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Superoxide Dismutase and Oxygen Toxicity in a Eukaryote

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Saccharomyces cerevisiae var. *ellipsoideus* contained 6.5 times more superoxide dismutase and 2.3 times more catalase when grown under 100% O_2 than when grown anaerobically. Growth under oxygen caused equal increases in both the cyanide-sensitive and the cyanide-insensitive superoxide dismutases of this organism. Experience with other eukaryotes has shown that cyanide sensitivity is a property of the cupro-zinc superoxide dismutase of the cytosol, whereas cyanide insensitivity is a property of the corresponding mangani-enzyme found in mitochondria. Cu^{2+}, which has been shown to increase the radioresistance of yeast, also caused an increase of both of the superoxide dismutases of *S. cerevisiae*. Yeast which had been grown under 1 atm of O_2 were more resistant toward the lethal effects of 20 atm of O_2 than were yeast which had been grown in the absence of O_2. *Escherichia coli* K-12 *his*$^-$ responded to growth under 1 atm of O_2 by increasing its content of catalase and of peroxidase, but not of superoxide dismutase. This contrasts with *E. coli* B, which was previously shown to respond to O_2 by a striking increase in superoxide dismutase. *E. coli* K-12 *his*$^-$ did not gain resistance toward 20 atm of O_2 because of having been grown under 1 atm of O_2. Once again, this contrasts with the behavior of *E. coli* B. These data indicate that, in both prokaryotes and in eukaryotes, superoxide dismutase is an important component of the defenses against oxygen toxicity.

The superoxide radical seems to be a common intermediate in the biological reduction of oxygen, and the enzyme superoxide dismutase, which catalyzes the reaction $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$, appears to be present in all oxygen-metabolizing cells (13, 14). These findings led to the proposal that O_2^- is an important agent of the toxicity of oxygen (14). This hypothesis has received some experimental support. Thus, the content of superoxide dismutases in *Escherichia coli* B and in *Streptococcus faecalis* was shown to be increased by exposure to oxygen, and increased intracellular levels of this enzyme did correlate with enhanced resistance toward the lethality of hyperbaric oxygen (8, 9). In contrast, the catalase of *E. coli* B was not affected by oxygen, and *S. faecalis* did not contain catalase under any conditions of growth. *Bacillus subtilis* provided an important control in that it responded to oxygen by increasing its content of catalase but not of superoxide dismutase, and *B. subtilis*, unlike *E. coli* B or *S. faecalis*, did not gain resistance toward hyperbaric oxygen because of prior exposure to oxygen. These results were taken as an indication that superoxide dismutase acts as an important defense against hyperbaric oxygen in these prokaryotes, whereas catalase does not (8, 9).

It seemed important to extend these studies to eukaryotes which contain distinct mitochondrial and cytosol superoxide dismutases. Several questions arise. Can augmentation of superoxide dismutase be demonstrated in a eukaryote? Do both mitochondrial and cytosol enzymes show such augmentation? Do increased levels of superoxide dismutase correlate with increased resistance toward hyperbaric oxygen in a eukaryotic cell? *Saccharomyces cerevisiae* was chosen as the test eukaryote, because it can be grown in the absence or in the presence of oxygen and can be conveniently manipulated. Furthermore, the yeast cupro-zinc superoxide dismutase, presumably derived from the cytosol, has been isolated and characterized (7), and extracts of yeast have been shown to contain both the cyanide-sensitive and the cyanide-insensitive types of superoxide dismutase (R. A. Weisiger, Ph.D. thesis, Duke University, Durham, N.C., 1973). Cyanide sensitivity is a characteristic of the cupro-zinc superoxide dismutase of eukaryotic cytosols; whereas cyanide insensitivity is characteristic of the mangani-enzyme found in mitochondria (18). The pres-

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ent report describes augmentations of the superoxide dismutases of *S. cerevisiae* and the enhanced resistance toward the lethality of hyperbaric oxygen which accompanied these augmentations. Extensions of earlier work (8, 9) on the bases of oxygen toxicity in prokaryotes are also presented.

