**Bacillus anthracis** thioredoxin systems – characterization and role as electron donors for ribonucleotide reductase

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**Keywords:**

Bacillus anthracis, Bacillus subtilis, redox, thioredoxin, thioredoxin reductase, ribonucleotide reductase, NrdH, bacillithiol, oxidative stress, RF cloning, autolysis, autoinduction

**Background:** **Bacillus anthracis** encodes several potential thioredoxin systems.

**Results:** Thioredoxin 1 was the most efficient disulfide reductase and was present at 60 times higher levels in *B. anthracis* compared to NrdH.

**Conclusion:** The major disulfide reductase and electron donor for ribonucleotide reductase was thioredoxin 1 rather than NrdH.

**Significance:** Understanding the thioredoxin systems in *B. anthracis* could form the basis for novel antimicrobial therapies.

**SUMMARY**

*Bacillus anthracis* is the causative agent of anthrax which is associated with a high mortality-rate. Like several medically important bacteria, *B. anthracis* lacks glutathione, but encodes many genes annotated as thioredoxins, thioredoxin reductases and glutaredoxin-like proteins. We have cloned, expressed and characterized three potential thioredoxins, two potential thioredoxin reductases and three glutaredoxin-like proteins. Of these, thioredoxin 1 (Trx1) and NrdH reduced insulin, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and the manganese-containing type Ib ribonucleotide reductase (RNR) from *B. anthracis* in presence of NADPH and thioredoxin reductase 1 (TR1), whereas thioredoxin 2 (Trx2) could only reduce DTNB. Potential TR2 was verified as an FAD-containing protein reducible by dithiothreithol, but not by NAD(P)H. The recently discovered monothiol bacillithiol did not work as a reductant for RNR, either directly, or via any of the redoxins. The catalytic efficiency of Trx1 was 3 and 20 times higher than that of Trx2 and NrdH, respectively, as substrates for TR1. Additionally, the catalytic efficiency of Trx1 as an electron-donor for RNR was sevenfold higher than that of NrdH. In extracts of *B. anthracis*, Trx1 was responsible for almost all of the disulfide reductase activity, whereas western-blots showed that the level of Trx1 was 15 and 60 times higher than that of Trx2 and NrdH, respectively. Our findings demonstrate that the most important general disulfide reductase system in *B. anthracis* is TR1/Trx1, and that Trx1 is the physiologically relevant electron-donor for RNR. This information may provide a basis for development of novel antimicrobial therapies targeting this severe pathogen.

**INTRODUCCION**

*Bacillus anthracis*, the causative agent of anthrax, is a gram-positive, sporforming rod (1). It belongs to the *Bacillus cereus* group and the same genus as the nonpathogenic *Bacillus subtilis*, which has been used extensively as a model organism (2). During the course of an infection, *B. anthracis* spores germinate inside macrophages and the vegetative cells rapidly divide in the host reaching high densities in blood during fulminant decease (3). This would imply that the bacterium needs efficient systems for synthesis of deoxyribonucleotides for replication and repair of DNA. Furthermore, the need for protection...
against oxidative stress imposed by the host immune system is obvious.

Thioredoxin was first identified as a reductant for *Escherichia coli* ribonucleotide reductase (RNR) (4). Since then, many more functions have been attributed to the thioredoxin system, which is composed of thioredoxin (Trx), thioredoxin reductase (TR), and NADPH (5). Trx, which is a major intracellular protein disulfide reductase, is also an electron donor for protein methionine sulfoxide reductase, Trx-dependent peroxidases and is a key player in antioxidant defense (6, 7).

Many organisms, including *E. coli* and man, also contain a glutaredoxin system composed of glutaredoxin (Grx), glutathione (GSH), glutathione reductase (GR) and NADPH. Grx was discovered in an *E. coli* mutant lacking Trx, but with a fully active ribonucleotide reduction and DNA synthesis activity (8). The Grx system shares a lot of functions with the Trx system, but has its own distinct roles in vivo (9).

The thioredoxin and glutaredoxin systems in *E. coli* have been the subject of much interest since their discoveries. Although *E. coli* encodes three ribonucleotide reductases, two thioredoxins and multiple glutaredoxins, it was shown that the type Ia RNR and either a functional thioredoxin system or a glutaredoxin system is needed to sustain aerobic growth and produce dNTP’s in vivo. Furthermore, the thioredoxin can be either Trx1 or Trx2 whereas in the absence of a thioredoxin system, Grx1 becomes essential (10–12). This is in stark contrast to the situation in *B. subtilis*, where both Trx1 and TR are essential (13, 14). Although the essentiality of Trx1 can be overcome by supplementing the growth medium with deoxyribonucleotides and cysteine or methionine, the growth is severely retarded and the sporulation efficiency is reduced more than five orders of magnitude (15). It has been observed that conditional knock-outs of Trx1 in *B. subtilis* are viable under certain conditions. However this can be attributed to leakage of the promoter and suppressor mutations (15–17). It was also shown that in *S. aureus*, TR appears to be essential (18).

*B. anthracis* and *B. subtilis* lack GSH, a feature they share with other well-known human pathogens such as *Staphylococcus aureus* and *Mycobacterium tuberculosis* (19, 20). Although *B. anthracis* lacks GSH, it does produce the recently described low molecular-weight glycosidic thiol, bacillithiol (BSH), albeit at approximately 30 times lower concentrations than GSH in *E. coli* (21). BSH appears to participate in sensing of peroxides and may substitute for GSH, but gene knockouts abolishing BSH synthesis in both *B. subtilis* (22) and *B. anthracis* (23) are viable without obvious growth defects. The absence of BSH does however affect sporulation efficiency in both bacteria as well as tolerance against high salt and low pH in *B. subtilis* (22, 23).

With few exceptions the genus *Bacillus*, including *B. anthracis*, *B. cereus* and *B. subtilis*, encodes an operon for the class Ib RNR (24). The *Bacillus* operon consists of three genes: the promoter-proximal *ndrI* followed by *ndrE* and *ndrF*. The RNR proper is a dimer of NrdE and NrdF homodimers, where the larger NrdE contains the active site and the smaller NrdF a dinuclear metallosite stabilizing a catalytically essential tyrosyl radical (25). NrdI is a flavodoxin essential for generation of the tyrosyl radical in the manganese form of NrdF (Mn-NrdF) (26, 27). In most other bacteria the class Ib RNR operon also comprises a Grx-like gene called *ndrH* (24). The NrdH-redxin is a specific reductant for class Ib RNR working via TR and NADPH (28). The equivalent *ndrH* gene in *Bacillus* is unlinked to the *ndrI-ndrE-ndrF* operon and located elsewhere on the chromosome. *B. cereus* NrdH was recently shown to be an efficient reductant of the Mn-forms of *B. cereus* and *B. anthracis* class Ib RNRs (29). It has been shown that both subunits of the type Ib ribonucleotide reductase is essential for *B. subtilis* (14) and given the absence of a type Ia RNR in *B. anthracis* (30) it would be reasonable to assume that the same holds true for this bacterium.

