Biochimica et Biophysica Acta 965 (1988) 46–51
Elsevier

BBA 22906

Oxygen-independent induction of enzyme activities related to oxygen metabolism in yeast by copper

Francesca Galiazzo [a], Alma Schiesser [c] and Giuseppe Rotilio [a,b]

[a] *Department of Biology, Tor Vergata University and* [b] *Center for Molecular Biology of CNR, Rome and* [c] *Institute of Microbiology and Agricultural Technology, University of Tuscia, Viterbo (Italy)*

(Received 31 August 1987)
(Revised manuscript received 14 December 1987)

Key words: Copper; Superoxide dismutase; Cytochrome oxidase; Antioxidant enzyme; (*S. cerevisiae*)

Aerobic growth of *Saccharomyces cerevisiae* in the presence of $CuSO_4$ (between 0.1 and 1 mM) caused a generalized induction of major enzyme activities involved in 'housekeeping' routes of oxygen metabolism (cytochrome oxidase, glutathione peroxidases and catalase) which were comparable to or higher than that observed with Cu,Zn-superoxide dismutase. Fumarase and glutathione transferase, tested as controls for oxygen-unrelated activities, were found to decrease under the same conditions. In the absence of oxygen, copper addition to yeast resulted in significant increases of Cu,Zn-superoxide dismutase and glutathione peroxidases and a slight increase of cytochrome oxidase, with catalase remaining undetectable irrespective of whether or not copper was present. Other metal ions tested (Mn^{2+}, Co^{2+}) were unable to produce such effects. It is concluded that copper has a general inducing effect on enzymes related to metabolism of oxygen and oxygen derivatives, which is mediated neither by formation of O_2^- and H_2O_2 nor by interaction with copper-specific apoproteins. These results point to a general role of copper as regulator of the expression of major enzyme activities involved in biological oxygen activation.

Introduction

Yeast has been shown to be tolerant to high concentrations of copper [1]. This has prompted several studies of the effects of copper addition or depletion on copper-containing enzymes of yeast. The results obtained have been related to copper metabolism, and in particular to the rate of incorporation, or depletion, of the metal ion into, or out of, major intracellular copper enzymes such as cytochrome oxidase (EC 1.9.3.1) and Cu,Zn-superoxide dismutase (EC 1.15.1.1) [2,3]. Copper can increase enzyme activity by multiple mechanisms. It may react as a one-electron redox-cycling metal ion with thiols and other reducing compounds of the cell and this may divert oxygen activation to a greater yield of O_2^- and H_2O_2. This metabolic alteration may result in turn in an adaptive increase of antioxidative enzymes that help remove O_2^- and H_2O_2 [4]. As far as copper enzymes are concerned, copper may also reconstitute pre-existing apoenzyme molecules or stimulate de novo biosynthesis of the apoproteins. In view of its potential interest as a natural antioxidant for industrial and medical applications, most studies have primarily been concerned with Cu,Zn-superoxide dismutase [3–6] and not focused on the just-mentioned more general aspects of the problem. An increase of the superoxide dismutase activity has generally been found, but it is not yet clear whether only the copper-containing super-

Correspondence: G. Rotilio, Department of Biology, Tor Vergata University, 00173 Rome, Italy.

oxide dismutase is increased by copper addition [4], or whether the Mn-containing superoxide dismutase [3] is also affected. Moreover, no experiments were made to test whether the copper effects are due to an increased O_2^- and H_2O_2 production, or to a direct interaction of the metal ion with the biosynthetic machinery. Finally, of other enzymes functionally related to superoxide dismutase, only catalase (EC 1.11.1.6) has been studied in this context [3,4,6]. In view of the many questions that still remain open in this regard, a wild-type strain of *Saccharomyces cerevisiae* was exposed to increasing copper concentrations with following strategy: (i) either aerobic or strictly anaerobic conditions were applied to the cultures; (ii) the glucose concentration was kept much lower than in other studies, in order to harvest yeast under conditions of active oxygen metabolism; (iii) Mn^{2+} and Co^{2+} were tested in the place of copper; (iv) all major enzyme activities involved in oxygen metabolism were assayed under any condition. The results obtained show that the copper effects are more general and not restricted to superoxide dismutase as might be inferred from previous data, are not shared by the other metal transition ions tested and are independent of the presence of oxygen.

