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Original Article

Extracellular Ascorbate Stabilization as a Result of Transplasma Electron Transfer in *Saccharomyces cerevisiae*

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The presence of yeast cells in the incubation medium prevents the oxidation of ascorbate catalyzed by copper ions. Ethanol increases ascorbate retention. Pyrazole, an alcohol dehydrogenase inhibitor, prevents ascorbate stabilization by cells. Chelation of copper ions does not account for stabilization, since oxidation rates with broken or boiled cells or conditioned media are similar to control rates in the absence of cells. Protoplast integrity is needed to reach optimal values of stabilization. Chloroquine, a known inhibitor of plasma membrane redox systems, inhibits the ascorbate stabilization, the inhibition being partially reversed by coenzyme Q_6. Chloroquine does not inhibit ferricyanide reduction. Growth of yeast in iron-deficient media to increase ferric ion reductase activity also increases the stabilization. In conclusion, extracellular ascorbate stabilization by yeast cells can reflect a coenzyme Q dependent transplasmalemma electron transfer which uses NADH as electron donor. Iron deficiency increases the ascorbate stabilization but the transmembrane ferricyanide reduction system can act independently of ascorbate stabilization.

KEY WORDS: Saccharomyces; ascorbate stabilization; plasma membrane.

INTRODUCTION

Electron transport across the plasma membrane has been described in both plant and animal cells. Functions described for this activity include (a) reduction of Fe(III) to supply Fe(II) for transport into the cell (Lesuisse and Labbe, 1992), (b) alteration of the redox state of the cytoplasm (Navas *et al.*, 1986) or activation of nuclear genes (Wenner *et al.*, 1988) to stimulate cell growth (Larm *et al.*, 1994), and (c) reduction of extracellular antioxidant compounds, e.g., tocopherol, after they have been oxidized by free radicals (Stocker and Suarna, 1993).

Genes involved in iron uptake by cells, including those related to extracellular reduction by a transmembrane redox system, have been cloned and sequenced in yeast (Roman *et al.*, 1993). Since genetic manipulation is greatly favored in yeast cells, they provide an excellent vehicle for further definition of transmembrane electron transport and its significance.

Ascorbate stabilization in the extracellular environment by both animal and plant cells has been described cells (Alcaín *et al.*, 1991; González-Reyes *et al.*, 1994). A steady-state ascorbate concentration in the cell surface (or around the cell wall) may be necessary to maintain an optimal redox state for cell metabolism or growth. It can also be hypothesized that ascorbate free radical (AFR) must be reduced to ascorbate to prevent dehydroascorbic acid (DHA) accumulation, since DHA has been reported to be toxic for living cells (Arrigoni, 1994). This maintenance of ascorbate in the reduced form has been attributed to a transplasma membrane NADH-AFR reductase.

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Activity of this type can be demonstrated with isolated plasma membrane (Navas *et al.*, 1988). The component enzymes of the AFR reductase have not been defined. Since AFR represents a natural oxidant outside the cell and reduction of the radical is important to maintain the antioxidant protection of the cells (Frei, 1994; Sharma and Buettner, 1993), this enzyme can be essential. In this study we will describe a system in yeast cells which keeps extracellular ascorbate reduced.

MATERIALS AND METHODS

Saccharomyces cerevisiae (AJ483 MATα leu his) was used in this work. Yeast cells were grown on YPD medium (2% peptone, 1% yeast extract, and 2% and glucose) incubated at 30°C with shaking. Ascorbate was not present in media used except when specifically added. In some experiments an iron-deficient medium was used which is similar to YPD except that medium is prepared according to Nicholas (1957).

Growth was monitored by determining the A_{660}, and the cultures were collected to A_{660} 2–3 in the last log phase. The cells were washed twice in 5 mM EDTA and once in cold water. The ascorbate oxidation was followed by direct reading at 265 nm, using an extinction coeficient of 14.5 mM^{-1} cm^{-1} at pH 7.4. Cells were immediately resuspended at 10^7 cells ml^{-1} in 0.1 M Tris-HCl buffer, pH 7.4, with 0.06 μM $CuSO_4$ and 0.15 mM ascorbate. The cells were discarded by centrifugation and only the supernatants were used to measure the ascorbate oxidation rates.

