Purification and Functional Analysis of the Mycobacterium leprae Thioredoxin/Thioredoxin Reductase Hybrid Protein*

Brigitte Wielens, Jacque Van Noort, Jan Wouter Drijfhout, Rienk Offringa, Arne Holmgren, and Tom H. M. Ottenhoff

From the Department of Immunohematology and Blood Bank, Leiden University Hospital, Rijnsburgerweg 10, Building 1 E30, 2333 AA Leiden, The Netherlands and the Medical Nobel Institute for Biochemistry, Karolinska Institute, S-171 77 Stockholm, Sweden

In Mycobacterium leprae, thioredoxin and thioredoxin reductase are expressed from a single gene. This results in the expression of a hybrid protein with subunits attached to each other by a hydrophilic peptide linker. In all other organisms studied so far, thioredoxin (Trx) and thioredoxin reductase (TR) are expressed as two separate proteins. This raises the question of whether the hybrid protein is enzymatically active and, if so, whether TR reduces its own Trx partner or alternatively a heterologous Trx subunit. To address this question, the hybrid TR/Trx protein of M. leprae as well as the individual parts of the hybrid gene coding for either TR or Trx were overexpressed in Escherichia coli and purified. The purified proteins were tested for their ability to catalyze NADPH-dependent insulin disulfide reduction. Here we show that the M. leprae hybrid protein is indeed enzymatically active. Compared with the enzymatic activity of the separately expressed Trx and TR proteins, the hybrid protein is shown to be more efficient, particularly at low equimolar concentrations. This suggests that the hybrid protein of M. leprae is active by itself and that its activity involves intramolecular interactions between the TR and Trx domains. The activity of the hybrid protein increases when exogenous TR or Trx is added, indicating an additional role for intermolecular interactions.

Experimental Procedures

Bacterial Strains and Plasmids—Strain M15 (8) was used as a host for pTrcHis (Invitrogen Corp., San Diego, CA) and derivatives. Strain XL-1 Blue (9) was used as a host for pCl20H (10) and derivatives. Bacterial strains were grown at 37°C or room temperature in LB medium and LB agar (11) supplemented with ampicillin (100 μg/ml) and kanamycin (10 μg/ml).

Subcloning of the Proteins in the pTrcHIS Expression Vector—Clone pBB0010 containing the gene coding for the TR/Trx protein was used as a template for PCR (5). The forward primer containing a PstI site annealed upstream of the start codon, the reversed primer annealed downstream of an internal EcoRI site, which is located 537 base pairs downstream of the start codon. The PCR product was digested with PstI and EcoRI and subcloned in pCl20H to create clone pCl/C537. Subsequently an EcoRI–XholI fragment of clone pBB0010 was used to clone the second part of the gene in pl/C537, creating pl/C/TR/Trx. This clone was digested with PstI and subcloned in pTrcHis-A to create pTrcHis/Trx.

For overexpression of the TR part of the protein, two stop codons were introduced in the gene using a reversed PCR primer with two stop codons and a HindIII site. The forward primer annealed upstream of the internal EcoRI site. The PCR fragment digested with EcoRI and HindII was subcloned in pl/C537 creating pl/C/Trx and subsequently subcloned in pTrcHis-A creating pHis/Trx.

Subcloning of the part of the gene coding for Trx was done by PCR where the forward primer contained a BamHI site and the reversed primer contained a HindIII site. The digested PCR product was cloned in pTrcHis-A creating pHis/Trx. The nucleotide sequence of all cloned PCR products was established for verification.

Purification of Proteins—The hybrid protein TR/Trx as well as the separate subunits, thioredoxin and thioredoxin reductase of M. leprae, were expressed as fusion proteins containing 6 histidine residues plus a 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 overnight culture at 22°C, the bacteria were centrifuged and lysed by sonication under non-denaturing conditions. The proteins were purified by using a nickel chelate affinity resin according to the recommendations of the supplier (QIAGEN, Chatsworth, CA). The purified fraction was dialyzed against 50 mM potassium phosphate, pH 7.0. The purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis on 12.5% gels as described (12).

Prolonged induction at lower temperatures was used to avoid the accumulation of insoluble aggregates that were found in the TR and TR/Trx recombinants when cultured at higher temperatures (e.g. 37°C). The accumulation of insoluble aggregates is in conflict with the findings of LaVallie et al. (13). In their commercially available Trx fusion system, a protein of interest is fused to the C terminus of Trx, and the fusion protein is supposed to remain in the soluble fraction and

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† To whom correspondence should be addressed. Tel: 31-71-261737; Fax: 31-71-216751; E-mail: Brigitte@uls200.leidenuniv.nl.

‡ The abbreviations used are: Trx, thioredoxin; TR, thioredoxin reductase; PCR, polymerase chain reaction.
Induction and Purification of Functionally Active Proteins—In order to produce a sufficient amount of the TR/Trx hybrid protein and the separate TR and Trx subunits, we subcloned the genes in the pTrxH1S expression vector. Mid-log cultures were induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside and cultured overnight at 22 °C. Prolonged induction at lower temperatures was used to avoid the accumulation of insoluble aggregates that were found in the TR and TR/Trx recombinants when cultured at higher temperatures (e.g. 37 °C).

