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Effect of the additional cysteine 503 of vancomycin-resistant *Enterococcus faecalis* (V583) alkylhydroperoxide reductase subunit F (AhpF) and the mechanism of AhpF and subunit C assembling

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Abstract

The vancomycin-resistant *Enterococcus faecalis* alkyl hydroperoxide reductase complex (AhpR) with its subunits AhpC (*EfAhpC*) and AhpF (*EfAhpF*) is of paramount importance to restore redox homeostasis. Therefore, knowledge about this defense system is essential to understand its antibiotic-resistance and survival in hosts. Recently, we described the crystallographic structures of *EfAhpC*, the two-fold thioredoxin-like domain of *EfAhpF*, the novel phenomenon of swapping of the catalytic domains of *EfAhpF* as well as the unique linker length, connecting the catalytically active N- and C-terminal domains of *EfAhpF*. Here, using mutagenesis and enzymatic studies, we reveal the effect of an additional third cysteine (C503) in *EfAhpF*, which might optimize the functional adaptation of the *E. faecalis* enzyme under various physiological conditions. The crystal structure and solution NMR data of the engineered C503A mutant of the thioredoxin-like domain of *EfAhpF* were used to describe alterations in the environment of the additional cysteine residue during modulation of the redox-state. To glean insight into the epitope and mechanism of *EfAhpF* and -AhpC interaction as well as the electron transfer from the thioredoxin-like domain of *EfAhpF* to AhpC, NMR-titration experiments were performed, showing a coordinated disappearance of peaks in the thioredoxin-like domain of *EfAhpF* in the presence of full length *EfAhpC*, and indicating a stable *EfAhpF*-AhpC-complex. Combined with docking studies, the interacting residues of *EfAhpF* were identified and a mechanism of electron transfer of the *EfAhpC* donor to the electron acceptor *EfAhpF* is described.

1. Introduction

Enterococcus faecalis is a ubiquitous and opportunistic gram-positive microorganism responsible for 90% of nosocomial infections [1]. Compounding the pathogenicity of *E. faecalis* is its rapid development of resistance towards antimicrobial agents, the most common being the Vancomycin-Resistant Enterococcus (VRE) [2-3]. *E. faecalis* has evolved numerous virulence factors that ensure its survivability under harsh conditions of including reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) inside macrophages [2,3]. The ability of *E. faecalis* to survive the intolerant ROS condition of the macrophage implies the presence of robust antioxidant systems [4] to which the Alkyl hydroperoxide reductase complex (AhpR) does belong too, which may serve as therapeutic targets for the prevention of early stages of infections [5, 6].

The *E. faecalis* AhpR system is driven by two proteins, a 2-Cys peroxiredoxin, AhpC (*EfAhpC*) and a thiol-dependent peroxiredoxin reductase, AhpF (*EfAhpF*) to catalyse the NADH-dependent reduction of hydrogen peroxide [4] (Fig. 1A). The catalytic centre of AhpC is composed of two active cysteine residues; peroxidatic (C_P) and reducing (C_R). The peroxidatic cysteine residue (C_P) of one subunit of AhpC first reacts with a molecule of hydrogen peroxide to form sulfenic acid. Then a reducing cysteine residue (C_R) of the adjacent unit of AhpC attacks the sulfenic acid bond, producing water molecule and oxidised AhpC [4, 7]. As demonstrated by EM-data and the crystallographic structure, *EfAhpC* forms a decamer independent to the redox state [7] (Fig. 1A). This is unlike AhpCs of gram-negative bacteria like *Escherichia coli* (*EcAhpC*) and *Staphylococcus aureus* (*StAhpC*), which form a stable decamer in the reduced form and lower weight oligomers in the oxidized state [8-10]. In comparison, besides the two typical catalytic cysteine residues C_P and C_R , *EfAhpC* has two additional cysteines, with C13 and C66 at the N-terminus, which are important for stabilization of the decameric ring and enzyme activity [7].

Oxidised AhpC is regenerated via the reducing action of AhpF [11]. The latter is a homodimeric protein which is composed of two domains with a flexible linker in-between [12, 13]. The electron donor NADH first docks at its binding site located at the pyridine nucleotide-disulfide oxidoreductase domain (CTD:Pyr_redox_2). This sets off cascading steps of electron transfer from the NADH molecule via FAD (flavin adenine dinucleotide), a prosthetic group to the first CXXC-motif located at the same domain. The second CXXC-motif residing at the two-fold thioredoxin-like domain (NTD_N/C) will then come close to the first reduced CXXC-motif and pick up the electron. This allows the reduced second CXXC-motif to regenerate the reducing potential of oxidised AhpCs [11, 13, 14].

We had first described the unique phenomenon of AhpF protein in *E. faecalis* (V583) bacteria (*EfAhpF*), highlighting the distinct structural features such as domain swapping, and the unusual long flexible linker connecting the catalytic NTD_N/C and CTD:Pyr_redox_2 domains [13]. Our recent crystallographic structure of the *EfAhpF* NTD_N/C part unravelled a unique loop-helix stretch (₄₀₉ILKDTEPAKELLYGIEKM₄₂₆) not present in homologue domains of other prokaryotic AhpFs. Deletion of the ₄₁₅PAKELLY₄₂₁-helix affected protein stability or attenuates peroxidase activity [13].

Besides the significant differences of *EfAhpF* compared to its counterpart in other AhpFs described above, *EfAhpF* harbours one additional cysteine, C503, in the NTD_N/C domain. In analogy to the two additional cysteines (C13, C66) of *EfAhpC*, relevant for stabilization of the decameric ring and enzyme activity [7], we sought to investigate the significance of C503 of *EfAhpF* using a complementary approach of mutagenesis, enzyme assays, crystallography, and NMR solution spectroscopy. In addition, the complex formation of *EfAhpF*-AhpC is closely studied here using NMR analysis. Coupled with our previous published results, these data will lead to a better understanding of the *E. faecalis* (V583) AhpR system in *E. faecalis* (V583) which can then be exploited as a therapeutic target.

2. Materials and Methods

2.1 Cloning of *EfAhpF*, *AhpC* and their mutants

Wild type (WT) *EfAhpF* and *EfAhpF*₃₅₄₋₅₆₀ were cloned into the pET9-d1-His6 vector [15] as described in the paper [13]. Cloning of *EfAhpC* and its single mutants C13N and C66V as well as double mutant C13N/C66V was performed as described recently by Pan et al. [7]. Full-length *EfAhpF* C503A and *EfAhpF*₃₅₄₋₅₆₀ C503A were cloned with forward primer (5' – GTCGTGGCGGCTGCCCAACGTATT – 3') (underlined indicates single amino acid substitution) and reverse primer (5' – ATCGGGACAGAAATGACATGTTAGTGAAACAAAGATTTC – 3'), using respective wild-type *EfAhpF* vector as template by non-overlapping site-directed mutagenesis. The PCR products were *DpnI*-digested before purified and transformed into *E. coli* DH5α cells.