Ascorbate stabilization is defined as the difference between the oxidation rate of ascorbate in the presence of cells (and after treatments as indicated) and the oxidation rate in media without cells. Cells were washed and resuspended in fresh buffer before the start of the treatments.

To obtain protoplasts, cells grown in YPD to A_{660} 2–2.5, were harvested by centrifugation and twice washed in cold water and 5 mM EDTA. Then, cells were resuspended in 1 M sorbitol buffer and 5 mM EDTA to 10^8 cells ml^{-1}, and 0.1 mM DTT and 1 mg ml^{-1} pronase E was added. This suspension was shaken for 30 min at 30°C and centrifuged. All cells were washed again with sorbitol buffer and resuspended to 10^8 cells ml^{-1} containing 20 U Lyticase 10^{-8} cells, and gently shaken at 37°C. To test the yield of cell protoplast, A_{660} was determined until it reached a value <10% of initial absorbance. Protoplasts were centrifuged 5 min at 250 *g* and washed twice in Tris-HCl buffer with 1 M sorbitol, pH 7.4, and resuspended in the same buffer to 10^7 cells ml^{-1} to assay.

Broken cells were obtained by shaking cells, 12 mg dry weight, with 10 ml glass beads (0.5 mm diameter) in a total volume of 15 ml of 10 mM Tris-HCl, pH 7.4, with 5 mM EDTA. The disruption was made at 4°C. This solution was centrifuged (1000 *g*, 10 min) to remove glass beads and whole cells. This technique provides a suspension of broken cells with a yield about 95%. Also, to obtain cell walls, the broken cells were centrifuged at 3000 *g*, 10 min. The supernatant was discarded and the pellet was resuspended in 0.1 M Tris-HCl buffer, pH 7.4, for assays.

NAD^+ and NADH determinations were carried out using the cycling assay as described by Matsumura and Miyachi (1980). Ferricyanide reductase assay was performed as described by Crane *et al.* (1982), but 50 mM citrate buffer, pH 6.5, was used. Cell viability was tested using the methylene blue staining (Lee *et al.*, 1981), and protein was determined according to Bradford (1976), with bovine γ-globulin as standard. All chemicals were purchased from Sigma Company (Spain).

RESULTS

Ascorbate undergoes a chemical oxidation at a steady rate in buffer at neutral pH when small amounts of copper were present (Buettner, 1993). Addition of yeast cells to the ascorbate solution inhibited the decrease in ascorbate (Fig. 1). The protection of ascorbate was maintained over at least four hours. As specified in Materials and Methods, measurements of ascorbate were always made in the supernatant after cells were discarded, preventing the interference of cells.

If buffer was conditioned by incubation with cells without ascorbate or copper for 4 h followed by removal of the cells, the rate of ascorbate oxidation was not decreased compared to the rate in fresh buffer (Fig. 1). Protein was not detected in conditioned buffers. If ascorbate was present during the conditioning, there was only a 25% decrease in the rate of ascorbate oxidation after 3 h compared to an 81% inhibition by cells (Fig. 1). In all cases cell numbers were similar. Stabilization by cells was proportional to cell concentration and did not show saturation even up to 10^8 cells; a minimum of 10^5 cells was needed to give significant protection (Fig. 2.).

