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Thiol-Dependent Peroxidases in *Mycobacterium tuberculosis* Antioxidant Defense

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1. Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) is the causative agent of tuberculosis disease. According to the Global Tuberculosis Control 2010 report of the World Health Organization http://www.who.int/tb/publications/global_report/en/, approximately one-third of the world's population is latently infected with *M. tuberculosis* and about two million people die of this disease every year. The emergence of multi- and extensively- drug resistant strains to the currently available drugs makes the development of new therapeutic strategies a priority. However, the mechanisms underlying pathogenesis, virulence and persistence of infections caused by *M. tuberculosis* are not completely understood (Nathan, 2009; Lawn & Zumla, 2011).

M. tuberculosis is one of the most successful human pathogens. It has evolved diverse strategies to ensure growth and survival inside the hostile environment of macrophages, its primary host cells (Ehrt & Schnappinger, 2009; Meena & Rajni, 2010). The molecular mechanisms of *M. tuberculosis* pathogenesis are under active investigation, since they could provide the basis for a rationalized drug design. These include inhibition of phagosome maturation into phagolysosomes (Armstrong & Hart, 1971; MacMicking *et al.*, 2003), inhibition of the acidification of *Mycobacterium*-harboring phagosomes (Sturgill-Koszycki *et al.*, 1994), DNA repair and protein repair or degradation (Boshoff *et al.*, 2003; Gandotra *et al.*, 2007; Lee *et al.*, 2009), as well as decomposition of cytotoxic reactive nitrogen and oxygen species formed upon phagocytosis (Nathan & Shiloh, 2000; Shiloh & Nathan, 2000; Bedard & Krause, 2007). These should be considered as complementary survival mechanisms. Herein, we will focus in the antioxidant systems of *M. tuberculosis*, and particularly, in thiol-dependent peroxidases.

2. Formation of reactive oxygen and nitrogen species by activated macrophages

Upon phagocytosis, NADPH oxidase (NADPHox) assembles into an enzymatically active complex that transfers electrons from NADPH to molecular oxygen producing superoxide

anion radical ($O_2^{\bullet-}$) inside the phagosomes (Babior, 1984; Groemping & Rittinger, 2005). The charged nature of this radical at physiological pH (hydroperoxyl radical ($HO_2^{\bullet}$) pK_a = 4.75, (Blelski & and Allen, 1977)) determines its low diffusion capability through membranes. In turn, INFγ-mediated induction of iNOS leads to the formation of nitric oxide ($^{\bullet}NO$), a small lipophilic moiety that can diffuse into the phagosome (Xie *et al.*, 1993; Martin *et al.*, 1994; MacMicking *et al.*, 1997). $O_2^{\bullet-}$ can spontaneously or enzymatically dismutate into hydrogen peroxide (H_2O_2)(De Groote *et al.*, 1997; Fridovich, 1997). Reactions of the latter species with reduced transition metal centers (particularly containing Fe^{2+} or Cu^{+}) yield the strong and non-selective oxidizing compound, hydroxyl radical ($^{\bullet}OH$) through Fenton reactions. Moreover, the diffusion-controlled reaction between $O_2^{\bullet-}$ and $^{\bullet}NO$ forms peroxynitrite[1], an

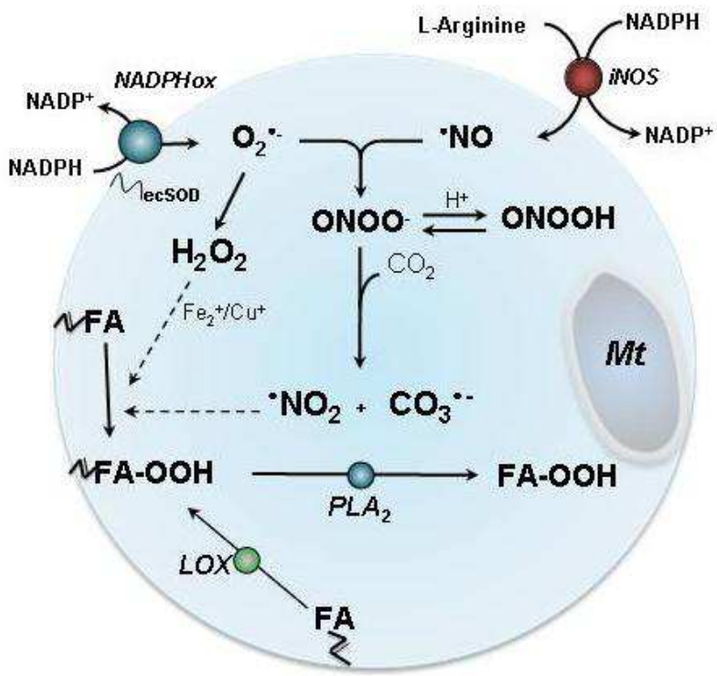

Fig. 1. **Peroxide sources in *M. tuberculosis* (*Mt*)-harboring phagosomes of activated macrophages.** Details on the pathways leading to the production of peroxides (H_2O_2, peroxynitrite and fatty acid hydroperoxides (FA-OOH), in bold) among other reactive nitrogen and oxygen species are given in the text. Dashed lines indicate reactions involving several steps and intermediates.

[1] IUPAC recommended names for peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH) are oxoperoxonitrate (1-) and hydrogen oxoperoxonitrate, respectively. The term peroxynitrite is used to refer to the sum of $ONOO^-$ and ONOOH.

