O₂-Dependent Methionine Auxotrophy in Cu,Zn Superoxide Dismutase-Deficient Mutants of Saccharomyces cerevisiae

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Mutant strains of the yeast Saccharomyces cerevisiae which lack functional Cu,Zn superoxide dismutase (SOD-1) do not grow aerobically unless supplemented with methionine. The molecular basis of this O₂-dependent auxotrophy in one of the mutants, Dscd1-1C, has been investigated. Sulfite supported anaerobic but not aerobic mutant growth. On the other hand, cystine and homocysteine supported aerobic growth while serine, O-acetylserine, and homoserine did not, indicating that the interconversion of cysteine and methionine (and homocysteine) was not impaired. Thiosulfate (S₂O₃²⁻) and sulfide (S²⁻) also supported aerobic growth; the activities of thiosulfate reductase and sulfhydrylase in the aerobic mutant strain were at wild-type levels. Although the levels of SO₃²⁻ and adenosine-5'-sulfate (the first intermediate in the SO₃²⁻ assimilation pathway) were elevated in the aerobically incubated mutant strain, this condition could be attributed to a decrease in protein synthesis caused by the de facto sulfur starvation and not to a block in the pathway. Therefore, the activation of SO₃²⁻ (to form 3'-phosphoadenosine-5'-phosphosulfate) appeared to be O₂ tolerant. Sulfite reductase activity and substrate concentrations ([NADPH] and [SO₃²⁻]) were not significantly different in aerobically grown mutant cultures and anaerobic cultures, indicating that SOD-1⁻ mutant strains could reductively assimilate sulfur oxides. However, the mutant strain exhibited an O₂-dependent sensitivity to SO₃²⁻ concentrations of <50 μM not exhibited by any SOD-1⁺ strain or by SOD-1⁻ strains supplemented with a cytosolic O₂⁻-scavenging activity. This result suggests that the aerobic reductive assimilation of SO₃²⁻ at the level of SO₃²⁻ may generate a cytotoxic compound(s) which persists in SOD-1⁻ yeast strains.

The univalent reduction of O₂ produces O₂⁻ (superoxide radical), H₂O₂, and OH⁻ (hydroxyl radical). It has been suggested that antioxidant enzymes which remove O₂⁻ or H₂O₂ in vitro play protective roles in vivo (6, 8). Superoxide dismutases (SODs) catalyze the dismutation of O₂⁻ to O₂ and H₂O₂, and are found in almost all aerobes, including the yeast Saccharomyces cerevisiae (6). Yeast has a cytosolic Cu,Zn SOD (SOD-1) and a mitochondrial Mn SOD (SOD-2). Mutation in or deletion or disruption of either the gene for SOD-1 (SOD1) or the gene for SOD-2 (SOD2) renders yeast O₂ sensitive. For example, an SOD2 disruption mutant (DL1-d) is growth inhibited in 100% O₂ (24) and cannot use a nonfermentable two-carbon source such as ethanol or acetate in air (25). Yeast mutants which lack a functional SOD-1 are also sensitive to O₂ (2). The sod1 allele in these mutants (2) has been mapped to SOD1 and has been designated sod1-1 (1; E. C. Chang, T. Bilinski, and D. J. Kosman, submitted for publication). These mutants exhibit only a 50% survival following a 30-min exposure to 100% O₂ and a nonparental Met and Lys auxotrophy when grown in air (2).

Although the specific O₂-related cellular damage in sod1-1-containing mutant strains which leads to this aerobic Met and Lys auxotrophy is not known, two observations suggest that the damage is probably in the cytosol. First, transformation of these mutants with an SOD1-containing plasmid (1) or addition of millimolar [Mn²⁺] to the growth medium (3), both of which treatments support a cytosolic O₂⁻-dismutation activity missing in these mutants, abolishes this auxotrophy. Second, this auxotrophy appears related to a defect in SO₃²⁻ assimilation (7), a metabolic process which is cytosolic in yeast (11). In this study, the O₂-dependent Met auxotrophy in one of these sod1-1 mutants, Dscd1-1C (referred to hereafter as 1C), was investigated in detail in an attempt to elucidate the biochemical basis for the apparent inability of aerobic SOD-1⁻ strains to reductively assimilate SO₃²⁻.

