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Original Contribution

The yeast cytosolic thioredoxins are involved in the regulation of methionine sulfoxide reductase A

Ingeborg Hanbauer [a], Jackob Moskovitz [b,*]

[a] *Laboratory of Biochemistry, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA*
[b] *Department of Pharmacology and Toxicology, Pharmacy School, University of Kansas, Lawrence, KS 66045, USA*

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Abstract

Previously we have shown that the binding complex formation of methionine sulfoxide reductase A (*msrA*) promoter and calcium phospholipid binding protein (CPBP) enhances *msrA* transcription and expression. The *msrA* promoter-CPBP-binding complex (P*msrA*-CPBP) formation was similar in Δ*trx1*, Δ*trx2*, and Δ*trx3* yeast strains and their control, with or without exposure to H_2O_2. In Δ*trx1*/Δ*trx2* double mutant the P*msrA*-CPBP was similar to its parent strain, following exposure to H_2O_2 for 30 min. However, a late-onset loss of P*msrA*-CPBP binding activity occurred following exposure to H_2O_2 for 24 hours. Hence, it was inferred that both Trx1 and Trx2 are involved in the P*msrA*-CPBP formation during prolonged oxidative stress conditions. In addition, the survival rate of the Δ*trx1*Δ*/trx2* double mutant was ~10% of its parent strain when exposed to H_2O_2. The MsrA activity was obliterated in Δ*trx1*/Δ*trx2* and Δ*trx1* strains and remained intact in the Δ*trx2* and Δ*trx3* strains. The *msrA mRNA* level in Δ*trx1* was significantly reduced in comparison to that of its control, slightly reduced in Δ*trx2*, and unchanged in Δ*trx3*, respectively. It is suggested that under normal growth conditions Trx1 is essential for *msrA* transcription and activity. Moreover, following long-term oxidative stress conditions, Trx1 and Trx2 appear to promote P*msrA*-CPBP-binding activity and cell survival.
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Keywords: Yeast; Methionine sulfoxide reductase; Thioredoxin; Oxidative stress; Transcription factors; Methionine oxidation; Free radicals

Introduction

Protein oxidation occurs with increasing frequency during aging-associated inflammatory and/or neurodegenerative diseases [1–3]. Among amino acids, methionine is among the most susceptible to oxidation. Its oxidized form, methionine sulfoxide (MetO), can be readily reverted to methionine by the methionine sulfoxide reductase (Msr) system [4]. MetO exist in two forms: *S*- and *R*-epimer, which can be reduced by MsrA and MsrB, respectively [5,6]. During oxidative stress, the repair function of both Msr isomers appears to play an important role in antioxidant defense [3,7–9]. In *msrA* yeast mutants, various oxidative stress conditions caused an increase of the free and protein-bound MetO levels relative to those of the wild-type parent strain [8]. The Msr activity is mediated by the thioredoxin (Trx) system that serves as an electron donor in the reduction process of oxidized methionine residues [10]. In contrast to human cells, yeast cells contain multiple Trx isomeric forms. The *trx1* and *trx2* genes encode the cytoplasmic Trx proteins whereas the *trx3* gene encodes a mitochondrial Trx [11,12].

Our previous work discovered that one of the factors involved in the transcriptional regulation of *msrA* in *Saccharomyces cerevisiae* is the calcium phospholipid binding protein (CPBP), a homolog of EF-1γ specifically bound to the *msrA* promoter [13]. Further studies revealed that deletion of the CPBP gene downregulated *msrA* mRNA and MsrA protein expression [13]. Although the role of the Trx-NADPH system as an electron donor for the catalytic activity of MsrA has been suggested [10] it is unclear whether Trx is required for the CPBP-*msrA* promoter-binding complex formation. The reduced form of Trx by itself was shown to interact with other proteins by creating redox-regulatory complexes [14]. Moreover, many transcription factors undergo redox-regulation by Trx that

Abbreviations: Msr, methionine sulfoxide reductase; Trx, thioredoxin; Trr, thioredoxin reductase; EMSA, electrophoretic mobility shift assay; CPBP, calcium phospholipid binding protein; Meto, methionine sulfoxide; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

* Corresponding author. Fax: +1 785 864 5219.
E-mail address: moskovij@ku.edu (J. Moskovitz).

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appears to be critical for the transcriptional control of oxidative stress response genes and modulation of cell function [15]. However, it is still unknown whether Trx binds to the *msrA* promoter or participates indirectly in the CPBP-*msrA* promoter-binding complex formation.

Generally, Trx1, Trx2, and Trx3 have a multifunctional task in cellular redox regulations. Since the Trx system plays an important role in maintaining the reductive environment of the cytoplasm in yeast [16], the present studies performed on Trx mutants may provide better insight on the role of Trx in the regulation of the MsrA expression. This study examines the effects of H_2O_2 on the *msrA* promoter activity in the various *trx* null mutants and their effects on the *msrA* transcription and activity.