2.2 Purification of WT and mutant proteins of *E. faecalis*

WT *E. faecalis* AhpR subunits AhpC and F as well as their mutants were transformed into *E. coli* BL21 (DE3) cells (Stratagene, USA) and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20 °C (*EfAhpF*) or 37 °C (*EfAhpC*) for protein production. Cells were lysed on ice by sonication for 3 × 1 min in buffer A (50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 0.8 mM DTT and 2 mM Pefabloc^{SC} (BIOMOL)). The cell lysate was centrifuged at 10 000 × g for 35 min before the supernatant was filtered (0.45 μm; Millipore) and incubated with Ni-NTA Agarose (Qiagen) for one hour at 4 °C. His₆-tagged protein was then eluted with an imidazole gradient (20 mM to 500 mM) in Buffer A. For *EfAhpC*, pooled fractions containing the protein of interest were applied onto a gel filtration column (SuperdexTM 200 HR 10/300 column, GE Healthcare) in buffer B (50 mM Tris/HCl, pH 7.5 and 200 mM NaCl). In the case of *EfAhpF*, fractions containing the recombinant protein were pooled and applied onto an anion chromatography column ResourceQ (6 ml). Fractions

containing recombinant protein were pooled and applied onto a gel filtration column (Superdex™ 200 HR 10/300 column, GE Healthcare) in buffer B (50 mM Tris/HCl, pH 7.5 and 200 mM NaCl), and the purified recombinant protein was pooled and concentrated. All other *EfAhpF* mutants were purified according to the WT *EfAhpF* purification protocol [13].

2.3 Crystallization of *EfAhpF*₃₅₄₋₅₆₀ C503A

Recombinant *EfAhpF*₃₅₄₋₅₆₀ (NTD_N/C) C503A mutant was concentrated to 5 mg/ml in a buffer composed of 50 mM Tris/HCl, pH 7.5 and 200 mM NaCl. Hexagonal-shaped crystals were grown in precipitant solution of 0.1 M Bis-Tris (pH 6.5), 1.5 M ammonium sulfate and 0.1 M sodium chloride by hanging-drop vapor-diffusion method. The final *EfAhpF*₃₅₄₋₅₆₀ C503A crystals were flash-frozen in liquid nitrogen at 100 K in crystallization buffer containing 0.1 M Bis-Tris (pH 6.5), 2.0 M ammonium sulfate, 0.1 M sodium chloride and 2.0 M Proline.

2.4 Data collection and structure determination of *EfAhpF*₃₅₄₋₅₆₀ C503A

X-ray diffraction data for the *EfAhpF*₃₅₄₋₅₆₀ C503A were collected at 100 K at beamline 13B1 of the National Synchrotron Radiation Research Centre (NSRRC, Hsinchu, Taiwan) using the ADSC Quantum 315 CCD detector. Data were collected as a series of 0.5° oscillation images covering a crystal rotation range of 100° on cryo-cooled crystals. A complete 2.5 Å data set was collected and the diffraction data were indexed, integrated and scaled using HKL2000 suite [16]. The results of data processing and data statistics are summarized in Table 1. The crystallographic structure was solved by molecular replacement method using WT *EfAhpF*₃₅₄₋₅₆₀ structure (PDB ID: 5H29) [13] as model in PHASER [17]. The starting model was improved by iterative cycles of model building manually using Coot [18] and refinement by REFMAC5 [19] of CCP4 suite. Refinement was done until

convergence and the geometry of the final model was validated with MolProbity [20]. This analysis indicated that the overall geometry of the final model ranked in the 100th percentile (MolProbity score of 1.23). The clash score for all atoms was 1.22 corresponding to a 100th percentile ranking of structures of comparable resolution. The data were cut off at 2.5 Å based on the CC_{1/2} statistics [20] (correlation coefficient between two random halves of the data set where CC_{1/2} > 10%) to determine the high-resolution cut-off for our data. Phenix [21] was used to compute CC_{1/2} (97.5% for the highest resolution shell and 99.9% for the entire data set), supporting our high-resolution cut-off determination. The figures were drawn using PyMOL [22] and structural comparison analysis was carried out using SUPERPOSE [23] from CCP4 suite [24, 25]. The coordinates and structure factors of *EfAhpF*₃₅₄₋₅₆₀ C503A at 2.5 Å have been deposited in the Protein Data Bank with accession code [6IL7].

2.5 NADH-dependent peroxidase assay

NADH-dependent peroxidase assay was monitored at 340 nm following the decrease in NADH absorbance using a stopped flow spectrophotometer Applied Photophysics SX20. The assay was carried out at 25 °C containing a 100 mM phosphate buffer at pH 7.0, 200 mM ammonium sulfate and 1 mM EDTA. One syringe arm contained a final concentration of 200 µM of NADH, 100 µM of H₂O₂, increasing concentration of AhpC [1 - 80 µM], and the other arm contained a final concentration of 100 nM WT *EfAhpF* or *EfAhpF* C503A mutant. The assay was done in triplicates. Michaelis-Menten plots were created using program Origin Pro 9.0 (OriginLab Corporation).

2.6 Ferrous oxidation xylenol orange (FOX) assay

Hydrogen peroxide reduction catalysed by *EfAhpC* in the presence of *EfAhpF* was measured in PeroxiDetect™ kit by Sigma-Aldrich®. The kit is based on the principle of

ferrous oxidation xylenol orange assay. 100 μ l of reaction mixture containing 50 mM HEPES buffer pH 7.0, 100 μ M H₂O₂, 250 μ M NADH, 15 μ M WT *EfAhpC* or *EfAhpC* mutants and 0.4 μ M WT *EfAhpF* or *EfAhpF* C503A were prepared from which 10 μ l of aliquots were removed at specific time intervals and added to 190 μ l of FOX reagent before incubating for at least 30 mins at room temperature. The absorbance of the sample was then measured at λ = 560 nm for detection of H₂O₂.