MATERIALS AND METHODS

Chemicals. Medium components were purchased from Difco Laboratories. The NaCl and CaCl₂ used for SO₃²⁻-free synthetic glucose (SG) medium were Analar (BDH) reagents obtained from Gallard-Schlesinger. All pyridine nucleotides and Ecteola cellulose (0.35 meq/g) were purchased from Sigma Chemical Co. [³⁵S]Na₂SO₄ (43 Ci/mg) was obtained from ICN Pharmaceuticals Inc. ³⁵S-labeled 3'-phosphoadenosine-5'-phosphosulfate ([³⁵S]PAPS) was obtained from Amersham Corp. All other reagents were reagent grade.

Yeast strains. The SOD-1⁻ mutant studied in detail in this study, 1C, was MATa arg4 leu1 sod1-1 (2). Complementary experiments were performed with other SOD-1⁻ strains: Dscd2-2C and Dscd1-4A, which also carry the sod1-1 allele (2), and EG1, an SOD1 null mutant generated by deletion and insertion of URA3 at the SOD1 locus (E. B. Gralla and J. S. Valentine, unpublished data). Transformants of Dscd2-2C and Dscd1-4A (Dscd2-2C⁺ and Dscd1-4A⁺, respectively) were used also; these carry a centromeric plasmid which contains wild-type SOD1 (1). This plasmid is designated [URA3 SOD1]. A strain lacking SOD-2 was used in one experiment; this strain, DL1-d, carries a disruption in SOD2 (24). The wild-type strain used as an SOD-1⁻ control was AS2-2A MATa trpl-2 MAL (19).

Medium for cell growth. The SO₃²⁻-free SG medium was
prepared as described elsewhere (Difco manual, 10th ed., Difco Laboratories, Detroit, 1984, p. 1136) except that all SO$_4^{2-}$-containing components were replaced by Cl$^-$ salts. Medium pH was buffered at 4.5 with succinate (for study of toxicity) and at 6.0 with phosphate. For anaerobic growth, 5 mg of ergosterol per liter and 1.4 g of inositol per liter were added. The yeast extract-peptone-glucose medium contained 1% (wt/vol) yeast extract, 2% (wt/vol) Bactopeptone, and 2% (wt/vol) glucose. Amino acids and nucleotides added for optimal growth of 1C and AS2-2A were as indicated previously (3).

**Cell growth.** Cell growth was monitored by turbidity measurement at 660 nm ($A_{660}$). For the strains used in these experiments, an $A_{660}$ of 1.0 was equivalent to 1.9 $\times$ 10$^{-1}$ to 2.2 $\times$ 10$^{-1}$ cells per ml. Culture stocks were pregrown in 1 mM SO$_4^{2-}$-SG medium. Prior to experiments, these stocks were washed three times with SO$_4^{2-}$-free SG medium, since any residual SO$_4^{2-}$ present in the medium inhibited Na$_2$So$_3$O$_3$ utilization (E. C. Chang and D. J. Kosman, unpublished results). In all pulse-labeling and sulfite reductase experiments, 250 $\mu$M Na$_2$So$_3$O$_3$ was added as the sole sulfur source. These washed cells were then diluted into fresh medium to an $A_{660}$ of 0.1 unless otherwise indicated in the text. Cells were grown at 30°C and harvested in log phase ($A_{660}$, 1.0) except in the growth yield experiments described elsewhere (see Tables 3 and 4). Growth yield was represented by the culture turbidity after overnight growth (ca. 20 h). For anaerobic growth, each 5-ml culture was flushed with N$_2$ for 30 min and then sealed with a rubber septum.

**Pulse labeling of cells and preparation of crude cell extracts.** Mutant strain 1C was pregrown in air or N$_2$ with Na$_2$So$_3$O$_3$ added as the sole sulfur source. Thiosulfate supported growth under both conditions but did not repress the reductive assimilation of sulfate (see below). Sulfate-free SG medium or the same medium flushed with N$_2$ was used to wash aerobically or anaerobically pregrown cultures, respectively. The washed, aerobically pregrown culture was suspended in SO$_4^{2-}$-free SG medium (pH 6) with all nutrients added for aerobic growth except Met unless otherwise indicated. This culture was labeled with [35S]SO$_4^{2-}$ (28 $\mu$Ci/ml of culture) for 30 min in air. The anaerobically pregrown culture was labeled similarly in N$_2$. After 30 min, the labeled cells were washed with lysis buffer (0.1 M phosphate [pH 7.8], 0.1% Triton X-100, and 1 mM EDTA) and the cell pellets were collected by centrifugation. The crude cell extracts were prepared by homogenization with glass beads (0.30 to 0.45 mm). The trichloroacetic acid-precipitable radioactivity was determined as described elsewhere (22).

