Site-directed mutagenesis reveals a novel catalytic mechanism of *Mycobacterium tuberculosis* alkylhydroperoxidase C

Radha CHAUHAN*† and Shekhar C. MANDE†[1]

*Institute of Microbial Technology, Sector 39-A, Chandigarh 160036, India, and †Centre for DNA Fingerprinting and Diagnostics, ECIL Road, Nacharam, Hyderabad 500076, India

Mycobacterium tuberculosis alkylhydroperoxidase C (AhpC) belongs to the peroxiredoxin family, but unusually contains three cysteine residues in its active site. It is overexpressed in isoniazid-resistant strains of *M. tuberculosis*. We demonstrate that AhpC is capable of acting as a general antioxidant by protecting a range of substrates including supercoiled DNA. Active-site Cys to Ala mutants show that all three cysteine residues are important for activity. Cys-61 plays a central role in activity and Cys-174 also appears to be crucial. Interestingly, the C174A mutant is inactive, but double mutant C174/176A shows significant revertant activity. Kinetic parameters indicate that the C176A mutant is active, although much less efficient. We suggest that *M. tuberculosis* AhpC therefore belongs to a novel peroxiredoxin family and might follow a unique disulphide-relay reaction mechanism.

Key words: antioxidant, dithiothreitol oxidation, glutamine synthetase enzyme, metal-catalysed oxidation.

INTRODUCTION

Peroxiredoxins (Prxs) belong to a family of peroxidases involved in cellular defence against oxidative and nitrosative stress. They play an important role in various physiological and pathological processes including metabolism, immunity, inflammation, cell signalling and apoptosis [1–3]. They have been shown to possess peroxynitrite reductase activity and participate in detoxification of reactive nitrogen intermediates [4,5]. Moreover, recent reports suggest that minor modification in a Prx sequence can alter the enzyme to act as a disulphide reductase [6]. In general Prxs reduce H_2O_2 to water and alkyl peroxides to corresponding alcohols. During this reaction they use flavin-dependent partners such as alkylhydroperoxidase (Ahp) F, thioredoxin reductase, NADH oxidase or glutathione reductase [7–10] for an electron-transfer reaction. They have been found to occur in a variety of organisms ranging from prokaryotes to mammals. Thus Prxs appear to have a broad range of functions and therefore constitute an interesting set of enzymes to study.

Prxs have been broadly classified into two classes, namely 1-Cys Prxs and 2-Cys Prxs, depending upon the involvement of the number of cysteine residues in their activity [11,12]. Reaction mechanisms of both 1-Cys and 2-Cys Prxs have been proposed from extensive biochemical studies carried out on them. 1-Cys Prxs contain a strictly conserved Cys residue in their N-terminal region, which forms the site for oxidation by peroxides. A cysteinyl-sulphenic acid is proposed to be formed during the catalytic reaction [13–15]. The 2-Cys Prxs possess an additional conserved Cys residue in their C-terminal region which forms an intersubunit disulphide bond along with the N-terminal Cys residue of another subunit [16,17]. The cysteine sulphenic acid in 1-Cys Prxs or the disulphide bond in 2-Cys Prxs formed during the reaction is reduced by a flavin-dependent enzyme, thus regenerating the enzyme for the next cycle.

Mycobacterium tuberculosis genome sequence has shown the presence of at least four Prxs, namely bacterioferritin co-migratory proteins (Bcp and BcpB), AhpE and AhpC [18]. Three of these belong to the 1-Cys Prx class, and one of them, AhpC, appears to belong to the 2-Cys Prx class. Interestingly, AhpC has been shown to be overexpressed in isoniazid-resistant strains of *M. tuberculosis* [19,20]. It has also been proved experimentally to harbour virulence [21]. Interestingly, unlike in other bacteria, the *M. tuberculosis* genome sequence does not possess the flavin-dependent redox enzyme adjacent to the *ahpC* gene.

We have earlier shown that *M. tuberculosis* AhpC is a decameric enzyme composed of five identical dimers [22]. The dimer formation is mainly due to intersubunit disulphide linkage, and the oligomeric interactions are dominated by ionic character. Interestingly the *M. tuberculosis* AhpC possesses three Cys residues, two of which occur very close together in the C-terminal region, at positions 174 and 176. The conserved N-terminal Cys residue occurs at position 61. Both the 174 and 176 Cys residues can form a disulphide bond with the conserved N-terminal Cys residue [22] and all the three Cys residues are important for activity [23].