In this study we have theoretically identified three potential Trx proteins, three potential Grx-like proteins, including the NrdH-redoxin mentioned above, and two potential TRs. We have characterized their activity as general disulfide reductase systems, as well as RNR reductants. Only three of the potential redoxins were able to reduce disulfides in presence of TR1 and NADPH with Trx1 as the most efficient substrate for TR1. Interestingly, we showed that the Trx1 system was a more efficient reductant of the Mn-form of *B. anthracis* class Ib RNR than the NrdH system. Furthermore, the Trx1 system was also the predominant reductase in crude extracts.
MATERIALS AND METHODS

General: B. anthracis Sterne 7700 (pXO1/pXO2), which is a non-toxigenic, non-encapsulated strain lacking the two virulence-plasmids, was obtained from the Swedish Defence Research Agency. pNIC28-BSA4 was a generous gift from Professor Opher Gileadi, University of Oxford and pMHT238Δ from Professor Brian G Fox, University of Wisconsin. The His-tagged and C-terminally truncated TEV-protease encoded in pMHT238Δ was expressed and purified essentially as described before (31). Primers were from Thermo Fischer Scientific, Phusion High-Fidelity PCR Master mix was from Finnzymes and Exonuclease I from Fermentas. E. coli XJb(DE3) Autolysis was from Zymo Research. Plasmid Mini-prep Kit was from Qiagen and sequencing was done by Eurofins MWG Operon. Ni-Sepharose 6 Fast Flow was from GE Healthcare, SDS-PAGE gels and equipment were from Invitrogen, whereas “Complete EDTA-free Protease Inhibitor” and DNase I was from Roche. Affi-Gel-601, DC Protein Assay Kit,ChemiDoc XRS imaging system and Quantity One were from Bio-Rad. Emulsifier Safe was from Perkin Elmer, and reduced bacillithiol (BSH) from Jema Biosciences. Other chemicals were generally from Sigma-Aldrich.

Bioinformatics/target selection: Genes encoding potential thioredoxin reductases, thioredoxins and NrdH-redoxins/glutaredoxin-like proteins were identified from the genome sequence of B. anthracis strain Ames (30) in the RefSeq database (32) using the following search-terms: “thioredoxin reductase” for thioredoxin reductase; (thioredoxin[Protein Name] OR thioredoxin family protein[Protein Name] OR thioredoxin, putative[Protein Name]) for thioredoxin; and (ndrH OR glutaredoxin BUT NOT ndrI) for NrdH-redoxins/grx-like. Sequences were manually inspected for the presence of a potential active site (CXXC) and were excluded when absent. Remaining genes were analyzed for predicted subcellular localization using PSORTb with default settings for Gram-positive bacteria (33). Eight positively scoring gene products with assumed cytosolic localization were included in the experimental part of this study.

Exonuclease I dependent Restriction Free cloning (ERF-cloning) of target genes: Target genes were cloned from genomic B. anthracis Sterne 7700 (pXO1/pXO2) DNA into pNIC28-BSA4 (34). Primers were designed based on the sequence of B. anthracis Ames, since this strain was the first to be whole-genome sequenced (30). For cloning, we developed an optimized version of the Restriction Free cloning method (35, 36). Our major improvements of the cloning procedure included substitution of the gel-purification of the PCR-products in favor of treatment with Exonuclease I for removal of excess primers. Furthermore DpnI-digestion was omitted since the SacB-fragment present in pNIC-BSA4 allows for negative selection against parental plasmid on plates containing 5% sucrose (34). The method will be referred to as ERF-cloning (Exonuclease I dependent Restriction Free cloning).

Primers were designed with the following sequences: 5’-gaacctgtactcaatccatg-F_R-3’ (forward) and 5’-gatcgatgacactttaacctgta-R_R-3’ (reverse), where F_R and R_R denotes any number of nucleotides complementary to the target sequence in the forward and reverse directions respectively, which gives a predicted T_m of approximately 55°C. Bold sequences represent vector-complementary sequences and were designed to give a predicted T_m of 65°C. Predictions of T_m were done according to the instructions provided by the manufacturer of the polymerase.

An initial PCR-step was performed, where PCR-reactions contained 1 ng/µl template DNA, a final concentration of 1x Phusion High Fidelity PCR Master Mix, and 0.5 µM each of forward and reverse primers. An annealing temperature of 55°C for the first 8 cycles and 72°C for the following 22 cycles was used. After amplification, the reaction-mixture was incubated for 45 minutes at 37°C with 2 units/µl Exonuclease I followed by a 15 min heat inactivation step at 80°C.

For the linear amplification step, where the fragment is inserted into the destination vector, the reaction mixture contained 5 µl of Exonuclease digested fragment, 1x Phusion High Fidelity PCR Master Mix and 4ng/µl destination vector (pNIC28-BSA4) in a final volume of 25 µl. Cycling was done using 60°C as annealing temperature with a total of 25 cycles.

The product from the linear amplification step was used directly to transform chemically competent E. coli Mach1 prepared essentially as

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described before (37) using heat-shock. Transformed cells were spread on LB-plates containing 50 µg/ml kanamycin for positive selection and 5% sucrose for negative selection against unmodified plasmid. Positive clones were identified by colony-PCR (34) and verified by sequencing.

**Protein expression and purification:** XJb(DE3) Autolysis, which is a BL21(DE3) derivative with a chromosomally integrated gene encoding lambda phage endolysin under the control of an arabinose-promoter, was used for protein expression. Proteins were expressed by auto-induction (38) at either 15°C or 20°C in 2xA medium (Autoinduction, Autolysis). Final medium composition was 1% peptone, 2% yeast-extract, 1% glycerol, 0.015% glucose, 0.6% lactose, 0.05% arabinose, 0.4% aspartic acid, 1% K$_2$HPO$_4$, 2 mM MgSO$_4$, 100 µg/ml kanamycin and 0.002% polypropylene glycol 2000 adjusted to a final pH of 7.5 using NaOH. Cultures were harvested by centrifugation, pellets were resuspended in hypotonic lysis buffer (50 mM Tris-HCl, pH 8.0, 1 tablet/50 ml buffer of “Complete EDTA-free protease inhibitor mix”, 20 µg/ml DNase I and 0.2 mM FAD when needed) and were stored at -20°C until use.

