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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.

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′	