In the current study we have attempted to answer two major questions. The first one addresses the general role of AhpC in isoniazid-resistant mycobacteria. Molecular genetics studies have established that isoniazid-resistant *M. tuberculosis* have a malfunctional catalase-peroxidase (KatG), the only peroxide-inducible gene in *M. tuberculosis* in the absence of functional OxyR [24,25]. In order to help mycobacteria surmount the oxidative pressure in the absence of the catalase-peroxidase, a compensatory mutation overexpresses AhpC, possibly to overcome oxidative stress [26]. We therefore addressed the question of whether mycobacterial AhpC has a general antioxidant property. In the present study we have demonstrated that mycobacterial AhpC can act as an antioxidant and protect DNA and other proteins from metal-catalysed oxidation (MCO) damage.

The second important issue is the role of three Cys residues in the activity of *M. tuberculosis* AhpC. We have generated all the seven possible combinations of Cys-to-Ala mutation and characterized the mutants to obtain functional insight into the active site of mycobacterial AhpC. Through these studies we show that all three Cys residues are important for its activity, and that Cys-61 and Cys-174 play a critical role but Cys-176 is also involved

Abbreviations used: Ahp, alkylhydroperoxidase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; GS, glutamine synthetase; MCO, metal-catalysed oxidation; Ni-NTA, Ni^{2+}-nitrilotriacetate; Prx, peroxiredoxin.

[1] To whom correspondence should be addressed (e-mail shekhar@www.cdfd.org.in).

in the catalytic cycle. Thus, *M. tuberculosis* AhpC belongs to a novel class of the Prx family.

EXPERIMENTAL

Materials

PAGE-purified oligonucleotides were obtained from BIOBASIC, Markham, Ontario, Canada. *Xho*I and *Sma*I restriction enzymes, T4 DNA polymerase and ligase were purchased from New England Biolabs (Beverly, MA, U.S.A.) and Amersham Biosciences. Transformer site-directed mutagenesis kit was supplied by Clontech (Palo Alto, CA, U.S.A.). Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose superflow resin and miniprep plasmid purification kit was from Qiagen (Chatsworth, CA, U.S.A.). Dithiothreitol (DTT), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), glutathione, t-butyl hydroperoxide, glutamine synthetase (GS) enzyme, ADP, trichloroacetic acid, ferrous ammonium sulphate, potassium thiocyanate, ferric chloride, hydroxylamine, potassium arsenite, $MnCl_2$, imidazole and all other chemicals were purchased from Sigma-Aldrich and were of the highest quality. A Shimadzu spectrophotometer 1601 was used for spectroscopic studies. Automated DNA sequencing was performed on an ABI Prism automated DNA sequencer.

Site-directed mutagenesis

Mutants of *ahpC* were generated by using the Transformer site-directed mutagenesis kit. Different primers used to obtain the mutants are listed in Table 1. Briefly, mutagenic and selection primers were simultaneously annealed to one strand of denatured pET23AhpCHis6 (pET23a plasmid containing the *M. tuberculosis ahpC* gene insert), followed by elongation and ligation under conditions recommended by the manufacturer. The selection primer was such that pET23AhpCHis6 lost a *Xho*I site while gaining a *Sma*I site. Thus selection was made by linearizing the parent vector by digesting with *Xho*I, before transforming the plasmid into the *mutS* strain of *Escherichia coli* and positive selection was obtained by *Sma*I digestion. To generate double cysteine mutants, at positions 174 and 176, the C174/176A primer, harbouring both mutations as shown in Table 1, was used. In order to generate a combination of cysteine mutants, C61A mutant plasmid was used as a template for site-directed mutagenesis and C61/174A, C61/176A and C61/174/176A mutants were generated. DNA sequencing of the whole gene confirmed all the mutations.

Protein overexpression and purification

Mutant plasmids were transformed into *E. coli* BL21(DE3) cells and protein expression was induced by isopropyl β-D-thiogalactoside. All mutants and wild-type recombinant AhpC were purified using Ni-NTA affinity chromatography as described earlier [22].

Gel-filtration chromatography

Amersham Biosciences SMART analytical system equipped with Superdex 200 PC 3.2/30 was used to determine the molecular mass of proteins. Tris/HCl buffer (50 mM, pH 7.4) containing 200 mM NaCl and 5% glycerol was used to equilibrate the column for at least 4 bed vol. A typical flow rate of 100 μl/min was maintained in all the runs. Absorbance at 280 nm was measured to monitor the elution of proteins from the column. Calibration of the column was performed using molecular-mass standards for gel filtration supplied by Sigma as described earlier [22].

Table 1 Primers used for site-directed mutagenesis

The mutations are underlined.

