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Entamoeba histolytica thioredoxin reductase: Molecular and functional characterization of its atypical properties

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ABSTRACT

Background: Entamoeba histolytica, an intestinal protozoan that is the causative agent of amoebiasis, is exposed to elevated amounts of highly toxic reactive oxygen and nitrogen species during tissue invasion. Thioredoxin reductase catalyzes the reversible transfer of reducing equivalents between NADPH and thioredoxin, a small protein that plays key metabolic functions in maintaining the intracellular redox balance.
Methods: The present work deals with *in vitro* steady state kinetic studies aimed to reach a better understanding of the kinetic and structural properties of thioredoxin reductase from *E. histolytica* (*Eh*TRXR).
Results: Our results support that native *Eh*TRXR is a homodimeric covalent protein that is able to catalyze the NAD(P)H-dependent reduction of amoebic thioredoxins and *S*-nitrosothiols. In addition, the enzyme exhibited NAD(P)H dependent oxidase activity, which generates hydrogen peroxide from molecular oxygen. The enzyme can reduce compounds like methylene blue, quinones, ferricyanide or nitro-derivatives; all alternative substrates displaying a relative high capacity to inhibit disulfide reductase activity of *Eh*TRXR.
Conclusions and general significance: Interestingly, *Eh*TRXR exhibited kinetic and structural properties that differ from other low molecular weight TRXR. The TRX system could play an important role in the parasite defense against reactive species. The latter should be critical during the extra intestinal phase of the amoebic infection. So far we know, this is the first in depth characterization of *Eh*TRXR activity and functionality.

1. Introduction

Amoebiasis is an intestinal infection widespread throughout the world, caused by the human pathogen *Entamoeba histolytica* [CDC, http://www.cdc.gov/]. The parasitic disease is the third leading cause of death in almost all countries where sewage and water quality are inadequate, causing 50 million clinical episodes of dysentery or amoebic liver abscess and ca. 100,000 deaths annually [WHO, http://www.who.int/en/]. 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. In this regard, processes involved in redox metabolism are of particular interest in *E. histolytica* [1,2]. It is known that the trofozoites of *E. histolytica* find in the anaerobic environment of the human gut a suitable place to live and multiply. However, during tissue invasion the microorganism is exposed to environments containing high levels of oxidizing reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as nitric oxide (NO) [3,4]. NO can diffuse into *E. histolytica* cells and react with species derived from cysteine (the major intracellular low molecular mass thiol [5]) to perform CySNO. This metabolite is thought to be critical for *S*-nitrosylation (addition of NO to cysteine residue's thiol group) or *S*-thiolation (addition of cysteine to cysteine residue's thiol group) of cellular proteins [6].

It has been proposed that exposure to high concentrations of NO or *S*-nitrosothiols render an imbalance in redox potential leading to *E. histolytica* growth inhibition. *In vitro* studies demonstrated the inhibitory effect of GSNO on the activity of cysteine proteases and alcohol dehydrogenase 2, enzymes considered as important virulence factor for the cytopathogenecity and survival of this parasite, respectively [7]. Although NO and *S*-nitrosothiols are toxic *in vitro*, the pathogen can still survive and multiply during tissue invasion, this suggests that *E. histolytica* possesses a detoxification system to cope with these toxic agents. All living forms have developed efficient enzymatic systems to resist damage generated by ROS and RNS. The redox cellular status is a crucial mediator for different metabolic processes acting in signaling and regulation of several metabolic and cellular processes [8].

The thioredoxin system plays an important role as an antioxidant mechanism. It can take part in: i) regulation of enzymatic activities, ii) repairing oxidized proteins, iii) affording reducing equivalents for

Abbreviations: TRXR, thioredoxin reductase; TRX, thioredoxin; GSNO, *S*-nitrosoglutathione; CySNO, *S*-nitrosocysteine; MBQ, 2-methyl-benzoquinone; DTBBQ, 2-5-ditert-buthyl-benzoquinone; DTNB, 5-5′-dithio-nitrobenzoic acid; MB, methylene blue; MV, methyl viologen; CDNB, 1-chloro-2,4-dinitrobenzene

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DNA synthesis; as well as cellular transcription, growth and apoptosis [9,10]. We have reported the functional characterization and immunolocalization of a thioredoxin system from *E. histolytica* [11,12]. After these findings the metabolic redox scenario operative in *E. histolytica* was revisited, proposing a central and critical role of the thioredoxin system. The redox metabolism in the parasite is complemented with other components such as *Eh*2CysPrx (or *Eh*p29), Fe-superoxide dismutase (Fe-SOD), rubrerythrin, cysteine, flavoprotein A, and a 34 kDa oxidoreductase (*Eh*p34) [11].

In this work, we present new kinetic, structural and thermodynamic properties exhibited by *Eh*TRXR. We characterized the NAD(P)H-dependent reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid, TRXs and *S*-nitrosothiols (such as GSNO and CySNO), gathering information that reinforces the right assignment structure-function for *Eh*TRXR and broads the functional relationship between the enzyme and the capacity of *E. histolytica* for coping with environmental oxidative conditions.

2. Materials and methods

2.1. Materials

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

2.2. Expression and purification

The pGEM-T Easy/*Eh*TRXR plasmid [11,12] and the pET28-a vector (Novagen) were digested with *Bam*HI and *Hind*III. Ligation to the pET28-a vector of the insert was performed using T4 DNA ligase for 16 h at 16 °C to generate the pET28/*Eh*TRXR plasmid. Preparation of plasmid DNA and subsequent restriction treatment were performed to check the correctness of the different constructs.