oxidizing and nitrating moiety (Ferrer-Sueta & Radi, 2009; Alvarez *et al.*, 2011). In the absence of direct targets, peroxynitrous acid (pK_a = 6.5-6.8, (Goldstein & Czapski, 1995; Pryor & Squadrito, 1995; Kissner *et al.*, 1997)) homolyses into nitrogen dioxide ($^{\bullet}NO_2$) and $^{\bullet}OH$ in 30% yields (k = 0.9 s^{-1} at pH 7.4 and 37 ^{o}C) (Goldstein & Czapski, 1995; Gerasimov & Lymar, 1999). However, the importance of this reaction *in vivo* is probably limited, since in cells, most peroxynitrite is expected to be involved in direct reactions. For instance, peroxynitrite can react with carbon dioxide (CO_2) present in mM concentrations in biological systems (k = 4.6 x 10^4 M^{-1} s^{-1} at pH 7.4 and 37 ^{o}C), leading to the formation of up to 35% carbonate ($CO_3^{\bullet-}$) and $^{\bullet}NO_2$ radicals, which are also oxidizing species (Lymar & Hurst, 1995; Denicola *et al.*, 1996; Bonini *et al.*, 1999; Augusto *et al.*, 2002). $^{\bullet}OH$ and $^{\bullet}NO_2$ can participate in lipid peroxidation reactions, resulting in fatty acid hydroperoxide formation (Barber & Thomas, 1978; Prutz *et al.*, 1985). These can also be synthesized by enzymatic mechanisms through lipoxygenase (LOX)-catalyzed reactions (Sevanian *et al.*, 1983). Fatty acid hydroperoxides can be released from membranes by the action of phospholipase A_2 (PLA_2)(Bonney *et al.*, 1985). Free arachidonic acid is toxic for *M. tuberculosis* acting in a synergistic way with reactive nitrogen species (Akaki *et al.*, 2000). Although the mechanism of synergism has not been resolved, the fact that free fatty acid- dependent toxicity to *Helicobacter pylori* increases in peroxidase-deficient strains indicates that fatty acid hydroperoxides could participate in cytotoxicity (Wang *et al.*, 2006). In summary, inside the phagosomes of activated macrophages, and among other reactive species, different peroxides can be formed, including H_2O_2, peroxynitrite and fatty acid hydroperoxides (Figure 1). All of these species have been reported to be cytotoxic against microorganisms including bacteria (Clifford & Repine, 1982; Denicola *et al.*, 1993; Hurst & Lymar, 1997; Evans *et al.*, 1998; Wang *et al.*, 2006). The enzymatic mechanisms that allow reactive nitrogen and oxygen species detoxification, in general, and peroxide reduction, in particular, thus enabling the bacterium to infect and persist inside the phagosome of activated macrophages, is a field of active investigation.

3. Singular aspects of the antioxidant defense systems of *M. tuberculosis*

The antioxidant defenses of *Mycobacterium tuberculosis* are unusual in many aspects. As most other Actinobacteria, it lacks glutathione, and contains millimolar concentration of 1-D-myo-inosityl-2-deoxy-2-(N-acetyl-L-cysteinyl)amino-D-glucopyranoside, or mycothiol (MSH), as main low molecular weight thiol (Newton & Fahey, 2002). MSH is maintained in the reduced form by mycothione reductase using NADPH as electron donor (Patel & Blanchard, 2001). It participates in drug detoxification pathways by forming adducts with alkylating agents and antibiotics that are subsequently cleaved by MSH S-conjugate amidase to generate a mercapturic acid (excreted outside the cell) and glucosamine inositol (used to regenerate MSH) (Newton *et al.*, 2000). MSH can function as a resource for metabolic precursors and for energy production (Bzymek *et al.*, 2007). Mycothiol-deficient *M. smegmatis* strains are more sensitive to $^{\bullet}NO$- and H_2O_2-mediated toxicity than wild type strains (Rawat *et al.*, 2002; Miller *et al.*, 2007). However, there is currently no evidence for MSH acting as a reducing substrate for any peroxidase. Mycobacteria, among other organisms, also synthesize ergothioneine, which is a thiourea derivative of histidine containing a sulfur atom in the imidazole ring. Its synthesis is increased in *M. smegmatis* mutants in MSH synthesis suggesting a compensation mechanism (Ta *et al.*, 2011), although the actual function of this unusual thiol remains to

be investigated (Seebeck, 2010). Related to enzymatic mechanisms of reactive oxygen species detoxification, *M. tuberculosis* expresses a Fe-dependent superoxide dismutase, SODA (Rv3846), which is released to the extracellular medium and is considered to be important for pathogenesis (Edwards *et al.*, 2001); it also express a Cu-dependent SODC (Rv0432) that is not essential for intracellular growth within macrophages and seems to play a minor role in pathogenicity (Dussurget *et al.*, 2001). *M. tuberculosis* contains different thioredoxin-related enzymes which are maintained at reduced state by thioredoxin reductase and NADPH (Jaeger *et al.*, 2004). In spite of the absence of glutathione, *M. tuberculosis* genome codifies for different glutaredoxin-like proteins whose functional role awaits further investigation (Cole *et al.*, 1998). The bacterium expresses a heme-dependent peroxidase (catalase peroxidase, KatG) and several thiol-dependent peroxidases of the peroxiredoxin (Prx) type (see below). Moreover, *M. tuberculosis* lacks a functional OxyR, that in *E. coli* controls the transcription of a regulon of ~ 20 antioxidant genes (Zahrt & Deretic, 2002). The regulation of oxidative stress responses in *M. tuberculosis* is at least partially dependent on the alternative sigma factor H/antisigma factor H, a zinc-thiolate redox sensor (Raman *et al.*, 2001).

4. Catalase peroxidase, the heme-dependent peroxidase of *M. tuberculosis*

M. tuberculosis constitutively expresses a catalase peroxidase (EC 1.11.1.6) (*Mt*KatG, Rv1908)(Diaz & Wayne, 1974). The enzyme has attracted considerable attention due to its role in the activation of the first line antituberculosis prodrug isonicotinic acid hydrazide (isoniazid, INH) and the fact that loss-of-function mutations are a major mechanism of resistance to INH (Zhang *et al.*, 1992). *In vitro* generated *Mt*KatG negative strains were non pathogenic. Virulent catalase-negative clinical isolates overexpressed the thiol-dependent peroxidase alkyl hydroperoxidase reductase C (AhpC), indicating the need of another peroxidase to assure protection of the pathogen against oxidizing species (Sherman *et al.*, 1996). More recently, a mechanism of INH resistance in *M. tuberculosis* through down-regulation of KatG was proposed based on the observation that mutations in the *furA*[2]*-katG* intergenic region conferred INH resistance (Ando *et al.*, 2011). The protein has been identified in the cytosol, membrane fraction and culture filtrates of *M. tuberculosis* (Gu *et al.*, 2003; Mawuenyega *et al.*, 2005; Malen *et al.*, 2007). It displays a broad peroxidase activity, as well as a high catalase activity (k_{cat}/K_M = 2 x 10^6 $M^{-1}s^{-1}$)(Johnsson *et al.*, 1997), catalyzing the dismutation of H_2O_2 into dioxygen and water. It also reduces peroxynitrite (k = 1.4 x 10^5 M^{-1} s^{-1} at pH 7.4 and 25 ^oC (Wengenack *et al.*, 1999)). The catalytic mechanism of H_2O_2 reduction by KatG involves the initial two-electron oxidation of the enzyme to compound I ((Fe IV=O)$^\bullet$). KatG contains a unique post-translational modification in the form of a three amino acid adduct (Met255-Tyr229-Trp107) with a specific role in the catalase reaction since mutation of any of the three residues virtually eliminates catalase but not peroxidase activity (Jakopitsch *et al.*, 2004; Ghiladi *et al.*, 2005). It has been proposed that catalase activity in KatG is associated with a radical formation in the Met-Tyr-Trp adduct, whereas during the peroxidase activity a tyrosyl radical is formed (Zhao *et al.*, 2010). In the case of peroxynitrite reduction, oxidation of resting state KatG to compound II (Fe IV=O) plus $^\bullet NO_2$ has been proposed (Wengenack *et al.*, 1999).