Primer location	Mutation	Mutagenic primer
ahpC	Cys-61 → Ala	5′-CAC GTT CGT GGC CCC TAC CGA-3′
ahpC	Cys-174 → Ala	5′-GAC GAG CTG GCT GCA TGC AAC TGG-3′
ahpC	Cys-176 → Ala	5′-GAG CTG TGC GCA GCC AAC TGG-3′
ahpC	Cys-174/Cys-176 → Ala	5′-GAG CTG GCT GCA GCC AAC TGG CGC-3′
pET23a	*Xho*I → *Sma*I	5′-GCG GCC GCA CCC GGG CAC CAC CAC C-3′

Thiol group estimation

To determine free thiol groups in protein, Ellman's reagent (DTNB) was used according to the procedure described by Riddles et al. [27]. DTNB was used to a final concentration of 2 mM in the reaction mixture containing 50 mM Tris/HCl and 250 mM NaCl buffer, pH 7.4. The reaction was carried out at 37 °C for 30 min. For denaturing conditions, 2% SDS was used and protein was incubated at 40 °C for 4 h. To reduce the protein, it was treated with 100 mM DTT along with 2% SDS at 40 °C for 4 h prior to adding DTNB.

Measurement of AhpC enzyme activity

Four different assays were used to characterize the activity of mycobacterial AhpC. In each of the four assays, protein concentration was adjusted so that absorbance could be measured in an optimal range. The four assays were as follows.

DTT oxidation assay

Enzyme (5.6 μM) was incubated for 10 min with 10 mM DTT and 2 mM t-butyl hydroperoxide in 50 mM potassium phosphate buffer, pH 7.5, containing 300 mM NaCl and 1 mM EDTA. The rate of DTT oxidation was measured spectrophotometrically at 310 nm at room temperature as described by Iyer and Klee [28].

Ferrithiocyanate assay

Enzyme (2.8 μM) was incubated with 10 mM DTT and 0.2 mM t-butyl hydroperoxide for 15 min at 37 °C. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 20%. The remaining amount of substrate was then measured as a red-coloured complex at 480 nm as described earlier [29].

GS protection antioxidant assay

The metal-catalysed oxidative inactivation of GS mediated by $DTT/Fe^{3+}/O_2$ was assayed as a function of the ability of AhpC to prevent inactivation of GS [30]. One unit of GS enzyme was incubated with different concentrations of AhpC along with $FeCl_3$ and DTT to final concentrations of 6 μM and 10 mM respectively. Incubation at 37 °C for 10 min was followed by addition of 2 ml of γ-glutamyl assay mix and further incubation at 37 °C for 15 min. The reaction was terminated with the addition of 1 ml of stop mix and the absorbance of coloured iron hydroxamate was measured at 540 nm on a spectrophotometer [30].

DNA supercoiling protection assay

To demonstrate the ability of AhpC and its mutants to protect supercoiled DNA, approx. 200 ng of pBSK plasmid DNA was

incubated with 14 μM protein along with 0.4 μM $FeCl_3$, 10 mM DTT, 100 mM ethanol and 2 mM H_2O_2. The reaction was carried out in a 15 μl vol. for 30 min at 37 °C. To stop the reaction EDTA was added to a final concentration of 10 mM and the whole sample was analysed on a 0.8 % agarose gel [31].

Measurement of steady-state kinetics

A DTT oxidation assay was used to calculate apparent K_m and k_{cat} values by varying t-butyl hydroperoxide concentration as a substrate. All the experiments were repeated at least three times and then the data were plotted on a double reciprocal plot.

RESULTS AND DISCUSSION

Rationale for generation of cysteine mutants of mycobacterial AhpC

The *M. tuberculosis* AhpC amino acid sequence contains three cysteines, at positions 61, 174 and 176. Cys-61, which has been referred to as the N-terminal cysteine, is strictly conserved in all the members of 1-Cys and 2-Cys Prx families. Cys-174, although absent in the 1-Cys Prx family, is conserved in the 2-Cys Prx family. Recently we demonstrated that Cys-176 could form an intersubunit disulphide linkage with Cys-61 of another subunit [22]. Therefore, to probe the role of cysteines in the activity of mycobacterial AhpC, the cysteines at positions 61, 174 and 176 were each mutated to alanines by site-directed mutagenesis. Combinations of the three cysteines were also used to generate C61/174A, C61/176A, C174/176A and C61/174/176A mutants. Mutations were confirmed by restriction digestion of the plasmid vector either with *Xho*I or *Sma*I. They were also independently confirmed by DNA sequencing of the entire gene. The mutant proteins were purified using Ni-NTA affinity chromatography, with yields of more than 80 mg/l of culture. They were found to be better than 98 % pure on Coomassie-stained gels.

