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Evidence for the Presence of a Second Electron Donor for the Cytoplasmic Thioredoxins in the Yeast *Saccharomyces cerevisiae*

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Abstract: In yeast, the cytoplasmic thioredoxin system is composed of NADPH, thioredoxin reductase-1 (*TRR1*) and 2 thioredoxin genes (*TRX1, TRX2*). In this study, using yeast knockout mutants for *TRR1*, *TRX1* and *TRX2* genes, the role of the thioredoxin system in methionine sulfoxide reduction was investigated. Cells lacking both *TRX1* and *TRX2* genes simultaneously were not able to reduce methionine sulfoxides to methionine; however, mutants missing the *TRR1* gene were able to reduce methionine sulfoxides to methionine, which showed that electrons could be transferred from NADPH to thioredoxins in the absence of *TRR1*. Similar results were observed for 3-phosphoadenosine 5-phosphosulfate reduction in the inorganic sulfate assimilation pathway. Results from both assays suggested that yeast cells have additional cytoplasmic thioredoxin reductase activity that could compensate for methionine sulfoxide reduction and sulfate assimilation in the absence of *TRR1*. This report also constitutes the first evidence that thioredoxins are the in vivo electron donors for methionine sulfoxide reductases in yeast.

Key Words: Thioredoxin reductase, thioredoxin, methionine, methionine sulfoxide, yeast, *Saccharomyces cerevisiae*

Maya (*Saccharomyces cerevisiae*) Sitoplazmik Tiyoredoksinleri için İkinci Bir Elektron Donörünün Varlığını Gösteren Deliller

Özet: Maya mantarında sitoplazmik tioredoksin sistemi NADPH, bir tane tiyoredoksin redüktaz (*TRR1*) ve iki tane tiyoredoksin (TRX1, *TRX2*) den oluşmaktadır. Bu çalışmada, genomundan *TRR1*, *TRX1* ve *TRX2* genleri çıkartılmış maya hücrelerinin metiyonin sülfoksit indirgeme kapasiteleri incelenmiştir. İki tiyoredoksini aynı anda çıkartılmış mutant hücreler metiyonin sulfoksiti indirgeyip metiyonin oluşturamazken, tiyoredoksin redüktaz-1 geni çıkartılmış hücreler oluşturabildiler. Bu maya mantarında metiyonin sülfoksit redüktazlar için gerekli olan elektronların tiyoredoksinlerden sağlandığını gösteren ilk çalışmadır. Ayrıca metiyonin sülfoksit indirgenmesi için gerekli olan elektronlar tiyoredoksin redüktaz-1 olmadan NADPH den tiyoredoksine transfer edilebildiler. Aynı sonuç sülfatın indirgenip methiyonin sentezinde kullanılmasında rol oynayan 3-fosfoadenozin 5-fosfosülfat redüktaz enzim aktivitesi içinde gözlemlendi. Her iki deneyin sonuçlarına göre, tiyoredoksin redüktaz-1 in yokluğunda tiyoredoksinler için gerekli olan elektronlar kimliği bilinmeyen ikinci bir elektron donörü tarafından sağlanmaktadır.

Anahtar Sözcükler: tiyoredoksin redüktaz, tiyoredoksin, metiyonin, metiyonin sülfoksit, maya hücresi, Saccharomyces cereviseae

Introduction

The thioredoxin system is 1 of the 2 major redox systems in bacteria and eukaryotes, which, together with the glutathione system, participates in the redox control of a great variety of biological processes. Thioredoxin reductase was first identified, based on its ability to reduce thiol dependent peroxidase (TSA1), in in vitro assays in the presence of thioredoxin. Thioredoxins are a group of small proteins widely distributed in both prokaryotes and eukaryotes. Thioredoxins contain a dithiol active site sequence of Cys-Gly-Pro-Cys (1), and participate in redox reactions through the reversible oxidation of its active center dithiol to a disulfide. Oxidized thioredoxin can be reduced by NADPH through the action of a flavoprotein, thioredoxin reductase (2). Thus, thioredoxin, thioredoxin reductase and NADPH form a disulfide reduction pathway called the thioredoxin system.

