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Review Article

The thioredoxin antioxidant system

Jun Lu*, Arne Holmgren*

Division of Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-171 77 Stockholm, Sweden

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ABSTRACT

The thioredoxin (Trx) system, which is composed of NADPH, thioredoxin reductase (TrxR), and thioredoxin, is a key antioxidant system in defense against oxidative stress through its disulfide reductase activity regulating protein dithiol/disulfide balance. The Trx system provides the electrons to thiol-dependent peroxidases (peroxiredoxins) to remove reactive oxygen and nitrogen species with a fast reaction rate. Trx antioxidant functions are also shown by involvement in DNA and protein repair by reducing ribonucleotide reductase, methionine sulfoxide reductases, and regulating the activity of many redox-sensitive transcription factors. Moreover, Trx systems play critical roles in the immune response, virus infection, and cell death via interaction with thioredoxin-interacting protein. In mammalian cells, the cytosolic and mitochondrial Trx systems, in which TrxRs are high molecular weight selenoenzymes, together with the glutathione-glutaredoxin (Grx) system (NADPH, glutathione reductase, GSH, and Grx) control the cellular redox environment. Recently mammalian thioredoxin and glutathione systems have been found to be able to provide the electrons crossly and to serve as a backup system for each other. In contrast, bacteria TrxRs are low molecular weight enzymes with a structure and reaction mechanism distinct from mammalian TrxR. Many bacterial species possess specific thiol-dependent antioxidant systems, and the significance of the Trx system in the defense against oxidative stress is different. Particularly, the absence of a GSH-Grx system in some pathogenic bacteria such as *Helicobacter pylori*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus* makes the bacterial Trx system essential for survival under oxidative stress. This provides an opportunity to kill these bacteria by targeting the TrxR-Trx system.

Contents

Abbreviations: AhpC, Alkyl hydroperoxide peroxidase subunit C; AhpF, Alkyl hydroperoxide peroxidase subunit F; *B. subtilis*, *Bacillus subtilis*; Bcp, Bacterioferritin comigratory protein; BSO, Buthionine sulfoximine; *E. coli*, *Escherichia coli*; *E. faecalis*, *Entercoccus faecalis*; FAD, Flavin adenine dinucleotide; HDAC, Histone deacetylases; *H. pylori*, *Helicobacter pylori*; HTLV-1, Human T-lymphotropic virus type I; GPx, Glutathione peroxidise; GR, Glutathione reductase; Grx, Glutaredoxin; GSH, Glutathione; GST, Glutathione transferase; KatA, Catalase; KatG, catalase peroxidase; Keap1, Kelch-like ECH-associated protein 1; *M. tuberculosis*, *Mycobacterium tuberculosis*; Mrx, Mycoredoxin; MSH, Mycothione; MsrA, Methionine-*S*-sulfoxide reductase; MsrB, Methionine-*O*-sulfoxide reductase; Mtr, Mycothione reductase; NADPH, Nicotinamide adenine dinucleotide phosphate; Nrf2, Nuclear factor erythroid-related factor 2; RNR, Ribonucleotide reductase; ROS, reactive oxygen species; PDI, Protein disulfide isomerase; Prx, Peroxiredoxin; *S. aureus*, *Staphylococcus aureus*; *S. pyogenes*, *Streptococcus pyogenes*; Sec, U, selenocysteine; SucB, Dihydrolipoamide succinyltransferase; TGR, Thioredoxin glutathione reductase; Tpx, Thiol peroxidase; Trx, Thioredoxin; TrxR, Thioredoxin reductase; TryR, Trypanothione reductase; TS_2, Trypanothione; TXNIP, Thioredoxin interacting protein; WT, Wild type

* Corresponding authors. Fax: 46 8 7284716/46 8 305193.
E-mail addresses: Jun.lu@ki.se (J. Lu), Arne.Holmgren@ki.se (A. Holmgren).

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Introduction

The thioredoxin system, comprising NADPH, thioredoxin reductase (TrxR), and thioredoxin (Trx), is a major disulfide reductase system which can provide electrons to a large range of enzymes and is found to be critical for DNA synthesis and defense against oxidative stress. Trx was originally discovered to be a reducing substrate of ribonucleotide reductase (RNR) [1], the essential enzyme catalyzing *de novo* synthesis of 2′-deoxyribonucleotides from corresponding ribonucleotides and is thus involved in DNA replication and repair (reviewed recently in [2]). In this review we will focus on the roles of the thioredoxin system as antioxidant in the defense against oxidative stress.

Structure and reaction mechanism of Trx and TrxR

Thioredoxins are typically 12 kDa small reductases, catalyzing protein disulfide/dithiol change with a conserved -CGPC- active site motif. Trx is ubiquitously distributed from archaea, bacteria to man. The structure of Trx is that five β-strands form the internal core of protein, and four α-helices and a short stretch of helix surround the central β-sheets. The active site disulfide is located after the β2-sheet and forms the N-terminal portion of α2 [3]. Many critical enzymes in the thiol-dependent antioxidant system have this thioredoxin fold structure, such as glutaredoxin [4], peroxiredoxin [5], and glutathione peroxidase [6].

Homodimeric flavoprotein TrxR is a member of the pyridine nucleotide-disulfide oxidoreductase family which includes TrxR, glutathione reductase (GR), trypanothione reductase (TryR), alkyl hydroperoxide reductase, lipoamide dehydrogenase, and mercuric reductase [7]. There are two classes of TrxRs, high molecular weight (*Mr*) TrxR with 55 kDa for each subunit and low *Mr* of TrxR with 35 kDa for each subunit. High *Mr* TrxRs are present in higher eukaryotes (Figs. 1 and 2) [8]. Three TrxRs are found in mammalian cells, cytosolic TrxR1, mitochondrial TrxR2, and a testis-specific thioredoxin glutathione reductase (TGR) [9] (Fig. 1). Mammalian TrxR1 and TrxR2 contain FAD and NADPH binding domains and an interface domain. TGR have these domains and an extra Grx domain in the N-terminus. The overall structures of mammalian TrxRs are similar to those of GR [10,11]. Most pyridine nucleotide disulfide reductases including GR, TrxR, and TryR possess the same N-terminal active site motif CVNVGC (Fig. 1). However, different than GR and TryR, mammalian TrxR have a C-terminal extension sequence containing Gly-Cys-Sec-Gly [10]. The two subunits of dimeric mammalian TrxRs form a head to tail pattern. The reaction mechanisms are also similar for these enzymes [10]. The electrons are transferred from NADPH to FAD, then to N-terminal redox-active dithiol motifs, subsequently to the selenenylsulfide of the other subunit, and finally to disulfide substrates in mammalian TrxR [10,11,12]. The electron transfer path of the other pyridine nucleotide-disulfide oxidoreductases containing an active site CVNVGC motif, e.g., GR and TryR, is also from NADPH→FAD→redox active site disulfide→ disulfide substrate (Fig. 1). Instead of using a CysSec-containing active site, the electrons are transferred from a CVNVGC motif to a cysteine-containing peptide GSH and trypanothione (TS_2) in GR and TryR, respectively. With the two active sites, mammalian TrxRs have a very broad range of substrates including proteins such as Trx, protein disulfide isomerase (PDI), and Grx2 and small molecules such as selenite and lipoic acid [14]. By providing the electrons to small molecules which can react with H_2O_2 directly, mammalian TrxR can function as an antioxidant [15,16].

Low *Mr* TrxRs are present in archaea, bacteria, fungi, and plants (Fig. 2). The structure of low *Mr* TrxRs is distinct with high *Mr* TrxRs. They have only an active site-containing CXXC motif, instead of a N-terminal CVNVGC active site motif and another C-terminal active site in high *Mr* TrxRs. They possess FAD and NADPH binding domains, but lack the interface domain. The active site is located in the NADPH binding domain, not in the FAD binding domain. The two globular domains are connected by a two-stranded β-sheet. The electron transfer process is NADPH→FAD→ redox active site C135ATC138 disulfide→Trx in *E. coli* TrxR. The catalysis of the enzyme involved a large conformation change. The NADPH domain rotates 67° toward the FAD domain to expose the buried cofactor to substrate [17]. In contrast, electron transfer from NADPH to the disulfide of substrate in mammalian TrxR does not involve a large conformational change [18]. The low *Mr* TrxR has narrow substrate specificity.