Cys-61 and Cys-174 are critical residues for oligomerization

The quaternary structure of the wild type as well as the mutants was assessed by gel-permeation chromatography. Chromatograms of four mutants are shown in Figure 1. Comparison with molecular-mass standards showed that C61A and C174A have elution volumes corresponding to dimeric AhpC. C176A on the other hand showed three peaks, one corresponding to the decameric AhpC and the others equivalent to dimeric and monomeric AhpC. Interestingly the double mutant C174/176A also showed two peaks for dimeric and monomeric proteins. Our earlier work on mycobacterial AhpC had indicated that recombinant mycobacterial AhpC is decameric in nature, the decamer being formed by five identical dimers. Two monomers in a dimer were shown to be linked by an intersubunit disulphide bond with dimer–dimer interactions being dominated by ionic character. Hence gel-filtration experiments of the cysteine mutants indicate that mutations at positions 61 and 174 have a profound effect on decameric aggregation of mycobacterial AhpC. While C61A and C174A are readily converted into a dimeric form, the C176A mutant shows only a partial conversion from the decameric state into the dimeric and monomeric states.

Cys-61 forms an intersubunit disulphide bond with Cys-174 as well as Cys-176

One of the intermediate stages in the 2-Cys Prx reaction mechanism is an intersubunit disulphide-bond formation. We therefore measured free cysteine content of the wild-type AhpC and its mutants by the method of DTNB titrations. As shown in

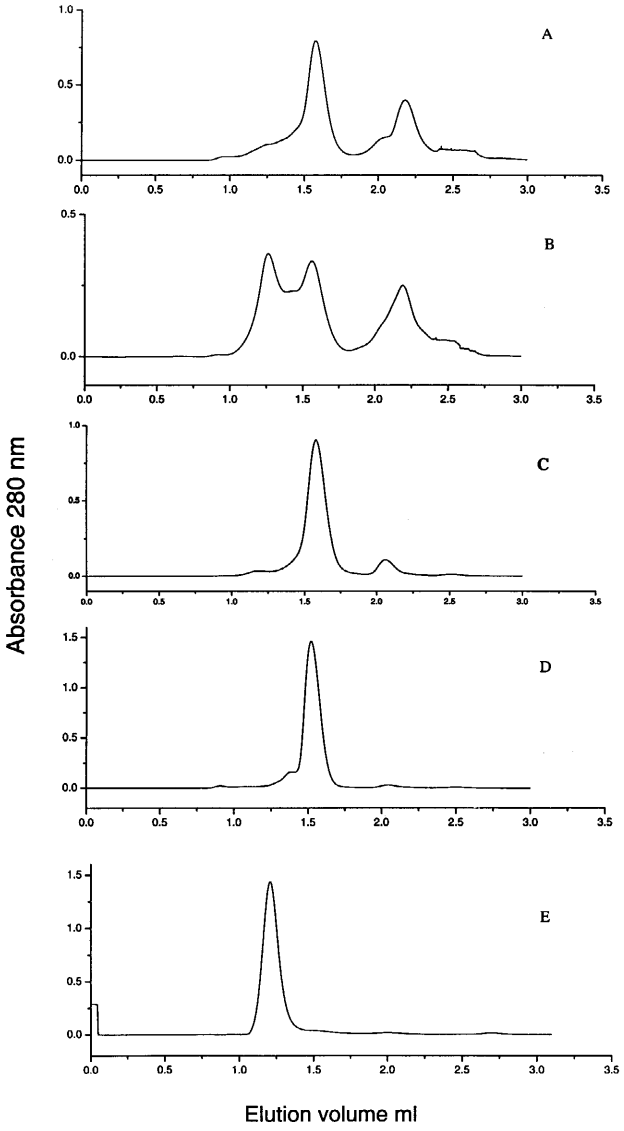

Figure 1 Quaternary structure of wild-type AhpC and its cysteine mutants

Wild-type AhpC and cysteine mutants of mycobacterial AhpC were subjected to gel-filtration chromatography as described in the Experimental section. Chromatograms of (**A**) C174/176A, (**B**) C176A, (**C**) C174A, (**D**) C61A and (**E**) wild-type AhpC protein are shown. C61A and C174A are dimeric proteins while C176A has decameric, dimeric and monomeric peaks. Wild-type AhpC corresponds to a decameric aggregation. Calibration of the column was performed using molecular-mass standards as described in [22].

Table 2, wild-type protein contains a single thiol group per enzyme molecule. The C174A and C176A mutants showed no free thiol per monomer, clearly indicating that Cys-61 is capable of forming a disulphide linkage with both Cys-174 and Cys-176. The C61A mutant on the other hand showed two thiol groups per enzyme molecule, suggesting that Cys-174 and Cys-176 residues do not form a disulphide linkage. Interestingly, under denaturing conditions, C61A showed no free cysteine residues, implying that Cys-174 and Cys-176 formed a disulphide bond when the enzyme was denatured.

To further reveal the role of disulphide bonds in aggregation of the mycobacterial AhpC, we carried out SDS/PAGE of the proteins under reducing and non-reducing conditions. Equal amounts of each mutant protein were treated with gel loading

Table 2 DTNB assay showing free thiol groups/enzyme molecule

Results are the means ± S.D. for three independent experiments.

