response, the amount of these enzymes increases to about 10-fold higher. In order for the cells to respond rapidly to external oxidative stresses, OxyR can detect H_2O_2 concentrations as low as ~ 100 -200 nM. The rapid response of OxyR is able to reduce the H_2O_2 to around 20 nM [45, 46]. The essentiality of OxyR in combating ROS was demonstrated by the mutational deletion of the *oxyR* gene, which led to a phenotype with 10- to 55-fold higher frequencies of spontaneous mutagenesis and oxidative damage to DNA [47]. Importantly, the over-expression of AhpR reduced the frequency of spontaneous mutagenesis in the *oxyR* deletion mutants [47].

1.3 Alkyl hydroperoxide reductase (AhpR) system

As explained earlier, the AhpR system is made up of the 57 kDa AhpF flavoprotein protein and a 21 kDa AhpC (Figure 1.4) [48]. Sequence alignments place AhpF among the thioredoxin reductase family of proteins. These group of proteins is known to be a flavoprotein pyridine branch of the disulphide oxidoreductase enzyme family different from other flavoprotein disulphide, like glutathione reductases, dihydrolipoamide reductases and mercuric reductases [49].

Figure 1.4: The proposed catalytic cycle of the AhpR system. The AhpR system is made up of the flavoprotein, AhpF(1HYU) and the peroxiredoxin, AhpC. AhpC exists as a catalytic dimer, with each dimer possessing a conserved peroxidatic cysteine (C_P) and a resolving cysteine (C_R). During catalysis, AhpC donates electrons to peroxides reducing it to water whilst the later becomes oxidized. The cycle is completed when AhpC becomes reduced by AhpF. Figure modified from [50].

There is a striking homology between the C-terminal domain (CTD) of AhpF and thioredoxin reductase (TrxR) (Figure 1.6). A sequence alignment revealed a 32 % homology [51]. However, AhpC does not show similarities to Trx in amino acid sequence alignments, but AhpC and other homologous proteins appear to be conserved throughout all kingdoms and belong to the family of peroxiredoxins [52]. AhpC and its peroxiredoxin family members are highly expressed in bacteria cells, especially in *E. coli*, where it's among the ten most abundant proteins [53]. Although variations exist in how different *E. coli* species cause disease, that is uropathogenic strains which cause symptomatic infections and asymptomatic bacteriuria strains, in the stressful environment of the urinary tracts where oxidative stress is high, most *E. coli* species response to the later in a similar manner. It has been suggested that the redox active cysteine disulfide of AhpF and AhpC are involved in the catalysis of hydroperoxide reduction (Figure 1.4) [54].

1.3.1 Alkyl hydroperoxide reductase subunit F (AhpF)

AhpF has three domains, an N-terminal domain (NTD) containing a redox-active disulfide centre, an FAD-binding domain (FAD) harbouring the redox active flavin, and an NADH-binding and redox-active disulfide-containing domain (NADH/SS) [49, 55, 56].

Historically (Figure 1.5), the AhpF gene was discovered during studies of *S. typhimurium* defences against oxidative stress and heat-shock proteins [57]. During infection, invasive *S. typhi* penetrate the underlying tissues of the intestinal epithelium where they are engulfed by macrophages. The macrophage is a bastion of ROS. For the *S. typhi* to survive and cause disease it should be able to defend itself against the ROS. In the studies to access *S. typhi* defences, it became resistant to peroxide killing when they were pre-exposed to low dosage of non-lethal H₂O₂. Analysis of the dominant H₂O₂-resistant strains by 2D gel electrophoresis reveals that 30 proteins were induced from which nine are under the regulation of the OxyR-regulon [57]. These nine proteins included the alkyl hydroperoxide reductase. Additionally, it was found that OxyR deletion was recessive and uninducible by H₂O₂ concerning the nine overexpressed proteins in the H₂O₂-resistant strains leading the researchers to suggest that OxyR is a positive regulatory element. It was not until 1989, that AhpR was purified by Storz *et al* [58]. By cloning and mapping the *ahp* gene responsible for encoding the two proteins, AhpF and AhpC, the group was able to overexpress and isolate both proteins. Subsequent deletion experiments of the *ahp* genes produced *E.*

coli and *S. typhi* strains being hypersensitive to cumene hydroperoxide killing [58]. As the insight into this unique protein system widens, attention was also paid to other homologue proteins. Chae *et al* in 1994, through the isolation of a thiol-specific enzyme (TSA) cDNA from rat brain and subsequent sequencing and comparison with *S. typhi* AhpF, revealed a much broader family of proteins [52]. In an attempt to decipher the roles of each domain of AhpF, Reynolds and Poole in 2000 [59],

Figure 1.5: Historical milestones in the understanding of the alkyl hydroperoxide subunit F. Christman *et al* reported for the first time, that *S. typhimurium* is able to adapt to hydrogen peroxide in ways similar to that of *E.coli*. By performing two-dimensional protein gels, about 30 proteins were found to be induced; among them was the novel alkyl hydroperoxide reductase [57]. In 1989, Storz *et al*, mapped and cloned the components of the AhpR, AhpF and AhpC. They found that deletion of *ahp* led to hypersensitivity to organic peroxides and disappearance of the two proteins from 2D gels [58]. Chae *et al* classified AhpF into a wider group of antioxidant enzymes which includes thioredoxin reductase [52]. In an attempt to delineate the functions of the N-terminal domain of AhpF, Reynolds and Poole engineered a chimeric protein consisting of the *E. coli* thioredoxin reductase (TrxR) fused with the N-terminal domain of *S. typhi* AhpF. This enables the TrxR to reduce AhpC [61]. The first crystallographic structure of *S. typhi* AhpF was solved in 2001 by Wood *et al*. In order to understand the dynamics of the NTD which consists of two fused thioredoxin molecules, Hall *et al*, performed NMR studies and determined that reduction and oxidation do not affect the structural integrity of the NTD [62].

engineered a chimeric protein where the NTD of *S. typhi*, consisting of a stretch of about 200 amino acids, was attached to *E. coli* thioredoxin reductase. Based on

previous observations that the CTD of AhpF and its homologue thioredoxin reductase are unable to reduce AhpC [60], the ability of the chimeric protein to restore AhpC reduction capabilities confirm that the NTD of AhpF has a direct role in AhpC reduction [59].

Figure 1.6: Crystal structures of (A) the C-terminal portion of AhpF from *E. coli* (EcAhpF₂₁₂₋₅₂₁, PDB: 1FL2) and (B) Thioredoxin Reductase (TrxR) from *E. coli* (PDB: 1TDF) [63, 64]. Both structures are shown in its monomeric state and they consist of two domains, the FAD-domain (yellow) and the NADH-domain (brown). FAD is in blue sticks.

The crystal structure of the C-terminal portion of AhpF from *E. coli* (EcAhpF₂₁₂₋₅₂₁) comprises of 311 residues (A212-A521), which correspond closely to the large proteolytic fragment from *StAhpF* (residues 203-521), confirmed the structural homology to TrxR (Figure 1.6) [63]. EcAhpF₂₁₂₋₅₂₁ consists of an FAD-domain and a NADH domain. Its redox active cysteines C345/C348 are located close to the isoalloxazine-ring of the FAD (Figure 1.7A) [63]. The NTD has a redox-active disulfide centre C129/C132 [52, 55, 65]. Both redox-active disulfide centres in AhpF (C345/348 and C129/C132) have the CXXC structural motif, which is also present in the Trx and TrxR form. Mutations in AhpF from *S. typhimurium*, involving the two disulfide centres C129S/C132S or C345S/C348S, highlight that both redox active disulfides are essential for the catalytic activity of AhpF, and support the role of C129/C132 in the NTD to mediate the electron transfer between C345/C348 to the

AhpC active disulfide C47/C166 (Figure 1.10) [60]. Additionally, the NTD is homologous to thioredoxin and protein disulfide oxidoreductase (PDO) (Figure 1.8), a thioredoxin-like (Trx-like) protein from *Pyrococcus furiosus* (PfPDO) (23% identical sequence), as described the AhpF crystal structure from *S. typhimurium* (PDB ID: 1HYU) [56, 65, 66]. The crystal structure of *S. typhimurium* as well as PfPDO revealed the existence of two fused thioredoxin-like folds in the NTD (Figure 1.8)

Figure 1.7: Crystal structures of the closed and open conformation of (A) *S. typhi* AhpF (PDB ID: 1HYU) [56] and (B) *E. coli* AhpF (PDB ID: 405Q) [67].

Figure 1.8 Cartoon representation of *P. furiosus* protein disulfide isomerase (PfPDO). The two thioredoxin moieties are coloured differently for clarity (PDB ID: 1ABL).

[56, 66]. In PfPDO, each thioredoxin folds consists of one active disulphide centre, whereas the NTD of *E. coli* comprises only one redox active disulfide centre, namely in the second thioredoxin fold with a distinct CXXC active site motif (Figure 1.9) [56, 66]. The *StAhpF* crystal structure reveals all 521 amino acids, highlighting all four regions (Figure 1.7). Electron transfer within AhpF starts from NADH and goes via FAD to the

redox active disulfide C345/C348 at the C-terminal portion and from this disulfide to

the N-terminal C129/C132 disulfide centre (Figure 1.10) [60]. The electron movement from AhpF to AhpC is predicted to occur via the redox centres C129/C132 of AhpF to the disulfide centre C47/C166 of AhpC (Figure 1.10) [59]. During my studies, the first crystallographic structure of the open conformation of *E. coli* AhpF was solved by our group (Figure 1.7B) [67] with the NTD rotated and translated by about 178° and 1.25 Å compared with the *S. typhimurium* crystal structure (Figure 1.7). Dip *et al*, therefore, proposed that during catalytic

Figure 1.9: Structural comparison of the archaea *PpPDO* (PDB ID: 1A8L) and the *E. coli* AhpF₁₋₂₁₂ (PDB ID: 4O5Q). The striking homology implies that the NTD of *EcAhpF* evolved from the PDO family of proteins.

activity, the NADH/SS-domain turns 67° toward the FAD. This movement brings a bound NADH to the *re*-face of the isoalloxazine ring of flavin for its oxidation (Figure 1.7) [68]. The rotation of the NADH/SS-domain brings also the reduced dithiol (C345/348) to the surface, where it might be in a position to reduce the NTD. After the reduction of the NTD, the NADH/SS turns 66° back, bringing C345/C348 back to the *re*-face of the flavin group, and the NAD⁺ to the surface for exchange with new NADH. The catalytic cycle of AhpF is complete after the hydride from NADH reaches the redox-active cysteines in NTD [56]. The NTD has two important roles. Firstly, it acts as a substrate for the C-terminal redox-active disulfide bond, and secondly, it acts as a protein disulfide reductase for redox active disulfide in AhpC [55, 56, 59, 68].

Figure 1.10: Electron transfer of the Alkyl hydroperoxide Reductase system. Electrons are transferred from NADH to FAD and subsequently to the CTD thiol centre C345/348. Conformational movement of the NTD brings its catalytic centre C129/132 to the CTD to receive the electrons. The NTD then donates the electrons to AhpC. This figure has been adopted with modification from Wood *et al.* 2001 [56].

1.3.2 Alkyl hydroperoxide reductase subunit C (AhpC)

The *E. coli* AhpC belongs to a larger group of thioredoxin-scaffold enzymes which scavenges peroxides and organic peroxides, and are known as peroxiredoxins (Prx) [69]. They are distributed through all domains of life indicating their ancient origins. Broadly speaking, Prx can be classified based on the location and number of catalytic cysteine residues; hence, there are typical 2-Cys, atypical 2-Cys and 1-Cys Prx (Figure 1.11). 2-Cys Prx exists functionally as a dimer. During the peroxidase reaction, a conserved peroxidatic cysteine (C_P) attacks a peroxide substrate, forming an intermediate sulfenic acid ($Cys-C_POH$). In the next phase a resolving cysteine (C_R) attacks the intermediate with the loss of a water molecule. The Prx becomes oxidised. The oxidised Prx is then reduced by a disulphide reductase-like AhpF [69]. Atypical 2-Cys Prx function similarly but they are monomeric and hence both the C_P and C_R are within the same monomer (Figure 1.11). 1-Cys Prx, on the other hand, lacks the C_R altogether and is reduced by a thiol-containing reductant [70]. Among all the classes of Prx, the most ubiquitous, abundant and well-studied is 2-Cys Prx [71]. They possess a unique C-terminal extension which bears the C_R (Figure 1.12). There has been no exact role attributed to the C-terminal tail. Variations have been observed in the lengths of Prxs with eukaryotic Prxs possessing a YF- motif at the extreme C-terminal tail. This has been associated with hyper oxidation at the C_P -position [70]. Therefore, even though most 2-Cys Prxs, especially the bacteria 2-Cys Prx which lacks this modification (Figure 1.12), has a higher efficiency towards H_2O_2 , ($10^7 M^{-1} s^{-1}$) [72],

Figure 1.11: The three mechanism that separates the classes of Prx. The initial peroxidative attack by the C_P is a common step among all the classes. But in the typical 2-Cys Prx, the basic unit is a homodimer with the resolving cysteine (C_R) separately borne on the unique C-terminal tail of the other monomer. In the case of atypical 2-Cys, the proteins are monomeric hence both C_P and C_R are in the same monomer. 1-Cys lacks the C_R. Whereas the oxidized typical and atypical 2-Cys Prx are reduced by a disulphide reductase (DS) that of 1-Cys Prx is reduced by a thiol-containing reductant (TS). Cysteine sulphurs are not shown for clarity. The figure was adapted from [69].

their eukaryotic counterparts are easily over-oxidized at high concentrations of H₂O₂ to generate sulphinic (-S_PO₂H) and sulphonic (-S_PO₃H) acids intermediates, the dead-end products that need to be reactivated by sulphiredoxin (Srx), an ATP-requiring oxidoreductase found in eukaryotes [72]. H₂O₂, despite its harmful effects, is used as signalling molecules in higher organisms and for that matter, higher organisms have evolved the eukaryotic Prx as molecular switches to sense benign H₂O₂ conditions from deleterious conditions through irreversible over-oxidation [73]. Thus, by comparing the crystal structures of a non-disulphide bond containing eukaryotic PrxII “fully folded” (FF) and disulphide bond containing PrxI “locally unfolded” (LU), the hypersensitivity can be understood. In the case of FF, the helix containing the conserved YF-motif packs closely to a conserved GGLG-motif (*coloured in wheat* in figure 1.13) to cordon off the C_P from C_R [69]. This arrangement allows more time for the C_PSOH intermediate to be further oxidized to C_PSO₂H. Additionally, the unfolding of the C_P-containing helix in the LU state exposes both catalytic centres to form a disulphide bond. This arrangement is absent in bacteria Prxs (Figure 1.12), hence, it will be extremely interesting to understand what role the C-terminus in bacteria Prx, especially the AhpR, plays and ascertain whether its contributes to its robustness and

higher efficiency.

Figure 1.12: Sequence comparison among the 2-Cys Prxs. 2-Cys Prxs are the most diversified and abundant group of Prx. The alignment shows representative groups of eight 2-Cys Prx. The unique C-terminal extension of the 2-Cys is indicated by a black box. The two catalytic cysteines are indicated by stars. Eukaryotic peroxiredoxin and *H. pylori* contain a conserved YF- and GGLG-motifs indicated by the green box and this makes them sensitive to excess H₂O₂.

Figure 1.13: Cartoon representations of the active site of eukaryotic PrxII (PDB ID: 1QMV) and PrxI (PDB ID: 1QQ2) showing the FF and LU arrangements that make them sensitive to the higher concentration of peroxides. In the FF, the YF-motif (shown as sticks) make contact with the GGLG motif (coloured in wheat) to stabilize and protects the fully folded C_P-containing helix (magenta), whilst in the LU formation, the unwinding of the helix exposes the C_P for disulphide bond formation. Figure adapted from [69].

1.3.3 Summary of the catalytic cycle of the AhpR system

In AhpC, both cysteines C47 (C_P) and C166 (C_R) are conserved. C47 is located at the N-terminal region within a conserved valosin-containing protein (VCP) motif. AhpF catalyses the hydride transfer from NADH via flavin and two different redox-active disulfide bridges to the dithiol centre of AhpC (Figure 1.10) [59]. The reduced AhpC is activated to scavenge peroxides (Figure 1.4 and 1.11). The peroxidatic cysteine (C_P) initiates the attack on hydroperoxides or alkyl hydroperoxides (ROOH) (Figure 1.11) [74]. During this attack, the thiol group (C_P -SH) of the peroxidatic cysteine (C47) is oxidised to sulphenic acid (C_P -SOH) with the release of H_2O . This sulphenic acid leads to a local unfolding of α 2-helix residues into the loop region at the active site, similar to eukaryotic Prx (figure 1.13) that causes C47 to come in close contact to C166 [55, 75]. The oxidation of C_P unwinds the α -helix allowing the other redox active C166, so called resolving cysteine (C_R) located at the C-terminus end of the neighbouring monomer, to convert sulphenic acid to an intermolecular disulphide bond (C_P S- SC_R) [75]. The disulphide bond is guided with a partial unfolding of the α -helix that contains C_P [74]. Therefore, during its catalytic cycle, AhpC alternates between locally unfolded when oxidised and fully folded when reduced [74]. The regeneration of the oxidised AhpC is carried out by the disulphide C129/C132 in the NTD of AhpF. It acts as an electron donor and reduces the disulfide bond between C_P and C_R to two catalytic active thiol groups [76]. Characterisation of AhpF and AhpC will give new insights into oxidative stress, since they are heavily involved in quite a number of diseases, such as rheumatoid arthritis, inflammatory bowel disorders, and atherosclerosis, and it is emerging as one of the most important causative agents of mutagenesis, tumor genesis, and aging [2, 77-80].

1.4 Goals of this thesis

The AhpR-system, consisting of AhpC and AhpF, is responsible for detoxifying the bacteria cells from hydrogen peroxide and organic peroxides [81]. As important as this enzyme system is in all domains of life, no attempt has been made to understand the mechanism of binding between the two components. Moreover, in higher organisms, the C-terminus of the AhpC has been implicated in peroxide sensing but no role has been attributed to the C-terminus of bacterial AhpC.

To gain insight into the role of the AhpC C-terminus, a series of C-terminally truncated mutants of the *Escherichia coli* protein were generated. Single amino acid

mutants at the C-terminus were also produced. It has been reported that the oxidized AhpC exists as a lower oligomeric form whilst upon reduction, it oligomerizes into a decamer [7, 69, 82]. By using dynamic light scattering (DLS), the oligomerization properties of these C-terminal mutants were examined under reducing conditions. Again, in order to examine the association between the C-terminal mutations and enzymatic activity, NADH-dependent peroxidative activity assays were performed for all the mutants. Furthermore, crystallographic studies were undertaken so as to throw light on the structural features of the C-terminus of AhpC.

Moving on to characterize the interaction between AhpF and AhpC, three constructs of AhpF, *EcAhpF*₁₋₁₉₆ (NTD), *EcAhpF*₁₋₂₁₂ (NTD + linker) and *EcAhpF*₂₁₂₋₅₂₁ (CTD) were generated. First, solution x-ray scattering (SAXS) studies were performed to ascertain the integrity and oligomeric state of each construct and then isothermal titration calorimetry (ITC) studies were performed to confirm, which domain of the AhpF interacts directly with AhpC. Furthermore, nuclear magnetic resonance (NMR) studies were performed on the AhpF domains and titrations were performed with AhpC so as to enable identification of the binding interface of the complex and subsequent understanding of the mechanism of the interaction that makes this enzyme system efficient. Throughout these studies, the role of the C-terminus will be carefully examined.