2.7 NMR analysis of *EfAhpF*₃₅₄₋₅₆₀

0.7 mM of ¹⁵N/¹³C double-labelled *EfAhpF*₃₁₄₋₅₆₀ was prepared in 50 mM sodium phosphate pH 6.8, 50 mM NaCl, 10% D₂O in the presence or absence of 2 mM TCEP (tris(2-carboxyethyl)phosphine) according to the purification method described above. NMR measurements were performed on 700 MHz Bruker Avance NMR spectrometer equipped with 5 mm triple-resonance (¹H/¹⁵N/¹³C) single axis gradient cryogenic probes at 298K.

To assign the backbone of *EfAhpF*₃₅₄₋₅₆₀, both uniform (traditional) and non-uniform sampling (NUS; [26]) techniques were employed to acquire the data. 2D-¹H-¹⁵N TROSY-HSQC data were collected with conventional linear sampling technique, whereas all triple resonance experiments, including TROSY-HNCACB, 3D-TROSY-HN(CO)CACB, CBCA(CO)NH, HNCA and HN(CO)CA data were collected using NUS techniques of the indirect dimension as 20% sampling rates and reconstructed using compressed sensing (CS) scheme using MDDNMR software [27,28]. All NMR-spectra were processed using NMRpipe [29] and analyzed with SPARKY program [30]. Both techniques enabled the assignment of 90% of the backbone resonances (N, CA, HN).

Titration of 0.3 mM uniformly ¹⁵N-labelled *EfAhpF*₃₅₄₋₅₆₀ or *EfAhpF*-C503A₃₅₄₋₅₆₀ with oxidized *EfAhpC* was performed to achieve incremental molar equivalents of 0.05 and 0.1. ¹H-¹⁵N TROSY-HSQC spectra were recorded with 2,048 complex data points in *t*₂ and 256

*t*₁ increments at molar ratios of 1 (*EfAhpF*) : 0 (*EfAhpC*), 1:0.05 and 1:0.1, respectively. Spectral width of 2,270 and 11,160 Hz were employed in F1(¹⁵N) and F2(¹H), respectively. The combined chemical shift perturbation, the peak intensity and line width informations were extracted to determine molecular interaction between two proteins.

2.8 High Ambiguity Driven DOCKing(HADDOCK) modeling of *EfAhpF*₃₅₄₋₅₆₀ and *EfAhpC*₁₋₁₆₇

Experimentally identified binding interfacial residues of *EfAhpF*₃₅₄₋₅₆₀, excluding the extra flexible loop from amino acids A314-Q353, were used to perform docking studies, and to model the complex of *EfAhpF*₃₅₄₋₅₆₀ and *EfAhpC*₁₋₁₆₇ using the HADDOCK webserver [31]. The experimentally determined crystal structures for the *EfAhpF*₃₅₄₋₅₆₀ and *EfAhpC*₁₋₁₆₇ dimer were used for docking simulation. Surface exposed residues experiencing intensity and linewidth changes 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 *EfAhpC*₁₋₁₆₇, a dimeric unit of coordinates from the crystal structure were submitted to the meta-PPISP for protein–protein interfacial residues prediction [32]. These predicted interfacial residues were set as “active” residues. These restraints were used as input for the HADDOCK webserver and default parameters were used for docking. The resulting structures were clustered by a cut-off value (7.5 Å). Finally, based on the HADDOCK score and total energy, the best model from the highest scoring cluster was selected for analysis. The model was visualized using the PyMOL software [22].

3. Results

3.1 Sequence analysis of *E. faecalis* AhpF's two-fold thioredoxin-like domain (NTD_N/C)

Inspection of the *EfAhpF* protein sequence showed that its two-fold thioredoxin-like domain (NTD_N/C) contains an additional cysteine residue at position 503 in helix $\alpha 6$ (Fig. 1B). To investigate whether the occurrence of this additional cysteine residue is predominant in AhpFs of other species, a protein sequence alignment of AhpF NTD_N/C domains of ten different species was performed. It was shown that domain swapping is a unique feature of *EfAhpF* where the NTD_N/C is located at the C-terminus and the pyridine nucleotide-disulfide oxidoreductase domain (CTD:Pyr_redox_2) is located at the N-terminus [13]. Therefore, AhpF NTD_N/C domains of five different species displaying the '*E. faecalis*-like' AhpF domain localization (C-terminus) and AhpF NTD_N/C domains of another five different species showing '*E. coli*-like' AhpF domain localization (N-terminus) were chosen for this sequence alignment. The alignment indicates that the additional cysteine at position 503 is conserved across several species belonging to the '*E. faecalis*-like' subgroup, *E. faecalis* (V583), *L. reuteri* (SD2112) and *C. stercorarium* (DSM8532), but is not present in the *Bacillus* species and all sequences of the '*E. coli*-like' subgroup (Fig. 1B, red box). Interestingly, both *B. bifidum* (BGN4) and *B. longum* (157F) NTD_N/C harbour two additional cysteine residues in position 496 and 609 according to *B. bifidum* (BGN4) numbering. To note, in all AhpF NTD_N/C domains of the species belonging to the '*E. coli*-like' subgroup the cysteine residue is replaced by a leucine residue.

3.2 Importance of the extra cysteine 503 in *EfAhpF*

To investigate the importance of the extra cysteine C503, this residue was mutated to alanine (C503A) in the full-length *EfAhpF* construct. Kinetic studies using NADH-based

oxidation assay on stopped-flow spectrometer was performed (Fig. 2). WT *EfAhpF* displays a k_{cat} of $46.1 \pm 4 \text{ s}^{-1}$ and a K_m (AhpC) of $24.5 \pm 5 \text{ }\mu\text{M}$, giving it an enzymatic efficiency, k_{cat}/K_m (AhpC) of $1.9 \times 10^6 \text{ M}^{-1}\cdot\text{s}^{-1}$ (Fig. 2B). In contrast, the *EfAhpF* C503A mutant has a similar k_{cat} of $49.2 \pm 6 \text{ s}^{-1}$ but an ~ 3 -fold higher K_m (AhpC) of $70.9 \pm 12 \text{ }\mu\text{M}$, which translates to a nearly 27-fold lower catalytic efficiency of *EfAhpF* C503A where k_{cat}/K_m (AhpC) is $0.7 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$ (Table. 2) (Fig. 2B). The increased K_m (AhpC) of *EfAhpF* C503A suggests that the mutation leads to a negative effect on its interaction with *EfAhpC*.

To further confirm that the C503 mutation affects the reduction of *EfAhpC*, a FOX assay was performed to measure the ability of *EfAhpF* C503A together with *EfAhpC* to reduce H_2O_2 (Fig. 3A). WT *EfAhpF* reduced $5 \text{ }\mu\text{M}$ of H_2O_2 in about 1 min., whereas *EfAhpF* C503A reduced the same concentration of H_2O_2 in about 3 mins. This correlates with the stopped-flow kinetic studies, showing that C503 is important for the catalytic activity of *EfAhpF*.

