The Cytoplasmic Cu,Zn Superoxide Dismutase of *Saccharomyces cerevisiae* Is Required for Resistance to Freeze-Thaw Stress

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The involvement of oxidative stress in freeze-thaw injury to yeast cells was analyzed using mutants defective in a range of antioxidant functions, including Cu,Zn superoxide dismutase (encoded by *SOD1*), Mn superoxide dismutase (*SOD2*), catalase A, catalase T, glutathione reductase, γ-glutamylcysteine synthetase and Yap1 transcription factor. Only those affecting superoxide dismutases showed decreased freeze-thaw tolerance, with the *sod1* mutant and the *sod1 sod2* double mutant being most affected. This indicated that superoxide anions were formed during freezing and thawing. This was confirmed since the *sod1* mutant could be made more resistant by treatment with the superoxide anion scavenger MnCl₂ or by freezing in the absence of oxygen, or by the generation of a rho⁰ petite. Increased expression of *SOD2* conferred freeze-thaw tolerance on the *sod1* mutant indicating the ability of the mitochondrial superoxide dismutase to compensate for the lack of the cytoplasmic enzyme. Free radicals generated as a result of freezing and thawing were detected in cells directly using electron paramagnetic resonance spectroscopy with either α-phenyl-N-tetra-butyl-nitronate or 5,5-dimethyl-1-pyrroline-N-oxide as spin trap. Highest levels were formed in the *sod1* and *sod1 sod2* mutant strains, but lower levels were detected in the wild type. The results show that oxidative stress causes major injury to cells during aerobic freezing and thawing and that this is mainly initiated in the cytoplasm by an oxidative burst of superoxide radicals formed from oxygen and electrons leaked from the mitochondrial electron transport chain.

Cryopreservation provides an excellent way of preserving living cells and storing them and has found wide application in medicine, agriculture, and food technology. However, the processes of freezing and thawing cause severe stress to cells and can lead to loss of viability. There is, therefore, a need to understand the molecular mechanisms that underlie freeze-thaw damage and how cells survive it or respond to prevent it. A number of hypotheses have been proposed to explain the damage caused by freezing and thawing; these are based on an analysis of the physical and chemical changes that occur. For example, cells are known to be damaged by physical changes associated with ice nucleation and dehydration (1). They can also be affected by accompanying changes in intracellular osmolarity and pH which lead to aggregation of macromolecules (2) and denaturation of proteins (3, 4). Oxidative damage has also been considered to be a factor since an oxidative burst has been predicted to occur during thawing (5), and this would lead to the generation of reactive oxygen species (ROS)¹ and oxidative damage to cellular components. This is supported by the observation that antioxidant defense systems of reptiles are activated by freezing stress (5) and that overexpression of superoxide dismutase enhances the freezing tolerance of transgenic Alfalfa (6). Moreover we have shown that yeast cells can become more resistant to freeze-thaw damage following treatment with a dose of hydrogen peroxide that causes cells to adopt to further peroxide stress (7). Here we examine the extent to which oxidative damage occurs during freezing and thawing of cells and directly demonstrate the generation of ROS.

ROS are generated as an unavoidable side reaction in living systems that rely on oxygen as the terminal electron acceptor during energy generation. One primary product of electron leakage from the respiratory chain is the superoxide anion (O₂⁻) generated by the one-electron reduction of O₂ (8, 9). This can also be formed from other enzymatic systems including xanthine oxidase or NADPH oxidase (8). The superoxide anion disturbs the redox balance of cells by reducing and releasing metal ions from metal ion-clustered proteins or is converted to other ROS including the peroxyl radical (HO₂) or H₂O₂ (10, 11). Reduced metal ions and H₂O₂ undergo the Fenton reaction generating one of the most reactive ROS, the hydroxyl radical ('OH). These ROS damage cells by reacting with many cellular molecules including proteins, lipids, and DNA (12, 13). The generation of ROS is accelerated as an oxidative burst following ischemia and reoxygenation since this stimulates the conversion of dehydrogenases to oxidases and the introduction of excess O₂ in the cell cytoplasm (14). Freezing and thawing may form an analogous situation since cells are isolated from O₂ following freezing and are re-oxygenated during thawing. This may be augmented by the attendant dehydration and rehydration processes that result from the balancing of vapor pressure

¹ The abbreviations used are: ROS, reactive oxygen species; O₂⁻, superoxide anion; HO₂, hydroperoxy radical; 'OH, hydroxyl radical; SOD, superoxide dismutase; YEPD, yeast extract peptone dextrose medium; SD, synthetic minimal dextrose medium; ONPG, α-nitrophenyl-β-D-galactoside; PBN, α-phenyl-N-tetra-butyl-nitronate; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; bp, base pair; mT, millitesla.