Thioredoxin activity was first identified in *S. cerevisiae* as a reducing agent for the reduction of sulfate (3,4). In yeast, 2 thioredoxins (*TRX1*, *TRX2*) and 1 thioredoxin reductase (*TRR1*) form the cytoplasmic thioredoxin

system. The 2 genes encoding the thioredoxins were cloned (5,6) and the cloned genes were used to generate and characterize a set of thioredoxin mutants. Characterization of mutants revealed that the 2 thioredoxin genes, *TRX1* and *TRX2*, are interchangeable and dispensable during normal growth. Loss of both thioredoxins results in methionine auxotrophy consistent with thioredoxin being the sole reductant for PAPS reductase, the enzyme which converts 3-phosphoadenosine 5-phosphosulfate (PAPS) to sulfite during sulfate assimilation in methionine synthesis (7,8).

The thioredoxin system is one of the major redox regulatory systems involved in oxidative stress tolerance in all organisms. Thioredoxin dependent peroxidases play an important role in scavenging hydrogen peroxide. Methionine sulfoxide reductases are another group of enzymes that uses electrons from the thioredoxin or glutathione system to reduce oxidatively damaged free or protein-bound methionines (9). Oxidation of methionines by free radicals results in the formation of 2 stereoisomers of methionine sulfoxide (MetO), methionine-R-sulfoxide (Met-R-O) and methionine-S-sulfoxide (Met-S-O). Yeast cells have at least 2 methionine sulfoxide reductases (MsrA and MsrB) that reduce both R and S isoforms of methionine sulfoxide stereo-specifically. MsrA uses Met-S-SO and MsrB uses Met-R-SO as substrates (10). It has not been shown whether the thioredoxin system or the glutaredoxin system gives electrons to MsrA and MsrB enzymes to reduce MetO to methionine. To address this question, the methionine sulfoxide reduction pathway was investigated in mutants lacking thioredoxin reductase and thioredoxins. Cells without thioredoxin genes were deficient in methionine sulfoxide activity, but cells without thioredoxin reductase-1 were able to reduce methionine sulfoxides. Similar results were observed for inorganic sulfate assimilation. Results from both assays showed that, in the absence of thioredoxin reductase, thioredoxins were reduced by another electron donor and were able to perform the downstream activities.

Materials and Methods

Yeast Strains, Growth and Media

Standard yeast genetic methods were applied for the analysis of strains and crosses. Cells were grown either in YPD (1% yeast extract, 2% peptone, 2% dextrose) medium or in YNB (yeast nitrogen base) medium containing the required supplements. The yeast strains are listed in Table 1. SY2626, a derivative of W303-1, was provided by G. Sprague (University of Oregon, Eugene, OR, USA). Strain EMY56-5D was a gift from E. Muller (University of Washington, Seattle, WA, USA). AKY501 was generated by a single-step replacement of the TRR2 gene by a 1800 bp KanMX module, and AKY502, double mutant for *Δtrr1 Δtrr2*, was isolated by replacement of the *TRR2* gene by a KanMX module in AKY36 cells.

Table 1. Yeast Strains

Strain	Genotype
W303-1A	*MAT-a ade2 ura3 leu2 trp1 his3 can1*
SY2626	*MAT-a ade2 ura3 leu2 trp1 his1 can1 bar1*
EMY56-D	*MAT-α ade2 ura3 leu2 trp1 his3 lys2:HIS3 Δtrx1:LYS2 Δ trx2:LEU2*
AKY326	*MAT- α ade2 ura3 leu2 trp1 his3 lys2 Δ trx1:LYS2 Δ trx2:LEU2*
AKY370	*MAT- α ade2 ura3 leu2 trp1 his3 lys2 Δ trr1:HIS3 Δ trx1:LYS2 Δ trx2:LEU2*
AKY401	*MAT-a ade2 ura3 leu2 trp1 his3 lys2 bar1*
AKY36	*MAT-a ade2 ura3 leu2 trp1 his3 lys2 Δ trr1:HIS3 bar1*
AKY39	*MAT-a ade2 ura3 leu2 trp1 his3 lys2 Δ trx1:LYS2 Δ trrx2:LEU2 bar1*
AKY40	*MAT-a ade2 ura3 leu2 trp1 his3 lys2 Δ trr1:HIS3 Δ trx1:LYS2 Δ trx2:LEU2 bar1*
AKY501	*MAT-a ura3 leu2 trp1 his3 Δ trr2:KANMX*
AKY502	*MAT-a ade2 ura3 leu2 trp1 his3 lys2 Δ trr1:HIS3 bar1 Δ trr2:KANMX*