**Ecteola cellulose anion-exchange chromatography.** The column was prepared as described elsewhere (26). The stepwise elution with (NH$_4$)$_2$HCO$_3$ was as suggested previously (17); authentic [35S]PAPS and [35S]SO$_4^{2-}$ were eluted from the column as standards. The radioactive crude extract (15 $\mu$l) was boiled for 15 s and then clarified by centrifugation. This supernatant (5 $\mu$l) was mixed with 5 $\mu$l of 10 mM triethanolamine (pH 7.3) and then loaded on the column. Eluant (1 ml) from each fraction was mixed with 10 ml of scintillation fluid for counting.

**Partial purification and assay of SRase.** The partial purification of sulfite reductase (SRase) was as described previously (29). A crude extract was prepared by homogenization with glass beads in 0.35 M phosphate (pH 7.3) containing 1 mM EDTA. Insoluble material was removed by centrifugation. (NH$_4$)$_2$SO$_4$ was added to 50% saturation. The precipitate was collected and dialyzed for 48 h against 0.35 M phosphate buffer. This dialyse was then concentrated in an ultrafiltration cell (Amicon Corp.) with a membrane filter cutoff of 10 kilodaltons. The activity of SRase was defined as the SO$_4^{2-}$-dependent disappearance of NADPH determined spectrophotometrically at 340 nm measured in the presence of 150 $\mu$M NADPH and 1 mM SO$_4^{2-}$ (29). One unit of activity was defined as 1 $\mu$mol of NADPH oxidized per min.

**Other assays.** Protein concentration was determined by the bicinchoninic acid assay (Pierce Chemical Co.) with bovine serum albumin as the standard (20). Separation of pyridine nucleotides using high-pressure liquid chromatography was as described elsewhere (12) except that a Zorbax Gold C$_8$ column (Du Pont Co.) was used. Cell samples for the analysis of SO$_4^{2-}$ were prepared in 10% trichloroacetic acid, and the precipitate was discarded by centrifugation. The assay for SO$_4^{2-}$ utilizing the Fuchsin method was as described elsewhere (13). The same (NH$_4$)$_2$SO$_4$ fraction used for measurement of SRase activity was also used for the sulfhydrolase and thiosulfate reductase assays, which involved measuring the appearance of -SH by using nitropruside (28) and the appearance of SO$_4^{2-}$ by using the Fuchsin method (4).

**RESULTS**

**Sulfate utilization and nutritional supplementation of intermediates in methionine biosynthesis.** The biosynthetic pathway of Met in yeast is illustrated in Fig. 1. Sulfate is first conjugated as a phosphoanhydride species, adenosine-5'-sulfate (APS), which is further activated by an ATP-dependent phosphorylation yielding PAPS. The SO$_4^{2-}$ in PAPS is reduced to S$^2-$ via the intermediate sulfur oxidation state (+4) represented by sulfate, SO$_3^{2-}$. The SO$_3^{2-}$ may be formed as a bound intermediate (23, 27). Sulfide is then incorporated into the carbon skeletal provided by O-acetylserine or O-acetylhomoserine in a reaction catalyzed by sulfhydrolase (Fig. 1, enzyme 7) to form Cys or homocysteine (HomoCys). The interconversion of Met, Cys, and HomoCys serves to maintain balanced pool sizes of the three thiol-containing amino acids. Yeast mutants defective in one or more steps of this pathway have been isolated (11, 14).

In air, SOD-1$^{-}$ strains like 1C do not grow unless Met is added (2, 7); that is, they are not able to grow aerobically with SO$_4^{2-}$ as the sole source of sulfur. The block in this assimilation was investigated by nutritional supplementation with pathway intermediates of Met biosynthesis. The aerobic Met requirement was satisfied by Cys or HomoCys indicating that the interconversion of Met and Cys occurred in SOD-1$^{-}$ strains when they were grown in air. Both S$^2-$ and SO$_3^{2-}$ also supported aerobic growth while the addition of Ser or O-acetylserine, the other substrates for sulfhydrolase, did not. Sulfide was generated from SO$_3^{2-}$ at the expense of a thiol like glutathione in a process initiated by thiosulfate reductase (5; Fig. 1, enzyme 16). The S$^2-$ produced could be utilized by sulfhydrolase to synthesize Cys. The activities of thiosulfate reductase and sulfhydrolase were essentially the same in mutant strain 1C grown aerobically or anaerobically and in an SOD-1$^{-}$ wild type. For example, the sulfhydrolase activities in aerobically grown mutant and wild-type strains were identical, with a $\Delta A_{643}$ of 1.5/min per mg of protein (which represents generation of RSH [28]).