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between intra- and extracellular ice systems during freezing and thawing (1).

Cells contain various enzymatic and non-enzymatic systems for detoxifying ROS (15–17). One main defense system is provided by superoxide dismutases that convert O$_2^-$ to H$_2$O$_2$ (18, 19) which is then disproportionated to water by catalases or peroxidases (20). Yeast has two types of superoxide dismutase (SOD). The Cu,Zn-SOD encoded by the sod1 gene is located in the cytoplasm; its level is constitutively high (about 1% of soluble protein in the cell) during fermentation and respiration (19). The Mn-SOD encoded by sod2 is located in the mitochondrial matrix, and from a low level in fermentative cells it is induced during respiration (19) or starvation (21). Other systems found in yeast include the cytoplasmic catalase encoded by CT1 (20), and the glutathione-based systems including glutathione itself and various peroxidases (22).

Here we have exploited the availability of mutations affecting oxidative stress response systems in the yeast Saccharomyces cerevisiae to characterize the nature and extent of oxidative stress encountered by cells during freezing and thawing. The mutations used included those in CT1, CT1, sod1, sod2, and yap1 which encodes a transcriptional activator required for stress-induced expression of several oxidative defense genes (13, 23), GSH1 which encodes γ-glutamyllysteine synthetase, and GLR1 encoding glutathione reductase (22). Cu,Zn-SOD and Mn-SOD were found to be involved in the recovery of cells from freeze-thaw injury, and this enabled an indication of the nature of the major species causing oxidative stress damage and where this damage occurred in the cells. The role of SODs in the process was further analyzed using mutations affecting mitochondrial activity, and the free radicals generated during freeze-thaw injury in wild-type and sod mutants have been detected and characterized using electron paramagnetic resonance (EPR) spectroscopy.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Condition**—190 Yeast strains used are described in Table I. The rho⁻ respiratory-deficient strains, 1753rho⁻ and KS105rho⁻, were generated by treating 1783 and KS105 with ethidium bromide (24). The gsh1 deletion mutant, JL-3, which was provided by J.-C. Lee (this laboratory) was isogenic to the wild-type strain, CY4 (25, 26). Strains deleted for the various antioxidant genes were made using the cta1::URA3 and ctt1::URA3 deletion plasmids donated by A. Hartig (Vienna) and the yap1::HIS3 deletion plasmid donated by W. S. Moye-Rowley (23).

Some strains were grown at 30 °C, with shaking at 180 rpm in 3 ml of medium in a 16 × 100-mm culture tube. For variation of aeration conditions, the methods of Longo et al. (27) were used. For high aeration of cultures, cells were grown at 30 °C, with shaking at 180 rpm, in 40 ml of medium in a 250-ml Erlenmeyer flask; and for low aeration of cultures, cells were incubated at 30 °C, without shaking, in 8 ml of medium in a 16 × 100-mm culture tube. These experiments were repeated with shaking of all cultures using 250-ml flasks and volumes of medium corresponding to those used in the above conditions, to minimize any problems arising from the differences in shaking; both experiments gave similar results. YEPD medium contained 2% glucose, 2% bactopeptone, and 1% yeast extract; and SD medium contained 2% glucose, 0.17% yeast nitrogen base (Difco), 0.5% ammonium sulfate (Oxoid), and auxotrophic requirements at 40 mg/liter where necessary. SD medium for anaerobic culture was supplemented with 0.1% Tween 80 and ergosterol at 30 mg/liter. Media were solidified by adding 2% agar. To avoid mutation of the sod strains they were stored on slopes in an anaerobic jar (Oxoid) which contained a gas-generating kit (Anaerobic system BR38; Oxoid).