Besides the above described thiol-dependent antioxidant enzymes, a low *Mr* TrxR homologue alkyl hydroperoxide peroxidase subunit F (AhpF) participates in the antioxidant process using its active site disulfide in some bacteria. AhpF transfers electrons directly to a peroxidase alkyl hydroperoxide peroxidase subunit C (ahpC) to remove alkyl hydroperoxide [19] (Fig. 2). Another pyridine nucleotide-disulfide oxidoreductase member, NADH peroxidase, can catalyze the reduction of hydrogen peroxide into water via a stable active site cysteine thiol/sulfenic acid (-SH/-SOH) redox couple. The reaction mechanism involves the reduction of the enzyme active site cysteine sulfenic acid to thiol and electron transfer from the thiol to H_2O_2 [7] (Fig. 2).

Thioredoxin antioxidant systems in mammalian cells

Antioxidant roles of the Trx system

In mammalian cells there are two major thiol-dependent antioxidant systems, the Trx and the glutathione antioxidant system. GSH is the most abundant nonprotein thiol in the mammalian cells. Mammalian cells possess two Trx systems, the cytosolic Trx1 and the mitochondrial Trx2 system (Fig. 3). Trx2 has only the two cysteines in its active site, whereas Trx1 has three additional extra cysteines, which play a role in the redox regulation of activity and NO signaling [15].

The antioxidant activity of the Trx system is mainly shown by transferring electrons to Prxs, MSRs, and some redox-sensitive transcription factors. Prx was first identified to be a Trx-dependent peroxidase in yeast [20]. There are six human Prxs distributed in various subcellular organelles, which can be classified as 2-Cys Prx isoform (Prx1–4), atypical 2-Cys Prx isoform (Prx5), and one 1-Cys Prx isoform (Fig. 4) [21]. The Trx system can transfer electrons to 2-Cys Prx isoforms and atypical 2-Cys Prx to remove H_2O_2, ROOH, and $ONOO^-$ [5,21]. These Prxs have two Cys residues in their active site, N-terminal peroxidatic Cys_P and C-terminal resolving Cys_R. Under neutral pH conditions, Cys_P is present in a thiolate (deprotonated) form because of its low pK_a value about 5–6 [21]. The catalytic mechanism involves two steps. First, the thiolate Cys_p reacts with hydrogen peroxide via a nucleophilic attack to form Cys_P sulfenic acid

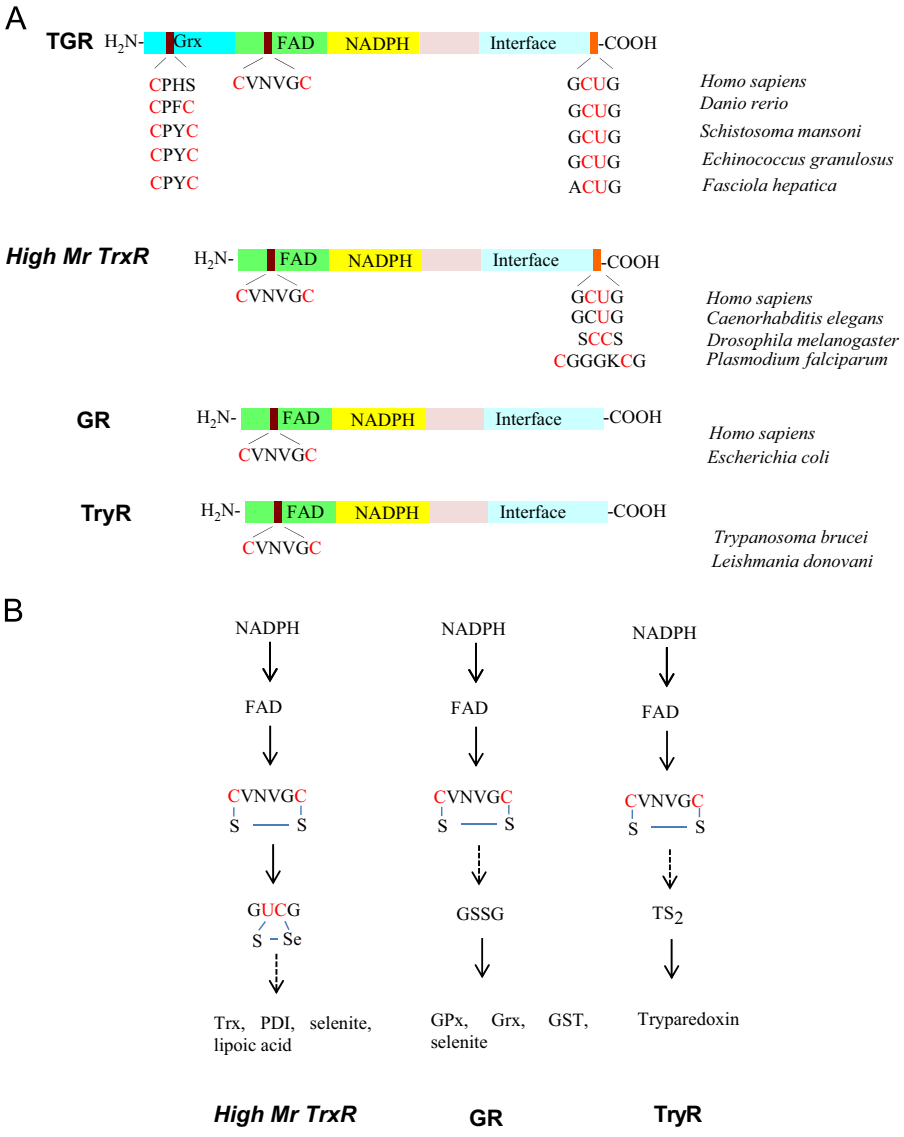

Fig. 1. Scheme of the active site and electron transfer process of high *Mr* TrxR and other related pyridine nucleotide-disulfide oxidoreductases. (A) Comparison of the active site of thioredoxin glutathione reductase (TGR), high *Mr* thioredoxin reductase (TrxR), glutathione reductase (GR), and trypanothione reductase (TryR). TGR is widely found or predicted in different species such as mammals like human testis, fish (*Danio rerio*), and parasites (*Schistosoma mansoni, Echinococcus granulosus*, *Fasciola hepatica*). High *Mr* TrxRs include mammalian cytosolic and mitochondrial TrxR, TrxRs from *Caenorhabditis elegans, Drosophila melanogaster,* and *Plasmodium falciparum.* GR is widely distributed in different organisms such as in human and bacteria, but some parasites possess a trypanothione system instead. The CVNVGC active site is highly conserved in these pyridine nucleotide-disulfide oxidoreductases. The high *Mr* TrxRs and TGR have another C-terminal active site. Moreover, TGRs contain an extra Grx active site. (B) Electron transfer process in high *Mr* TrxR, GR, and TryR-mediated processes. The electron transfer in these disulfide oxidoreductases is from NADPH to FAD, then to the active site Cys, and finally to the substrates.

with release of water. Second, the C-terminal resolving Cys_R will react with Cys_P sulfenic acid to form an intermolecular disulfide bond for typical 2-Cys Prxs, and an intramolecular disulfide bond for atypical 2-Cys Prx. The recycling of Prx is performed when these disulfide bonds are reduced by the Trx system to become the active form again (Fig. 4) [5,22,23].