Finally, by employing a high ionic buffer system (ammonium sulphate), I have stabilized the oligomeric forms of the AhpC under oxidizing conditions and also captured the full-length AhpF in complex with the AhpC. This will go a long way to giving additional insight into the AhpR-systems.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals

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

2.1.1.1 Buffers 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)
¹⁵ NH ₄ Cl	Cambridge Isotopes Lab (USA)
LB Media	BD (Sparks, MD, USA)

2.1.1.2 Electrophoresis Chemicals

(Agarose, SDS, Glycine, APS etc.)	Bio-Rad (Hercules, CA, USA)
Antibiotics	Calbiochem, Sigma and Gibco
(Invitrogen)	
IPTG	Fermentas (<u>Vilnius, Lithuania</u>)
BSA	GERBU (Heidelberg, Germany)

2.1.2 Molecular biology materials

Genomic DNA	<i>Escherichia coli</i> (Bl21 strain)
Primers	1 st Base and Research Biolabs (Singapore)
Peptides	NTU proteomics core facility, School of Biological Sciences,

Nanyang Technological University, Singapore

Pfu DNA Polymerase	Fermentas (Glen Burnie, MD, USA)
<i>NcoI</i> , <i>SacI</i>	Fermentas and New England Biolabs
T4 DNA Ligase	Fermentas and NEB
Precision Proteases	(GE Healthcare, Uppsala, Sweden)
Miniprep Plasmid Kit	Qiagen (Hilden, Germany)
Nucleobond AX mediprep Kit	MN & Co (Düren, Germany)
<i>Escherichia coli</i> expression strains	DH5 α , BL21 (DE3)
pET-9d1	Grüber <i>et al.</i> , 2002 [83]

2.1.3 Chromatography materials

2.1.4 Gel filtration

Superdex 75 HR (10/30)	GE Healthcare (Uppsala, Sweden)
Superdex 200 HR (10/30)	GE Healthcare (Uppsala, Sweden)

2.1.5 Instruments and accessories

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

2.1.6 Protein concentration, estimation

Centriprep YM10	Millipore (Co-cork, Ireland)
Amicon ultra (3-30kDa)	Millipore (Co-cork, Ireland)
BCA Assay Kit	Pierce (Rockford, IL, USA)

2.1.7 Other instrumentation

PCR Thermocycler:	
Biometra T personal	Biometra
Biometra T gradient	Biometra
Sonoplus Sonicator	Bendelin
Micropulser Electroporator	Bio-Rad (Hercules, CA, USA)
	Ultraspec 2100 Pro-
Spectrophotometer	Amersham Biosciences
BioSpec-Nano	Shimadzu, Tokyo, Japan

2.1.8

Vector NTI 10.3.0

SPARKY

Topspin 2.1

CYANA 2.1

TALOS

MOLMOL

PyMOL v0.99

HKL2000 package

Refmac5 (CCP4 package)

MOLREP

PHASER

CNS

Coot

Ramachandran plot

PROCHECK

Quantity One

PRIMUS

Gasbor

SUBCOMB

UCSF Chimera

MEGA 6.0

Computer software

Invitrogen

Goddard and Kneller, 1997 [84]

Bruker Biospin

Günthert *et al.*, 1997 [85]Cornilescu *et al.*, 1999 [86]Koradi *et al.*, 1996 [87]

DeLano Scientific LLC, USA [88]

Zbyszek and Wladek, 1997 [89]

Murshudov *et al.*, 1997 [90]

Vagin and Taplyakov, 1997 [91]

McCOY *et al.*, 2007 [92]Brunger *et al.*, 1998 [93]

Emsley and Cowtan, 2004 [94]

Ramachandran *et al.*, 1963 [95]Laskowski *et al.*, 1993 [96]

Bio-Rad (Hercules, CA, USA)

Svergun, 1993 [97]

Svergun *et al.*, 2001 [98]Svergun *et al.*, 2001 [98]Pettersen *et al.*, 2004 [99]Tamura *et al.*, 2013 [100]

2.2 Cloning of *EcAhpC* C-terminal mutants and the N-terminal domain of *EcAhpF*

To amplify the mutant coding region, oligonucleotide primers incorporating *NcoI* or *SacI* restriction sites (underlined), were designed.

Construct		Oligonucleotide sequence 5'-3'
<i>EcAhpC</i>	forward	CATGCCATGGCAATGTCCTTGATTAACA
	reverse	GCGAGCTCGTTAGATTTTACCAACCAGGTC
<i>EcAhpC</i> ₁₋₁₈₅	forward	CATGCCATGGCAATGTCCTTGATTAACA
	reverse	GCGAGCTCTTAACCAACCAGGTCCAGAGAC
<i>EcAhpC</i> ₁₋₁₈₂	forward	CATGCCATGGCAATGTCCTTGATTAACA
	reverse	GTTGAGCTCTTAGTCCAGAGACGGAGCCCAG
<i>EcAhpC</i> ₁₋₁₇₇	forward	CATGCCATGGCAATGTCCTTGATTAACA
	reverse	GCGAGCTCTTACAGAGTTGCTTCACCTTC
<i>EcAhpF</i> ₁₋₂₁₂	forward	CATTCCATGGCAATGCTCGACACAAATATG
	reverse	GTCGAGCTCTTAATCAATTTTGGCAACGAT
<i>EcAhpC</i> ₁₋₁₉₆	forward	CATTCCATGGCAATGCTCGACACAAATATG
	reverse	GTCGAGCTCTTAATCAATTTTGGCAACGAT

E. coli genomic and plasmid DNA were used as templates for the polymerase chain reaction (PCR). The PCR was set up in a total volume of 50 µl on ice as mentioned below with the appropriate concentration of each constituent as:

Reagents	Amount
Pfu buffer (10x)	5 µl
DNTP's (2 mM)	1.5 µl
Primers (100 µM)	2 x 0.5 µl
Template (genomic DNA)	1 µl
MilliQ water	40.5 µl
Pfu DNA polymerase	1 µl

All reagents were pipetted into a PCR tube kept on ice all the time, mixing of the reagents was done by short centrifugation. PCR thermocycler (Biometra T personal) was preheated to 95 °C before 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 min
Cycle denaturation	96 °C	30 sec
Annealing	58 °C	45 sec
Extension	72 °C	60 sec
Final extension	72 °C	5 min
End	4 °C	

Cycle denaturation, annealing and extension steps were repeated for another 29 cycles. The total time taken for the PCR program was 1 h 43 min 49 sec.

After amplification, the PCR product was stored at -20° C, 5 µl of which was then applied onto an analytical agarose gel (1.5%) to check its quality. The gel was kept in ethidium bromide solution for 20 min and observed under UV light. The size of the PCR product was confirmed with the appropriate DNA marker. The remaining reaction mixture was applied onto a 1.5% preparative agarose gel. The portions corresponding to the correct PCR

Figure 2.1: Modified pET-9d (+) vector showing a full map and unique multiple cloning site (MCS) that has been used to clone various genes [83].

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 – 50 µl water or Tris-HCl buffer of pH 8.0. 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 using the appropriate amount of *Nco*I and *Sac*I enzymes. After overnight digestion, the reaction mixture was purified by the enzyme reaction purification kit from QIAGEN. Subsequently, the ligation reaction between vector (Figure 2.1) and amplified PCR product was set up at room temperature. The reagents used were as follows:

Ligase buffer (10x)	1.5 µl
Vector (V)	50-100 ng
Insert (I)	Variable (1-5 times of vector)
T4 Ligase	1 µl
MilliQ water	variable
Total	15 µl

The ligation mixture was kept at room temperature for 2 hours. The reaction was terminated by precipitation of DNA from the rest of the reaction mixture by adding 85 µl of Milli-Q water to make a final volume of 100 µl. 1 ml of butanol was added to the mixture and mixed thoroughly. Then the mixture was centrifuged at 13,000 x g (using Eppendorf Mini-centrifuge) for 10 minutes and the supernatant was discarded gently. The pellet was resuspended in 150 µl of 70% ethanol and centrifuged again for 5 minutes. Supernatant was again removed and the pellet was re-dissolved in 10 µl of elution buffer (10 mM Tris-HCl, pH 8.5; Elution Buffer (EB) , QIAGEN) or Milli-Q water. 5 – 10 µl of 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 incubated at 37 °C. 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 ligated insert. The size of insert and vector were compared with appropriate controls and markers. The verified plasmid was finally transformed into *E. coli* BL21 (DE3) cells for protein production. The cells were grown on 30 µg/ml kanamycin-containing Luria-Bertoni (LB) agar plates. *E. coli* BL21 or Rosetta-gamiTM2 (DE3) expression strains were purchased from Novagene (Darmstadt, Germany).

2.2.1 Electroporation transformation

Reagents

SOB media: 20 g/l Tryptone
5 g/l Yeast Extract
0.58 g/l NaCl
0.18 g/l KCl
10 ml Mg-solution (1 M MgCl₂·6H₂O, 1 M MgSO₄)

Electroporation was performed for the transformation of the plasmid into electro-competent cells, which were prepared as per the protocol from the Current Protocols in Molecular Biology (Wiley InterScience) manual. 1 – 50 ng of plasmid was added to 80

– 100 μ l of competent cells and incubated on ice for 5 – 10 min. Electroporation was done at a constant voltage of 2500 V (Micropulser Electroporator, Bio-Rad). The cuvette was then immediately placed back on the ice for 1 min., following the addition of LB or Super Optimal Broth with Catabolite repression, SOC (SOB + 100 mM Glucose) media to the total volume of 1 ml. The mixture was incubated at 37 °C for 1 h. Appropriate dilutions were prepared before plating the cells on antibiotic selection plates.

2.3 Production and purification protein constructs

2.3.1 Induction test

Reagents for 4 x lysis buffer:	250 mM Tris-HCl, pH 6.8
	9.2% SDS
	40% Glycerol
	0.2% Bromophenol Blue
	1 mM DTT

At least 3-4 single colonies were randomly picked. They were grown to OD₆₀₀ of 0.6 - 0.7 at 37 °C with shaking at 170 – 200 rpm (Infors HT Minitron shaker). Final concentrations of 0.5-1 mM IPTG were then added to start induction. The mixture was kept at 30 – 37 °C for 2 – 3 h. The concentration of Isopropyl β -D-1-thiogalactopyranoside (IPTG) depends on the characteristics of protein being produced and the conditions that have been optimised for the respective protein. Respective controls were left uninduced under identical conditions. Cells were pelleted down and resuspended in 60 μ l of lysis buffer in the presence of 1 mM DTT. The resuspended cell mass was heated at 95 °C for 5 min and 15 – 20 μ l of each sample was loaded onto a 17% SDS polyacrylamide gel. The expression was more in the induced cultures ((+) supplemented with IPTG) than uninduced cultures ((-) not supplemented with IPTG).

2.3.2 Solubility tests for recombinant proteins

Following the successful induction of proteins with appropriate concentration of IPTG at the optimised temperature and shaking conditions, the solubility of the produced proteins was tested in various buffers. 50 ml of culture was grown and induced with IPTG. Cells were pelleted into five equal fractions by centrifuging at 10000 x g for 10 min. Freshly prepared protease inhibitors Pefabloc^{SC} in water and PMSF (dissolved in isopropanol) were added to final concentrations of 2 – 8 mM (Pefabloc^{SC}) or 1 – 2 mM (PMSF). The reducing agent DTT (1 mM) was used in the

preparation. Resuspension was sonicated at 15% power with KE 76 tip of sonicator (Bandelin Sonoplus) three times for one min each, with a cooling interval of two min between each cycle. The supernatant was then separated from the pellet by centrifugation at 10,000 x g and transferred to a fresh tube. All the steps were carried on the ice. 10 – 15 μ l of pellet and supernatant were loaded onto a 17% SDS gel [101].

2.3.3 Purification of Alkyl hydroperoxide Reductase subunit F (AhpF) N-terminal domains (*EcAhpF*₁₋₁₉₆ and *EcAhpF*₁₋₂₁₂) from *E. coli*

To express the individual proteins, cultures were grown in LB medium containing kanamycin (30 μ g/ml) for about 6 h at 310 K until an optical density OD₆₀₀ of 0.6–0.7 was reached. To induce the production of proteins, cultures were supplemented with IPTG to a final concentration of 1 mM, followed by incubation for 4 h at 310 K.

Cells producing the respective *EcAhpF*₁₋₂₁₂ and *EcAhpF*₁₋₁₉₆ were lysed on ice by sonication with an ultrasonic homogenizer (Bandelin, KE76 tip) for 3 x 1 min in buffer A (50 mM Tris-HCl (pH 7.5) 200 mM NaCl, 2 mM PMSF, 1 mM Pefabloc^{SC} and 0.8 mM DTT). After sonication, the cell lysates were centrifuged at 10 000 x g for 35 min at 277 K. The resulting supernatant was passed through a filter (0.45 μ m; Millipore) and supplemented with Ni²⁺-NTA resin pre-equilibrated in the lysis buffers. The His-tagged proteins were allowed to bind to the matrix for 1.5 h at 277 K by mixing on a sample rotator (Neolab). To avoid any traces of residual DTT from the lysis buffer, the Ni²⁺-NTA beads was thoroughly washed with 10 column volumes of respective buffers without DTT and was subsequently eluted with an imidazole gradient (0–250 mM). Subsequently, the fractions containing *EcAhpF*₁₋₂₁₂ and *EcAhpF*₁₋₁₉₆ were identified by SDS–PAGE [101] and applied onto a Resource Q column (6 ml, GE healthcare). Both *EcAhpF*₁₋₂₁₂ and *EcAhpF*₁₋₁₉₆ eluted with the flow through. The flow-through was concentrated and further purified by gel filtration chromatography using a Superdex 75 HR 10/30 column (GE Healthcare) with a buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl. The purity and homogeneity of *EcAhpF* samples were analyzed as described in section 2.3.3. Protein concentrations were determined by bicinchoninic acid assay (BCA; Pierce, Rockford, IL., USA) as well as absorbance at 280 nm in a spectrophotometer (BioSpec-Nano, Shimadzu) using the extinction coefficients 10 005 M⁻¹ cm⁻¹.

2.3.4 Purification of Alkyl hydroperoxide Reductase subunit C (AhpC) C-terminal mutants from *E. coli*

Figure 2.2; Schematic representation of the generated C-terminal constructs of *EcAhpC*. The C-terminal residues under studies are highlighted in brown.

For the production of *EcAhpC* constructs (Figure 2.2), *E. coli* strain BL21 (DE3) was used. The first purification step of the recombinant His₆-AhpC involves a Ni-NTA resin column as described in section 2.3.3. To purify the oxidised form of recombinant mutants of

EcAhpC, no DTT was used in all following buffers. To avoid remaining DTT from the lysis buffer A, the Ni²⁺-NTA beads was initially washed with 10 column volumes of lysis buffers without DTT and subsequently eluted with an imidazole gradient (0–300 mM). Lysis buffer A of AhpC purification under oxidising condition contains: 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM PMSF and 2 mM Pefabloc^{SC} (BIOMOL). The second step employed ion-exchange chromatography carried out with ResourceTM Q column (GE Healthcare) to clear impurities that bind to the column, whereas the *EcAhpC* remains in the flow through. The flow through were pooled and concentrated using Centricon YM-10 (10 kDa molecular mass cut-off) spin concentrators (Millipore). To obtain highly purified *EcAhpC* and to remove the final impurities gel filtration chromatography was employed using a Superdex 75 HR 10/30 column (GE Healthcare) and a buffer of 50 mM Tris-HCl, pH 7.5, 200 mM NaCl.

To obtain *EcAhpC* in its reduced form, all three columns Ni-NTA, ResourceTM Q (GE Healthcare) and Superdex 75 HR 10/30 (GE Healthcare) steps were similar to that used for the oxidising condition, except buffer A of the purification contains additional 0.8 mM DTT in the Ni-NTA column step and 1 mM DTT in both ResourceTM Q column (GE Healthcare) and Superdex 75 HR 10/30 column (GE Healthcare) steps. The purity and homogeneity of *EcAhpC* samples in oxidised and reduced forms were verified as described in section 2.3.3. Protein concentrations were determined by the bicinchoninic acid assay (BCA; Pierce, Rockford, IL., USA) as well as absorbance at 280 nm in a spectrophotometer (BioSpec-Nano, Shimadzu) using extinction

coefficients $24\,075\text{ M}^{-1}\text{cm}^{-1}$ for AhpC in the oxidised form and $23\,950\text{ M}^{-1}\text{cm}^{-1}$ in its reduced form.

2.4 Quantification of proteins by bicinchoninic acid (BCA) method

BSA is used as a standard for protein quantification by the BCA method. The BCA reagent is available in kit form from Pierce (Rockford, IL, USA.) and instructions for measuring protein concentration were followed. In general, two dilutions of protein samples were taken and each was measured in triplicate to avoid any error in the measurement. Optical density was measured at 562 nm against the blank. The standard curve with 50 $\mu\text{g/ml}$, 125 $\mu\text{g/ml}$, 200 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$ of BSA was then drawn from the OD_{562} . The fitting equation was calculated from the drawn curve. The concentrations of the proteins were obtained based on the derived equation.

2.5 Circular dichroism spectroscopy

Circular dichroism spectroscopy measures the difference between the absorption of right- and left-handed circularly polarized light, arising due to the chirality of a particular molecule under consideration. Circular dichroism can be used to determine the secondary structure contents of protein in far UV range, where the peptide bond acts as an active chromophore. Steady state CD spectra were measured in 0.1 mm quartz Hellma cell (60 μl volume) at 18 °C on CHIRASCAN spectropolarimeter (Applied Photophysics) instrument at a step resolution of 1 nm from 180 – 260 nm far UV range, of spectrum under continuous purging of N_2 -gas. Protein samples were buffer exchanged, reduced or oxidised by appropriate protocols before measurements. Each protein sample was measured in triplicate at a concentration of 2 mg/ml, with the buffer reading taken before and after measuring the protein sample. The averaged buffer base line was subtracted from the average protein values using the Chirascan software. Initial degree $\text{cm}^2\text{dmol}^{-1}$ units were converted into mean molar residue ellipticity (θ) with the Chirascan software by giving appropriate cell path length, molecular weight, the number of amino acid residues and protein concentration values. The final plotting was done in MS Excel (2003). In order to analyze the CD spectrum, the following algorithms were used: Varselec [102], Selcon [103], Contin [104], K2D [105]. All methods are incorporated into the program Dicroprot [106] and Neural Net [107].

2.6 Dynamic light scattering (DLS)

Dynamic light scattering (DLS) of *EcAhpF* as well as reduced and oxidised *EcAhpC* were carried out using the Malvern Zetasizer Nano ZS spectrophotometer. DLS was measured in a low-volume quartz batch cuvette (ZEN2112, Malvern Instruments) using 12 μ l of 1-10 mg/ml of protein sample. After 60 s equilibration time, the backscattering at 173° was detected for all proteins, accordingly. Scattering intensities were analyzed by using the inbuilt software, Zetasizer to calculate the hydrodynamic diameter (D_H), size, and volume distribution.

2.7 NADH-dependent peroxidase activity

The NADH-dependent peroxidase assay was monitored at 340 nm following the decrease in NADH absorbance. The assay was carried out at 25° C in a total volume of 100 μ l containing 300 μ M of NADH, 1 mM of hydrogen peroxide, 1 μ M of both *EcAhpC* and *EcAhpF* and 50 mM of phosphate buffer at pH 7.0, containing 100 mM of ammonium sulfate and 0.5 mM EDTA. All the reaction mixtures were added to the reaction buffer except the NADH, which is added at the end to start the reaction. The background reaction measured for all mixtures without the *EcAhpF* was taken as a control. To ascertain the time points required for subsequent experiments, the time required for maximum NADH oxidation activity was monitored in the presence of *EcAhpC*. Saturation was observed at 6 min. The NADH oxidation was then measured with multi-wavelength scanning ranging from 280-540 nm at two different time scales. The first measurement was carried out immediately after all the reaction mixtures were added and the second measurement was done after 6 min, where the NADH oxidation has been found to be saturated for *EcAhpC*.

