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**RESEARCH LETTER**

**Mycobacteriophage L5Gp56, a novel member of the NrdH family of redoxins**

Prithwiraj Kirtania, Bidisha Bhattacharya & Sujoy K. Das Gupta

Department of Microbiology, Bose Institute, Kolkata, India

**Correspondence:** Sujoy K. Das Gupta, Bose Institute, Department of Microbiology, P1/12 C.I.T. Scheme VIIIM, Kolkata 700054, India. Tel.: +91 33 23559416; fax: +91 33 2553886; e-mail: sujoy@jcbose.ac.in

**Present address:** Bidisha Bhattacharya, NEIDL, Boston University, 620 Albany Street, Boston, MA 02118, USA

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**Abstract**

Mycobacteriophage L5 gene 56 encodes a putative thioredoxin family protein. Phylogenetic analysis revealed that Gp56 and related proteins are distantly related to NrdH—a glutaredoxin homolog which has thioredoxin-like properties. To understand its function, the recombinant version of the protein was biochemically characterized. For the sake of comparison, a mycobacterial thioredoxin, TrxB, was included in the study. Results show that Gp56 can be reduced by dithiothreitol, but only at a higher concentration as compared with TrxB, indicating that the standard redox potential of Gp56 is lower than that of TrxB. The reduced protein can subsequently act as a reductant of protein disulfide bonds. Gp56 can be reduced by NADPH with the help of thioredoxin reductase (TrxR) but less efficiently as compared with TrxB. The abilities of Gp56 and TrxB to reduce Gp50, the L5-encoded ribonucleotide reductase, was examined. While both are capable of executing this function, the former needs more reducing equivalents in the process as compared with the latter. This study shows that L5Gp56 represents a new class of NrdH-like proteins that function optimally in a reducing environment.

**Introduction**

Thioredoxins constitute a family of proteins that function as protein disulfide reductants (Holmgren, 1985). These proteins can catalyse oxidoreductase reactions by a thiol disulfide exchange mechanism involving two redox active cysteines separated by two amino acids (−CXXC−) (Chivers et al., 1997). The characteristic feature of thioredoxins is the presence of a structural motif known as thioredoxin fold (Martin, 1995). This fold is present in five classes of proteins: thioredoxin (Trx), glutaredoxin (Grx), NrdH, glutathione peroxidase (GP) and DshA. Of these, the first three function as reductants whereas the last, DshA, functions as an oxidant. Whether the protein will function as a reductant or an oxidant depends to a large extent on the sequence of the two amino acids within the −CXXC− motif. Just by changing these it is possible to alter the redox potential of the proteins and for this reason the −CXXC− sequence is sometimes referred to as the redox rheostat (Chivers et al., 1997).

Mycobacteriophages have been used extensively as ‘tool-boxes’ for the genetic engineering of mycobacteria (Hatfull & Sarkis, 1993). Over the years, the genomes of a large number of these phages have been sequenced. Based on sequence homologies, mycobacteriophages have been grouped into several clusters designated as A–I (Hatfull et al., 2010). Within each cluster, there may be subclusters. Phages that belong to subcluster A2, which includes L5 and D29, possess several genes encoding enzymes involved in the generation of deoxyribonucleotide precursors for DNA synthesis. These are a ribonucleotide reductase class II (RNRII) (Gp50), a thymidylate synthase X or ThyX (Gp48) and a putative dCMP deaminase (Gp56.1). In a previous investigation, this laboratory has characterized Gp50 and 48 (Bhattacharya et al., 2008). Evidence indicated that these proteins were synthesized following phage infection, and that, as in the case of phage T4 (Wheeler et al., 1996), they functioned in close association with each other. A fundamental requirement for ribonucleotide reduction is the availability of the reducing power which is supplied by thioredoxins.
Thioredoxin reductase regenerates the reduced form of thioredoxin using NADPH as the source of redox equivalents. Mycobacteriophage L5 encodes a putative NrdH class protein (Gp56) and also an RNRII (Gp50). Therefore, it was hypothesized that the former may assist the latter in bringing about ribonucleotide reduction. To examine this possibility gene 56 was overexpressed in *Escherichia coli*. Following purification, the recombinant protein was biochemically characterized. It was found that the protein could function as a reductant for the phage-encoded RNRII provided the environment was sufficiently reducing.