Materials and Methods

Chemicals

Bovine serum albumin, xanthine, xanthine oxidase, cytochrome *c*, cumene hydroperoxide, Tween 80, ergosterol, cycloheximide and 1-chloro-2,4-dinitrobenzene were purchased from Sigma Chemical Co., St. Louis, MO. Sodium azide and hydrogen peroxide were from Merck, Darmstadt. Glutathione (reduced), glutathione reductase and NADPH were obtained from Boehringer Mannheim. Zymolyase (100 000 U/g) was from Seikagaku Kogyo Co. Ltd., Tokyo. Potassium cyanide and chloramphenicol were from Fluka, Buchs. Yeast extract was obtained from Difco, Detroit. All other materials were of reagent grade and obtained from the best available commercial sources.

Organisms and media

A wild-type D273-10B strain of *S. cerevisiae* was used. The basal growth medium contained 0.5% (w/v) glucose, 1% (w/v) yeast extract, 1% (w/v) NH_4Cl, 0.03% (w/v) $MgSO_4$, 0.09% (w/v) K_2HPO_4, 0.22% (w/v) KH_2PO_4. Under anaerobic culture conditions, the medium was supplemented with 1 g/l of Tween 80 and 30 mg/l of ergosterol to allow synthesis of sterols in the absence of oxygen [7]. Copper was measured with a Perkin-Elmer atomic absorption spectrometer model 4000. The copper concentration of the culture medium as such was 4 μM. The copper-loaded media were prepared by adding increasing amounts of $CuSO_4$ to the culture medium so as to reach the desired concentration. At concentrations higher than 1 mM, copper showed a toxic effect by inhibiting the yeast growth.

Growth conditions

An overnight culture grown in the basal medium for 17 h was used as the inoculum. Aerobic growth was performed in a rotatory shaker (Orbit Environ-shaker, Lab-Line Instrument, Melrose Park) at 30 °C and 180 rpm, with a ratio of flask volume to medium volume of 4 : 1. Anaerobic growth conditions were obtained by growing cultures at 30 °C in an anaerobic chamber (Anaerobic Glove Cabinet model 1024, Forma Scientific, Marietta, OH) in which $[O_2]$ was maintained at less than 10 ppm. Culture media before inoculation were equilibrated in the same anaerobic chamber for 24 h. Before exposure to air, the cultures were incubated for 10 min with 2 g/l chloramphenicol and 100 mg/l cycloheximide, to prevent aerobic growth and protein synthesis.

Preparation of cell extracts

Cells were harvested from yeast cultures grown into the late exponential phase, by centrifugation at 4 °C for 20 min at 2500 × *g*, and washed twice with cold distilled water. Cells from 1 liter of culture were incubated for 20 min at 22 °C in 50 ml of a solution containing 0.64 M 2-mercaptoethanol and 0.025 M EDTA. Cells were then washed in 70 ml cold sorbitol medium (1.1 M sorbitol, 0.05 M potassium phosphate buffer, pH 7.7). The washed cells were incubated for 40 min at 37 °C in 100 ml of sorbitol medium containing 1.5 mg of zymolyase/g wet weight of cells. Spheroplasts were collected by centrifugation at 6000 × *g* for 15 min and washed with sorbitol

medium. The pellet was suspended in a 3 ml cold solution containing 1.1 M sorbitol and 0.01 M Tris-HCl (pH 7.4) and the suspension was sonicated in an ethanol ice bath with a Branson model B-12 sonicator at a 40 W power for 3 min at 30-s intervals. Cell debris was removed by centrifugation at $7800 \times g$ for 15 min. The supernatant was extensively dialyzed against the same buffer to remove excess free copper. A fraction of the dialyzed extract was utilized for cytochrome oxidase activity assay. The dialyzed extract was then clarified at $35000 \times g$ for 20 min and used for other enzyme determinations. Protein was determined by the method of Bradford [8].

