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Overexpression of Peroxisomal Malate Dehydrogenase *MDH3* Gene Enhances Cell Death on H_2O_2 Stress in the *ald5* Mutant of *Saccharomyces cerevisiae*

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Abstract. Mitochondrial aldehyde dehydrogenase ALD5 of *Saccharomyces cerevisiae* is involved in the biosynthesis of mitochondrial electron transport chain, and the *ald5* mutant is incompetent for respiration. With use of the mutant, we examined the detoxication of H_2O_2 generation by fatty acid β-oxidation in peroxisome. The *ald5* mutant (AKD321), as well as the 746 ρ^0 mutant, was more resistant to H_2O_2 stress than the wild type. However, overexpression of the *MDH3* gene that was involved in the reoxidation of NADH during fatty acid β-oxidation caused a decrease in cell viability of AKD321 to H_2O_2 stress, while the 746 ρ^0 mutant had no such effect. Intracellular H_2O_2 concentration increased approximately fourfold in *MDH3* overexpressing *ald5* strain (MD3-AKD321), compared with AKD321. The peroxisomal catalase activity of MD3-AKD321 decreased by 83% to that of AKD321. And also, the overexpression of MDH3 had only a weak effect in *MDH3* overexpressing 746 ρ^0 strain, decreasing by 14% to that of 746 ρ^0 mutant. The increased palmitoyl CoA oxidation by overexpression of *MDH3* gene was the same in both strains. Under conditions of MDH3 overexpression, peroxisomal catalase (CTA1) appears to be a limiting factor to oxidative stress. These observations point to an important, as yet unidentified, role of mitochondrial aldehyde dehydrogenase (ALD5) to endogeneous oxidative stress in peroxisome.

In aerobically growing cells, elimination of oxygen-free radicals and other reactive oxygen species such as hydrogen peroxide is important to protect all cellular components [16, 21]. Reactive oxygen species are produced as byproducts from incomplete reduction of molecular oxygen in the mitochondrial electron transport chain during respiration [1, 18, 24]. Also, hydrogen peroxide occurs by β-oxidation of fatty acid even in peroxisome. This reaction, the acyl-CoA oxidase reaction, involves participation of an FAD cofactor, although the reaction differs from its mitochondrial counterpart in that the electrons are transferred directly to O_2. Living cells can respond to avoid oxidative damage via the synthesis of proteins including the enzymes, such as catalase, superoxide dismutase, and gluthathione peroxidase [8, 27]. Most of the studies to oxidative stress of the yeast *Saccharomyces cerevisiae* have focused on mitochondria, so attention has not been paid to peroxisome as yet. In this work, when fatty acid β-oxidation was accelerated in the peroxisome, we investigated how yeast cells would adapt to it.

In *S. cerevisiae*, mitochondrial aldehyde dehydrogenase ALD5 is a minor enzyme and is involved in the biosynthesis of the mitochondrial electron transport chain [11, 26]. The *ald5* mutant differs from the ρ^0 mutant in the normality of mitochondrial DNA, although both mutants are respiratory incompetent. We examined the difference between both mutants in response to oxidative stress in peroxisomes. The peroxisomal malate dehydrogenase (MDH3) was identified by Steffan and McAllister-Henn [23] and was involved in the reoxidation of NADH generated during fatty acid β-oxidation [25]. Therefore, a possible approach for investigating the effect of oxidative stress on the peroxisome is to overexpress the *MDH3* gene.

Saccharomyces cerevisiae has two catalases; catalase A (CTA1), peroxisomal enzyme, and catalase T (CTT1), a cytoplasmic enzyme [10]. The double mutant

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of catalases in *S. cerevisiae* was more sensitive to H_2O_2 than the wild-type and single mutants [7]. However, the expression of *CTA1* was unaffected in response to H_2O_2, in contrast to *CTT1* [4].

This paper describes that the overexpression of peroxisomal *MDH3* gene might have an influence on the activity of peroxisomal catalase that is dependent on the existence of mitochondrial aldehyde dehydrogenase ALD5.

