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Glutathione disulfide and S-nitrosoglutathione detoxification by *Mycobacterium tuberculosis* thioredoxin system

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ABSTRACT

***Mycobacterium tuberculosis* resides within alveolar macrophages. These phagocytes produce reactive nitrogen and oxygen intermediates to combat the invading pathogens. The macrophage glutathione (GSH) pool reduces nitric oxide (NO) to S-nitrosoglutathione (GSNO). Both glutathione disulfide (GSSG) and GSNO possess mycobactericidal activities in vitro. In this study we demonstrate that *M. tuberculosis* thioredoxin system, comprises of thioredoxin reductase B2 and thioredoxin C reduces the oxidized form of the intracellular mycothiol (MSSM) and is able to efficiently reduce GSSG and GSNO in vitro. Our study suggests that the thioredoxin system provide a general reduction mechanism to cope with oxidative stress associated with the microbe's metabolism as well as to detoxify xenobiotics produced by the host.**
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1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis, is a human intracellular pathogen responsible for two million deaths worldwide per annum [1]. *M. tuberculosis* infects, resides, and multiplies in alveolar macrophages and causes the formation of granulomas, which surround the bacilli to limit their dispersion. Within granulomas and phagosomes, *M. tuberculosis* is exposed to reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs), which generate a toxic environment aimed to kill the pathogen. In turn, *M. tuberculosis* employs multiple strategies to combat oxidative stress, which include a catalase/peroxidase enzyme (KatG) [2], superoxide dismutase [3] and a thiol based detoxification response [4].

In eukaryotes, glutathione (GSH) maintains a reducing environment within the cytoplasm, and is a key component of the cellular defense against oxygen toxicity [4]. *M. tuberculosis* lacks GSH and instead uses mycothiol (MSH), which functions as the mycobacteria's main anti-oxidant defense [4].

Thioredoxin systems [5] are key ubiquitous thiol-disulfide oxidoreductases. The thioredoxin system is composed of thioredoxins and thioredoxin reductases, which transfer electrons from NADPH to terminal oxidized substrates and protein disulfides [6]. Thioredoxin systems are essential for various metabolic pathways including the maintenance of a reduced state, DNA synthesis, and transcription regulation in cells [7]. Thioredoxins are small proteins, which possess a conserved CXXC catalytic motif that forms an active center dithiol and undergoes reversible oxidation. The redox cascade is based on the NADPH oxidation by thioredoxin reductases, which mediates reduction of oxidized thioredoxins [Trx-$(S)_2$] and forms reduced thioredoxin-$(SH)_2$. Finally, this reduced thioredoxin reduces a terminal substrate at the end of the cascade.

The *M. tuberculosis* genome encodes three thioredoxins (TrxA, TrxB1 and TrxC) and a single copy of the thioredoxin reductase TrxB2 (TrxR) [8]. The system comprising TrxR and TrxC was shown to reduce dinitrobenzene (DTNB), and hydroperoxides [9]. The thioredoxin system contributes to the pathogen's defense against ROIs [2,5] by the reduction of the alkyl hydroperoxidase (ahpC). The thioredoxin system also plays a role in hydroperoxide and peroxynitrite detoxification by supplying electrons to the thiol peroxidase Tpx [5].

Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); GSH, glutathione; GS-mB, bimane derivative of GSH; GSSG, glutathione disulfide; GSNO, S-nitrosoglutathione; LMW, low molecular weight; mBBr, monobromobimane; MSH, mycothiol; MS-mB, bimane derivative of mycothiol; MSSM, oxidized mycothiol (mycothione); NEM, *n*-ethylmaleimide; NO, nitric oxide; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates

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Upon infection by *M. tuberculosis*, macrophages induce both nitric oxide (NO) and GSH production. NO was shown to inhibit *M. tuberculosis* growth both in vitro and in vivo [10,11]. GSH protects macrophages against the toxic effect of ROIs and RNIs that are produced by the host in response to infection [12]. NO reacts with GSH leading to generation of S-nitrosoglutathione (GSNO). Interestingly, both GSNO and glutathione disulfide (GSSG) were shown to be toxic to *M. tuberculosis* [13]. Mechanistically it has been assumed that exposure to high concentrations of GSSG and GSNO renders an imbalance in redox potential leading to *M. tuberculosis* growth inhibition [14]. Although GSSG and GSNO are bactericidal to *M. tuberculosis* in vitro, the pathogen can still survive and multiply inside macrophages. This suggests that *M. tuberculosis* possesses a detoxification system to cope with these toxic agents.