[2] FurA is a negative regulator of KatG expression in *Mycobacterium smegmatis* (Zahrt *et al.*, 2001)

In addition to *Mt*KatG, the genome of *M. tuberculosis* codifies for a putative lignin peroxidase (Rv1900c) and other putative non-heme non-thiol -dependent peroxidases whose functional characterization is lacking (Cole *et al*., 1998)(http://www.webtb.org/).

5. Thiol-dependent peroxidases of *M. tuberculosis*

5.1 Thiol-dependent peroxidases

Peroxidases with catalytic activities dependent on critical cysteine residues are called thiol-dependent peroxidases. These enzymes catalyze the reduction of H_2O_2, organic hydroperoxides and/or peroxynitrous acid (ONOOH) to water, organic alcohols and nitrite, respectively, at the expense of a reducing substrate, usually thioredoxin (Trx) or a Trx-related protein, via a double-displacement or ping-pong kinetic mechanism (Flohe *et al*., 2003; Wood *et al*., 2003; Trujillo *et al*., 2007).

$$\begin{matrix} \text{ROOH} \\ H_2O_2 + \\ \text{ONOOH} \end{matrix} \quad Trx(SH)_2 \xrightarrow[\text{peroxidase}]{\text{Thiol}} H_2O + \begin{matrix} \text{ROH} \\ H_2O \\ NO_2^- + H^+ \end{matrix} + TrxS_2 \qquad (1)$$

where ROOH is organic peroxide; ONOOH is peroxynitrous acid; NO_2^- is nitrite; ROH is organic alcohol; $Trx(SH)_2$ is reduced thioredoxin and $TrxS_2$ is oxidized thioredoxin.

The oxidizing part of the catalytic cycle involves a SN_2 reaction occurring through a nucleophilic attack of the deprotonated thiol at the so called peroxidatic cysteine residue (C_P) on one of the peroxide oxygens. In the transition state, the negative charge is distributed among the two oxygen and the sulfur atoms, and the reaction is completed by the break of the peroxide bond forming an alcoxide as leaving group, which may protonate depending on its basicity. Thus, the thiolate in C_P suffers a two-electron oxidation to sulfenic acid (E-SOH).

$$\begin{matrix} \text{ROOH} \\ H_2O_2 + \\ \text{ONOOH} \end{matrix} \quad C_P\text{-}S^- \longrightarrow \begin{matrix} \text{ROH} \\ H_2O \\ NO_2^- + H^+ \end{matrix} + C_P\text{-}SO^- \qquad (2)$$

The rest of the catalytic cycle differs depending on the kind of thiol-dependent peroxidase. In most cases, it consists on the formation of a disulfide bridge through the reaction between the sulfenic acid intermediate in C_P and another cysteine residue, which is called the resolving cysteine residue (C_R), which is then reduced by thioredoxin (Trx) (or another thiol-disulfide oxidoreductase protein) that is maintained at reduced state by thioredoxin reductase and NADPH (Poole, 2007). For all thiol-dependent peroxidases tested so far, the acidity constants of the peroxidatic thiols are quite high (pK_a ~ ‹5 - 6.3, (Bryk *et al*., 2000; Ogusucu *et al*., 2007; Trujillo *et al*., 2007; Nelson *et al*., 2008; Hugo *et al*., 2009)). Thus, under physiological conditions they are expected to be mostly under thiolate form, the reactive species. However, the rate constants of reactions of C_P in thiol-dependent peroxidases with peroxide substrates are several orders of magnitude faster than the corresponding reactions of low molecular weight or most protein thiolates, indicating the existence of protein factors involved in specific peroxide reduction by these enzymes that are only starting to be unraveled (Trujillo *et al*., 2007; Flohe *et al*., 2010; Hall *et al*., 2010; Ferrer-Sueta *et al*., 2011).

Other intriguing aspect related to thiol-dependent peroxidase catalytic mechanism is the molecular mechanisms of the oxidizing substrate specificity: although in most cases thiol-dependent peroxidases can catalyze the reduction of a broad range of peroxides, preferential substrates vary, and do not reflect the expected trend that correlates thiolate reactivity with leaving group pK_a[3] (Trujillo *et al.*, 2007) that was reported for the reactivities of other thiolate with peroxides (Trindade *et al.*, 2006; Trujillo *et al.*, 2007).

Thiol-dependent peroxidases can be classified into two main groups[4] based on sequence homology: glutathione peroxidases (Gpxs) and peroxiredoxins (Prxs). Since there are not genes for enzymes of the GPx type in *M. tuberculosis* genome, but there are several members of the Prx family, we will focus in the latter group of enzymes through the rest of this chapter.

5.2 Peroxiredoxins (EC 1.11.1.15)

Prxs are a family of thioredoxin-scaffold enzymes with thiol-dependent peroxidase activity (Chae *et al.*, 1994). They are ubiquitous, present in all living kingdoms and in different cellular compartments. They are also abundant, with concentrations usually in the μM range (Hofmann *et al.*, 2002). Due to their peroxidase activity, these enzymes play a role in antioxidant defenses. Moreover, at the light of the signaling role ascribed to H_2O_2 and other peroxides, Prxs are also regarded as key players in redox signaling processes and regulation of transcription factors (Rhee *et al.*, 2005; Hall *et al.*, 2009; Brigelius-Flohe & Flohe, 2011; Rhee & Woo, 2011). Peroxiredoxins have been functionally classified into 1-Cys Prxs and 2-cysteine Prxs according to the number of cysteine residues that participate in catalysis (Poole, 2007). The first part of the catalytic cycle is common for all kinds of Prxs and consists on the reduction of the peroxide substrate with concomitant oxidation of the C_P to a sulfenic acid derivative. In the case of 1-Cys Prxs, this sulfenic acid is reduced by different reducing pathways that depend on the particular 1-Cys Prx and that for most of them are still unclear. In 2-Cys Prxs, the sulfenic acid in C_P reacts with another Cys residue also required for catalysis, C_R that can be either in the same or in a different protein subunit (atypical or typical 2-Cys Prxs, respectively), forming a disulfide bridge that is reduced by Trx or a Trx-related protein. More recently, a Prx classification base on sequence homology has been proposed in the peroxiredoxin classification index (PREX) database (http://csb.wfu.edu/prex/index.php)(Nelson *et al.*, 2011; Soito *et al.*, 2011). Subfamilies thus identified are denoted by the name of one or more canonical member, as indicated below:

- **Alkyl hydroperoxide reductase C (AhpC) - Peroxiredoxin 1 (Prx1)**. This subfamily is both the largest and the most widely distributed, with members found in archaea, bacteria, and all classes of eukaryotes These proteins are functionally classified as typical 2-Cys Prxs.