2.8 Solution X-ray scattering experiments and data analysis for *EcAhpF*₁₋₁₉₆, *EcAhpF*₁₋₂₁₂, *EcAhpC*₂₁₂₋₅₂₁ and the *EcAhpC-EcAhpF* complex

Small angle X-ray scattering (SAXS) data of the different forms of *EcAhpC* as well as for the *EcAhpC:EcAhpF* complex were collected with the BRUKER NANOSTAR SAXS instrument, equipped with a Metal-jet X-ray source and the VANTEC 2000-detector system. The X-ray radiation was generated from a liquid gallium alloy with microfocus X-ray source ($K\alpha = 1.3414 \text{ \AA}$ with a potential of 70 kV and a current of 2.857 mA). The X-rays were filtered through Montel mirrors and collimated by a two pinhole system. The sample to detector distance was set at 67 cm and the sample chamber and X-ray paths were evacuated [11,31]. This setup covers of

the momentum transfer range $0.016 < s < 0.4 \text{ \AA}^{-1}$ ($s = 4\pi \sin(\theta)/\lambda$, where 2θ is the scattering angle). SAXS experiments were carried out at 15 °C with different protein concentrations in respective buffers, a sample volume of 40 μl in a vacuum tight quartz capillary. Corresponding to each protein sample, data were collected for a buffer under identical experimental conditions, providing a background scattering curve. The data were collected for 30 min and for each measurement a total of six frames at 5 min intervals were recorded. The data were flood-fielded, spatial corrected and processed using the built-in SAXS software. The data were tested for possible radiation damage by comparing the six data frames, which indicated no change. The scattering of the corresponding buffer was subtracted and the difference curves normalized for the concentration as well as initial intensity. All the data processing steps were performed using the program package PRIMUS [32]. All the experimental data obtained were analyzed for aggregation using the Guinier region [33]. The forward scattering $I(0)$ and the radius of gyration, R_g were computed using the Guinier approximation assuming that at very small angles ($s < 1.3/R_g$) the intensity is represented as $I(s) = I(0) \cdot \exp(-(sR_g)^2/3)$ [33]. These parameters were also computed from the extended scattering patterns using the indirect transform package GNOM [34], which provides the distance distribution function $p(r)$ hence the maximal particle dimension, D_{max} , and R_g . Low-resolution models of the protein were built by the program GASBOR [37] considering low angle data ($s < 0.3 \text{ \AA}^{-1}$). *Ab initio* solution shapes were obtained by superposition of ten independent model reconstructions with the program package SUBCOMP [38] and building an averaged model from the most probable one using the DAMAVER program [39]. Comparison of the experimental scattering curve with the theoretical scattering curves calculated from available high-resolution crystal structures was performed with CRY SOL [40]. The models were then used in OLIGOMER [32] to find the best fit to a multi-component mixture of proteins. Rigid-body modeling for oxidized- and reduced forms of decameric *EcAhpC* were performed using the software CORAL [41]. The rigid-body modeling of the *EcAhpC*₁₋₁₈₇: *EcAhpF* complex was performed with the SASREF [8].

2.9 Sequence alignment analysis

The amino acid sequences of the *EcAhpF*₁₋₂₁₂ and 2-Cys peroxiredoxin proteins were sourced from the National Center for Biotechnology Information databases [108]. These sequences were used to extract distantly related sequences using the PSI-BLAST algorithm. The selected sequences were then aligned using the

CLUSTERO algorithm included in the JalView program [109]. The aligned sequences were visualized using ESPript [110].

2.10 *In silico* High Ambiguity Driven DOCKing (HADDOCK) modeling

Experimentally identified binding interfacial residues were used to perform docking studies to model the complex of *EcAhpF*₁₋₂₁₂ and *EcAhpF*₁₋₁₇₂ using the HADDOCK webserver [111, 112]. The coordinates for the *EcAhpF*₁₋₂₁₂ were fetched from the Protein Data Bank (PDB ID; 4o5q; [67]). 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 *EcAhpC*₁₋₁₇₂, a dimeric unit of coordinates from the PDB database (PDB ID; 4QL9) [50] was renumbered to treat each monomer as a unique entity. The prepared coordinates were submitted to the meta-PPISP server for protein–protein interfacial residues prediction [113]. These predicted interfacial residues were set as “active” residues. Briefly, the submitted subunits were placed in space with an approximate distance of 25 Å and a complex is formed through rigid body energy minimization, which resulted in an initial 1000 structures. The top 200 lowest energy models from the above minimization were subjected to a simulated annealing with a torsion angle space procedure and subsequent flexible refinement in explicit solvents. Models which fulfilled the interface RMSD cutoff of 5 Å were clustered into seven groups. The best model in the highest scoring cluster was selected for analysis. The model was visualized using the PyMOL software package [114].

2.11 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is a powerful technique used to study the structural and functional aspects of macromolecules such as proteins. Using NMR technique, the solution structure of a protein can be determined. NMR can be used to study biochemical and biophysical properties such as protein-ligand interactions to demonstrate new binding partners as well as to map the binding regions down to a single amino acid level. Since hydrogen is one of the most sensitive and abundant NMR active nuclei besides other isotopes such as ¹⁵N, ¹³C, and ³¹P, it can be observed in magnetic resonance. Any nuclei with mass number, which exhibits net intrinsic

magnetic momentum and angular momentum, can be defined as NMR active. All NMR experiments were collected on Bruker Avance 700 MHz machines at the SBS NMR-core facility.

2.11.1 ^{15}N single and ^{13}C ^{15}N double labeling of proteins

Reagents for 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
	Antibiotic
	LB Media

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 as explained in section 2.3.3 using established protocols. The clones were grown in M9 minimal media to ensure the labelling of the expressed protein. 30 ml of the overnight culture was pelleted down at low centrifugal force at room temperature and washed with minimal media and then transferred to a large volume of minimal media with OD_{600} of 0.1. The culture was grown (37 °C, 180 rpm) till OD_{600} of 0.6 - 0.7 was achieved. The culture was then induced with 1 mM IPTG for 3 – 5 h or alternatively induced overnight at 20 °C. Cells were harvested and frozen in liquid nitrogen and stored at – 80 °C.

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

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). Unlabelled or labelled protein samples in phosphate buffer, pH 6.8 were used in the presence of 10% D_2O (v/v) to record the spectra. For 2D experiments like HSQC (Heteronuclear Single Quantum Coherence), ^{15}N labelled proteins in 90% H_2O and 10% D_2O in 25 mM phosphate buffer (pH 6.8) was used to collect data. Other 2D experiments used in the study were 2D NOESY (Nuclear Overhauser Effect spectroscopy) and 2D TOCSY (Total correlation spectroscopy) for peptide data collection. Different parameters including temperature, buffer and protein

concentration were optimised before making final measurements and 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 0 ppm using the methyl signal of DSS (2, 2-dimethyl-2-silapentane-5-sulphonate (Cambridge Isotope Laboratories) as an external reference.

2.11.3 Non-Uniform Sampling (NUS) NMR spectroscopy and titration

NMR experiments were performed at 293 K on a 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. The uniformly labeled *EcAhpF*₁₋₂₁₂ was concentrated to 0.4 mM in 50 mM phosphate buffer at pH 6.8, 50 mM NaCl, 5 mM EDTA and 5% D_2O . To assign the backbone of *EcAhpF*₁₋₂₁₂, both uniform (traditional) and non-uniform (NUS; [115]) sampling techniques were employed to acquire the data. 2D- ^1H - ^{15}N HSQC, 3D-HNCACB, 3D-CBCACONH were collected with the linear sampling technique, whereas 3D-HNCO, 3D-HNCA and 3D-HN(CO)CA data were collected using non-uniform sampling techniques. Traditional NMR-spectra were processed using NMRpipe [116] and analyzed with the SPARKY program [117]. NUS-data points were collected at 10% of the uniform data points. The NUS schedules were generated and integrated into the pulse program by using nusPGSv3 [118, 119] where data point selection is based on Poisson Gap generation. The data were finally processed by applying the iterative soft thresholding (hmsIST) together with NMRpipe [118, 119]. Both techniques enabled the assignment of 90% of the backbone resonances.

Titration of 0.3 mM of labeled *EcAhpF*₁₋₂₁₂ with oxidized *EcAhpC*₁₋₁₈₇ and the truncated *EcAhpC*₁₋₁₇₂ was performed against the latter two to achieve incremental molar equivalents of 0.08, 0.1, 0.3, 0.5 and 1. To calculate the combined chemical shift, the intensity information was extracted after integration using Gaussian fit method in the SPARKY software package.

2.12 Crystallisation of *E.coli* AhpC₁₋₁₈₂

Recombinant *E.coli* AhpC with concentration of 10 mg/ml was used to set up crystals drops in Hampton Research crystal screens HR-110 and HR-112 and Emerald biosystems Wizard screen 1 and Wizard screen 2, in hanging drop plates with 2 μl

droplet size per well in 1:1 μ l ratio of AhpC in buffer B (50 mM Tris-HCl, pH 7.5, 200 mM NaCl) and precipitant solution at 18 °C. Initial needle bundles were observed in Hampton research crystal screen 2 #25 (HR2#25) after two days and in Hampton research crystal screen 2 #23 (HR2#23), small crystal formation after 14 days. Both conditions were similar in their composition: HR2#25 contains 1.8 M ammonium sulphate, 100 mM MES (2- (*N*-morpholino) ethane sulfonic acid), pH 6.5, and 10 mM cobalt chloride, whereas HR2#23 includes 1.6 M ammonium sulphate, 100 mM MES, pH 6.5, and 10 % dioxane. Both conditions were optimised simultaneously. The ammonium sulphate concentration, protein concentration, dioxane concentration, pH, and temperature were optimised to improve the growth and diffraction quality of AhpC crystals. The first larger size crystals that showed reflections in the diffraction image using the in-house X-ray source (Rigaku) were observed under the condition 1.6 M ammonium sulphate, 100 mM MES, pH 6.5, and 5 % dioxane using 8 mg/ml protein concentration.

The diffraction quality of *Ec*AhpC crystals was 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/MSO). All the diffraction data were indexed, integrated and scaled using the HKL2000 suite program under the supervision of Dr. Kamariah Neelegandan [89].

2.13 Data collection and structure determination

Together with Dr. Kamariah Neelegandan, single-wavelength datasets for *E. coli* AhpC crystals were collected at 140 K on beamline 13B1 at the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan) using ADSC Quantum 315 CCD detector. The staff at beamline 13B1 (NSRRC) kindly provided technical assistance. All the diffraction data were indexed, integrated and scaled using the HKL2000 suite package [89]. All the crystals belong to space group C2 and have similar unit cell parameters.

Single wavelength datasets of AhpC from *E. coli* were also collected at the protein crystallography beamline S06 PX at the Swiss Light Source (SLS) with a PILATUS 6M detector by Dr. Neelagandan Kamariah. Data sets were collected as a series of 0.2° oscillation images with a 0.2 s exposure time with a detector distance of 500 mm. All diffraction data reduction, including indexing, integration, and scaling were carried out with the iMosflm program [120].

Initial phases for AhpC structure determination from *E. coli* were obtained by molecular replacement method using the oxidised *EcAhpC* (PDB ID: 4O5Q) [121] using the program PHASER [92]. The Chainsaw software was used for model editing (Stein, 2008). Rigid-body refinement was carried out followed by difference Fourier syntheses calculations. Inspection of the $F_O - F_C$ and $2F_O - F_C$ maps reveal no clashes between the monomeric chains. The preliminary AhpC structure from *EcAhpC* clearly showed electron density corresponding to additional amino acids. Since the crystal structure of all five *StAhpC* monomer chains in an asymmetric unit consist of 162 amino acids, amino acids were manually added to the N- and C-terminus of the *EcAhpC* structure during iterative refinement and model building using the software COOT [94] and Refmac5 [90]. The final density of 167 amino acids from the 187 amino acids full length was observed in *EcAhpC*. The missing 20 amino acids located at the C-terminal segment reflects a highly flexible part as evident by the high B-factors $>70 \text{ \AA}^2$ for C_α atoms from C-terminal residue 163-167. The final geometry of the *E. coli* AhpC structure was checked with PROCHECK [96] and figures was created using the program PyMOL [88]. Structural comparison analyses were carried out using the SUPERPOSE program [122] as included in the CCP4 suite.

3. Results

3.1 Alkyl hydroperoxide Reductase subunit C (AhpC) C- terminal mutants and crystal structure of *EcAhpC*₁₋₁₈₂

3.1.1 Cloning, production and purification of oxidized AhpC C-terminal mutants from *E. coli*

Figure 3.1: PCR gene product amplification of *EcAhpC*₁₋₁₈₅ (555 bp) (lane 2). PCR products of *EcAhpC*₁₋₁₈₂ (546 bp) (lane 3) and *EcAhpC*₁₋₁₇₇ (531 bp) (lane 4). Lane 1 and lane 5 are loaded with 100 bp and 1 kb markers respectively. DNA sample of 5 μ l was loaded on an agarose gel (1.2%).

In the eukaryotic homologue of bacterial AhpC, the C-terminus has been found to be hypersensitive to high levels of peroxides hence it has been implicated in peroxide signalling [69]. However, such phenomenon is absent in the robust and highly efficient bacterial AhpC. In order to ascertain the role of the bacterial AhpC, C-terminal truncations have been generated. The *EcAhpC*₁₋₁₇₇, *EcAhpC*₁₋₁₈₂, and *EcAhpC*₁₋₁₈₅ gene products (Figure 3.1) were cloned into the pET9d-6His plasmid. Induction tests (Figure 3.2) and solubility tests were performed to

establish the presence and fidelity of the induced proteins. The single amino acid truncate and mutants (*EcAhpC*₁₋₁₈₆, *EcAhpC*_{K186A}, and *EcAhpC*_{I187G}) were generated by Claudia Beushausen from our laboratory. The next step was to express the cloned genes and purify the recombinant proteins in either the reduced or oxidized state.

Figure 3.2: SDS-PAGE showing the expression of the recombinant *EcAhpC*₁₋₁₈₅ (A), *EcAhpC*₁₋₁₈₂ and (B), *EcAhpC*₁₋₁₇₇ (C)). Proteins were induced with IPTG to a final concentration of 1 mM. The + and – signs indicate the presence and absence of IPTG, respectively. It can be observed that even in the absence of IPTG some amount of protein is produced.

To purify the protein in the oxidized state, steps were taken to avoid any reducing agent in the next step. During the Ni^{2+} -NTA step, the beads bound with the

protein were initially washed with 10 column volumes of buffers without DTT and subsequently eluted with an imidazole gradient (from 0 to 500 mM). Buffer A which contains 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM PMSF and 2 mM Pefabloc^{SC} (BIOMOL), was used for the purification. Afterwards, ion-exchange chromatography was carried out using ResourceTM Q column (GE Healthcare) to clear impurities that eluted with the target protein. The proteins were collected from the Resource Q column and were concentrated. Further purification using an S75 Superdex column were performed yielding pure proteins as shown in SDS gel section below.

Figure 3.3: Superdex S75 purification chromatograms of *EcAhpC* C-terminal truncations. (A) *EcAhpC*₁₋₁₈₅ (B) *EcAhpC*₁₋₁₈₂ and (C) *EcAhpC*₁₋₁₇₇. The inset shows a 17% SDS-PAGE gel of the final protein products from S75 Superdex column.

3.1.2 Dynamic light scattering of C-terminal mutants of *E. coli* AhpC

Oxidized AhpC exists as a mixture of oligomers, whilst reduced AhpC exists as a decamer [75]. In order to examine the effect of C-terminal mutations on the oligomerization of the reduced AhpC, DLS experiments were performed on the generated truncates using the Malvern Zetasizer Nano ZS spectrophotometer as described in section 2.6. After 60 s equilibration time, the backscattering at 173° was

detected for full-length *EcAhpC* revealing a diameter of 11.1 nm and an average molecular weight of 223 kDa $\pm$ 48 kDa, which is in line with a decamer configuration. However, the scattering for the *EcAhpC*₁₋₁₇₂ reveals a diameter of 6.06 nm with an average M.W of 38 $\pm$ 6 kDa, consistent with a dimeric unit of AhpC.

Figure 3.4: Dynamic light scattering experiments of C-terminally truncated *E. coli* AhpC. Under reducing condition, the full-length *EcAhpC* exists as a decamer shown in all figures as dashed curves. Plotted against (A) *EcAhpC*₁₋₁₇₂ (B) *EcAhpC*₁₋₁₇₇ (C) *EcAhpC*₁₋₁₈₂ (D) *EcAhpC*₁₋₁₈₅ (E) *EcAhpC*₁₋₁₈₆. In all constructs, there is a breakdown of the decamer assembly with *EcAhpC*₁₋₁₇₂ having the highest breakdown and *EcAhpC*₁₋₁₈₆ having the least.

Similarly, *EcAhpC*₁₋₁₇₇ (Figure 3.4B) and *EcAhpC*₁₋₁₈₂ (Figure 3.4C) reveal a dimer configuration with diameters of 6.5 nm and 6.1 nm and average molecular weights of 47 kDa $\pm$ 8 kDa and 41.9 kDa $\pm$ 6 kDa, respectively. Even though, *EcAhpC*₁₋₁₈₅

(Figure 3.4D) and *EcAhpC*₁₋₁₈₆ (Figure 3.4E) truncations destabilized the decameric ring of the reduced AhpC, they formed tetramers, with average molecular weights of $80.2 \text{ kDa} \pm 14.9 \text{ kDa}$ and $99.6 \pm 19.6 \text{ kDa}$, respectively, rather than dimers. Altogether, this reveals a direct relationship between the C-terminus and oligomerization.

3.1.3 NADH-dependent peroxidase activity assay of AhpC mutants from *E. coli*

In order to examine the effect of mutations of the C-terminus of *EcAhpC* on overall enzymatic activity, an NADH-dependent assay was performed where the decrease in NADH absorbance is monitored at 340 nm. Due to the ability of NADH to differentially absorb at this wavelength (340 nm), the transfer of electrons between the NADH via FAD and the C345/C348 catalytic centres at the AhpF C-terminal domain results in the decrease in NADH absorbance at 340 nm. In the presence of a viable AhpC, this reduction is maximum after 6 mins (Figure 3.5) [50]. During peroxidase activity; electrons are transferred from the C-terminal catalytic centre of AhpF,

Figure 3.5: The NADH dependent peroxidase activity was measured in the presence of *EcAhpC*, *EcAhpF* and H_2O_2 . Catalytic activity in presence of full length *EcAhpC* (red line) and the absence of *EcAhpC* (black line) served as a control. The data demonstrate that the NADH oxidase activity was saturated at 6 min.

C345/C348 through its N-terminal catalytic centre C129/C132 and subsequently to the AhpC catalytic centre, C44/C166. To demonstrate the conversion of NADH to NAD^+ , different wavelengths were scanned from 280-540 nm at two-time points, 0 mins and an optimised time point where NADH usage is

maximum. The assay was carried out as described in section 2.7. To calculate the second time point, measurements were taken in the presence and absence of *EcAhpC*₁₋₁₈₇. In the presence of *EcAhpC*₁₋₁₈₇, the absorbance dropped significantly and reached saturation after 6 minutes of the reaction (Figure 3.5). The result clearly indicated that for H_2O_2 reduction and maximum NADH-oxidation, both *EcAhpF* and *EcAhpC* are required. In order to compare the catalytic activity of different truncated *EcAhpC* proteins with the full-length *EcAhpC* protein, the NADH-oxidation was measured

Figure 3.6: The NADH-dependent peroxidase activity of truncated proteins *EcAhpC*₁₋₁₇₇ (green), *EcAhpC*₁₋₁₈₂ (magenta) and *EcAhpC*₁₋₁₈₅ (blue) compared to full length *EcAhpC* (red) and *EcAhpC*₁₋₁₇₂ (purple), were measured at 0 min (continuous line) and 6 min (dotted line), respectively. Maximum catalytic activity was observed only in the presence of full-length *EcAhpC*.

Figure 3.7: The NADH-dependent peroxidase activity measured for *EcAhpC* and the mutant proteins *EcAhpC*_{K186A} and *EcAhpC*_{I187G} were observed in a range of 280-540 nm at 0 min and 6 min. The data presented are average of three independent measurements, and error bars represent the standard deviation.

directly after incubation as well as after saturation (6 min) (Figure 3.6). Maximum catalytic activity was observed in the presence of *EcAhpC*, indicating an active AhpR complex formation. However, the spectra of the truncated *EcAhpC*₁₋₁₇₂, *EcAhpC*₁₋₁₇₇, *EcAhpC*₁₋₁₈₂ and *EcAhpC*₁₋₁₈₅ proteins resembled that of *EcAhpF* alone (control), reflecting that no active AhpR complex was assembled (Figure 3.6). In order to

investigate the effect of the last two residues (K186 and I187) residues of the extreme C-terminus of *EcAhpC* on enzymatic activity, the *EcAhpC*_{1187G} mutant, as well as the *EcAhpCK186A* mutant, were generated and studied. Similar to the truncated *EcAhpC* proteins, substituting the very C-terminal residues K186 and I187 of *EcAhpC* resulted in an enzymatically non-active AhpR complex (Figure. 3.7).

