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# Identification and Functional Characterization of Thioredoxin of *Mycobacterium tuberculosis*

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**We have previously described a *Mycobacterium tuberculosis* protein designated MPT46 that was present in culture filtrates. Here we report that the MPT46 protein is thioredoxin of *M. tuberculosis*. MPT46 is recognized by antibodies to thioredoxin (Trx) of *Escherichia coli*, and antibodies to MPT46 recognize *Mycobacterium leprae* Trx. Moreover, MPT46 was shown to have enzymatic activity identical to that of Trx of other species, such as its ability to reduce insulin. These findings identify MPT46 as a functionally active Trx.**

*Mycobacterium tuberculosis* is a major cause of mortality worldwide. The high incidence of tuberculosis in AIDS patients has led to increased spread of tuberculosis. Several new *M. tuberculosis* strains have appeared to be multidrug resistant (4, 10). These facts point to the need for new and better methods for control of tuberculosis which require more knowledge about pathogenic mechanisms of *M. tuberculosis.*

One important issue is the question of how this acid-fast bacterium can survive inside mononuclear phagocytes where it has to deal with the triggered oxidative burst and the hydrolase-rich acidic environment within the endosomal-lysosomal compartment. It has been shown that the mycobacterium-containing vacuoles are less acidic than neighboring lysosomes. A lack of proton-ATPase subunits was found that could explain the lack of acidification in these vacuolar compartments (11, 15).

In addition to inhibiting phagolysosome fusion and lysosome acidification, *M. tuberculosis* may protect itself against superoxide radicals by secreting superoxide dismutase, an enzyme that inactivates toxic radicals generated by activated macrophages (16). A protein with similar scavenging activity is thioredoxin, a 12-kDa protein present in all prokaryotic and eukaryotic cells. Trx forms together with thioredoxin reductase and NADPH a redox active system which donates electrons to a wide variety of different metabolic processes (2). In addition, the reduced form is a general protein disulfide reductant which can reactivate proteins that have been oxidized by $H_2O_2$ (3). Trx in eukaryotic cells has been shown to reduce reactive oxygen species, thereby protecting the cell against oxidative stress (8). In this study we describe the identification and functional characterization of Trx of *M. tuberculosis.*

Previously, an antiserum, K23, was raised against a 5-week-old culture filtrate of *M. tuberculosis* that displayed no significant lysis. A number of individual proteins present in this culture filtrate were subsequently purified and their molecular masses and N-terminal amino acid sequences were reported for identification purposes (9). We have shown previously that a recombinant *Mycobacterium leprae* clone, designated L2, was recognized by K23 (13). This clone was subsequently shown to encode Trx and thioredoxin reductase of *M. leprae* (14). In this same study, the N-terminal part of the gene coding for Trx of *M. tuberculosis* was sequenced. Interestingly, the deduced amino acid sequence of the *M. tuberculosis* Trx gene was found to be highly homologous to the five N-terminal amino acids of MPT46 (RDSEK), one of the proteins present in the *M. tuberculosis* culture fluid (9). We therefore determined the first 20 N-terminal amino acids of MPT46 by automatic Edman degradation with an Applied Biosystems 477A gas-phase sequencer and an on-line 120A phenylthiohydantoin amino acid analyzer. The analysis showed that the sequence is indeed almost identical to the deduced amino acid sequence of the N terminus of Trx of *M. tuberculosis* (14) (Fig. 1). The first amino acid as reported by Nagai et al. (9) was found to be an R, but after rechecking the sequence, this amino acid appeared to be in fact a T. The MPT46 and *M. tuberculosis* Trx sequences differ in a single amino acid in position 13, where in MPT46 a glycine was found and in the DNA sequence a triplet coding for aspartic acid was found. This difference is probably due to an amino acid sequencing error. It is most likely an aspartic acid because all mycobacterial strains sequenced in our previous study (14) contain an aspartic acid at position 13.