MATERIALS AND METHODS

S. cerevisiae var. *ellipsoideus* (ATCC 560) and *E. coli* K-12 *his*$^-$ (ATCC 25290) were obtained from the American Type Culture Collection, Rockville, Md. Yeast extract and Trypticase soy broth were products of Becton, Dickinson and Co., and agar was purchased from Difco, Inc. Puromycin hydrochloride was obtained from Nutritional Biochemicals, Inc. Yeast were grown in the semisynthetic medium of Gesswagner et al. (6). Inocula of 0.2 → 0.5% of the total broth volume were used, and growth was accomplished at 34 C on a gyratory shaker for 24 to 48 h. When the yeast were to be grown anaerobically, the Gesswagner medium was supplemented with 1 g of Tween 80 and 30 mg of ergosterol per liter in order to compensate for the inability of these cells to synthesize sterols in the absence of oxygen (15). *E. coli* K-12 *his*$^-$ was grown in a soy-yeast extract broth as previously described (8), and growth was monitored turbidimetrically (11). Yeast were disrupted, after suspension in 10 ml of 0.05 M potassium phosphate, 1×10^{-4} M ethylenediaminetetraacetic acid (EDTA), pH, 7.8, with a Branson model W185 sonifier for 20 min at a power setting of 100 W. Cell suspensions were kept in an ethanol-ice bath during sonication, and power was applied intermittently, 5 min on followed by 3 min off, to avoid overheating. *E. coli* K-12 *his*$^-$ cells were disrupted under similar conditions, except that the power was applied at 90 W for only 3 min. Cell debris was removed by centrifugation, and the resultant soluble cell extracts were assayed for superoxide dimutase (12), catalase (3), peroxidase (19), and total protein (16). Isozymes of superoxide dismutase were visualized by applying an activity stain (1) to 10% polyacrylamide electropherograms (4). The cupro-zinc superoxide dismutase, which is characteristic of eukaryotic cytosols (5), was distinguished from the mangani-superoxide dismutase, which is characteristic of mitochondria (17, 18), by including 1 mM cyanide in the solutions used in the activity staining procedure (2). Bands of superoxide dismutase activity on polyacrylamide gels were quantitated by linear densitometry at 560 nm on a Gilford model 240 spectrophotometer equipped with a gel scanner.

The resistance of yeast to hyperbaric oxygen was assessed by suspending cells to a density of 10^7/ml in the Gesswagner medium (6) to which 1.5 mg of puromycin had been added per ml. These cell suspensions were placed in stainless-steel bombs and were exposed to 20 atm of O_2 at 37 C. Magnetic stirring was maintained to ensure rapid equilibration of oxygen between the gas and liquid phases. At intervals, samples were removed, diluted, and plated onto Gesswagner medium solidified with 1.5% agar. After incubation at 34 C for 48 h, colonies were counted. Puromycin was used, during exposure to hyperbaric oxygen, to inhibit protein synthesis and thus to prevent induction of superoxide dismutase during the test period. The bomb chambers were depressurized gradually to minimize cell disruption, and controls were performed by utilizing 20 atm of N_2 in place of O_2. The resistance of *E. coli* K-12 *his*$^-$ to hyperbaric O_2 was assessed as previously described (8).

RESULTS

Induction by oxygen of the superoxide dismutases of yeast. *S. cerevisiae* was grown anaerobically, aerobically, or under 100% O_2, and soluble extracts of these cells were assayed for superoxide dismutase and for catalase. Both of these enzymes were augmented by oxygen, but superoxide dismutase responded more dramatically (Table 1). Thus, the level of superoxide dismutase was increased 6.5-fold by growth under 100% O_2, whereas the level of catalase increased only 2.3-fold under the same conditions. Disk-gel electrophoresis demonstrated three bands of superoxide dismutase activity in extracts of yeast. The major band, which accounted for 70% of the total activity, was cyanide sensitive and exhibited mobility identical to that of the cupro-zinc superoxide dismutase which has been isolated from yeast (7). The two minor bands were not inhibited by 1 mM cyanide and, on this basis, resemble the superoxide dismutases found in mitochondria (17) and in prokaryotes (10). The relative proportions of the cyanide-sensitive and the cyanide-insensitive superoxide dismutases were the same whether the cells had been grown anaerobically or under 100% O_2. It is thus clear that O_2 augmented all of the superoxide dismutases of *S. cerevisiae* to an equal degree.