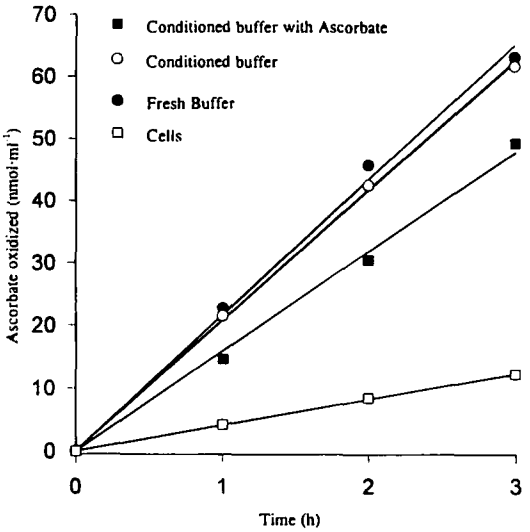

Fig. 1. Ascorbate oxidation in several buffers. The conditioned buffers were obtained by incubating 10^7 cells ml^{-1} in 0.1 M Tris-HCl buffer, pH 7.4, with or without 0.15 mM ascorbate for 4 h. Cells were removed by centrifugation and the supernatants were filtered through cut-off filters of 10 kD. The assay was made as described. Protein was not detected (n = 3, SD ≤ 5%).

The age of the cell culture also affected the rate of ascorbate stabilization, with the maximum effect being observed during the log phase of growth (Fig. 3). Specific activities of ascorbate stabilization were higher in this figure compared with other data included in Fig. 2 and Table III. These later experiments included a preincubation in buffer for 1 h before the assay. Cells in buffer progressively lost its property of ascorbate stabilization. In fact, specific activity calculated for cells immediately after separation of growth media was 14 ± 1 nmol·10^{-7} cells h^{-1}, and 10 ± 0.7 and 8 ± 0.5 30 min and 60 min, respectively, after separation of growth media and maintenance in buffer.

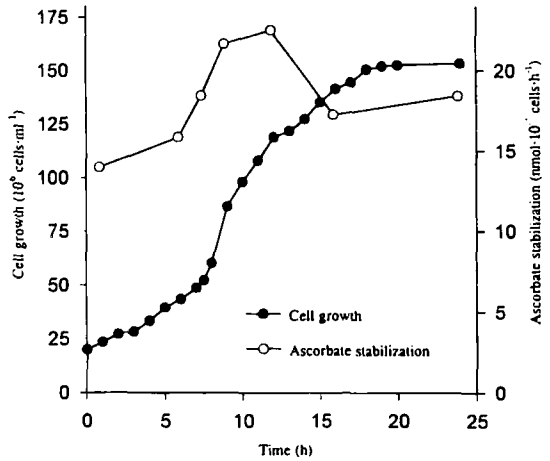

Fig. 3. Ascorbate stabilization compared with the cell culture age. The culture was started with 2 × 10^7 cells ml^{-1}. All assays were made with 10^7 cells ml^{-1} (n = 3, SD ≤ 10%).

Dead cells did not protect ascorbate. Cells heated to 90°C for 15 min had less than 15% of the inhibition of ascorbate oxidation observed with untreated cells (Fig. 4).

Ascorbate stabilization rates depended on the extracellular concentration of ascorbate. Highest stabi-

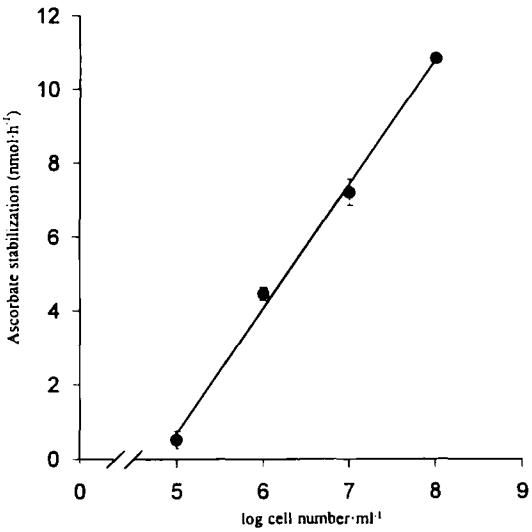

Fig. 2. Ascorbate stabilization in the presence of different amounts of yeast cells. The assay was achieved with the indicated concentrations of cells (n = 3, SD ≤ 5%).