Antiserum raised against MPT46 (obtained by a standard immunization procedure [1]) and antiserum to Trx of *Escherichia coli* (a gift of A. Holmgren, Karolinska Institute, Stockholm, Sweden) were used for immunoblot analysis of MPT46, recombinant Trx of *M. leprae*, and Trx of *E. coli*. Protein samples (0.2 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (5). The proteins were transferred to polyscreen membranes (NEN Research Products, Boston, Mass.) by using a Bio-Rad mini transblot apparatus according to the recommendations of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Membranes were incubated with antiserum for 1 h and washed twice for 15 min each time with phosphate-buffered sodium chloride containing 1% Tween 20 (PBS-Tween). After 30 min of incubation with a horseradish peroxidase-labelled secondary antibody, the blots were washed again twice for 15 min each time with PBS-Tween. The blots were developed with a solution consisting of PBS, 0.03% $H_2O_2$, 40 μg of imidazole per ml, and 40 μg of 3,3′-diaminobenzidine tetrahydrochloride dihydrate per ml. Trx of *M. leprae*, which is

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| | |
|---|---|
| MPT46 | T D S E K S A T I K V T G A S F A T x V |
| TUB Trx | T D S E K S A T I K V T D A S F A T D V |

FIG. 1. N-terminal amino acid sequences of MPT46 and deduced amino acid sequence of thioredoxin of *M. tuberculosis* (TUB Trx).

used in the immunoblot analysis as a control mycobacterial Trx, was expressed as a fusion protein containing six histidine residues plus another 24-amino-acid linker attached to its N terminus (pTrcHIS vector; Invitrogen Corp.). Expression in *E. coli* was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. After culturing the bacteria overnight at 20°C, they were centrifuged and lysed by sonication under nondenaturing conditions. The proteins were purified by using a nickel-chelate affinity resin according to the recommendations of the supplier (Qiagen, Chatsworth, Calif.). The purified fraction was dialyzed against 50 mM potassium phosphate buffer, pH 7.0. Endogenous Trx of *E. coli* was a kind gift of A. Holmgren.

The immunoblot analysis showed that antiserum to the *E. coli* Trx indeed recognized MPT46 as well as the control *M. leprae* Trx. Thus, this antiserum cross-reacts with determinants on Trx molecules of several species. The anti-MPT46 antiserum recognizes *M. leprae* Trx and MPT46 but not *E. coli* Trx and therefore recognizes determinants unique to the mycobacterial Trx. This latter feature is useful in distinguishing endogenous *E. coli* Trx from the mycobacterial Trx protein when recombinant mycobacterial Trx proteins are overproduced in *E. coli.*

The Coomassie blue-stained gel and also the immunoblot (Fig. 2) show that the mycobacterial Trx proteins have a slightly higher molecular mass than *E. coli* Trx. The molecular weights of *E. coli* and *M. leprae* Trx, calculated from the deduced amino acid sequences, are 11,675 and 12,530, respectively. The molecular mass of MPT46 determined by SDS-PAGE as described by Nagai et al. (9) was 14 kDa. By mass spectrometry, the molecular mass of MPT46 was shown to be 12,397 Da (with a deviation of 0.1%), which is close to the molecular mass of the *M. leprae* Trx.

To investigate whether the MPT46 is functionally active, we performed an insulin reduction assay as previously described (7). In the insulin reduction assay, the rate of conversion of NADPH to $NADP^+$ is measured by the decrease in $A_{340}$. In this process, electrons from NADPH are transferred to thioredoxin reductase, which reduces Trx; the reduced form of Trx in turn reduces insulin, thereby separating the A and B chains. For this assay, we used recombinant thioredoxin reductase of