[3] pK_a value of the alkoxide formed upon peroxide reduction

[4] Other thiol-dependent peroxidases non-structurally related to Gpxs and Prxs exist. For example, in many bacteria, a thiol-dependent organic hydroperoxide reductase (Ohr) is involved in organic hydroperoxide detoxification. However, the ohr gene is absent in *M. tuberculosis* genome

- **Bacterioferritin comigratory protein (Bcp)- Peroxiredoxin Q (PrxQ) (for *Escherichia coli* Bcp and plant PrxQ, respectively).** Present mostly in bacteria, also in yeast and plants but not in mammals. They can function either as atypical 2-Cys Prxs or as 1-Cys Prxs.
- **Thiol peroxidase (Tpx) (for *E. coli* Tpx).** Tpx subfamily members are all bacterial and are almost exclusively classified as atypical 2-Cys Prxs.
- **Peroxiredoxin 5 (for *Homo sapiens* Prx5).** Members of this subfamily are found from bacteria to mammals with members present in plants, fungi, and yeast. They are functionally classified as either 1-Cys Prxs or atypical 2-Cys Prxs.
- **Peroxiredoxin 6 (for *H. sapiens* Prx6).** Members of this subfamily are found in bacteria, plants, yeast and mammals. In general, they function as 1-Cys Prxs.
- **Alkyl hydroperoxide reductase E (AhpE) (for *M. tuberculosis* AhpE).** Found in aerobic gram-positive bacteria of the order Actinomycetes and some archaea. AhpE from *Mycobacterium tuberculosis* has been functionally classified as a 1-Cys Prx, but information regarding the catalytic mechanisms of other members of this group is lacking.

Further information regarding this sequence-based classification of Prxs can be found in the PREX database and references therein.

Peroxiredoxins are now known to be, at least in some cases, very efficient peroxidases (Trujillo *et al.*, 2007; Parsonage *et al.*, 2008; Manta *et al.*, 2009). The local sequence motif at the active site, ProXXXThrXXCys, is very conserved among different Prx subfamilies, although Thr is replaced by Ser in few known Prx sequences and a peroxidatic selenocysteine (Sec) instead of Cys has been reported in a Prx from *Eubacterium acidamidophilum* (Sohling *et al.*, 2001; Hofmann *et al.*, 2002; Poole, 2007; Nelson *et al.*, 2011). Prxs also contain a highly conserved Arg. These conserved residues along with several backbone interactions determine a low pK_a value of C_P and contribute to the catalytic mechanism of Prxs, in which transition state stabilization has been proposed to be involved (Hall *et al.*, 2010), although the precise mechanism of catalysis is still to be unraveled. Prx concentrations in different cells and tissues are frequently regulated, and usually increase under conditions of oxidative stress. Moreover, their catalytic activities are also regulated by different mechanisms, including protein phosphorylation (Chang *et al.*, 2002; Woo *et al.*, 2010) and inactivation due to overoxidation of the C_P, which involves the two- electron oxidation of the sulfenic form of the enzyme to sulfinic acid (Yang *et al.*, 2002). Recent data from our group indicated that the mechanism of C_P overoxidation is similar to that of oxidation, with the deprotonated sulfenate (or its tautomeric sulfoxide form) and the protonated peroxide as the reacting species (Hugo *et al.*, 2009; Reyes *et al.*, 2011).

$$\begin{matrix} \text{ROOH} \\ \text{H}_2\text{O}_2 + \\ \text{ONOOH} \end{matrix} \quad \text{E-SO}^- \longrightarrow \begin{matrix} \text{ROH} \\ \text{H}_2\text{O} \\ \text{NO}_2^- + \text{H}^+ \end{matrix} + \text{E-SO}_2^- \qquad (3)$$

In 2-Cys Prxs, the susceptibility to overoxidation depends on the structural GGLG and YF motifs present mostly in eukaryotic 2-Cys Prxs (Yang *et al.*, 2002) but also in some prokaryotic organisms including cyanobacteria (Pascual *et al.*, 2010). These structural motifs make disulfide formation with C_R to occur at a slower rate and thus, 2-Cys Prxs that possess them are more prone to oxidative inactivation (Wood *et al.*, 2003). Cysteine sulfinic acid,

previously considered as an irreversible post-transductional modification, is now known to be reversed by enzymatic mechanisms in different 2-Cys Prxs (Chang *et al.*, 2004; Iglesias-Baena *et al.*, 2011) and has been suggested to be involved in signaling processes (Iglesias-Baena *et al.*, 2010). Moreover, overoxidized forms of some members of the Prx family gained function as molecular chaperones (Moon *et al.*, 2005; Lim *et al.*, 2008).

5.3 Peroxiredoxins from *M. tuberculosis*

The genome of *M. tuberculosis* codifies for different thiol-dependent peroxidases of the Prx type, namely AhpC, TPx, AhpE, and two putative Bcps proteins (Cole *et al.*, 1998), which have been detected in the cytosolic, membrane and culture medium fractions (Figure 2). We will describe below the main functional characteristics of *M. tuberculosis* Prxs as well as reported evidences of their participation in peroxide detoxification in cellular or animal models of tuberculosis disease.