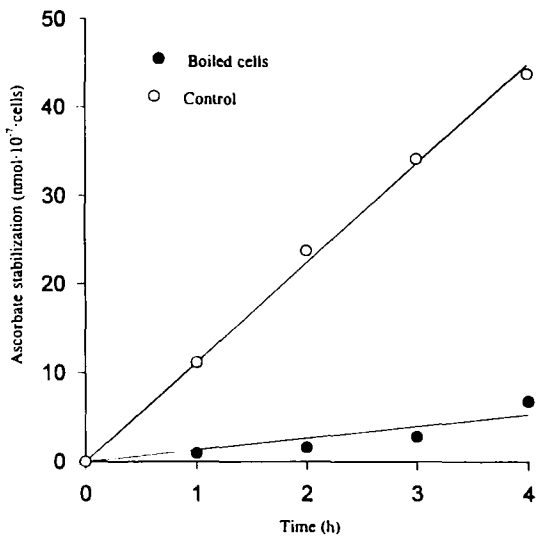

Fig. 4. Ascorbate stabilization in the presence of boiled cells. Experiments were conducted with 10^7 cells ml^{-1} (n = 3, SD ≤ 3%).

Table I. Effect of Ascorbate Concentration on Ascorbate Stabilization[a]

Treatments	Ascorbate stabilization (nmol 10^{-7} cells h^{-1})
50 μM Ascorbate	10.22 ± 0.06
100 μM Ascorbate	14.75 ± 0.77
1 mM Ascorbate	92.48 ± 0.49
10 mM Ascorbate	341.87 ± 3.24

[a] Assay was achieved with indicated concentration of ascorbate and 10^7 cells ml^{-1}. Mean values ± SD are from three separate experiments.

lization rates were observed when 10 mM ascorbate was used (Table I). To check if ascorbate stabilization was mediated by its uptake by cells, we measured the production of ascorbate by cells incubated in the presence of DHA. After 4 hours, no ascorbate was detected in growth media.

Chloroquine is an inhibitor of plasma membrane electron transport which also inhibited the stabilization of ascorbate by yeast cells. The inhibition was achieved by incubation of the cells with chloroquine for 60 min followed by separation of the cells and resuspension in assay medium. Chloroquine was a strong selective inhibitor of ascorbate protection but not of ferricyanide reduction (Table II). CoQ_6 reverted chloroquine inhibition, and stimulated the ascorbate stabilization.

Other inhibitors such as capsaicin and dicumarol had no effect on ascorbate stabilization but inhibited ferricyanide reduction by 32 and 21%, respectively.

Sulfydryl reagents (PHMB and NEM) did not inhibit the ascorbate stabilization, although NEM inhibited ferricyanide reduction by 15%. Adriamycin did not inhibit ascorbate stabilization nor ferricyanide reduction. In all cases, cell viability was not affected after 1-h treatments with inhibitors.

Ethanol increased the stabilization of ascorbate, but this was not due to the direct effect of ethanol on Cu^{2+}-dependent ascorbate oxidation because ethanol excess was eliminated by cell washing before the assay. Further, ethanol did not affect the Cu^{2+}-dependent chemical oxidation of ascorbate (Table III). This increase was prevented by pyrazole, which is also a good inhibitor of ascorbate stabilization if it was added without ethanol to yeast cells (Table III). NADH content of cells was significantly increased in the presence of ethanol, and was disminished when pyrazole was present (Table III). The incubation of cells with both ethanol and pyrazole partially abolished the inhibitory effect of the latter compound. Pyrazole itself did not affect cell viability, being about 96% compared to 97% of control cells. Also, addition of 100 mM pyrazole to 1 ml Tris-HCl buffer, pH 7.4, did not change the pH.

The stabilization of ascorbate was also increased when cells were grown under iron-deficient conditions (Fig. 5).

In order to check if cell integrity is necessary for the ascorbate stabilization, we have determined the activity in protoplasts, broken cells, and cell walls. Protoplasts obtained from yeast cells protected ascorbate from oxidation (77% as effective as whole cells). This decrease can be due to the process of cell wall digestion, which can modify surface protein(s). Activities measured in cell walls and broken cells were 0.4 and 2% of whole cells, respectively.