In this study, we show that the thioredoxin system of *M. tuberculosis* possesses oxidoreductase activity towards various low molecular weight (LMW) thiols. We provide evidence that this system has the capacity to reduce GSSG and GSNO in vitro. This finding suggests that the thioredoxin system may potentially protect *M. tuberculosis* against these antimycobacterial compounds. We also demonstrate that this system efficiently reduces the oxidized form of mycothiol (MSSM) in vitro, which is suggestive of the potential role of the thioredoxin system to restore MSH levels under oxidative stress.

2. Materials and methods

2.1. Gene cloning and production of recombinant proteins

The *trxR*, *trxC* and *tpx* genes were amplified by PCR from genomic DNA of *M. tuberculosis* H37Rv using the primers (Operon) listed in Table 1. The *trxC* and *tpx* genes were ligated to pET-22b (Novagen) after digestion with NdeI and HindIII. The resulting constructs were transformed into *Escherichia coli* DH5α and confirmed by sequencing. The *trxC*/pET and *tpx*/pET constructs were subsequently transformed into *E. coli* BL-21 (DE3) for protein expression and cultured in Luria–Bertani (EMD) medium supplemented with 100 μg/ml ampicillin (Fisher). *trxR* was ligated to the *E. coli*/*Mycobacterium* shuttle vector pALACE [15] after digestion with AflII and ClaI. Once the construct was confirmed by sequencing, it was electroporated into *Mycobacterium smegmatis* mc^2155 and cultured in Middlebrook 7H9 (BD) supplemented with 0.05% Tween 20 (Fisher), 1% glucose, and 50 μg/ml hygromycin (Roche).

Protein expression in *E. coli* and *M. smegmatis* was performed according to published protocols [16,17]. Recombinant proteins were purified as his-tagged proteins using Ni-NTA affinity chromatography (Qiagen) following the manufacturer protocols. Eluted proteins were dialyzed overnight in 50 μM Tris–HCl pH 7.5, 1 mM dithiothreitol, 5% glycerol, and stored at −20 °C.

2.2. Enzymatic assays

The reaction mixture contained 100 μM phosphate buffer pH 7.4, 1 mM EDTA (Sigma), 450 μM NADPH (ICN), and purified proteins at a final concentration of 1 μM in a final volume of 0.6 ml. GSSG (Sigma) and GSNO (Sigma) were assayed at 26 μM, while H_2O_2 was used at a final concentration of 5 μM. A thiol-containing crude extract from *M. smegmatis* (6 mM as assayed by HPLC [18]) at a final concentration of 26 μM was used to evaluate the activity of the system towards MSSM. The activity of the thioredoxin system was measured by spectrophotometry in two procedures. First procedure is based on the assay described by Holmgren [19], which measures the decrease in A_{340} during the first minute of reaction every 10 s. The amount of NADPH oxidized was calculated as nmoles/min and according to the relation $\Delta_{340} \times 0.6/6.2$. In the second procedure, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was the substrate at a final concentration of 26 μM [20], and followed by measuring the increase in A_{412}. One unit of activity was calculated according to the relation $\Delta_{412} \times 0.6/13.6 \times 2$ [21]. All the experiments were performed in triplicate.

