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Original Contribution

Functional characterization of methionine sulfoxide reductase A from *Trypanosoma* spp.

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ABSTRACT

Methionine is an amino acid susceptible to being oxidized to methionine sulfoxide (MetSO). The reduction of MetSO to methionine is catalyzed by methionine sulfoxide reductase (MSR), an enzyme present in almost all organisms. In trypanosomatids, the study of antioxidant systems has been mainly focused on the involvement of trypanothione, a specific redox component in these organisms. However, no information is available concerning their mechanisms for repairing oxidized proteins, which would be relevant for the survival of these pathogens in the various stages of their life cycle. We report the molecular cloning of three genes encoding a putative A-type MSR in trypanosomatids. The genes were expressed in *Escherichia coli,* and the corresponding recombinant proteins were purified and functionally characterized. The enzymes were specific for L-Met(*S*)SO reduction, using *Trypanosoma cruzi* tryparedoxin I as the reducing substrate. Each enzyme migrated in electrophoresis with a particular profile reflecting the differences they exhibit in superficial charge. The in vivo presence of the enzymes was evidenced by immunological detection in replicative stages of *T. cruzi* and *Trypanosoma brucei.* The results support the occurrence of a metabolic pathway in *Trypanosoma* spp. involved in the critical function of repairing oxidized macromolecules.

All aerobic organisms are exposed to reactive oxygen species (ROS) generated during cellular respiration and able to react with numerous macromolecules [1]. There is evidence about the relationships between oxidative stress and cellular damage, aging, and several pathological situations such as arthritis and Alzheimer, cardiovascular, and other neurodegenerative diseases [2]. Hence, several protective systems (catalase, peroxidase, superoxide dismutase, and glutathione-associated enzymes) help the cell to eliminate or minimize these injurious molecular species [1]. Additionally, there are enzymes responsible for DNA or protein rescue after the occurrence of oxidative damage. It is known that protein oxidation can generate conformational changes and, in some cases, loss of function [3]. The amino acids most susceptible to oxidation are cysteine, histidine, tryptophan, tyrosine, and methionine [3]. Oxidation of methionine to methionine sulfoxide (MetSO) produces a mixture of epimers on the sulfur atom [Met(*S*)SO and Met(*R*)SO] [4], affecting the biological function of the oxidized protein [3]. Consequently, the occurrence of a system for reducing MetSO to Met does matter in the cell. Such a reaction is catalyzed by the enzyme methionine sulfoxide reductase (MSR), which is present in almost all organisms [2,4]. The enzyme protects the organism from oxidative damage, being essentially involved in resistance against abiotic and biotic stress in plants and animals [5,6]. It can also act as a virulence factor in some bacterial pathogens such as *Neisseria gonorrhoeae* [7], *Staphylococcus aureus* [8], and *Mycobacterium tuberculosis* [9].

Despite the relevance of MSR to the biochemistry and cellular physiology of various organisms, the enzyme has never been characterized in protozoa, especially in those of medical and veterinary importance. At present, two unrelated classes of MSR have been described in several organisms: MSRA, which is stereospecific to the *S* isomer of the sulfur atom in the sulfoxide group [4,10], and MSRB, which is specific to the *R* isomer [4,8]. Several crystalline structures of MSRA and MSRB from various sources have been solved [4]. Both MSRA and MSRB present similar three-step catalytic mechanisms [4]. During the first (reductive) step a sulfenic group is formed on the catalytic cysteine of the enzyme with a concomitant regeneration of Met. Afterward, a second cysteine (resolutive cysteine) attacks the intermediary sulfenic group generating an intrachain disulfide bridge, with elimination of a water molecule.

Abbreviations: GSH, reduced glutathione; L-MetSO, L-methionine sulfoxide; MSR, methionine sulfoxide reductase; TR, trypanothione reductase; TXNI, tryparedoxin I; $T(SH)_2$, reduced trypanothione.

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Finally, the enzyme is reduced, preferentially by thioredoxin (TRX), in the last stage of the catalytic cycle.

Trypanosomatids are unicellular organisms of the order Kinetoplastida that parasitize a wide variety of invertebrate and vertebrate hosts. [11]. The most relevant specimens for human and animal health belong to two genera, *Trypanosoma* and *Leishmania,* which produce around half a million human deaths annually worldwide. In sub-Saharan countries, *T. brucei rhodesiense* and *T. brucei gambiense* are the causative agents of African sleeping sickness, and Nagana cattle disease is caused by *T. brucei brucei* [11]. In Latin America, *T. cruzi* is responsible for Chagas disease [11]. Trypanosomatids represent one of the earliest branches of the eukaryotic evolution [12]. The parasites show biochemical peculiarities among living organisms, such as a unique thiol redox metabolism [12]. Genome sequencing projects of *T. brucei* and *T. cruzi* revealed that trypanosomatids lack genes coding for glutathione reductase, TRX reductase, catalase, and selenocysteine-containing glutathione peroxidases [13].

Although in most eukaryotic organisms the glutathione (GSH) and TRX systems are the major components responsible for maintaining the intracellular thiol redox homeostasis, in trypanosomatids redox metabolism mainly is based on a low-molecular-mass dithiol, trypanothione [N_1,N_8-bis(glutathionyl) spermidine; $T(SH)_2$] [12]. For this, trypanothione reductase (TR) catalyzes the NADPH-dependent reduction of oxidized trypanothione (TS_2) to the reduced form $T(SH)_2$, which drives peroxide detoxification through two proteins working together: tryparedoxin (TXN) and a peroxiredoxin (PRX). The trypanothione-dependent system protects the parasite against oxidative damage, as well as poisoning by heavy metals, and also provides reduction equivalents for deoxyribonucleotide synthesis [11]. $T(SH)_2$ is the specific reductant of TXN, a multipurpose redox protein belonging to the TRX superfamily [14]. TXN is a specific reducing substrate for diverse PRXs found in the parasite, including two typical Cys–PRXs and a glutathione peroxidase-like protein [15].