The supplementation results, which were characteristic of all strains tested which lack SOD-1 activity (1C, Dscd2-2C, Dscd1-4A, and EG1; see Materials and Methods), indicated that the block in aerobic Met biosynthesis in these mutant strains was in the activation and reductive assimilation of SO$_4^{2-}$ and not in the incorporation of reduced sulfur into the appropriate carbon skeleton.
Sulfate activation in the SOD-1" mutant strains. To assess whether the activation of SO_4^{2-} to PAPS in the mutant strain 1C was O_2-sensitive, the distribution of the intermediates in the SO_4^{2-} activation pathway was investigated. Aerobic and anaerobic mutant cultures pregrown on S_2O_3^{2-} as the sulfur source were pulse labeled with [35S]SO_4^{2-} in the presence of all required nutrients except Met (or S_2O_3^{2-}). Crude, heat-treated cell extracts were analyzed by Ecteola cellulose anion-exchange chromatography, which separated the SO_4^{2-} activation metabolites. In 30 min, aerobically grown mutant cells accumulated three times more labeled SO_4^{2-} and APS than anaerobically grown cultures (Table 1). The relative amounts of labeled SO_4^{2-}, APS, and PAPS in an SOD-1" wild-type strain were similar to those in the anaerobically grown mutant; i.e., each had 5% of the total radioactivity loaded (data not shown).

The elevated SO_4^{2-} and APS did not appear to be due to a differential growth arrest (i.e., within the 30-min labeling period) in the aerobic culture (which cannot grow on SO_4^{2-}), since this culture did exhibit a normal doubling time for up to 2.5 h in the presence of added [35S]SO_4^{2-}. Another explanation for the accumulation of SO_4^{2-} and APS detected in the aerobic culture was that ATP sulfurylase (Fig. 1, enzyme 2) or APS kinase (Fig. 1, enzyme 3) or both were limiting under these conditions. In a separate experiment, however, another sodl" mutant (Dscdl-4A) grown anaerobically or aerobically in the absence of all required amino acids exhibited levels of SO_4^{2-} and APS which each represented ca. 15% of the total radioactivity loaded (cf. the 5% exhibited for 1C in air [Table 1]). Consequently, such accumulation of intermediates could result from a suppression of protein synthesis due to a lack of one or more amino acids, including Met and Cys. This analysis does suffer from its failure to distinguish among potential metabolite pools which might be differentially affected in cultures grown under air. Nonetheless, the data do not clearly support a model which includes a significant inhibition of SO_4^{2-} activation in the SOD-1" mutant strain. A feature of this chromatographic procedure to note is that the recovery of the total [35S] applied to the column was lower in extracts derived from cultures which supported significant protein synthesis (for example, anaerobic versus aerobic cultures; Table 1). This feature was apparently due to the strong binding to the Ecteola of (heat-stable) labeled polypeptides (17).

Reductive assimilation of sulfur oxides in SOD-1" mutant strains. Sulfite (SO_3^{2-}) did not remove the requirement for Met in aerobic cultures of mutants carrying the sodl" allele, but may be due to a metabolic or nutritional change in the mutants. This is supported by the finding that approximately 50% of the total radioactivity loaded from [35S]SO_4^{2-} was recovered from the aerobically pregrown sample, while the recovery from the anaerobically pregrown sample was ca. 50% (see text).