Resuspended cultures were thawed, 10 units/ml Benzonase was added, cultures were freeze-thawed once and, when needed, briefly sonicated on ice to ensure complete lysis. Imidazole and thawed once and, when needed, briefly sonicated Benzonase was added, cultures were freeze-thawed once and, when needed, briefly sonicated. Cultures were freeze-thawed once and, when needed, briefly sonicated.

Proteins were purified by Immobilized Metal Affinity Chromatography (IMAC) followed by TEV-protease cleavage and subtractive IMAC (39). Concentrations of potential redoxins were calculated based on extinction coefficients at 280 nm as predicted using ProtParam (40). Concentrations of potential TR1 and TR2 were calculated based on the extinction coefficient of 13 600 M$^{-1}$cm$^{-1}$ for TNB at 412 nm and theoretical extinction coefficients for the proteins listed in Table 1, and were expressed as mol converted substrate per mol enzyme and second (s$^{-1}$). Velocities were plotted and K$_m$ and kcat-values were determined by non-linear regression using GraphPad Prism 5.

**Screening of potential redoxins and thioredoxin reductases:** Initial characterization was conducted using the DTNB- and insulin-assays (42), where the potential thioredoxin reductases were screened against the potential redoxins. The final reaction mixture contained 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 µM NADPH, 50 nM potential thioredoxin reductase, 5 µM potential reductase and either 1 mM DTNB or 320 µM insulin in a final volume of 200 µl in 96-well plates. In the DTNB-assay, activity was measured by following the increase in A$_{412nm}$ during the first 3 minutes whereas the decrease in A$_{340nm}$ was followed during 15 minutes in the insulin-assay. Each screening-assay was run in duplicate.

**Kinetic characterization of redoxins with disulfide reductase activity:** Combinations which showed activity in the initial screen were further characterized using the DTNB-assay with an NADPH-regenerating system adapted from (43). The final reaction mixture contained 100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 µM NADPH, 2 mM glucose-6-phosphate, 0.2 units/ml glucose-6-phosphate dehydrogenase, 0.1 mg/ml BSA and 1 mM DTNB in a final volume of 200 µl in micro-titer plates. Thioredoxin reductase was kept constant at 15 nM whereas redoxin concentrations were varied in the range 2-100 µM for Trx1 (BA4758), 5-150 µM for Trx2 (BA4945) and 5-200 µM for NrdH (BA4201). Each series was run in duplicate. Velocities were calculated based on the extinction coefficient of 13 600 M$^{-1}$cm$^{-1}$ for TNB at 412 nm and theoretical extinction coefficients for the proteins listed in Table 1, and were expressed as mol converted substrate per mol enzyme and second (s$^{-1}$). Velocities were plotted and K$_m$ and kcat-values were determined by non-linear regression using GraphPad Prism 5.

**Screening of potential redoxins and BSH as reducing systems for RNR:** Activity of *B. anthracis* RNR was measured by monitoring the conversion of [$^3$H]-CDP to [$^3$H]-dCDP. The reaction mixtures included 50 mM Tris-HCl, pH 7.5, 20 mM Mg(CH$_3$COO)$_2$, 0.2 mM dATP, 0.8 mM [$^3$H]-CDP, and 1 mM DTT or 0.5 mM BSH. The chosen concentration of DTT is sufficient as a reductant for the redoxins while giving a relatively low RNR activity in the absence of presence of NrdI was done as described in (29). Reconstitution of the iron form of NrdF (Fe-NrdF) was conducted as described earlier (41).

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redoxin. Since redoxins interact with the NrdE component of RNR, the NrdF component was kept in excess (2.5-fold) in all reactions to ensure that the 0.75 µM NrdE dimer is engaged in NrdE:NrdF complexes. Unless otherwise specified the concentrations of the redoxins were kept at 10 µM. In reactions where TR/NADPH substituted for DTT concentrations were 0.5 µM TR1 and 1 mM NADPH. These reaction mixtures contained ≤13 µM DTT from the storage buffer of the diluted NrdE.

Reactions were started by addition of [3H]-CDP, incubated for 10 minutes at 37°C, and were stopped by boiling for 5 min. After cooling and centrifugation, deoxyribonucleotides and ribonucleotides in the supernatant were separated on a 1 ml boronate column (Affi-Gel-601), using a modification of the protocol described in(44). After application of the sample, 150 µl Ambic buffer (15 mM MgCl₂, 25 mM NH₄CO₃, 25 mM NH₄(CO₃)₂, pH 8.9) was added and effluent was discarded. The formed deoxyribonucleotides were then eluted with 1 ml Ambic buffer. The eluted sample was mixed with 10 ml Emulsifier Safe before scintillation counting.

One enzyme unit (U) is defined as one nmol dCDP produced per minute at 37°C. The specific activity is given as U per mg of NrdE.

**Kinetic characterization of redoxins with RNR reducing activity:** In further kinetic studies Trx1 and NrdH were treated as apparent substrates for RNR in the presence of either DTT or TR1/NADPH. Reaction mixtures were as described above with a 2.5-fold excess of Mn-NrdF over NrdE. When included, the DTT concentration was 1 mM, whereas the concentrations of TR1 was 500 nM and NADPH 1 mM. Each series was run in duplicate. Velocities were calculated based on the formation of [3H]-dCDP and were expressed as mol converted substrate per mol enzyme and second (s⁻¹). Velocities were plotted and Kₘ- and kₗ₉⁻values were determined by non-linear regression using Prism 5.

**Preparation of B. anthracis lysates:** B. anthracis Sterne 7700 (pXO1/pXO2) was grown aerobically in LB-medium to an OD₆₀₀ of 0.45, harvested by centrifugation and was stored frozen until use. After thawing, OD₆₀₀ was adjusted to 20 with 50mM Tris-HCl pH 7.5, 1mM EDTA, and 1 tablet of Complete EDTA-free protease inhibitor mix per 50 ml buffer. Cells were freeze-thawed once and disrupted by sonication. The lysate was cleared by centrifugation and was sterile-filtered twice through 0.2µm syringe-filters. One portion of the lysate was stored at -20°C until use, whereas the rest was dialyzed overnight against 50mM Tris-HCl pH 7.5, 100 mM NaCl, 5% glycerol, 1 mM EDTA before storage. Total protein concentration was determined according to the method of Lowry, using the DC Protein Assay Kit with BSA as a standard.