Materials and Methods

Yeast strains and plasmid. Yeast strains of *Saccharomyces cerevisiae* used in this study are derived from DBY 746 (MATα *his3 leu2-3,112 trp1-289 ura3-52*) obtained from The Yeast Genetic Stock Center. AKD321 was made by disruption of ALD5 in strain DBY 746, as described previously [11]. *Petite* strains were generated by treatment with ethidium bromide [20]. Both ρ^0 and *ald5* haploid strains were transformed with the *MDH3* overexpressing plasmid, pRS426-MDH3. Plasmid pRS426-MDH3 was constructed by cloning the *MDH3* gene into the *Xho*I/*Sal*I sites of multi-copy vector of pRS426. The *MDH3* gene was amplified from the DBY746 genome by polymerase chain reaction using the forward primer 5′-TTAGATTAGAGGGAAATA-AATTGCA-3′ and the reverse primer 5′-TCTCATGATTTTTT-TCGGT-3′. The 3.8-kb PCR product was digested with *Xho*I and *Sal*I and was cloned into *Xho*I/*Sal*I sites of pRS426. Yeast transformations were performed with the improved lithium acetate procedure [5].

Media and culture conditions. Yeast strains were grown in either YPD medium (1% yeast extract, 2% polypeptone, 2% glucose) or YNB medium (0.67% yeast nitrogen base, 2% glucose) supplemented with appropriate amino acids and base. Media were solidified by the addition of 2% agar. Growth was followed by monitoring the turbidity at 600 nm.

Hydrogen peroxide treatment. Cells were inoculated to a density of 10^5 cells/mL and were grown to the exponential phase (about 10^7 cells/mL) in YNB medium, were harvested by centrifugation, and were resuspended in 0.1 M potassium phosphate buffer (pH 7.5) to obtain the initial $OD_{600} = 0.1$. A lethal H_2O_2 survival assay was performed with aliquots of cell suspension by addition of final concentrations of 2 mM H_2O_2. Cells were maintained at 30°C for 1 h with shaking. Cell survival was monitored by plating serial dilutions of samples from each treatment onto YPD plates. Where indicated, hemin was added to the growth medium at concentrations of 4, 10, and 30 mg/L, respectively.

Preparation of peroxisomal fraction. Peroxisomal fractionations were modified by the method of Kapperli et al. [9]. Late exponential phase cells were harvested by centrifugation and washed with 0.1 M Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, and 1 M sorbitol (buffer A). The cell wall was digested with Zymolyase 100-T at a concentration of 1 mg/mL in buffer A. Incubation was performed with gentle shaking for 1 h at 30°C. The spheroplasts were washed twice in buffer A, then resuspended in 0.1 M Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, and 0.25 M sucrose, and lysed by a short sonic treatment. The resulting homogenate was centrifuged at 4000 *g* for 10 min, and the supernatant was designated as cell-free extract. The cell-free extract was centrifuged at 12,000 *g* for 15 min, which mainly sediments the mitochondria. The postmitochondrial supernatant was centrifuged at 25,000 *g* for 15 min. Solid $CaCl_2$ was added to the resulting supernatant up to a concentration of 16 mM. The peroxisomal fraction was then collected by centrifugation at 15,000 *g* for 15 min. The sediment was washed in 0.1 M Tris-HCl containing 0.15 M KCl, pH 8.0, and centrifuged at 25,000 *g* for 15 min. The sediment (peroxisomes) was resuspended in 0.1 M Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, and 0.25 M sucrose.

Measurement of hydrogen peroxide. For determination of hydrogen peroxide concentration in crude extracts, cells were grown to the late exponential phase and were disrupted with glass beads in a MSK cell homogenizer (B. Braun, Melsungen). The homogenate was centrifuged at 12,000 *g* for 15 min, and the supernatant was used for measurement of hydrogen peroxide. The quantitative assay of hydrogen peroxide, and indirectly of superoxide, was performed by the method of Rapoport et al. [19]. Ten-microliter aiquots of the supernatants of crude extracts were added to 1 mL of 10 mM Hepes buffer, pH 7.2, and 100 mM KCl including 6% methanol, 10 U/mL superoxide dismutase, and 200 U/mL catalase that are necessary for conversion of superoxide to H_2O_2 and subsequent oxidation of methanol to formaldehyde. The reaction mixture was incubated at 37°C for 20 min and was stopped by adding two reaction volumes of Nash reagent and immersing immediately in a 60°C bath for 10 min. The reaction mixture was then transferred to a cuvette, and fluorescence was measured with a Japan Spectroscopic FP-770 spectrofluorophotometer (excitation and emission wavelengths of 412 and 505 nm, respectively). In all experiments, control readings were performed with all reaction components, except the enzymes of superoxide dismutase and catalase, and their fluorescence was subtracted from the sample readings.