Protein	Treatment ...	Number of free thiol groups/enzyme molecule		
		Normal conditions	2% SDS + 40 °C for 4 h	2% SDS + 40 °C + 100 mM DTT
AhpC:C61A		1.5 ± 0.28	0.0	2.12 ± 0.43
AhpC:C174A		0.14 ± 0.17	0.123 ± 0.12	1.92 ± 0.7
AhpC:C176A		0.145 ± 0.19	0.21 ± 0.19	1.82 ± 0.2
AhpC:C174/176A		0.825 ± 0.183	1.29 ± 0.18	1.38 ± 0.78
Wild-type AhpC		0.77 ± 0.2	0.695 ± 0.3	2.8 ± 0.78

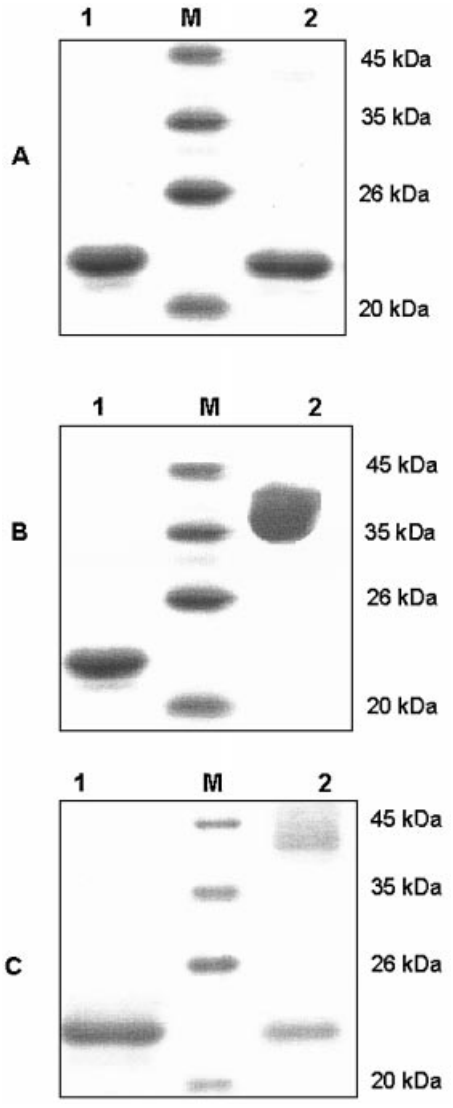

Figure 2 Disulphide-bond formation probed by SDS/PAGE (10% gels) under reducing and non-reducing conditions

M indicates molecular-mass markers in all the scans. (**A**) Reduced C61A protein in lane 1 and oxidized protein in lane 2. (**B**) C174A mutant protein in lane 1 shown under reducing conditions and in lane 2 under non-reducing conditions. (**C**) C176A mutant in reducing conditions in lane 1, and in non-reducing conditions in lane 2. The gels clearly indicate the absence of an intersubunit disulphide bond in the C61A mutant. Whereas Cys residues 61 and 176 can form a strong disulphide linkage (**B**), those at positions 61 and 174 appear to form only a partial linkage as suggested by two bands in the gel (**C**).

Table 3 Effect of cysteine mutations on enzyme activity

Results are the means ± S.D. for three independent experiments.

Mutant	DTT oxidation activity (%)	Thiocyanate assay activity (%)
Wild-type AhpC	100 ± 8	100 ± 20
C61A	Not detectable	Not detectable
C174A	2.15 ± 2.4	Not detectable
C176A	46.24 ± 2.4	65.75 ± 15
C174/176A	43.01 ± 3.2	26.5 ± 12.5

buffer with or without 2-mercaptoethanol. The results of this SDS/PAGE analysis are shown in Figure 2. These experiments indicated that the C61A protein has no intersubunit disulphide bond under both reducing and non-reducing conditions, since the mutant has the same mobility in either condition (Figure 2A). C174A, however, exhibited distinctly different mobility on SDS/PAGE under reducing and non-reducing conditions (Figure 2B). The two species of proteins correspond to the monomeric and dimeric forms of the AhpC enzyme. Hence there is clearly an intersubunit disulphide bond (Cys-61–Cys-176) between the two monomers of C174A mutant. C176A mutant protein showed two bands, corresponding to monomeric and dimeric molecular masses under non-reducing conditions (Figure 2C). This indicates that C176A has mixed populations of monomers and intersubunit disulphide-linked dimers (Cys-61–Cys-174). The two species appear to exist in a dynamic equilibrium.