Augmentation by Cu^{2+} of the superoxide dismutases of yeast. Because Cu^{2+} is a component of at least one of the superoxide dismutases

TABLE 1. *Superoxide dismutase and catalase levels of S. cerevisiae as a function of oxygen tension*[a]

Growth condition	Superoxide dismutase (U/mg of protein)	Catalase (U/mg of protein)
Nitrogen	1.3	0.7
20% Oxygen	2.5	—[b]
100% Oxygen	8.6	1.6

[a] *S. cerevisiae* was grown under the conditions described and was prepared and assayed as described in Materials and Methods. Superoxide dismutase and catalase activities were also assayed as described in Materials and Methods.

[b] Not assayed.

of yeast, it appeared possible that addition of Cu^{2+} to the growth medium might cause an increase in the amount of this enzyme in these cells. Yeast were grown aerobically in the Gesswagner (6) medium with variable additions of $CuSO_4$. Soluble extracts of these cells were dialyzed for 18 h against 400 volumes of cold 0.05 M potassium phosphate and 1×10^{-4} M EDTA, pH 7.8, to remove free Cu^{2+} and were then assayed for superoxide dismutase. Cu^{2+} did lead to increased amounts of superoxide dismutase in *S. cerevisiae* (Fig. 1). Thus, 7.85×10^{-4} M $CuSO_4$ caused a 2.8-fold increase in this activity while having no significant effect on the amount of catalase. Densitometric scanning of disk-gel electropherograms demonstrated that Cu^{2+} caused equal increases in both the cyanide-sensitive and the cyanide-insensitive superoxide dismutases. This was not the effect anticipated, because the cyanide-sensitive enzyme contains Cu^{2+} (7), whereas the cyanide-insensitive superoxide dismutases studied thus far (10, 17, 18) do not contain Cu^{2+}

Superoxide dismutase and the survival of S. cerevisiae in oxygen. Cells grown anaerobically and those grown under 100% O_2 were compared with respect to their ability to survive exposure to 20 atm of O_2. Line 1 in Fig. 2 demonstrates that yeast which had been grown anaerobically were killed rapidly by exposure to 20 atm of O_2. In contrast, cells which had been grown under 1 atm of O_2 (line 2) were much more resistant toward the lethality of hyperbaric O_2. Controls, in which cells were exposed to 20 atm of N_2 (lines 3 and 4) were also performed and demonstrated that there was only a small lethality due to pressure changes or to puromycin, or both. It is clear that growth of the yeast under 100% O_2 did confer resistance, toward 20 atm of O_2. Earlier experiments, in which *E. coli* B and *B. subtilis* were compared, demonstrated that augmentation of catalase did not confer resistance toward 20 atm of O_2, whereas augmentation of superoxide dismutase did confer such resistance (8, 9). In the present case, it seems most likely therefore that the enhanced resistance toward the lethality of 20 atm of O_2 exhibited by the oxygen-grown yeast correlated with their increased content of superoxide dismutase.

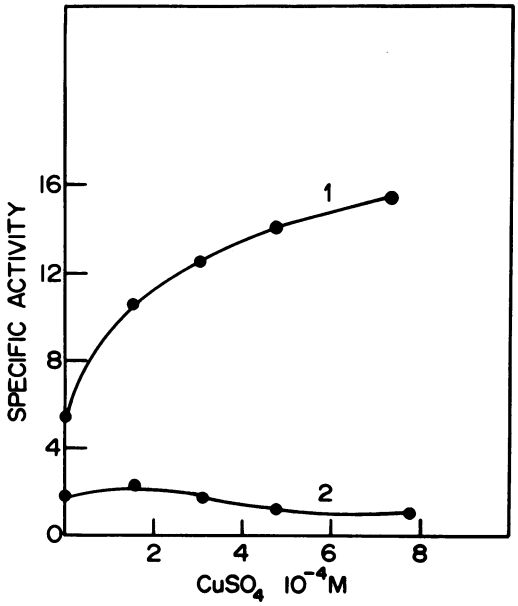

FIG. 1. *Effect of Cu^{2+} on the level of superoxide dismutase in S. cerevisiae. The yeast were grown aerobically at 34 C in Gesswagner (6) medium supplemented with $CuSO_4$ as shown. The cells were harvested in early stationary phase, and soluble extracts were prepared, dialyzed, and assayed for catalase and for superoxide dismutase as described in Materials and Methods. Line 1 shows that superoxide dismutase was induced by Cu^{2+}. Line 2 demonstrates that catalase was not induced by Cu^{2+}.*