2.3. Biochemical assays using HPLC analyses

A typical reaction for kinetic studies contained the concentrations 26, 40, and 57 μM of each substrate. All the biochemical assays were performed in triplicate. Formation of products from each substrate was analyzed by HPLC according to published protocols [18]. Briefly, reactions were performed at 30 °C, and aliquots of 200 μl were taken at time 0, 10, and 60 min. These aliquots were treated at 60 °C with the fluorescent alkylating agent monobromobimane (mBBr) to produce the fluorescent bimane derivatives (S-conjugates) MS-mB and GS-mB from MSSM and GSSG/GSNO, respectively. The treated samples were centrifugated at 14000 rpm for 3 min and subsequently acidified with 2 μl of 5 M methanesulphonic acid (Sigma). At each time point an aliquot of each reaction was taken for treatment with *n*-ethylmaleimide

Table 1
Strains, plasmids and oligonucleotides used in this work.

Strain, plasmid/oligonucleotides	Characteristics	Source
Strain		
E. coli DH5α		Invitrogen
E. coli BL21		Invitrogen
M. smegmatis	mc^2155	ATCC 700084
Plasmids		
pALACE	*ace* promoter, $hygromycin^R$	[15]
pET-22b	P_{T7}-based expression vector	Novagen
trxR/pALACE	*Rv3913* within AflII/ClaI restriction sites	This work
trxC/pET-22b	*Rv3914* within NdeI/HindIII restriction sites	This work
tpx/pET-22b	*Rv1932* within NdeI/HindIII restriction sites	This work
Oligonucleotides used in this work		
trxR-forward	5′-aaacttaagatgaccgccccgcctgt-3′	
trxR-reverse	5′-aaaatatcgattcatcgttgtgctcctatca-3′	
trxC-forward	5′-aattctagacatatgaccgattccgagaagt-3′	
trxC-reverse	5′-aaaaagcttgttgaggttgggaacc-3′	
tpx-forward	5′-aattctagacatatggcacagataaccctgc-3′	
tpx-reverse	5′-aaaaagcttggcgcccagcgcg-3′	
trxC-Cys^{40}Ser-forward	5′-acatggtgtggacctagcaagatggtagcgccc-3′	
trxC-Cys^{40}Ser-reverse	5′-gggcgctaccatcttgctaggtccacaccatgt-3′	

(NEM) (Sigma) (negative control), which binds and blocks all available thiol groups [22]. Samples were then resolved on reverse phase Symmetry C18 column (250 mm × 4.6 mm) (Waters) using 0.25% glacial acetic acid pH 3.6 (solvent A), and methanol (solvent B). Separation of products was carried out by using 10% solvent B for 10 min, 18% solvent B for 15 min, and 27% solvent B for another 22 min. The amount of product produced was calculated using a calibration curve.

2.4. Generation of a catalytic-defective TrxC

A catalytic-defective TrxC was constructed by replacing the cysteine at position 40 with serine using the primers listed in Table 1. The mutation $Cys^{40}Ser$ was performed by oligonucleotide overlapping according to published protocols [23]. This mutation renders TrxC unable to transfer electrons to a final electron acceptor. The mutation was confirmed by sequencing.

3. Results

3.1. Production of recombinant proteins

His-tagged TrxR (35 kDa), TrxC (12 kDa), TrxC-$Cys^{40}Ser$ (12 kDa), and Tpx (16.8 kDa) were produced as recombinant soluble proteins and purified using affinity chromatography. Fig. 2 shows that all proteins migrated according to the expected size.

3.2. Reductase activity of recombinant thioredoxin system

DTNB and H_2O_2 act as substrates for the thioredoxin system [5,9,24], and were used as positive controls to test the performance of our system. When DTNB was used as the terminal electron acceptor, the thioredoxin system was able to oxidize NADPH (Table 2). Upon addition of Tpx to the reaction, an approximate 50% increase in NADPH oxidation was observed. TrxR and its combination with Tpx showed NADPH oxidation, which is in agreement with previous reports [5,24]. Individual enzymes did not show any activity except for TrxR, which shows a 90% decrease in the reductase activity as compared to the thioredoxin system. No activity was measured when the parental TrxC was replaced with the mutated $Cys^{40}Ser$ TrxC, as this catalytic-defective enzyme is not able to transfer electrons to the final electron acceptor.