Hydrogen peroxide scavenging by Prx is a very fast process; the reaction rate can range up to 10^7–$10^8\ M^{-1}\ s^{-1}$ [24–26]. The reaction rate between hydrogen peroxide and normal thiolate in small molecules or proteins is in the range of 0.89–500 $M^{-1}\ s^{-1}$, although the pK_a values of the thiol in some of the proteins such as Trx or PTP1B are lower or similar to those in Prxs [27,28]. The reason may be because of the special structure of Prx enhancing the catalytic process [29,30] (Fig. 4). An active site PXXXTXXC and a distant Arg are conserved in the Prx (Fig. 4). When thiolate Cys_p attacks hydrogen peroxide, it acts as a S_N2 nucleophilic reaction. The Arg, Pro, and Thr in these positions stabilize the transition state of the reaction by the hydrogen bonds and cause Prx to have a remarkably high catalytic efficiency [29,30]. The reaction rates of Prxs are at levels similar to those of the well-known antioxidant enzymes glutathione peroxidase ($10^8\ M^{-1}\ s^{-1}$) [31] and catalase ($10^7\ M^{-1}\ s^{-1}$) [32,33]. Considering that Prx is one of the most abundant proteins in the mammalian cells, Prxs should be a key player in removing ROS and defending against oxidative stress. The Prxs are widely distributed in different suborganelles, which is the same as GPxs, and may help them to scavenge H_2O_2 locally and control signal transduction more specifically [34].

Methionine sulfoxide reductases (Msr) are another type of antioxidant enzyme which obtains the electrons from the thioredoxin system (Fig. 3A) [35,36]. Free methionine and protein methionine can be oxidized to methionine sulfoxide under oxidative stress and the protein function can be affected by the oxidation. MsrA and MsrB repair the free and protein-bound *S*- and *R*-methionine sulfoxides back to methionine, respectively,

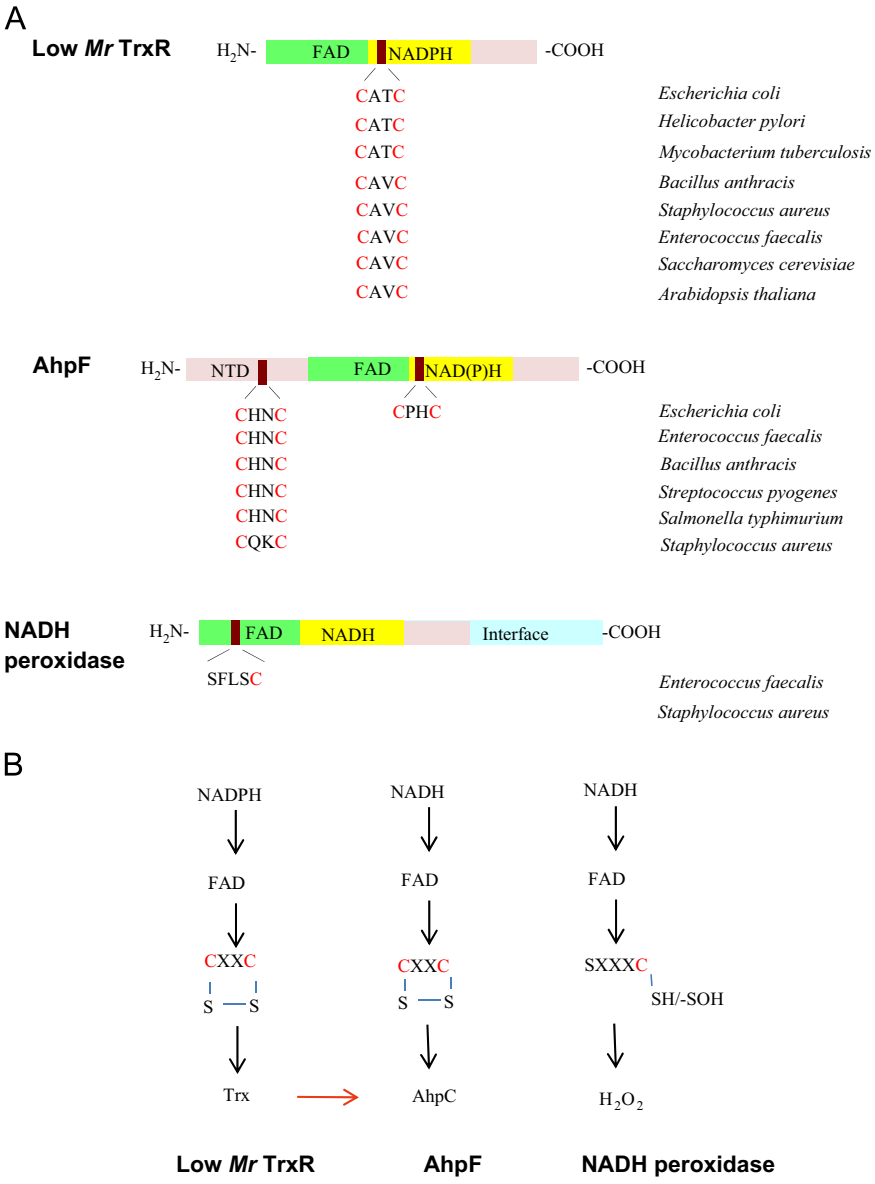

Fig. 2. Scheme of the active site and electron transfer process of low *Mr* TrxR and other related pyridine nucleotide-disulfide oxidoreductases. (A) Comparison of the active site of low *Mr* thioredoxin reductase (TrxR), alkyl hydroperoxide reductase subunit F (AhpF), and NADH peroxidase. CXXC active site is conserved in low *Mr* TrxR and AhpF and located in the NAD(P)H domain, different with FAD binding domain location of CVNVGC active site in high *Mr* TrxR. AhpF has another CXXC containing N-terminal domain. (B) Electron transfer process in low *Mr* TrxR, AhpF and NADH peroxidase-mediated processes. AhpC obtains the electrons from AhpF, but in some bacteria like *H. pylori* and *M. tuberculosis* AhpC can obtain the electrons from the Trx system.

and thus indirectly participate in the removal of reactive oxygen species [37,38]. Besides providing the electrons to the antioxidant enzymes, Trx is involved in redox signaling by regulating the activities of many transcription factors (Fig. 3A) [39,40].

Cross-talk between GSH and thioredoxin system

The GSH system, the other major thiol-dependent antioxidant system in mammalian cells, participates in the defense against oxidative stress via the efficient removal of various ROS by glutathione peroxidase (Fig. 3A) [41,42]. GSH together with Grxs can also regulate protein function by reversible protein *S*-glutathionylation under oxidative stress (Fig. 3A) [43,44]. Although the Trx and GSH systems have many overlapping functions, in most cases they were believed to work in parallel. Recently accumulating evidence shows that there is much cross-talk between the two systems. TrxR was known to be the only physiological reductant able to reduce the Trx. However, the down-regulation of TrxR1 by siRNA or using a specific inhibitor such as aurothioglucose did not change the Trx1 redox status and affect cell viability [45–47], indicating that there is a backup system to reduce Trx. The combination of inhibition of TrxR by aurothioglucose and the depletion of GSH by treatment with ebselen (a glutathione peroxidase mimic) or buthionine sulfoximine (BSO) (an inhibitor of γ-glutamylcysteine synthetase (γ-GCS)) shows very strong synergistic effects on the oxidation of Trx1, the production of ROS, and cell death. Moreover, GSH and Grx at physiological concentration levels can reduce oxidized Trx *in vitro*, indicating that GSH-Grx is the backup of TrxR to provide electrons to Trx [47]. On the other hand, the thioredoxin system can be an alternative system for reducing oxidized GSH [48]. Moreover, it is also found that mitochondrial Grx2 is the substrate of TrxR [49]. More recently, both mitochondrial Trx2 and Grx2 were revealed to contribute to reduce mitochondrial located Prx3 [50].