Effects of oxygen on E. coli K-12 his⁻. Soluble extracts of this organism, grown under various tensions of oxygen, were assayed for superoxide dismutase, catalase, and peroxidase. Table 2 presents the results of these measurements. Growth of *E. coli* K-12 *his⁻* under oxygen caused an approximate doubling of catalase and of peroxidase, but no increase in superoxide dismutase. This is remarkable in view of the fact that *E. coli* B exhibited a pronounced augmentation of superoxide dismutase by oxygen, but no augmentation of catalase (8, 9). If superoxide dismutase is an important element of the defenses against oxygen toxicity, then *E. coli* K-12 *his⁻*, unlike *S. cerevisiae* or *E. coli* B, should gain little resistance toward the lethality of hyperbaric O_2 from growth under 1.0 atm of O_2. This was tested and the results are presented in Fig. 3. Anaerobically grown *E. coli* K-12 *his⁻* (line 1) was only slightly more susceptible to the cidal effects of 20 atm of O_2 than were comparable cells grown under 1.0 atm of O_2 (line 2). Lines 3 and 4 demonstrate that exposure to 20 Atm of N_2 under otherwise identical conditions caused no significant mortality. *E. coli* B, when grown under nitrogen, yields extracts which contain less superoxide dismutase than *E. coli* K-12 *his⁻* (3.4 units/mg as opposed to 6.0 units/mg). Line 5 in Fig. 3 shows that *E. coli* B, which had been cultured anaerobically, was more susceptible to the lethal effects of 20 atm of O_2 than was *E. coli* K-12 *his⁻*.

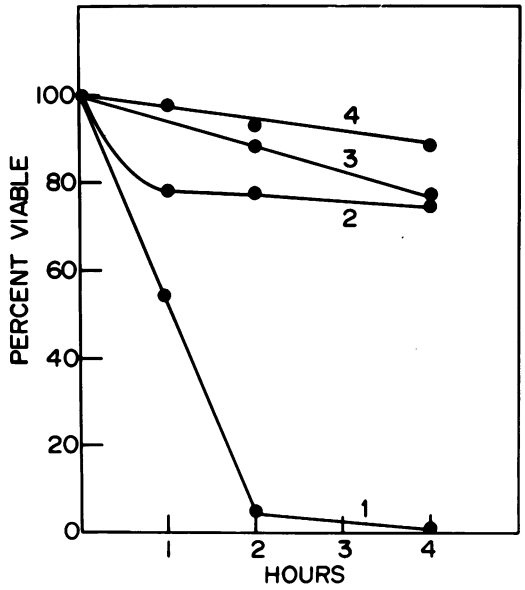

FIG. 2. *Effects of hyperbaric oxygen on S. cerevisiae. The yeast were grown anaerobically or under 1.0 atm of O_2 and then were exposed to 20 atm of O_2 or of N_2 at 34 C in the presence of 1.5 mg of puromycin per ml. At intervals, samples were removed and diluted and plated onto agar for the counting of viable cells. Line 1 demonstrates that yeast which had been grown anaerobically and which contained only 1.3 units of superoxide dismutase per mg of soluble protein were rapidly killed by 20 atm of O_2. Line 3 shows that yeast which contained 8.6 units of superoxide dismutase per mg of soluble protein, by virtue of growth under 1 atm of O_2, were much more resistant to 20 atm of O_2. Lines 2 and 4 present the survival curves for anaerobically-grown and oxygen-grown cells, respectively, under 20 atm of N_2.*

TABLE 2. *Catalase, peroxidase, and superoxide dismutase levels in E. coli K-12 his⁻ as a function of oxygen tension*[a]

Growth condition	Catalase	Peroxidase (U/mg of protein)	Superoxide dismutase (U/mg of protein)
Anaerobic	6.9	0.008	6.0
20% Oxygen ..	13.3	0.012	6.1
100% Oxygen ..	13.2	0.012	5.5

[a] Cells were grown under the conditions of aeration listed, and enzyme levels were assayed on fresh, crude-cell sonic extracts, which had not been frozen, as described in Materials and Methods.