Fig. 1 shows the expression of TRx, TR, and TR/Trx by the recombinant strains before (lanes 1) and after over-night induction at 22 °C (lanes 2). Lanes 3 shows the proteins after purification on a nickel affinity column.

Molecular Weight of the Recombinant TR/Trx Hybrid Protein—The calculated molecular weight of the recombinant TR/Trx hybrid protein containing the His-tag is 54,019 (4991 of which are contribute by the attached His-tag). On a Superdex 200 HR column equilibrated with 0.1 M Tris-HCl, pH 7.5, the TR/Trx hybrid protein was eluted between bovine serum albumin (Mr = 68,000) and aldolase (Mr = 158,000). This might be indicative for the existence of a TR/Trx dimer.

Enzyme Activity of the M. leprae Hybrid Protein and of Separately Expressed M. leprae Trx and TR—To investigate whether the M. leprae TR/Trx hybrid protein has enzymatic activity similar to the separately expressed subunits, we performed an insulin reduction assay. Equimolar amounts of the hybrid protein TR/Trx or separate TR and Trx proteins were tested in a concentration range of 0.1 – 4 μM. Results in Fig. 2A show that especially at low concentrations the TR/Trx hybrid protein is more efficient. At higher concentrations the difference in efficiency between the hybrid protein system and separate TR and Trx proteins decreases. Fig. 2B focuses on the difference in efficiency between the separate M. leprae TR and Trx and the hybrid protein in a low concentration range of 0.1 – 1.6 μM. For the hybrid protein an increase in the protein concentration gives a linear increase in the amount of NADPH oxidized, whereas this is not the case for the separately expressed proteins. The ratio between the activity of TR/Trx and TR + Trx is high when low concentrations of the proteins are used but decreases with increasing protein concentrations (Table I). The relative high efficiency of the hybrid protein at low protein concentrations is indicative for intramolecular activity.

Ability of the Hybrid Protein to Accept Exogenous Trx—To investigate whether, besides the intramolecular activity mentioned above, the hybrid protein could also act via an intermolecular pathway, we measured the increase in enzyme activity upon addition of exogenous Trx. Fixed amounts of recombinant M. leprae TR or TR/Trx were used (0.5 and 1.0 μM). When Trx was added in a concentration range of 0.1 – 4 μM, an increase in the enzyme activity was measured for both the hybrid protein system and the separately expressed TR. Fig. 3 shows that although the effect of the addition of increasing amounts of Trx is similar for TR and TR/Trx, the activity of the TR/Trx hybrid protein is affected to a larger extent, especially at lower Trx concentrations where the slope of the curve is much steeper for the TR/Trx hybrid protein. Again the TR/Trx hybrid protein is by far more efficient then an equivalent amount of TR, even when TRx concentrations as high as 6.4 μM are used. These data show that, although TR/Trx is active by itself, additional Trx...
can increase the activity of the hybrid protein, which indicates that the TR part of the hybrid protein is able to form active complexes with exogenous Trx.

Ability of the Hybrid Protein to Accept Exogenous TR—Fig. 4 shows that the addition of TR to the TR/Trx hybrid protein also increases the enzyme activity. Comparison of the effects of extra TR and Trx on the activity of the TR/Trx hybrid protein shows that the positive effect of TR levels off between 1.6 and 3.2 μM, whereas additional TR positively affects the enzyme activity even beyond 6.4 μM. These findings may be explained in terms of the necessity of TR to undergo a conformational change in order to be able to complex Trx, as was suggested by Waksman et al. (15) for E. coli TR. The availability of TR in the proper conformational state may thus be a limiting factor in catalysis. Therefore an increase in exogenous TR (i.e., more TR molecules that are in the proper conformational state for Trx complexation) will affect enzymatic activity of the TR/Trx system over a wide concentration range.

Taken together, these data indicate that the TRx of the hybrid protein can accept electrons from exogenous TR, whereas TR of the hybrid protein can use exogenous Trx to donate electrons to. Therefore, the enzyme activity of the TR/Trx hybrid protein appears to involve both intramolecular and intermolecular interactions.

The results in this paper show that the M. leprae strain 10.3294/T1015412 protein TR/Trx, which is a thioredoxin and thioredoxin reductase hybrid protein, is functionally active. Comparison of the thioredoxin reductase part of the protein with that of E. coli shows that it is elongated at the C terminus by 22 amino acids that continue into the start of thioredoxin. Apparently, this 22-amino acid spacer gives the protein enough flexibility to permit interaction between the TR and Trx entities of this protein. Alternatively, the spacer does not inhibit intermolecular interactions of either the TR or the Trx part of the hybrid protein with free Trx and TR, respectively.

So far no crystals of the TR enzyme complexed with Trx have been obtained. A crystal structure of the reduced state of this M. leprae TR/Trx hybrid protein might shed more light on the interaction between the two subunits.

Combining thioredoxin and thioredoxin reductase in one hybrid protein may be of advantage when the intracellular concentration is low and intramolecular interactions are favored. M. leprae resides inside macrophages, where it has to deal with strongly triggered oxidative burst. Because Trx has been shown to reduce reactive oxygen species (16), this redox hybrid system might contribute to the escape mechanisms of the bacterium. We have preliminary results that indicate that the TR/Trx protein can exert its function outside the bacterial cell in the macrophage environment where the linkage of TR to Trx in a hybrid form might be particularly advantageous. We are currently investigating how M. leprae benefits from this unique redox system.

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