Materials and methods

Plasmids and bacterial strains

Cloning and overexpression of genes was done using *E. coli* strains, either XL1Blue or BL 21(DE3). A pQE30- (Qiagen) based recombinant plasmid expressing mycobacteriophage L5 gene 56 was constructed in this study, whereas a similar construct expressing gene 50 was done earlier (Bhattacharya et al., 2008). Recombinant plasmids pSCM1102 and pSC1105, based on pET23A, that express the genes for thioredoxin B (TrxB) and thioredoxin (Gp50), respectively, were a gift of Dr S. C. Mande [Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad, India] (Akif et al., 2008).

Gene cloning, expression and affinity purification of recombinant proteins

The gene for mycobacteriophage L5Gp56 was amplified using the forward primer 56FP (GGGGATCCATGTTAGGG AGACCGATA) and reverse primer 56RP (CCCAAGCTT CATATGTGACGCATA) having sites for the restriction enzymes BamHI and HindIII (underlined), respectively. The PCR amplicon was cloned in the expression vector pQE30. Following transformation into *E. coli* XL1, blue gene expression was induced by the addition of isopropyl β-D–1-thiogalactopyranoside (IPTG). The hexahistidine-tagged recombinant proteins were then isolated by Ni-NTA affinity chromatography as described by Bhattacharya et al. (2008).

Phylogenetic analysis and homology modelling

Homology models were created using the online server I-TASSER (Roy et al., 2010). The PDB structure that gave best results was used as a reference for determining the secondary structures. CLUSTAL W-based alignments of multiple sequences were performed by using MEGA 4.0 (Tamura et al., 2007). Pairwise and multiple-alignment penalties of 10 and 0.1 were used for gap opening and extension, respectively, or as stated. The weight matrices chosen were either PAM or BLOSUM. Phylogenetic trees were constructed by using these alignments with MEGA 4.0. The secondary structure features were incorporated into the final alignment by using the online software ESPRIT 3.0 (Gouet et al., 1999).

Insulin aggregation assay

The assays were performed essentially as described by Holmgren (1979). The assay mixture (100 μL), contained 39 μL of insulin (from 2 mg mL\(^{-1}\) stock), 2 mM EDTA, and either Gp56 or TrxB at a final concentration of 10 μM in 100 mM potassium phosphate buffer pH 7.2. Aggregation induced by the addition of dithiothreitol (2 mM or as mentioned) was monitored spectrophotometrically by measuring the optical density at 650 nm at regular intervals.

Thioredoxin reductase assays

NADPH-dependent reduction of Gp56 and TrxB was assayed by using the reagent 5-5 dithiobis [nitrobenzoic acid] (DTNB). The assay mixture contained 1 mM DTNB, 2 mM EDTA, 0.5 mM NADPH, 50 μM either Gp50 or TrxB, and 100 mM potassium phosphate buffer, pH 7.2. The reaction was started by the addition of TrxR at a final concentration of 10 μM. DTNB reduction was monitored by measuring the optical density at 412 nm (OD\(_{412}\) nm).

Ribonucleotide reductase assay

This assay was performed as previously described in an earlier study (Blakley, 1966; Bhattacharya et al., 2008). The reaction mixture (50 μL) contained 1 mM ATP, 10 μM 5‘-deoxyadenosylcobalamine (coenzyme B\(_{12}\)), 1 mM EDTA, 50 mM potassium phosphate buffer, pH 7.2, 5 μM Gp50, 2 mM dithiothreitol (or as stated) and finally Gp56 or TrxB at the indicated concentrations. The reaction mixture was incubated for 1 h at 37 °C followed by treatment with 2-chloroacetamide (0.4 M) and boiling for 10–15 min. Subsequently, diphenylamine (four volumes of the original reaction mixture) was added and kept at 37 °C for 4 h for colour development. Conversion of ATP to dATP was monitored by recording the optical density at 595 nm (OD\(_{595}\) nm).