GSH-Grx serving as the backup of TrxR is also shown by the compensatory effects of the GSH system when TrxR activity decreases under conditions such as selenium deficiency, Sec

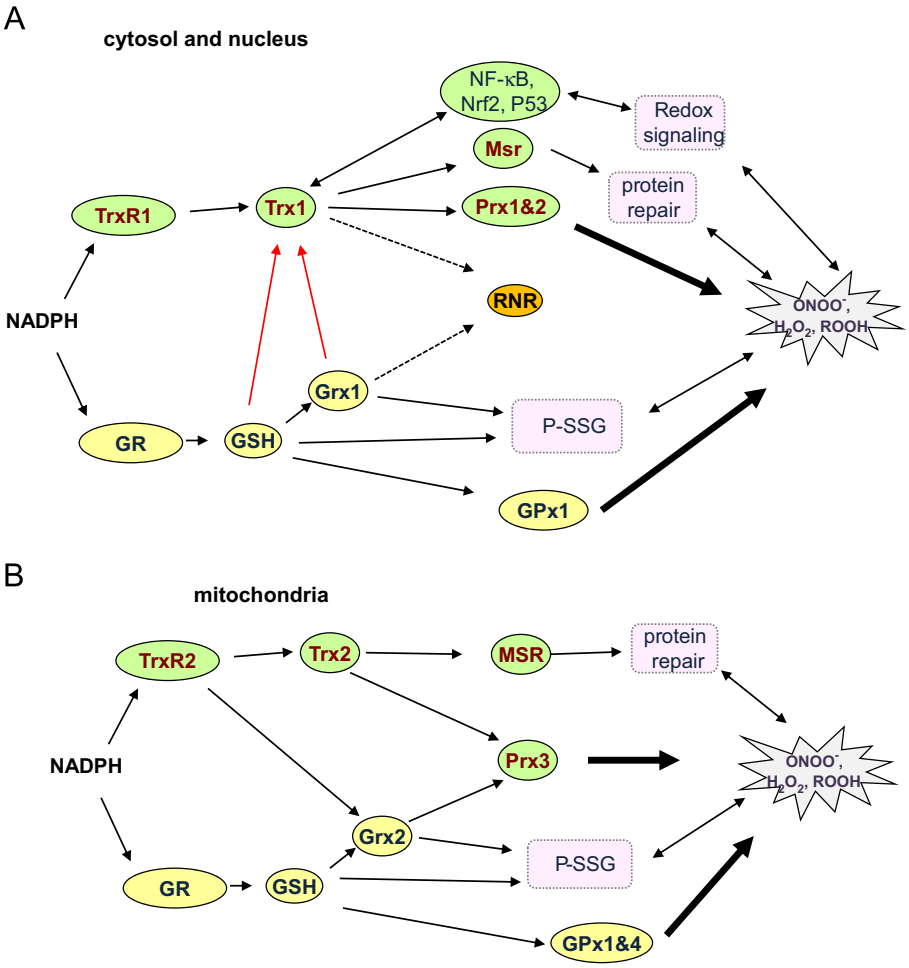

Fig. 3. Thioredoxin and glutathione antioxidant systems in mammalian cells. Thioredoxin and glutathione systems are the two major thiol-dependent antioxidant systems in mammalian cells. (A) Mammalian thiol-dependent redox system in cytosol, nucleus. Thioredoxin system provide the electron to thioredoxin-dependent peroxidases (Prx1&2), which can efficiently remove reactive oxygen species as glutathione peroxidase (GPx1). Moreover, thioredoxin reduces methionine sulfoxide reductases and is involved in the repair of oxidized proteins. Trx regulated the activities of many oxidative-sensitive transcription factors such as NF-κB, Nrf-2, and P53 and thus is involved in the redox signaling. The GSH system can serve as a backup system to reduce thioredoxin when the electron transfer pathway from TrxR1 is blocked. (B) Mammalian mitochondria thiol-dependent redox systems. Mitochondrial Grx2 can be reduced by mitochondria TrxR2 and GSH. Mitochondrial Prx3 can be reduced by both mitochondrial Trx2 and Grx2. The thick black lines represent the direct reaction between the protein and reactive oxygen species in a fast reaction rate, which is also shown in the other figures.

synthesis machinery deficiency, TrxR inhibition, and TrxR knock-out or knockdown [51–53]. This may be because the inactivation of TrxR triggers the nuclear factor erythroid-related factor 2 (Nrf2) activation via the direct regulation of Kelch-like ECH-associated protein 1 (Keap1) disulfide formation and Nrf2 stabilization or mediation of hydrogen peroxide level [54]. Besides Nrf2, the activity of many other redox-related transcription factors including Ref-1, NF-κB, P53, and HIF1α and signaling factors such as ASK1 and PTP1B are mediated by thioredoxin or GSH-Grx systems [40,55].

Regulation of thioredoxin by thioredoxin interacting protein

One of the important endogenous molecules to interact with Trx is thioredoxin interacting protein (TXNIP, TBP2, VDUP1), which is a negative regulator of Trx function [56]. Cys63 and Cys247 in TXNIP can form the mixed disulfide bond with Trx active site thiols and suppress the activity of Trx and result in oxidative stress [57]. TXNIP is located in the nucleus under normal conditions. In response to oxidative stress TXNIP can shuttle into cytosol or mitochondria, which binds and oxidizes Trx1/Trx2, reducing the binding of Trx1/Trx2 with ASK1 and resulting in a ASK1-mediated signaling pathway [52,58,59]. Trx has also been revealed to regulate nucleocytoplasmic shuttling of class II histone deacetylases (HDAC) together with TXNIP [60]. On oxidative stress caused by hypertrophic stimuli, HDAC4 becomes oxidized and moves from the nucleus to the cytosol, Cys667/Cys669 in HDAC4 and Cys274/Cys276 in DnaJb5, and forms intramolecular disulfide bonds. Trx1 can reduce the critical Cys in HDAC4 by forming a multiprotein complex including DnaJb5, TXNIP, and importin α, and shuttling back to the nucleus [60]. Very interestingly, mitochondria from the heart of TXNIP knock-out mice are shown to be functionally and structurally altered, but this gene deletion protects the myocardium from ischemia-reperfusion injury. This may be because TXNIP-KO reprograms the glucose metabolism to a more anaerobic glycolysis and/or the release of Trx2 to scavenging ROS more efficiently [61].

Accumulating evidence shows that TXNIP is a critical player in glucose metabolism [61–64] and therefore closely linked in prediabetics and diabetics [64,65], though TXNIP may exert its role with redox-dependent and redox-independent mechanisms [66]. TXNIP inhibits adipogenesis directly and the binding of thioredoxin facilitates the stability of TXNIP protein [67]. TXNIP is a critical mediator for high glucose-induced ROS generation and mitochondrial pathway apoptosis in β-cells [68,69].