Similarly, the thioredoxin system oxidized NADPH when H_2O_2 was used as a terminal substrate. An increase of 35% in NADPH oxidation was measured when Tpx was added to the system. No activity was measured when either individual enzymes or combination of TrxR and Tpx were tested. Moreover, $Cys^{40}Ser$ TrxC was not able to transfer electrons to the terminal acceptor substrate H_2O_2.

Table 2
NADPH-dependent disulfide reductase activity of *M. tuberculosis* thioredoxin system.

Protein	DTNB	H_2O_2
TrxR	42.9 ± 7.1	N.D.
TrxC	N.D.	N.D.
Tpx	N.D.	N.D.
TrxR + TrxC	406 ± 20	130 ± 5
TrxR + $Cys^{40}Ser$ TrxC	63 ± 6.6	N.D.
TrxR + Tpx	92 ± 2.4	N.D.
TrxC + Tpx	N.D.	N.D.
TrxR + TrxC + Tpx	606 ± 26	177 ± 3.6
TrxR + $Cys^{40}Ser$ TrxC + Tpx	83.3 ± 2.3	N.D.

Results are expressed as nmoles of NADPH oxidized/min ml mg protein.
N.D. = No Detected. Shown is the mean ± S.E. of three independent experiments.

3.3. MSSM, GSSG and GSNO are substrates of thioredoxin system

Since MSH is the major LMW thiol within the mycobacterial cell, we tested whether the thioredoxin system is able to reduce MSSM (Fig. 1). Results from NADPH oxidation assay showed that the thioredoxin system was able to recruit MSSM as the final electron acceptor (Table 3). Addition of Tpx to the reaction led to a decrease in NADPH oxidation towards MSSM. No reductase activity was detected when either individual enzymes or $Cys^{40}Ser$ TrxC were used (data not shown).

The activity of the thioredoxin system towards GSSG and GSNO (Fig. 1) was assessed by the NADPH oxidation assay. Results showed that the thioredoxin system was able to transfer electrons from NADPH to both GSSG and GSNO (Table 3). Upon addition of Tpx to the system, a decrease in NADPH oxidation was measured towards both substrates. No NADPH oxidation was detected when either individual enzymes or $Cys^{40}Ser$ TrxC were used (data not shown). Thus, we concluded that the thioredoxin system comprised of TrxR and TrxC has the capacity to transfer electrons from NADPH to the three examined substrates GSSG, GSNO, and MSSM.

3.4. Direct measurements of product formation

HPLC analysis was performed [18] to directly measure the amount of product formed in the enzymatic reaction, and to comparatively assess the oxidoreductase activity of the thioredoxin

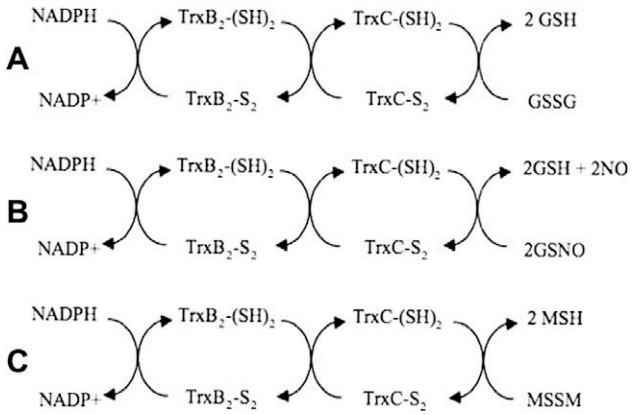

Fig. 1. Scheme of NADPH-dependent transfer of electrons to the terminal acceptors mediated by thioredoxin system. (A) NADPH-driven reduction of GSSG, (B) breakdown of GSNO, (C) reduction of MSSM.