**Antibodies against Trx1, Trx2 and NrdH, neutralization assays and Western blots:** Hyperimmune rabbit antisera raised against pure Trx1, Trx2 and NrdH from one animal per antigen were obtained from Innovagen (Lund, Sweden). In the primary immunization, antigens were mixed with Freund's complete adjuvant, whereas Freund's incomplete adjuvant was used for the subsequent boosts. Total immunoglobulins were purified by the caprylic acid–ammonium sulfate method (45). After dialysis against antibody-buffer (25 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 150 mM NaCl, 25 % glycerol) preparations were adjusted to the original serum volume and were stored frozen until use.

For neutralization experiments, 15µl aliquots of the dialysed B. anthracis lysate (6.5µg/µl total protein) were mixed with 35µl buffer BSA-buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mg/ml BSA) and 50µl of the antibody-preparations. To the samples without lysate, an equal amount of BSA-buffer was added and to samples without antibody, an equal volume of antibody-buffer was added. Mixtures were incubated at 37°C for 30 minutes, and DTNB-assays were run (at room-temp) as previously described with the modification that BSA was kept at 0.5 mg/ml and TR at a final concentration of 300 nM. Samples were run in quadruplicate. The background from TR and lysate was combined and subtracted from the activities.

For western-blots, samples of the undialyzed lysate were separated on SDS-gels in parallel with standards of the respective redoxin and transferred to 0.22µm nitrocellulose membranes. As primary antibodies, antisera was used without prior purification, at 4000 times dilution for anti-Trx1 and 2000 times dilution for anti-Trx2 and anti-NrdH. HRP-conjugated goat anti-rabbit IgG
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was used at 3000 times dilution as secondary antibody. Blots were imaged using a ChemiDoc XRS imaging system and densitometric analysis was done using Quantity One.

**Multiple amino acid sequence alignment of redoxins:** Relevant redoxin sequences from *B. anthracis*, *B. subtilis*, *S. aureus* and *E. coli* were sampled manually. NrdH-redoxin sequences were sampled from RNRdb (24). Other sequences included in the multiple alignment were sampled by separate psiBLAST of the redoxin sequences from *B. anthracis* and from *E. coli*. Multiple sequence alignment was performed by Clustal (46), and the radial phylogram was generated in Dendroscope (47).

**RESULTS**

*The Bacillus anthracis genome encodes several potential thioredoxin reductase, thioredoxin and glutaredoxin-like proteins:* A search among the annotated genes in *Bacillus anthracis* Ames for potential thioredoxin reductases, thioredoxins and NrdH/glutaredoxins resulted in several hits with typical CXXC active site sequence motifs (Table 1). Two potential thioredoxin reductases denoted TR1 (BA5387) and TR2 (BA2768), three potential thioredoxins denoted Trx1 (BA4758), Trx2 (BA4945), and Trx3 (BA5225), two potential NrdH-redoxins denoted NrdH (BA4201) and NrdH2 (BA5371), and one hypothetical protein similar to the arsenate reductase family denoted ArsC (BA5229) were cloned, expressed and purified. Two additional potential thioredoxins (BA1779, BA0757) were predicted to be extracellular and therefore not included in this study.

The potential TR1 has a CAVC active site motif corresponding to the CATC found in *E. coli* TR (48), and the potential TR2 has a CYPC active site motif normally associated with glutaredoxins (9). Of the potential thioredoxins, only Trx1 has the archetypical CPGC active site motif (49), whereas Trx2 has an unusual CPDC active site motif previously found in *Helicobacter pylori* Trx2 (50), and Trx3 a CGTC motif also found in the potential ArsC protein. Of the potential NrdH-redoxins only NrdH has a typical CPPC active site motif (51), whereas NrdH2 has an unusual CGLC motif. The potential ArsC protein was included in this study, because it lacks other conserved features of an arsenate reductase (52), but retains the CGTC motif.

**ERF-cloning is an efficient method for parallel cloning of multiple genes:** All targets were efficiently amplified in the primary PCR-step and the transformation typically generated 50-200 colonies on each plate. A total of 21 colonies, from eight different targets, were screened by colony-PCR and 19 were positive, yielding a 90% per-colony success-rate. 13 colonies were sent for sequencing and all contained the expected sequences yielding a 100% per-target success rate. Although the experimental set is small, the figures suggest that the ERF-cloning methodology could be efficiently used in both high throughput as well as a low – medium throughput laboratories. In fact, the whole process from initial PCR to colonies on plates, can easily be finished in 24 hours while still retaining the flexibility of the original RF-cloning methodology.

**Expression and purification of targets:** The cultures typically saturated at an OD$_{600}$ of 15-25 and bacteria were generally efficiently lysed by a simple freeze-thaw treatment in hypotonic buffer. All targets were purified in adequate quantities from 50-250 ml culture. Yields ranged from approximately 7.5 mg/litre culture for NrdH2 up to 800mg/litre culture for TR2. Proteins were generally purified to >95% purity after TEV-cleavage of the His-tag and subtractive IMAC. NrdH2 resisted TEV-cleavage and reached approximately 70% purity after the IMAC. Trx2 was also inefficiently cleaved by TEV-protease, but still reached >95% purity after a single IMAC-step (Data not shown).

**Both potential thioredoxin reductases were FAD-containing proteins:** When the potential thioredoxin reductases TR1 and TR2 were expressed, the cell-pellets after harvest were green indicating expression of flavin-containing proteins. Previous investigations of other bacterial thioredoxin reductases have shown that the FAD-synthesis cannot keep up at high levels of recombinant protein expression and that this can be overcome by supplementing the cofactor upon lysis of the cells (53, 54). Therefore, this approach was used routinely for both potential TR1 and TR2. Both proteins were purified as distinctly yellow but differed significantly in their UV-vis spectra. TR1 exhibited a spectrum with absorption peaks at 383 nm and 456 nm, typical of bacterial thioredoxin reductases (43), whereas TR2 had absorption peaks at 375 nm and 447 nm respectively (Fig 1). To identify the nature of the
cofactor in TR2, we purified the protein in the presence of FAD or FMN as well as without added cofactor. All three preparations contained FAD as determined by ESI-MS, albeit at a sevenfold lower cofactor/protein ratio in the fractions without added FAD (data not shown). Reduction of TR1 with NADPH was fast and resulted in a colourless sample (Fig. 1, inset). This is in contrast to TR2 which did not react with the obligate two-electron donor NADPH even after prolonged incubation (data not shown), indicating that NADPH is not the physiological electron donor for TR2. Instead, TR2 could be reduced with DTT (Fig. 1, inset). Interestingly DTT did not reduce TR1 beyond the semiquinone state, i.e. one-electron reduced state of FAD. This reduction was slow and the neutral radical had an absorption maximum at 576 nm. Upon admittance of oxygen the reoxidation of FAD occurred immediately (Fig 1, inset).