Enzyme assays

Cytochrome oxidase [9], catalase [10], glutathione peroxidase (EC 1.11.1.9) [11], glutathione transferase (EC 2.5.1.18) [12] and fumarase (EC 4.2.1.2) [13] activities were determined as previously reported. Cytochrome oxidase activity was found to be the same irrespective of the presence in the assay mixture of detergent such as 1% or 2% Tween 80 to solubilize the enzyme. The selenium-dependent glutathione peroxidase activity was assayed with either H_2O_2 or cumene hydroperoxide as substrate. The difference between the two activities (cumene $- H_2O_2$) was taken as indicative of selenium-independent glutathione peroxidase activity, which is known to be negligible with H_2O_2 [11]. Specific activity was expressed as μmol of substrate transformed per min per mg protein. Superoxide dismutase activity was assayed and expressed as units/mg protein according to the cytochrome *c*/xanthine oxidase/xanthine method [14]. The test was performed in the presence of $1 \cdot 10^{-5}$ M KCN to inhibit cytochrome oxidase and peroxidase activities. The relative amount of Cu,Zn- and Mn-superoxide dismutase was analyzed in the presence of $3 \cdot 10^{-3}$ M cyanide to suppress Cu,Zn-superoxide dismutase activity in both the solution and activity-stained gel electrophoresis [14,15]. An LKB UltroScan XL Laser densitometer was used to quantify the superoxide dismutase isoenzymes on gels, in the presence of KCN [16]. Enzyme activities were assayed using a Lambda 9 Perkin Elmer spectrophotometer. All determinations reported in the text are the average of at least three independent preparations, except for the anaerobic conditions, which were investigated with a single set of experiments.

Results

Effect of copper on enzyme activities of aerobic yeast

The effect of copper on some enzyme activities of aerobic yeast that are directly involved in O_2 metabolism (superoxide dismutase, cytochrome oxidase, catalase, selenium-dependent and selenium-independent glutathione peroxidase) is reported in Table I. All these enzyme activities increased in the range $(1-10) \cdot 10^{-4}$ M copper, with the exception of Mn-superoxide dismutase which was unaffected by copper addition. In contrast, the level of two enzymes, selected as representative of enzyme activities not involving either oxygen or a derivative of oxygen reduction as substrate, i.e., fumarase and GSH transferase, decreased significantly as a function of the same copper concentrations. An increase of the total amount of soluble protein was also observed in the presence of copper and this makes even more significant the observed increases of specific enzyme activities.

Effect of other transition metal ions

The effect of copper was found to be irreproducible by substituting either Mn^{2+} or Co^{2+}. Cobalt addition resulted in cell death. Manganese addition in the same concentration range resulted in approximately 50% decrease of all the enzyme activities tested, with the exception of Mn-superoxide dismutase, which increased by approximately 30%.

Effect of copper under anaerobic conditions

The growth of yeast under strictly anaerobic conditions resulted in a 50% decrease of total protein. Total superoxide dismutase activity (Table II) was 7% of that measured in air. Cytochrome-*c* oxidase activity of anaerobically grown yeast was 3.2% of that of aerobically grown cells, as expected from previous results. In fact, it is known that 'promitochondria' of anaerobically grown cells lack this enzymatic activity [17] and oxygen is necessary to restore it, probably by inducing proper assembly of enzyme subunits [18]. Catalase and selenium-dependent glutathione per-

oxidase were not detectable, while the selenium-independent glutathione peroxidase activity was found to be much higher than in aerobic cells.

The effects of two different concentrations of $CuSO_4$ added to the culture medium under anaerobic conditions are reported in Table III. An

TABLE I

EFFECT OF ADDITION OF COPPER TO YEAST ENZYMES

S. cerevisiae was grown aerobically at 30 °C in the presence of the indicated copper concentrations. The cells were harvested in the late-exponential phase of growth and extracts prepared from them were dialyzed and assayed for selected enzyme activities. The data are represented as means ± S.D. of three separate experiments.

$[Cu^{2+}]$ ($\times 10^4$ M)	U/mg protein		μmol/min per mg protein					
	Cu,Zn-superoxide dismutase	Mn-superoxide dismutase	cytochrome oxidase	catalase	fumarase	Se-dependent GSH peroxidase [a]	Se-independent GSH peroxidase [a]	GSH transferase [a]
0.04	21.2 ± 2.0	12.6 ± 2.2	0.14 ± 0.01	25.6 ± 3.5	0.76 ± 0.01	0.33 ± 0.08	3.52 ± 0.17	4.0 ± 0.2
1.00	26.4 ± 0.8	13.2 ± 1.0	0.16 ± 0.02	60.6 ± 6.3	0.38 ± 0.01	0.58 ± 0.17	4.25 ± 0.25	3.0 ± 0.1
2.50	31.1 ± 1.6	12.0 ± 1.0	0.19 ± 0.01	65.7 ± 8.3	0.37 ± 0.02	0.80 ± 0.20	4.71 ± 0.22	2.0 ± 0.1
5.00	31.4 ± 0.5	12.3 ± 2.7	0.20 ± 0.01	73.3 ± 6.4	0.33 ± 0.01	1.15 ± 0.06	7.23 ± 0.38	1.9 ± 0.1
10.00	35.2 ± 4.2	14.0 ± 2.0	0.26 ± 0.01	121.2 ± 16.6	0.25 ± 0.02	1.20 ± 0.03	10.50 ± 0.80	1.7 ± 0.2

[a] Units of enzyme activity are $10^3 \times$(μmol/min per mg protein).