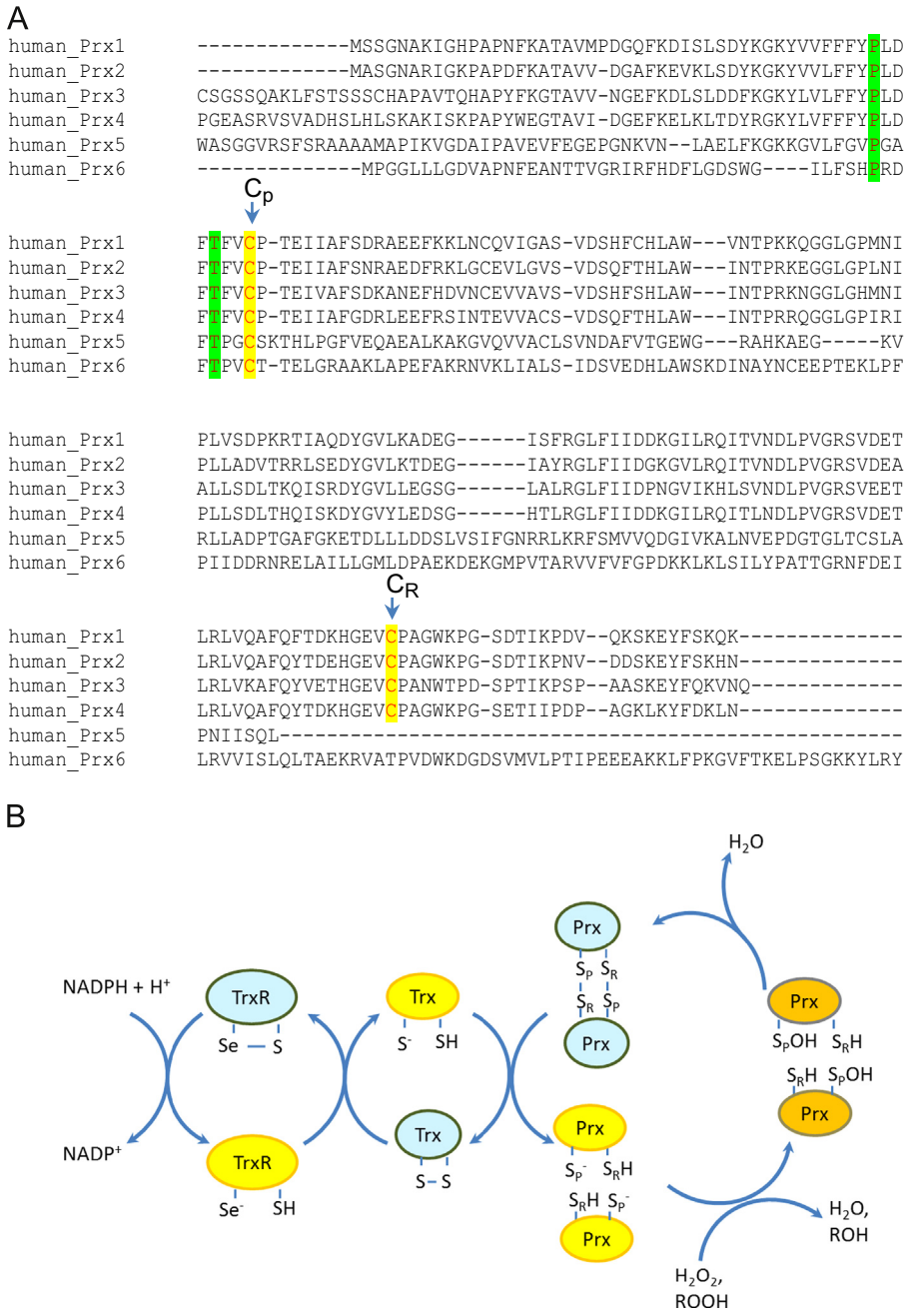

Fig. 4. Multiple sequence alignment of human Prxs and the reaction mechanism of typical 2-Cys peroxidase. (A) Multiple sequence alignment of six human Prxs. Protein sequences of human Prx1 (gi:13937907), Prx2 (gi:12804327), Prx3 (gi:12803699), Prx4 (gi:119619402), Prx5 (gi:109731385), and Prx6 (gi:4758638) were obtained from the PubMed Protein Database and the multiple sequence alignments were performed by ClustalW2. The conserved active site PXXXTXXC and resolving Cys_R in typical 2-Cys Prxs are highlighted. (B) Scheme of reaction mechanism of typical 2-Cys peroxidase. Under neutral pH conditions, deprotonated Cys_P reacts with H_2O_2 or ROOH via a nucleophilic attack to form Cys_P sulfenic acid with the release of water or ROH. Then, the C-terminal resolving Cys_R in the other subunit will react with Cys_P sulfenic acid and form an intermolecular disulfide bonds. These disulfide bonds are reduced by the Trx system to obtain the active form again.

Role of the thioredoxin system in immune response and cancer

Trx is expressed and secreted by human T-lymphotropic virus type I (HTLV-1)- and Epstein-Barr virus (EBV)-transformed and regulatory T cells, and associated with retroviral infections [70–72]. Trx is shown to be a growth promoting factor for several lymphoid cells with synergistic effects with cytokines such as IL-1 and IL-2 [71,73]. Moreover, Trx is secreted by $CD4^+$ T cells and can reduce the disulfide in domain 2 of CD4, which is required for the entry of HIV-I into susceptible cells [74]. TXNIP is important for the growth regulation of T cells and the loss of TXNIP expression may be a key event in the progression for adult T cell leukemia (ATL) leukemogenesis upon the infection of HTLV-1 [75]. TXNIP also is a key player in the regulation of T cell sensitivity to glucocorticoid during HTLV-1 infection [76].

TXNIP has also been found to bind to NLRP3, a major component of NLRP3 inflammasome, controlling the activation of the innate immune system in response to ROS production [77]. Trx binds to TXNIP in the steady state. On stimulation of inflammasome activators, ROS will be produced, resulting in the dissociation of TXNIP from Trx and the binding to NLRP3. Then the NLRP3 inflammasome is activated, and the active, mature interleukin 1β

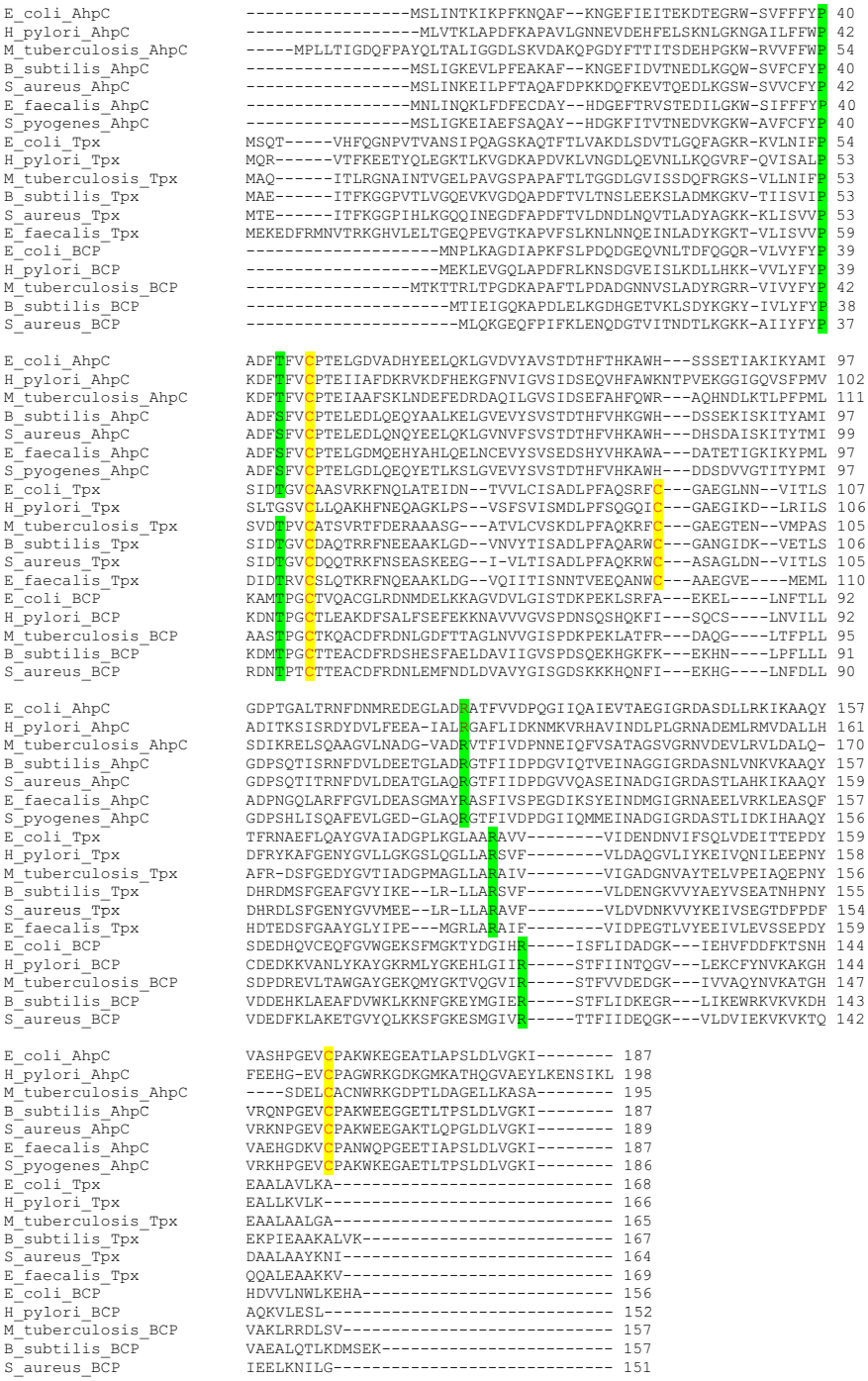

Fig. 5. Multiple sequence alignment of bacterial thiol-dependent peroxidases. Protein sequences of *E. coli* AhpC (gi:388476709), *H. pylori* AhpC (gi:58198721), *M. tuberculosis* AhpC (gi:1172078), *B. subtilis* AhpC (gi:1064782), *S. aureus* AhpC (gi:15926082), *E. faecalis* AhpC (gi:29377215), *S. pyogenes* AhpC (gi:383494707), *E. coli* Tpx (gi:191171080), *H. pylori* Tpx (gi:385222481), *M. tuberculosis* Tpx (gi:15609069), *B. subtilis* Tpx (gi:16080001), *S. aureus* Tpx (gi:15927290), *E. faecalis* Tpx (gi:293388508), *E. coli* BCP (gi:388478516), *H. pylori* BCP (gi:15611194), *M. tuberculosis* BCP (gi:13882332), *B. subtilis* BCP (gi:50812202), and *S. aureus* BCP (gi:387603195) were obtained from the PubMed Protein Database and the multiple sequence alignments were performed by ClustalW2. The conserved active site PXXXT(S)XXC and resolving Cys_R in typical or atypical 2-Cys Prxs are highlighted.