Sulfate Assimilation Assay

Overnight yeast cultures were diluted to 0.2 OD_{600} and 10 µl of cell suspension was applied to spots. Each plate contained 0.1 mM of ammonium sulfate. Cells were allowed to grow for 3 days at 30 °C, and the plates were photographed.

Methionine Sulfoxide Reductase Assay

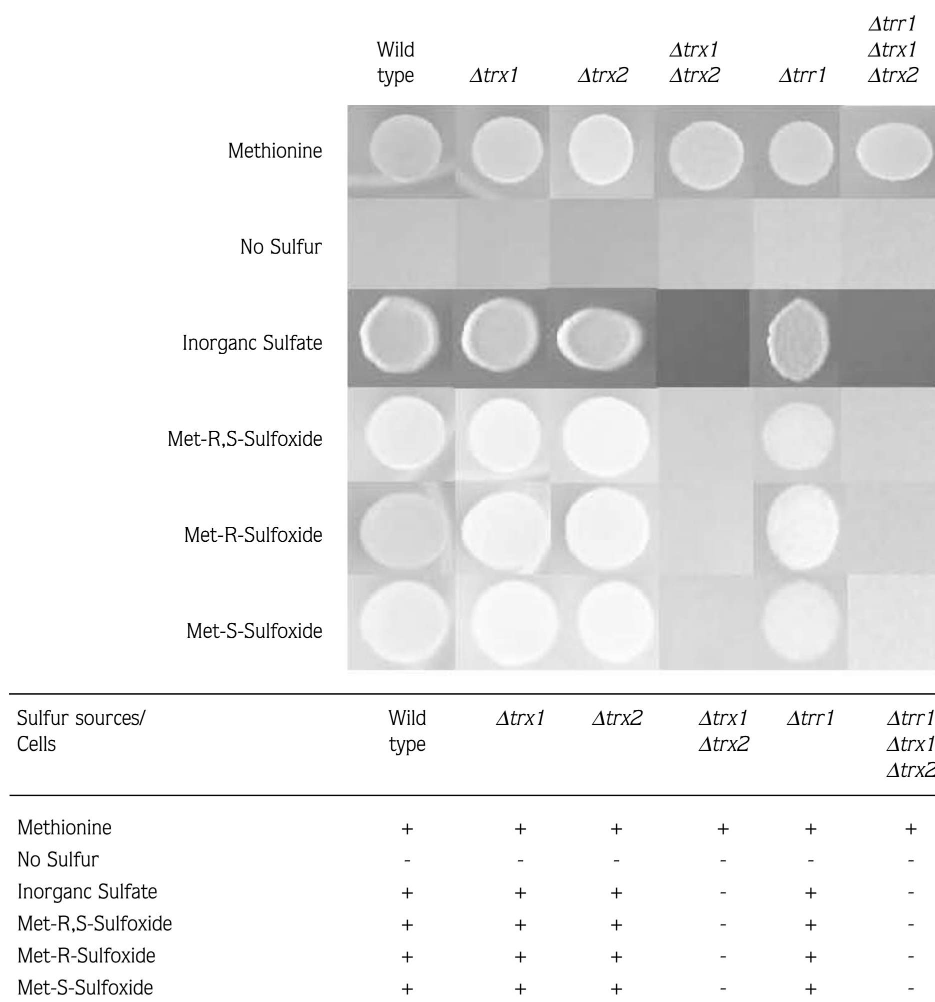
Overnight yeast cultures were diluted to 0.2 OD_{600} and 10 µl of cell suspension was applied to spots. Each plate contained 0.14 mM of methionine or indicated methionine sulfoxides. Cells were allowed to grow for 3 days at 30 °C, and the plates were photographed. L-Methionine-S-sulfoxide and L-methionine-R-sulfoxide

were prepared from L-methionine-R,S-sulfoxide (Sigma) according to the method described by Lavine (11).

