Nuclear Thiol Peroxidase as a Functional Alkyl-hydroperoxide Reductase Necessary for Stationary Phase Growth of Saccharomyces cerevisiae*

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Yeast nucleus-localized thiol peroxidase (nTPx) was characterized as a functional peroxidase. There are two cysteine residues in nTPx. Replacement of Cys-106 or Cys-111 with serine resulted in a complete loss of thioredoxin-linked peroxidase activity. However, when their activities were measured in terms of the ability to inhibit oxidation of glutamine synthetase, C111S showed the same antioxidant activity as the wild type protein. SDS-PAGE gel analysis revealed that only C111S existed as the dimer form. In addition to the identification of Cys-106 as the primary catalytic site, these data suggest the formation of the intradisulfide bond as a part of the catalytic cycle between nTPx and thioredoxin. nTPx preferentially reduced alkyl-hydroperoxides rather than H2O2. Furthermore, a nTPx mutant strain showed higher sensitivity toward alkyl-hydroperoxide than hydrogen peroxide. Also, reduction of the viability of nTPx mutant strain against various oxidants supports an antioxidant role for nTPx.

EXPERIMENTAL PROCEDURES

Strains and Media—S. cerevisiae strains used in this study were grown in rich medium (YPD; 1% yeast extract, 2% glucose and YPGE; instead of glucose, 3% glycerol plus 1% ethanol) or in synthetic minimal medium supplemented with the appropriate nutrients. The tor1 mutant strain NH349-3d (MATa, leu2-3,112, ura3-52, rme1, trpl1, his4, GAL1, HMLa, tor1::LEU2-4) and its parent strain JK9-3da (MATa, leu2-3,112, ura3-52, rme1, trpl1, his4, GAL1, HMLa) were kindly provided by Dr. Michael N. Hall (University of Basel, Switzerland) (17). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Biozentrum, Switzerland). The ras1 mutant (MATa, leu2-3, 112, ura3-52, trp1-D36, ade2-101, lys2-801, spo11-1::LEU2–4), ras2 mutant (MATa, leu2-2, ura3-52, trp1-D36, ade2-101, lys2-801, spo11-1::LEU2–4), and their parent strain YPK9 (MATa, leu2-3, ura3-52, trp1-D36, ade2-101, lys2-801) were kindly donated by Dr. S. Michael Jazwinski (Louisiana State University, New Orleans, LA). The wild type strain SEY2612 (MATa, leu2-3, ura3-52, his3-d200, lys2-801, spo11-1, su-29, mat-1) and its isogenic yap1 mutant SM13 (ysi1::HisG) was kindly given by Dr. Steen T. Coleman (University of Iowa, Iowa City, Iowa). The wild type strain JDF7–7C (MATa, ura3-52, leu2, trpa, K’ ) used in promoter study is our laboratory stock. The msn2/4 double mutant strain YM24 (MATa, ade2, can1, leu2, ura3, msn2-3::HIS3, msn4-1::TRP1) and its wild type W303-1A were kindly provided by Dr. Michael Jacquet (University of Paris, Paris, France). The wild type strains JD7 (MATa, ura3-52, leu2, trpa, K’ ) used in promoter study is our laboratory stock. The null mutant of nTPx was a laboratory stock used in previous work (22).

Cloning and Mutagenesis of nTPx—The nTPx gene was amplified from yeast genomic DNA by PCR with two pairs of primers covering whole coding sequence. The forward primer has an NdeI site and, the reverse primer has a BamHI site for cloning. The following primers were used for the PCR of the ntpx gene, and enzyme sites are underlined: 5'-CGATGGATCCATG GGT GAA GCA CTA CGT AG (forward) and 5'-CCGCGGATCCTTATATTATTTTGACACCAGACC) (reverse). The resulting PCR products were cloned into Escherichia coli expression plasmid pET7 (Novagen, Madison, WI). For substitution of two cysteine residues of nTPx, PCR-based strategy was employed to introduce nucleotide substitution at defined location (23). For replacement of putative functional cysteines of nTPx with serine, the respective cysteine codon was changed to serine codon. Three mutant proteins (C106S, C111S, and C106S/C111S) were generated by the standard PCR-mediated site-directed mutagenesis with complementary primers containing a 1-base pair mismatch that converts the codon for cysteine to the codon for serine. The mutated PCR products were ligated into pET7-Digested with NdeI and BamHI.