(IL-1β) can be produced and secreted after the cleavage from pro-IL-1β precursor by caspase-1 [77]. TXNIP is also shown to be induced by endoplasmic reticulum (ER) stress, to activate IL-1β production via NLRP3 inflammasome, and to mediate ER stress-induced β cell death [78]. These results indicate that TXNIP may be a potential target against diabetes, cancer, or infection by altering the activation of the host innate response.

Since TrxR and Trx are overexpressed in many cancer cells and the growth of cancer cells is shown to be more reliable on the Trx system, TrxR emerges as a therapeutical drug target [52,79]. However, TrxR1 was not essential for cell proliferation. The hepatocyte-specific TrxR1 knockout mice lived as normal mice [80] and the cancer cells without TrxR1 survived *in vivo* [81]. One viable strategy is to convert the TrxR from an antioxidant to a pro-oxidant to produce ROS and lead cancer cells into apoptosis [52]. Considering the overlapping function of the Trx and the GSH system, we proposed the combination of the inhibition of the two systems as an anticancer strategy [82]. This is verified by the observation that

TrxR1 knockout cancer cells were highly susceptible to GSH depletion *in vitro* and *in vivo* [81]. The combination of the treatment with TrxR inhibitor nedaplatin together with BSO greatly enhanced the cancer therapeutical effects *in vivo* without causing renal toxicity [83], indicating that the combination of modulation of both Trx and GSH systems is a promising anticancer strategy.

Thioredoxin antioxidant systems in bacteria

Since bacteria live in various environments, different bacteria are equipped with various types of antioxidant systems [84]. The thiol-dependent peroxidases include bacterioferritin comigratory protein (BCP), thiol peroxidase (Tpx), and AhpC [84]. AhpC is classified as 2-Cys Prx with conserved N-terminal peroxidatic Cys_P and C-terminal resolving Cys_R and widely distributed from prokaryote to eukaryotes with a high catalytic efficiency, e.g., $>10^7\ M^{-1}\ s^{-1}$ in *Salmonella typhimurium* [85] (Fig. 5). Tpx is a type of atypical 2-Cys Prx and BCP is a 1-Cys Prx. These thiol-dependent peroxidases have been widely found in many bacteria, such as *Escherichia coli, Helicobacter pylori, Mycobacterium tuberculosis, Bacillus subtilis,* and *Staphylococcus aureus*. Peroxidatic Cys_P containing the sequence PxxxT(S)XXC and remote Arg are conserved in almost all the peroxidases (Fig. 5).

Thioredoxin- and glutathione-dependent antioxidant systems and catalase are the major antioxidant systems. Thioredoxin is ubiquitous in bacteria, whereas the GSH antioxidant system or catalase is lacking in some specific bacteria. Here we classify the bacteria into three types according to their antioxidant system for further discussion of the roles of Trx antioxidant in bacteria. The first type is the bacteria with Trx, GSH systems, and catalase; the second type is GSH-negative bacteria; and the third is catalase-negative bacteria.

Bacteria with Trx, GSH systems, and catalase

Most gram-negative bacteria belong to this type, possessing both Trx and GSH systems, same as mammalian cells. *E. coli* has been extensively studied and is a modern bacteria with Trx, GSH systems, and catalase. There are one TrxR (encoded by *trxB*), two Trxs (Trx1 and Trx2, encoded by *trxA* and *trxC*), and three major thiol peroxidases (BCP, Tpx, and AhpC) in *E. coli* [86]. 2-Cys peroxidase AhpC obtains the electrons from AhpF [87] (Fig. 6). Same as in mammalian cells, Trx1 in *E. coli* is involved in protein repair by providing the electrons to *E. coli* Msr [1], which participates in the protection of *E. coli* against oxidative damage from reactive nitrogen intermediates [88].

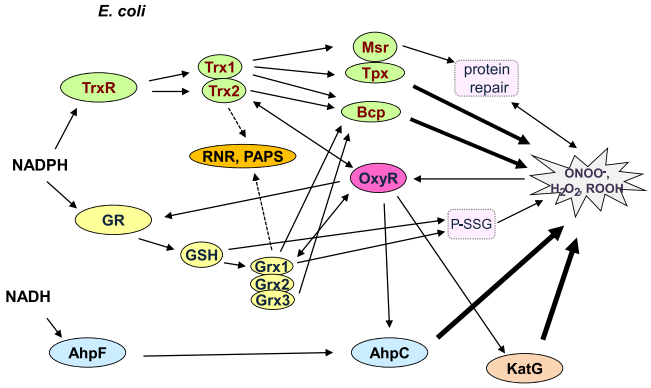

Fig. 6. Scheme of antioxidant systems in *E. coli*. Trx, GSH antioxidant systems, and catalase are present in *E. coli*. The thioredoxin antioxidant system in the bacterium contains one TrxR, two Trxs (Trx1 and Trx2), and three major thiol peroxidases (BCP, Tpx, and AhpC). *E. coli* OxyR transcription factor regulates the expression of many antioxidant enzymes including AhpC, catalase, GR, Trx2, and Grx1.

E. coli peroxidases BCP and Tpx showed different properties in obtaining the electrons from reductase systems to scavenge ROS [89–91]. *E. coli* Trx1 acts as a specific reductase for homodimeric Tpx, which contains an intrasubunit disulfide in oxidized form [90]. *E. coli* Trx1 and Tpx formed a Michaelis complex with a catalytic efficiency of $3 \times 10^6\ M^{-1}\ s^{-1}$. The Trx1-linked peroxidase activity shows efficiency at $4 \times 10^4\ M^{-1}\ s^{-1}$ for H_2O_2 and $7.7 \times 10^6\ M^{-1}\ s^{-1}$ for cumene hydroperoxide. The other reductases including *E. coli* Trx2, Grx1, and AhpF did not have the capacity to reduce Tpx [90]. In contrast, monomeric *E. coli* BCP together with Trx1 catalyses hydrogen peroxide with apparent V_{max}/K_m of $1.4 \times 10^4\ M^{-1}\ s^{-1}$. Other reductants in *E. coli* such as Trx2, Grx1, and Grx3 can also provide the electrons to BCP [89]. The BCP null mutant grows slower than the wild type under aerobic conditions and is hypersensitive to oxidative stress [91].

The presence of the GSH-Grx system in *E. coli* provides a strong backup for the Trx system. In fact, Grx was discovered by investigation of the substitutes to reduce RNR in a Trx-deficient mutant [1,2]. GSH and Grxs in *E. coli* participate in the antioxidant process by deglutathionylation as in mammalian cells. Notably, the activation of *E. coli* OxyR transcription factor which regulates the expression of many antioxidant enzymes such as AhpC, catalase, GR, Trx2, and Grx1 is controlled by forming a disulfide bond between Cys199 and Cys208 [92,93]. Both Trx system and GSH-Grx1 can reduce this disulfide bond in OxyR *in vitro*, and OxyR is deactivated by Grx1 and GR *in vivo* (Fig. 6), forming an autoregulating response process [92].

Since GSH provides sufficient complementary effects for the Trx functions, the Trx system is not essential for bacterial growth. However, *E. coli* requires either a functional Trx or a GSH-Grx system to grow well under aerobic conditions. The *E. coli* strain with both TrxR and GR deletion and the strain with both TrxR and γ-glutamylcysteine synthetase grow very poor without reductants [94]. Interestingly, the expression of *E. coli* Trx2 was activated in response to oxidative stress, which is regulated by OxyR. But the *E. coli* strain without any Trxs was shown to be more resistant to oxidative stress. This may be because the loss of Trxs resulted in a disulfide bond formation in OxyR and activated the expression of other antioxidant enzymes such as AhpC and catalase which have a higher capacity to remove H_2O_2 [95]. This result indicates that the thioredoxin system is not necessary to defend against oxidative stress in *E. coli,* but is critical for keeping cellular protein disulfide/dithiol redox control.