**TR1 was an NADPH-dependent thioredoxin reductase whereas the potential TR2 was not:**

We started the screen by testing all possible combinations of thioredoxin reductases and thioredoxins/NrdH-redoxins in micro-plate format using the DTNB- and the insulin-assays. In both assays, TR1 worked as a reductase for Trx1 and NrdH, but not for Trx3, NrdH2 and ArsC. In contrast, when TR1 was used as a reductase for Trx2 it was only active in the DTNB-assay and not in the insulin assay. The potential TR2 could not work as a reductase for any of the six tested redoxins in any assay, neither in presence of NADPH nor NADH.

**Trx1 as the most efficient substrate for TR1:**

Further characterizations of Trx1, Trx2 and NrdH were conducted using the DTNB-assay and an NADPH-regenerating system (43) (Fig. 2). The $K_m$-value of TR1 for Trx1 was 8.4 $\mu$M and the $k_{cat}$ (3.5 s$^{-1}$, giving a catalytic efficiency ($k_{cat}/K_m$) of 1.6x10$^7$ M$^{-1}$s$^{-1}$ (Table 2), which was almost 5 times lower than that of the E. coli system (43). The catalytic efficiency for *B. anthracis* TR1 as a reductant of its Trx2 was approximately one third of that using Trx1, mainly due to an increase in $K_m$ (2.3-fold), but also a modest decrease in $k_{cat}$. Using *Ba*NrdH as a substrate, the catalytic efficiency was only about 5% of that using *Ba*Trx1 due to a major increase in $K_m$ (10-fold) as well as a decrease in $k_{cat}$ (2-fold). As a comparison, the $K_m$ of *E. coli* TR for its NrdH (CVQC active-site) is 2.5-fold lower (1.1 $\mu$M) and the $V_{max}$ is 89% of that for *E. coli* Trx1 (28).

The previously published data for the more closely related NrdH from *S. aureus*, does not permit a direct comparison, but the activity appears to be low (51).

**Trx1 and NrdH as the only efficient electron donors for RNR: In vivo thioredoxins are involved in a wide range of redox reactions in most organisms (5), whereas NrdH-redoxins are restricted to bacteria and seem to be exclusively involved in reduction of class Ib RNRs (12). To identify physiologically relevant electron donors for *B. anthracis* class Ib RNR, all of the six redoxins were screened in a RNR activity assay against both the manganese- and the iron-form of RNR. Electrons were supplied from DTT to avoid any potential redoxin-TR mismatch. Using Mn-RNR, the activity was increased more than eight-fold in presence of excess Trx1 and about 5-fold in presence of excess NrdH. None of the other four redoxins stimulated the DTT-induced activity of the Mn-RNR form. Using Fe-RNR, none of the redoxins showed any relevant increase in activity compared to the DTT control (Fig. 3).

**Bacillithiol was not active as an electron donor for *B. anthracis* RNR:**

The question arose whether bacillithiol (BSH) (21) could be a biological counterpart to the nonphysiological reductant DTT for reduction of class Ib RNR. Hence we tested the effect of BSH as a direct or an indirect (via any of the six redoxins) electron donor for *B. anthracis* RNR. The chosen concentration of BSH (0.5 mM) is more than double the concentration found in actively growing *B. anthracis* (21). No activity was detected with BSH alone or in combination with any of the six redoxins (Fig. 3).

**Trx1 as the most efficient electron donor for RNR:**

After the initial characterization, we studied the interaction between Trx1/NrdH and RNR by keeping the concentration of RNR constant and varying the concentrations of the redoxins, thus treating the reduced redoxins as substrates for the oxidized RNR. The specific activities of RNR in presence of Trx1 were 62.4 U/mg when electrons were supplied via DTT and 54.3 U/mg when TR/NADPH was used as ultimate electron donor system. The RNR specific activities in presence of NrdH were 43.6 U/mg (DTT) and 33.6 U/mg (TR/NADPH). Activities were thus 15% and 30% higher, respectively, when electrons were supplied via...
DTT as compared to TR1/NADPH (Fig. 4), perhaps reflecting the general oxidation-sensitivity of RNR (55, 56). In addition, a slight inhibition was seen at the highest concentration of NrdH (20 μM compared to 10 μM) with both electron donor systems (data not shown). The apparent $K_p$-values for Trx1 were found to be 0.49 μM and 0.54 μM respectively and the $k_{cat}$ 0.17 s$^{-1}$ and 0.15 s$^{-1}$ giving catalytic efficiencies of $3.5 \times 10^2$ M$^{-1}$s$^{-1}$ and $2.8 \times 10^2$ M$^{-1}$s$^{-1}$. The catalytic efficiencies for NrdH were approximately 15% of that, predominantly due to the 5- to 6-fold increase in $K_m$ (Table 3).

**Trx1 as the major disulfide reductase in extracts of B. anthracis:** To put the previous findings in a physiological context, we investigated the relative contribution of the different redoxins to the total DTNB-reducing activity in extracts from *B. anthracis* cells grown aerobically and harvested in log-phase. When lysates were pre-incubated with anti Trx1 antibodies, only 2% of the activity of the untreated control remained ($P < 0.0001$). Anti Trx1 gave no inhibition of activity with any of the other redoxins in pure form. When instead anti NrdH was used, the difference compared to the untreated control was small (7%) and statistically insignificant ($P = 0.22$) (Fig 5). The inhibition observed matched the values found when anti NrdH was used with pure Trx1. Anti Trx2 showed no neutralizing activity – neither with pure proteins nor bacterial extracts (data not shown).

To further investigate the levels of the different redoxins we developed quantitative Western-bLOTS with the pure redoxins as standards (Fig 6). In a lysate of exponentially growing *B. anthracis*, Trx1 was present at 0.71 ng/µg (± 0.06, n= 6), Trx2 at 0.049 ng/µg (± 0.008, n = 7) and NrdH at 0.012 ng/µg (± 0.007, n= 4) total protein. Since the His-tag could not be easily removed from recombinant Trx2 the standards differ from the native protein by 2.5 kDa. The native Trx2 band was identified as the only band present in the blots below the recombinant standards. Thus, in *B. anthracis* Trx1 was present at 15 and 60 times higher concentrations than Trx2 and NrdH, respectively.