5.3.1 Alkyl hydroperoxide reductase C (*Mt*AhpC, Rv2428)

AhpCs are thiol-dependent peroxidase member of the AhpC-Prx1 subfamily of Prxs. *Mt*AhpC is functionally classified as a typical 2-Cys Prx, although site directed mutagenesis experiments revealed that it has three instead of two Cys residues involved in catalysis: C_P (Cys 61), the putative C_R (Cys 174) and a third Cys (Cys 176) whose role in catalysis is not completely clear but could provide an alternative route of disulfide bond formation (Guimaraes *et al.*, 2005). Whereas Cys 61 plays a central role in catalysis, the ehzyme remains partially functional in the absence of Cys 174 and 176 and possible adopts a 1-Cys-like mechanism (Chauhan & Mande, 2002; Koshkin *et al.*, 2004). *Mt*AhpC has been detected both in the bacterial cytosol (Covert *et al.*, 2001) and as a membrane associated protein (Gu *et al.*, 2003). AhpC forms part of bacterial alkyl hydroperoxide reductase (Ahp) system (Storz *et al.*, 1987). In enterobacteria, this system commonly consists of two components, AhpC and a flavin-containing disulfide reductase (AhpF) that reduces AhpC at NADH expense, and both enzymes are jointly up-regulated under oxidative stress conditions targeting the oxyR regulon (Tartaglia *et al.*, 1989). However, AhpF is lacking in all mycobacteria. In this context, two reducing systems for *M. tuberculosis* AhpC (*Mt*AhpC) have been proposed. Firstly, alkyl hydroperoxide reductase D (AhpD), that contains a CXXC motif, can reduce *Mt*AhpC. The *ahpD* gene is found immediately downstream of *ahpC*, in the position occupied by *ahpF* in *S. typhimurium* genome, and both proteins are controlled by the same promoter (Hillas *et al.*, 2000). Oxidized AhpD is regenerated by dihydrolipoamide acyltransferase (DlaT); in turn, dihydrolipoamide dehydrogenase (Lpd) mediates the reduction of DlaT at NADH expense and completes the catalytic cycle (Bryk *et al.*, 2002). *dlaT* (Rv2215) encodes the E2 component of the piruvate deshydrogenase complex, and *lpdC* (Rv0462), the only functional Lpd in *M. tuberculosis* (Argyrou & Blanchard, 2001), most probably codifies the E3 components of the piruvate deshydrogenase complex (Tian *et al.*, 2005). Secondly, thioredoxin C (TrxC), but not thioredoxin B (TrxB) or A (TrxA), was also able to act as AhpC reducing substrates (Jaeger *et al.*, 2004), and the catalytic cycle is completed by thioredoxin reductase (*Mt*TR) and NADPH. Catalytic efficiency of TrxC-mediated AhpC reduction was ~ 100 fold lower than that measured using AhpD as reducing substrate (2.5×10^4 *versus* 2.7×10^6 $M^{-1}s^{-1}$, respectively)(Jaeger *et al.*, 2004). However, the preferential reducing substrate would be determined not only by catalytic efficiencies but also by the steady-state concentrations of

reducing substrates at reduced state. *Mt*TrxC is consistently seen as a major spot in bacterial proteomes while the spot corresponding to *Mt*AhpD is of much lower intensity (Jungblut *et al.*, 1999; Mollenkopf *et al.*, 1999), indicating a lower concentration of *Mt*AhpD compared to *Mt*TrxC in these cells. Moreover, *Mt*TR is also an abundant protein in Mycobacteria (Jungblut *et al.*, 1999; Mollenkopf *et al.*, 1999), and it is expected to keep TrxC at reduced state as long as NADPH is not limiting (Jaeger *et al.*, 2004). These data suggest that, despite the lower catalytic efficiency of *Mt*TrxC compared to *Mt*AhpD in *Mt*AhpC reduction, both enzymatic pathways could be contributing to *Mt*AhpC-mediated peroxide detoxification *in vivo*. Concerning the oxidizing substrate specificity, AhpC are broad-spectrum peroxidases that catalyze the reduction of H_2O_2, organic hydroperoxides and peroxynitrite. The catalytic efficiency of *t*-BuOOH reduction (an artificial hydroperoxide used as a mimic of natural organic hydroperoxides) by *Mt*AhpC was reported as ~ 10^4 M^{-1} s^{-1} (Jaeger *et al.*, 2004). The enzyme could also reduce another artificial organic hydroperoxide, cumene hydroxperoxide at similar rates. H_2O_2 and linoleic acid hydroperoxides, but not phosphatidylcholine hydroperoxide, were also reduced by *Mt*AhpC. This enzyme, together with other bacterial AhpC enzymes, where the first Prxs for which a peroxynitrite reductase activity was demonstrated (k = 1.3 x 10^6 M^{-1} s^{-1} at pH 6.85 and RT (Bryk *et al.*, 2000)). H_2O_2 was a preferential substrate of *Mt*AhpC, although precise activity measurements were difficult to estimate due to the basal activity of the TR/Trx system (Jaeger *et al.*, 2004). In the case of another AhpC protein (from *Salmonella typhimurium*) the catalytic efficiency for H_2O_2 reduction was reported as 3.7 x 10^7 M^{-1} s^{-1} (Parsonage *et al.*, 2008). Thus, oxidizing substrate selectivity of bacterial AhpC seems to follow the same trend as for other members of the AhpC-Prx1 subfamily, where reduction of peroxynitrite is somewhat slower than that of H_2O_2 at near-physiological pHs, and occur with similar pH independent rate constants[5] (Manta *et al.*, 2009). There is no data regarding the pK_a of C_P and redox potential of AhpC from *M. tuberculosis*. The pK_a values of C_P in *S. tiphymurium* AhpC was first determined as < 5 (Bryk *et al.*, 2000) and more recently reported as 5.8 (Nelson *et al.*, 2008), indicating that C_P would be mostly deprotonated at physiological pH. The midpoint reduction potential of the enzyme was reported as -178 ± 0.4 mV, somehow lower than that reported for mammalian Prx 3 ($E^{o\prime}$ = -290 mV) (Cox *et al.*, 2010) and plant 2-Cys Prxs and PrxQ ($E^{o\prime}$ = −288 to −325 mV)(Dietz *et al.*, 2006). Data regarding redox potential of *Mt*AhpD is lacking. In turn, *M. tuberculosis* Trx redox potentials have not been investigated so far, but redox potential of other bacterial Trxs has been reported to be low (-270 mV for *E. coli* Trx (Krause *et al.*, 1991). Since the standard midpoint reduction potential for H_2O_2 reduction to water and for ONOOH reduction to nitrite and water are 1.77 and 1.6 V, respectively (Latimer, 1938; Koppenol & Kissner, 1998), the thermodynamic driving forces would highly favor the flux of electrons from Trxs to these peroxides through AhpC. In addition to its peroxidase

[5] According to the mechanism of reaction in which the thiolate form of the C_P reacts with the protonated peroxide, and considering that all the reported pK_a values of Prxs including AhpC are < 6.3 (*68*), (*70*), (*71*), and that the pK_a for the first H_2O_2 deprotonation is far above physiological pH, the pH-independent rate constant for H_2O_2-mediated Prx oxidation is practically the same as the rate constant determined at physiological pH. However, the pK_a value of peroxynitrous acid is around 6.8 (reported values of ONOOH pK_a = 6.5-6.8 (*23*), (*24*), (*25*)) and therefore, only 50 % or 20 % of peroxynitrite would be as be protonated at pH 6.8 or 7.4, respectively. Thus, the pH-independent rate constant of Prx oxidation by peroxynitrite would be 2 or 5 times higher than the value determined at pH 6.8 or 7.4.

activity, some bacterial AhpCs have other functions: *Helicobacter pylori* AhpC can form high molecular weight aggregates with chaperone activity under oxidative stress conditions (Huang *et al.*, 2010). Moreover, AhpC from some Gram negative microorganisms show a deglutathionylating activity that depends on C_R rather than on C_P (Yamamoto *et al.*, 2008).