GSH-negative bacteria

The glutathione system is absent in many gram-positive and some gram-negative bacteria, such as *H. pylori, M. tuberculosis, B. subtilis, Bacteroides fragilis,* and *Lactobacillus casei* [96–101]. This property renders the Trx system essential for cellular thiol/disulfide balance and survival under oxidative stress in many of the bacteria.

The three peroxidases Bcp, Tpx, and AhpC are all found in *H. pylori* and *M. tuberculosis.* Different than *E. coli*, there is no AhpF homologue in *H. pylori* and *M. tuberculosis*. Instead, the Trx system is the electron donor for AhpC, BCP, and Tpx in *H. pylori* [102,103] (Fig. 7A). Moreover, functional OxyR is lacking in *H. pylori* and *M. tuberculosis* [99,104], which make the regulation of the defense system against oxidative attack in these bacteria different than that in *E. coli* and many other eubacteria.

Helicobacter pylori

H. pylori is a microaerophilic bacterium which colonizes in human gastric mucosae and is the causative agent of peptic ulcer

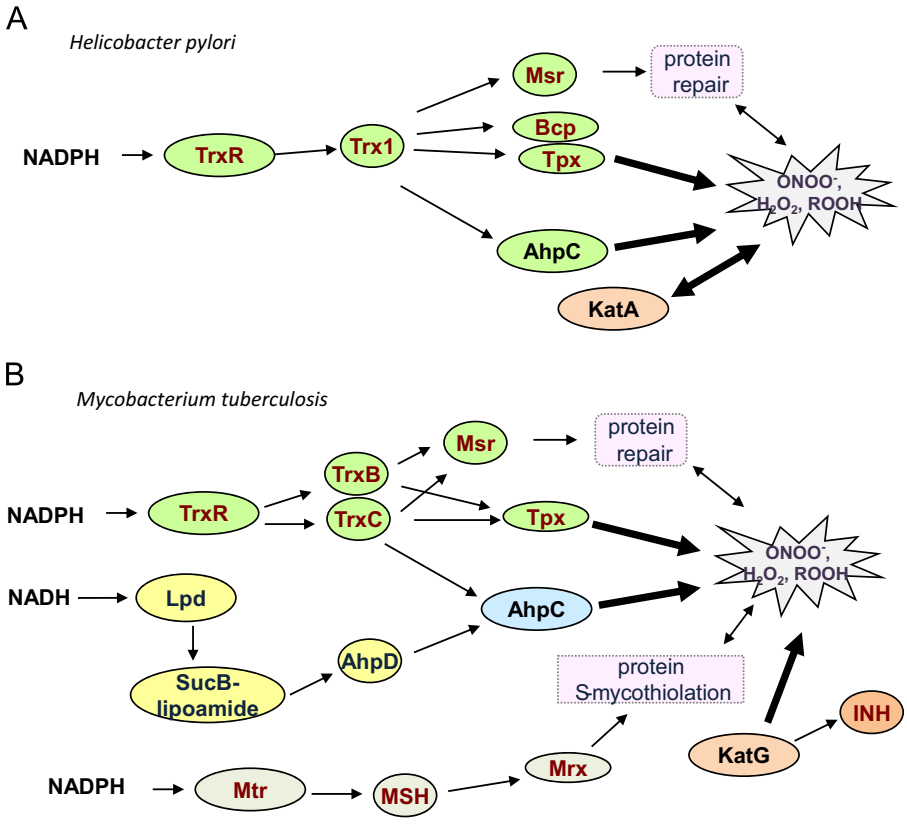

Fig. 7. Scheme of thioredoxin antioxidant system in GSH-negative bacteria *H. pylori* and *M. tuberculosis*. (A) Antioxidant system in *H. pylori*. *H. pylori* TrxR and Trx1 compose a physiological functional Trx system. The *H. pylori* Trx system can provide the electrons to BCP, Tpx, AhpC, and Msr to defend against oxidative stress. The *H. pylori* Trx system and catalase are the two major antioxidant systems for removing reactive oxygen species and reactive nitrogen intermediates. (B) Antioxidant systems in *M. tuberculosis*. The functional *M. tuberculosis* Trx system is composed of TrxR, TrxB, and TrxC. The Trx system can provide the electrons to Msr, Tpx, and AhpC. The other antioxidant systems including *M. tuberculosis* lipoamide reductase, dihydrolipoamide succinyltransferase, AhpD, and catalase peroxidase (KatG), and the mycothiol system also contribute to the defense against oxidative stress.

and chronic gastritis. *H. pylori* has one TrxR [105] and two Trxs, Trx1 with classic active motif CGPC and Trx2 with an unusual active site motif CPDC [106]. The reduction of *H. pylori* Trx1 by *H. pylori* TrxR was shown to have the same level of catalytic efficiency as the reduction *E. coli* Trx1 by *E. coli* TrxR. K_{cat}/K_m values of the reduction of Trx1 by TrxR in *H. pylori* and *E. coli* are 3.3×10^6 and $9.1 \times 10^6\ M^{-1}\ s^{-1}$, respectively [102]. *H. pylori* and *E. coli* TrxRs share many structure similarities [105] and can provide electrons to Trx1 from both bacteria [102]. *H. pylori* Trx1 but not Trx2 can provide the electron to AhpC, with infinite V_{max} and K_m values and a reaction rate of $1 \times 10^5\ M^{-1}\ s^{-1}$ [102]. *H. pylori* AhpC cannot be reduced by AhpF from *Salmonella typhimurium*, an efficient reductant for *S. typhimurium* AhpC, indicating that the reduction of *H. pylori* AhpC by Trx1 is very specific [102]. Though *H. pylori* AhpC is very critical for *H. pylori* viability [102], *ahpC* mutants were generated under low oxygen conditions [107]. The *H. pylori* A*hpC* mutants were more sensitive than the wild type to chemicals causing oxidative stress including hydrogen peroxide, tBOOH, cumene hydroperoxides, and paraquat [107,108]. Notably, the lipid peroxide level in *H. pylori* A*hpC* mutants is about three times more than that in the wild type [108,109], which may be the agents to inactivate catalase in A*hpC* mutants [109]. Since the *H. pylori* infection process causes the activation of host neutrophils and the elevation of production of a large amount of ROS/RNS, none of the *H. pylori AhpC* mutants colonized mouse stomachs, which may be due to the critical role of AhpC against oxidative stress [110].

The roles of other *H. pylori* thiol-dependent peroxidases in the defense against oxidative stress have been investigated [111]. *H. pylori* Tpx showed a significant role in resistance against hydrogen peroxide and superoxide. In contrast, *H. pylori* Bcp which can obtain electrons from the thioredoxin system may prefer to have linoleic acid hydroperoxide as the substrate [103,111]. In consistence with the antioxidant activity, *H. pylori tpx* mutant colonized only 5% of the inoculated mice, whereas 78% of the inoculated mice were colonized with the wild-type strain [110]. *H. pylori* Bcp also had significant roles in the bacterial colonization, particularly in long-term colonization [103].

H. pylori methionine sulfoxide reductase, another important enzyme in the Trx antioxidant system, also had a critical role in the bacterial long-term colonization [112]. *H. pylori* Msr is a fused protein with both MsrA- and MsrB-like domains and can obtain the electrons from Trx1, but not Trx2 [113]. Notably, *H. pylori* Msr display a synergistic effect with GroEL in repairing methionine-oxidized catalase, another critical antioxidant enzyme constituting 4–5% of the total *H. pylori* protein levels [114].

Taken together, a functional *H. pylori* Trx system is necessary for *H. pylori* survival under microaerobic conditions because of its roles in the protection of bacteria from oxidative stress. In agreement with the reducing capacity of Trx1 and Trx2 for the thiol peroxidases and Msr, the *H. pylori trx*1 mutant was more sensitive to oxidative and nitrosative stress than the wild type, whereas mutation of *trx*2 only led to a slight sensitivity to oxidative stress [111].