3.1.4 Crystallisation, data collection and structure determination of *EcAhpC*

The available wild-type structure of the *EcAhpC* lacks information beyond the cysteine disulphide bond (C166). This is due to the high flexibility of the C-terminal tail [50]. By truncating the C-terminus, the flexibility is likely to decrease and hence enable the crystallization of some C-terminal residues. Crystallisation techniques have been performed on all three mutant proteins *EcAhpC*₁₋₁₇₇, *EcAhpC*₁₋₁₈₂ and *EcAhpC*₁₋₁₈₅. Microcrystals were observed in 1.8 M ammonium sulfate, 100 mM MES, pH 6.5. These microcrystals were then seeded into similar crystal screen conditions. Larger crystals were observed within 3 days of microseeding (Figures. 3.8, 3.9 and 3.10). Crystals were examined in-house and the best diffracting crystal was *EcAhpC*₁₋₁₈₂ (Figure 3.9).

*EcAhpC*₁₋₁₈₂ crystals were then used for data collection. The crystals were quickly soaked into cryoprotectant solution containing 25% (w/v) glycerol and flash-cooled in liquid nitrogen at 100 K. With the assistance of Dr. Neelagandan Kamariah from our laboratory, single wavelength datasets for *EcAhpC*₁₋₁₈₂ were collected at 140 K on beamline 13B1 at the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan) using a single frozen crystal of *EcAhpC*₁₋₁₈₂. Using the ADSC

Figure 3.8: Representative pictures of (A) *EcAhpC*₁₋₁₈₅ crystals were observed in 0.1 M MES pH 6.5, 1.8 M ammonium sulphate, pH 6.5 in a hanging drop. Protein concentration was 8 mg/ml and (B) a zoomed-in figure of (A).

Figure 3.9: Representative pictures of (A) *EcAhpC*₁₋₁₈₂ crystals were observed in 0.1 M MES pH 6.5, 1.8 M ammonium sulphate, pH 6.5 in a hanging drop. Protein concentration was 8 mg/ml and (B) a zoomed-in figure of (A).

Figure 3.10: Representative pictures of (A) *EcAhpC*₁₋₁₇₇ crystals were observed in 0.1 M MES pH 6.5, 1.8 M ammonium sulphate, pH 6.5 in a hanging drop. Protein concentration was 8 mg/ml and (B) a zoomed-in figure of (A).

Figure 3.11: Representative diffraction image of *EcAhpC*₁₋₁₈₂.

Quantum 315 CCD detector, 100° of data were collected with 0.5° oscillation (20 s per 0.5° frame).

Data sets were collected as a series of 0.2° oscillation images with 0.2 s exposure time at a detector distance of 500 mm. The representative diffraction pattern of the *EcAhpC*₁₋₁₈₂ crystal is presented in figure 3.11. All diffraction data reduction, including indexing, integration and scaling were carried out in the iMosflm program [120]. The results of data processing and data statistics for *EcAhpC*₁₋₁₈₂ are summarized in Table 3.1. *EcAhpC*₁₋₁₈₂ was crystallised in a trigonal system with the space group P3₁21 with unit cell parameters $a = 136.91$, $b = 136.91$ and $c = 147.81$ Å. The crystals diffracted to 3.4 Å resolution. Assuming five molecules in the asymmetric unit, the solvent content of the AhpC crystal is 68.0 % with a Matthews coefficient (V_m) of 3.8 Å³ per Dalton. The log-likelihood gain (LLG) of 3436 and Z-score for the fast rotational function (RFZ) of 13.8 and for the fast translational function (TFZ) of 114.1 in Phaser, indicating a good solution. To improve the electron density by solvent flattening and to reduce model bias that may occur in a crystal structure below 3 Å, the program RESOLVE was used for prime and switch phasing. The preliminary structure was subsequently used for 30 cycles of rigid-body refinement using the program Refmac5 [90], obtaining an overall R-factor of 21.39 % and free R-factor of 23.51 % (Table 3.1). The initial structure reflects five molecules in one asymmetric unit, where each molecule consisted of 162 amino acids. The overlay of $F_O - F_C$ and $2F_O - F_C$ maps (Figure 3.12) of *EcAhpC* shows green electron density to amino acids at the C-terminus. Iterative cycles of model building and refinement were performed in a combined manner using the programs COOT [94] and REFMAC5 [90] of the CCP4 suite (1994). Each cycle of the model building allowed the addition of amino acid to its corresponding green density of the C-terminal region. All data are summarized in Table 3.1.

3.1.5 Crystal structure of AhpC₁₈₂ from *E. coli*

The crystal structure of *EcAhpC*₁₋₁₈₂ was solved at 3.4 Å resolution and revealed five molecules (A-E) in the asymmetric unit that form a half ring shape conformation (Figure 3.13). The full ring, decamer structure is generated by its crystallographic two-fold symmetry operation ($A'E'$). The entire ring complex consists of five homodimers (α_2)₅, with an outer diameter of 123.7 Å x 115.4 Å calculated using MOLEMAN2 [125] and an inner diameter of 55 Å and 60 Å measured between opposite molecules A/E' and C/C' using Pymol [88]. The thickness of the ring complex corresponds to a monomer molecule and is 50 Å. The *E. coli* AhpC₁₋₁₈₂ structure shows clear electron density for all the main chain atoms except for the C-

terminus residues beyond P166, which is highly disordered. The molecules A, C and D showed clear and continuous electron density up to P167, whereas in molecule B it is

Figure 3.12: *Top:* The 2Fo-Fc density map around the C_P disulphide is revealed and bonded with C_R contoured at 1 σ . *Below* omit map demonstrating the presence of P167

up to C166 and in molecule E up to V165. Each AhpC monomer consists of seven β -sheets located in the centre of the molecule, flanked at one side by four and the other side by two α -helices. Furthermore, each monomer contains two cysteine residues namely the peroxidative cysteine (C_P47) and the resolving cysteine (C_R166). In this *EcAhpC*₁₋₁₈₂ structure, electron densities are clearly visible up to C_R166 in A, B, C and D chains. The residues after C_R166 are mostly disordered in all the chains. The monomer molecules in each dimer are related by a two-fold symmetry and no significant structural differences were observed between each monomer in a dimer structure (Figure 3.14). The dimer structure reflects an oval shape with dimensions of 67.2 Å x 47.9 Å x 36.6 Å, calculated using MOLEMAN2 (Figure 3.14) [125]. The two molecules of each dimer are related

to a two-fold symmetry and are tightly connected to each other. The dimer interface is formed between β 7 and β 7' of each monomer. The β 7 and β 7' interface is stabilised by hydrogen bonds between the residues D119, Q132, I134, E135, V136, A138, R150, and G142 (Figure 3.14). The active site is composed of the intermolecular disulphide bond between the C_P47 from one monomer with C_R166 from another monomer, interacting in a head to tail manner (Figure 3.14).

Figure 3.13: Crystal structure of oxidised AhpC₁₋₁₈₂ from *E. coli*. Decamer (α_2)₅ of AhpC. Each asymmetric unit consists of five molecules (A-E), depicted in different colour and its crystallographic symmetry mates, depicted with an apostrophe (A'-E'). Dimer formations are between A-B, C-D, and E-E'.

Figure 3.14: Dimer structure of oxidised *EcAhpC*₁₋₁₈₂ between the molecules A (brown) and B (yellow). The redox active cysteines (C47/C166' and C47'/C166) are depicted green. The dimer formation between chains A and B is also stabilised along both β 7-sheets with hydrogen bonds, highlighted in arrows.

Figure 3.15: Oligomer interface of *EcAhpC*₁₋₁₈₂. The dimer-dimer interface between chain B (*brown*) and chain C (*cyan*) reflects no hydrogen bond or salt bridges, rather patches of hydrophobic residues formed by F21, F43, F45, F77, A81 and W82 of each monomer.

The two intermolecular redox-active disulphide bonds formed by C47 and C166' contribute additional stabilisation to the dimer interface (Figure 3.14). C_p47 is located close to the N-terminus of α 2-helix and is part of the so-called C_p-loop. In the oxidised form of *EcAhpC*₁₋₁₈₂, the C_p is unwound to form a flexible loop, which exposes C_p47 to the resolving cysteine C_R166'. C_R166' is located at the very C-terminus of each chain of the crystal structure and is partially exposed. The oligomeric assembly at the dimer-dimer interfaces for example between chain B and chain C are formed by hydrophobic residues F21, A41, F43, F45, F77, A81, W82, P100, G116 and L117. This interface consists of hydrophobic interactions (Figure 3.15).

Table 3.1: Statistics of crystallographic data collection and refinement for EcAhpC₁₋₁₈₂

Data statistics	EcAhpC ₁₋₁₈₂
Wavelength (Å)	1.000
Crystal-to-detector distance (mm)	500
Rotation range per image (°)	0.2
Total rotation range (°)	100
Exposure time per image (s)	0.2
Space group	P3 ₁ 21
Unit cell parameters (Å,°)	
<i>a</i> =	136.91
<i>b</i> =	136.91
<i>c</i> =	147.81
γ =	120
Molecules in asymmetric unit	5
Solvent content (%)	68.0
Resolution limits (Å)	30.0-3.40 (3.52-3.40)
No. of reflections	195044
Unique reflections	21757
Multiplicity	2.3 (2.1)
Completeness (%)	99.8 (100.0)
R _{merge} (%)	10.0 (49.0)
<I//s(I)>	10.2 (2.5)
Refinement statistics	
R-factor (%)	21.39 (27.59)
R-free (%)	23.51 (33.37)
Ramachandran statistics	
Favoured region (%)	96.9
Allowed region (%)	2.2
Outlier region	0.9
R.M.S. deviations	
Bond lengths (Å)	0.007
Bond angles (°)	0.71
Overall B values (Å ²)	
Mean B value	55.2

^a R-factor = $\sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o and F_c are measured and calculated structure factors, respectively. ^b R-free = $\sum ||F_o| - |F_c|| / \sum |F_o|$, calculated from 5% of the reflections selected randomly and omitted from the refinement process.

3.2 Solution shapes of *EcAhpF*₁₋₁₉₆, *EcAhpF*₁₋₂₁₂ and *EcAhpF*₂₁₂₋₅₂₁ and interaction studies with *EcAhpC*

Solution shapes of *EcAhpF*₁₋₁₉₆, *EcAhpF*₁₋₂₁₂ and *EcAhpF*₂₁₂₋₅₂₁ and interaction studies with *EcAhpC*

3.2.1 Production of *EcAhpF*₁₋₁₉₆ and *EcAhpF*₁₋₂₁₂

It has been proposed that *EcAhpF* interacts with *EcAhpC* via the N-terminal domain of the former [81]. In an attempt to understand the mechanism of binding of *EcAhpF* to *EcAhpC*, the three constructs, *EcAhpF*₁₋₁₉₆ (NTD), *EcAhpF*₁₋₂₁₂ (NTD + linker) and *EcAhpF*₂₁₂₋₅₂₁ (CTD) of *EcAhpF* were generated (Figure 3.16), cloned and purified as described in section 2.3. Briefly, the genes of these constructs were amplified by PCR from the *E. coli* genomic DNA. The PCR products were successfully inserted into the pET9b vector [83] which were then transferred to the BL 21 *E. coli* (DE3) expression strain as described in section 2.3. Induction tests were performed to ensure the presence of the protein after which solubility tests were performed. The

Figure 3.16: PCR gene product amplification of *EcAhpF*₁₋₁₉₆ (600 bp) (lane 2). PCR products of *EcAhpF*₁₋₂₁₂ (636 bp) (lane 3). Lane 1 is loaded with 1 kb marker. DNA sample of 5 μ l was loaded on an agarose gel (1.2%).

solubility test confirms highly soluble proteins of ~22 kDa and 24 kDa of *EcAhpF*₁₋₁₉₆ and *EcAhpF*₁₋₂₁₂, respectively. With the use of the 6 x His-tag on the protein, the expressed proteins were purified by Ni-NTA affinity column purification (Figures 3.17A and B). Both proteins eluted in the 50-200 mM imidazole fractions. To ensure a highly homogenous protein sample, only fractions 75-150 mM were pooled together, diluted, filtered and applied to a Resource Q column (6 ml). The proteins of interest eluted in the flow-through and was then concentrated and finally applied to the S75 Superdex purification step (Figure 3.17C and D). The high purity of the purified protein permitted that SAXS experiments were performed on the individual mutants to ascertain their structural fidelity and solution behaviour. All SAXS data were collected with the assistance of Dr. N. Kamariah and data analysis was also assisted by Dr. M.S. Subramanian from our laboratory. Samples were prepared as described in section 2.3.

3.2.2 Solution shapes of *EcAhpF*₁₋₁₉₆ and *EcAhpF*₁₋₂₁₂

The solution shapes of the N-terminal domain of *E. coli* AhpF without the linker (*EcAhpF*₁₋₁₉₆) and with the linker region, which connects the N-terminal and the C-terminal domains of *E. coli* AhpF, *EcAhpF*₁₋₂₁₂, were studied by SAXS experiments.

Figure 3.17: Purification of *EcAhpF*₁₋₁₉₆ and *EcAhpF*₁₋₂₁₂ (A) The purification was performed in buffer 50 mM Tris-HCl, pH 7.5, 200 mM NaCl using an imidazole step gradient (7 – 250 mM) for elution of Ni²⁺-NTA bound protein. Fractions from 75 – 150 mM imidazole were pooled together for further purification steps. (B) Similar treatment for *EcAhpF*₁₋₂₁₂ (C) *EcAhpF*₁₋₁₉₆ eluted as a single peak in a Superdex S75 (Inset: SDS-PAGE of the selected peaks in the graph) (D) S75 Superdex profile of *EcAhpF*₁₋₂₁₂.

SAXS patterns of both proteins were recorded at protein concentrations of 2.0, 5.8 and 8.5 mg/ml, which showed similar final composite scattering curves (Figure 3.18A and 3.21A). The scattering plot of *EcAhpF*₁₋₂₁₂ is displayed for both merged (Figure 3.21C) and unmerged (Figure 3.21A) data to enable better assessment of any concentration-dependent effect. The Guinier plots at low angles for both *EcAhpF*₁₋₁₉₆ (Figure 3.18B) and *EcAhpF*₁₋₂₁₂ (Figure 3.21A *inset*) are linear and revealed good data quality with no indication of protein aggregation. Furthermore, the radius of gyration (R_g) values from the Guinier approximation for both *EcAhpF*₁₋₂₁₂ and *EcAhpF*₁₋₁₉₆ were consistent at the three concentrations measured with a value of 17.4 ± 0.5 Å and 15.8 ± 0.03 ,

respectively. Again, the distance distribution functions ($p(r)$) were similarly shaped for the concentrations used for *EcAhpF*₁₋₁₉₆ (Figure 3.19A) and *EcAhpF*₁₋₂₁₂ (Figure 3.21B) and the maximum particle dimensions were calculated as $D_{max} = 50.4 \pm 0.1 \text{ \AA}$ and $D_{max} = 60.3 \pm 1.2 \text{ \AA}$, which compared well with the crystal structure (PDB ID: 4O5Q) [67] maximum dimension of 45.4 $\text{\AA}$ and 59.6 $\text{\AA}$, respectively. The theoretical scattering curve computed for the atomic structure of *EcAhpF*₁₋₁₉₆ and *EcAhpF*₁₋₂₁₂ [67] fits well with the experimental scattering curve with a χ^2 -value of 1.2 for the merged data of *EcAhpF*₁₋₂₁₂ (Figure 3.21C), whilst the χ^2 -value for *EcAhpF*₁₋₁₉₆ was 1.12, indicating in both cases that the recombinant proteins retain their overall structure like in the entire *EcAhpF*. To examine the flexibilities of the proteins, normalized Kratky plots were prepared for both proteins. As observed in Figure 3.19B and Figure 3.21B (inset), both proteins are generally globular with limited flexibilities, with the *EcAhpF*₁₋₁₉₆ having the least flexibility (Figure 3.19B). *Ab initio* reconstruction of the gross shapes of *EcAhpF*₁₋₁₉₆ and

Figure 3.18: (A) Scattering curve of *EcAhpF*₁₋₁₉₆ and (B) The Guinier plot of the protein displaying linearity at a low angle. The red and green lines represent the scattering curves for the concentrations 5.8 mg/ml and 8.5 mg/ml.

Figure 3.19: Small-angle X-ray scattering analysis of the *EcAhpF*₁₋₁₉₆ (A) Distance distribution function of *EcAhpF*₁₋₁₉₆ (B) Kratky plot for *EcAhpF*₁₋₂₁₂ (orange) in comparison with lysozyme (cyan); the insignificant difference indicates limited flexibility for *EcAhpF*₁₋₁₉₆.

Figure 3.20: The solution shape of *EcAhpF*₁₋₁₉₆ calculated by GASBOR program is revealed in different orientations with the superimposed crystal structure (PDB ID: 4O5Q) [67].

*EcAhpF*₁₋₂₁₂ were performed and they had a good fit to the experimental data in the entire scattering range with discrepancies of $\chi^2 = 1.12$ and $\chi^2 = 1.2$, respectively. Ten independent reconstructions produced similar envelopes with normalized spatial discrepancy (NSD) of 0.73 ± 0.01 for *EcAhpF*₁₋₂₁₂ and 0.81 ± 0.03 for *EcAhpF*₁₋₁₉₆ with the average structures shown in Figures 3.21D and 3.20, respectively. The *EcAhpF*₁₋₁₉₆ shape reveals the globular domain with dimensions of 37.0×49.6 Å and the shape of *EcAhpF*₁₋₂₁₂ reveal the globular domain with an extended tail with dimensions of 37.0×62.7 Å. The globular part of *EcAhpF*₁₋₂₁₂ overlays nicely with the *EcAhpF*₁₋₁₉₆ shape (Figure 3.22) and the extended tail represents the linker region including amino acids 197 to 212. The known atomic structure of *EcAhpF*₁₋₁₉₆ and *EcAhpF*₁₋₂₁₂ from the entire *EcAhpF* (PDB ID: 4O5Q) [67] compares well with respective solution structures with an RMSDs of 0.77 Å and 0.9 Å, respectively, demonstrating a high-quality match between the solution and crystal structure. All SAXS-data statistics are shown in Table 3.3.

3.2.3 Solution shape of *EcAhpF*₂₁₂₋₅₂₁ (CTD)

From the literature, it is known that *EcAhpF* is a functional dimer [126]. But so far the only evidence of the dimer formation has been from crystal structures [82]. Due to the dynamics of the AhpF [67], it is difficult to capture the intact dimer in solution. This has raised concerns about the physiological meaningfulness of the crystallographic dimer. From

Figure 3.21: Solution X-ray scattering studies of *EcAhpF*₁₋₂₁₂. (A) Small angle X-ray scattering pattern (○) and its corresponding experimental fitting curves (—; black: experimental, gray: calculated) for the concentrations 2.0 (red), 5.8 (green) and 8.5 (blue) mg/ml. The scattering curves of *EcAhpF*₁₋₂₁₂ are displayed in logarithmic units for clarity. (inset) Guinier plots show linearity for the concentrations used, indicating no aggregation. (B) Pair-distance distribution function $P(r)$ of *EcAhpF*₁₋₂₁₂ at 2.0 (red), 5.8 (green) and 8.5 (blue) mg/ml. (inset) Normalized Kratky plot of *EcAhpF*₁₋₂₁₂ SAXS-data at 8.5 mg/ml (red) and the standard lysozyme data at 5 mg/ml (blue). The scattering pattern of *EcAhpF*₁₋₂₁₂ exhibits a bell-shaped profile with a maximum value of 1.23 for $sR_g=1.96$, which is shifting slightly right with respect to the normal value of 1.104 for $sR_g=\sqrt{3}$ for globular proteins, indicating a small asymmetry and/or flexibility of the protein. (C) The fit of the scattering profile of the crystal structure of *EcAhpF*₁₋₂₁₂ with the best χ -value of 1.2 (red) to the experimental scattering pattern of *EcAhpF*₁₋₂₁₂ of the merged data. (D) Superposition of the solution shape with the crystal structure of *EcAhpF*₁₋₂₁₂ from the entire *EcAhpF* (entry 4O5Q) [67].

the knowledge that the CTD of *EcAhpF* is the dimerization domain, SAXS experiments were performed with *EcAhpF*₂₁₂₋₅₂₁. All SAXS-data are shown in Table 2.3. The scattering curves (Figure 3.23A) and the linearity of the Guinier plots (Figure 3.23B) at low angles indicate a good data quality and no protein aggregation. From the distance distribution curve (Figure 3.24A), the maximum dimension, D_{max} for *EcAhpF*₂₁₂₋₅₂₁ was calculated to be 86.8 ± 0.1 Å. This is consistent with the crystal structure [67] maximum dimension of 96.2 Å. The low-resolution shape of *EcAhpF*₂₁₂₋₅₂₁ had a good fit to the experimental data with the entire scattering range and has a discrepancy of $\chi^2 = 1.42$. The *EcAhpF*₂₁₂₋₅₂₁ shape without the linker (Figure 3.25)

reveals the first head to tail dimeric form of with the overall dimension of 62.2 x 89.5 Å. This first solution dimer confirms that the crystallographic dimer is not an artefact and that the dimerization of the AhpF may be physiologically important.