Materials and methods

Yeast strains and media

The diploid homozygous mutant strains Δ*trx1,*Δ*trx2,*Δ*trx3,* and their parent strain BY4743 (wt1) were obtained from Open Biosystems Clone Resources [http://openbiosystem.com]. The wild-type strain EMY60 (wt2) and its isogenic double knockout mutant EMY63 (Δ*trx1*: : *TRP1,* Δ *trx2*: : LEU2) were provided by Dr. E. Muller, University of Washington, Seattle, WA). Media and culture conditions were chosen following yeast genetic methods described by Sherman [17].

H_2O_2 sensitivity

The yeast strains were grown in YPD media at 30°C until A_{600} = 1.0 was reached. The cells were harvested, the pellets were resuspended in 0.1× phosphate-buffered saline, and the cell suspensions of A_{600} = 0.1 were exposed for 30 min or 24 h to various concentrations of H_2O_2 (1–50 mM). Aliquots from each suspension were transferred to fresh YPD agar medium and incubated at 30 °C. The number of formed colonies represented the percentile of cell survival. Nuclear extracts were made from the corresponding strains grown in the presence or absence of H_2O_2 following 30 min and 24 h of incubation time.

Nuclear protein extract

The parent and mutant yeast strains were grown in YPD media for the length of time indicated. After centrifugation for 5 min at 5000 rpm (Sorvall SS34) the pellet was washed with 1 M sorbitol containing 50 mM potassium phosphate, 10 mM $MgCl_2$, 20 mM dithiothreitol (OTT), and 0.5 mM phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor cocktail III (Boehringer). The pellet was resuspended in 1 M sorbitol containing 25 mM potassium phosphate, 10 mM $MgCl_2$, 2 mM dithiothreitol, 25 mM sodium succinate, and 0.5 mM PMSF and incubated at 30°C with zymolase (final concentration 4 mg/ml zymolase 20-T, ICN Biomedicals) until protoplast formation had occurred. The protoplasts were homogenized, in the above described buffer containing 0.2% Triton X-100, and centrifuged at 3500 rpm for 5 min. The resuspended cells, in the above buffer containing 0.5% Triton X-100, were disrupted with glass beads and centrifuged for 15 min in a Beckman microfuge at maximum speed.

Electrophoretic mobility shift assay (EMSA)

^{32}P-labeled-double-stranded *msrA*-promoter oligonucleotides consensus sequence was synthesized by PCR using *Taq* DNA polymerase (Promega), ^{32}P α-dCTP (0.1 μci/50 μl final incubation volume, ICN Biomed), 0.02 mM dCTP, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, and synthetic oligonucleotide primers (BioSynthesis Inc.; PCR cycle: 5 min at 94°C followed by 33 cycles of 30 s at 94°C, 60 s at 50°C, and 60 s at 72°C). The PCR products were purified with the Wizard PCR Preps DNA Purification System (Promega). The EMSA reaction mixture contained 1× gel shift buffer (Promega), yeast nuclear protein solution (20 μg protein/sample), and ^{32}P-labeled DNA (~100,000 cpm). The mixtures were incubated at 24°C for 20 min. To identify calcium phospholipid binding protein (CPBP) in the DNA-binding complex a postincubation with rabbit polyclonal anti-CPBP antibody was performed using EMSA assay [13].

Determination of msrA activity

The ability of MsrA to reduce protein-bound MetO was assayed using a mixture of 200 μM dabsyl-methionine sulfoxide as substrate, 20 mM DTT, 25 mM Tris–HCl (pH 7.0), and yeast protein extract. Following incubation for 30 min at 37°C, the reaction mixture was monitored for the formation of dabsyl–methionine using an HPLC reverse-phase column (C-18) separation, as previously described [8].

RNA isolation and dot blot analysis

Total RNA was extracted with RNAwiz kit (Ambion) according to the manufacturer's instructions. The total RNA content was determined spectrophotometrically ($A_{260/280}$: 2.047–2.1495); 20 μg total RNA was spotted on BrightStar membrane (Ambion) and processed according to the manufacturer's instructions. ^{32}P-labeled *msrA* coding region was used as probe.

Results

H_2O_2 sensitivity

Under fermentative media conditions (YPD), the H_2O_2 sensitivity of the Δ*trx1,* Δ*trx2*, and Δ*trx3* strains was only slightly or not affected relative to that of the wild-type parent strain BY4743, referred to as wt1 (Table 1, Δ*trx1,* Δ*trx2*, or Δ*trx3* had 15, 23%, or no reduction in H_2O_2 resistant, relative to wt1, respectively*)*. In contrast, the *Δtrx1/Δtrx2* double mutant grown in YPD showed 10-fold higher sensitivity to H_2O_2 than its parent strain EMY60, referred to as wt2 (Table 1, reduction of 90% in H_2O_2 resistant, relative to wt2). Interestingly, the wt2 strain was more resistant to H_2O_2 (IC_{50} = 40 mM) than the wt1.