**DISCUSSION**

Novel classes of antibiotics are in high demand and one of the important steps in development of new antimicrobials is the characterization of novel potential drug-targets (57). Given the lack of glutathione in many medically relevant bacteria including *M. tuberculosis*, *S. aureus* and *H. pylori* (19, 20), we wanted to take a closer look at the “redox-situation” in *B. anthracis* which also lacks glutathione but instead has bacillithiol (21). Although in *B. subtilis* Trx1 and TR are essential (13–15), it was tempting to speculate that redundancies might exist in *B. anthracis* enabling this obligate pathogen to cope with the environmental stress imposed by the host immune-system and to allow for the rapid replication seen during fulminant disease. Therefore, we combined bioinformatics with enzymological methods and measurements of proteins levels, in an attempt to characterize the flow of electrons from NADPH via thioredoxin reductase, thioredoxins/NrdH-redoxins to their terminal substrates with a special emphasis on the type Ib ribonucleotide reductase of *B. anthracis*.

Initial searches revealed that there were a large number of genes annotated as potential thioredoxin reductases, thioredoxins and NrdH-redoxins although, several of them lacked a potential active-site (CXXC) and some appeared to be extracellular. Therefore, we applied the presence of a the essential active-site motif (CXXC) and a presumed cytosolic localization as additional inclusion-criteria. We arrived with a final list of two potential thioredoxin reductases, three potential thioredoxins and three potential NrdH-redoxins which were studied experimentally. This resembles the situation in *B. subtilis*, which, on a gene-level, appears to have multiple thioredoxins and thioredoxin reductases (58).

Class Ib RNR is restricted to a few bacterial phyla - *Actinobacteria*, *Firmicutes*, and alpha- and gamma-*Proteobacteria* - and is absent from all sequenced *Archaea* and *Eukaryota* (24). The classical Ib RNR operon in *Actinobacteria*, *Proteobacteria*, and some *Firmicutes* comprises the genes *nrdE* and *nrdF* for the RNR proper, *nrdf* for the flavodoxin needed to activate the RNR, and *nrdf* for the physiological glutaredoxin-like reductant of the RNR, and the genes are generally arranged in the order *nrdf-nrdll-nrdE-nrdF* from the promoter proximal end. In contrast, *B. anthracis* and almost all other members of the *Bacillus* genus encode an *nrdI-nrdE-nrdF* operon that lacks a nearby *nrdf* gene. A glutaredoxin-like gene denoted *nrdf* (with a CPPC active site motif) is found elsewhere in the...
B. anthracis genome, and a conserved homolog of this gene is found in all Firmicutes. However, B. anthracis nrdH has low similarity (ca 30%) to the nrdH gene (with a CXQC motif) in the Firmicutes genomes that carry a classical Ib RNR operon, e.g. the Lactobacillus, Streptococcus, a few B. thuringiensis species and the B. subtilis prophage. The closest homolog to B. anthracis NrdH outside Firmicutes is a glutaredoxin in Acidobacterium capsulatum (lacking a class Ib operon) with 48% identity and a CPPC active site sequence.

A sequence similarity comparison of the B. anthracis NrdH, shows that it is strikingly different from most other NrdHs and groups with other Bacillales NrdHs (Fig. 7). The NrdHs that are encoded within a classical nrdHIEF operon form a separate clan. Similarly, B. anthracis Trx1 groups with other Trx1 proteins from related species, but is rather distant from other Trx1 proteins even though it has an archetypical CPGC active site. In contrast, B. anthracis Trx2 and similar redoxins in related species group with other potential Trx2 proteins. The Trx-like B. anthracis protein called Trx3 has no obvious counterpart among the thioredoxin/glutaredoxin proteins, and the redoxin-like protein called B. anthracis NrdH2 groups with a fraction of glutaredoxins that are rather different from the psi-clans formed with E. coli Grx1-Grx4 as input. The ArsC-like protein of B. anthracis groups together with homologous proteins from its close relatives B. subtilis and S. aureus in a distinct ArsC-like clan. All in all Figure 7 illustrates that whereas B. anthracis Trx2 groups together with other potential Trx2, both Trx1 and NrdH in B. anthracis are more distant from the archetypical redoxin classes, perhaps reflecting their specific role as electron donors for the class Ib RNR encoded in an operon that lacks an adjacent redoxin gene.

The finding that only Trx1, Trx2 and NrdH work as substrates for TR1 suggest a significantly lower complexity than anticipated from the bioinformatic investigation. We interpret the inability of Trx2 to react with insulin (a model for protein disulfides), whereas it readily reacts with DTNB (a model for low molecular weight disulfides), as a sign that it might not act as a general protein disulfide reductase in B. anthracis. Rather this result suggests that it might react with some specific, unknown substrate. In H. pylori on the other hand, the protein called Trx2 is active in both the insulin- and DTNB-assays respectively (59), despite a CPDC active-site, identical to the active-site found in B. anthracis Trx2. The major differences in catalytic efficiencies in favor of BaTrx1 compared to BaTrx2 and BaNrdH as a substrate for BaTR1, suggest that the major protein-disulfide reductase in B. anthracis is Trx1. This would hold true unless the relative protein levels of the other redoxins were significantly higher.

NrdH from B. cereus has recently been shown to stimulate the RNR activity in the manganese form of the enzyme from both B. anthracis and B. cereus (29). When we screened all the six redoxins (at 10µM) as electron-donors for Mn-RNR, only Trx1 and NrdH stimulated the activity 8- and 5-fold, respectively. When the two redoxins were treated as substrates for the oxidized RNR, the catalytic efficiency of NrdH was only about 15% of that for Trx1. When instead the Fe-RNR was used, none of the redoxins stimulated the activity, further corroborating the notion that type Ib ribonucleotide reductases are indeed manganese-containing enzymes (29, 60-63).

Earlier studies on electron-donors for other Ib RNRs have only tested the iron-loaded form. E. coli, NrdH was found to give a $K_m$ of 0.3-0.6 or 1.2-1.6 µM depending on if the electrons were supplied by either DTT or TR/NADPH, whereas thioredoxin 1 was inactive (28). Aharonowitz and colleagues found that both S. aureus NrdH and Trx1 were active as electron-donors for its type Ib ribonucleotide reductase. However, the activity was modest and no estimation of $K_m$ and $V_{max}$ values was conducted (51).

We have also considered other reducing systems for class Ib RNR in B. anthracis which lacks GSH but instead contains the recently described monothiol BSH (21). The finding that BSH is inactive both as a direct and indirect (through the six tested redoxins) electron-donor for RNR in vitro strongly suggest that BSH does not fulfill this function. Admittedly, we cannot exclude that electrons are shuttled from BSH to RNR via any unknown redoxin. However, this seems less likely given the essentiality of both Trx1 and TR in B. subtilis (13, 14).