Results

Yeast cells are able to uptake and process inorganic sulfate to synthesize sulfur containing amino acids. In the absence of an available sulfur source, which could be either inorganic sulfate or sulfur containing amino acids, cells cease dividing and die. Reduction of oxidized methionine (methionine sulfoxide) in the media also provides a good source of sulfur in the form of methionine.

To elucidate whether methionine sulfoxide reductases get electrons from the thioredoxin system to reduce methionine sulfoxides to methionine, cells lacking the components of the thioredoxin system were generated and tested for growth on methionine sulfoxides as the only sulfur source (Figure 1). In the presence of methionine as the sulfur source, WT, thioredoxin reductase-1 mutant (*Δtrr1*), thioredoxin-1 mutant (*Δtrx1*), thioredoxin-2 mutant (*Δtrx2*), thioredoxin double mutant (*Δtrx1Δtrx2*) and a triple mutant lacking thioredoxin reductase and both thioredoxins (*Δtrr1Δtrx1Δtrx2*) were able to grow. As predicted, in the absence of any sulfur source no growth was observed. In the presence of a racemic mixture of methionine R and S sulfoxides or purified R and S isoforms, although WT, *Δtrr1*, *Δtrx1* and *Δtrx2* cells were able to grow, mutants missing both thioredoxins



Sulfur sources/ Cells	Wild type	*Δtrx1*	*Δtrx2*	*Δtrx1* *Δtrx2*	*Δtrr1*	*Δtrr1* *Δtrx1* *Δtrx2*
Methionine	+	+	+	+	+	+
No Sulfur	-	-	-	-	-	-
Inorganc Sulfate	+	+	+	-	+	-
Met-R,S-Sulfoxide	+	+	+	-	+	-
Met-R-Sulfoxide	+	+	+	-	+	-
Met-S-Sulfoxide	+	+	+	-	+	-

Figure 1. Growth of wild-type and mutant cells in the presence of different sources of sulfur. Approximately 50,000 cells were inoculated on to each spot. Plates received 0.1 mM of shown sulfur sources.

simultaneously (*Δtrx1Δtrx2* and *Δtrr1Δtrx1Δtrx2*) were not able to grow. These data suggested that the thioredoxins were required for methionine sulfoxide reductases; however, the absence of thioredoxin reductase-1 did not block the electron flow from NADPH to methionine sulfoxide reductases.

Similar results were observed for the reduction of inorganic sulfate to sulfite in methionine biosynthesis. Thioredoxin double mutant cells (*Δtrx1Δtrx2* and *Δtrr1Δtrx1Δtrx2*) did not grow on inorganic sulfate, whereas thioredoxin reductase mutant cells (*ΔTRR1*) did.

To elucidate whether mitochondrial thioredoxin reductase (*trr2*) might be the putatative electron donor for cytoplasmic thioredoxins in *Δtrr1* cells, we isolated (Figure 2a) and tested a double mutant missing both *TRR1* and *TRR2* genes. These cells were able to grow on inorganic sulfate without any phenotypes (Figure 2b), which shows that thioredoxins get electrons from an unidentified source to reduce sulfate to synthesize methionine.

Discussion

Two phenotypic aspects were analyzed in mutants lacking thioredoxin reductase-1 (*TRR1*) and thioredoxin genes (*TRX1*, *TRX2*). Although the dependence of sulfate