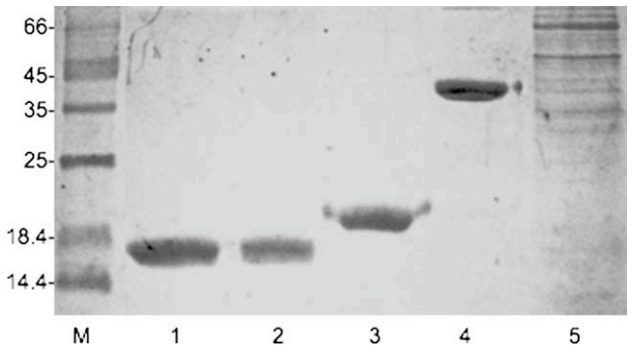

Fig. 2. Purification of redox proteins using affinity Ni-NTA affinity chromatography. Elution fractions corresponding to purifications by affinity chromatography were resolved on 15% SDS–PAGE and Commassie blue stained. Lanes: (M) Protein marker, (1) wt TrxC, (2) $Cys^{40}Ser$ mutated TrxC, (3) Tpx, (4) TrxR, (5) uninduced culture (representative). Molecular weight of protein standards is indicated on left side.

Table 3
Specific reductase activity of the *M. tuberculosis* thioredoxin system towards selected substrates.

	GSSG	GSNO	MSSM
NADPH oxidation^			
TrxR + TrxC	139 ± 8.3	120 ± 3	50 ± 5.6
TrxR + TrxC + Tpx	70 ± 6.6	63 ± 2	15 ± 1.3
HPLC analysis^^			
TrxR + TrxC	150 ± 46	103 ± 27	49 ± 3
TrxR + TrxC + Tpx	103 ± 16	90 ± 29	18 ± 2

Results are expressed as: ^nmoles of NADPH oxidized/min ml mg enzyme, and ^^nmoles of thiol produced/min ml mg protein. Shown is the mean ± S.E. of three independent experiments.

system towards GSSG, GSNO and MSSM. In the presence of TrxC, TrxR, and NADPH, GSH was produced from both GSSG and GSNO (Fig. 3 and Table 3). The thioredoxin system was able to produce 150 and 103 nmoles of GSH/ml min mg of protein when GSSG and GSNO were used, respectively. However, upon addition of Tpx to the system, a decrease in the rate of GSH formation was observed. In the case of MSSM, 49 nmoles of MSH/ml min mg of protein were formed, but addition of Tpx to the system decreased the rate of MSH formation. In addition, in all cases where $Cys^{40}Ser$ TrxC was used, neither GSH nor MSH formation was detected (data not shown).

3.5. Determination of kinetic parameters

To compare the efficiency of the thioredoxin system towards the selected substrates, we determined the kinetic parameters of the reactions. Kinetic results listed in Table 4 show that MSSM has the highest affinity to the thioredoxin system. The thioredoxin system showed a similar turnover number towards GSSG and GSNO with 352 and 298 molecules of substrate converted to GSH min^{-1}, respectively, while 60 molecules of MSSM were converted to MSH min^{-1} by the system. When Tpx was added to the system, 168 and 245 molecules of GSSG and GSNO were converted to GSH min^{-1}, while 46 molecules of MSSM were converted to MSH min^{-1}.