TABLE II

EFFECT OF O_2 ON ENZYMES INVOLVED IN O_2 METABOLISM

Yeast cells were grown in the presence or absence of oxygen in the late-exponential phase of growth. Extracts obtained from the cells were assayed for enzyme activities. The values reported were obtained from a single set of experiments.

	μmol/min per mg protein				U/mg protein
	cytochrome oxidase [a]	Se-dependent GSH peroxidase [a]	Se-independent GSH peroxidase [a]	catalase	superoxide dismutase
Aerobiosis	116.0	0.3	3.9	21.2	51.4
Anaerobiosis	3.7	< 0.1	29.0	< 0.1	3.6

[a] Units of enzyme activity are $10^3 \times$(μmol/min per mg protein).

TABLE III

EFFECT OF COPPER ON SUPEROXIDE DISMUTASE AND 'OXYGEN-RELATED' ENZYMES IN ANAEROBIC CONDITIONS

Yeast cells were grown in the presence or in the absence of oxygen and, where indicated, copper was added to the culture medium. Enzyme activities were assayed on the soluble cell extracts. The values reported were obtained from a single set of experiments.

	μmol/min per mg protein				U/mg protein
	cytochrome oxidase [a]	Se-dependent GSH peroxidase [a]	Se-independent GSH peroxidase [a]	catalase	superoxide dismutase
Aerobiosis	102.0	0.3	3.7	20.0	51.0
Anaerobiosis	3.5	< 0.1	28.4	< 0.1	3.7
Anaerobiosis + Cu^{2+} ($1.0 \cdot 10^{-4}$ M)	4.2	0.9	36.6	< 0.1	8.4
Anaerobiosis + Cu^{2+} ($2.5 \cdot 10^{-4}$ M)	4.6	3.0	63.0	< 0.1	8.8

[a] Units of enzyme activity are $10^3 \times$(μmol/min per mg protein).

approximately 2-fold increase of total superoxide dismutase activity was detectable in the presence of $1 \cdot 10^{-4}$ M copper and remained at the same level at the higher Cu^{2+} concentration. Activity-stained electrophoretic gels demonstrated that Mn-superoxide dismutase activity was not present in the absence of oxygen even after copper addition. Copper did not significantly increase the cytochrome oxidase activity but had a pronounced inductive effect on selenium-dependent and selenium-independent glutathione peroxidase activities. Catalase was undetectable both in the presence and absence of copper. The amount of soluble protein was furtherly decreased by the presence of copper, although no other cytotoxic effects were observed.

Discussion

Previous studies have already shown that copper enhances the content of Cu,Zn-superoxide dismutase in aerobically grown yeast [3–6], especially in cytochrome *c*-deficient mutants [4]. The latter result suggested that the copper effect could be due, at least in part, to an increased flux of O_2^- as a consequence of redox reactions of the metal ion with reducing compounds of the cell. The data presented here confirm the effect of Cu^{2+} on Cu,Zn-superoxide dismutase content of aerobic yeast and add significant novel information on more than one aspect which had been overlooked in previous work.

(1) The increase of superoxide dismutase activity regards only the Cu,Zn-isoenzyme (Table I), as clearly shown by both solution and electrophoretic assays.

(2) The increase of activity is not restricted to this enzyme, but involves all other enzymes tested in the class having O_2 or an O_2 reduction intermediate as substrates: cytochrome oxidase, catalase, selenium-dependent and selenium-independent glutathione peroxidase. This increase is even more significant when compared to the decrease observed for other enzymes belonging to classes not directly involved in O_2 metabolism, such as fumarase and GSH transferase. It is also important to note that the relative increase of Cu,Zn-superoxide dismutase (1.7-times) is less than that of other enzymes, thus emphasizing the lack of specificity of the copper effect on Cu,Zn-superoxide dismutase.