**Plasmid Construction and Yeast Transformation**—Molecular techniques were carried out as described (28, 29). The SOD1 gene was isolated by polymerase chain reaction amplification of total yeast DNA with specific oligonucleotides (TCTTCTGCTAGACTTGCTCCATT), and 235 bp downstream untranslated region was cloned into the SacI/Umu1 site of the plasmid pRS425 vector (30). The SOD2::lacZ fusion construct containing 558 bp of upstream untranslated region of SOD2 and 235 bp of coding sequence has been described (21). Yeast transformation was performed using the lithium acetate method (31). Integrative plasmids were linearized by cleavage at the StuI site within the URA3 gene prior to transformation. Correct single copy integration was checked by Southern blot analysis (data not shown). Transformants of sod1 mutant strain were maintained anaerobically as described above.

**β-Galactosidase Assays—**Assays for β-galactosidase were carried out using o-nitrophenyl-β-D-galactoside (ONPG) as substrate as described previously (32). Specific activity is expressed as nanomoles of ONPG hydrolyzed per min⁻¹ mg⁻¹ protein. Protein concentrations were measured by the Bio-Rad assay method as indicated by the manufacturer. All determinations were the average of three independent experiments.

**Freezing and Thawing Conditions**—Cells were harvested by centrifugation and washed in 0.1 M sodium phosphate buffer (pH 7.0) and resuspended in 588 bp of upstream untranslated region of SOD2 and 235 bp of coding sequence has been described (21). Yeast transformation was performed using the lithium acetate method (31). Integrative plasmids were linearized by cleavage at the StuI site within the URA3 gene prior to transformation. Correct single copy integration was checked by Southern blot analysis (data not shown). Transformants of sod1 mutant strain were maintained anaerobically as described above.

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were harvested and washed in 0.1 M sodium phosphate buffer (pH 7.0). Cell density was determined by measuring optical density and by colony counting. Cells were then suspended to approximately 2×10⁶ cells/ml in 500 μl of the same buffer containing spin trap agents, either 50 μM α-phenyl-N-tetralylnitrore (PBN) or 100 μM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). Samples were frozen at −20 °C for 2 h and thawed as described above. Thawed samples were then either transferred to an EPR flat cell or extracted into toluene. Toluene extracts were bubbled with argon in cylindrical cells for 10 min prior to EPR measurement. The increase in amplitude with time of the EPR signal of the radicals was measured and expressed in arbitrary units per about 10⁶ (PBN), modulation amplitude 0.2 mT (DMPO) or 0.1 mT (PBN), conversion time 81 ms, time constant 81 ms (DMPO) or 163 ms (PBN), sweep time 83 s, center field 347.5 mT, field scan 8 mT, power 31 milliwatts (DMPO) or 50 milliwatts (PBN), frequency 9.722 GHz, temperature 294 K, with 8 scans averaged.

RESULTS
Freeze-Thaw Injuy Is Most Extensive in Mutants with Defective Cytoplasmic Cu,Zn-SOD—We have previously shown that H₂O₂ pretreatment could induce freeze-thaw tolerance of yeast cells (7). Since such treatment induces cells to adapt to oxidative stress, this indicated that ROS generated during freezing and thawing may cause lethal damage to the cell. To test this hypothesis, and determine which oxidative stress response systems are involved in protecting cells from freeze-thaw damage, the tolerance of a range of mutants affected in various aspects of the oxidative stress response was examined. The mutants examined included those with null mutations in YAP1, GSH1, GLR1, CTA1, CTT1, SOD1, and SOD2, as well as the sod1 sod2 double mutant. YAP1 encodes a transcriptional activator of several genes involved in oxidative stress and detoxification of toxic compounds (13). GSH1 and GLR1 are responsible for producing and regenerating, respectively, the important antioxidant glutathione (22). CTA1 encodes the peroxisomal catalase A and CTT1 the yeast cytosolic catalase which scavenges H₂O₂ (20), whereas the SOD1 and SOD2 gene products are the superoxide dismutase genes that scavenge O₂⁻ (18, 19). The freeze-thaw tolerance of each mutant was compared with its isogenic wild type after growth to the post-diauxic shift phase (48 h in YEPD) since wild-type cells have been shown to be more resistant in this phase (7). The mutants affected in the SOD genes showed a much greater sensitivity to freeze-thaw stress than their isogenic wild-type strain 1783, whereas the freeze-thaw sensitivity of the other oxidative stress mutants was much less affected relative to their wild-type strain CY4 (Fig. 1). This sensitivity was very apparent in the sod1 and sod1 sod2 double-mutant strains although the sod2, gsh1, and glr1 mutants were slightly more sensitive than their isogenic wild-type strains. Since the SOD1 gene product is located in the cytoplasm, this indicates that freeze-thaw damage to cells involves superoxide radicals rather than H₂O₂ or compounds for which glutathione-based defense systems are important and that prevention of the damage to cells is more dependent on the cytoplasmic Cu,Zn-SOD than the mitochondrial Mn-SOD.