Enzme assay. Malate dehydrogenase activity was measured as the oxaloacetate-dependent rate of NADH oxidation in assay mixtures containing 0.1 M KPO_4, pH 7.4, 0.12 mM NADH, and 0.33 mM oxaloacetate [23]. One unit of the enzyme activity is the amount of enzyme producing 1 μmol of NAD^+ per min. Catalase activity was assayed by measuring the decomposition of H_2O_2 at 240 nm. One unit of the enzyme activity was expressed as 1 μmol of H_2O_2 decomposed/min per mg of protein [2]. Fatty acid β-oxidation activities were determined in peroxisomal lysates in buffer containing 0.175 mM Tris-HCl, pH 8.5, 50 μM palmitoyl-CoA, and 50 μM FAD. Oxygen uptake was measured polarographically with a Clark electrode in a closed chamber at 25°C. The activity of β-oxidation was determined from the slope and a plot of O_2 concentration versus time. Protein concentration was measured by the method of Lowry et al. [13] with bovine serum albumin as a standard.

Results

Increasing the sensitivity to hydrogen peroxide by the *ald5* mutant overexpressing *MDH3*. As expected, it was observed that the *ald 5* mutant (AKD321) as well as the ρ^0 mutant (746 ρ^0) was more resistant to H_2O_2 than the wild-type (Fig. 1A). Since the *ald5* mutant is defective in respiration, these results indicate that the absence of respiratory function was due to the increased resistance to H_2O_2 in the same way as with lipid hydroperoxide, as described previously [3]. A better understanding of oxidative stress is necessary for living yeast cells to consider not only the exogenous adaptive response, but also the response to the oxidative stress generating intracellularly. Thereupon, we examined the adaptive response of the yeast to H_2O_2 that may occur in peroxisomes with overexpression of the *MDH3* gene.