(3) Other transition metals tested did not give the same effects. Manganese effects, in particular, were opposite to those of copper, leading to an increase of Mn-superoxide dismutase and a decrease of the other oxygen-related enzyme activities.

(4) Anaerobic conditions produced a general decrease of total protein and of all enzyme activities involved in O_2 metabolism. The only exception was the selenium-independent GSH peroxidase; this activity, however, is known to reflect the presence of some GSH transferase isoenzymes [19], which are not affected by the availability of oxygen. Copper is still effective in the absence of air, with relative increases that are comparable to those measured in the presence of air. An interesting exception is the increase of selenium-dependent GSH peroxidase, which is approximately one order of magnitude higher in the absence of air. This large effect may be related to the low activity of this enzyme in the yeast strain used and to the absence under anaerobic conditions of the other H_2O_2-removing enzyme, catalase.

In conclusion, copper induces a more general response of the cell as far as protein synthesis and specific activities of O_2-related enzymes are concerned, rather than a specific increase of Cu,Zn-superoxide dismutase. This influence of copper on the activity of this class of enzymes appears to be independent of the role of copper as a prosthetic group and as a catalyst of intracellular reactions of O_2 with reductants leading to increased O_2^- and H_2O_2 fluxes. It is therefore likely that copper regulates the biosynthesis of these enzymes by some direct influence on gene expression. This conclusion is supported by the specificity of its effects, with regard to other transition metals tested in this work, and other stimulating conditions such as heat shock, which has recently been reported to increase peroxidase but not superoxide dismutase activity in *Neurospora* [20]. As far as the response of each enzyme of oxygen metabolism is concerned, selenium-dependent GSH peroxidase appeared to be the most responsive to copper itself, irrespective of whether or not oxygen was present, while Cu,Zn-superoxide dismutase and catalase were responsive to both copper and

oxygen, with catalase being more selective for O_2. This response selectivity may lead to the isolation, under the conditions described here, of mutant strains in which the different enzymes are specifically induced or repressed and which may individually respond to oxidative stress in a way reflecting the role of each enzyme.

Acknowledgments

We are grateful to Dr. Luigi Allievi (Department of Food Science Technology and Microbiology of Milan University), for providing assistance in the preparation of anaerobic experiments. We would like to thank Ms. Patrizia Civitareale for technical assistance. This work was supported in part by 'Progetto Strategico Biotecnologie' of CNR.

References

1 Naiki, N. and Yamagata, S. (1976) Plant Cell Physiol. 17, 1281–1295.
2 Keyhani, E. and Keyhani, J. (1975) Arch. Biochem. Biophys. 167, 596–602.
3 Gregory, E.M., Goscin, S.A. and Fridovich, I. (1974) J. Bacteriol. 117, 456–460.
4 Lee, F.J. and Hassan, H.M. (1985) J. Free Radic. Biol. Med. 1, 319–325.
5 Naiki, N. (1980) Plant Cell Physiol. 21, 775–783.
6 Greco, M.A., Magner, W., Hrab, D. and Kosman, D. (1986) in Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine (Rotilio, G., ed.), pp. 296–299, Elsevier, Amsterdam.
7 Park, L.W. and Starr, P.R. (1963) J. Cell. Comp. Physiol. 61, 61–65
8 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
9 Cooperstein, S.J. and Lazarrow, A.J. (1951) J. Biol. Chem. 189, 665–669.
10 Luck, H. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), pp. 885–888, Academic Press, New York.
11 Lawrence, A.R. and Burk, R.F. (1976) Biochem. Biophys. Res. Commun. 71, 350–357.
12 Habig, W.H., Pabst, M.J. and Jacoby, W.B. (1974) J. Biol. Chem. 249, 7130–7139.
13 Massey, V. (1955) Methods Enzymol. 1, 729–735.
14 Crapo, J.D., McCord, J.M. and Fridovich, I. (1978) Methods Enzymol. 53, 382–393.
15 Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
16 Hassan, H.M. and Fridovich, I. (1977) J. Bacteriol. 129, 15–19.
17 Criddle, R.S. and Schatz, G. (1969) Biochemistry 8, 322–334.
18 Woodrow, W.G. and Schatz, G. (1979) J. Biol. Chem. 254, 6088–6093.
19 Mannervik, B. (1985) Methods Enzymol. 113, 490–495.
20 Kapoor, M. and Lewis, J. (1987) Biochem. Biophys. Res. Commun. 147, 904–910.