Size exclusion chromatography indicated that wild-type *Mt*AhpC performs as a heterogeneous mixture of oligomers under non-reducing conditions, whereas under reduced state the enzyme is a homogeneous oligomer formed by 10- or 12-subunits. The C176S mutant form of AhpC is dimeric under oxidized state, and forms oligomers of 10-12 subunits upon reduction. The crystallographic structure of C176S *Mt*AhpC trapped as an intermediate of its catalytic cycle (where condensation had already occurred but still the enzyme was under its oligomeric form) was consistent with the formation of a ring shaped oligomer of 12 subunits, a hexamer of dimers (Guimaraes *et al.*, 2005). The relationship between *Mt*AhpC oligomerisation and activity has not been addressed. In the case of *Salnonella typhimurium* AhpC, decameric under reduced state, the analysis of mutated forms of the enzyme at the decamer-building interface indicated that the oligomerization is quite important, but not essential to activity (Parsonage *et al.*, 2005).

The role of *Mt*AhpC in the detoxification of peroxides *in vivo* was first suggested by the fact that pathogenic, INH-resistant strains lacking KatG over-expressed *Mt*AhpC, which would represent a compensatory mechanism allowing the bacteria to get rid of cytotoxic peroxides (Sherman *et al.*, 1996). Overexpression of MtAhpC in those strains was associated to mutations in the gene promoter (Wilson & Collins, 1996). Thus, *Mt*AhpC was proposed as a potential drug target. However, data obtained using *M. tuberculosis* strains lacking *Mt*AhpC are not straightforward. AhpC expression in virulent strains of *M. tuberculosis* grown *in vitro* was repressed and increased under conditions of static growth, probably reflecting adaptation of the bacterium during its infection cycle (Springer *et al.*, 2001). AhpC expression was also induced by hypoxia (Sherman *et al.*, 2001). *S. typhimurim* lacking ahpC became hypersusceptible to reactive nitrogen species and *Mt*AhpC complemented the defect. The enzyme also protected human cells from toxicity caused by reactive nitrogen species (Chen *et al.*, 1998). Whereas inactivation of *Mt*AhpC caused no effect on bacterial growth during acute infection in mice and had no effect on *in vitro* sensitivity to H_2O_2, it caused an increase susceptibility to organic hydroperoxide and peroxynitrite-mediated toxicity (Springer *et al.*, 2001; Master *et al.*, 2002). Inactivation of *Mt*AhpC caused a decrease in the survival of *M. tuberculosis* in non-stimulated macrophages but not in macrophages stimulated with interferon-γ (Master *et al.*, 2002). Strains lacking DlaT showed retarded growth, were highly susceptible to killing by acidified nitrite *in vitro*, showed decreased intracellular survival during macrophage infection and were less virulent in a mouse model of tuberculosis (Shi & Ehrt, 2006). Overall, these data indicate the importance of both *Mt*AhpC and *Mt*DlaT, its reductant through *Mt*AhpD, for *M. tuberculosis* to overcome oxidative stress encountered inside its primary host cells and to establish a successful infection.

5.3.2 Thiol peroxidase (*Mt*TPx, Rv1932)

The second Prx from *M. tuberculosis* to be identified belonged to the TPx subfamily (Jaeger *et al.*, 2004), enzymes widely distributed among Gram-positive and Gram-negative bacteria. In the case of *E. coli* Tpx, the enzyme is localized in the periplasmic space. In *M. tuberculosis*,

TPx was firstly characterized as an extracellular antigen that induces a strong proliferative response in animals (Weldingh *et al.*, 1998) . *Mt*TPx was repeatedly found in culture filtrates; it has also been found associated to membranes and in cytosolic fractions (Rosenkrands *et al.*, 2000; Covert *et al.*, 2001; Malen *et al.*, 2007; Malen *et al.*, 2010).

TPxs are atypical 2-Cys Prxs. They typically contain three cysteine residues where Cys60 is C_P, C93 is C_R and Cys80[6] is catalytically irrelevant. However, site directed mutagenesis studies revealed that *Mt*TPx lacking Cys 93 remained active for a limited period of time before getting inactivated by C_P overoxidation to sulfinic acid, and therefore the role of Cys93 is likely the formation of an intramolecular disulfide with the sulfenic acid in C_P and to avoid C_P overoxidation under conditions of restricted availability of reducing substrates (Trujillo *et al.*, 2006). *Mt*TPx reacts very rapidly with peroxynitrite (k = 1.5 x 10^7 M^{-1} s^{-1} at pH 7.4 and 25 ^oC)[7]. Reduction of *t*-BuOOH was slower (k ~ 1 x 10^5 M^{-1} s^{-1} at pH 7.4 and 25 ^oC). Reduction of H_2O_2 was faster than that of *t*-BuOOH, although the exact number was difficult to estimate. The enzyme was hardly active towards linolenic acid hydroperoxide and could not reduce phosphatidylcholine hydroperoxide. Concerning the reductive part of the catalytic cycle, both *Mt*TrxB and *Mt*TrxC reduced *Mt*TPx with similar catalytic efficiencies (4.6 and 5.8 x 10^4 M^{-1} s^{-1}, respectively). Since according to proteomic data currently available *Mt*Trx C would be much more abundant than *Mt*TrxB, the former would play a major role as *Mt*TPx reducing substrate. Mycothiol plus mycothione reductase/NADPH were not able to reduce *Mt*TPx (Jaeger *et al.*, 2004).

The crystallography structure of *Mt*Tpx (Rho *et al.*, 2006) and on the inactive mutant C60S *Mt*Px (Stehr *et al.*, 2006), as for other bacterial TPxs, indicated that Cys60 in *Mt*TPx forms part of a typical catalytic triad with Thr57 and Arg130. The enzyme is dimeric both in the crystal structure and in solution (Rho *et al.*, 2006; Stehr *et al.*, 2006). In C60S *Mt*TPx, a cocrystallized acetate molecule interacted with Ser60, Arg130 and Thr57 (Stehr *et al.*, 2006). Similarly, the wild type enzyme also showed anions near the active site. Co-crystallization with anions is frequently observed in Prxs; it has been proposed the existence of an anion-binding site in the neighborhood of reactive thiols in proteins, that could participate in transition state stabilization and thus, in the acceleration of peroxides reduction in general (Hall *et al.*, 2010; Ferrer-Sueta *et al.*, 2011).