Expression and Purification of Recombinant TPx Proteins—Transformed E. coli cultures were cultured at 37 °C overnight in LB medium supplemented with ampicillin (100 μg/ml) and then transferred to fresh medium to the ratio of 1:200. When the optical density of the culture at 600 nm reached 0.4, isopropyl-1-thio-β-D-galactopyranoside was added to final concentration of 0.5 mM. After induction for 3 h, the cells were harvested by centrifugation and stored at −70 °C until use. The frozen cells were suspended in 50 mM Tris-HCl (pH 8.0) containing 2 mM phenylmethylsulfonyl fluoride and 1 mM EDTA and disrupted. The supernatants after centrifugation at 18,000 rpm using a SS-34 rotor at 4 °C were then dialyzed against 10 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, and 10 mM serine, were generated by the standard PCR-mediated site-directed mutagenesis with serine, the respective cysteine codon was changed to serine codon. Three mutant proteins (C106S, C111S, and C106S/C111S) were generated by the standard PCR-mediated site-directed mutagenesis with complementary primers containing a 1-base pair mismatch that converts the codon for cysteine to the codon for serine. The mutated PCR products were ligated into pET7-Digested with NdeI and BamHI.

Chemical Modification of nTPx with N-Ethylmaleimide—The TPx was preincubated in the absence or presence of 1 mM DTT at 30 °C for 30 min, and then chemical modification was carried out in a 100-μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 2.0 mM Na2S, and 10 mM N-ethylmaleimide at 30 °C for 2 h as previously described. To remove unreacted reagents, the reaction mixture was extensively dialyzed against 10 mM Tris-HCl (pH 7.4) at 4 °C.

Dithiothreitol-Dependent Antioxidant Activity—The antioxidant activity was determined by measuring the activity to protect the inactivation of E. coli glutamine synthetase (GS) by a thiolic metal-catalyzed oxidation system (DTPV/Fe2+O2-). The resulting recombinant (C106S, C111S, and C106S/C111S) and wild type proteins were homogeneously purified from the corresponding recombinants (Fig. 1). We determined the antioxidant activity of each recombinant protein in terms of its ability to protect against the inactivation of GS by thiolic metal-catalyzed oxidation system (Fig. 2A). In addition to the double-mutated nTPx, the C106S protein also did not show any antioxidant activity. In the presence of the Trx system (Trx, Trx reductase, and NADPH), the peroxidase activities of mutant proteins were also determined indirectly by monitoring the decrease of absorbance at 340 nm (Fig. 2B). In this assay system, in addition to C106S, C111S also did not significantly exert Trx-linked peroxidase activity. The activity analysis of three mutant proteins (C106S, C111S, and C106S/C111S) in two assay systems suggests that the Cys-106 is a primary catalytic site for the antioxidant reaction and that Cys-111 acts as a part of the catalytic cycle between nTPx and Trx via the formation of an intramolecular disulfide bond.