The DTNB assays, SDS/PAGE experiments and gel-permeation studies together demonstrate a complex feature of the mutants and the wild-type enzyme. While the C61A mutant exists as a dimer, the dimeric form appears to be stabilized by non-covalent interactions and not through an intersubunit disulphide linkage. The C174A mutant is also dimeric in nature but, unlike the C61A mutant, has an intersubunit Cys-61–Cys-176 disulphide link. Non-reducing SDS/PAGE showed that a predominant population of the mutant protein has an oxidized disulphide link. This observation is further corroborated by gel-permeation chromatogram, where it migrates as a homogenous dimer (Figure 1). On the other hand, the C176A mutant can occur in a dynamic equilibrium of monomer, dimer and decamer. The dimers are crosslinked by a Cys-61–Cys-174 disulphide bridge. Thus in the absence of Cys-174 the protein readily forms a Cys-61–Cys-176 disulphide bond, while a lack of Cys-176 leads to only a fraction of the protein being linked by a Cys-61–Cys-174 disulphide bond.

AhpC is a general antioxidant

Four different types of assay were used to confirm the activity of AhpC mutants, as described in the Experimental section. The rationale behind using different assays for enzymic characterization was to explore the role of mycobacterial AhpC as a peroxidase as well as a general antioxidant. Activity of the proteins with Cys-61 mutated (C61/174A, C61/176A and C61/174/176A) was similar to the C61A mutant in all the assays described. Therefore, comparative results are described only for C61A, C174A, C176A and C174/176A mutants. Results of all the four assays are discussed below.

DTT oxidation activity

Table 3 shows comparison of DTT oxidation capability of four mutants, namely C61A, C174A, C176A and C174/176A, in the

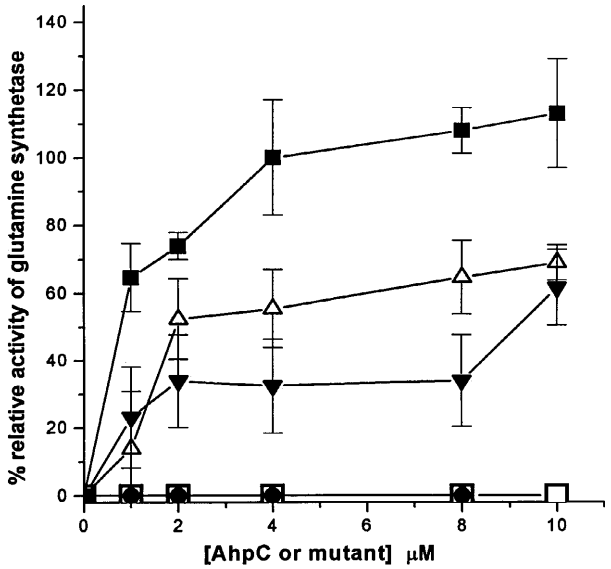

Figure 3 GS protection activity of AhpC and its mutants

The protection of GS from MCO damage was measured in the presence of various concentrations of wild-type protein (■), or C61A (□), C174A (●), C176A (△) and C174/176A (▼) mutants, as described in the Experimental section. The extent of protection is expressed as a percentage relative to the inactivation apparent in the absence of AhpC. Data shown are the means for three independent experiments; the error bars represent ±S.D.

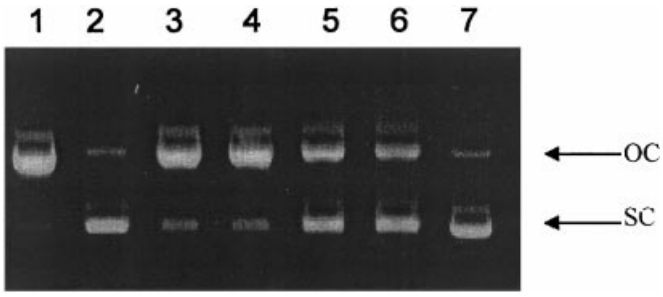

Figure 4 Protection of plasmid DNA supercoiling

Agarose (0.8%) gel scan shows two forms of plasmid DNA band. OC indicates nicked or open coiled DNA and SC denotes supercoiled DNA. DNA in lanes 1–6 was incubated with the MCO system and proteins as described in the Experimental section. Lane 1 shows pBSK DNA in the absence of any protein; lanes 2–6 respectively show pBSK DNA incubated with wild-type AhpC, and the C61A, C174A, C176A and C174/176A mutants. Lane 7 shows the plasmid DNA only (without the MCO system).

Table 4 Kinetic characterization of wild-type AhpC and mutant proteins

Results are the means ± S.D. for three independent experiments. ND, not detectable.