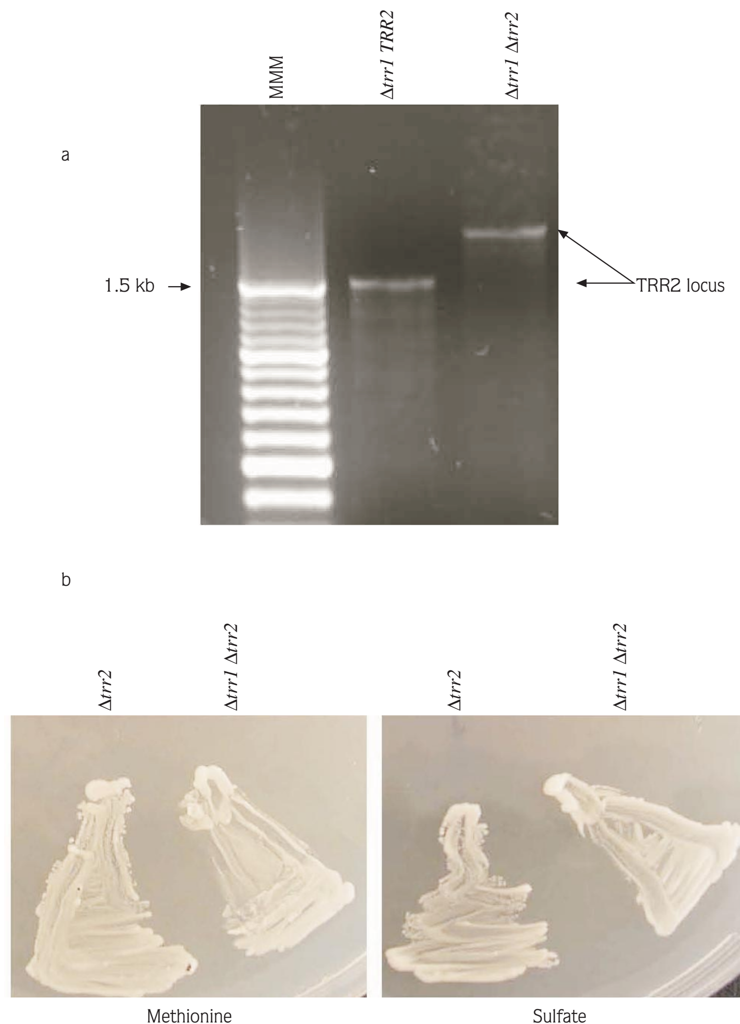


Figure 2. Deletion of *TRR2* gene and sulfate reduction test of *Δtrr2* mutants. a) Wild-type TRR2 locus (1439 bp) was replaced by KanMX module (1800 bp) in *Δtrr1* cells to isolate a *Δtrr1 Δtrr2* double mutant. b) Sulfate assimilation of *Δtrr2* and *Δtrr1Δtrr2* cells. Cells were inoculated on synthetic media containing either methionine or ammonium sulfate as the sole source of sulfur.

assimilation on the thioredoxin system has been well documented previously, this is the first report showing that the thioredoxin system is the proximal electron donor for methionine sulfoxide reductases in yeast. In many redox regulated pathways, the thioredoxin and the glutaredoxin systems crosstalk and the absence of either system does not provide any significant change in phenotypes (12). However, the methionine sulfoxide reduction pathway uses up electrons coming from the thioredoxin system only, and the status of the glutaredoxin system has no effect on this pathway since the thioredoxin double mutants, which have a functional glutathione system, are not viable on methionine sulfoxides.

In the absence of *TRR1*, electron flow from NADH to thioredoxin via an alternative electron carrier was detected for the methionine sulfoxide reduction pathway, which was confirmed by the sulfate assimilation capability of *TRR1* mutant cells. Thus, the results represented here suggest that there is a second electron donor (SED) for cytoplasmic thioredoxins in yeast (Figure 3) of unknown identity.

Bioinformatic analyses to find the SED identity yielded no results. The only protein that showed significant homology to the *TRR1* gene was the mitochondrial thioredoxin reductase gene (TRR2) in the entire yeast genome. Yeast, like most eukaryotes, contains a complete mitochondrial thioredoxin system including a thioredoxin (TRX3) and a thioredoxin reductase (TRR2), which is thought to function in protection against oxidative stress generated during the respiratory metabolism (13). Since the TRR2 gene product is a mitochondrial protein, it is not expected to interact with cytoplasmic thioredoxins. Our results obtained from *Δtrr2* and *Δtrr1Δtrr2* mutants also confirmed that *Trr2* is not involved in cytoplasmic thioredoxin reduction.