Members of the TSA/AhpC family can be divided into two subgroups, such as one-cysteine and two-cysteine groups according to the number of conserved cysteines within the protein.
The two Cys-containing protein exists as a homodimer via an intermolecular disulfide bond. Previously, we suggested that except for nTPx, all of the yeast TPx isoforms exist as the dimer (22). Nuclear TPx was detected at the molecular mass corresponding to that of monomer regardless of the presence or absence of DTT (22). Analysis of nTPx mutant proteins on a nonreducing and reducing SDS-PAGE gel showed that in contrast to other mutant and wild type proteins, most of the C111S proteins exist in the dimer form (Fig. 1). The existence of C111S is noteworthy that in contrast to other yeast TPx isoforms (22), nTPx more easily reduced bulky hydroperoxide such as cumene-hydroperoxide containing benzyl group as the alkyl moiety. The preference for bulky substrate suggests that nTPx may be designed to remove the hydroperoxide linked to bulky groups such as the base groups of DNA. Collectively, these data demonstrate that nTPx is a functional thiol peroxidase exerting alkyl-hydroperoxide reductase activity.

Physiology of nTPx Null Mutant Strain—To investigate the in vivo antioxidant function of nTPx, we imposed various oxidants on nTPx null mutant (nTPxΔ) growing aerobiocally on LB medium containing varying concentrations of 4-NQO. The growth of nTPxΔ was considerably retarded by 4-NQO, a DNA oxidation agent, when compared with the isogenic strain (Fig. 3). To test the viability of nTPx mutant toward oxidative stress, we exposed various oxidants to the mutant. As expected, nTPxΔ was much more sensitive to paraquat, diamide, and 4-NQO than the isogenic strain (Fig. 4). In addition, the sensitivity of nTPxΔ to various peroxides was examined. As shown in Fig. 5, nTPxΔ showed higher sensitivity toward alkyl-hydroperoxide such as t-butyl-hydroperoxide and cumene-hydroperoxide than the wild strain but did not toward H2O2, indicating the in vivo function of nTPx as an alkyl-hydroperoxide reductase. The alkyl-hydroperoxide-specific sensitivity of nTPxΔ can be explained in terms of its alkyl hydroperoxide-selective kinetic properties shown in Fig. 2C. Furthermore, the reduction of the viability of nTPxΔ mutant toward various oxidants supports an in vivo antioxidant role for nTPx.

Transcription of nTPx Gene Is Turned On at the Diauxic Shift—When the cells are cultured in a liquid-rich medium in which the major carbon source is a fermentable carbohydrate (e.g. YPD), they exhibited two distinct growth phases, followed by a stationary phase in which cells cease to divide. During the first phase, cells meet their energy requirements primarily by fermentation. The second growth phase is initiated when cells exhaust most of the fermentable carbon source, undergo major physiological change, and begin to grow at a much slower rate. The shift between these two phases is called the diauxic shift (a switch from fermentative to oxidative metabolism) (26). Several genes such as HSP26 are transcriptionally induced during the diauxic shift, in which dramatic changes in gene expression occur (27). Therefore, it is necessary to monitor the transcriptional activity of nTPx during growth.

To follow a growth-dependent transcription of nTPx gene, we monitored the transcriptional activity of nTPx as function of the growth. Comparison of growth curve before the diauxic shift with their corresponding transcriptional activity (Fig. 6A) indicates that the transcriptional activity of nTPx is not activated in the log phase cells. Therefore, this result suggests the possibility that nTPx gene could be transcriptionally induced during the diauxic shift. To test the possibility, we measured the possible transcriptional activation of nTPx gene upon changing the carbon source from fermentable carbohydrate (i.e. glucose) to nonfermentable carbohydrate (glycerol plus ethanol). As shown in Fig. 6B, the transcriptional activity in the yeast growing on a nonfermentable carbon was about four times higher than that of the yeast on a fermentable carbon when the activities were measured at the mid-log phase growth. Furthermore, the transcriptional activities of Tor1Δ were much higher when compared with those of the isogenic strain (Fig. 6B). Rapamycin forms a complex with FKBP12 that inhibits components of signal transduction pathways, named TOR (target of rapamycin) pathway. The target of the complex was first identified in yeast as the Tor1p and Tor2p (28). The loss of TOR function at the diauxic shift induces several other physiological changes characteristic of starved cells entering