As an initial step to try and confirm that the superoxide radicals were involved, we tested the effect on the freeze-thaw-sensitive sod1 mutant of pretreatment with MnCl₂, which is known to act as a O₂⁻ scavenger (33, 34). From Fig. 2 it can be seen that pretreatment with 4 mM MnCl₂ for 30 min led to a 3-fold increase in the survival of the sod1 strain. The same treatment also increased the freezing tolerance of the wild-type strain as might be expected if free radicals were generated during freezing and thawing. MgCl₂ used at the same concentration as a divalent metal ion control did not induce any freezing tolerance (data not shown). We next examined the effects of overexpressing SOD1 and SOD2 in the sod1 mutant. Although the effect of SOD1 gene in multiple copies was not significantly different (Fig. 3A; compare pRS425SOD1 with pRS425), interestingly overexpression of SOD2 (YEp13SOD2 with YEpl3) led to a significant increase in survival approaching that of the wild-type strain. This indicates that higher levels of SOD in the mitochondrial compartment can compensate for the lack of the cytoplasmic enzyme; this is discussed later. The failure of the multi-copy SOD1 vector to improve the tolerance of the sod1 mutant may have been due to H₂O₂ produced due to the augmented activity of the cytoplasmic superoxide dismutase since in Escherichia coli and Drosophila melanogaster overproduction of superoxide dismutase increased sensitivity to oxidative stress caused by paraquat (35, 36). We attempted to resolve this point by using sod1 strains transformed with multiple copy vectors carrying SOD1 and CTT1, but the interpretation of these results was hampered by the differences in growth rates of the various single and double mutants.

FIG. 1. Freeze-thaw resistance of oxidative mutants. Strains were grown on YEPD medium for 2 days, and freeze-thaw tolerance was determined by measuring viability after exposure to −20 °C. CY4 is the wild-type strain of CY7 (glr1), JY29 (gsh1), JCA1 (cta1), and JCT1 (ctt1). 1783 is the wild-type strain of KS105 (sod1), JS001(sod1 sod2), and JS002 (sod2). Percentage survival is expressed relative to the culture viability immediately prior to freezing (%). Data shown are from triplicates from a representative experiment. Error bars represent the standard error of the measurements. Experiments were repeated at least 3 times with similar results.

FIG. 2. MnCl₂ pretreatment of the sod1 mutant rescues freeze-thaw sensitivity. Cells of strain 1783 and KS105 at early exponential growth phase (A₅₀₀ of 1) were pretreated with either 2 or 4 mM MnCl₂ for 30 min at 30 °C prior to the freeze-thaw process. Freeze-thaw tolerance was determined by measuring viability after exposure to −20 °C. Percentage survival is expressed relative to the culture viability immediately prior to freezing (%). Data shown are means of triplicate experiments. Error bars represent the standard error of the means.
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Fig. 3. Overexpression of SOD1, SOD2, or CTT1 in the sod1 mutant. A, transformants were grown on SD medium for 2 days, and freeze-thaw tolerance was determined by measuring viability after exposure to −20 °C. Percentage survival is expressed relative to the culture viability immediately prior to freezing (%). Data shown are means of triplicate experiments. Error bars represent the standard error of the means (p value obtained for the double transformants was 0.022). Experiments were repeated at least twice. B, the resistance to paraquat of a 1-day culture of each transformant was determined by spotting 5 μl of each diluted fraction (A500 = 0.1) onto YEPD plates containing 0.2 mM paraquat. Experiments were repeated at least three times with similar results. A YEPD control plate showed similar growth of all strains in the absence of paraquat.