Reduction of proteins by dithiothreitol

A gel electrophoresis-based assay (Go & Jones, 2009) was performed to investigate the extent to which dithiothreitol
reduces either Gp56 or TrxB. Purified Gp56 and TrxB were treated with different concentrations of dithiothreitol (0–1 mM). Briefly, the proteins (20 μM, final concentration) were incubated with increasing concentrations of dithiothreitol at room temperature in 100 mM phosphate buffer for 20 min. Then, 15 μL of 15 mM 4-acetoamido-4′-melleimidylstilbene-2,2′-disulfonic acid (AMS) was then added and kept for 3 h in the dark at room temperature for stabilization of the thiols formed after dithiothreitol treatment. The degree of reduction was measured by analysing the proteins in a 15% nonreducing polyacrylamide gel electrophoresis (PAGE). The AMS-treated oxidized and reduced forms of the proteins have different mobilities. For the sake of control, the protein was either completely oxidized using 10 mM H2O2 or completely reduced using 10 mM dithiothreitol. Coomassie blue-stained band intensities were measured in a Versa Doc imaging system (BioRad).

Results

Phylogenetic analysis of Gp56

Homology modeling indicated that the protein was structurally related to the NrdH from Bacillus cereus (Rohr et al., 2013) the crystal structure of which is available in PDB (3ZIJ). Comparison of the secondary structures predicted from the homology model indicated that overall the thioredoxin fold (Martin, 1995) was conserved in these proteins (Fig. 1a). However, the interesting feature

![Fig. 1.](image-url)
is that the sequence of the redox rheostat (CXXC) of Gp56 (CKPC) was different from that of *E. coli* NrdH (CVQC). The sequence homology analysis therefore suggests that although Gp56 is a part of the NrdH family, it may have unique redox properties. Phylogenetic analysis (Fig. 1b) revealed that NrdH proteins can be divided into three groups, the first of which is represented by the *E. coli* NrdH, an ortholog of which is also present in *M. tuberculosis*. The second group comprises NrdH sequences derived from mycobacteriophages of the A2 and A9 subclusters while the third identified earlier (Rabinovitch *et al.*, 2010) comprises sequences from *B. cereus* and *Staphylococcus aureus*. Interestingly, subcluster A2 and A9 phages also possess genes encoding an RNR class II (L5Gp50) and a thymidylate synthase of the X type (L5Gp48) (www.phagesdb.org). This observation indicates that the three A2/A9 family proteins (Gp50, Gp56 and Gp48) are functionally related.

In NrdH class proteins, the \( \beta_4 \)-loop-\( \alpha_3 \) segment and the motif \( 61\text{WSGFRP(D/E)}67 \) located in this region are highly conserved (Jordan *et al.*, 1997). Homology modeling predicts that in L5Gp56, this secondary structure element is different (Fig. 2). The \( \beta_4 \) region is unstructured and, as a result, the loop extends from \( \beta_3 \) directly to \( \alpha_3 \) (Fig. 2c). The conserved motif \( 61\text{WSGFRP(D/E)}67 \) (Stehr *et al.*, 2001; Phulera & Mande, 2013), which is responsible for forming an intricate network of hydrogen bonds, is also absent. The C-terminal region of L5 Gp56 is therefore likely to be more flexible as compared with its bacterial counterparts. In contrast to L5 Gp56, the Wildcat and cluster E-derived phage NrdH proteins possess this conserved sequence motif (Fig. 1a).

**Insulin aggregation assay to determine the reducing capacity of L5Gp56**

To estimate the ability of Gp56 to perform as a reductant, insulin aggregation assays were performed (Holmgren, 1979). Gp56 and the mycobacterial thioredoxin, TrxB (Akif *et al.*, 2008), were purified to near homogeneity (Fig. 3a) and the resulting protein samples were added to the reaction mixture containing dithiothreitol (2 mM) and insulin. Insulin aggregation was then monitored spectrophotometrically. The results (Fig. 3b) indicate that whereas TrxB reduced insulin in the presence of 2 mM dithiothreitol rapidly, Gp56 failed to do so. However, when the dithiothreitol concentration was raised to 7.5 mM by the addition of additional amounts of dithiothreitol, Gp56 performed on a par with TrxB. At this high concentration of dithiothreitol, aggregation in the absence of protein was also observed, but this happened only after a further lag period. The results suggest that the reduction of Gp56 requires a higher concentration of dithiothreitol in comparison with TrxB.