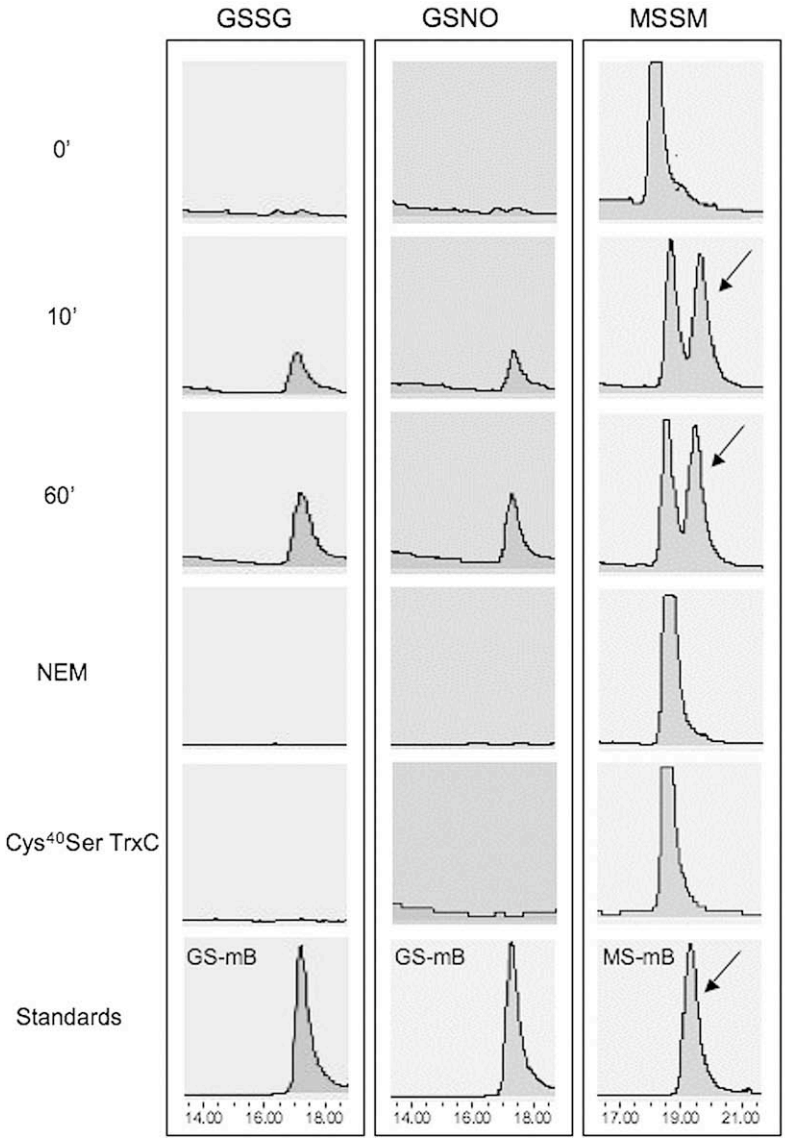

Fig. 3. Time-dependent formation of thiols by thioredoxin systems. The formation of thiols over time was monitored by HPLC. Reactions were incubated at 37 °C for 60 min in presence of 26 μM of each substrate. Reactions were stopped, acidified, labeled with mBBr, and separated in HPLC. Arrows indicate the position of MS-mB. NEM is a specific thiol-blocking compound and was used as negative control.

Table 4
Steady-state kinetic parameters of substrate conversion.

Substrate	K_M (μM)	K_{cat} (min^{-1})	K_{cat}/K_M (min^{-1} μM^{-1})
GSSG			
TrxR + TrxC	8.8 ± 2.7	352	40
TrxR + TrxC + Tpx	1.5 ± 0.9	168	112
GSNO			
TrxR + TrxC	3.7 ± 0.9	298	80
TrxR + TrxC + Tpx	1.6 ± 0.13	245	153
MSSM			
TrxR + TrxC	0.8 ± 0.1	60	75
TrxR + TrxC + Tpx	0.4 ± 0.18	46	115

Shown is the mean ± S.E. of three independent experiments.

Table 5
Kinetic parameter comparison of different thioredoxin systems.

Substrate	Organism	K_{cat} (min^{-1})	K_{cat}/K_M (min^{-1} μM^{-1})	Reference
GSSG	*M. tuberculosis*	352	40	This work
	Plasmodium falciparum	<0.2	<0.00001	[32]
GSNO	*M. tuberculosis*	298	80	This work
	Human TrxC	36	0.6	[27]
	Plasmodium falciparum	9.4	0.3	[32]
MSSM	*M. tuberculosis* thioredoxin system	60	75	This work
	M. tuberculosis mycothiol reductase	64	55	[33]

4. Discussion

Using both an NADPH oxidation assay and HPLC analysis of product formation, we showed that the *M. tuberculosis* thioredoxin system is able to transfer electrons in vitro to various substrates including the intracellular substrate MSSM and the antimycobacterial GSSG and GSNO.