transformants and the inability to obtain strains carrying both vectors alone as a control. On transforming the sod1 mutant containing the SOD1 multi-copy vector with another vector carrying CTT1, to remove excess H2O2, a 2-fold increase in survival was seen compared with that of the sod1 mutant transformed with the SOD1 multi-copy vector and the control plasmid pRS426. The overexpression of CTT1 alone in the sod1 mutant did not affect its freeze-thaw tolerance (data not shown). To confirm that the SOD1 and SOD2 genes were overexpressed sufficiently in the various constructs to affect cellular superoxide levels, they were tested for their sensitivity to paraquat on YEPD plates. From Fig. 3B it can be seen that the SOD1 and SOD2 constructs were resistant to paraquat and that overexpression of both SOD1 and CTT1 conferred the greatest resistance. Overexpression of both of the SOD en-
zymes was also demonstrated by separating them from cell extracts using polyacrylamide gel electrophoresis and activity staining using nitro blue tetrazolium (data not shown).

The results with the sod1 mutant indicated that the cytoplasmic Cu,Zn-SOD is more important for freeze-thaw resistance than other oxidative defense systems, and this raised the question of whether the freeze-thaw sensitivity of the sod1 mutants was due to accumulated oxidative damage during prior culture, or to O2− generation during freezing or thawing.

Superoxide Dismutase Is Required during the Freeze-Thaw Process—To investigate whether freeze-thaw stress sensitivity of the sod1 mutants was due to the accumulated oxidative damage affecting cellular integrity, or to generation of O2− during the freeze-thaw process, the sod1 mutants and the wild-type strain were grown under high or low aeration culture conditions, and changes in freeze-thaw tolerance of the cells were followed. If the freeze-thaw sensitivity of the sod1 mutants was due to the accumulated damage, then high aeration culture would decrease freezing tolerance by increased generation of ROS, and low aeration would do the opposite. Under low aeration conditions the wild-type strain and the sod1 mutants (except for the double-mutant) showed decreased freeze-thaw tolerance, whereas under high aeration all strains except for the double mutant showed an increase in freeze-thaw tolerance (Fig. 4). This indicated that the freeze-thaw sensitivity of the sod1 mutants was probably not due to the accumulation of oxidative damage during growth but was due to the lack of superoxide dismutase activity during the freeze-thaw process. High aeration may increase freeze-thaw tolerance by inducing the remaining superoxide dismutase activity in each of the singly mutant strains since this protection was not observed in the sod1 sod2 double mutant. This is addressed later. The general result led us to test the effect of the oxidative burst induced by the freeze-thaw process on cell survival.

The Presence of Oxygen during Freeze-thaw Is Necessary for Damage to Cells—Since the sensitivity of the sod1 mutants was probably due to decreased superoxide dismutase activity during the freeze-thaw process itself, we tested whether an oxygen-dependent oxidative burst occurs during the freezing and thawing. The generation of ROS, specifically O2−, is proportional to the amount of O2 available, and the electrons leaked from the respiratory chain or other enzymatic reactions since the generation of O2− is a first-order reaction with respect to the concentration of O2 or the electrons leaked (37). Hence, we predicted that restricting the availability of O2 should decrease generation of O2− and this should rescue the sod1 mutant cells from damage if an oxidative burst occurs during the freeze-thaw process. We observed that freezing and thawing the cells anaerobically rescued the sod1 mutant which showed a similar level of freeze-thaw tolerance to that of the wild-type strain (Fig. 5A). This indicated that the freeze-thaw sensitivity of sod1 mutants is due to O2− generated during an oxidative burst caused by the freeze-thaw process and raised the question of the mechanism by which O2− is generated in cells during this process.