Figure 3.22: Averaged *ab initio* model *EcAhpF*₁₋₁₉₆ (cream) is docked into the averaged *ab initio* model of *EcAhpF*₁₋₂₁₂. The docked structures are revealed in different orientations.

Figure 3.23: (A) Scattering curve of *EcAhF*₂₁₂₋₅₂₁ and (B) The Guinier plot for *EcAhF*₂₁₂₋₅₂₁ displaying linearity at low angle.

Figure 3.24: Small-angle X-ray scattering analysis of the *EcAhpF*₂₁₂₋₅₂₁ (A) Distance distribution function of *EcAhpF*₂₁₂₋₅₂₁ (B) Kratky plot for *EcAhpF*₂₁₂₋₅₂₁ (brown) in comparison with lysozyme (cyan); the insignificant difference indicates limited flexibility for *EcAhpF*₂₁₂₋₅₂₁.

Figure 3.25: The solution shape of *EcAhpF*₂₁₂₋₅₂₁ calculated by the GASBOR program is revealed in different orientations with the superimposed crystal structure (PDB ID: 4O5Q) [67].

3.2.4 *EcAhpC* binds to the N-terminal domain of *EcAhpF*

Although the NTD of bacterial AhpF has been proposed to bind AhpC, no attempts were made to characterize this interaction. Here isothermal titration calorimetry (ITC) was used to demonstrate that *EcAhpC* interacts via the NTD of AhpF and to determine the binding constant of the interaction. Firstly, the recombinant

Figure 3.26: Affinity measurement using Isothermal titration calorimetry. Representative ITC profiles are shown for (A) *EcAhpF*₂₁₂₋₅₂₁ and (B) *EcAhpF*₁₋₁₉₆ with *EcAhpC*. The top panel in the figures shows the injection profile after baseline correction and the bottom panel shows the integration (heat release) for each injection. (C) Binding signature of the interaction of *EcAhpF*₁₋₁₉₆ with *EcAhpC*. 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 entropic driven.

E. coli proteins AhpF₁₋₁₉₆ and AhpF₂₁₂₋₅₂₁ were produced as described earlier in this chapter, including the residues 1-196 of the N-terminal domain and amino acids 212-521 of the C-terminal domain of *EcAhpF*. As shown in the ITC experiments (Figure 3.26A), no interaction of *EcAhpF*₂₁₂₋₅₂₁ with *EcAhpC* could be observed in the injection profile of *EcAhpF*₂₁₂₋₅₂₁ after baseline correction (top panel) with the profile of heat release/mole of injected subunits (bottom panel). In contrast, after titration of *EcAhpF*₁₋₁₉₆ with *EcAhpC*, an overall positive heat can be detected, indicating an endothermic reaction (Figure 3.26B). The free energy of the reaction (ΔG) is a function of both the enthalpic contribution (ΔH) and entropic contribution ($-T\Delta S$).

From the binding signature of the interaction (Figure 3.26C), it is clear that this reaction is mainly entropic driven since the free energy comes mainly from (-TΔS). By using a single site model equation, the binding isotherm could be fitted nicely and reflects an equimolar binding of *EcAhpF*-NTD with *EcAhpC*. The dissociation constant (K_d) of about 3.2 μM indicates a moderate binding between these two proteins. Table 3.2 shows the binding parameters of the interaction

Table 3.2 Binding affinity measurements for *EcAhpF*₂₁₂₋₅₂₁ and *EcAhpC* using isothermal titration calorimetry.

	K_a ($\times 10^6 \text{ M}^{-1}$)	K_d (μM)	ΔH (kcal/mol)	TΔS (kcal/mol)	ΔG (kcal/mol)
<i>EcAhpF</i> ₂₁₂₋₅₂₁ + <i>EcAhpC</i>	0.31 ± 0.09	3.2	2.86 ± 0.11	10.3	- 7.50

Table 3.3: Data collection and scattering derived parameters for subunit *EcAhpF* construct using SAXS

Data collection parameters	<i>EcAhpF</i> ₁₋₁₉₆	<i>EcAhpF</i> ₁₋₂₁₂	<i>EcAhpF</i> ₂₁₂₋₅₂₁
Instrument (source & detector)	BRUKER NANOSTAR with MetalJet eXcillum VANTEC-2000		
Beam geometry	100 µm slit		
Wavelength (Å)	1.3414		
q range (Å ⁻¹)	0.015-0.4		
Exposure time (min)	30 (6 frames x 5 min)		
Temperature (K)	288		
Concentration range (mg/ml)	2.0-8.5	2.0 – 8.5	2.0 – 8.5
Structural parameters*			
I(0) (cm ⁻¹) [from P(r)]	456 ± 13.8	376 ± 2	360 ± 3.0
R _g (Å) [from P(r)]	15.9 ± 0.6	17.6 ± 0.6	26.3 ± 0.3
I(0) (cm ⁻¹) (from Guinier)	459 ± 15.2	375 ± 3	367.88 ± 3.19
R _g (Å) (from Guinier)	18.30 ± 0.25	17.4 ± 0.5	29.56 ± 0.32
D _{max} (Å)	50.4 ± 0.1	60.3 ± 1.2	89.8 ± 0.1
Porod volume estimate (Å ³)	~30625	~27303	~48551
Dry volume calculated from sequence (Å ³)	~31734	28279	~50660
Molecular mass determination (kDa)*			
Calculated monomeric MM from sequence	~22	~23	~35
MM from I(0)	20 ± 6	21	34 ± 6
MM from excluded volume	21 ± 6	22	33 ± 6
Software employed			
Primary data reduction	BRUKER SAXS		
Data processing	PRIMUS		
<i>Ab initio</i> analysis	GASBOR	GASBOR	GASBOR
Validation and averaging	DAMAVAR		
Computation of model intensities	CRY SOL		
Three-dimensional graphics representations	PyMOL		

3.3 Insights into the C-terminal binding epitope and solution dynamics of the *EcAhpF*₁₋₂₁₂-*EcAhpC* complex

3.3.1 NMR studies of the *EcAhpF*₁₋₂₁₂-*EcAhpC* complex

The *EcAhpR* system consists of the peroxiredoxin, *AhpC* and the peroxiredoxin reductase, *AhpF*. Together, the two enzymes scavenge harmful hydrogen peroxides and organic peroxides. The *EcAhpF* is made up of three distinct domains, the N-terminal domain, the C-terminal domain and the linker region [67]. In section 3.2 of this thesis, ITC was used to confirm that the *EcAhpF* interacts via the NTD with its partner protein, *EcAhpC*. ITC data also revealed that the CTD of *AhpF* does not partake in the interaction with *EcAhpC*. In this chapter, solution nuclear magnetic resonance is employed to understand the binding mechanism of the *EcAhpF*₁₋₂₁₂-*EcAhpC* interaction. To begin with, 1D NMR spectrum was acquired for the purified *EcAhpF*₁₋₂₁₂ in 50 mM phosphate, 50 mM NaCl, pH 6.8. The 1D spectrum (Figure 3.27) indicate 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 3.27).

Figure 3.27: 1D NMR spectrum of *EcAhpF*₁₋₂₁₂. One-dimensional NMR spectrum of *EcAhpF*₁₋₂₁₂ 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.

In order to obtain a well-defined 2D ¹⁵N HSQC spectrum, the protein was optimised concerning buffer (Figure 3.14) and temperature (Figure 3.15) conditions. In phosphate buffer, additional 10 peaks were obtained, which did not exist in the case of Tris-HCL buffer

Figure 3.28: ^1H - ^{15}N HSQC spectrum of *EcAhpF*₁₋₂₁₂ acquired in different buffer conditions. (A) 2D HSQC NMR spectrum of *EcAhpF*₁₋₂₁₂ recorded in 50 mM Tris-HCL, 200 mM NaCl buffer, pH 7.5 on Bruker Avance 700 MHz machine at 298 K (B) and in 50 mM phosphate, 50 mM NaCl, pH 6.8. Peaks coloured in green in (B) are extra peaks observed in phosphate buffer.

Figure 3.29: ^1H - ^{15}N HSQC spectrum of *EcAhpF*₁₋₂₁₂ acquired at different temperatures. (A) 2D HSQC NMR spectrum of *EcAhpF*₁₋₂₁₂ recorded in 50 mM phosphate buffer, pH 6.8, 50 mM NaCl on Bruker Avance 700 MHz machine at (A) 303 K and (B) 293 K.

(Figure 3.14B). This is not surprising as the use of a non-protonated buffer system (phosphate) at lower pH, hence, slower exchangeable amide protons enhances spectra quality. Nevertheless, to ensure that the best spectra are obtained for downstream

experiments, the temperature was optimised for the phosphate buffer as the temperature can directly affect the spectra line width quality. However, no significant difference was observed for the two temperatures of 303 K (Figure 3.29A) and 293 K (Figure 3.29B) compared with the room temperature spectrum (Figure 3.28B), hence, the 3D NMR acquisition was therefore carried out in 50 mM phosphate, 50 mM NaCl, pH 6.8 at 298 K.

3.3.2 Backbone resonance assignment of *EcAhpF*₁₋₂₁₂

As can be seen from Figure 3.28B, a well-dispersed ¹H-¹⁵N HSQC-spectrum of *EcAhpF*₁₋₂₁₂ was observed. This implies, that a proper folding of the protein has been achieved and that the protein is very likely monodispersed in solution. As explained earlier on, the *E. coli* AhpF is made up of three distinct domains, the AhpC-binding NTD, the CTD and a linker region connecting the two domains. This linker region allows flexible movements of the NTD relative to the CTD allowing better coordination and efficient electron transfer between the two domains (PDB ID; 4O5Q) [67]. In the construct in this experiment (*EcAhpF*₁₋₂₁₂) the linker region from 197-212 was included and the inherent flexibility of this region was manifested on the NMR time scale. High mobility of the linker region gave intense and sharp peaks (Figure 3.30), which in some cases overlap with other

Figure 3.30: Pseudo-3D orientation of ¹H-¹⁵N HSQC spectrum of *EcAhpF*₁₋₂₁₂ acquired in 50 mM phosphate buffer, pH 6.8, 50 mM NaCl on Bruker Avance 700 MHz machine at 298 K, showing the relative intensities of the peaks. This shows that a region of the protein, likely, the linker region is independently flexible relative to rest of the protein.

less intense signals. However, this did not affect the overall quality of the spectrum as seen in Figure 3.28B. The traditional 3D data set was acquired as described in section 2.11.2. Sequential backbone assignment, initially employing the CBCACONH and HNCACB experiments, enabled the assignment of 160 out of 202 observable peaks of the 212-residue *EcAhpF*₁₋₂₁₂ (excluding proline and sidechains). Using a non-uniform sampling strategy (NUS) [115], another set of 3D-data were acquired (NUS_HNCA, NUS_HNCOCA, and NUS_HNCO) (Figure 3.31). These additional data sets enabled backbone assignment of 185 amino acids (Figure 3.32A). The secondary structure prediction of *EcAhpF*₁₋₂₁₂ based on assigned $\text{C}\alpha$ - and $\text{C}\beta$ -chemical shifts, subtracted from their random coil values ($\Delta\text{C}\alpha$ - $\Delta\text{C}\beta$), is highly consistent with crystal structure [67] and reveals 7 α -helices and 8 β -sheets (Figure 3.32B).

Figure 3.31 Sequential assignment of the backbone resonances of *EcAhpF*₁₋₂₁₂. Representative strips are shown from a Non-Uniform Sampling (NUS) 3D HNCA. Sequential assignment indicated by *arrows* is shown for residues K32-I36. Data were acquired at 298K in a 700 MHz machine equipped with a triple resonance cryoprobe.

Figure 3.32: NMR spectroscopic characterization of *E. coli* AhpF₁₋₂₁₂ (A) ¹H-¹⁵N- HSQC spectrum of *E. coli* AhpF₁₋₂₁₂. Side chain amide resonances are connected by lines. (B) The secondary structure elements from the crystal structure of *Ec*AhpF are shown above the secondary chemical shift plots. The scores were calculated from $\Delta C^{\alpha}-\Delta C^{\beta}$ based on backbone assignment of *Ec*AhpF₁₋₂₁₂. Contiguous positive and negative chemical shift index values correspond to α -helix and β -strand, respectively.

3.3.3 NMR titration studies of *EcAhpF*₁₋₂₁₂-*EcAhpC*₁₋₁₈₇

With the backbone assignment of *EcAhpF*₁₋₂₁₂ complete, titration studies of *EcAhpF*₁₋₂₁₂ with the full-length *EcAhpC* was performed in an attempt to elucidate the binding epitope of the interaction. The ¹H-¹⁵N-HSQC spectra obtained for the labeled *EcAhpF*₁₋₂₁₂ protein alone and in the presence of increasing amount of the partner protein, *EcAhpC*, exhibit characteristics reminiscent of a long-lived complex (Figure 3.33). Thus, the majority of peaks disappear in a coordinated fashion, suggesting a global decrease in tumbling. Starting from a molar equivalent of 0.08 of *EcAhpC* (Figure 3.33B), the cross-peaks of the *EcAhpF*₁₋₂₁₂ gradually disappeared (Figure 3.33B-F), until no further signal changes were observed. Since the *EcAhpC* forms mixed oligomers in solution (>40 kDa), the abovementioned observation indicates a long-lived complex. The remaining signal mostly emanates from amino acid residues in the highly flexible linker region, 197-212 (Figure 3.33F). This disappearance is typical of interacting proteins, where the isotopically labeled protein is relatively small, whereas the binding partner is larger (> 40 kDa) [127]. In that case, if the interaction is long-lived, the binding of the larger protein to the isotopically labelled protein significantly increases the R₂ transverse relaxation time of the complex, leading to line broadening and disappearance of cross-peak resonances. In other words, the larger protein dictates the tumbling motion of the smaller protein post-binding. The cross-peaks representing residues from the flexible linker region exhibits a local tumbling motion independent of the global motion of the complex and hence it is not affected by the disappearance. This observation of a slowly dissociating complex was reported for long-lived protein complexes [127, 128].

3.3.4 Transient complex of *EcAhpF*₁₋₂₁₂-*EcAhpC*₁₋₁₇₂

The important role of the C-terminus in the reduced *EcAhpC* has been examined in oligomerization and subsequently enzyme catalysis in sections 3.12 and 3.13, respectively [50]. To establish the role of the C-terminus in the formation of the stable *EcAhpC*-*EcAhpF*₁₋₂₁₂ complex described in section 3.3.3, and subsequently examine the interacting residues of the NTD in more detail, NMR-titration experiments of *EcAhpF*₁₋₂₁₂ with the C-terminally truncated *EcAhpC*₁₋₁₇₂ protein has been performed. Unlike the full-length *EcAhpC* titration, the addition of similar equimolar concentrations of the truncated *EcAhpC*₁₋₁₇₂ did not yield any coordinated peak disappearance in the observed ¹H-¹⁵N HSQC spectra, as shown in Figure 3.34.

Figure 3:33: Interaction of EcAhpC with EcAhpF₁₋₂₁₂ as studied by NMR. Summary of gradual disappearance of the EcAhpF₁₋₂₁₂ cross-peaks in the presence of increasing amount of its larger binding partner, EcAhpC (A) HSQC of 0.3 mM of EcAhpF₁₋₂₁₂ alone and in the presence of (B) 0.08 molar excess of EcAhpC, (C) 0.1 molar excess of EcAhpC, (D) 0.3 molar excess of EcAhpC, (E) 0.5 molar excess of EcAhpC, (F) 1 molar excess of EcAhpC. The residual cross-peaks in the HSQC-spectrum are from the flexible linker region of EcAhpF₁₋₂₁₂.

However, by superimposing the resulting individual HSQCs, varying per residue peak perturbations were observed (Figure 3.34). Some residues have undergone classical intermediate chemical exchange. For example, residue D155 (Figures 3.34-3.36), which gradually shifts, broadens and reappears toward saturation with *EcAhpC*₁₋₁₇₂. Other residues undergoing fast to intermediate chemical exchange are the amino acids A31, F181, C129, F175 and I154 (Figures 3.34-3.36). The interchange between split and single peaks in the absence and presence of *EcAhpF*₁₋₁₇₂ as observed in the case of residues A79, T158, I163, Y125 and V135 (Figure 3.37) demonstrates that these residues undergo conformational exchange upon binding [129]. In some cases, an initial distribution of the conformational states of the residues is shifted to a predominant state as can be seen from the line shape analysis of residues I163 and T158 (Figure 3.37). The intensity plot in Figure 3.35A show reduction and gain of intensity, in the presence of 0.1 molar equiv., 0.3 molar equiv. and 1 molar equiv. of *EcAhpC*₁₋₁₇₂. Intensity gain or loss is associated with motion and conformational flexibility of residues at the binding interface [129]. This dynamic nature of the *EcAhpC*₁₋₁₇₂-*EcAhpF*₁₋₂₁₂ binding interface contrast with the static interaction observed in section 3.3.3.

Figure 3.34: NMR analysis of *EcAhpC*₁₋₁₇₂ and *EcAhpF*₁₋₂₁₂ (A) superposition of the ¹H-¹⁵N HSQC-spectrum of *EcAhpF*₁₋₂₁₂ alone (red) and in the presence of increasing amount of *EcAhpC*₁₋₁₇₂. (Blue-0.08:1), (turquoise-0.1:1), (orange-0.3:1), (magenta-0.5:1) and (green-1:1) ¹H-¹⁵N HSQC-spectra was obtained on a Bruker avance III 700 MHz machine at 298K.

Figure 3.35: (A) Intensity plot based on the ratio of peak resonance changes of bound to unbound (I/I_0) for three titration points (green: -0.1, blue: -0.3 and brown: -1). A cutoff point was established based on the 1δ from the average values (B) Combined ^1H and ^{15}N -chemical shift difference between the ^1H - ^{15}N HSQC resonances of *EcAhpF*₁₋₂₁₂ alone and in the presence of 1 molar excess of *EcAhpC*. Chemical shift change above the horizontal line ($\sigma + 1\delta$) is considered significant.

Figure 3.36: Expanded region shows cross-peaks undergoing mainly fast to intermediate chemical exchange perturbations and conformational exchanges. Peaks are encircled to show that they are from the same residue. *EcAhpF*₁₋₂₁₂ alone (*red*) and in the presence of increasing amount of *EcAhpF*₁₋₂₁₂. (*Blue*-0.08:1), (*turquoise*-0.1:1), (*orange*- 0.3:1), (*magenta*- 0.5:1) and (*green*-1:1).

Figure 3.37: Conformational perturbation of *EcAhpF*₁₋₂₁₂ residues upon *EcAhpC*₁₋₁₇₂ titration. Each line represents the titration point with the arrow on the right showing the increasing molar equivalent from 01 to 1. Spectral line analysis was performed using LineShapeKin software package [130].