Our recent studies revealed, that extra cysteine residues from *EfAhpC* (C13 and C66) have an important role in the *EfAhpR* system as mutation of these cysteine residues to its equivalent residues in *E. coli* AhpC (C13N and C66V) adversely affected the *EfAhpR* system to reduce H_2O_2 [7]. Here, *EfAhpF* C503A was analysed by the FOX assay in the presence of the *EfAhpC* mutants C13N (*EfAhpC* C13N), *EfAhpC* C66V, and the double mutant *EfAhpC* C13N/C66, respectively (Fig. 3B). When compared to *EfAhpC*-AhpF C503A, the mutant ensemble *EfAhpC* C66V-AhpF C503A revealed a reduction in H_2O_2 decomposition which became even more prominent for mutant complexes *EfAhpC* C13N-AhpF C503A followed by *EfAhpC* C13N/C66-AhpF C503A, indicating an additive effect on the *EfAhpC* cysteine mutants' ability to reduce H_2O_2 .

3.3 Crystallographic structure of the *EfAhpF*₃₅₄₋₅₆₀ C503A mutant

To investigate whether the substitution of C503 to alanine causes a structural change in *EfAhpF* NTD_N/C, we crystallized *EfAhpF*₃₅₄₋₅₆₀ C503A in the same crystallisation solution as WT *EfAhpF*₃₅₄₋₅₆₀ [13]. Crystals grew to the same cubic space group P4₁32 and the determined atomic structure was refined to 2.5 Å resolution. The final R-factor and R-free (calculated with 5% of reflections that were not included in the refinement) were 21.47% and 26.72%, respectively (Table 1). There is one molecule of *EfAhpF*₃₅₄₋₅₆₀ C503A in the asymmetric unit with a solvent content of 72.06%. The *EfAhpF*₃₅₄₋₅₆₀ C503A monomer contained 207 amino acid residues with almost all main chain residues fitting well into the electron density. The *EfAhpF*₃₅₄₋₅₆₀ C503A monomer also contained three molecules of SO₄ and 67 molecules of water (Table 1). The overall structure of *EfAhpF*₃₅₄₋₅₆₀ C503A is shown in figure 4A. The side chain density for all the amino acids is very well resolved. The average B-factor was calculated to be 61.02 Å². Analysis of the stereo-chemical quality of the final model by PROCHECK has identified that 98.1% of all the residues are within the favoured regions of the Ramachandran plot, 1.9% are within the generously allowed regions. There were no residues in the disallowed region.

When *EfAhpF*₃₅₄₋₅₆₀ C503A was overlaid with WT *EfAhpF*₃₅₄₋₅₆₀, it aligned well with an r.m.s.d of 0.1 Å for 155 Cα atoms (Fig. 4B). Careful inspection of the structural superimposition of both crystallographic structures did not reveal any structural alterations due to the mutation of C503.

3.4 Redox-dependent modulation within *EfAhpF*₃₅₄₋₅₆₀ C503A

While the atomic structure of *EfAhpF*₃₅₄₋₅₆₀ C503A did not show a change due to the mutation made, the question was addressed whether redox processes may alter the position of C503 and neighbouring residues in solution. Therefore, WT *EfAhpF*₃₅₄₋₅₆₀ was studied in

NMR solution studies in the presence and absence of the reducing agent TCEP. As shown in figure 5A, uniformly labelled *EfAhpF*₃₅₄₋₅₆₀ resulted in a well-dispersed ¹H-¹⁵N HSQC-spectrum of the protein (Fig. 5), indicating proper folding and monodispersity of the recombinant protein. High mobility of the linker region (A314 to Q353), which is included in this construct, gave intense and sharp peaks, which in some cases overlap with other less intense signals. However, this did not affect the overall quality of the spectrum. Sequential backbone assignment based on the triple resonance NMR data, including HNCACB, HNCOCACB, HNCA and HNCOCA enabled the assignment of ¹H and ¹⁵N resonances for 204 residues out of 234 non-proline residues of *EfAhpF*₃₅₄₋₅₆₀. The secondary structure prediction of *EfAhpF*₃₅₄₋₅₆₀ was performed for the residues W354-A560 based on assigned C α -chemical shifts, which were subtracted from their random coil values (Δ C α). The prediction is highly consistent with the *EfAhpF*₃₅₄₋₅₆₀ crystal structure and reveals eight α -helices and nine β -strands (Fig. 5B).

In comparison, addition of TCEP caused chemical shifts in the ¹H-¹⁵N HSQC-spectrum of *EfAhpF*₃₅₄₋₅₆₀ (Supplementary Figure S1 and Fig. 5C). Based on the chemical shift perturbation (CSP) analysis between reduced and oxidized *EfAhpF*₃₁₄₋₅₆₀, the residues W354, S428, G445, G449, E451-N453, F488, C496, I499-C503, I506, H512, V518, V535, D541, and G548 undergo significant alteration (>0.1) due to an alteration of the redox-state (Fig. 5D). As shown in figure 5E, the identified residues of *EfAhpF*₃₅₄₋₅₆₀ involved in redox-dependent modulation form an epitope based on the neighbouring helices α 4 and α 6 as well as the β -sheets 5 and 6, including C503 of helix α 6.

3.5 NMR characterisation of a stable *EfAhpC-EfAhpF*₃₅₄₋₅₆₀ complex formation

Previous studies have shown that AhpF transfers electrons to AhpC through interaction with its thioredoxin-like domain (NTD_N/C) [9, 33]. To understand this interaction and

decipher the binding epitope, NMR titration experiments were performed, in which uniformly labelled *EfAhpF*₃₅₄₋₅₆₀ was titrated against *EfAhpC*. The highly resolved and dispersed NMR-spectrum of *EfAhpF*₃₅₄₋₅₆₀ mentioned above (Fig. 5A) enabled titration experiments to be performed. The ¹H-¹⁵N-TROSY-HSQC spectrum was obtained for the labelled *EfAhpF*₃₅₄₋₅₆₀ protein alone as well as in the presence of increasing amounts of the partner protein *EfAhpC* (Fig. 6A). During titration experiments, we observed the gradual disappearance or decrease of overall cross peak intensities and line broadening in the ¹H-¹⁵N-TROSY-HSQC spectra (Fig. 6A-B). Due to the large molecular weight of the *EfAhpF*₃₅₄₋₅₆₀-*EfAhpC* complex, the majority of *EfAhpF*-NTD peaks gradually disappeared, suggesting a global decrease in tumbling, consistent with a stable interaction. Since *EfAhpC* forms a decamer in solution (about 210 kDa), the above mentioned observation is consistent with a stable complex. However, peaks from amino acid residues in the highly flexible linker region, residues, A314-Q353 showed relatively higher intensities compared with the core part of the protein (W354-A560), indicating that the flexible linker region of the *EfAhpF* construct was not affected by the interaction with *EfAhpC* and exhibits a local tumbling motion independent of the global motion of the complex.