**TABLE 1. Analysis of [35S]-labeled intermediates of SO_4^{2-} activation in SOD-1" mutant**

<table>
<thead>
<tr>
<th>mM (NH_4)HCO_3</th>
<th>Standard eluted</th>
<th>% of total [35S] loaded ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
<td>N_2</td>
</tr>
<tr>
<td>0 (H_2O)</td>
<td>NC</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>5</td>
<td>NC</td>
<td>26 ± 0</td>
</tr>
<tr>
<td>20</td>
<td>SO_4^{2-}</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>60</td>
<td>APS</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>140</td>
<td>PAPS</td>
<td>6 ± 1</td>
</tr>
</tbody>
</table>

*Mutant 1C was pregrown aerobically or anaerobically to an A_660~ of 1.0 in SO_4^{2-}-free SG medium supplemented with 250 μM S_2O_3^{2-}. The cultures were washed, suspended in fresh SO_4^{2-}-free SG medium, and labeled for 30 min with [35S]SO_4^{2-} in air (for aerobically pregrown cultures) or in N_2 (for anaerobically pregrown cultures). The boiled, crude cell extracts prepared from these cultures subsequently were separated on Ecteola cellulose.

*Authentic SO_4^{2-} and PAPS were eluted in 20 and 140 mM (NH_4)HCO_3, respectively, as described previously (20). APS was eluted with 60 mM (NH_4)HCO_3 (17). NC, Not characterized. This label may be associated with sulfated organic compounds (17).

*Total radioactivity eluted at different (NH_4)HCO_3 concentrations was normalized with respect to the total radioactivity loaded. Standard error of the mean is for two separate experiments. Approximately 80% of the radioactivity loaded was recovered from the aerobically pregrown sample, while the recovery from the anaerobically pregrown sample was ca. 50% (see text).
although it could do so in anaerobic ones (see below). This result suggested that the enzyme catalyzing the reduction of \( \text{SO}_2^{2-} \) to \( \text{S}^{2-} \), SRase (Fig. 1, enzyme 5), was inhibited in cultures grown under air. Therefore, the SRase activity was measured in soluble cell extracts derived from the mutant strain 1C grown aerobically and anaerobically (Table 2). With \( \text{Na}_2\text{SO}_4 \) as the sole source of sulfur, SRase activities from aerobically and anaerobically (\( \text{N}_2 \)) grown mutant cultures were essentially the same, i.e., \( \pm 0.7 \) U/g of protein. An SRase activity in a wild-type strain of \( \text{S}^{2-} \) has been reported (29). These results indicated that SRase in aerobic mutant cells was fully active when assayed in vitro. Note that with Met in the culture medium, the SRase activity decreased from ca. 11 to 1 U/g as expected, because of end product repression (11, 14). In contrast, \( \text{Na}_2\text{SO}_4 \) as the sole sulfur source did not repress SRase activity and therefore can be used as a sulfur source which does not down-regulate the Met biosynthetic pathway.

To determine if any \( \text{O}_2 \)-dependent inactivation of SRase could be detected, the following experiment was carried out with wild-type and mutant strains. Cultures were grown anaerobically, washed free of supplements, and then suspended in fresh medium lacking Trp (wild type) or Lys and Met (mutant) to suppress new protein synthesis. The two cultures were divided; one half of each culture was incubated aerobically, and the other half was incubated anaerobically. After 4 h under air, the SRase activity measured in the mutant strain was 81% and that in the wild-type strain was 61% of the \( t_0 \) control. Under \( \text{N}_2 \), the SRase activity in the mutant strain was 82% and that in the wild-type strain was 85% of the \( t_0 \) control. Thus, SRase in the mutant strain did not appear unusually sensitive to \( \text{O}_2 \). However, these assays were performed on an ammonium sulfate fraction (29) and not on the total cell extract. Thus, the possibility that SRase was inhibited (reversibly) in vivo by some species lost during this fractionation can not be excluded.

The levels of the substrates (\( \text{SO}_3^{2-} \) and NADPH) for SRase in the mutant strain 1C under air were examined also. Wild-type and mutant strains were pregrown anaerobically and then incubated in SG medium in air or \( \text{N}_2 \) for 4 h; cell extracts were prepared. The \( \text{SO}_3^{2-} \) levels measured in the aerobically and anaerobically incubated wild-type strains were 10.4 and 9.4 pmol/10^6 cells, respectively; the values for the mutant strain were 8.1 and 10.8 pmol/10^6 cells. The concentration of sulfite in the cells was estimated on the basis of an assumed cell volume of 60 \( \mu \text{m}^3 \) (21); the concentration ranged from 135 to 180 \( \mu \text{M} \) for these four cultures. For the analysis of NADPH, the mutant strain was pregrown in \( \text{N}_2 \) and then suspended and incubated in air or \( \text{N}_2 \) for 12 h. The NADPH levels measured in aerobically and anaerobically incubated cultures were 3.2 and 5.3 pmol/10^6 cells, respectively, or ca. 55 and 85 \( \mu \text{M} \). The \( K_m \) values for \( \text{SO}_3^{2-} \) and NADPH measured in vitro were 10 and 17 \( \mu \text{M} \), respectively (29). Thus, the differences observed in these substrate levels under air were not likely to have been kinetically significant.