Mycobacter tuberculosis

M. tuberculosis is the leading infectious bacteria to cause human death. The infection process involved resides in alveolar mononuclear phagocytes. Thus the bacteria need to use various antioxidant systems to defend against the reactive oxygen and

nitrogen species produced by the activated macrophage [115]. In *M. tuberculosis*, there are one TrxR [116] and three Trxs (TrxA, TrxB, and TrxC) [117–119]. The redox potentials of *M. tuberculosis* TrxA, TrxB, and TrxC are −248, −262, and −269 mV, respectively. TrxA cannot obtain the electron from TrxR, which indicates that only TrxB and TrxC are the biological active disulfide reductases [118]. *M. tuberculosis* Tpx can obtain electrons from both TrxB and TrxC, whereas AhpC can be reduced by TrxC [120]. AhpC can also obtain the electrons from AhpD, and the oxidized AhpD is reduced by dihydrolipoamide succinyltransferase (a lipoamide containing protein), lipoamide reductase, and NADH [121,122] (Fig. 7B).

M. tuberculosis AhpC was proposed to be a compensatory antioxidant enzyme for the mutation of catalase peroxidase (KatG), the cause of the resistance to the front line antitubercular drug isoniazid (INH) [123,124]. *M. tuberculosis* KatG is a heme-dependent peroxidase and protects the bacteria from attack from various peroxides including peroxynitrite. At the same time, KatG is responsible for converting INH into the bioactive form, the clinically significant INH-resistant *M. tuberculosis* is commonly involved in the mutation or missing of the *katG* gene. *ahpC* mutant *M. tuberculosis* is more sensitive to peroxynitrite and has a decreased survival in macrophages [125]. However, whether AhpC is a key virulence factor is still controversial since some of the mutations of *katG* resulting in the INH resistance mantain catalase peroxidase activity to some degree [126] and inactivation of ahpC did not affect bacterial growth during acute infection in mice [127].

Another thiol-dependent peroxidase in *M. tuberculosis Tpx*, which can act as one-Cys peroxiredoxin [97], is a predominant antioxidant to defend against oxidative and nitrosative stress [128]. Moreover, another substrate of the thioredoxin system, *M. tuberculosis* MsrA and MsrB, protects the bacteria from the killing by nitrite and hypochlorite [129].

The homologue of the *OxyR* gene in *M. tuberculosis* contains numerous deletions and frameshifts, which make the *OxyR* gene nonfunctional [104]. Alternatively, the expression of TrxR and Trx (TrxB and TrxC) is regulated by SigH [130–132]. Under reductive conditions, SigH is bound with RshA and the transcription of TrxR and Trx is inhibited. While in oxidative stress, SigH is released from the complex and binds with the promoter of TrxR, TrxB, and TrxC and activates their transcription [132]. Very interestingly, TrxR appears to be essential for the growth for another GSH-negative bacteria *Staphylococcus aureus* [98]. The transcription of TrxR is regulated by several transcription factors including SigB [98], PerR [133], and SarA [134].

Instead of GSH, there are other small thiol molecules such as mycothiol or bacillithiol in some of the bacteria [135,136] (Fig. 7B),

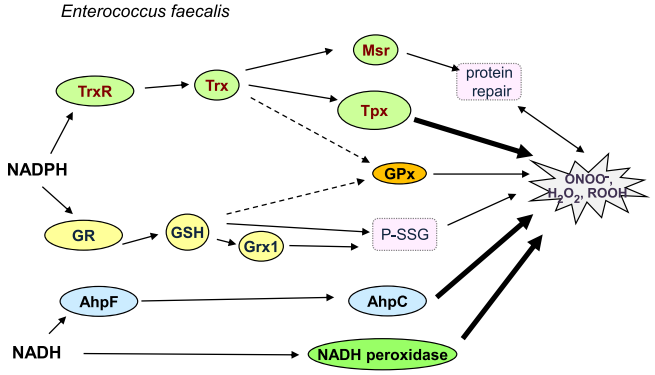

Fig. 8. Scheme of the thioredoxin antioxidant system in *Enterococcus faecalis*. Catalase (KatA) in *E. faecalis* is not functional without heme supplement, but the bacterium contains three peroxidases (AhpC, Tpx, and NADH peroxidase). Tpx is the most important antioxidant that protects the bacteria in macrophages among them. Moreover, MsrA and MsrB are virulence factors for *E. faecalis*.

which is critical for the oxidative stress defense [137]. The mycothiol system may exert its antioxidant function by reducing *S*-mycothiolated mixed disulfide [137]. No thiol-coupled peroxidases have been found and small thiol molecules such as bacillithiol do not support the Trx disulfide reducing activity [138], which indicate that the thioredoxin system exerts indispensable antioxidant functions in these bacteria.

Catalase-negative bacteria

Catalase (KatA) is widely distributed in various organisms, but many streptococci do not have this enzyme. Because of the lack of heme-dependent catalase, the antioxidant function is mainly dependent on a thiol-dependent system, which makes the bacteria easy to be attacked by thiol reacting agents. This type of bacteria like *Streptococcus pyogenes* has both Trx and GSH systems as *E. coli*, but the major thiol-dependent peroxidase system is not so clear [139]. In *S. pyogenes* mammalian GPx orthologues have been found, which are also present in many bacteria based on their sequence homology. For this bacterial GPx, the Gln and Trp residues in the active site of mammalian GPxs are well conserved, but bacterial GPxs have a Cys in their active site instead of Sec residue in the active site of mammalian SecGPxs. This type of CysGPx is also found in eukaryotes such as *Drosophila melanogaster. D. melanogaster* CysGPx prefers to obtain the electron from the thioredoxin system rather than GSH, and has a high efficiency for removing H_2O_2 with a reaction rate of $10^6\,M^{-1}\,s^{-1}$ [140]. Whether the thioredoxin system is the electron source for bacterial CysGPxs is not known, but CysGPx is present in some GSH-negative bacteria such as *Bacillus subtilis* and *S. aureus*. The thioredoxin system may be a good candidate for providing eletrons to bacterial CysGPx. Compared to wild-type *S. pyogenes,* CysGPx deletion mutant *S. pyogenes* was shown to be more sensitive to the oxidative stress caused by paraquat, but not hydrogen peroxide [139]. Moreover, CysGPx acts as a virulence factor in the mice model and may contribute to the adaption to oxidative stress caused by the inflammatory response [141].

Entercoccus faecalis contains catalase, but the enzyme can be active only when the bacterium is supplied with heme since the enzymes to synthesize heme are lacking in this bacterium [142,143]. In the presence of heme, catalase in *E. faecalis* can contribute to the resistance against hydrogen peroxide [142,143]. The physiological roles of three peroxidases (AhpC, Tpx, and NADH peroxidase) in *E. faecalis* have been investigated [144]. Tpx was shown to be the most important antioxidant for protecting the bacteria in macrophages and acted as a key virulence factor for *E. faecalis* [144]. Moreover, MsrA and MsrB are critical for the removal of H_2O_2 and act as virulence factors for *E. faecalis* [145], indicating that the thioredoxin antioxidant system is critical for survival in the hosts Fig. 8.

Conclusions

In summary, organisms are equipped with a diversity of thiol-dependent antioxidant systems, which coordinate removal of reactive oxygen and nitrogen species. The significance of Trx systems in protecting the cells against oxidative stress in different organisms varies. In mammalian cells Trx and GSH-Grx systems can act as a backup system for each other. In many pathogenic bacteria the GSH system is lacking, which confers on the thioredoxin system an essential role for growth and survival under oxidative conditions. Moreover, the distinct differences of TrxR in structure and reaction mechanisms between bacteria and mammals cause TrxR to emerge as a novel antibiotic target. Most recently, we have found that the stroke drug ebselen is an inhibitor of TrxR and can inhibit the growth of bacteria lacking GSH [146]. This explains the ebselen

antibacterial mechanism [147] and also opens a door to development of a new antibiotic against GSH-negative pathogens.

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