Overexpression of the *MDH3* gene had no effect on

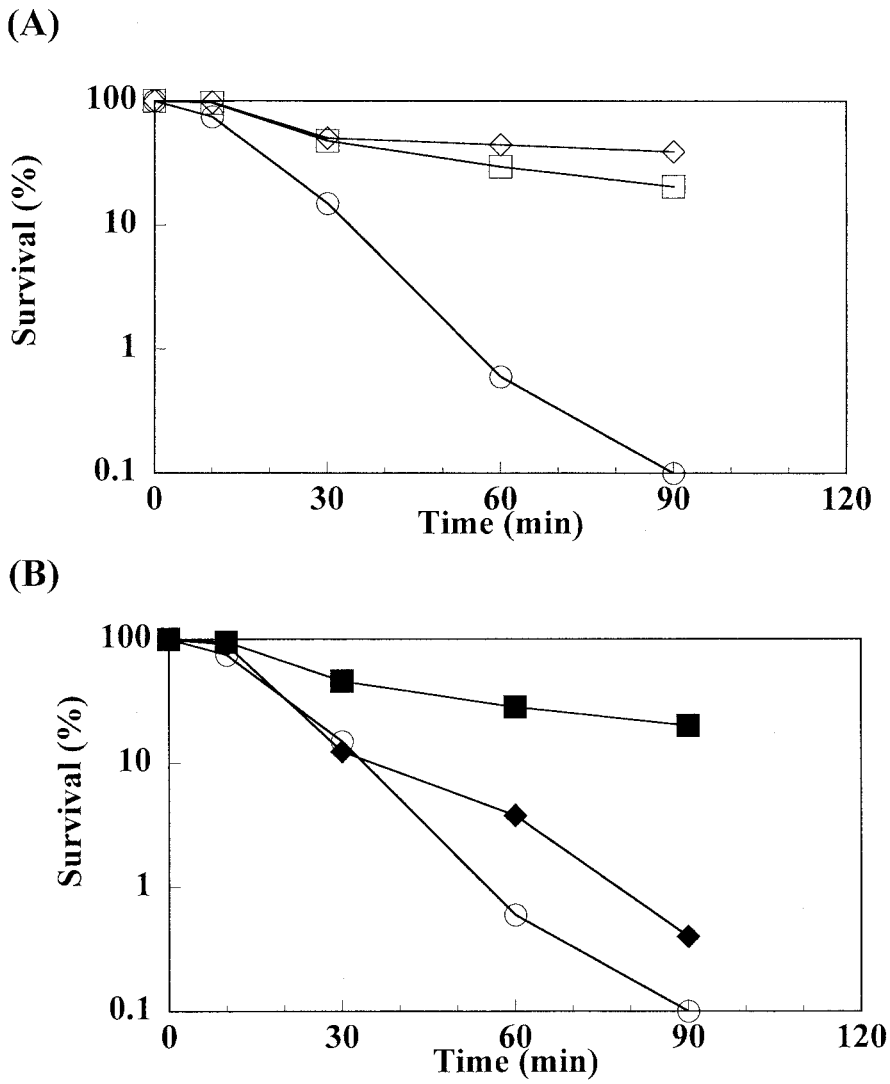

Fig. 1. Sensitivity to hydrogen peroxide. (A) Strains DBY746 (○), DBY746 ρ^0(□), and AKD321(◇) and (B) strains DBY746 (○), MD3-746 ρ^0(■), and MD3-AKD321(◆) were grown to exponential phase in YNB medium and treated with 2 mM H_2O_2 at 30°C. Cells were diluted and plated in triplicate onto YPD medium to monitor cell viability. Percentage survival is expressed relative to the untreated control culture (100%). Data are means of triplicates from a representative experiment.

the sensitivity to H_2O_2 in 746 ρ^0 mutant, while the significant decrease in cell viability of AKD321 was caused by it (Fig. 1B). These results suggest that regulation other than mitochondrial respiratory function will work on the detoxication of H_2O_2 when the *MDH3* gene is overexpressed.

Effect of overexpression of *MDH3* on the intracellular contents of H_2O_2. We examined whether changes of the intracellular concentrations of H_2O_2 would be present with overexpression of the *MDH3* gene. In 746 ρ^0 and AKD321, overexpression of the *MDH3* gene led to an approximately 1.4-fold and 4-fold increase in the concentrations of H_2O_2 in crude extracts, respectively (Fig. 2). This observation was thought to be due to the progress of fatty acid β-oxidation with overexpression of the *MDH3* gene. However, the level of increased H_2O_2 concentration was different between MD3-746 ρ^0 (14.2 μmol/mg protein) and MD3-AKD321 (44.3 μmol/mg protein). The difference might result from the difference of cell viability to exogeneous H_2O_2 stress in both strains.

Effect of overexpression of *MDH3* gene on fatty acid β-oxidation and catalase activity. To ascertain why overexpression of the *MDH3* gene brought about the significant increase in intracellular H_2O_2 concentrations in MD3-AKD321, we investigated the oxidation of palmitoyl CoA and catalase activity in peroxisomes. As shown in Table 1, palmitoyl CoA oxidation increased approximately 1.4-fold in both MD3-AKD321 and MD3-746 ρ^0, compared with the corresponding control strains. The catalase activity of MD3-AKD321 decreased by 83% to that of AKD321, while that of MD3-746 ρ^0