O2− Is Generated Mainly from the Mitochondrial Respiratory Chain during the Oxidative Burst Caused by the Freeze-Thaw Process—We investigated which cellular system is the main source of electrons to reduce O2 supplied during the oxidative burst. Blocking or removing of cellular sources of electron leakage should also rescue cells from freeze-thaw damage. To see if the mitochondrial electron transport chain is the major site of electron leakage, we generated respiratory-incompetent strains (Rho0) of the wild-type and sod1 mutant strains since electron leakage from the mitochondria does not occur in a Rho0 petite (38). Once the sod1 mutant strain was made petite,
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Fig. 5. Oxygen and functional mitochondria are involved in freeze-thaw damage. The effects of oxygen in the freezing buffer or of mitochondrial activity on the freeze-thaw tolerance were tested by freezing and thawing cells aerobically or anaerobically in buffer deoxygenated with argon (A). Open symbols represent aerobic conditions and closed symbols anaerobic conditions. Circles indicate the KS105 sod1 mutant and squares the wild-type 1783. B, using respiratory-deficient (Rho0) cells. Open symbols represent grande strains and closed symbols the isogenic petite; the strains are as indicated above. Percentage survival is expressed relative to the culture viability immediately prior to freezing (%). Data shown are from triplicate measurements from a representative experiment. Error bars represent the standard error of the measurements. Experiments were repeated twice with similar results.

A Complementary Relationship Exists between Cytoplasmic Cu,Zn-SOD and Mitochondrial Mn-SOD—The major site of electron leakage during the freeze-thaw process (Fig. 5B) indicating that the mitochondrial electron transport chain is the major site of electron leakage during the freeze-thaw process. The hyperfine coupling constant of the observed signal in the cytoplasmic Cu,Zn-SOD despite the difference in their cellular location.

Characterization of Radical Signals Generated by Freeze-Thaw Stress—The data presented above clearly implicate \( \text{O}_2^\cdot \) or species arising from this radical, as damaging agents in freeze-thaw injury. In order to confirm the generation of radical intermediates during this process, EPR spectroscopy using the spin traps DMPO and PBN was employed, since this can directly detect radical species. Inclusion of DMPO in cell cultures subjected to a standard freeze-thaw cycle resulted in the detection of radical signals from both the sod1 sod2 and the sod1 mutant (Fig. 7; left) but not from the sod2 mutant or the wild-type cells subject to an identical treatment; no signals were observed in non-frozen control samples from any of these cells. The hyperfine coupling constant of the observed signal (\( \alpha(N) = \alpha(H) = 1.49 \text{ mT} \)) identifies this signal as being due to the species DMPO-OH. This species is known to arise via a number of different pathways including direct trapping of \( \cdot \text{OH} \), via trapping of \( \text{O}_2^\cdot \) and subsequent rapid decay of the DMPO-OH adduct, as well as oxidation of \( \cdot \text{O}_2 \) by \( \text{H}_2 \text{O}_2 \) and subsequent hydration of the Radical cation (37). Although the exact pathway that gives rise to this species cannot be ascertained with certainty from the available data, these results are in accord with the generation of free radical species in both the sod1 mutant and sod1 sod2 double mutant. Similar pathways may occur in the sod2 mutant or wild-type cells during freeze-thaw cycles, but the free radical burden is reduced by the Cu,Zn-SOD.
to low levels. There was no evidence for any type of cell producing detectable free radicals in the absence of freezing.

Further experiments employed the spin trap PBN. As with DMPO, evidence was obtained for the generation of radical species during freeze-thaw cycles. Again no radical adduct signals were obtained under the conditions employed with any of the non-frozen cell types (wild-type, sod1 mutant, and sod1 sod2 double mutant) and only weak ones with wild-type cells subject to a standard freeze-thaw cycle. Weak signals were also observed with the sod2 mutant and stronger signals (i.e., higher radical adduct concentrations) with both the sod1 mutant and the sod1 sod2 double-mutant strains (Fig. 7; right). In these latter cases, the observed signals could be analyzed in terms of the presence of two species: an oxidation product of the spin trap (tBuNHO, a(N) = a(H) ~ 1.44 mT) and (at least one) spin adduct species with a(H) ~ 0.40 mT. The non-symmetrical nature of these spin adduct signals indicated that several species with similar, but non-identical, hyperfine coupling constants were present. Although these spin adduct species could not be definitively identified from these parameters, they are clearly not the ‘OH or O2•− adducts that have much smaller a(H) splittings (39) and are likely to be a secondary species arising from reaction of the initial radical(s) with intracellular material. Attempts to extract these spin adducts into organic solvents, such as toluene, to aid spectroscopic analysis proved unsuccessful; this implies that the radicals