M. tuberculosis strains lacking functional *Mt*TPx had a lower peroxidase activity than their wild type counterparts, indicating that the enzyme importantly contributes to the total peroxidase activity in *M. tuberculosis*. Moreover, *Mt*TPx mutants were more sensitive to H_2O_2 and •NO-mediated toxicity, but the effect was recovered when they were complemented with the tpx gene. Strains lacking *Mt*TPx failed to grow and survive in macrophages, particularly after activation by interferon-γ. Growth was significantly restored in the macrophages from iNOS knockout mice. This is consistent with the ability of the enzyme to rapidly reduce peroxynitrite *in vitro*. Moreover, strains lacking *Mt*TPx

[6] Cysteine numbers correspond to the sequence in TPx from *M. tuberculosis*.

[7] The pK_a value of C_P in *Mt*TPx or other bacterial TPx has not been reported previously. Considering a pK_a value of <6.3, as for all other Prxs investigated so far, more than 90 % of C_P would be as thiolate and 20% of peroxynitrite as ONOOH at pH 7.4. Thus, the pH-independent rate constant of C_P oxidation by peroxynitrite would be 5 times higher than the value determined at pH 7.4, 7.5 x 10^7 $M^{-1}s^{-1}$. It would be even higher if the pK_a of C_P of *Mt*TPx was > 6.3.

failed to initiate an acute infection and to maintain a persistent infection, and were less virulent than wild type strains (Hu & Coates, 2009). In the *M. bovis* strain BCG, TPx is induced in response to exposure to diamide, an agent that causes thiol oxidation (Dosanjh *et al.*, 2005).

5.3.3 Alkyl hydroperoxide reductase E (*Mt*AhpE, Rv2238c)

The genome of *M. tuberculosis* also codifies for a one-cysteine Prx, alkyl hydroperoxide reductase E, which is highly conserved among many Mycobacteria (Cole *et al.*, 1998; Passardi *et al.*, 2007). *Mt*AhpE belongs to a novel family of Prxs, comprising bacterial and archaean AhpE and AhpE-like enzymes (Passardi *et al.*, 2007; Soito *et al.*, 2011). This protein has been identified in the membrane fraction of *M. tuberculosis* H37Rv using a proteomics approach (Gu *et al.*, 2003). The expression of *Mt*AhpE increases during the dormant phase of tuberculosis disease (Murphy & Brown, 2007). Although *Mt*AhpE shows greater sequence similarity with mammalian typical two-Cys Prxs than with one-Cys Prxs (Passardi *et al.*, 2007; Soito *et al.*, 2011), it has only one Cys residue and functions by a one-Cys mechanism. Accordingly, in the oxidized form of the enzyme C_P is as sulfenic acid, as revealed by crystallographic studies and by mass spectrometry analysis (Li *et al.*, 2005; Hugo *et al.*, 2009). We have reported the peroxidase activity of *Mt*AhpE, being the first member of the AhpE family to be functionally characterized (Hugo *et al.*, 2009). The physiological reducing substrate(s) for *Mt*AhpE (as well as AhpE-like Prxs) is/are still unknown, but its catalytic activity was demonstrated using the artificial substrates dithiotreitol (DTT) and thionitrobenzoic acid (TNB). Neither N-acetylcysteine nor glutathione could reduce oxidized *Mt*AhpE but led to mixed disulfides formation. Concerning oxidizing substrate specificity, *Mt*AhpE reduces peroxynitrite three orders of magnitude faster than H_2O_2 (1.9×10^7 *versus* 8.2×10^4 M^{-1} s^{-1} at pH 7.4 and 25 ^oC, respectively[8]). These rate constants were measured directly by taking advantage of the decrease in Trp-dependent fluorescence intensity that the enzyme exhibits upon oxidation. Moreover, the kinetics of peroxide-mediated inactivation by overoxidation of C_P to sulfinic acid was measured following the increase in the enzyme's intrinsic fluorescence intensity (k = 40 $M^{-1}s^{-1}$ for H_2O_2–mediated overoxidation)(Hugo *et al.*, 2009). This value was very similar to that previously calculated for mammalian Prx 1 oxidative inactivation by H_2O_2 (57 M^{-1} s^{-1}) (Wood *et al.*, 2003; Stone, 2004). The pK_a of the thiol (in reduced *Mt*AhpE) and of the sulfenic acid (in oxidized *Mt*AhpE) were reported to be 5.2 and 6.6, respectively. Thus, taking into account the intrabacterial pH of wild-type *M. tuberculosis* (6.8–7.5 (Vandal *et al.*, 2008)), >95 % of the reduced and >50 % of the oxidized form of C_P in *Mt*AhpE would be deprotonated, and therefore, at their reactive forms with peroxides (Hugo *et al.*, 2009). More recently, we have performed a comprehensive study on *Mt*AhpE oxidizing substrate specificity as well as on its oxidative inactivation (Reyes *et al.*, 2011). For most peroxides tested, oxidation as well as oxidative inactivation rates

[8] Considering a mechanism of reaction where thiolate and sulfenate as well as protonated peroxides are the reactive species, the reported pK_a values of the thiol and sulfenic acid in reduced and oxidized *Mt*AhpE (Hugo *et al.*, 2009) and the pK_a of the H_2O_2 and peroxynitrite above indicated, pH independent rates constants can be calculated as very similar (for H_2O_2) and ~ 5 fold higher (for peroxynitrite) that the corresponding values measured at pH 7.4.

correlated with leaving group pK_a, indicating that both reactions occur by similar mechanisms, i.e. reaction of the thiolate or sulfenate anion at C_P with the protonated peroxide. In contrast, the hydroperoxide at position 15 of arachidonic acid (15-HpETE) and linolenic acid-derived hydroperoxides reacted surprisingly fast, with rate constants of ~10^8 and ~10^5 M^{-1} s^{-1} for *Mt*AhpE oxidation and overoxidation, respectively. The molecular basis for the fast reactivity of *Mt*AhpE with fatty acid hydroperoxides is intriguing. The quaternary structure of *Mt*AhpE in solution is tightly regulated by the oxidation state of the C_P, the enzyme being a dimer under reduced state and slowly forming high molecular weight aggregates upon oxidation (Hugo *et al.*, 2009). Analysis of the reported crystallographic structure of the protein under reduced state (Li *et al.*, 2005) showed a hydrophobic grove present in the dimeric enzyme, and formed by residues from both subunits, which is proposed as an anchoring site for fatty acid hydroperoxide binding (Reyes *et al.*, 2011). These data set *Mt*AhpE (and probably other AhpE-like Prxs) as potential Prxs specialized for fatty acid hydroperoxide detoxification. However, the roles of *Mt*AhpE in reduction of these or other peroxides *in vivo*, as well as in macrophage infection or bacterial virulence, remain to be investigated.