DISCUSSION

The stabilization of ascorbate by yeast cells in a buffer solution in the presence of copper to generate AFR resembles the effects seen with animal (Alcaín *et al.*, 1991) and plant cells (González-Reyes *et al.*,

Table II. Role of CoQ_6 and Chloroquine on Ascorbate Stabilization and Ferricyanide Reduction[a]

Treatments	Ascorbate stabilization (nmol 10^{-7} cells h^{-1})	Percent	HCF(III) reductase (nmol mg cells h^{-1})	Percent
Control	15.81 ± 0.57	100	184.6 ± 9.7	100
40 μM Chloroquine	11.18 ± 0.40	70	169.5 ± 7.1	92
60 μM CoQ_6	30.18 ± 0.37	190	ND	ND
40 μM Chloroquine + 60 μM CoQ_6[b]	28.09 ± 0.34	177	ND	ND

[a] 10_7 cells ml^{-1} were used in ascorbate stabilization assay and 0.66 mg in ferricyanide reduction assay.

[b] Cells were incubated with the inhibitor for 1 h, then washed with buffer and assayed in the presence of CoQ_6. N.D.: not determined. Mean values ± SD are from three separate experiments.

Table III. Effect of Ethanol and Pyrazol on Ascorbate Stabilization[a]

Treatments	NAD^+/ NADH rate	Ascorbate stabilization (nmol 10^{-7} cells h^{-1})	Percent
Control	1.87 ± 0.06	8.24 ± 0.54	100
Ethanol 1%	1.13 ± 0.05	14.32 ± 0.21	174
Pyrazole 100 mM	2.52 ± 0.11	0.45 ± 0.18	5
Ethanol 1% + pyrazole 100 mM	1.69 ± 0.09	8.37 ± 0.31	102

[a] All samples (including control) were incubated 1 hr at 30°C, and twice washed in cold water and buffer. The assay was made as described in Materials and Methods. Mean ± SD are from three separate experiments. Cu^{2+}-mediated chemical oxidation was of 33 nmol h^{-1} in the absence of ethanol and 34 nmol h^{-1} in the presence of ethanol.

1994). The basis for this stabilization has been discussed (Rodríguez-Aguilera and Navas, 1994) and the primary effect is attributed to a plasma membrane AFR reduction which uses internal NADH as electron donor (Navas *et al.*, 1994; Pethig *et al.*, 1985).

The results with yeast cells support this conclusion. The increase in activity with ethanol, which increases NADH in yeast, and the suppression of activity by inhibition of alcohol dehydrogenase by pyrazole are consistent with NADH as source of electrons. The pyrazole effect indicates that NADH produced by alcohol dehydrogenase can be the principal substrate. The inhibitory effect of 100 mM pyrazole on alcohol dehydrogenase of plants (Dahse *et al.*, 1994) and yeast (Crane *et al.*, 1982) is well known. This effect is apparently due to chelation by pyrazole of Zn contained in the tetrameric alcohol dehydrogenase (Magonet *et al.*, 1992), although secondary effects on other enzymes cannot be discarded.

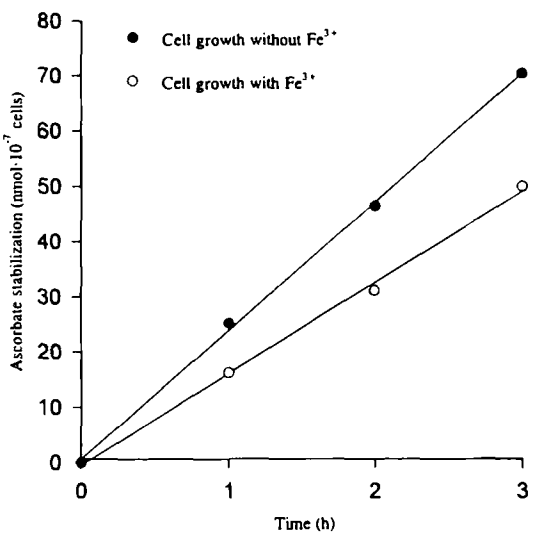

Fig. 5. Ascorbate stabilization rates in cells grown with or without Fe^{3+} in culture medium. Experiments were conducted with 10^7 cells ml^{-1} (n = 3, SD ≤ 4%).