Fig. 7. Generation of free radicals during freezing and thawing. Left, EPR spectra were observed on incubation of wild-type and mutant strains (10⁶ in 500 μl) with the spin trap DMPO (100 mM) before (A) or after (B–E) a single freeze-thaw cycle (see “Experimental Procedures” for further details). A, 1783 wild-type strain not subjected to freezing and thawing; B, wild-type cells after freeze-thaw cycle; C, KS105 sod1 mutant after freeze-thaw cycle; D, JS002 sod2 mutant after freeze-thaw cycle; E, JS001 sod1 sod2 double mutant after freeze-thaw cycle. Signals in C and E are indicated by ♦ assigned to DMPO-OH arising from either direct trapping of ‘OH, decomposition of DMPO-OOH or oxidation of the spin trap (see text for further details). Fluctuation in the base line in each case is due to the broad metal ion absorptions. Right, same as left except using the spin trap PBN (50 mM). A, 1783 wild-type strain not subjected to freezing and thawing; B, wild-type cells after freeze-thaw cycle; C, KS105 sod1 mutant after freeze-thaw cycle; D, JS002 sod2 mutant after freeze-thaw cycle; E, JS001 sod1 sod2 double mutant after freeze-thaw cycle. Signals indicated by ○ are assigned to a PBN spin trap adduct arising from trapping of a secondary radical (see text for further details); signals indicated by ▼ are assigned to an oxidation product of the spin trap. Fluctuation in the base line in each case due to the broad metal ion absorptions.
that had been added to the trap were hydrophilic species and, on the basis of the isotopic lines detected, of relatively low molecular mass.

DISCUSSION

The results of this study show the involvement of Cu,Zn-SOD and Mn-SOD in the freeze-thaw stress survival of yeast cells. They confirm in a more direct way that oxidative stress is a cause of lethal damage to cells, as has been suggested in other systems (5, 6), and further show that the main damage in aerobic systems occurs in the cytoplasmic compartment and results from generation of \( \text{O}_2^- \). Of all the genes involved in oxidative stress defense systems tested including \( \text{GSH1}, \text{GLRI}, \text{YAP1}, \text{CTA1}, \text{CTT1}, \text{SOD1}, \) and \( \text{SOD2} \) the \( \text{SOD} \) genes, which scavenge mainly superoxides, were found to be more involved in the defense against the damage caused by freeze-thaw stress. GLRI, and \( \text{GSH1} \), which regulate glutathione metabolism, are less involved during the freeze-thaw process since the deletion of these genes did not significantly affect freeze-thaw tolerance nor did overexpression of \( \text{GSH1} \) or extracellular provision of glutathione (data not shown). The result with the \( \text{YAP1} \) mutant was surprising since \( \text{yap1} \) mutants are sensitive to most other forms stress tested (13, 23, 40). The deletion of \( \text{CTA1} \) or \( \text{CTT1} \) did not affect freeze-thaw tolerance of yeast cells, indicating that \( \text{H}_2\text{O}_2 \) is not generated extensively or that absence of catalases can be compensated by other peroxidases in the cell (11). Taken together, these results highlight that the rapid removal of \( \text{O}_2^- \) is the most important step for the oxidative defense of cells during freeze-thaw stress. These results need to be considered in the light of the fact that \( \text{sod1} \) deletion strains have pleiotropic behavior with respect to gene regulation and other aspects of their physiology such as decreased invertase production, changes in the cell division cycle (19), and changes in divalent ions and metal ion homeostasis (50). However, the demonstration that the effects of the \( \text{sod1} \) mutation can be reversed by removal of oxygen, the finding that \( \text{MnCl}_2 \) treatment also improves the freezing tolerance of the wild-type as well as of the \( \text{sod1} \) mutant, and the direct demonstration of free radicals in cells following freezing and thawing indicate that oxidative damage due to free radical generation is a major component of freeze-thaw damage.