DISCUSSION

Augmentation of superoxide dismutase by O_2 and a concomitant increase in resistance toward hyperbaric oxygen, which had previously been seen in *E. coli* B and in *S. faecalis* (8, 9), has now been observed in the eukaryote *S. cerevisiae*. Extracts of this yeast contained cyanide-sensitive and cyanide-insensitive types of superoxide dismutase. Both of these isoenzymes were equally augmented by O_2. The cyanide-sensitive superoxide dismutase of yeast is known (7) to be a cupro-zinc enzyme, which is very similar to those isolated from other eukaryotes and which is localized in the cytosol (5). The cyanide-insensitive activity is derived from the mitochondria (R. A. Weisiger, Ph.D. thesis, Duke University, Durham, N.C., 1973). It is of interest that both of these activities are augmented by oxygen. It appears likely that increased concentrations of O_2 lead to increased rates of production of O_2^- in both the mitochondria and the cytosol of yeast and that the induction of superoxide dismutase in both of these compartments of the cell provides a defense against the reactivity of these radicals.

Because Cu^{2+} is a component of the cyanide-sensitive cytosol enzyme, we might have anticipated that it could cause an increase in the level of this enzyme. In fact, Cu^{2+} added to the growth medium caused an increase in both the

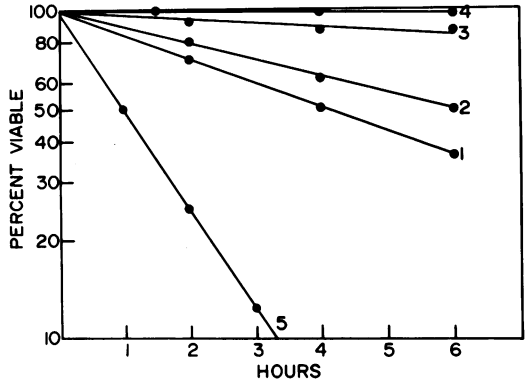

FIG. 3. *Effects of hyperbaric oxygen on E. coli K_{12} his⁻ and on E. coli B. E. coli K-12 his⁻ was grown under 100% N_2 or under 100% O_2, harvested in late log phase, and suspended in fresh medium containing 0.5 mg of puromycin per ml. They were then exposed to 20 atm of O_2 or N_2, and survival curves were constructed by sampling, diluting, and plating onto nutrient agar. Line 1 demonstrates that E. coli K-12 his⁻ which had been grown under 1.0 atm of N_2 were only slightly more susceptible to the lethality of 20 atm of O_2 than comparable cells grown under 1.0 atm of O_2 (line 2). Lines 3 and 4 record the minimal effects of 20 atm of N_2 on cells grown under 1.0 atm of N_2 and 1.0 atm of O_2, respectively. Line 5 demonstrates that E. coli B when grown under 1.0 atm of N_2 was more susceptible to the lethality of 20 atm of O_2 than comparably grown E. coli K-12 his⁻.*

cyanide-sensitive and the cyanide-insensitive superoxide dismutases of yeast. Previous work has shown that the cyanide-insensitive superoxide dismutase of eukaryotes is due to a mangani-enzyme localized in mitochondria (17). Because this enzyme contains no Cu^{2+} its augmentation by Cu^{2+} could not have been due to incorporation of this metal into an apo-enzyme. Another explanation is clearly required. The augmentation of both types of superoxide dismutase by Cu^{2+} suggests that the effects of Cu^{2+} augmentation may have been due to an increased flux of O_2^- in the Cu^{2+}-enriched cells. Thus, Cu^{2+} is both a potent catalyst of oxidations and a component of several oxidases. If the actual inducer of superoxide dismutases were O_2^- or some reaction product formed from O_2^-, then Cu^{2+} could indirectly augment superoxide dismutases by increasing the rate of production of O_2^-.

Previous studies with *S. faecalis*, *E. coli* B, and *B. subtilis* (8, 9) have indicated that superoxide dismutase is a major element of the defenses against the toxicity of oxygen. The remarkable observation that *E. coli* K-12 *his*$^-$, unlike *E. coli* B, does not respond to O_2 by augmentation of superoxide dismutase provided another control to buttress this proposal. Thus, *E. coli* K-12 *his*$^-$ did not show increases of superoxide dismutase when grown under 1.0 atm of O_2 and did not gain significant resistance toward 20 atm of O_2 from growth under 1.0 atm of O_2. In contrast *E. coli* B, which did show augmentation of this enzyme when grown under 1.0 atm of O_2, did gain such resistance.

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