**Reduction of Gp56 with NADPH**

NrdH proteins are known to be related to glutaredoxins, but their function is similar to thioredoxin (Jordan *et al.*, 1997). These proteins can be reduced by thioredoxin reductases. To examine whether thioredoxin reductase can reduce Gp56, an assay was carried out in which either TrxB or Gp56 was subjected to reduction by TrxR using NADPH as the source of reducing equivalents. The extent of reduction of the redox proteins was monitored using DTNB. The results (Fig. 4) show that the time kinetics of reduction of TrxB and Gp56 were similar although the maximum level of reduction obtained was less for Gp56 as compared with TrxB. The results indicate that TrxR can reduce Gp56 although the efficiency was lower compared with when thioredoxin was used.

**Dithiothreitol titration to determine differences in redox potential between TrxB and NrdH**

In the case of thioredoxin, reduction can be monitored biophysically using fluorescence-based methods. A

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**Fig. 2.** Comparison of the predicted model for Gp56 (c) with the crystal structures of the NrdH proteins of either *Bacillus cereus* (3ZIJ) (a) or *Escherichia coli* (1H75) (b). The Gp56 model was derived using the crystal structure 3ZIJ as template. The \( Q \) mean score of the model thus derived was 0.2. The secondary structure elements \( \beta_4 \) and \( \alpha_3 \) are indicated. The unstructured region in Gp56 corresponding to the \( \beta_4 \) region is indicated with an interrogation mark.
tryptophanyl residue close to the redox rheostat is highly sensitive to the redox status of the protein (Holmgren, 1972). However, in case of Gp56 no such redox-sensitive fluorophore exists. Hence another strategy had to be designed to monitor the redox state of the protein in a given condition. AMS is a reagent that can stabilize thiol groups, resulting in an increase in the molecular weight by 500 Da per thiol group. To examine whether AMS treatment can differentiate the oxidized and reduced forms of Gp56, this protein and TrxB were either fully oxidized (using H₂O₂) or reduced (using dithiothreitol) and then reacted with AMS. The modified proteins thus obtained were analysed using 15% PAGE. The results (Fig. 5, boxed region) show that the AMS-treated oxidized and reduced forms migrate differently in the gel. As expected the completely reduced form in which both the thiols apparently became modified migrated more slowly due to an increase in the molecular mass as compared with the oxidized forms. Dithiothreitol titrations were then performed using Gp56 and TrxB (Fig. 5). The results show that the intensity of the band corresponding to the reduced protein increased in a dose-dependent manner. Apart from the major band corresponding to the fully reduced protein, a minor band having intermediate mobility was also observed. This band possibly represents a partially modified protein in which one of the two thiols had reacted with AMS. The reduction profiles for TrxB and Gp56 were, however, different. The concentration of dithiothreitol necessary to reduce Gp56 was greater as compared with that of TrxB. Hence, Gp56 has a lower standard reduction potential compared with TrxB.

Ribonucleotide reduction by Gp56

The central objective of this study was to investigate whether Gp56 can supply reducing equivalents to the phage-encoded ribonucleotide reductase. To examine this, ribonucleotide reduction assays were performed using Gp50 and either Gp56 or TrxB, in the presence of varying

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![Fig. 3. (a) 10% Sodium dodecyl sulfate PAGE analysis of purified proteins, either TrxB or L5Gp56. Two different quantities were loaded (1× or 2×). The sizes of the proteins in kDa are indicated on the left. (b) Dithiothreitol-induced Insulin aggregation kinetics, in the absence (open circles) or presence of either thioredoxin (black diamond) or Gp56 (black circles). Reactions were initiated with 2 mM dithiothreitol. Turbidity was monitored at 650 nm. At the point indicated by an arrow an additional amount of dithiothreitol was added (final concentration 7.5 mM) to the Gp56- and dithiothreitol-only assay tubes.](image)

![Fig. 4. Reduction of TrxB (black diamond) and Gp56 (black circle) by TrxR using NADPH as donor of reducing equivalents. The extent of reduction was determined by using the reagent DTNB. Traces marked by open circles represent DTNB assay performed in the absence of redoxins. The reaction scheme is shown above.](image)