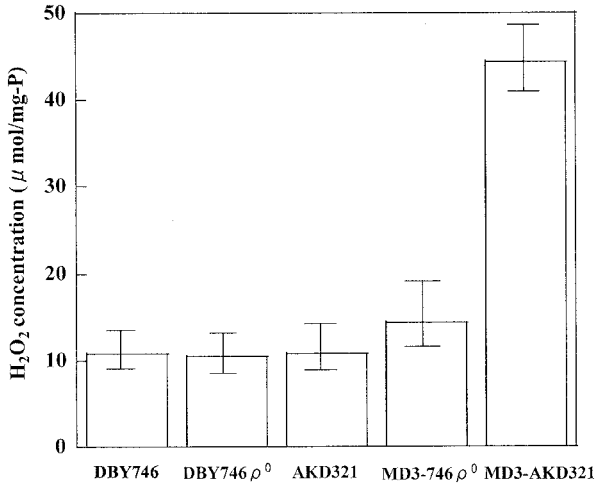

Fig. 2. H_2O_2 concentrations in strains DBY746, DBY746 ρ^0, AKD321, MD3-746 ρ^0, and MD3-AKD321. Hydrogen peroxide was measured in crude extracts from the strains. Each bar represents the mean ± SE of the results of two experiments.

decreased by 14% to that of 746 ρ^0. Thus, the extreme sensitivity of H_2O_2 stress in MD3-AKD321 was due to the reduction of peroxisomal catalase activity.

Effect of heme on the sensitivity to hydrogen peroxide in *ald5* mutant. Peroxisomal catalase A is a heme-containing enzyme. Heme is known for its ability to bind oxygen reversibly in heme-containing enzymes. Therefore, we examined whether the increased sensitivity to H_2O_2 stress in MD3-AKD321 was due to the decreased activity of catalase as the result of the deficiency of heme. In MD3-AKD321, the reduced sensitivity of H_2O_2 stress was observed in the presence of hemin in the medium, while in AKD321, addition of hemin had no effect on it (Fig. 3). These results suggest that the decreased activity of peroxisomal catalase caused by overexpression of the *MDH3* gene was partially due to the lack of heme.

Discussion

In our previous study, we found that the yeast mutant lacking mitochondrial aldehyde dehydrogenase (ALD5) is respiratory incompetent and has few cytochromes [11]. The *ald5* mutant differs from *petite* mutant (ρ^0) that has normal cytochome *c* content, as well as the wild-type. The *ald5* mutant was insensitive to exogeneous H_2O_2, whereas the overexpressing *MDH3-ald5* strain was more sensitive to it. Since the ρ^0 mutant had no effect of overexpression of *MDH3* gene on the sensitivity to H_2O_2, the cytochrome *c* content was thought to be important to adapt the endogeneous oxidative stress induced with overproduction of MDH3. Certainly, overexpression of the *MDH3* gene induced peroxisomal β-oxidation, and as a result, intracellular H_2O_2 concentration increased. Cytochrome *c* is the electron transfer protein [12], and the reduction of the cytochrome *c* content in the *ald5* mutant may disturb electron transfer systems and introduce cell death to endogeneous oxidative stress such as overexpression of *MDH3* gene.

This study has elucidated the functions of various cellular responses with respect to endogeneous oxidative stress. One of the functions is that the reduction of peroxisomal catalase (CTA1) is caused by overexpression of *MDH3* gene, in particular, in the *ald5* mutant. Overexpression of the *MDH3* gene might lead to a detrimental effect of the peroxisomal redox balance in terms of the NAD/NADH, because MDH3 is involved in the reoxidation of NADH generated with fatty acid β-oxidation, and the peroxisomal membrane is impermeable to NAD(H) under in vivo conditions [25].

In *Saccharomyces cerevisiae*, cytosolic catalase (CTT1) was stimulated with exposure to exogenous H_2O_2, but not peroxisomal catalase (CTA1). The induction of the *CTA1* gene was present at the stationary phase [4], and a double mutant unable to produce either CTA1 or CTT1 was more sensitive to H_2O_2 than the wild-type or single mutant at the stationary phase [7]. Also, mutants lacking both catalases exhibit a decreased life span in 55% oxygen [17]. We observed the notable reduction of life span in MH3-AKD321, which had low activity of peroxisomal catalase (data not shown). Therefore, CTA1 seemed to be needed for reaching a normal life span.