3.6 Binding interface of the *EfAhpF*₃₅₄₋₅₆₀-AhpC complex

To investigate the molecular interaction between *EfAhpF*₃₅₄₋₅₆₀ and *EfAhpC* in more detail, NMR-titration data were analyzed in terms of the peak intensities and the line width changes of the NMR spectra between *EfAhpF* and *EfAhpC* in the ratio 1:0 and 1:0.1, respectively. The NMR spectra show the significant disappearances or decrease of some cross peak intensities in the ¹H-¹⁵N TROSY-HSQC spectra compared with other residues (Fig. 6A-B). Plots of intensity ratio (I_0/I_1) before (I_0) and after (I_1) for AhpC (I_0/I_1) and increased line width for indirect ¹⁵N dimension at a molar ratio 1:0.1 are shown in the figure

7A-B. They reveal the candidates of the binding surface of *EfAhpF*₃₅₄₋₅₆₀ when interacting with *EfAhpC*. Significant changes in intensity ratio and linewidth over standard deviation were observed for the backbone resonances of residues G449-L455 (Fig. 7A), indicating that these residues are considered as directly involved in the interaction with *EfAhpC*. Amino acids F355, R360, Q362, F367, I444, I446, G449-L455, Q467, A502, and I506 show intensity changes reflected by the standard deviation values after addition of *EfAhpC* (Fig. 7A). In addition, the residues including L363, E385, F397, S428, L432, I441, I446, G449-L455, Q467, K475, S490, V500, E525, S534, and M552 reveal increased ¹⁵N line-widths over than standard deviation (Fig. 7B).

Based on the NMR intensity changes and linewidth analysis data, the binding area inside *EfAhpF*, which interacts with *EfAhpC*, was mapped on the crystal structure of *EfAhpF*₃₅₄₋₅₆₀ as shown in figure 7C, reflecting that surface exposed residues form a well-defined interaction surface around the active site cysteine residue C493. Most surface exposed residues (G449-L455), which show relatively high intensity changes and line width increase form a clustered interaction cleft, indicating that these residues may be strongly related to *EfAhpF*-AhpC interaction. Residues I441, I446, S448-L455, L490, T492, S521, K527, M531, S532, V533, clustered closely to the catalytically active C493, might also directly participate in *EfAhpF*-AhpC assembling. In addition, amino acids around the binding epitope such as W354, F355, R360, Q362, S364, Q467 as well as S385, F396, S399, G436, T437, K401, A456, V461, G476, R483, K484, E486, N510, E525, L526, M552 and E554 could be indirectly related to protein-protein interaction as a result of the slight conformational changes of *EfAhpF* caused by *EfAhpC*.

3.7 The role of C503 in *EfAhpF*-AhpC interaction

To shed light into the role of C503 in *EfAhpF*-AhpC interaction, NMR titration experiments using mutant *EfAhpF*₃₅₄₋₅₆₀ C503A and *EfAhpC* (molar ratio 1:0.1) were performed. As displayed in figures 8A-C, overall changes of decreasing intensity ratios and increasing line width (¹⁵N line width) were observed. Residues G449-N453 of *EfAhpF*₃₅₄₋₅₆₀ C503A show relatively high intensity ratios compared to other residues, however, the relative intensity changes dramatically decreased (~40%) compared to the WT protein (Fig. 7A-B), indicating overall decreased interaction between *EfAhpF*₃₅₄₋₅₆₀ C503A and *EfAhpC*. In addition, the residues showing changes of ¹⁵N line width within the *EfAhpF*₃₅₄₋₅₆₀ C503A mutant after addition of *EfAhpC* significantly changed (Fig. 7A) when compared to WT (Fig. 5D), indicating that the substitution of C503A affects the interaction between *EfAhpF* and -AhpC by modulation of protein conformation and dynamic properties. Moreover, ¹H-¹⁵N-HSQC spectra show disappearance and significant CSPs of the resonances around C503, including residues C496, A501, Q504, A507, S508, N510, H512 and E514 (Fig. 8A). These observations are supporting that the C503A induce changes in conformational and dynamic properties of the residues located around C503, resulting in modulation of the interaction between *EfAhpF* and -AhpC. In conclusion, it is considered that C503 is not directly involved in protein-protein interaction, but affects the interaction between *EfAhpF* and -AhpC by the formation of a molecular interaction network with neighbouring residues.

3.8 NMR based Docking studies of an *EfAhpF*-AhpC complex

To investigate the complex formation of *EfAhpF*-AhpC, the obtained restraints based on the NMR titration experiments and prediction of the interaction residues, were used to drive a HADDOCK-docking simulation [31] as described under Materials and Methods. The top scoring HADDOCK model from the highest scoring cluster was selected (HADDOCK Score

-107.8 $\pm$ -6.4; Restraint violation energy: 162.4 $\pm$ 50.16 Kcal/mol; Buried surface area: 2,403.2 $\pm$ 190.8 Å²; Z-score: -0.7). The resulting *EfAhpF*₃₅₄₋₅₆₀-*AhpC*₁₋₁₆₇ complex model (Fig. 9) indicates the spatial proximity of the regions including the two catalytic cysteine residues C493 and C496 in *EfAhpF*₃₅₄₋₅₆₀ with the *EfAhpC*₁₋₁₆₇ dimer. The *EfAhpC*₁₋₁₆₇ dimer contacts this interface mainly with regions including the two helices $\alpha 2'$ of the 1st monomer and $\alpha 6$ of the 2nd monomer (Fig. 9). Helix $\alpha 6$, followed by a loop (residues 161-167) region holding C_R (C166), and $\alpha 2'$ helix holding C_P (C47) are linked to form a protein-protein interaction scaffold, which fits into the groove on the binding surface of *EfAhpF*₃₅₄₋₅₆₀ (Fig. 9). Residues including G448-L455, S490-F495 comprise the major interaction cleft, forming contact with the binding scaffold of the *EfAhpC*₁₋₁₆₇ dimer.