![Fig. 5. Redox titration of TrxB and Gp56. Increasing amounts of dithiothreitol were added to achieve the final concentrations as indicated at the top. The reduced and oxidized forms after AMS stabilization were separated by 15% non-reducing PAGE. For the sake of controls, the proteins were treated with either 10 mM H₂O₂ or dithiothreitol for either complete oxidation (Ox) or reduction (Red), respectively. The resulting proteins were run alongside the experimental samples (boxed region of the gel).](image)
concentrations of dithiothreitol. The results indicate that both TrxB and Gp56 could function as the redox partner for Gp50 (Fig. 6a and b). However, the optimum concentrations differed, as is evident when the background activity due to dithiothreitol alone was subtracted (Fig. 6c). The optimum dithiothreitol concentration for TrxB was 2 mM, whereas for Gp56 it was 7.5 mM.

**Discussion**

Gp56 of mycobacteriophage L5 (Hatfull & Sarkis, 1993), a well-studied mycobacteriophage representing the A2 family, has been investigated phylogenetically as well as biochemically. Sequence comparisons with other NrdH family proteins indicate that the mycobacteriophage proteins constitute a distinct clade within the family. In a previous study (Rabinovitch et al., 2010) the NrdH family was divided into two phylogenetic groups represented by the *E. coli* and *S. aureus* proteins, respectively. In this study, it is proposed to introduce a third group comprising the NrdH proteins of A2 and A9 mycobacteriophages. NrdHs from mycobacteriophages belonging to other clusters, however, do not belong to this group, an example being the one derived from mycobacteriophage Wildcat that is clearly more related to the *E. coli* protein rather than those from A2/A9 phages.

An important feature of the mycobacteriophage NrdH proteins examined here is that in most of them the redox rheostat is CKPC, which is different from that of the *E. coli* NrdH (CVQC). Interestingly even in the cluster E-derived NrdH sequences, which are very similar to the *E. coli* protein, the rheostat sequence is CKPC and not CVQC. Hence for some unknown reason the CKPC sequence is preferred in the case of mycobacteriophage NrdH. Homology modeling confirms that the thioredoxin fold is also present in L5 Gp50. The basic structure is similar to NrdH of *E. coli* (Stehr et al., 2001) and *M. tuberculosis* (Phulera & Mande, 2013). However, the C-terminal region of L5 NrdH encompassing the β4-loop-α3 domain appears to be structurally different.

The results presented in this study show that the phage-encoded RNRII can use both Gp56 and TrxB as reductants, but more dithiothreitol was found to be required in the case of Gp56 as compared with TrxB. The inability of dithiothreitol to reduce Gp56 efficiently indicates that at equilibrium the oxidized form is more preferred as compared with the reduced form. Gp56 is therefore likely to be more reducing as it tends to be present more in the oxidized form than the reduced in comparison with TrxB.

Why mycobacteriophage L5 should encode a thioredoxin class protein (Gp56) when a similar one (TrxB) is synthesized by the host is an intriguing question. The phage-encoded protein may be needed because following infection by mycobacteriophages L5/D29 host protein synthesis stops, (Hatfull & Sarkis, 1993) and thus essential enzymes including TrxB are likely to become limiting. Such a shift from host to phage enzymes has been demonstrated for thymidylate synthase (Bhattacharya et al., 2008). Another intriguing question is why Gp56 has low redox potential, and if so how does it help. To answer this question it is necessary to consider that in the environment mycobacteria encounter microaerobic conditions and that various *Mycobacterium* species have
developed elaborate systems to survive under such conditions (Boon & Dick, 2012). Mycobacteriophages grow on mycobacteria and therefore they too must have developed similar systems. The fact that L5 and related mycobacteriophage encode a class II RNR (Gp50) that is known to function in the absence of oxygen (Fontecave, 1998, Bhattacharya et al., 2008) supports such a possibility. Gp56, the likely reducing partner for Gp50, thus may have evolved to function under low redox conditions. The observation made in this study that Gp56 assists Gp50 as efficiently as TrxB or perhaps better at higher concentration of dithiothreitol indicates that such an explanation is tenable. Further studies are, however, needed to unravel the precise role of Gp56. One can always argue that many of the phage proteins may have had some function in the past and that their present existence is vestigial in nature.

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References