![Fig. 1. SDS-PAGE (A) and Western blot (B) analyses of nTPx proteins. A, lane 5 shows size markers (14.4, 21.5, 31.0, 45, 66.2, and 97.4 kDa from the bottom). Lanes 1–4 show the reducing SDS-PAGE, and lanes 6–9 show nonreducing SDS-PAGE. Lanes 1 and 6, wild type nTPx; lanes 2 and 7, C106S mutant protein; lanes 3 and 8, C111S; lanes 4 and 9, C106S/C111S, a double mutant protein. The corresponding Western blot is shown in B. The dimer form band is designated d, and the monomeric form band is designated m.](http://www.jbc.org/)

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the stationary phase. Inhibition of the TOR signaling pathway by a lack of fermentable carbon induces a set of proteins necessary for the stationary phase yeasts (29, 30). Taken together with the function of the TOR signaling pathway described above, these results suggest that the transcription of nTPx is turned on at the diauxic shift.

The increase of the nTPx transcript upon deletion of Tor1 suggests that the transcription of nTPx is under down-regulation of TOR pathway. To demonstrate negative control on transactivation of the nTPx promoter by TOR pathway, we monitored the transcriptional activity of the nTPx promoter in tor1 mutants throughout the yeast growth cycles. Figs. 6B and 7A show that disruption of Tor1 resulted in a dramatic increase, which is consistent with the Northern blot analysis (Fig. 7A, inset). Taken together, these results demonstrate that Tor1p suppresses the transactivation of nTPx via activation of the TOR pathway.

In S. cerevisiae, Ras proteins, which encode GTP-binding proteins, are activated by both growth signals (e.g. glucose) (31) and stress signals (e.g. UV radiation and starvation) (32–34). The unregulated Ras/cAMP pathway suppresses the activation of a large number of stress-related genes (32–34). Ras proteins activate the cAMP-dependent protein kinase A activity, which in turn turns on TOR pathway before the diauxic growth. We have demonstrated the negative control of the TOR signaling pathway on the expression of nTPx in response to glucose starvation (Fig. 8). To elucidate the transactivation of nTPx under negative control of Ras/cAMP pathway, we determined the transactivational ability of nTPx in Ras1 and Ras2 mutants...
Antioxidant Function of Nuclear Thiol Peroxidase in Yeast

**Fig. 5.** The sensitivity of the nTPx null mutant toward various peroxides. The wild type (W) and null mutants (M) were compared for their ability to grow on YPD plates containing increasing concentrations of peroxides (H₂O₂, HOOH; t-butyl-hydroperoxide, tBOOH; cumene-hydroperoxide, COOH) (H₂O₂, 1, 2, and 3 mM; t-butyl-hydroperoxide, 0.2, 0.3, and 0.4 mM; cumene-hydroperoxide, 0.05, 0.1, and 0.15 mM, from left to right). For each strain, 10 μl of overnight culture diluted to 20, 5, or 1 × 10⁵ cells (from spot 1 to spot 5) were spotted on plates. Growth was monitored after 2–4 days.

**Fig. 6.** Transcriptional activity of nTPx promoter. lacZ fusion vectors containing nTPx promoter as the promoter of lacZ structural gene were transformed in strains JK9-3da, the wild type of Tor1 mutant strain. The cells were cultured in synthetic minimal medium and harvested to determine the activity of the expressed β-galactosidase (β-Gal) at the indicated times (A). The growth was determined in terms of the increase of optical density at 600 nm. In the experiments shown in B, the cells (JK9-3da and Tor1 mutant) were cultured in synthetic minimal medium and harvested at 5 A 600 nm to determine activity of the expressed β-galactosidase in the presence of glucose (2%) (fermentable carbon source) or glycerol (3%) plus ethanol (EtOH, 1%) as a nonfermentable carbon source. The values represent the averages of the five independent experiments.