Enzyme	K_m (mM)	V_{max} (μM oxidized DTT/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Wild-type AhpC	5.38 ± 1.31	66.66 ± 2.89	0.111 ± 0.017	20.632 ± 3.72
AhpC:C176A	14.4 ± 2.3	128.33 ± 7.64	0.213 ± 0.013	14.79 ± 5.54
AhpC:C174/176A	7.83 ± 0.7	80.5 ± 3.28	0.133 ± 0.005	16.985 ± 7.14
AhpC:C174A	ND	ND	ND	ND
AhpC:C61A	ND	ND	ND	ND

presence of the substrate t-butyl hydroperoxide. This assay showed that C176A retained substantial activity in comparison with the wild-type enzyme. In contrast, the C61A mutant was completely inactive. Similarly, the C174A mutant also showed very little or negligible activity. Interestingly, the double mutant C174/176A showed significant activity, thus acting as a revertant of the C174A mutation. The results of this assay clearly indicate that Cys-61 and Cys-174 are required for enzymic reaction.

Ferrithiocyanate assay

Direct measurement of removal of the substrate t-butyl hydroperoxide was estimated by thiocyanate assay as described in the Experimental section. It was found that C61A and C174A have no measurable activity (Table 3) while the mutant C176A was less effective than the wild type in removal of the substrate. The double mutant C174/176A also diminished the peroxidase activity, as shown in Table 3, but still acted as a C174A mutation revertant.

GS protection assay

In the presence of DTT as an electron donor, Fe^{3+} can catalyse the reduction of O_2 to generate H_2O_2. The generated H_2O_2 has been shown to damage a number of enzymes including GS [32–35]. Any enzyme that eliminates H_2O_2 can prevent this damage. We used a DTT-mediated MCO system to demonstrate that mycobacterial AhpC can prevent GS damage by MCO (Figure 3). As expected the C61A and C174A mutants did not contribute to MCO damage protection. On the other hand, C176A and C174/176A showed protection although to a much lesser extent than the wild-type AhpC. These results suggest that mycobacterial AhpC might have general antioxidant properties. The results also established that an efficient reaction involves all three cysteine residues.

DNA supercoiling assay

Supercoiled DNA can form nicks in the presence of oxidative radicals, which can be easily observed on agarose gels. We therefore used the MCO system to generate H_2O_2 and assayed for damage in supercoiled plasmid DNA. When the activities of mycobacterial AhpC and its mutants were compared, the wild-type mycobacterial AhpC was seen to protect the plasmid DNA. However, C61A and C174A were unable to protect the DNA supercoiling from peroxide damage. As was observed in the GS protection experiments, C176A and C174/176A mutants also showed protection of DNA supercoiling but to a lesser magnitude than the wild-type enzyme (Figure 4). Thus both the GS protection and DNA supercoiling assays support the fact that mycobacterial AhpC can act as an antioxidant for DNA and proteins.

All three cysteine residues participate in enzymic activity

Wild-type recombinant AhpC and its mutants were characterized kinetically by DTT oxidation assay. K_m and k_{cat} values were determined for the peroxidase reaction using t-butyl hydroperoxide as a substrate. These kinetic parameters are presented in Table 4. Kinetic parameters of C61A and C174A could not be calculated as both showed little or no measurable DTT oxidation activity. C176A, although active, had different kinetic parameters from the wild type, as K_m increased approx. 2.4-fold and k_{cat}