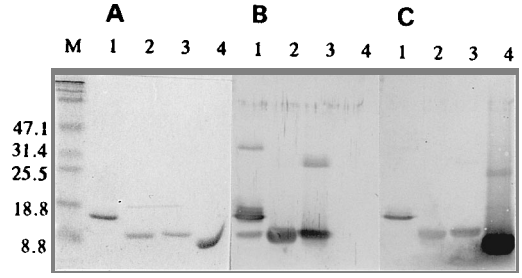


FIG. 2. SDS-PAGE followed by Coomassie blue staining (A) or immunoblotting with antiserum to *E. coli* Trx (B) or antiserum to MPT46 (C). Lanes 1, recombinant *M. leprae* Trx containing the His tag; lanes 2, recombinant *M. leprae* Trx cleaved with enterokinase to remove the His tag; lanes 3, MPT46; lanes 4, *E. coli* Trx; lane M, molecular mass standards. Molecular masses (in kilodaltons) are indicated to the left of the gel.

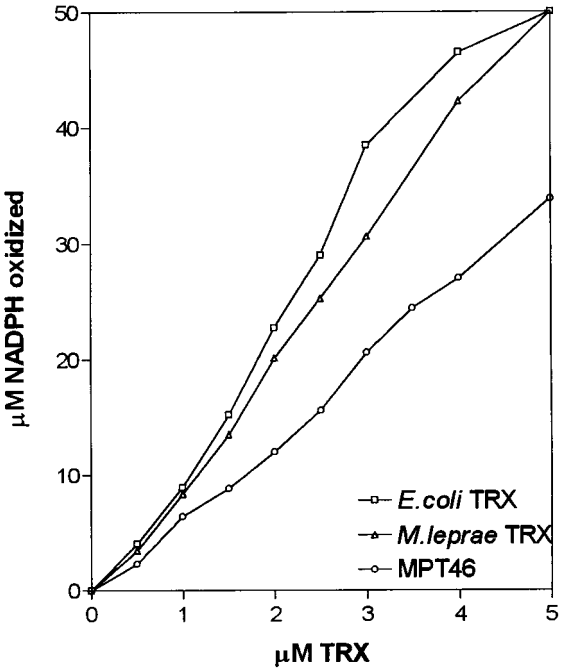


FIG. 3. Insulin reduction assay results. Equimolar concentrations of *M. leprae* thioredoxin reductase (0.5 to 5 μM) are used in combination with thioredoxin from *E. coli* or *M. leprae* or MPT46 in a total volume of 0.5 ml. The decrease in $A_{340}$ was monitored over time, and the amount of NADPH oxidized was calculated with a molar extinction coefficient of 6,200 $M^{-1}$ $cm^{-1}$.

*M. leprae* (isolated by the same procedure as that described for *M. leprae* Trx), since the *E. coli* thioredoxin reductase has a narrow substrate specificity and does not accept mycobacterial Trx. The *M. leprae* reductase was used in different concentrations together with MPT46 or Trx of *E. coli* or its own substrate, *M. leprae* Trx. All three proteins were shown to have similar activities, although the redox activities of *E. coli* and *M. leprae* were somewhat higher than that of MPT46 (Fig. 3). These results demonstrate that our purified MPT46 is a functionally active Trx and possesses redox activity.

In conclusion, the N-terminal sequence identity, the serological recognition, and the enzymatic analysis of MPT46 show that this protein is the functionally active thioredoxin of *M. tuberculosis*. The fact that MPT46 is present in the culture filtrate might suggest that this protein is secreted. However, in an extensive study describing a localization index for distinguishing between extracellular and intracellular antigens of *M. tuberculosis*, MPT46 could not unequivocally be categorized as either an intracellular or extracellular antigen (12). In *E. coli*, thioredoxin is located at the adhesion sites and can be released from the bacterium upon osmotic shock (6). In mammalian cells, thioredoxin is secreted (3). If the *M. tuberculosis* thioredoxin is indeed a secreted protein, it can easily exert its free radical scavenging activity inside the macrophage. Inside the mycobacterium, Trx can regenerate proteins damaged by oxidative stress.

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