*Eh*TRXR, *Eh*TRX8 and *Eh*TRX41 were expressed in *Escherichia coli* BL21 (DE3) as His-tag (N-terminal) recombinant proteins, and they were chromatographically purified as previously described [11,12].

2.3. Protein methods

SDS-PAGE was carry out using the Bio-Rad minigel equipment, basically according to previously described methods [13,14]. Protein concentrations were determined by the method of Bradford [15], utilizing BSA as standard.

2.4. Determination of the molecular mass by gel filtration chromatography

The determination of the native molecular mass of proteins was performed by gel filtration chromatography in a Superdex 200 HR Tricorn column (GE). The calibration curve was constructed using the logarithm of the molecular masses (log MM) vs. the distribution coefficients (K_{av}) measured for each molecular mass standard: Thyroglobulin (669 kDa), Ferritin (440 kDa), Aldolase (158 kDa), Conalbumin (75 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), Ribonuclease A (13.7 kDa) and Aprotinin (6.5 kDa) (Gel Filtration Calibration Kit-GE).

2.5. Preparation of S-nitrosothiols

GSNO or CySNO were prepared as previously described [16] by nitrosation under acid conditions. Briefly, equal volumes of glutathione or cysteine (both 200 mM) and sodium nitrite (200 mM) were incubated in the presence of 10 mM HCl on ice for 30 min. GSNO or CySNO were stabilized by addition of 1 mM EDTA at pH 7.0. GSNO or CySNO were freshly prepared and stored on ice in the dark. The concentration of GSNO and CySNO was estimated by measuring absorbance at 332 nm, using molar absorption coefficients of 0.92 mM^{-1} cm^{-1} and 0.75 mM^{-1} cm^{-1}, respectively [16].

2.6. EhTRXR assay and kinetic analysis

All enzymatic assays were performed spectrophotometrically at 30 °C using a Multiskan Ascent one-channel vertical light path filter photometer (Thermo Electron Co.). The general assay medium (GAM) contained 50 mM potassium phosphate, pH 7.0, and 2 mM EDTA, over which specific additions were made for each of the enzymes. In all the cases the final volume was of 50 μl.

TRXR activity was measured by monitoring NADPH oxidation at 340 nm with the addition to the GAM of 0.3 mM NADPH, 0.13 mM bovine insulin, 0.15–30 μM TRXs, and 0.1–1 μM *Eh*TRXR.

Activity for 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) reductase was measured by monitoring the production of thionitrobenzoate at 405 nm after complementing the GAM with 0.3 mM NADPH, 0.078–10 mM DTNB, and 0.1–1 μM *Eh*TRXR. Activity was calculated using the molar extinction coefficient at 405 nm of 13.8 mM^{-1} cm^{-1} and considering that 1 mol of NADPH yields 2 mol of thionitrobenzoate [17].

Quinone reductase activity was measured by monitoring the oxidation of NADPH at 340 nm in a reaction mixture comprising GAM supplemented with 0.3 mM NADPH, 2–500 μM 2-methyl-benzoquinone or 2′-5′-di-tert-buthyl-benzoquinone, and 0.1–1 μM *Eh*TRXR.

Diaforase activity was measured by monitoring the oxidation of NADPH at 340 nm by addition to the GAM of 0.3 mM NADPH, 2–1000 μM ferricyanide or methylene blue, and 0.1–1 μM *Eh*TRXR.

Nitroreductase activity was measured by monitoring the oxidation of NADPH at 340 nm by supplementing GAM with 0.3 mM NADPH, 10–1000 μM 1-chloro-2,4-dinitrobenzene or methylviologen, and 0.1–1 μM *Eh*TRXR.

Hydrogen peroxide production due to NAD(P)H oxidase activity of the enzyme was determinate with the ferrithiocyanate method [18].

All kinetic data were plotted as initial velocity ($\mu M \cdot min^{-1}$) 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%. In the study of inhibitors, IC_{50} refers to the concentration of the inhibitor giving 50% of the initial activity.

2.7. Determination of macroscopic redox potential of EhTRXR

The redox potential of *Eh*TRXR was determined by steady-state kinetics as described elsewhere [19,20]. All measurements were carried out in 50 mM potassium phosphate, pH 7.0, 2 mM EDTA at 30 °C. When *Ec*TRX_{Ox} (0.3–30 μM) was the electron acceptor, reaction rates were monitored by the decrease in absorbance at 340 nm due to oxidation of NADPH (1.5–300 μM) at 340 nm. In the reverse direction, where $NADP^+$ (7–1000 μM) was the electron acceptor, the increase in absorbance at 340 nm due to reduction of $NADP^+$ in the presence de of *Ec*TRX_{Red} (0.3–30 μM) was monitored. A constant level of *Ec*TRX_{Red} was maintained including 5 mM DTT in the buffer. Direct reductions of $NADP^+$ by *Eh*TRXR in presence of 5 mM DTT but without *Ec*TRX_{Red} were recorded for background correction. The steady-state kinetic parameter $k_{cat} \cdot K_m^{-1}$ represents the apparent second-order rate constant of the substrate-free enzyme reaction. Using Haldane equation, the equilibrium constants can be calculated, and the redox potential of the enzyme is then obtained from the Nernst equation [21]. A value of −320 mV and −283 mV were used as standard redox potential of $NADP^+$/NADPH and *Ec*TRX_{Ox}/*Ec*TRX_{Red} couples, respectively [19,20].