To be able to draw accurate conclusions about the physiological roles of the different redoxins in B. anthracis, information about the relative levels...
was needed. Therefore, we used antibodies raised against the respective redoxins to neutralize the enzymatic activity in cell-lysates. This showed that almost all of the DTNB-reducing activity could be attributed to Trx1. Even though the DTNB-reducing activity does not necessarily reflect general protein-disulfide reducing activity or RNR reduction, the combination with the previous enzymatic data implies that Trx1 is indeed the predominant protein disulfide reductase and the most important electron donor for \textit{B. anthracis} RNR. This was further corroborated by the Western-blot data which showed that Trx1 was present at up to 60 and 15 times higher concentrations than Trx2 and NrdH, respectively.

In conclusion, we show that \textit{B. anthracis}, the causative agent of anthrax, has one thioredoxin reductase, two thioredoxins and one NrdH-redoxin. Catalytic parameters, enzymatic activities and relative protein-levels in cell-extracts demonstrate that the most important general disulfide reductase system in \textit{B. anthracis} is composed of TR1 and Trx1 and that Trx1 is the physiologically relevant electron-donor for RNR. The results presented herein, combined with the essentiality of the corresponding genes in related species (16, 17, 19), implies that \textit{B. anthracis} TR1 and Trx1 constitute attractive drug-targets.

ACKNOWLEDGEMENTS

Associate Professor Åke Engström, Mass Spectrometry Facility, Uppsala University, is acknowledged for his help with cofactor identification in potential TR2.

References

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Footnotes

*This investigation was supported by grants from Swedish Research Council (grant number 3529 to AH and 2978 to BMS), Swedish Cancer Society (grant number 961 to AH and 814 to BMS), K.A. Wallenberg Foundation (to AH) and Karolinska Institutet (in form of a fellowship from the MD-PhD program to TNG).

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The abbreviations used are: Trx, thioredoxin; TR, thioredoxin reductase; Grx, glutaredoxin; GR, glutathione reductase; RNR, ribonucleotide reductase; NrdE, large component of class Ib ribonucleotide reductase; NrdF, small component of class Ib ribonucleotide reductase; Fe-NrdF, iron-containing NrdF; Mn-NrdF, manganese-containing NrdF; NrdH, glutaredoxin-like reductant of class Ib ribonucleotide reductase; NrdI, flavodoxin-like activator of Mn-NrdF; BSH, reduced bacillithiol; DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid) ; IMAC, immobilized metal affinity chromatography; TEV protease, Tobacco Etch Virus protease; ERF-cloning, Exonuclease I dependent Restriction Free cloning.

Tables

Table 1. Overview of the experimental set. Listed below are genes identified in the primary bioinformatic search which encodes a potential CXXC active site and with a predicted cytosolic localization. These genes were included in the experimental part of the study. Molecular weights and extinction coefficients at 280nm (for proteins without FAD) were predicted using ProtParam.

<table>
<thead>
<tr>
<th>NCBI annotation</th>
<th>Locus tag</th>
<th>Potential active site</th>
<th>Potential gene product</th>
<th>Predicted extinction coefficient (M⁻¹ cm⁻¹)</th>
<th>Predicted MW (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioredoxin reductase</td>
<td>BA5387</td>
<td>CAVC</td>
<td>TR1</td>
<td>11300 (456 nm)</td>
<td>34669.4</td>
</tr>
<tr>
<td>Thioredoxin reductase</td>
<td>BA2768</td>
<td>CPYC</td>
<td>TR2</td>
<td>11300 (448 nm)</td>
<td>33777.2</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>BA4758</td>
<td>CGPC</td>
<td>Trx1</td>
<td>12615 (280 nm)</td>
<td>11565.3</td>
</tr>
<tr>
<td>Thioredoxin family protein</td>
<td>BA4945</td>
<td>CPDC</td>
<td>Trx2</td>
<td>14565 (280 nm)</td>
<td>15024.7</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>BA5225</td>
<td>CGTC</td>
<td>Trx3</td>
<td>16055 (280 nm)</td>
<td>11719.7</td>
</tr>
<tr>
<td>Glutaredoxin family protein</td>
<td>BA4201</td>
<td>CPDC</td>
<td>NrdH</td>
<td>7575 (280 nm)</td>
<td>9109.4</td>
</tr>
<tr>
<td>Glutaredoxin family protein</td>
<td>BA5371</td>
<td>CGLC</td>
<td>NrdH2</td>
<td>10555 (280 nm)</td>
<td>12033.7</td>
</tr>
<tr>
<td>Hypothetical protein</td>
<td>BA5229</td>
<td>CGTC</td>
<td>ArsC</td>
<td>21555 (280 nm)</td>
<td>16720.9</td>
</tr>
</tbody>
</table>

Table 2. Kinetic parameters for different redoxins as substrates for thioredoxin reductase. Kinetic parameters were determined from the Michaelis-Menten plot in Fig. 2 using nonlinear regression. Ba denotes B. anthracis and Ec denotes E. coli. *obtained from (43).

<table>
<thead>
<tr>
<th>Protein</th>
<th>k_{cat} (s⁻¹) ±SE</th>
<th>K_{m} (µM) ±SE</th>
<th>k_{cat}/K_{m} (M⁻¹s⁻¹)</th>
<th>Relative catalytic efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaTrx1 (BA4758)</td>
<td>13.5 ± 0.15</td>
<td>8.41 ± 0.32</td>
<td>1.61 x 10⁷</td>
<td>1</td>
</tr>
<tr>
<td>BaTrx2 (BA4945)</td>
<td>10.9 ± 0.24</td>
<td>19.2 ± 1.39</td>
<td>5.68 x 10⁷</td>
<td>0.35</td>
</tr>
<tr>
<td>BaNrdH (BA4201)</td>
<td>7.41 ± 0.25</td>
<td>85.5 ± 5.98</td>
<td>8.66 x 10⁷</td>
<td>0.054</td>
</tr>
<tr>
<td>EcTrx1/EcTR*</td>
<td>22</td>
<td>2.89</td>
<td>7.61 x 10⁷</td>
<td>4.7</td>
</tr>
</tbody>
</table>
Table 3. Kinetic parameters for the different RNR – redoxin combinations. Relative catalytic efficiencies compares Trx1-DTT with NrdH-DTT and Trx1-TR with NrdH-TR.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$V_{\text{max}}$ (U/mg) ± SE</th>
<th>$k_{\text{cat}}$ (s$^{-1}$) ± SE</th>
<th>$k_{\text{cat}}/K_{m}$ (M$^{-1}$s$^{-1}$)</th>
<th>Relative catalytic efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trx1-DTT</td>
<td>65.2 ± 1.1</td>
<td>0.174 ± 0.003</td>
<td>3.54 x 10$^2$</td>
<td>1</td>
</tr>
<tr>
<td>Trx1-TR</td>
<td>56.1 ± 0.86</td>
<td>0.149 ± 0.002</td>
<td>2.75 x 10$^2$</td>
<td>1</td>
</tr>
<tr>
<td>NrdH-DTT</td>
<td>56.4 ± 2.4</td>
<td>0.150 ± 0.006</td>
<td>5.60 x 10$^1$</td>
<td>0.16</td>
</tr>
<tr>
<td>NrdH-TR</td>
<td>44.7 ± 1.6</td>
<td>0.119 ± 0.004</td>
<td>3.64 x 10$^1$</td>
<td>0.13</td>
</tr>
</tbody>
</table>
Bacillus anthracis thioredoxin systems