Grant et al. has shown that glutathione and catalase provide overlapping defense system against hydrogen peroxide in *S. cerevisiae* [6]. Glutathione disulfide is reduced to a reduced form of glutathione by glutathione reductase in the presence of NADPH. Interestingly, mitochondrial ALD5 utilized both NAD and NADP as cofactors, and the enzyme activity preferred NADP to NAD [26]. In addition, binding NADPH and heme to peroxisomal catalase A was indispensable to the increased activity [14]. Thus, ALD5 might play a role in not only the biosynthesis of heme, which is an essential component of the cytochromes [11], but also in the supply of NADPH for performing the efficient action of peroxisomal catalase A. We thought heme deficiency might be one of the reasons why the reduction of catalase activity was caused by overexpression of *MDH3* gene in the *ald5* mutant. The expression of *CTA1* is markedly decreased in the absence of heme [22]. In fact, in MD3-AKD321, a small reduction of sensitivity to H_2O_2 was observed in the presence of hemin. Considering that, NADPH may be needed for adequate recovery of adaptation to H_2O_2 stress.

Minard and McAlister-Henn suggest that cellular

Table 1. Enzymatic activities of malate dehydrogenase, palmitoyl-CoA β-oxidation, catalase in peroxisomal extracts of strains DBY746, DBY746 ρ^0, AKD321, MH3-746 ρ^0, and MD3-AKD321

Strains	Malate dehydrogenase (mU/mg of protein)	Palymitoyl-CoA β-oxidation (μM O_2/min/mg of protein)	Catalase (mU/mg of protein)
DBY746	30.2 ± 3.8	0.81 ± 0.10	2.58 ± 0.34
DBY746 ρ^0	28.1 ± 2.8	0.73 ± 0.09	3.47 ± 0.30
AKD321	26.0 ± 3.4	0.80 ± 0.11	2.95 ± 0.23
MD3-746 ρ^0	31.7 ± 3.0	1.03 ± 0.14	2.99 ± 0.29
MD3-AKD321	35.3 ± 3.3	1.15 ± 0.16	0.50 ± 0.13

The values are means ± SE from two experiments.

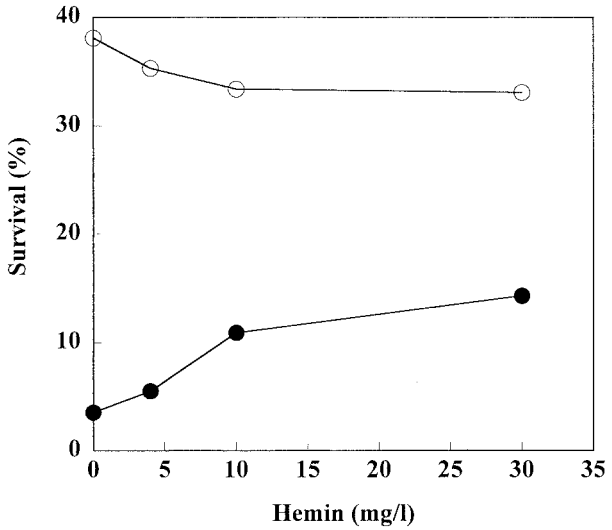

Fig. 3. Effect of hemin on sensitivity to hydrogen peroxide in the strains AKD321 and MD3-AKD321. Strains AKD321(○) and MD3-AKD321(●) were grown to exponential phase in YNB medium containing various concentrations (4,10, and 30 mg/L) of hemin and treated with 2 mM H_2O_2 at 30°C for 60 min. Cells were diluted and plated in triplicate onto YPD medium to monitor cell viability. Percentage survival is expressed relative to the untreated control culture (100%). Data are means of triplicates from a representative experiment.

mechanisms dependent on NADPH are crucial metabolic antioxidants [15]. In this context, it is of interest to examine the effects of the generation of NADPH by mitochondrial ALD5. Nevertheless, the relation of peroxisomal MDH3 and CTA1 remains to be clarified. This study allowed us to evaluate the sensitivity of hydrogen peroxide with endogeneous oxidative stress in peroxisomes, but, insofar as we could determine, there was no regulatory pathway to adapt oxidative stress between mitochondria and peroxisomes. Further study of endogeneous and exogeneous metabolic oxidative stress in yeast cells may shed more light on the functional pathway with respect to detoxication of reactive oxygen species.

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