Prx	reductant	pK_a of C_P	$k_{2\ H2O2}$ (M^{-1} s^{-1})	$k_{2\ ONOOH}$ (M^{-1} s^{-1})	$k_{2\ t\text{-}BuOOH}$ (M^{-1} s^{-1})	$k_{2\ LOOH}$ (M^{-1} s^{-1})
AhpC	AhpD, TrxC	[a]5.8 (C_P-SH)	[a]3.7 x 10^7	[b]1.3 x 10^6	1-2.3 x 10^4	[c]6.9 x 10^3
TPx	TrxB, TrxC	ND	ND	1.5 x 10^7	0.9-3.4 x10^5	0
AhpE	ND	5.2 (C_P-SH) 6.6 (C_P-SOH)	8.2 x 10^4	1.9 x 10^7	8 x10^3	[d]1.8 x 10^8 [e]2.7 x 10^8

[a]For *St*AhpC; [b]At pH 6.85 and RT; [c] Calculated from (Jaeger *et al.*, 2004; Parsonage *et al.*, 2008), for linoleic acid hydroperoxide; [d]For 15-HpETE; [e]For α-linolenic acid hydroperoxide; ND is non determined. In the case of H_2O_2 reduction by *Mt*AhpC and *Mt*TPx, reactions were faster than with *t*-BuOOH, but precise rate constants were difficult to estimate (Jaeger *et al.*, 2004).

Table 1. Functional data on Prxs from *M. tuberculosis*: acidity constants, reducing substrates and kinetics of peroxide reduction.

5.3.4 Bacterioferritin comigratory proteins (Bcp, Rv2521; BcpB, Rv1608c)

The genome of *M. tuberculosis* also codifies for two putative Prxs of the Bcp type (Cole & Barrell, 1998). Evidence for the first Bcp (Rv2125) expression at a protein level exists, both in the membrane fraction (Gu *et al.*, 2003) and in the cytosol of H37Rv strains (Mawuenyega *et al.*, 2005). The protein has been shown to be target of modification by the small protein Pup, a post-translational modification that targets proteins for degradation by the *M. tuberculosis* proteosome (Pearce *et al.*, 2006; Festa *et al.*, 2010). To note, pupylation and proteosome function are essential for the virulence of this bacterium, for reasons still unknown (Darwin *et al.*, 2003; Gandotra *et al.*, 2007). In the case of BcpB (Rv1608c), it was identified associated to the membrane fraction of *M. tuberculosis* H37Rv (Gu *et al.*, 2003). The genes for both putative Bcps are considered as non-essential according to mutagenesis analysis in H37Rv strain (Sassetti *et al.*, 2003). Structural and functional data regarding both putative Bcps from *M. tuberculosis* and their role in infection processes await further investigation.

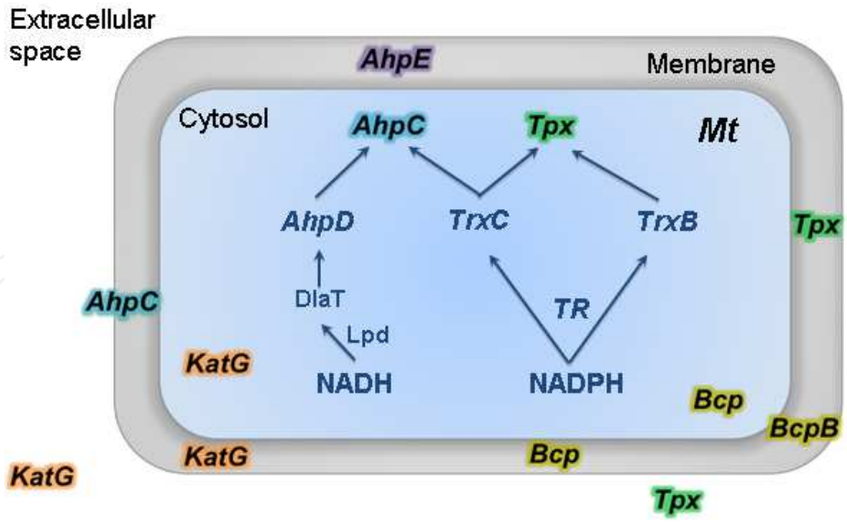

Fig. 2. **Cellular localization and reducing substrates of peroxidases from *M. tuberculosis*.** The five Prxs and the heme peroxidase KatG have distinct, although overlapping cellular distributions. *Mt*KatG (orange) has been found in the cytosol, membrane and extracellular space. *Mt*AhpC (blue) is a cytosolic enzyme also that was also found associated to the bacterial membrane. *Mt*TPx (green) was detected in culture media repeatedly. It has also been found in membrane fractions and in the cytosol. *Mt*AhpE (violet), and the putative BcpB and Bcp (yellow) were detected in cell membrane fractions, and the latter also in the cytosol. Reducing systems for *Mt*AhpC and *Mt*Tpx (in grey) are shown without considering their cellular localization. *Mt*AhpE and *Mt*Bcps reducing substrates are still unknown.

6. Conclusions

M. tuberculosis is an extremely successful pathogen, despite of being exposed to cytotoxic peroxides formed inside the phagosome of activated macrophages, its primary host cells. The bacterium expresses a heme-dependent peroxidase, KatG, and various thiol-dependent peroxidases of the Prx type. From the data reviewed herein, it becomes clear that Prxs from *M. tuberculosis* differ in cellular location, and have diverse oxidizing and reducing substrate specificities, that may explain in part the presence of different subfamilies of Prxs in the bacterium. Available data indicate that at least two of them (*Mt*AhpC and *Mt*TPx) play a role in pathogenesis. The third one, *Mt*AhpE, has an outstanding reactivity with fatty-acid derived hydroperoxides, but since natural reducing substrate(s) has not been identified so far, its peroxidase catalytic activity *in vivo* remains to be confirmed. Similarly, further investigation is required to characterize the two putative Bcp proteins from *M. tuberculosis.*

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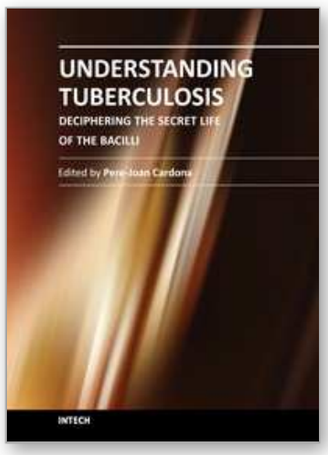

Understanding Tuberculosis - Deciphering the Secret Life of the Bacilli

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Mycobacterium tuberculosis, as recent investigations demonstrate, has a complex signaling expression, which allows its close interaction with the environment and one of its most renowned properties: the ability to persist for long periods of time under a non-replicative status. Although this skill is well characterized in other bacteria, the intrinsically very slow growth rate of Mycobium tuberculosis, together with a very thick and complex cell wall, makes this pathogen specially adapted to the stress that could be generated by the host against them. In this book, different aspects of these properties are displayed by specialists in the field.

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