4. Discussion

4.1 Unique features of the *E. faecalis* (V583) AhpR system

Like the pathogenic *Mycobacterium tuberculosis*, vancomycin-resistant *E. faecalis* (V583) represents a unique AhpR ensemble that evolved a variety of structural variations combined with diverse mechanism to tackle stress caused by ROS. Unlike the well-studied *EcAhpC* and *StAhpC*, which form a stable decamer in the reduced form and lower weight oligomers in the oxidized state [8-10], the *Mycobacterial* AhpC (*MtbAhpC*) forms a dodecamer in a reduced state [34,35], while *EfAhpC* forms a decamer independent to its redox state [7]. The gram-negative *EcAhpC* and *StAhpC* contain two typical catalytic cysteine residues C_P and C_R [8-10], while *MtbAhpC* harbours a third cysteine C176, proposed to reduce the disulphide between C_P61 and C_R174 [34]. The gram-positive *EfAhpC* has two additional cysteines (C13, C66), being relevant for stabilization of the decameric ring and enzyme activity [7].

The electron donor subunit of *EfAhpC*, *EfAhpF*, extends these variations for niche adaptation by swapping of the usually N-terminal domain to the C-terminus (NTD_N/C

domain) and the CTD:Pyr_redox_2 domain to the N-terminus, a phenomenon discussed by the concept of circular permutation, resulting in improved catalytic activity and altered substrate or ligand binding affinity [36]. Despite the domain swapping, the linker connecting the N- and C-terminal domains in *EfAhpF* is longer and more diverse in sequence compared to its gram-negative counterparts [13]. Linker mutant studies demonstrated that the length of the linker is required for optimal catalytic activity [13]. In addition, our recent *EfAhpF*- [13] as well as the presented *EfAhpF* C503A mutant structure (Fig. 4) revealed the additional ₄₀₉ILKDTEPAKELLYGIEKM₄₂₆-loop-helix stretch. Complete and partial deletion of this α 3-helix affected protein stability and activity, respectively, identifying Y421 as a critical residue [13]. The unique AhpF α 3-helix is proposed to interact with the C-terminus of AhpC, thereby providing efficient electron transfer for AhpC for the final reduction of H₂O₂. Here, we present for the first time that substitution of the extra C503 in *EfAhpF* increases the K_m for AhpC ($70.9 \pm 12 \mu\text{M}$), resulting in a nearly 27-fold lower catalytic efficiency ($k_{cat}/K_m = 0.7 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$ (Table. 2)). These data are in line with a significant reduction in H₂O₂ consumption, indicating the relevance of C503 in electron transfer from the electron donor NADH via the electron centres of *EfAhpF* to the peroxidatic (C_p47) and resolving (C_R166') cysteine residues in the catalytic centre of *EfAhpC*, where H₂O₂ is catalysed. Recently, we showed, that substitution of the additional cysteine residues C13 and C66 affect *EfAhpC* ring stabilization, which was directly related to a reduction of NADH-oxidation and H₂O₂ consumption [7]. The increasing reduction in H₂O₂ decomposition of the mutant ensemble *EfAhpC* C66V-AhpF C503A followed by *EfAhpC* C13N-AhpF C503A and *EfAhpC* C13N/C66-AhpF C503A, respectively (Fig. 3), confirms an additive effect and reflects that these specific *EfAhpC*-AhpF cysteines are a prerequisite for proper ensemble formation and maximum oxidation of its substrate NADH and the reduction of H₂O₂.

4.2 Important residues of *EfAhpF*₃₅₄₋₅₆₀ for the redox process

The NMR solution redox experiment of *EfAhpF*₃₅₄₋₅₆₀ sheds light into the residues of the two-fold thioredoxin-like domain (NTD_N/C) which are affected by the process of electron transfer (Fig. 5). The CSP analysis revealed that the residues W354, S428, G445, G449, E451-N453, F488, C496, I499-C503, I506, H512, V518, V535, D641, and G533 undergo significant alteration due to redox-dependent modulation (Fig. 5C-D). These residues do not only include the extra cysteine C503 but also its close neighbourhood I499 to A502, and I506, which together provide an area formed by the neighbouring helices $\alpha 4$ and $\alpha 6$ as well as the β -sheets 5 and 6 (Fig. 5E). While the comparison of the atomic structures of WT- and mutant *EfAhpF*₃₅₄₋₅₆₀ C503A nicely demonstrated that the C503 to A mutation does not alter the secondary or tertiary structure of the protein, the NADH oxidation assay and NMR data identify unequivocally its important role for the redox processes within *EfAhpF*₃₅₄₋₅₆₀.

4.3 Critical binding epitope of *EfAhpF*₃₅₄₋₅₆₀ for assembling with *EfAhpC*

The electron transfer pathway after NADH oxidation in the pyridine nucleotide-disulfide oxidoreductase domain (CTD:Pyr_redox_2) of *EfAhpF* is dictated by conformational changes in the CTD:Pyr_redox_2 as well as by the movement of *EfAhpF*₃₁₄₋₅₆₀ relative to the CTD:Pyr_redox_2 that enable the redox process between both disulfide centers (C134/C137 and C493/C496) [14,33]. The subsequent rearrangement of *EfAhpF*₃₅₄₋₅₆₀ to an extended form of *EfAhpF* brings its disulfide center C493/C496 in proximity to the redox-active cysteines of *EfAhpC*. The NMR titration experiment of full length *EfAhpC* and labelled *EfAhpF*₃₅₄₋₅₆₀ demonstrate that both *EfAhpR* subunits interact in a more dynamic and shorter-lived complex (Fig. 6A-D). We recently predicted that the unravelled groove in the atomic structure of *EfAhpF*₃₅₄₋₅₆₀ wrapping around the protein would provide an area for the packing of the C-terminal tail of *EfAhpC* [13] as demonstrated for the *E. coli* AhpC [14]. While the C-terminus

of *EfAhpC* would bind to the backside of *EfAhpF*₃₅₄₋₅₆₀, the disulfide center C493/C496 of *EfAhpF*₃₅₄₋₅₆₀ would come in neighbourhood to the catalytic disulphide (C_p47-C_R166) of bound *EfAhpC*, and thereby providing rapid electron transfer. The titration and docking data presented extend this prediction by identifying the amino acids G448-L455, S490-F495 comprising the major interaction cleft of *EfAhpF*₃₅₄₋₅₆₀. Interestingly, some of these sequence segments were shown to be involved in the redox process within *EfAhpF*₃₅₄₋₅₆₀ as well. As highlighted by the inset of figure 7C, residues within the proximity of residues V500, S502, the additional C503, and I506, were found to undergo alterations during reduction of the disulfide center C493/C496 of *EfAhpF*₃₅₄₋₅₆₀.