In order to identify the binding interface of the *EcAhpC*–*EcAhpF*₁₋₂₁₂ interaction, all affected residues were mapped onto the crystal structure of *EcAhpF*₁₋₂₁₂ (Figure 3.38) [67]. The surface exposed residues form a well-defined patch around the active site residue C129. The distribution of the affected residues delineates an interface for the *EcAhpC*₁₋₁₇₂–*EcAhpF*₁₋₂₁₂ complex. The potential binding sites of the unlabelled *EcAhpC*₁₋₁₇₂ were predicted using the meta-server, meta-PPSIP (Figure 3.39) [113]. The obtained restraints were then used to drive an HADDOCK-docking simulation [111] as described in section 2.10. The HADDOCK statistics table for the highest scoring clusters is shown in Table 3.4. The top scoring model from the highest scoring cluster was selected (HADDOCK Score: -66 ± -5.7 ;

Figure 3.38: Surface representation of *EcAhpF*₁₋₂₁₂ displaying residues undergoing fast to intermediate chemical exchange and/or conformational exchange (forest green) and residues, whose intensity were affected by the presence of *EcAhpC*₁₋₁₇₂ (pale green). The cysteine catalytic site of *EcAhpF*₁₋₂₁₂ is represented in red. Different orientation of the *EcAhpF*₁₋₂₁₂, showing interfacial residues perturbations (*right*) and long-range perturbations and also a groove that runs across the backside of the protein (*left*).

41:A, 43:A, 44:A, 45:A, 46:A, 47:A, 48:A, 49:A, 139:A, 140:A, 161:A, 162:A, 163:A, 164:A, 165:A, 166:A, 167:A, 210:B, 211:B, 212:B, 213:B, 214:B, 215:B, 216:B, 307:B, 324:B, 325:B, 326:B, 327:B, 328:B, 329:B, 330:B, 331:B, 332:B, 333:B

Figure 3.39: Prediction by meta-PPISP: a metamethod for Protein-Protein Interaction Site Prediction used in HADDOCK modelling. Figure derived from PDB file (PDB ID: 4O5Q) [50] in which meta-PPISP prediction scores are stored as B-factors. The predicted interface residues are (shown as residueID:chainID, separated by comma). Colours represent the two epitopes in the dimer unit. The residues in red were submitted to the HADDOCK server.

Restraint violation energy:

285.9 ± 52.55 Kcal/mol;

Buried surface area: 2357.2

± 98.2 Å; Z-score: -0.7).

The binding orientation of the *EcAhpC*₁₋₁₇₂–*EcAhpF*₁₋₂₁₂ shown in Figure 3.40 gives insight about the interacting sites of both proteins. The spatial proximity of the two catalytic sites indicates that the *EcAhpC*₁₋₁₇₂ dimer contacts this interface mainly with two helices α6 and α2' (Figure 3.40A-C).

Helix α6 followed by a loop (160-165 aa) region holding

C_R and $\alpha 2'$ helix holding C_P is linked to forms scaffold which fits into the “arch-like” groove on the binding surface of *EcAhpF*₁₋₂₁₂ (Figure 3.38). In addition, the backside of the *EcAhpF*₁₋₂₁₂ structure is characterized by a well-defined groove that runs across the protein (Figure 3.38). Since the disulphide bond comes very close to this groove, we were able to manually dock the short piece of C-terminal peptide region (P167 to E172) of *EcAhpC* into it (Figure 3.40A). Furthermore, the entire C-terminus could be docked into the observed backside groove (Fig. 3.40B). The manually docked C-terminal tail was submitted to Rosetta’s FlexPepDock [131], an algorithm that is able to refine an initial starting structure of a peptide-protein complex, to a near-native model of the interaction, and the top 200 minimized structures of which the top 10 were chosen is displayed in Figure 3.42.

Figure 3:40: The best results of HADDOCK runs displaying the *EcAhpF*₁₋₂₁₂-*EcAhpC*₁₋₁₇₂ complex. (A) The top view shows the *EcAhpF*₁₋₂₁₂-*EcAhpC*₁₋₁₇₂ complex, where the short peptide from residue P167 to E172 of the *EcAhpC*₁₋₁₇₂ (brown; PDB ID: 4ql9) [50] is manually docked into the back surface groove of the *EcAhpF*₁₋₂₁₂ structure. Inset figure shows the proximity of the two catalytic cysteines (B) Docking of the entire C-terminus onto the backside groove of *EcAhpF*₁₋₂₁₂. The major helical contacts of *EcAhpC* were labelled as $\alpha 6$ and $\alpha 2'$, with the $\alpha 2'$ provided by the second monomer of the dimer unit. (C) Side view of the complex highlighting the $\alpha 6$ and $\alpha 2'$ helices in of *EcAhpC* in contact with *EcAhpF*₁₋₂₁₂. (D) Surface representation of the entire C-terminal region docked into the backside groove of the *EcAhpF*₁₋₂₁₂.

Figure 3.41: Orientation of the *EcAhpF*₁₋₂₁₂ relative to the full length *EcAhpC* (PDB ID: 4ql9)[50]. (A) The top view (B) the side view and (C) the lateral view shows the *EcAhpC* C-terminus wrapping around *EcAhpF*₁₋₂₁₂ along the C-terminal binding groove.

Table 3.4: Statistics for the best model in the top two clusters obtained by HADDOCK docking

Parameters		Cluster I	Cluster II
Structure number	30	17	
HADDOCK score	-66 ± -5.7	-40 ± 5.5	
Backbone r.m.s.d	1.3 ± 0.2	2.1 ± 0.4	
Restraint violations energy	285.9 ± 52.55 Kcal/mol	305 ± 62.1 Kcal/mol	
Buried surface area	2357.2 ± 98.2 Å	± 87	
Z-score	- 0.7	-0.2	
RAMPAGE statistics(%)			
Favoured region	96.5	94.7	
Allowed region	3.2	4.3	
Outlier region	0.4	1.0

Figure 3.42: Rosetta FlexPepDock analysis of the C-terminal peptide (P167-I187) in complex with *EcAhpF*₁₋₂₁₂ [67]. (A) A plot of the energy landscape of the top 200 models sampled by FlexPepDock. (B) Overlay of the top 10 models created by FlexPepDock [131].

The high complementarity of the peptide to the backside groove gives credence to the obtained model. Figure 3.41 displays the orientation of the NTD of AhpF in complex with the decamer ring of *EcAhpC* (PDB ID; 4ql9) [50]. This observation, where the C-terminal extension wraps around the NTD (Figure 3.40D, 3.41 and 3.42B), may explain the stability and long resident time of the full-length *EcAhpC* interaction with *EcAhpF*₁₋₂₁₂. Such binding has been reported in the crystal structures of a 2-Prx with sulphiredoxin, where the C-terminus of the 2-Prx wraps around the later [132].

3.3.5 The AhpF-AhpC interaction is redox-dependent

To examine whether the redox state of AhpC affects its interaction with *EcAhpF*₁₋₂₁₂, ITC was performed under oxidizing and reducing conditions. As shown in Figure 3.43A, titration of *EcAhpC* with *EcAhpF*₁₋₂₁₂ resulted in an overall positive heat, indicating an endothermic reaction similar to the observed interaction with *EcAhpF*₁₋₁₉₆ (Figure 3.26). However, in the presence of increasing amounts of reducing agent, 2 mM (Figure 3.43B) and 5 mM (Figure 3.43C), the interaction was drastically reduced with 5 mM completely terminating the interaction. This observation brings into focus the importance of the conformational changes at the active site during catalytic activity.

Figure 3.43: Representative ITC profiles are shown for *EcAhpF*₁₋₂₁₂ with *EcAhpC*. (A) ITC titration of *EcAhpF*₁₋₂₁₂ vs *EcAhpC* in the presence of (B) 2 mM TCEP and (C) *EcAhpF*₁₋₂₁₂ vs *EcAhpC* in the presence of 5 mM TCEP. The top panels in the ITC profile show the injection after baseline correction and the bottom panels show the integration (heat release) for each injection. The solid lines in the bottom panel reveal the fit of the data to a function based on a one-site binding model.

3.4 Effect of ionic strength on *EcAhpF*–*EcAhpC* complex formation

Effect of ionic strength on *EcAhpF*–*EcAhpC* complex formation

3.4.1 Ammonium sulphate increases the catalytic efficiency of the AhpR system

Ionic strength played an essential role in shaping the evolution of life [133]. From the salty oceans to the salty soil, macromolecules from bacteria have evolved to function in varying ionic strength. The cell dependency on ionic strength is seen by the presence of abundant charge molecules in the cell, for example, charged phospholipids, nucleic acids, and proteins. This intricate balance of ionic strength and protein evolution explains why proteins from non-halophiles like *E. coli* are still affected ionic strength.

In *Amphibacillus xylanus*, ammonium sulphate was used to study the complex between NADH oxidase, an AhpF homologue, and AhpC [134]. To gain more insight into *EcAhpR* system, a similar buffer system has been employed. In the NADH-dependent assay in Figure 3.44, the decrease in NADH absorbance in the presence of *EcAhpC* indicates the completion of the catalytic cycle as the former becomes depleted. In the presence of 100 mM ammonium sulphate (AS), the absorbance saturation was reached in 6.5 min. But since the complex will be studied at a higher concentration (320 mM), further assays were performed to examine the influence of increasing AS concentration (200 mM and 320 mM AS) on the structural integrity of the protein. In both cases, saturation was achieved by 6 min and 5 min, respectively

Figure 3.44: NADH-dependent assay of *EcAhpC*-*EcAhpF* complex in increasing concentration of ammonium sulphate. The assay measures catalytic activity in standard 100 mM ammonium sulphate (green), in the absence of AhpC (—) and in the presence of AhpC (---). The data show that NADH oxidase activity was saturated at approximately 6.5 min. In the presence of 200 mM ammonium sulphate (brown), saturation reached at 6 min whilst in the presence of 320 mM ammonium sulphate saturation reached at 5 min, indicating a concentration dependent NADH oxidase activity increase.

(Figure 3.44). This confirms that not only do the proteins maintain their integrity in 320 mM AS, but their activity is also enhanced. Having confirmed that increasing ammonium sulphate concentrations do not affect the fidelity of the AhpR enzymes but rather increase its efficiency, an attempt was made to capture the AhpR complex in solution. To prepare the complex between the *EcAhpF*-*EcAhpC*, the proteins were independently buffer exchanged into 50 mM HEPES pH 7.0, 320 mM ammonium sulphate using a 10 kDa Amicon® centricon and incubated together in a 1:5 molar ratio, respectively for 2 hours. The mixture was then passed through a Superdex S200 column which was pre-equilibrated with 50 mM HEPES pH 7.0, 320 mM ammonium sulphate, to separate the complex from unbound fractions. The formed complex was used for further studies. Control experiments were performed by injecting individual *EcAhpC*₁₋₁₈₇ and *EcAhpF* under the identical buffer conditions.

Figure 3.45: Size exclusion chromatography (SEC) of the *EcAhpF*-*EcAhpC* complex. Normalized chromatograph of the elution profile of the *EcAhpF*-*EcAhpC* (brown), *EcAhpC* (blue) and *EcAhpF* (green). The experiment was performed in 50 mM HEPES, 320 mM ammonium sulphate, pH 7.0 buffer and run at a flow rate of 0.3 ml/min. A 200 μ l of protein was injected into a 500 μ l loop individually and as a complex. The shift in elution volume of *EcAhpC* from 13.0 ml to 12.7 ml in the presence of *EcAhpF* indicates a complex formation. *Inset:* The apex peaks of each chromatograph were loaded onto a 17% SDS-PAGE to confirm the constituent of the elution.

The individual proteins of *EcAhpC*₁₋₁₈₇ and *EcAhpF* were eluting at 13.0 ml and 13.9 ml, respectively, whereas the *E. coli* AhpR complex of the two proteins eluted earlier at 12.7 ml (Figure 3.45). To confirm the constituents of the peaks, the apex peak of each of the chromatographs were examined by a 17% SDS-PAGE (*inset*). The results confirm an *EcAhpF*-*EcAhpC* complex formation.

3.4.2 Dynamic light scattering studies of *EcAhpF*-*EcAhpC*₁₋₁₈₇ in ammonium sulphate

In furtherance of the gel filtration experiments, DLS experiments were performed to analyse the effect of increasing ionic strength on oligomerization and complex formation.

Figure. 3.46: Dynamic light scattering experiments of *E. coli* AhpC. DLS measurement of (A) AhpC and (B) AhpF in Tris-HCL (black) buffer and ammonium sulphate (red) buffer show the influence of ionic strength in the oligomerization of AhpC. (C) Comparison of *EcAhpF*-*EcAhpC* complex formation in Tris-HCL (green) and ammonium sulphate (blue) buffers. (D) Superimposition of ammonium sulphate DLS analysis of *EcAhpC* (black), *EcAhpF* (red) and *EcAhpF*-*EcAhpC* complex (blue).

From Figure 3.46A, DLS shows that in the presence of ammonium sulphate, there is an increase in the diameter of the *EcAhpC* species solution. This directly indicates that the ammonium sulphate influences the oligomerization of the *EcAhpC* to a functional decamer. However, there was no influence of the ammonium sulphate on the oligomeric state of *EcAhpF* (Fig. 3.46B). To ascertain the effect of ammonium sulphate on complex formation, the complexes were prepared under two different buffer conditions, 50 mM Tris-HCL, 200 mM NaCl, pH 7.5 and 50 mM HEPES, 320 mM AS, pH 7.0. As it can be observed from Figure 3.46C, there was no complex

formation under Tris-HCL conditions, whereas under AS-conditions, the increased in hydrodynamic ratio indicates a complex formation (Figure 3.46C and D).

In section 3.1.2 [50], It was demonstrated that the C-terminal tail of AhpC is critical for oligomerization, catalytic activity and binding to the NTD of AhpF under near physiological conditions (50 mM Tris-HCL, 200 mM NaCl, pH 7.5). To examine the importance of the C-terminus in complex formation under ammonium sulphate conditions, I attempted to prepare a complex of the C-terminally truncated *EcAhpC*₁₋₁₇₂ and *EcAhpF*. Unlike the full length *EcAhpC* in which the complex formation led to the slowing of the diffusional properties of the *EcAhpC*-*EcAhpF* species, thus slowing the relaxation of the autocorrelation curve in the DLS experiment (Figure 3.47A), in the case of the truncated protein, there was no significant changes in both the size and autocorrelation function (Figure 3.47B). This underscores, once again, the importance of the C-terminal tail of the *EcAhpC* as an anchor in the AhpR complex.

Figure 3.47: Influence of *EcAhpC* C-terminus in the *EcAhpF*-*EcAhpC* complex formation under ammonium sulphate condition (A) Correlation functions of DLS studies of *EcAhpF*-*EcAhpC* complex under 50 mM HEPES, 320 mM AS, pH 7.0 showing a slowed relaxation of the autocorrelation curve (red) compared with *EcAhpC* alone (black). Inset represent the size distribution of the complex and individual proteins. (B) Correlation functions of DLS studies of *EcAhpF*-*EcAhpC*₁₋₁₇₂ (red) showing no significant change as compared with *EcAhpC*₁₋₁₇₂ (black).

3.4.3 SAXS studies of *EcAhpC* in ammonium sulphate

To confirm the behavior of *EcAhpR* proteins in AS, SAXS patterns of *EcAhpC* and the C-terminal truncated *EcAhpC*₁₋₁₇₂ were measured at two different protein concentrations (4 and 6 mg/ml). Firstly, the protein was measured in a buffer containing 50 mM Tris-HCL pH 7.5, 200 mM NaCl to yield the final composite scattering curve as shown in Figures 3.48A and 3.48B. The data and data analysis

have been performed with Dr. Neelagandan Kamariah from our laboratory. The Guinier plots at low angles are linear and reveals good data quality

Figure 3.48: SAXS experiments of $EcAhpC_{1-172}$ and $EcAhpC$ in 50 mM Tris-HCL, 200 mM NaCl, pH 7.5. Composite scattering curve for (A) $EcAhpC_{1-172}$ and (B) $EcAhpC$. The Guinier plots at low angles are linear shown as an inset. Distance distribution curve for (C) $EcAhpC_{1-172}$ and (D) $EcAhpC_{1-187}$ with the later exhibiting a concentration dependent oligomerization (arrows in B). CRY SOL [135] and OLIGOMER [136] calculations for (E) $EcAhpC_{1-172}$ and (F) $EcAhpC$.

with no indication of protein aggregation (Figure 3.48A and B, inset). The radius of gyration (R_g) values from the Guinier approximation were measured with values of 38.95 ± 1.24 Å, 41.27 ± 0.54 Å for 4 mg/ml and 42.30 ± 0.66 Å, 47.29 ± 0.63 Å for 6 mg/ml for the $EcAhpC_{1-172}$ and $EcAhpC$, respectively. It can be observed that

$EcAhpC_{1-187}$ exhibited a concentration-dependent oligomerization as is shown at the low angle of the scattering curve and slight deviation in the Guinier plot (Figure 3.48B). The maximum particle dimensions (D_{max}) of the molecules were estimated to be $123.56 \pm 5 \text{ \AA}$, $126.07 \pm 5 \text{ \AA}$ for 4 mg/ml and $130.54 \pm 5 \text{ \AA}$, $150.89 \pm 5 \text{ \AA}$, for 6 mg/ml for the $EcAhpC_{1-172}$ and $EcAhpC$, respectively (Figures 3.48C and D).

In furtherance, SAXS was then used to examine the solution behavior of $EcAhpC$ in a 50 mM HEPES pH 7.0, 320 mM of ammonium sulfate using similar protein concentrations as done before for Tris-HCL buffer. The final composite scattering curves for $EcAhpC_{1-172}$ and $EcAhpC$ is shown in Figure 3.49A and B, respectively. The most striking difference

Figure 3.49 Solution X-ray scattering of $EcAhpC_{1-172}$ and $EcAhpC$ in 50 mM HEPES, 320 mM ammonium sulphate, pH 7.0. Composite scattering curves for $EcAhpC_{1-172}$ (A) and (B) $EcAhpC$. The Guinier plots at low angles are linear, shown as an *inset*. Distance distribution curves for (C) $EcAhpC_{1-172}$ and (D) $EcAhpC$.

here is that unlike the Tris-buffer, there was no concentration-dependent effect. Inspection of the Guinier plots reveals a good data quality and no protein aggregation (*inset* of Figure 3.49A and B). The radius of gyration (R_g) values from the Guinier

approximation was consistent for the two concentrations measured with a value of 46.38 ± 0.54 Å and 48.19 ± 0.44 Å for the *EcAhpC*₁₋₁₇₂ and *EcAhpC*, respectively. The distance distribution functions ($p(r)$) were shown in Figures 3.49C and D and the maximum particle dimensions (D_{max}) were estimated to be 135.1 ± 6 Å and 150.25 ± 3 Å for *EcAhpC*₁₋₁₇₂ and *EcAhpC*₁₋₁₈₇, respectively. The bimodal feature observed in the $p(r)$ function reveals the ring formation of *EcAhpC* in solution. The molecular weight estimated based on porod volume calculated using the DATPOROD [136] and from the V_c value [137] are 173 kDa, 198 kDa and 174 kDa, 194 kDa for *EcAhpC*₁₋₁₇₂ and *EcAhpC*, respectively and validate the decamer formation in solution.