The integrity of cells is necessary to provide a substrate for the reduction. Broken cells do not provide NADH for the enzyme, so activity is lost even though all chelation capacity is present. The stabilization requires intact cells or protoplasts and is negligible with cell walls or broken cells. The activity is proportional to cell concentration over a wide range and is not saturated at higher cell concentration as would be expected if the cells were removing copper from the media.

The electron transport remains when the cell wall is removed, which is consistent with a redox system in the plasma membrane. Lack of protection by isolated cell wall fractions shows that removal of the copper or other autooxidation catalyst is not the basis for the preservation. This is consistent with the lack of activity by heat-killed cells.

Conditioning of the media by adding and removing cells also does not produce the stabilization, which shows that the yeast cells do not stop the oxidation by removal of copper or secretion of antioxidants.

With increase in ascorbate concentration, there should be an increase in AFR concentration. The increase in specific activity of the cells in preventing a decline in ascorbate is consistent with a higher concentration of AFR (Buettner and Jurkiewicz, 1993).

The rate of transmembrane ferricyanide reduction by yeast cells increases during the log phase of growth and declines when growth stops (Crane *et al.*, 1982). A similar effect is seen with ascorbate stabilization except that the extent of increase during growth is reduced.

Iron deficiency also activates not only transmembrane Fe(III) reduction, but also ferricyanide reduction. Fe(III) reduction increases 10-fold, whereas ferricya-

nide reduction increases less (Dancis *et al.*, 1990). The increase of 40% in ascorbate stabilization by iron-deficient yeasts is similar to the reported change in ferricyanide reduction. Quantitative differences suggest that ascorbate stabilization is probably not based on activity of the FRE1 or FRE2 products necessary for Fe(III) reduction, although the stimulation of the ascorbate stabilization by iron starvation may indicate a common regulatory signal to those described for iron reduction.

The effects of the inhibitors are consistent with electron transport across the plasma membrane. However, there are significant differences between sensitivity of the ferricyanide reduction and the ascorbate stabilization to several inhibitors, suggesting that both mechanism are probably due to independent redox systems as has been previously suggested for NADH-ferricyanide and NADH-AFR reductases (Villalba *et al.*, 1993).

Ascorbate stabilization was inhibited by chloroquine, and CoQ_6 reversed the inhibition. The CoQ_6 protective effect may indicate that ascorbate stabilization is dependent on a redox enzyme complex including a quinone-like compound needed for maximal performance. CoQ_{10} is required for the transplasma membrane redox system of animal cells (Sun *et al.*, 1992). Actually, a NADH-CoQ_{10} reductase has been purified from liver plasma membrane and is suggested to be involved as the first component of a multienzyme redox system responsible for the AFR reduction by NADH (Villalba *et al.*, 1995). This suggestion is further supported by the fact that NADH-AFR reductase from animal plasma membrane cannot be solubilized by detergents, although NADH-ferricyanide and NADH-quinone reductases are successfully solubilized (Villalba *et al.*, 1993, 1995). CoQ_6 is the main ubiquinone homologue present in yeasts (Umizawa and Kishi, 1989), and a similar function could be assumed for it in the transplasma electron transport to that observed for CoQ_{10}.

In conclusion, ascorbate stabilization by yeast cells may be due to electron transport across the plasma membrane. A mechanism involving cytosolic NADH as electron donor and extracellular AFR as electron acceptor, similar to that described for animal and plant cells, may be responsible for the ascorbate stabilization, in such a way that an optimal extracellular redox state can be maintained with the consequent protection against oxidative damage or possible toxic DHA effects.

This enzyme system involved in AFR reduction is apparently different from that described for the ferricyanide reduction, and probably represents a multienzyme complex where quinone-like compound(s) is(are) important components. Attempts to characterize the plasma membrane NADH-AFR reductase from yeast cells are currently in progress.

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