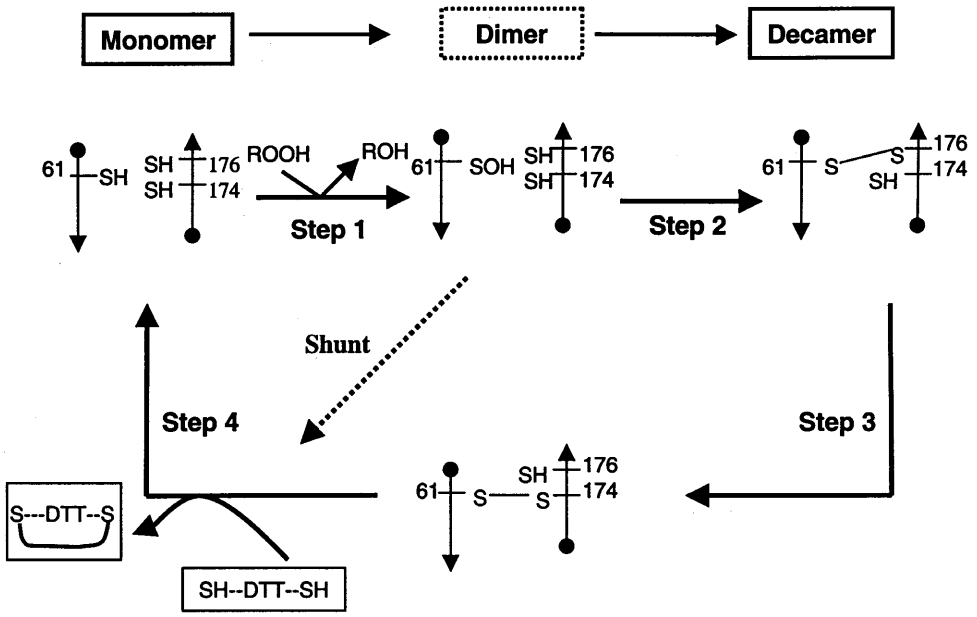

Figure 5 Proposed reaction mechanism for the action of mycobacterial AhpC

Step 1, attack on Cys-61 by peroxide substrate (ROOH) to form oxidized intermediate and alcohol (ROH) as products; step 2, formation of intersubunit disulphide bond between Cys-61 and Cys-176; step 3, formation of Cys-61–Cys-174 intersubunit disulphide bond; step 4, reduction of intersubunit disulphide bond with DTT. Shunt path is a direct reduction of the oxidized Cys-61 intermediate by DTT, which is observed in the C174/176A mutant. The fully reduced enzyme might be dimeric, whereas the oxidized enzyme might be decameric in nature.

doubled. The overall efficiency of the enzyme, as judged by the k_{cat}/K_m value, was reduced approx. 1.5-fold. Thus the C176A mutant showed lower efficiency as a result of increased turnover number as well as lower affinity to the substrate in comparison with the wild-type AhpC. The C174/176A mutant showed approximately the same k_{cat} as the wild-type enzyme but the K_m increased 1.4-fold.

Determination of the kinetic parameters of different mutants reveals a fascinating feature of *M. tuberculosis* AhpC. Whereas the N-terminal cysteine (Cys-61) assumes a central role in catalysis, the two C-terminal cysteines, 174 and 176, are also important for activity. In the absence of 174 and 176, the enzyme interestingly remains functional, and possibly adopts a 1-Cys Prx-type mechanism. A similar observation was also made by Chae et al. [8]. Similarly, in the absence of Cys-176 the enzyme possibly adopts a mechanism identical to 2-Cys Prx. Based on site-directed mutagenesis, enzymic characterization of the mutants, DTNB assays and SDS/PAGE experiments, we are able to propose the possible reaction mechanism of this important enzyme. The ability of the double mutant C174/176A to reduce peroxide substrates suggests that Cys-61 might be the primary site of peroxide reduction in the wild-type enzyme. The intermediate formed by Cys-61 oxidation can then be stabilized by the neighbouring subunit's Cys-176 to form a Cys-61–Cys-176 disulphide bond. Extrinsic reducing agents cannot regenerate the enzyme for the next reaction cycle, as suggested by the inactive C174A mutant. It is possible that the Cys-61–Cys-176 disulphide bond is solvent-inaccessible. The Cys-61–Cys-176 disulphide bond can, however, be exchanged by forming a Cys-61–Cys-174 disulphide bond. Extrinsic agents have the ability to reduce this disulphide, as also observed in the C176A mutant, and can then regenerate the enzyme for further reaction cycles. Thus a complex disulphide-relay mechanism might exist in the activity of *M. tuberculosis* AhpC, as illustrated in Figure 5.

Table 5 The role of Cys residues in oligomerization and enzyme activity

Cys residues	Oligomeric state	Activity
61, 174, 176 (wild-type)	Decamer	Active
61, 174 (C176A mutant)	Monomer, dimer, decamer	Partially active
61, 176 (C174A mutant)	Disulphide-linked dimer	Inactive
174, 176 (C61A mutant)	Dimer	Inactive
61 (C174/176A mutant)	Dimer, monomer	Partially active

Participation of the three Cys residues in oligomerization and activity is summarized in Table 5. A number of conformational and oligomerization changes evidently occur during the reaction cycle. We had earlier demonstrated these conformational changes through spectroscopic studies and that the wild-type protein is decameric in nature [22]. The present work shows that the C61A and C174A mutants cannot form decamers and are clearly inactive. The Cys-61–Cys-174 disulphide bond might be an essential determinant of decamerization, and thus activity. Thus the activity of the protein seems to be intricately related to conformational changes, intersubunit disulphide-bond formation and oligomerization, all of which might be simultaneously occurring events.

The proposed disulphide-relay reaction mechanism is distinct from the 1-Cys Prxs and 2-Cys Prxs, and therefore *M. tuberculosis* AhpC might belong to a novel Prx class. Relative solvent exposure of Cys-174 in the three-dimensional model of the enzyme suggests that *M. tuberculosis* AhpC might be able to use small molecule thiols more efficiently, rather than protein molecules, as its redox

partner (results not shown). Clearly more experiments will be needed to establish this intricate and unique disulphide-relay mechanism.

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