Since the \( \text{sod1} \) mutant showed higher sensitivity to freeze-thaw stress than the \( \text{sod2} \) mutant, it seems likely that the main lethal damage occurs in the cytoplasm. Where then does the \( \text{O}_2^- \) originate? The reactivity of \( \text{O}_2^- \) depends on its environment since it is relatively stable in a hydrophobic environment and can therefore continue to cause damage while it rapidly disappears in a hydrophilic environment (41); it is relatively less reactive than other ROS such as \( \text{HO}_2^- \) or \( \cdot \text{OH} \) in aqueous systems (11). Hence, whereas the oxidative damage during freeze-thaw stress may be initiated by \( \text{O}_2^- \), it is not likely that all the subsequent effects in the cell are due directly to \( \text{O}_2^- \), and some cell damage will also result from the secondary ROS which are formed spontaneously from \( \text{O}_2^- \). Spontaneous disproportionation of \( \text{O}_2^- \) into \( \text{HO}_2^- \) (pKa = 4.8) or \( \text{H}_2\text{O}_2 \) can occur, particularly at low pH (42, 43). Since we showed that \( \text{O}_2^- \) appears to be generated largely by the mitochondrial respiratory chain, these disproportionation reactions may be possible due to a low pH maintained near the mitochondrial membrane by the proton gradient. Moreover, there are drastic changes in intracellular pH and osmolarity during freezing and thawing, which form an acidic intracellular environment (44).

The EPR spectroscopy showed that free radical species are generated by the freeze-thaw process and were consistent with \( \text{O}_2^- \) formation initiating oxidative stress during the process. Although the use of the spin trap agent, DMPO, showed the presence of a signal from \( \cdot \text{OH} \) in the \( \text{sod1} \) and the \( \text{sod1 sod2} \) double-mutant strains, this signal probably resulted from the spontaneous decomposition of DMPO-OH adduct to DMPO-OH since \( \text{O}_2^- \) is very unstable even after adduct formation. This has been seen when DMPO was used to detect the radical signals after mendadiene treatment (45). The spontaneous disproportionation of \( \text{O}_2^- \) into \( \text{H}_2\text{O}_2 \) and subsequent generation of \( \cdot \text{OH} \) by the Fenton reaction is unlikely since overexpression of \( \text{CTT1} \) did not rescue the \( \text{sod1} \) mutant from freeze-thaw stress or from oxidative stress caused by paraquat, indicating that in the absence of Cu,Zn-SOD spontaneous disproportionation of \( \text{O}_2^- \) into \( \text{H}_2\text{O}_2 \) does not occur efficiently. The use of another spin trap agent PBN, which has a broad spectrum for radicals, showed significant generation of two different radical signals in the \( \text{sod1} \) mutant strain and the \( \text{sod1 sod2} \) double mutant strain but less in the \( \text{sod2} \) mutant and the wild-type strain which showed only a weak radical signal. This correlates with the viability changes shown by these \( \text{sod} \) mutants. All of our observations indicate that the radical chain reactions appear to occur mainly in the cytosol, and some water-soluble components may be the main targets of the chain reactions.

We conclude that the mitochondrial SOD can compensate for absence of the other despite a difference in their cellular localization. This raises a question about the direction of \( \text{O}_2^- \) generation in mitochondria. Since \( \text{O}_2^- \) is negatively charged, it cannot pass through the mitochondrial inner membrane by simple diffusion; however, it may be transported into the cytosol through an anion channel (46). It is also known that \( \text{O}_2^- \) is generated bidirectionally, both into the cytosol and the mitochondrial matrix (47). Intracellular damage caused by freezing and thawing may disturb the ability of the cell to sequester \( \text{O}_2^- \) in the mitochondrial matrix, and once the \( \text{O}_2^- \) level is beyond the capacity of the available Mn-SOD activity it may move out to the cytosol where the main damage is initiated. Overexpression of the \( \text{SOD2} \) gene would then alleviate this problem. It is not likely that the \( \text{de novo} \) precursor to Mn-SOD is active in the cytosol since removal of the signal peptide during the incorporation into mitochondrial matrix is a prerequisite for the formation of active Mn-SOD (48).

Here, we report that oxidative stress contributes to damage to cells during the freeze-thaw process, that the damage is probably caused by \( \text{O}_2^- \), and can be prevented by the activity of the cytoplasmic SOD. The mechanisms whereby the radical propagates cell damage leading to death during the freeze-thaw process remain to be elucidated. Our work has provided a foundation to investigate these mechanisms in detail.

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