Although mechanistically similar, many thiol-dependent redox processes are catalyzed by structurally distinct families of enzymes, which are difficult to identify by the available protein function prediction programs. Even within the most studied family of thiol-dependent enzymes, thioredoxin-fold proteins, intergroup homology analyses can rarely identify functional relationships (14). Thus, the SED activity for cytoplasmic thioredoxins in yeast is probably performed by a protein that does not show a thioredoxin fold structure.

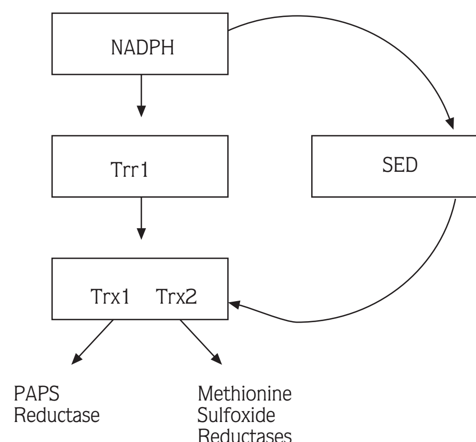


Figure 3. Model for SED action. Second electron donor bypasses the activity of *TRR1* and shuttles electrons to thioredoxins.

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References

1. Holmgren A, Slaby I. *Thioredoxin-C': mechanism of noncovalent complementation and reactions of the refolded complex and the active site containing fragment with thioredoxin reductase.* Biochemistry 18: 5591-9, 1979.
2. Moore EC, Reichard P, Thelander L. *Enzymatic synthesis of deoxyribonucleotides.* J Biol Chem 239: 3445-3452, 1964.
3. Black S, Harte EM, Hudson B et al. *A specific enzymatic reduction of L (-) methionine sulfoxide and a related nonspecific reduction of disulfides.* J Biol Chem 235: 2910-2916, 1960.

4. Wilson LG, Asahi T, Bandurski RS. *Yeast sulfate reducing system*. J Biol Chem 236: 1822-1829, 1961.

5. Muller EGD. *Thioredoxin deficiency in yeast prolongs S phase and shortens the G1 interval of the cell cycle*. J Biol Chem 266: 9194-9202, 1991.

6. Gan ZR. *Yeast thioredoxin genes*. J Biol Chem 266: 1692-6, 1991.

7. Muller EG. *Thioredoxin deficiency in yeast prolongs S phase and shortens the G1 interval of the cell cycle*. J Biol Chem 266: 9194-202, 1991.

8. Holmgren A, Bjornstedt M. *Thioredoxin and thioredoxin reductase.* Methods Enzymol 252:199-208, 1995.

9. Kryukov GV, Kumar RA, Koc A et al. *Selenoprotein R is a zinc-containing stereo-specific methionine sulfoxide reductase.* Proc Natl Acad Sci USA 99 p. 4245-50, 2002.

10. Koç A, Gash AP, Rutherford JC et al. Methionine sulfoxide reductase regulation of yeast lifespan reveals reactive oxygen species-dependent and -independent components of aging. PNAS 101: 7999-8004, 2004

11. Kumar RA, Koc A, Cerny RL et al. *Reaction mechanism, evolutionary analysis, and role of zinc in Drosophila methionine-R-sulfoxide reductase*. J Biol Chem 277: 37527-35, 2002.

12. Trotter EW, Grant CM. *Overlapping roles of the cytoplasmic and mitochondrial redox regulatory systems in the yeast Saccharomyces cerevisiae.* Eukaryot Cell 4: 392-400, 2005.

13. Pedrajas JR, Kosmidou E, Miranda VA et al. *Identification and functional characterization of a novel mitochondrial thioredoxin system in Saccharomyces cerevisiae.* J. Biol. Chem 274: 6366-73, 1999.

14. Fomenko DE, Gladyshev VN. *CxxS: fold-independent redox motif revealed by genome-wide searches for thiol/disulfide oxidoreductase function.* Protein Sci 11: 2285-96, 2002.