(FIG. 7B). The induction pattern of the transcriptional activities in Ras mutants shows that in contrast to the wild type cell (YPK9), the transactivation of nTPx in Ras1Δ and Ras2Δ occurs during early log phase ras1Δ and ras2Δ cells (Fig. 7B). Each disruption of Ras1 and Ras2 resulted in an increase in the transcriptional activity with ~3- and ~10-fold inductions, respectively, even in the mid-log phase cells (cells cultured for 12 h), which is consistent with the Northern blot analysis (Fig. 7B, inset). Taken together, these results indicate that the transactivation of nTPx gene at the diauxic shift resulted from the derepression of the negative control of the Ras/cAMP-TOR pathways.

**nTPx Is Inducible in Response to Oxidative Stress**—In yeast, Yap1p and Msn2p/4p have been known to be a transcriptional factor in response to oxidative stress. Yap1-mediated transcription can be activated by oxidative stress (35), and the activation is attributed to oxidative stress-induced nuclear localization of the Yap1 involving the nuclear export receptor Crm1 (Xpo 1) (36, 37). Another important transcriptional factor, Msn2p, and the partially redundant factor Msn4p are key regulators of oxidative stress-responsive genes expression (38). In addition to their involvement in the oxidative stress response, they are also implicated in the control of the multiple stress responses to carbon source starvation, osmotic stress, and heat stress (38). We performed semi-quantitative studies of nTPx transcripts in yeast mutants lacking Yap1p and Msn2p/4p (i.e. yap1Δ and msn2p/4pΔ, respectively). To test whether Msn2p/4p and Yap1p are involved in the induction of nTPx, the transcriptional activity of nTPx in msn2p/4pΔ and yap1Δ strains were examined. Fig. 8 shows that the transcriptional activity in the msn2p/4pΔ strain is ~50% of that of the isogenic strain, but the activity in the Yap1Δ strain is not changed when compared with the parent type strain, which indicates that Msn2p/4p acts as a transcriptional factor, but Yap1p does not.

nTPx (YIL010W/DOT5) was suggested to be involved in the derepression of telemelic silencing (39), and its mRNA was ~3-fold induced after exposure to alkylating agent, methylmethanesulfonate (40). However, the transcriptional response of nTPx upon oxidative stress as an antioxidant was not investigated. To examine the transcriptional response of nTPx to oxidative stress, Northern blots were performed using total RNA from exponentially growing yeast (W303-1a) in the presence of varying concentrations of H₂O₂ and diamide (Fig. 9A). The nTPx RNA level was not greatly but significantly induced upon H₂O₂ and diamide. The induction was considerably reduced in the Msn2Δ strain (Fig. 9B) compared with its wild type strain, which is consistent with the transcriptional activity data shown in Fig. 8B. Taken together, these data suggest that nTPx is an inducible protein in response to oxidative stress and that Msn2p/4p acts as one of the transcriptional factors to induce the expression of nTPx in response to oxidative stress.
Nuclear TPx Acts as an Antioxidant Necessary for Stationary Phase Survival of Yeast Cells

We have demonstrated that the transcription of nTPx is activated at the diauxic shift, which is strictly negatively regulated by Ras-TOR signaling pathway (Fig. 7). The transcriptional activation at the diauxic shift suggests that nTPx takes an important antioxidant role in stationary phase growth, and thus nTPx is one of the antioxidants necessary for stationary phase growth. To address the antioxidant function of nTPx in stationary phase yeast cells, we explored the effect of nTPx mutation on the survival in the stationary phase. Nuclear TPx mutant and its parent strain as a control were grown to the stationary phase under the condition of high aeration for 3 days and then were washed and resuspended in water, as described under “Experimental Procedures.” The viability of the stationary phase cells was then determined as a function of time (Fig. 10A). The wild type strain maintained viability of ~50% for 16 days, whereas the nTPx mutant died much sooner. Only ~10% of the nTPxΔ strain survived for the same time. The 5-fold decrease in the viability of nTPxΔ indicates that nTPx acts an important antioxidant necessary for stationary phase survival of yeast cells.