To summarize, our study proved the special role of the additional cysteine residue (C503) present in the two-fold thioredoxin-like domain (NTD_N/C) of *EfAhpF* and that there are alterations in its proximity during redox-state modulation. Moreover, we were able to identify amino acid residues of *EfAhpF* involved in the interaction with its partner protein *EfAhpC* and, therefore, to describe the mechanism of electron transfer from *EfAhpF* to *EfAhpC* in detail. The data presented here enhance the knowledge of the key enzyme complex AhpR important for ROS defense and ROS homeostasis. The knowledge of systems' special features, the identification of species-specific structures, epitopes and mechanisms, is an excellent basis for future drug development.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article

Author contributions

YKT performed most of the experiments, analyzed the data and drafted the manuscript. JS collected and analyzed the NMR data and drafted the manuscript. AMB collected and analyzed the crystallography data and drafted the manuscript. NK collected and analyzed stopped flow data and drafted the manuscript. AG purified labelled and unlabelled proteins and helped editing of the manuscript. FE and BE helped perform the computational analysis and editing of the manuscript. GG conceived the study and analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

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The abbreviations used are: H₂O₂, hydrogen peroxide; AhpR, alkyl hydroperoxide reductase; AhpF, alkyl hydroperoxide reductase subunit F; AhpC, alkyl hydroperoxide reductase subunit C; C_P, peroxidatic cysteine; C_R, resolving cysteine; NADH, nicotinamide adenine nucleotide; CTD:Pyr_redox_2, pyridine nucleotide-disulfide oxidoreductase domain; NTD_N/C, thioredoxin-like domain; FAD, flavin adenine nucleotide; *EfAhpF*, *E. faecalis* AhpF; TCEP, tris(2-carboxyethyl)phosphine

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627 **Table 1:** Data collection and refinement statistics

Wavelength (Å)	1.00
Space group	P4 ₁ 32
Unit cell parameters (Å)	$a = b = c = 134.101$ $\alpha = \beta = \gamma = 90^\circ$
Resolution range (Å)	30-2.5
Solvent content (%)	72.06
Total number of reflections	166,667
Number of unique reflections	14,035
I/ $\sigma(I)$ ^a	22.4 (7.3)
Completeness (%)	99.7 (99.7)
R _{merge} ^b (%)	6.9 (25.2)
Redundancy	11.2 (11.7)

628

Refinement Statistics

R factor ^c (%)	21.47
R free ^d (%)	26.72

Number of amino acid residues	207
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Ramachandran statistics

Favored (%)	98.1
Generously (%)	1.9
Disallowed (%)	0.0

R.M.S. Deviations

Bond lengths (Å)	0.006
Bond angles (°)	1.088

Mean atomic B values

Overall	61.02
Wilson B Factor	48.63

CC1/2	99.9 (97.5)
CC*	100 (99.4)

Clash score	1.22
Molprobit score:	1.23

629

630 ^a Values in parentheses refer to the corresponding values of the highest resolution shell (2.59 – 2.5 Å).

631 ^b $R_{\text{merge}} = \sum_i |I_h - \bar{I}_h| / \sum_i I_h$, where I_h is the mean intensity for reflection h .

632 ^c R-factor = $\sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o and F_c are measured and calculated structure factors, respectively.

633 ^d R-free = $\sum ||F_o| - |F_c|| / \sum |F_o|$, calculated from 5% of the reflections selected randomly and omitted during refinement.

634

635 **Table 2:** Kinetic constants for WT *EfAhpF* and mutant *EfAhpF* C503A.

<i>EfAhpF</i>	k_{cat} (s ⁻¹)	K_m (AhpC) (μM)	k_{cat}/K_m (AhpC)(M ⁻¹ ·s ⁻¹)
WT	46.1 ± 4	24.5 ± 5	1.9 × 10 ⁶
C503A	49.2 ± 6	70.9 ± 12	0.7 × 10 ⁵

636

Figure Legends

Figure 1. The proposed catalytic cycle of the *EfAhpC*-AhpF ensemble and multiple sequence alignment of the NTD_N/C domain of subunit AhpF. (A) *EfAhpC* exists as a decameric ring in both oxidized and reduced states [7]. In the crystal structure of the reduced *EfAhpC*₁₋₁₇₂ [7], the C_p loop exists in two different conformations as shown in the *inset boxes*. In the reduced state of *EfAhpC* the peroxidative cysteine C_p in the fully folded (FF) conformation attacks H₂O₂ and reduces it to H₂O. In this process, C_p is converted into sulfenic acid (C_p-SOH form). The resolving cysteine, C_R, then attacks the C_p-SOH form to generate an intermolecular disulphide bond. Subsequently, this disulphide bonded form of C_p undergoes a conformational change to a locally unfolded form (LU). This disulphide is then resolved by the interacting partner, AhpF. The recently determined low resolution solution structure of the dimeric *EfAhpF* with the crystal structure of *EfAhpF*₃₅₄₋₅₆₀ [13] inside. The unique loop in *EfAhpF*₃₅₄₋₅₆₀ with the short helix ₄₀₉ILKDTEPAKELLYGIEKM₄₂₆ is marked with an asterisk. (B) Multiple sequence alignment of the NTD_N/C domain of subunit AhpF from ten different species was performed using T-coffee algorithm [37] embedded within Jalview program [38]. The secondary structure elements of *EfAhpF*₃₅₄₋₅₆₀ are shown above the sequence. Conserved residues are highlighted in *blue*. The CXXC conserved motif is outlined in *green* box. *EfAhpF* C503 and its equivalent residues from other species are outlined in *red* box.

Figure 2. Enzymatic characterization of WT *EfAhpF* and its mutant. Michaelis-Menten plot of 0.5 – 40 μM of *EfAhpC* WT with 50 nM of (A) *EfAhpF* WT or (B) *EfAhpF* C503A. Data shown are mean of triplicates.

Figure 3. FOX assay of WT *EfAhpF* (brown) and *EfAhpF* C503A (blue) with *EfAhpC* (A). Data shown are mean of triplicates. (B) FOX assay data of *EfAhpF* C503A with WT *EfAhpC* (brown), *EfAhpC* C13N (red), C66V (green) and double-mutant C13N/C66V (blue). Data shown are mean of triplicates.