Characterization of the redox metabolism in trypanosomatids is far from being completed [16]. Most of the work carried out in this area emphasizes the analysis of ROS detoxification; but the maintenance of the cellular homeostasis is based not only on detoxification pathways but also on the repair of the oxidative damage of nucleic acids, lipids, and proteins. In this work we describe an enzymatic system involved in the repair of oxidized proteins in *T. cruzi* and *T. brucei*. Our results improve the knowledge and understanding of redox metabolism in parasites of the genus *Trypanosoma* and add value to the genome project database, identifying the occurrence of functional proteins involved in key metabolic routes.

Materials and methods

Materials

Bacteriological medium components were from Britania Laboratories. *Taq* DNA polymerase and the restriction enzymes were from Promega. Trypanothione disulfide was acquired from Bachem. All other reagents and chemicals were of the highest quality commercially available from Sigma. The L-Met(*S*)SO and L-Met(*R*)SO enantiomers were prepared according to Holland et al. [51].

Protozoa and culture procedure

For this study *T. cruzi* CL-Brener cells were used. Epimastigote cells were cultivated axenically at 28 °C in LIT medium supplemented with 10% (w/v) bovine fetal serum and 20 μg ml^{-1} hemin, as was previously reported [17]. Metacyclic trypomastigotes were obtained from axenic cultures under differentiating conditions [18]. Amastigote cells were developed in Vero cell cultures, as previously [19].

Bacteria and plasmids

Escherichia coli Top 10 F′ and *E. coli* BL21(DE3) cells (Invitrogen) were utilized in routine plasmid construction and expression assays. The vector pGEM-T Easy (Promega) was selected for cloning and sequencing purposes. The expression vector was pRSET-A (Invitrogen). DNA manipulation, *E. coli* culture, and transformation were performed according to standard protocols [20].

Molecular cloning of tcrmsr10, tcrmsr180, and tbrmsr genes

Genomic DNAs of the parasites were obtained from epimastigote cells grown at the logarithmic phase as previously described [20]. Genes from *T. cruzi* (*tcrmsr10* and *tcrmsr180*) and *T. brucei* (*tbrmsr*) were amplified from their respective genomic DNA by PCR, using the following oligonucleotide primer pairs designed from known spliced sequences (Pathogen Sequencing Unit, Sanger Institute, Wellcome Trust, http://www.genedb.org/): *Tcr*MSR10 For, *GGATCC*ATGGCTTCTGGTGTTC CAGCG; *Tcr*MSR180 For, *GGATCC*ATGGCTTCTGGTGCTCCAGCG; *Tcr*MSR Rev, *AAGCTT*TTCACCAATGAATTCGGTGCG; *Tbr*MSR For, *GGATCC*AT GAACCCAAATGCTGTTGC; and *Tbr*MSR Rev, *AAGCTT*TCACCAGTAGA GACGGTGTG. Each PCR was performed under the following conditions: 94 °C for 10 min; 30 cycles of 94 °C for 1 min, 60 °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. Fidelity and correctness of each gene were confirmed on both strands by complete sequencing (Macrogen, South Korea).

Construction of the expression vectors

pGEM-T Easy plasmids containing the cloned genes and the pRSET-A vector (Invitrogen) were digested with *Bam*HI and *Hin*dIII. Restriction fragments were purified by gel extraction after gel electrophoresis. Ligation to the pRSET-A vector of each insert was performed using T4 DNA ligase for 16 h at 16 °C. Competent *E. coli* BL21(DE3) cells were transformed with the respective construct. Transformed cells were selected in agar plates containing Luria–Bertani broth (10 g l^{-1} NaCl, 5 g l^{-1} yeast extract, 10 g l^{-1} peptone, pH 7.4) supplemented with ampicillin (100 μg ml^{-1}). Preparation of plasmid DNA and subsequent *Bam*HI/*Hin*dIII digestion were performed to check correctness of the constructs.

Overexpression and purification of recombinant proteins

Single colonies of *E. coli* BL21(DE3) transformed with the respective recombinant plasmid were selected. Overnight cultures were diluted 1/100 in fresh medium (TB broth: 12 g l^{-1} peptone, 24 g l^{-1} yeast extract, 4 ml l^{-1} glycerol, 2.3 g l^{-1} KH_2PO_4, and 12.5 g l^{-1} K_2HPO_4, pH 7.0, supplemented with 100 μg ml^{-1} ampicillin) and grown under identical conditions to exponential phase, OD_{600} 0.6. The expression of the respective recombinant protein was induced with 0.5 mM IPTG, followed by incubation at 25 °C. After 4 h, cells were harvested and stored at −20 °C. Purification of each recombinant protein was performed using a Co^{2+}–IDA–Sepharose resin (GE Healthcare). Briefly, the bacterial pellet was resuspended in binding buffer (20 mM Tris–HCl, pH 8.0, 300 mM NaCl) and disrupted by sonication. The lysate was centrifuged (10,000 *g*, 30 min) to remove cell debris. The resultant crude extract was loaded onto a Co^{2+}–IDA–Sepharose column that had been equilibrated with binding buffer. After being washed with 10 bead volumes of binding buffer plus 10 mM imidazole, the recombinant protein was eluted with elution buffer (20 mM Tris–HCl, pH 8.0, 300 mM NaCl, 500 mM imidazole). Purified enzyme fractions were pooled, concentrated by ultrafiltration, and stored at −80 °C in 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 4% (v/v) glycerol. Under the specified storage conditions, the recombinant proteins were stable for at least