It has been suggested that a thioredoxin-dependent reaction provides an alternative reduction of the \( \text{SO}_3^{2-} \) on PAPs to \( \text{S}^{2-} \); at the least, reduction of this sulfate to \( \text{SO}_3^{2-} \) has been shown to depend on the thioredoxin system (16). A deficiency in the thioredoxin system could have contributed to the inability of the mutant strain to reductively assimilate \( \text{SO}_3^{2-} \). To test this possibility, advantage was taken of the fact that methionine sulfoxide in \( S. \text{cerevisiae} \) is reduced by Met by an enzyme complex consisting of methionine sulfoxide reductase (Fig. 1, enzyme 17), thioredoxin, and thioredoxin reductase (10, 16). In fact, methionine sulfoxide supported aerobic mutant growth, indicating that thioredoxin and thioredoxin reductase were functional in this strain under these conditions. In summary, all in vitro analyses (and the latter in vivo observation) indicated that the mutant strain grown under air possessed the ability to reduce \( \text{SO}_3^{2-} \) to \( \text{SO}_2^{2-} \) and \( \text{SO}_3^{2-} \) to \( \text{S}^{2-} \).

### Table 2. Activity of sulfite reductase in aerobically and anaerobically grown SOD-1− mutants

<table>
<thead>
<tr>
<th>Sulfur source (mM)</th>
<th>SRase activitya (U/g of protein ± SD)</th>
<th>N2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Na}_2\text{SO}_4 ) (1.0)</td>
<td>NG 11.1 ± 0.3</td>
<td>85%</td>
</tr>
<tr>
<td>( \text{Na}_2\text{SO}_3 ) (0.25)</td>
<td>8.3 ± 0.2</td>
<td>6.2 ± 0.3</td>
</tr>
<tr>
<td>Methionine (0.4)</td>
<td>0.5 ± 0.1</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

a Mutant 1C was grown aerobically (Air) or anaerobically (\( \text{N}_2 \)) in \( \text{SO}_3^{2-} \)-free SG medium (pH 6.0) with the indicated supplements. SRase was partially purified and assayed as described in Materials and Methods. Values are means from four experiments. NG, No growth.

### Table 3. Comparison of sulfite-supported growth in wild-type and SOD-1− mutant strains

<table>
<thead>
<tr>
<th>mM ( \text{Na}_2\text{SO}_3 )</th>
<th>WT</th>
<th>Mutant</th>
<th>WT</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13</td>
<td>7</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>0.1</td>
<td>88</td>
<td>9</td>
<td>74</td>
<td>92</td>
</tr>
<tr>
<td>0.25</td>
<td>66</td>
<td>5</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>0.5</td>
<td>8</td>
<td>2</td>
<td>76</td>
<td>93</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>ND</td>
<td>68</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>&lt;1</td>
<td>ND</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

a Wild-type AS2-2A (WT) and mutant 1C were inoculated to an \( A_{600} \) of 0.1 in \( \text{SO}_3^{2-} \)-free SG medium (pH 4.5), and the \( \text{Na}_2\text{SO}_3 \) was added as indicated. Cultures were grown either in air or under nitrogen.

b After 20 h of growth at 30°C, Control cultures contained either 1 mM \( \text{SO}_3^{2-} \) or 0.4 mM Met for wild-type and mutant cultures, respectively. ND, Not determined.
TABLE 4. SO$_2^-$ tolerance of mutant strains supplemented with
cytosolic O$_2^-$-scavenging activity$^a$

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>Source of cytosolic O$_2^-$-scavenging activity</th>
<th>% of control cell density$^c$ ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrying sod-l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dscd2-2C$^+$</td>
<td>[URA3 SOD1]$^d$</td>
<td>87 ± 6</td>
</tr>
<tr>
<td>Dscd1-4A$^+$</td>
<td>[URA3 SOD1]$^d$</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>1C</td>
<td>4 mM Mn$^{2+}$</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>Dscd1-4A</td>
<td>4 mM Mn$^{2+}$</td>
<td>100 ± 6</td>
</tr>
</tbody>
</table>

| DIL1-d (lacking SOD-2)$^d$ | Genomic SODI | 100 ± 5 |

$^a$ Strains were inoculated to an $A_{600}$ of 0.1 in SO$_2^-$-free SG medium (pH 4.5 or 6.0) containing 0.1 mM Na$_2$SO$_4$ and nutritional supplements as required by genotype. MnCl$_2$ was added to the medium as indicated. Cultures were grown at 30°C under air for 20 to 24 h.