Figure 4. Cartoon representation of one monomer of the *EfAhpF*₃₅₄₋₅₆₀ C503A mutant structure shown in pale green, the α -helices and β -strands are labelled (A). Catalytic site cysteines 493 and 496 including the disulfide bond are shown in yellow spheres. The C503A mutation is shown in red spheres. (B) *EfAhpF*₃₅₄₋₅₆₀ C503A, pale green is overlapped with WT *EfAhpF*₃₅₄₋₅₆₀, blue (PDB ID: 5H29). Catalytic site cysteines 493 and 496, including the disulfide bond, are shown in yellow spheres. C503A mutation is shown in red spheres.

Figure 5. NMR spectroscopic characterization of *EfAhpF*₃₁₄₋₅₆₀. (A) ¹⁵N-¹H TROSY-HSQC-spectrum of *EfAhpF*₃₅₄₋₅₆₀. The spectrum was recorded at 298K on a Bruker Avance 700 MHz spectrometer. The assignments for resolved backbone residues are labelled with in letter amino acid code and residue number. (B) The secondary structural elements crystal structure of *EfAhpF*₃₅₄₋₅₆₀ from the experimentally determined crystal structure. The scores were calculated from $\Delta C\alpha$ values based on backbone assignment of *EfAhpF*₃₅₄₋₅₆₀. Contiguous positive and negative chemical shift index values correspond to α -helices and β -strands, respectively. (C) Selected sections of some *EfAhpF*₃₅₄₋₅₆₀ residues showing cross-peaks and undergoing significant changes of chemical shifts during redox modulation. (D) Chemical shift perturbation (CSP) analysis between reduced and oxidized *EfAhpF*₃₅₄₋₅₆₀. Weighted CSPs for the ¹⁵N and ¹H resonance of reduced and oxidized *EfAhpF*₃₅₄₋₅₆₀ residues revealing CSP above 0.1 ppm (solid line) are labeled as one-letter code. The weighted CSPs between reduced and oxidized *EfAhpF*₃₅₄₋₅₆₀ for backbone ¹⁵N and ¹HN were calculated by the

formula $\Delta\delta = [(\Delta N/5)^2 + (\Delta HN)^2]^{0.5}$. The average weighted CSP(Avg) and Avg+standard deviation(delta) values were displayed by dashed lines, respectively. (E) (top) Surface representation of *EfAhpF*₃₅₄₋₅₆₀ displaying residues showing significant (CSP>0.1, red) and moderate (0.05<CSP<0.1, green) CSP. (bottom) Ribbon representation of CSP analysis. The region which undergoes significant CSP is marked as circle. Amino acid C503, which is located in this region, is shown by stick representation and indicated by a line.

Figure 6. Interaction of *EfAhpC* with *EfAhpF*₃₅₄₋₅₆₀ as studied by NMR. (A) Summary of gradual disappearance and decrease of intensities of the *EcAhpF*₃₅₄₋₅₆₀ cross-peaks in the presence of increasing amount of its larger binding partner. A molar ratio of *EfAhpF*₃₅₄₋₅₆₀ to *EfAhpC* of 1:0 (left), 1:0.05 (middle), and 1:0.1 (right) were used. (B) Selected sections for some residues show cross-peaks which undergo significant decrease of peak intensities after addition of *EfAhpC*.

Figure 7. NMR analysis of *EfAhpF*₃₅₄₋₅₆₀ and *EfAhpC*. The residues of flexible linker regions were excluded from the analysis. (A) A plot of changes of peak intensity based on the ratio (I_O/I_I) at a molar ratio of 1:0.1. The average value of the intensity ratio and standard deviation were shown as dotted line and solid box, respectively. The residues showing higher intensity ratio than standard deviation are labelled (B) Changes of ¹⁵N linewidth between the ¹H-¹⁵N TROSY-HSQC resonances of *EfAhpF*₃₅₄₋₅₆₀ alone and in the presence of *EfAhpC* at a molar ratio 1:0.1. The average value of the linewidth changes (ΔL) and standard deviation were shown as dotted line and solid box, respectively. (C) Surface representation of *EfAhpF*₃₅₄₋₅₆₀ displaying residues showing significant and moderate decreased intensities (cyan and green cyan), and residues showing significant (over than standard deviation) and moderate (over

than average) increase of linewidths (*green and light green*). The cysteine catalytic site of *EfAhpF*₃₁₄₋₅₆₀ is shown in *red*.

Figure 8. NMR analysis of mutant *EfAhpF*₃₅₄₋₅₆₀ C503A. (A) CSP analysis of WT and mutant *EfAhpF*₃₅₄₋₅₆₀ C503A. Calculation of weighted CSPs and analysis for the ¹⁵N- and ¹H resonance of WT and *EfAhpF*₃₅₄₋₅₆₀ C503A were performed as described above. The average weighted CSP(Avg) and Avg+standard deviation(delta) values were displayed by dashed lines. Residues over than standard deviations are labelled as one letter code. (B) A plot of changes of peak intensity based on the ratio (I_0/I_I) at a molar ratio of 1:0.1. The average value of the intensity ratio and standard deviation were shown as dotted line and solid box, respectively. The residues showing higher intensity ratios than standard deviation are labelled. (C) Changes of ¹⁵N linewidth between the ¹H-¹⁵N TROSY-HSQC resonances of *EfAhpF*₃₅₄₋₅₆₀ C503A alone and in the presence of *EfAhpC* at a molar ratio 1:0.1. The average value of the linewidth changes (ΔL) and standard deviation were revealed as dotted line and solid box, respectively.

Figure 9. The best result of HADDOCK docking displaying the *EfAhpF*₃₅₄₋₅₆₀-AhpC₁₋₁₆₇ ensemble. (A) Surface and ribbon representation of the *EfAhpF*₃₅₄₋₅₆₀-AhpC₁₋₁₆₇ complex. The major binding contacts of the *EfAhpC*₁₋₁₆₇ dimer unit, including helical segment $\alpha 2'$ (1st monomer) and $\alpha 6$ (2nd monomer) are labelled and displayed as *blue*. The catalytic C47 and C166 of *EfAhpC*₁₋₁₆₇ are presented as sphere model. (B) The complex tilted at 30°. (C) Extended section of the complex highlighting the catalytic residues C493 (*red*, *EfAhpF*₃₅₄₋₃₆₀) and C47 (1st monomer of *EfAhpC*₁₋₁₆₇).

Figure 1A

(B)

Figure 1B

Figures 2A-B

Figures 3A-B

Figures 4A-B

Figures 5A-B

Figures 5C-D

Figure 5E

Figures 6A-B

Figures 7A-B

Figures 7C

Figure 8A-B

Figure 8C

Figure 9