The theoretical scattering curves of a decameric *EcAhpC*₁₋₁₈₇ (PDB ID: 4O5R) and the lower order oligomeric forms like dimer, tetramer etc., respectively, were computed and compared with the experimental solution X-ray scattering data of different forms of *EcAhpC* using the program CRY SOL [135] and OLIGOMER [136]. The oxidized *EcAhpC*₁₋₁₈₇ and *EcAhpC*₁₋₁₇₂ measured under 50 mM Tris-HCL pH 7.5, 200 mM NaCl had a better fit with χ^2 values of 1.62 and 1.33, respectively, with a mixture of different oligomeric conformations, thus 83% tetramer and 17% decamer for *EcAhpC* (Figure 3.48F) and 72% tetramer and 28% decamer for *EcAhpC*₁₋₁₇₂ (Figure 3.48E) present in solution. This is consistent with reported data, that the AhpC exists as a mixture of oligomers in solution [82]. However, such fit with the same mixture of oligomers cannot be obtained for the curves in the presence of ammonium sulfate as shown in Figures 3.50E and 3.50F where the χ^2 -values were calculated to be 5.74 and 4.38, for *EcAhpC* and *EcAhpC*₁₋₁₇₂, respectively. This observation is understandable as the ammonium sulphate has driven the AhpC into full decamers. The *ab initio* shapes for *EcAhpC*₁₋₁₈₇ and *EcAhpC*₁₋₁₇₂ measured under ammonium sulphate conditions were calculated with GASBOR using the P321 symmetry and the constraint of an oblate shape. The average structure of ten independent reconstructions is shown in Figure 3.50A and 3.50C with normalized spatial discrepancy (NSD) values being 0.90 ± 0.01 and 0.93 ± 0.01 for the *EcAhpC* and *EcAhpC*₁₋₁₇₂, respectively. This yielded the doughnut-like decameric ring structure of *EcAhpC* with a good fit to the experimental data (Figure 3.50B and D). The crystal structure of the decameric oxidized *EcAhpC* (PDB ID: 4O5R) [50] is well embedded into the *ab initio* model (Figure 3.50A and C). However, the larger χ^2 -values of 5.74 and 4.38 for *EcAhpC* and *EcAhpC*₁₋₁₇₂, respectively, observed

Figure 3.50: Solution X-ray scattering analyses of *EcAhpC*₁₋₁₇₂ and *EcAhpC* in 50 mM HEPES, 320 mM ammonium sulphate, pH 7.0. *Ab initio* shape calculations for (A) *EcAhpC* and (C) *EcAhpC*₁₋₁₇₂ using GASBOR [136]. Theoretical scattering curves fitted to experimental data for (B) *EcAhpC* and (D) *EcAhpC*₁₋₁₇₂. Discrepancies in the calculated and experimental data gives higher χ^2 -values for (black), with and (red), without the flexible region. Modelling using CORAL [138] for (E) *EcAhpC* and (F) *EcAhpC*₁₋₁₇₂.

between the calculated and experimental data were likely due to the C-terminal residues (167-187) of the decameric oxidized *EcAhpC* as it is highly disordered and

not observed in the crystallographic structure (section 3.1.5) [50, 67, 139], and hence contributes significantly to their discrepancies (Figure 3.50E and F).

Figure 3.51: CORAL rigid-body modelling of the flexible C-terminal tail and the N-terminal 6x-His-tag for (A) *EcAhpC* and (B) *EcAhpC*₁₋₁₇₂.

To resolve these discrepancies, a rigid-body modelling of the decameric *EcAhpC* (PDB ID: 4O5R) using the SAXS data obtained for the *EcAhpC* and *EcAhpC*₁₋₁₇₂ were performed using the program CORAL [138]. This program performs modeling of complexes against the experimental SAXS data employing a combined rigid body for the atomic structures of the domains and the *ab initio* modeling of the flexible regions,

which here are the C-terminal residues (167-187 and 167-172, for *EcAhpC* and *EcAhpC*₁₋₁₇₂, respectively). In addition, the N-terminal 6 x His-tag residues were included for the modeling. As shown in Figures 3.51A and B, the addition of the flexible regions in the CORAL models, significantly enhances the χ^2 -values of the fits, that is 0.85 and 1.1 for *EcAhpC* (Figure. 3.50E) and *EcAhpC*₁₋₁₇₂(Figure 3.50F), respectively. The different conformations observed for the C-terminus of *EcAhpC* in rigid body modeling demonstrate that the very C-terminus is highly flexible and exposed in solution, hence, may explain the ability to make contact with the NTD of AhpF (Figure 3.51A and B).

3.4.4 SAXS studies of the *EcAhpF*-*EcAhpC* complex in ammonium sulphate

The SAXS pattern of the purified *EcAhpC*-*EcAhpF* complex yielded the final composite scattering curve shown in Figure 3.52, which did not reveal a significant concentration effect. Inspection of the low angle of the Guinier plots reveals a good data quality and no protein aggregation (*inset* of figure 3.52). The *EcAhpC*-*EcAhpF* complex has a radius of gyration (R_g) of 55.72 ± 2.17 and a maximum dimension D_{max} of 179.1 ± 4.01 Å (Figure 3.52B). The R_g and D_{max} values are higher than the free *EcAhpC*₁₋₁₈₇, 46.4 ± 0.54 Å and 135.1 ± 6.20 Å, measured under identical buffer condition (Figure 3.52B). The molecular

Figure 3.52: Solution X-ray scattering of *EcAhpF*-*EcAhpC* complex in 50 mM HEPES, 320 mM ammonium sulphate, pH 7.0. The two concentrations used are represented as *red* and *purple* (A) Composite scattering curve for the *EcAhpF*-*EcAhpC* complex The Guinier plots at low angles are linear shown as an inset. (B) Distance distribution curve for the *EcAhpF*-*EcAhpC* complex.

weight estimated based on porod volume calculated using the DATPOROD [136] and from the V_c -value are 288 kDa and 280 kDa, respectively. These values necessitate

the complex formation in solution. To model the complex, we need to understand the solution behaviour of both proteins. The DLS study demonstrated that there was no influence of the ammonium sulphate of the oligomeric state of *EcAhpF* (Figure 3.46B). The major challenge in modelling

Figure 3.53: SASREF rigid-body modelling for possible binding orientation of the *EcAhpF* to *EcAhpC*. (A) Calculated theoretical fit (model C-blue; model B-magenta and model D-red) to experimental data. Modelling with extended *EcAhpF* (B), (C) and (D) yielded χ^2 -values of 0.61, 3.76 and 2.65, respectively. The arrow in (A) shows the discrepancies of the models from experimental scattering curves at a low angle.

complexes with SAXS is in predicting the conformations in which flexible proteins like *EcAhpF* exist in solution. Recent studies reveal the inherent flexibility of *EcAhpF* in solution, in which the N-terminal domain (NTD) possibly adopts multiple

conformations in solution to facilitate the electron transfer from the C-terminal domain dithiol centre to the disulphide centre of AhpC [140]. The closed conformation enables the electron transfer between the redox active centres of the NTD and NADH domains and the extended conformation is responsible for the preferential binding of *EcAhpC* [67].

Figure 3.54 SASREF rigid-body modelling for possible binding orientation of the *EcAhpF* to *EcAhpC*. (A) Calculated theoretical fit (model C-blue; model B-magenta and model D-red) to experimental data. Modelling with SAXS dimeric *EcAhpF* (B), (C) and (D) yielded χ^2 -values of 0.53, 0.58 and 0.80, respectively.

The rigid body modeling of the *EcAhpF*-*EcAhpC* complex was performed by using the program SASREF [136]. The decameric crystal structure of oxidized *EcAhpC* (PDB ID: 4O5R) [50] and the two different conformations of the extended

EcAhpF; the highly extended conformation obtained from the crystal structure (PDB ID: 4O5Q) [67] and the extended conformation found by SAXS to be the most predominant conformation in solution were used in the modelling [140]. Since the molecular weights estimates based on SAXS, DLS and SEC suggest a decameric *EcAhpC* (MW ~200 kDa) and dimeric *AhpF* (MW ~120 kDa) complex species, the question arises whether one can model a dimeric *EcAhpF* to be accommodated on the decameric *EcAhpC*. The SASREF allows user input for contact surfaces and also the molecular symmetry. In this case, the contacts between the active site residues, C166 of *EcAhpC* and C129 of *EcAhpF*, with the distance of ~10 Å was used as constraints. In order to predict the complex, two strategies were adopted. The first strategy was to use the fully extended *EcAhpF* structure, solved by crystallography (Figure 3.53) and the second strategy was to use the extended conformation solved by SAXS which is predominantly found in solution (Figure 3.54). In both cases, either one or two NTDs of *AhpF* are made to contact the decamer. In Figure 3.53, all the obtained models do not fit well with the experimental curves with model C (Figure 3.54C) being the least accommodated with most deviation at the low angle (shown with an *arrow* in Figure 3.54A) and χ^2 -value of 3.76. This is followed by model B (Figure 3.54B) which had a χ^2 -value of 2.65. The best of the three models is model D (Figure 3.54D) with the χ^2 -value of 0.61. In the second scenario (Figure 3.54), with the SAXS obtained *EcAhpF* dimer, all the three models yielded a good χ^2 -values of 0.53, 0.58 and 0.80 (Figure 3.54A) for models B (Figure 3.54B), model C (Figure 3.54C) and model D (Figure 3.54D). This observation makes the second scenario the most likely orientation of the full-length *AhpF* on the decameric *AhpC*.

4. Discussion

4.1 Overall catalytic efficiency of peroxiredoxins

Peroxiredoxins represent one of the most efficient enzyme systems responsible for redox homeostasis in both eukaryotes and bacteria [6]. In eukaryotes, it has been suggested that they are responsible for scavenging ~90 % of mitochondrial H_2O_2 [141] with the catalytic turnover rate of $\sim 10^7 \text{ M}^{-1}\text{s}^{-1}$ [6]. In bacteria, the catalytic efficiency was also measured to be $4.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ in *Salmonella typhimurium* [7]. This makes the bacterial peroxiredoxins critical scavenging enzymes in systems that lack more efficient peroxidases [142]. Some factors responsible for the efficiencies of the peroxiredoxins have been elucidated. Notable is the influence of the oligomeric state of AhpC on enzyme catalysis. Oligomerization of AhpC during catalysis has been found to enhance the overall activity of the AhpR system [7, 50]. In the oxidized state, the forces holding the basic dimeric unit together are mainly hydrophobic and hydrogen bonding. In *StAhpC*, residue T77 forms an important hydrogen bond with residue D73 of the neighbouring dimeric unit via water bridge. Mutations that interrupt this bond (T77I, T77D) (Figure 4.1) was found to destabilize the decamerization of AhpC and subsequently a 2-fold reduction in catalytic activity [7].

Figure 4.1: Destabilization of the decamerization interface through mutation. (A) Native structure with T77 (1YEP). (B) Substitution of T77 with aspartate (1YEX) introduced high mobilities for residues harbouring the active site loop (not shown). Even though the side-chain carboxylate group displaces the water molecule bridging the hydrogen bonding in the wild-type to form a direct hydrogen bond with D73, this was not found to be stabilizing. (C) Substitution of T77 with Isoleucine (1YFO) prevented the formation of a hydrogen bond with D73 hence destabilizes the decamer interface [75].

Another factor considered to be responsible for catalytic efficiency is the stabilization of the negatively charged active site C_p-thiolate by conserved residues through the lowering of the pK_a of the later, thereby enhancing its nucleophilicity towards peroxides [6]. All the factors examined so far encompass the AhpC-peroxide phase of the catalytic cycle of the AhpR system. Since the AhpR is a two-protein system (AhpC-AhpF), it is imperative to understand the mechanism of the *Ec*AhpC-*Ec*AhpF interactions in order to appreciate the overall catalytic efficiency.

In this thesis various biophysical techniques have been employed to examine the importance of the C-terminal tail of AhpC in catalysis and also its binding to AhpF. The role of ionic strength in oligomerization of AhpC, complex formation, and overall catalysis is examined.

4.2 The role of *Ec*AhpC C-terminus in enzyme catalysis

The first implication of the peroxiredoxin C-terminus in enzyme catalysis was made by Koo *et al.* [143]. By truncating the C-terminal domain of the peroxiredoxin, thioredoxin peroxidase of *Schizosaccharomyces pombe*, they stipulated that the C-terminal extension, especially the lysine residues, plays critical roles in the inactivation and reactivation of YL-motif-containing peroxiredoxins in the presence of H₂O₂. To examine the effect of the C-terminal extension in *Ec*Prx and other non-YF containing Prx, C-terminal truncations have been generated (*Ec*AhpC₁₋₁₇₂, *Ec*AhpC₁₋₁₇₇, *Ec*AhpC₁₋₁₈₂, *Ec*AhpC₁₋₁₈₅ and *Ec*AhpC₁₋₁₈₆). Attempts to crystallize the above-mentioned constructs were made and crystals of *Ec*AhpC₁₋₁₈₂ enabled the elucidation of the oxidized crystal structure (Figure 3.13). As with the full-length protein, the high flexibility of the C-terminal tail makes it difficult to be seen in crystal structures (Figure 4.2). Attempts to crystallize the reduced form of the protein with the reducing agents TCEP and DTT were unsuccessful. However, by comparing the solved crystal structure of *Ec*AhpF₁₋₁₈₂ in this thesis (4QL9) [50] with the reduced homologue protein *St*AhpC (C47S) (1N8J) [144], a better understanding of the role of C-terminus in oligomerization has been made. Furthermore, insight has been obtained as to what forces are holding the quaternary structure (dimer and decamer) of the oxidized and reduced AhpC together. It will be more interesting to know especially in the reduced state where all cysteines exist as thiols and for that matter, there are no disulphides to hold the monomers together.

Regarding the dimeric unit, observations from both the oxidized and reduced structures reveal that they are held together by inter hydrogen bonding between the $\beta 7$ and $\beta 7'$ sheets of each monomer (Figure 4.2). The extensive interactions across the interface may explain why the dimeric units are still held together in the absence of a disulphide bond. This interaction is common in both the oxidized and reduced forms of AhpC. However, the most noticeable difference is observed in the C-terminal tail of AhpC. The formation of a disulphide bond between C47 and C166 is due to a local unwinding (LU) of the $\alpha 2$ -helix exposing the former to the formation of the $C_P S-SC_R$ with the later [70]. The high flexibility of the C-terminus makes it difficult to observe any residue beyond C166. However, in the reduced state, where the $\alpha 2$ -helix assumes a fully folded conformation (FU), separating both the peroxidatic cysteine ($C_P 47$) and resolving cysteine ($C_R 166$) by about 10 Å. This separation disallows the formation of a disulphide bond. The C-terminus, however, is observable in the crystal structure due to its limited flexibility. There are basically two sets of interactions with the C-terminus that give rise to this limited flexibility. The first set of interactions are occurring in the region between C166-A168, where these residues interact with residues G139', G142', R143' and T49' from the other monomer of the dimeric unit (Figure 4.2).

Figure 4.2 Comparison of the oxidized (PDB ID: 4QL9) [50] and reduced (PDB ID: 1N8J) [144]. The oxidized AhpC is shown in light blue and light red. Disulphides are shown in the *sphere*. The reduced AhpC is shown in colours *cyan* and *green*. The hydrogen bonds that hold the dimer together are also shown.

These hydrogen bonds interactions may help to further stabilize the dimeric unit. Most interestingly, the second set of interactions which occurs at the extreme C-terminus may help explain the observed stability of the decameric structure of the reduced AhpC. At the decameric interface of all five dimer units, the very C-terminus is observed embedded in the interface (Figure 4.3A). Careful examination reveals a “hook-like” structure at the extreme termini of the AhpC tails. This “hook-like” structure is held together by a salt bridge interact-

Figure 4.3 The role of the AhpC (1N8J) C-terminus in oligomer interface stabilization (A) The presences of the extreme C-terminal hook-like tail at the interfaces of the decamer. (B) *left*; reveals polar interactions, that the “hook-like” tail makes with the decamer interface; *right*; a salt-bridge interaction between residue K186 and D182 stabilizes the hook structure which ensures subsequent polar contacts of the remaining residues with the decamer interface. (C) Detailed polar contacts of the C-terminus tail at the decamer interface.

-ion between the lysine (K186) and aspartate (D182) residues both of which are conserved (Figure 4.3B; *right*). This salt-bridge has made it possible for further interactions to occur between the C-terminal hook and the decameric interface residues (Figure 4.3 B-C). Thus, residue L183 interacts with G185 and with F77' and T78' via a water-mediated hydrogen bond (Figure 4.3C). G185 also interacts with S85' via water-mediated hydrogen bond. The last residue of the C-terminus, I187 interacts via its carboxyl group with S86' directly, and to W82' through a water-mediated hydrogen bonding. The overall interactions at this interface may explain why the C-terminus has a stabilizing effect at the decamer interface. This phenomenon has been reported in other non-related proteins like the legume Bowman-Birk Inhibitors (BBI), where a salt bridge between D76 and K71 generates a similar "hook-like" knob at the C-terminus, making it possible for other interactions to occur [145]. This was found to be critical to the dimerization of the BBI protein. In *EcAhpC*, it is not surprising why truncations at the very C-terminus of *EcAhpC*₁₋₁₇₇, *EcAhpC*₁₋₁₈₂, *EcAhpC*₁₋₁₈₅ and *EcAhpC*₁₋₁₈₆ were detrimental to AhpC decamer formation in the reduced state (section 3.1.2) [50]. The fact that NADH-dependent peroxidase activity shows a drastic reduction in catalytic activities of all C-terminal mutants and truncates confirms the importance of this region to overall catalytic efficiency (section 3.1.3).

4.3. Binding mechanism of the AhpR system of *E. coli*

The role of the C-terminal tail in oligomerization of the reduced *EcAhpC* and overall efficiency of the AhpR system has been established (section 3.12 and 3.13). Since the oxidized AhpC has to be reduced by the AhpF [50, 81, 144], it is imperative to understand the mechanism and interaction between the AhpC and the AhpF. It was speculated that AhpF may interact via its NTD with the AhpC [127]. To empirically prove this assertion, two constructs of the NTD domain of *EcAhpF* were generated, with (*EcAhpF*₁₋₂₁₂) and without linker (*EcAhpF*₁₋₁₉₆). Another construct consisting of the C-terminal domain (*EcAhpF*₂₁₂₋₅₂₁) was also generated. Whereas the CTD does not interact with *EcAhpC*, the NTD constructs were found to interact with *EcAhpC* with a K_d of 3.2 μ M. This affinity although moderate can be considered high for a redox protein considering the fact that most redox proteins bind transiently so as to ensure continuous electron transfer among partner proteins [146, 147]. Another observation uncovered by the ITC titration is the entropic nature of the interaction. The free energy of the interaction (ΔG) is provided mainly by the entropy component ($-T\Delta S$)

rather than the enthalpy component. This implies that the binding interface is probably hydrophobic in nature and taken together with the high affinity, the interface is likely broad. To identify the residues directly involved in the interactions NMR titrations were performed. Titration of *EcAhpF*₁₋₂₁₂ with the full-length *EcAhpC* resulted in a spectra characteristic of a long-lived complex (Figure 3.33), due to the coordinated disappearance of peaks upon titration. This stability of the *EcAhpF*₁₋₂₁₂-*EcAhpC* complex is consistent with results obtained from surface plasmon resonance measurement of the *Leishmania* homologue protein system, tryparedoxin-tryparedoxin reductase where the dissociation rate k_{off} was $5 \times 10^{-5} \text{ s}^{-1}$ [148]. Titrating the C-terminally truncated *EcAhpC*₁₋₁₇₂, however, prevented the coordinated disappearance (Figure 3.34) but instead enabled per residue perturbation to be observed. This is indicative of a highly dynamic binding system and it also directly implicates the C-terminus in the establishment of the long-lived complex between the *EcAhpF*₁₋₂₁₂-*EcAhpC*. By using the NMR constraints obtained from the *EcAhpC*₁₋₁₇₂ truncated protein, important epitopes and a plausible binding site for the C-terminal tail has been revealed (Figure 3.40 and Figure 4.4) through docking simulation.

4.4 Important binding epitopes of the *EcAhpC*-*EcAhpF* complex is unveiled

Using NMR, it has been established that the C-terminus influences the dissociation rate of the AhpR-complex. Titration with truncated *EcAhpC*₁₋₁₇₂ yielded residue perturbations that enabled HADDOCK simulation. The HADDOCK results revealed that *EcAhpC* binds *EcAhpF*₁₋₂₁₂ via a distinct epitope (Figure 3.40). The epitope consists of the helix $\alpha 6$ from one monomer of *EcAhpC* dimer and helix $\alpha 2'$ from the remaining monomer, these two helices are linked by the loop region S160-V165. The contribution of each monomer of the AhpC dimer to the formation of the binding epitope as observed above underscores the essentiality of the dimer as a basic functional unit [67, 82]. Besides the identified core binding domain, the docking simulation has revealed a reasonable binding site for the flexible C-terminal tail of *EcAhpC* on *EcAhpF*. A groove runs across the backside of the *EcAhpF*₁₋₂₁₂ crystal structure (Figure 3.24). The docked *EcAhpF*₁₋₂₁₂-*EcAhpC*₁₇₂ complex is such that the disulphide bond formation between C_R166' and C_P47, shown as a *brown sphere* in Figure 3.40, comes close to the groove allowing manual docking of the short peptide ¹⁶⁷PAKWKE¹⁷² into the early part of the groove (Figure 3.40A). In figure 3.40B (*cartoon*) and figure 3.40D (*surface representation*) it is shown that the entire C-

terminal peptide $^{167}\text{PAKWKEGEATLAPSLDLVGKI}^{187}$ can be well-accommodated in the groove. The peptide in the minimized *EcAhpF*₁₋₂₁₂-peptide complex can equally be well accommodated (Figure 3.42). This mode of binding is also reported in eukaryotic Prx. The Prx-Srx [132] complex in figure 4.4B revealed that the C-terminal region of Prx pack at the backside of the Srx for stabilization of the complex. In both the proposed *EcAhpF*₁₋₂₁₂-*EcAhpC* and the Prx-Srx complex, the thioredoxin β sheets play an important role to create the groove for the C-terminal tail (Figure 4.4. (Inset)).