Figure legends

Figure 1. UV-vis absorption spectra of TR1 and TR2. In the oxidized spectrum (large picture), TR1 had absorption peaks at 383 and 456 nm, typical for bacterial thioredoxin reductases, whereas TR2 had peaks at 375 and 448 nm respectively. The inset shows the spectra after reduction of the thioredoxin reductases with 10 times excess of the reductant NADPH and 25 and 12.5 times DTT for TR1 and TR2, respectively. The spectrum with TR1 and NADPH is cut at 390 nm for clarity due to the excess of NADPH. DTT did not reduce TR1 beyond the semiquinone form whereas TR2 could be two-electron reduced. The concentration of the TR peptides was approximately 10 µM.

Figure 2. Michaelis-Menten plot of B. anthracis redoxins as substrates for TR1. Thioredoxin reductase was kept constant at 15 nM whereas redoxin concentrations were varied in the range 2-100 µM for Trx1, 5-150 µM for Trx2 and 5-200 µM for NrdH using the DTNB-assay. Each point represents the mean of two experiments with SD indicated by error bars. Curves were fitted using nonlinear regression.

Figure 3. Screening of different redoxins as electron-donors for RNR. All six redoxins were screened as electron donors for B. anthracis RNR using CDP as a substrate. The redoxins were screened against both the manganese- (Mn-NrdF) and the iron- (Fe-NrdF) form of NrdF. Electrons were supplied by either 1 mM DTT or 0.5 mM BSH. The group Ctrl represents the control with terminal electron donor (DTT or BSH), without added redoxin. Activities are expressed as relative activities compared to Mn-NrdF + DTT + Trx1 and are plotted as the mean of duplicates.

Figure 4. Michaelis-Menten plot of RNR activity as a function of Trx1 and NrdH concentration in different reducing systems. Concentrations of redoxins were varied in the range 0-20 µM for Trx1 and 0-10 µM for NrdH. Specific activities with DTT or TR1 and NADPH are expressed as U/mg NrdE. The activity of RNR with 1mM DTT alone has been subtracted from the Trx1-DTT and NrdH-DTT series. Points represent the mean of duplicates with SD indicated by error bars and curves were fitted using nonlinear regression.

Figure 5. Neutralizing activity of antibodies against redoxins in dialysed extracts of exponentially growing B. anthracis. Dialyzed lysates of B. anthracis Sterne 7700 was incubated with rabbit antibodies raised against the respective pure proteins. After incubation, activity was measured using the DTNB-assay. Activity is expressed as the fractional activity of the untreated control and is plotted as the mean of quadruplicates with SEM indicated by error bars.

Figure 6. Western blot of Trx1, Trx2 and NrdH in lysates of exponentially growing B. anthracis. Cleared lysates were separated alongside with pure protein standards and were subjected to Western-blotting followed by densitometric analysis. The amount of protein is given below each lane and is expressed as µg total protein for the lysate samples and ng protein for the pure protein standards. In the case of Trx2, the native protein is expected to be 2.5 kDa smaller than the 6xHis-TEV-Trx2 standard and is identified as the only band present below the recombinant standard. The unspecific band is present at a position suggesting a size of approximately 1 kDa larger than the recombinant Trx2. Trx1 was present at 0.71(±0.06) ng/µg, Trx2 at 0.049(±0.008) ng/µg and NrdH at 0.012(±0.007) ng/µg total protein.
**Figure 7.** Sequence similarity comparison between selected members of the thioredoxin/glutaredoxin protein family. The *B. anthracis* thioredoxin/glutaredoxin members listed in Table 1 and the *E. coli* proteins Trx1-Trx2 plus Grx1-Grx4 were each subjected to psi-Blast, and the 200 top candidates after 6-12 iterations were included in the comparison. In addition all NrdH proteins listed in RNRdb were included prior to a ClustalX run with 1700 individual entries (after manual deletion of duplicates). Thioredxins (blue colors), glutaredoxins (green colors), NrdH redoxins (red), ArsC proteins (gray). Sequences that are specified are also marked in black; characterized proteins with redoxin function (bold); homologous sequences in related species (bold italics), characterized proteins without redoxin function (plain), homologous sequences in related species (italics)
Figures

Figure 1
Figure 2

![Graph showing the velocity of different redoxin proteins as a function of redoxin concentration.](image-url)
Figure 3
Figure 4

![Graph showing specific activity vs. [Redoxin] (μM) for various conditions. The graph includes lines for Trx1-DTT, Trx1-TR, NrdH-DTT, and NrdH-TR.]
Figure 5

No antibody  Anti Trx1  Anti NrdH

Relative activity

P = 0.22
P < 0.0001
Figure 6

Bacillus anthracis thioredoxin systems

Lysate Standards Lysate

Trx1

22µg 22µg 2.5ng 5ng 7.5ng 10ng 15ng 20ng 22µg

Trx2

44µg 44µg 0.4ng 0.8ng 1.6ng 2.4ng 4ng 6ng 44µg

NrdH

66µg 66µg 0.3ng 0.5ng 1ng 1.5ng 2ng 3ng 66µg

Unspecific
Recombinant
Native
Figure 7
Bacillus anthracis thioredoxin systems - characterization and role as electron donors for ribonucleotide reductase.
Tomas N. Gustafsson, Margareta Sahlin, Jun Lu, Britt-Marie Sjoberg and Arne Holmgren

J. Biol. Chem. published online September 25, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.413427

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