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Original Contribution

Immunolocalization and enzymatic functional characterization of the thioredoxin system in *Entamoeba histolytica*

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ABSTRACT

The components of the redox metabolism in *Entamoeba histolytica* have been recently revisited by Arias et al. (*Free Radic. Biol. Med.* 42:1496–1505; 2007), after the identification and characterization of a thioredoxin-linked system. The present work deals with studies performed for a better understanding of the localization and identification of different components of the redox machinery present in the parasite. The gene encoding for amoebic thioredoxin 8 was cloned and the recombinant protein typified as having properties similar to those of thioredoxin 41. The ability of these thioredoxins and the specific reductase to assemble a system utilizing NADPH to metabolize hydroperoxides in association with a peroxiredoxin has been kinetically characterized. The peroxiredoxin behaved as a typical 2 cysteine enzyme, exhibiting a ping-pong mechanism with hyperbolic saturation kinetics for thioredoxin 8 (K_m = 3.8 μM), thioredoxin 41 (K_m = 3.1 μM), and *tert*-butyl hydroperoxide (K_m about 35 μM). Moreover, the tandem system involving thioredoxin reductase and either thioredoxin proved to be operative for reducing low molecular weight disulfides, including putative physiological substrates as cystine and oxidized trypanothione. Thioredoxin reductase and thioredoxin 41 (by association also the functional redox system) have been immunolocalized underlying the plasma membrane in *Entamoeba histolytica* cells. These findings suggest an important role for the metabolic pathway involving thioredoxin as a redox interchanger, which could be critical for the maintenance and virulence of the parasite when exposed to highly toxic reactive oxygen species.

Introduction

Amoebiasis is an intestinal infection widespread throughout the world and caused by the human pathogen *Entamoeba histolytica*. The parasitic disease is the third leading cause of death in almost all countries where sewage and water quality are inadequate [1–3], causing 50 million clinical episodes of dysentery or amoebic liver abscess and ca. 100,000 deaths annually [4,5]. Identification and functional characterization of molecular targets are relevant matters for the rational design of new therapeutic drugs, which could improve the treatment of the disease [3,5–11]. In this regard, processes involved in redox metabolism are of particular interest in *E. histolytica* [7–11].

We have recently reported [12] the molecular cloning of two genes from *E. histolytica* encoding for thioredoxin reductase (*Eh*TRXR) and thioredoxin 41 (*Eh*TRX41), followed by their expression, purification, and functional characterization of the recombinant proteins. After these findings, the metabolic redox scenario operative in *E. histolytica* was revisited and the occurrence of a system (the *Eh*TRXR/TRX system) operating with a two cysteines peroxiredoxin (*Eh*p29; now more properly abbreviated *Eh*2CysPrx) has been proposed [12]. This metabolic pathway is complemented with other components such as Fe-superoxide dismutase (Fe-SOD), rubrerythrin, cysteine, flavoprotein A, and a 34-kDa oxidoreductase (*Eh*p34) [13]. In addition, it has been established that *E. histolytica* lacks or has insignificant amounts of glutathione and its associated enzymes [14,15], with cysteine being the major intracellular thiol [15,16]. Another metabolite reported to be present in *E. histolytica* is trypanothione [N^1,N^8-bis-(gluthationyl)-spermidine] [8,11,17], a thiol compound that in trypanosomatids is involved in the detoxification of reactive oxygen species, and alternatively can transfer reduction equivalents to TRX as well [18].

It is of relevance to further characterize the *Eh*TRXR/TRX system, especially with respect to the identification of all of its components, its cellular localization, kinetic properties, and the identification of different associated metabolites. This in-depth study is a prerequisite to a better understanding of the functionality of the redox system

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Abbreviations: BSA, bovine serum albumin; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TS_2, trypanothione.

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when the parasite is challenged under oxidative stress conditions. We present herein the cellular localization of *Eh*TRXR and *Eh*TRX41 in *E. histolytica* trophozoites, together with a kinetic study of *Eh*2CysPrx. In addition, we identify TRX8 as another component that is able to work together with TRXR, after having properties similar to those of TRX41. We also characterize the NADPH-dependent activity of the system associated with the reduction of low molecular weight disulfides, metabolites that are thought to be critical for maintaining a reduced intracellular redox potential [15,16]. The present study reinforces the view that the *Eh*TRXR/TRX system is a key molecular target for the design of new therapeutic drugs for amoebiasis.

Materials and methods

Materials

Bacteriological media components were from Britania Laboratories (Rosario, Argentina). All other reagents and chemicals were of the highest quality commercially available.

E. histolytica cultivation

Trophozoites of *E. histolytica* strain HM1:IMSS were axenically cultured at 36.5 °C in TYI-S-33 medium supplemented with 12% heat-inactivated adult bovine serum and 2% Diamond's vitamin [19].

Molecular cloning of trx8 from E. histolytica

The gene was amplified by PCR using *E. histolytica* genomic DNA as a template and primers designed after spliced sequences reported in the Wellcome Trust, Sanger Institute, Pathogen Sequencing Unit: http://www.genedb.org/. The specific primers were TRX8Fow, 5′-GGATCCATGGCTGTACTTCATATTAAC-3′; and TRX8Rev, 5′-AAGCTTTTATGCTGTTTCAACCATTTG-3′. The PCR was performed under the following conditions: 94 °C for 10 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and then 72 °C for 10 min. The PCR product was subsequently purified and ligated into the pGEM-T Easy vector (Promega) to facilitate further work. The fidelity and correctness of the gene were confirmed on both strands by complete sequencing. The construct and the pRSET-A vector (Invitrogen) were digested with *Bam*HI and *Hind*III. Ligation to the pRSET-A vector of the insert was performed using T4 DNA ligase for 16 h at 16 °C. Preparation of plasmid DNA and subsequent *Bam*HI/*Hind*III restriction treatment were performed to check the correctness of the different constructs.