$^b$ Control cultures contained 10 mM Na$_2$SO$_4$ as the sole sulfur source, which supported wild-type growth of all strains under the conditions indicated. Values are means for four separate pairs sets.

$^c$ Carries a centromeric plasmid which contains wild-type copies of URA3 and SOD1 (1, 7).

$^d$ Carries a disruption in SOD2 (24) and exhibits wild-type SOD-1 activity but no O$_2^-$-dependent autotrophy (24, 25).

µM SO$_2^-$ at pH 4.5 exhibited 100% survival (but no growth); at 200 µM SO$_2^-$, survival was >2%, while at 500 µM SO$_2^-$, mutant killing was complete. In contrast, 2 mM SO$_2^-$ was required for 50% killing of the SOD-1$^+$ wild-type strain AS2-2A; complete killing required 5 mM SO$_2^-$.

The controls for these survival experiments were sulfur-starved cultures, i.e., cultures in sulfur-free media. Thus, the killing observed was due to SO$_2^-$ toxicity and not to the sulfur starvation of a mutant strain which could not utilize sulfite.

Cytosolic O$_2^-$-dismutation activity, aerobic Met auxotrophy and sulfate intolerance in SOD-1$^+$ mutant strains. Mutant strains carrying the sod-l allele transformed with an SODI-containing CEN plasmid (1) (e.g., Dscd1-4A$^+$ and Dscd2-2C$^+$) grow normally in air on SO$_2^-$ as the sole source of sulfur (7; Chang et al., unpublished results). The aerobic auxotrophy in SOD-1$^+$ strains is also rescued by the addition of >2 mM Mn$^{2+}$ to the growth medium (3). Both of these treatments restore the cytosolic O$_2^-$-scavenging activity lacking in these mutant strains (1, 3; 7; Chang et al., unpublished results). Both of these treatments reduced or eliminated the aerobically SO$_2^-$ hypersensitivity exhibited by these strains as well (Table 4). These results suggest a strong correlation between the aerobic Met auxotrophy and the SO$_2^-$ sensitivity in the SOD-1$^+$ strains. In contrast, the inability to utilize SO$_2^-$ and the hypersensitivity to SO$_2^-$ are not phenotypes shared by a mutant strain which lacks SOD-2 activity. The SOD-2$^-$ strain (DIL1-d) utilized SO$_2^-$ as the sole sulfur source under air (Table 4). In summary, the O$_2^-$ sensitivity of SO$_2^-$ assimilation, leading to an aerobic Met auxotrophy, and the O$_2^-$ dependence of SO$_2^-$ toxicity appear to be inherently linked to the specific absence of the cytosolic O$_2^-$ dismutation activity in SOD-1$^+$ strains.

DISCUSSION

As shown previously (1, 2, 7), mutant strains of S. cerevisiae which lack SOD-1 activity require Met for aerobic growth. Data presented herein show that S$_2$O$_2^-$ and S$^2$- supported aerobic growth also. Furthermore, in vitro assay demonstrated that an SOD-1$^+$ mutant strain contained functional thiocysteate reductase (generating S$^2$-) and sulfhydrylase (generating Cys or HomoCys from S$^2$-). The inference drawn from these results is that the production of S$^2$- from SO$_2^-$ was impaired in aerobically grown mutant cultures. Since this metabolic insufficiency was exhibited in the presence of O$_2$ only, a role for O$_2$-derived or -dependent free radicals in this pathophysiology was indicated. Since both S$^2$- and S$_2$O$_2^-$ are redox active, they could have protected the mutant in part by removing O$_2$. Also, other radicals. In fact, S$_2$O$_2^-$ did exhibit an O$_2^-$-scavenging activity in the nitrite-based SOD assay used (data not shown). Growth phenotypes in the presence of other redox-active thiol compounds did not confirm this hypothesis. Glutathione supported aerobic growth; it would be metabolized to release cysteine in yeast, of course (11). In contrast, neither dithiothreitol nor β-mercaptoethanol was effective, although this negative result could be due to an impermeance of the yeast plasma membrane to these species. Since both are neutral, uncharged molecules, however, this possibility is not likely. Thus, the inability of SOD-1$^+$ strains to utilize SO$_2^-$ rendered them dependent on sulfur added at the level of S$^2$- when grown aerobically.