Figure 4.4 Role of the AhpC C-terminus in the binding of peroxiredoxins and their partner proteins. (A) The proposed binding mode of bacterial AhpC and the NTD of *EcAhpF* where the C-terminus of the former wraps around the backside of the later. (B) Crystal structure (PDB ID:2RII)[132] of the eukaryotic peroxiredoxin in complex with sulfiredoxin where the former uses the C-terminus to wrap around the back surface of the later. The insets underscore the importance of the anti-parallel beta sheets in defining the backside groove.

Jonsson and colleagues stipulated that the active site interfaces of the complex alone are insufficient for prolonged interactions and hence the necessity of the packing described above [149]. In this thesis, it has been established that the C-terminal packing at the backside of AhpF prolongs the half-life of the *EcAhpC*-*EcAhpF* complex in solution and that this may be necessary for overall catalytic activity and it may be conserved among Prx and peroxiredoxin reductase interactions. The broad observations make it plausible to suggest that the C-terminal packing may represent an

Figure 4.5 Amino acid residue conservation in the EcAhpF₁₋₂₁₂ domain. (A) Multiple sequence alignment of the EcAhpF₁₋₂₁₂ domain of *E. coli* with other species. Strictly conserved and highly conserved residues are boxed in blue. Residues with identity of 100% are shown with a red background and conservation of 90% or higher are shown with a white background.

Figure 4.6: The surfaces conservation of *EcAhpF*₁₋₂₁₂ are mapped on the crystal structure. To illustrate the surface conservation of the region around the catalytic sites, two different orientations have been shown. The backside groove is indicated on the structure(PDB ID: 4O5Q) [67]

important step required for efficient catalysis to take place in the 2-Cys Prxs. Moreover, sequence alignments indicate that the identified core binding interface is evolutionary conserved as can be seen in Figure 4.5 and Figure 4.6. Residues between F120 and N139 near the catalytic sites and a second region $_{77}\text{FAGxPxGHEFxT/SSLxLALL}_{94}$ in the multiple sequence alignment of the *EcAhpF*₁₋₂₁₂ with representative sequences from the bacterial domain are highly conserved and form a contiguous patch on the surface of the protein as shown in Figure 4.6. These regions and other conserved residues may have co-evolved to participate in binding of the N-terminal domain of *EcAhpF* with *EcAhpC*. Additionally, the strictly conserved residues on *EcAhpF*₁₋₂₁₂ form a hydrophobic core (Figure 4.6 (*left*)) on the binding interface. Surface electrostatic calculation (Figure 4.7) also reveals an hydrophobic patch at the binding interface. This hydrophobicity may play an important role in the *EcAhpC-EcAhpF* interaction as seen in the endothermic profile from the isothermal titration calorimetry studies in Figure 3.12. Furthermore, studies of several redox protein complexes have revealed that hydrophobic interaction is essential for the formation of a well-defined complex [150]. This is because unlike electrostatic interactions which can occur over a long range and hence can lead to non-specific complexes, two proteins interacting hydrophobically are very intimate and this is good for redox proteins as per Marcus theory of electron transfer, which states that the probability for an electron to be transferred from a donor to an acceptor during a transition state decreases with increasing distance between the two centres [151]. So to benefit from both interactions some proteins limit the hydrophobic patch to the entry

Figure 4.7: The electrostatic surface potential of the *EcAhpF*₁₋₂₁₂. The hydrophobic patch on the proposed binding surface explains the endothermic nature of the *EcAhpF*₁₋₂₁₂ and *EcAhpC* interaction (PDB ID: 4O5Q) [67].

and exit port of electrons and surround them with polar and charged residues. So in effect, the electrostatic interaction will speed up association rate whilst the hydrophobic interaction ensures rapid electron transfer.

4.5 An efficient “bind and release” mechanism mediated by the *EcAhpC* C-terminal conformational change

By comparing the observed binding epitope of the AhpR system in the crystal structure of the oxidized and reduced *EcAhpC*, it can be observed that there is a significant conformational change, which occurs during oxidation and reduction (Figure 4.8). Using ITC, it has been confirmed that reduction of the binding epitope of *EcAhpC* abolishes binding with the NTD of *EcAhpF* altogether (Figure 3.43). This observation may be a critical step in the catalytic cycle. There have been attempts to understand the kinetics of the *EcAhpF* and *EcAhpC* binding step [152-154]. In *Salmonella typhimurium*, the rate constant of the electron transfer between a prereduced S128W NTD and the AhpC was calculated to be $2.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ [154]. This translates into an apparent rate of electron transfer of approximately 70 s^{-1} using equation 1.

$$k_{app} = k_2 [\text{S128W NTD}] \quad (1)$$

where k_2 is the true second order rate constant ($2.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) and k_{app} is the pseudo-first order rate constant with the concentration of S128W NTD protein deliberately set to be greater than AhpC [154]. This rate of electron transfer is likely to be similar to that of the homologous *E. coli* NTD and AhpC. The caveat here is that to

ensure efficient electron transfer between the two redox centres in *EcAhpC* and *EcAhpF*, the dissociation rate (k_{off}) of the complex should be slow enough to allow electrons to be transferred efficiently without decreasing the overall catalytic turnover rate. In other words, k_{off} cannot be greater than the rate of electron transfer but should be fast enough not to be a rate limiting step. But it is observed from the NMR studies that the dissociation is slow due to the packing of the

C-terminal of *EcAhpC* onto the backside of *EcAhpF*. This may ensure an efficient electron transfer between the redox centres (Figure 4.9B). But to ensure that the slow k_{off} does not become a rate limiting step, the AhpR-system may have evolved a release mechanism mediated by the well-described C-terminal conformational change of *EcAhpC* upon reduction, hence the absence of binding in the ITC titration under reducing conditions (Figure 4.8 and 4.9B) [67, 155].

Figure 4.8: Binding thermodynamics and the redox-state of *EcAhpC*. The proposed binding epitope of *EcAhpC* in its intact oxidized state (*left*) and in its reduced state (*right*) (PDB ID: 1N8J), showing the conformational change accompanying its reduction. The C-terminal region in the crystal structure of the oxidized AhpC is missing due to its high flexibility. The superimposed structures are shown *below*.

In other words, during the conformational change where the catalytic disulphide ($C_P S-SC_R$) bond is reduced the cysteines “swing” about 10 Å away from each other likely accompanied by the unpacking of the extreme C-terminal tail from the back surface of the NTD and subsequent release of the proteins from each other. The released proteins undergo a regeneration step (Figure 4.9C-D)), where the NTD of AhpF receive

Figure 4.9: Proposed binding and release mechanism of the AhpR cycle. (A) In the oxidized state, the C-terminus of *EcAhpC* is highly flexible and residues following the disulphide bond (*spheres*) could not be determined in the crystallographic structure (PDB ID: 4O5R). The interaction between the NTD of *EcAhpF* and *EcAhpC* is specific and long-lived as a result of a well-defined core binding epitope of *EcAhpC* and the packing of the *EcAhpC* C-terminus on the backside of the NTD. (B) This is to ensure efficient transfer of electrons between their catalytic centers (*inset*). Upon reduction, *EcAhpC* undergoes conformational change at the C-terminus as shown by the superimposition of the reduced (*turquoise*) and oxidized (*brown*) structures (C). This disruption of the binding epitope is likely accompanied by the unpacking of the C-terminus from the backside of the NTD, leading to the release of the proteins from each other. (D) The released proteins are shown undergoing the regeneration steps, where the now reduced *EcAhpC* is able to scavenge peroxides and the oxidized NTD is proposed to assume a closed conformation, in order to receive electrons from its C-terminal domain of *EcAhpF*.

electrons from NADH via the C-terminal domain and AhpC becomes oxidised by donating its electrons to scavenge ROOH. This mode of interaction represents a very efficient binding mechanism where electrons are efficiently transferred without compromising the catalytic turnover rate.

In the future, mutations at the backside of the NTD will be made, and their effects analysed both *in vitro* and *in vivo*.

4.6 Structural evolution of AhpF towards a highly specific, well-coordinated and efficient enzyme.

EcAhpF is composed of an NTD and a CTD (Figure 4.5A) [50, 56]. The NTD which interacts directly with the AhpC belongs to a unique group of thioredoxin-like proteins which include the archaea protein disulphide oxidoreductases (PDO) and the bacteria glutaredoxins [155]. Structural analysis revealed that this class of thioredoxin-like proteins is made of two thioredoxin folds [156]. Thus, a gene duplication and fusion might have occurred in an ancient thioredoxin molecule. Using the MEGA software [100], a phylogenetic tree has been generated using representative homologue proteins from archaea and bacteria (Figure 4.10). Since the most ancient of the proteins currently known is the archaea PDO, it is highly likely that an ancient hyperthermophilic archaea thioredoxin may have undergone a gene duplication and fusion event (Figure 4.10). It has been proposed that this duplication followed by the covalent linkage of the two thioredoxin folds may have been a strategic stabilization attempt as oligomerization was used by hyperthermophilic organisms to stabilize its proteins under the extreme conditions in which they are found [157, 158]. At this juncture each of the thioredoxin folds, namely, the “a” and the “b” folds, retain their catalytic –CXXC– motifs as is seen in the *Pyrococcus furiosus* PDO and are also broadly specific for other proteins as they possess oxidative, reductive (fold “a” catalytic site) and isomerase (both fold “a” and fold “b”) functionalities [159]. Down the evolutionary tree, it is highly likely that this thermostable protein was horizontally passed on from archaea to other thermophilic bacteria like the *T. terrenum*, *A. aeolicus* etc. (Figure 4.10). This archaeon to bacteria transfer may have set the stage for the evolution of this unique protein fold in non-extremophile bacteria like *E. coli* accompanied by significant structural and functional changes. Structural comparison reveals that the fold “b” has lost the –CXXC– catalytic moiety and in essence lost its isomerase activity. Some major structural changes of the archaea to bacteria transition

Figure 4.10: Molecular phylogenetic analysis by Maximum Likelihood method for the "NTD" of AhpF. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis involved 8 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 185 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. A gene duplication of a thioredoxin moiety gave rise to a two-fold PDO which subsequently evolved into ancestral protein of the present-day "NTD" domain, which upon fusing with a thioredoxin reductase homologue protein gave rise to AhpF[100].

are the shortening of the $\beta 7$ -coil- $\beta 8$ region (Figure 4.10A (region I)) and the loss of a helical region (Figure 4.10A (region II)) between the beta sheets $\beta 2$ and $\beta 3$ in the case of the “NTD” of *EcAhpF*. These together with other structural changes have refined the observed binding groove of the AhpC C-terminus at the backside of the NTD (Figure 4.10B-D). It is possible that the bacterial AhpC may have co-evolved with the NTD to ensure such a binding complementarity and hence the narrow and high specificity of the NTD for the bacterial AhpC. The fusion of the “NTD” with a thioredoxin reductase homologue (“CTD”) to form the AhpF ensures better coordination of electron transfer among the partner proteins[67]. Thus, after the NTD has reduced the AhpC, it becomes oxidized and needs to be replenished by a reductase. The presence of a dedicated reductase appended to it ensures faster electron transfer and higher overall catalytic efficiency.

Figure 4.11: Structural changes and evolution of the backside groove of the NTD of AhpF. (A) Superimposition of the crystal structures of distant homologue proteins of the NTD of *EcAhpF*. A major structural change of the archaea-bacteria transition is the shortening of the $\beta 7$ -coil- $\beta 8$ region (region I). Further evolution resulted in the lost $\alpha 3$ (region II). Comparing the topology of the backside groove of the archaea (B) thermophilic bacteria (C) and “NTD” of *EcAhpF* (D) reveals the definition of a distinct groove over time.

4.7 The influence of iconicity on oligomerization and enzyme activity

It has been established that the reduced AhpC forms a decamer and this is stabilized by the C-terminal tail. Whereas the importance of the C-terminal tail has been established in the binding to the NTD, its high flexibility in the free AhpC in solution makes it difficult to observed from the crystallographic structure. Ionic strength has been implicated in oligomerization and higher activity of peroxiredoxins and their reductases [134, 160]. To gain insight into this property, a 320 mM ammonium sulphate buffer system has been employed. In the absence of ammonium sulphate, AhpC exists as lower oligomers (Figure 3.29). However, the presence of ammonium sulphate drives the oligomerization of AhpC into a decamer (Figure 3.46).

The polar nature of the decamer interface implies that the presence of salt molecules will compete for water molecules which keep the decamer interface in solution thus shifting the dimer to decamer equilibrium more to the decamer. Using SAXS the first insight into the solution state of AhpC has been obtained. By using CRY SOL, insight into the flexible C-terminal tail is revealed (Figure 4.12). The highly exposed nature of the C-terminus makes it possible for easy contact with the NTD in solution. In furtherance, a stable complex between the full-length *EcAhpC* and *EcAhpF* has been obtained in solution (Figure 3.29C-D). A model of the complex in solution has been made as described in the results (Figure 3.34). The orientation of the NTD is similar to the docking experiment obtained from the NMR experiment. Most interestingly, when the C-terminal truncated *EcAhpC*₁₋₁₇₂ was used instead of the *EcAhpC*, no stable complex was formed even in the presence of ammonium sulphate salt (figure 3.29C). This observation once again underscores the importance of the C-terminal in stabilizing the *EcAhpF*-*EcAhpC* complex.

Figure 4.12 A novel insight into the flexible C-terminal tail of oxidized *EcAhpC* from the modelled using CRY SOL. The *red* beads represent the C-terminus and the *green* beads represent the N-terminal 6xHis-tag. The solution-exposed and accessibility of the C-terminal tail ensure makes it possible for it to directly make contact with the NTD in solution.

5. Conclusions

Conclusions

The AhpR of bacteria is a robust and efficient enzyme complex that scavenges hydrogen peroxide and is also a dedicated quencher of organic peroxides [1]. Whereas interest in this enzyme system is growing, mechanistic understanding of the efficiency of this enzyme system is still lacking. In this thesis, systematic efforts have been made to unravel the role of the C-terminus of AhpC and to understand the binding mechanism of the AhpR-complex.

By generating C-terminally truncated constructs of *EcAhpC*, it has been uncovered that the C-terminus of AhpC regulates its oligomerization under reducing conditions. Thus, the truncations and mutations destabilize the decameric forms in solution. Critically examining the reduced structure of *EcAhpC* reveals a salt-bridge-stabilized “hook-like” structure at the extreme end of the C-terminus making it possible for other C-terminal residues to interact and stabilize the decameric interface. Furthermore, NADH-dependent peroxidase activity assays revealed a reduction of catalytic efficiency of the C-terminal mutants. *EcAhpF* constructs were also generated and the solution shapes of each domain have been confirmed by employing SAXS. Importantly the first solution structure of the CTD dimer was observed. By employing ITC, I have empirically proven that the AhpF interacts with the AhpC via its NTD and the interaction between the NTD and the AhpC is entropically driven with a K_d of 3.2 μM . By assigning the residues of the N-terminal domain using NMR, I have performed NMR titration studies with both the full-length AhpC and the C-terminal truncate of AhpC (*EcAhpC*₁₋₁₇₂). Titration with the full length resulted in a coordinated disappearance of peaks revealing a long-lived complex (slow K_{off}), whereas the absence of the C-terminus of *EcAhpC* created a dynamic interaction with per residue perturbations. Perturbation restraints obtained were used to drive a docking simulation between the *EcAhpC*₁₋₁₇₂ and the *EcAhpF*₁₋₂₁₂ from this a plausible binding site of the C-terminal tail of *EcAhpC* was revealed in which it wraps around the backside groove of the NTD of AhpF. Furthermore, the interaction between the AhpC and AhpF has been revealed to be redox-dependant, where only oxidised AhpC is able to interact with the NTD of AhpF. Taken together, a “grab and release” mechanism has been revealed, where AhpC binds and wraps its C-terminal tail around the NTD of AhpF to slow the dissociation for effective electron transfer to occur, and a release mechanism mediated by a conformational change of the AhpC epitope upon reduction. This

mechanism ensures the reduction and/or elimination of the probabilistic and leaky nature of electron transfer without compromising catalytic turnover.

In addition, by employing high ionic buffer systems, new insight into the solution structure of the oxidized AhpC and its C-terminus has been obtained, the latter of which was lacking in available crystallographic structures. The observed solution-exposed C-terminus makes it possible for the C-terminus to make contact with the NTD of AhpF, confirming the proposed mechanism of AhpC-AhpF binding. Finally, the first low-resolution solution complex of the full-length *EcAhpC* and *EcAhpF* has been obtained.

6. **References**

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7. Author's publication related to the projects

1. Dip, P. V., Kamariah, N., Subramanian Manimekalai, M. S., **Nartey, W.**, Balakrishna, A. M., Eisenhaber, F., Eisenhaber, B., Grüber, G. "Structure, mechanism and ensemble formation of the alkyl hydroperoxide reductase subunits AhpC and AhpF from *Escherichia coli*." *Acta Crystallogr D Biol Crystallogr*, 2014. **70**(Pt 11): 2848-2862.
2. *Dip, P. V., *Kamariah, N., ***Nartey, W.**, Beushausen, C., Kostyuchenko, V. A., Ng, T. S., Lok, S. M., Saw, W. G., Eisenhaber, F., Eisenhaber, B., Grüber, G., "Key roles of the *Escherichia coli* AhpC C-terminus in assembly and catalysis of alkyl hydroperoxide reductase, an enzyme essential for the alleviation of oxidative stress." *Biochim Biophys Acta*, 2014. **1837**(12): 1932-1943
3. Kamariah, N., Subramanian Manimekalai, M. S., **Nartey, W.**, Eisenhaber, F., Eisenhaber, B., Grüber, G. (2015) *Crystallographic and solution studies of NAD (+) - and NADH-bound alkyl hydroperoxide reductase subunit F (AhpF) from Escherichia coli provide insight into sequential enzymatic steps*. *Biochim Biophys Acta*, 2015. **1847**(10): p. 1139-1152.
4. **Nartey, W.**, Basak, S, Kamariah, N., Subramanian Manimekalai, M. S., Scott, R., Wagner, G., Eisenhaber, B., Eisenhaber, F., Grüber, G. *NMR studies reveal a novel grab and release mechanism for efficient catalysis of the bacterial 2-Cys peroxiredoxin machinery*. *Febs J*, 2015 **282**(23):4620-38
5. *Kamariah, N., ***Nartey, W.**, Eisenhaber, B., Eisenhaber, F., Grüber, G, Low-resolution solution structure of an enzymatic active AhpC₁₀:AhpF₂ ensemble of the *Escherichia coli* Alkyl hydroperoxide Reductase. *J Struct Biol*, 2015 **193**: 13-22

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8. Posters and Abstracts

1. **Nartey, W.**, Basak, S, Kamariah, N., and Grüber, G, "Mechanistic understanding of the interaction between the N-terminal domain of alkyl hydroperoxide reductase subunit F and C" (2014). The 7th Asia-Pacific Organization for Cell Biology Congress and American Society for Cell Biology, Singapore.
2. **Nartey, W.**, Basak, S, Kamariah, N., and Grüber, G, "Probing the interaction between the N-terminal domain of *Escherichia coli* AhpF and AhpC, 17th European Bioenergetics Conference (EBEC), Lisbon University of Lisboa, Portugal from 9th – 14th Sep. 2014, , 07/2014; **1837**:e63-e64 DOI: 10.1016/j.bbabbio.2014.05.242

9. Awards

Singapore International Graduate Award (SINGA). (Scholar Ref Number: 20112012A10907)