Expression and purification

*Eh*TRXR, *Eh*TRX8, *Eh*TRX41, and *Eh*2CysPrx were expressed in *Escherichia coli* as His-tag (N-terminal) recombinant proteins, and chromatographically purified as previously described [12]. Briefly, single colonies of *E. coli* BL21(DE3) transformed with the respective recombinant plasmid were selected. Overnight cultures were diluted 1/100 in fresh media (LB broth supplemented with 100 μg/ml ampicillin) and grew under identical conditions to the exponential phase, OD_{600} of 0.6. The expression of the recombinant proteins was induced with 0.5 mM IPTG, followed by incubation at 28 °C. After 16 h the cells were harvested and stored at -20 °C. Purification was performed using a Co^{2+}-IDA-agarose resin (Invitrogen). Desalting was performed on Bio-Gel P chromatography columns (Bio-Rad).

Protein methods

Protein content was measured after Bradford [20], utilizing BSA as a standard.

Cell-free extracts and purified proteins were analyzed electrophoretically by SDS–PAGE according to [21]. Coomassie brilliant blue was used to stain protein bands. Western blotting was performed after standard techniques [22]. Proteins in the gel were blotted onto PVDF membranes using a Mini-ProteanII (Bio-Rad) apparatus. The membrane was blocked overnight at 4 °C, subsequently incubated with primary antibody at room temperature for 1 h, and then incubated with a HRP-conjugated anti-rabbit secondary antibody for 1 h. Detection was carried out with 3,3′-diaminobenzidine and hydrogen peroxide (Sigma) in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl.

Reduction of disulfides by the EhTRXR/TRX system

Disulfide reduction was followed by means of a coupled assay system where, in the first reaction, *Eh*TRX8 or *Eh*TRX41 is reduced by *Eh*TRXR and NADPH. After the enzymatic reaction has run to completion, the disulfide (RSSR) is reduced by the respective TRX. Thus, disulfide reductase activity was determined by monitoring, at 30°C, the oxidation of NADPH at 340 nm in a reaction mixture (final volume of 250 μl) containing 50 mM potassium phosphate, pH 7.0, 2 mM EDTA, 300 μM NADPH, 1 μM *Eh*TRXR, 0–10 μM *Eh*TRX8 or *Eh*TRX41, and the different RSSR: 0–350 μM bovine insulin, 0–2000 μM oxidized glutathione (GSSG), 0–2000 μM cystine, or 0–200 μM oxidized trypanothione (TS_2).

Eh2CysPrx assay and kinetic analysis

*Eh*2CysPrx activity was measured by monitoring the NADPH oxidation at 340 nm and 30 °C. The standard assay mixture contained (in a final volume of 250 μl) 50 mM potassium phosphate, pH 7.0, 2 mM EDTA, 300 μM NADPH, 2 μM *Eh*TRXR, 20 μM *Eh*TRX8 or *Eh*TRX41, 1 μM *Eh*2CysPrx, and 500 μM *t*-bOOH. For kinetic analysis, the assay was performed using 0–500 μM *t*-bOOH and 0–30 μM *Eh*TRX8 or *Eh*TRX41.

The kinetic data were plotted as initial velocity (μM min^{-1}) versus substrate concentration. The kinetic parameters were acquired by fitting the data with a nonlinear least-squares formula and the Michaelis-Menten equation using the program Origin. Kinetic constants are the mean of at least three independent sets of data, and they are reproducible within ±10%.

Determination of redox potential of TRXs

Redox potential for TRXs was determined by following changes in the absorbance at 340 nm and 30°C. *Eh*TRX8 or *Eh*TRX41 (5–30 μM) was mixed with 36 μM NADPH in a total volume of 250 μl of 50 mM potassium phosphate, pH 7.0, 2 mM EDTA, followed by the addition first of 1 μM *Eh*TRXR and then an excess of $NADP^+$ (1.2 mM) as described previously [23]. Redox potentials were calculated according to the Nernst equation, based in the reactants concentration in the equilibrium. A value of −320 mV was used as redox potential of NADPH.

Confocal laser scanning microscopy

Cells culture were chilled for 15 min to detach trophozoites, harvested by centrifugation at 500 *g* at room temperature for 10 min, and washed twice with phosphate-buffered saline, PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, pH 7.4), to remove residual medium components. The cells were fixed with 4% paraformaldehyde and permeabilized for 1 h at room temperature in PBS, 0.05% Triton X-100, and 3% BSA. The cells were then incubated for 1 h at 37 °C with rabbit polyclonal antibodies against *Eh*TRXR or *Eh*TRX41 diluted 1:100 in PBS, 0.01% Triton X-100, and 1% BSA, followed by goat anti-rabbit secondary antibody labeled with fluorescein isothiocyanate (FITC) (final dilution of 1:1000; ICN Biomedicals) for 1 h at 37 °C.