This O$_2$-dependent requirement for S$^2$- could have been due to one of three possibilities: whether the oxidation of SO$_2^-$ to PAPS or (ii) SRate, which reduces SO$_2^-$ to S$^2$-, could have been sensitive to O$_2$ or a (radical) species derived from or dependent on O$_2$, or (iii) the metabolism of the SO$_2^-$, either free or bound (23, 27), derived from SO$_2^-$ could have been inhibited to growth in the absence of a cytosolic O$_2^-$-scavenging activity. The data support the last possibility.

First, although analysis of intermediates in the SO$_4^{2-}$ assimilation pathway showed that the levels of SO$_2^-$ and APS were elevated when the mutant strain was aerobically incubated with SO$_2^-$ as the sole sulfur source, this result did not confirm the presence of a pathway before the downstream step of APS. This is, anaerobically grown mutant cells labeled with $^{35}$S$^{2-}$ in the absence of the amino acids required for growth, i.e., for protein synthesis, exhibited a similar pattern of accumulation of intermediates. Therefore, this pattern may be characteristic of inhibited protein synthesis in yeast in general. Consequently, strong evidence that either ATP sulfurylase or APS kinase was sensitive to O$_2$ was lacking.

Second, in vitro assay demonstrated that the mutant strain incubated aerobically contained functional SRate. Furthermore, although the cellular [NADPH] fell by ca. 40% in aerobically grown mutant cells, [NADPH] remained greater than that of S$^2$- at the time of the experiment, which is consistent with a block of the SRate reaction. This result was obtained in the presence of SO$_2^-$ and PAPS, supporting the idea that the O$_2$- dependent O$_2^-$ toxicity is due to some other agent. However, the data do not provide a simple explanation of why the mutant strain is S$^2$- sensitive.

The third possibility remains as our working hypothesis. The data show that SOD-1$^+$ strains are sensitive to microsecond [SO$_2^-$] in an O$_2$-dependent fashion, whereas SOD-1$^+$ strains are not. Since SO$_2^-$ in some form is an obligate intermediate in the reduction of SO$_2^-$ to S$^2$-, a correlation between the aerobic SO$_2^-$ hypersensitivity and Met auxotrophy is suggested. The physiologic basis of this correlation may be that the assimilation of SO$_4^{2-}$ (11, 14), the manifestations of SO$_2^-$ toxicity (9), and SOD-1 are all located in the cytosol of yeast. However, the molecular basis of this correlation is not yet clear. The sulfite toxicity (cell killing) in wild-type (SOD-1$^+$) yeast strains observed at SO$_2^-$ concentrations of >1 mM has been linked to the inhibition by sulfite of glyceraldehyde-3-phosphate dehydrogenase and a resulting decrease in cellular ATP (9). However, these effects are reported to be O$_2$ independent and do not depend on active respiration (mitochondrial electron transport).
transport) (9, 18), indicating that the underlying chemistry does not involve an oxyradical directly or as an initiator or product of the univalent oxidation of SO$_3^{2-}$ (15). In contrast, the strains tested here which lack any cytoplasmic O$_2$-scavenging activity exhibit an aerobic sensitivity to SO$_3^{2-}$ concentrations of <50 nM which causes growth arrest only, not cell killing; under N$_2$, however, they exhibit the growth inhibition associated with cell killing only at SO$_3^{2-}$ concentrations of >1 mM; i.e., they behave like aerobic wild-type cultures. These results suggest that in *S. cerevisiae* an oxyradical-based mechanism of SO$_3^{2-}$ toxicity can occur. This mechanism is quantitatively significant only in the absence of a cytoplasmic O$_2$-scavenging activity and results in a phenotype functionally different from SO$_3^{2-}$ toxicity in SOD$^{-1}$ strains.

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LITERATURE CITED