To demonstrate further the antioxidant function of nTPx in the stationary phase growth rather than in exponential phase growth, 4-NQO as an oxidant was subjected to stationary phase and log phase nTPx mutant cells, and the cell viabilities were investigated (Fig. 10B). Exponential and stationary growth
Phase cells were plated on the YPD plate containing 0.5 mM 4-NQO, and the cell viability was measured in terms of the number of the survival colonies. For the wild type strain, the stationary phase cells survived longer than the log phase cells, which is consistent with the fact that stationary phase cells are more resistant to oxidative stress. However, as seen in the experiment with stationary phase cells (Fig. 10B), the cell viabilities of the nTPx mutant cells were 3-fold decreased compared with the viability of the stationary phase parent strain, which suggests the antioxidant function of nTPx in the stationary phase growth rather than in exponential phase growth. The model carcinogen 4-NQO can exert genotoxic potential via the generation of reactive oxygen species (ROS). Therefore, the high susceptibility of nTPx mutant toward a DNA-damaging agent such as 4-NQO can elucidate the antioxidant function of nTPx to protect against DNA oxidation by ROS.

**DISCUSSION**

As far as we know, nTPx is the first reported thiol peroxidase to be located in the nucleus, which implies that nTPx could act as a peroxidase to protect oxidative damage of DNA against ROS. Despite the physiological significance as a nuclear antioxidant, there is no evidence supporting *in vivo* function of nTPx as an antioxidant. In this work, we have investigated the *in vivo* function of nTPx in *S. cerevisiae*. Several lines of data demonstrate that nTPx acts as a functional alkyl-hydroperoxide thiol peroxidase necessary for stationary phase growth of *S. cerevisiae*: (i) nuclear TPx acts as a Trx-linked alkyl-hydroperoxi-
Antioxidant Function of Nuclear Thiol Peroxidase in Yeast

24643

oxide thiol peroxidase; (ii) nuclear TPx null mutant strain showed a higher sensitivity toward various oxidants; (iii) transcription of nTPx gene is turned on at the diauxic shift; (iv) the nTPx RNA level is induced upon oxidative stress; and (v) nuclear TPx acts an antioxidant necessary for the stationary phase survival of yeast cells.

Nuclear localization of nTPx indicates the antioxidant role in the nucleus. Despite the higher sensitivity of nTPxΔ toward various oxidants including alkyl-hydroperoxide such as t-butyl-hydroperoxide and cumene-hydroperoxide, the insensitivity of nTPxΔ toward H2O2 suggests its in vivo physiological function as an alkyl-hydroperoxide peroxidase. These results imply the antioxidant function of nTPx as an alkyl-hydroperoxide peroxidase to prevent from DNA oxidation against alkyl-hydroperoxide. It has been known that as yeast grows older, oxidative stress is gradually increased, and then stationary phase yeast cells suffer from the high level of ROS. In the stationary phase in which glucose as a carbon source is depleted, yeast begin to use ethanol and other secondary metabolites such as lipid as energy sources. In that situation, ROS including alkyl hydroperoxide such as fatty acid hydroperoxide should be highly accumulated in the cells. Therefore, during the post-diauxic shift, protection of DNA oxidation against ROS is essential for cell survival. The induction of nTPx at the diauxic shift suggests that nTPx acts as an important alkyl hydroperoxide peroxidase to maintain the yeast cells during stationary phase growth. The significant decrease in the viability of nTPxΔ compared with the isogenic strain supports the idea that nTPx acts an antioxidant necessary for stationary phase survival in the aerobic life of yeast.

In addition to the severe growth retardation of nTPxΔ in the presence of 4-NQO, a DNA-damaging oxidant, it is noteworthy that in contrast to other yeast isoenzymes (cTPx I, cTPx II, cTPx III, and mTPx III) (22), nTPx is more active toward bulky hydroperoxides such as DNA base-anchored hydroperoxide, an antioxidant necessary for stationary phase survival in the stationary phase survival of yeast cells.

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