According to our results, the number of nmoles of NADPH oxidized by the thioredoxin system towards the candidate substrates was consistent with the number of nmoles of GSH or MSH formed in the enzymatic reactions as measured by HPLC analysis (Table 3). Also, higher levels of NADPH oxidation and product formation towards GSSG and GSNO were observed by the thioredoxin system in the absence of Tpx. This observation is consistent with the higher K_{cat} values of the thioredoxin system, which converts more molecules of substrate to product in the absence of Tpx. One explanation of the lower activity of the system once Tpx was supplemented to the thioredoxin system is the formation of disulfide bridges between Tpx-Cys^{60}, TrxR-Cys^{31} and TrxC-Cys^{37} [25]. Then, since the substrate of Tpx (H_2O_2) is absent in this reaction, we suggest that there is a transient binding of Tpx to TrxC. This eventually reduces the number of available catalytic sites of TrxC thus decreasing the thioredoxin activity in the presence of Tpx.

Our study suggests a novel function for the *M. tuberculosis* thioredoxin system acting as a GSNO reducing system. Denitrosylation of GSNO by human thioredoxins has been studied by both the Holmgren and Stoyanovsky laboratories. Holmgren's group has reported a homolytic breakdown of GSNO generating GSH and NO [27], while Stoyanovsky has proposed a heterolytic breakdown of GSNO producing GSH and nitroxyl (HNO) [28]. However both groups suggest that other cellular nitrosylated proteins may serve as substrates for the thioredoxin system. Earlier attempts to elucidate the mechanism of GSNO breakdown in *M. tuberculosis* suggested that the only enzyme in *M. tuberculosis* with nitrosothiol reductase activity is S-nitrosomycothiol reductase (MscR), which is responsible for S-nitrosomycothiol reduction [26]. However, MscR is unable to utilize GSNO as a substrate.

As mentioned earlier, we present evidence that the *M. tuberculosis* thioredoxin system catalyzes the denitrosylation of GSNO, resulting in the formation of GSH. The consequence of this breakdown and the mechanism of inactivation of NO intermediates released from GSNO inside the pathogen remains to be elucidated. Production of the toxic NO as the result of GSNO breakdown seems paradoxical, due to the toxicity of NO [29]. However, bearing in mind the short half life of NO, and our previous studies showing that mycothiol efficiently protects mycobacteria against its bactericidal effect [11], NO is considered much less toxic compared to GSNO [30].

The mycobacterial thioredoxin system shows higher kinetic parameters (K_{cat} and K_{cat}/K_M) values compared to other thioredoxin systems, e.g. human thioredoxins or the protozoan parasite *Plasmodium falciparum* thioredoxin systems (Table 5). This observation suggests that the mycobacterial thioredoxin system is able to efficiently reduce GSSG and its nitroso derivative GSNO of the host.

Under oxidative stress MSH becomes oxidized and forms the oxidized MSSM disulfide. Mycothiol reductase (Mtr) reduces MSSM and maintains MSH levels [31]. However, Mtr is not an essential gene in *M. tuberculosis*, suggesting that other electron donors can restore MSH. Here, we show that the thioredoxin system reduces MSSM, with an efficiency comparable to Mtr.

In conclusion, our in vitro results show that the thioredoxin system of *M. tuberculosis* functions as a general reduction system, which is able to efficiently reduce the LMW disulfides GSSG and MSSM, and dissimilate GSNO. Our findings suggest that this system may potentially be involved in the detoxification of mycobactericidal compounds such as GSSG and GSNO, which are produced by macrophages to limit mycobacterial growth within the infected host. Additional work is in progress to characterize the activity of the thioredoxin system in infected macrophages.

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