Coenzyme Q₆ and Iron Reduction Are Responsible for the Extracellular Ascorbate Stabilization at the Plasma Membrane of Saccharomyces cerevisiae

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Yeast plasma membrane contains an electron transport system that maintains ascorbate in its reduced form in the apoplast. Reduction of ascorbate free radical by this system is comprised of two activities, one of them dependent on coenzyme Q₆ (CoQ₆). Strains with defects in CoQ₆ synthesis exhibit decreased capacity for ascorbate stabilization compared with wild type or with atp2 or cor1 respiratory-deficient mutant strains. Both CoQ₆ content in plasma membranes and ascorbate stabilization were increased during log phase growth. The addition of exogenous CoQ₆ to whole cells resulted in its incorporation in the plasma membrane, produced levels of CoQ₆ in the coq2 mutant strain that were 2-fold higher than in the wild type, and increased ascorbate stabilization activity in both strains, although it was higher in the coq3 mutant than in wild type. Other antioxidants, such as benzoquinone or α-tocopherol, did not change ascorbate stabilization.

The CoQ₆-independent reduction of ascorbate free radical was not due to copper uptake, pH changes or to the presence of CoQ₆ biosynthetic intermediates, but decreased to undetectable levels when coq3 mutant strains were cultured in media supplemented with ferric iron. Plasma membrane CoQ₆ levels were unchanged by either the presence or absence of iron in wild type, atp2, or cor1 strains. Ascorbate stabilization appears to be a function of the yeast plasma membrane, which is partially based on an electron transfer chain in which CoQ₆ is the central electron carrier, whereas the remainder is independent of CoQ₆ and other antioxidants but is dependent on the iron-regulated ferric reductase complex.

All aerobic organisms are exposed to the toxic effects of reactive oxygen species (ROS).¹ These are produced during normal metabolism and can also be generated by exposure to pro-oxidant compounds, an increase in oxygen pressure, or exposure to ionizing radiation (1). These ROS produce damage to many cellular components, affecting the function of lipids, proteins, and nucleic acids. However, in normal conditions, cells have a number of defense systems to avoid or minimize these problems. A good example is Saccharomyces cerevisiae, which has at least 14 proteins that participate in ROS protection (1, 2). The majority of anti-ROS mechanisms act inside the cell; however, little is known about mechanisms that protect against oxidative reduction. Some metabolic reactions involved in metal uptake produce superoxide at the apoplast (3), such as iron reduction, which is regulated by the presence or absence of iron in the culture medium (4). These ROS at the plasma membrane initiate lipid peroxidation and generate a wide array of oxidation products including shorter fatty-acyl chains. Such products impair membrane function and structural integrity and increase the membrane fluidity. The plasma membrane must have a defense system to scavenge free radicals and repair oxidative damage. A good candidate may be the redox couple ascorbate-ubiquinone. Ascorbate is a first order antioxidant and, because it scavenges free radicals in the aqueous phase of cells, is considered to be the terminal small molecule antioxidant in biological systems (5). Although ascorbate is a very efficient inhibitor of the lipid peroxidation process, it cannot inactivate the free radical effects within the plasma membrane (6). Recently, we showed that yeast cells have the ability to reduce ascorbate free radical by an enzymatic mechanism that depends on NADH as the electron donor and is inhibited by ubiquinone antagonists, such as chloroquine (7). Ubiquinone is a hydrophobic redox molecule located in different membranes, including the plasma membrane in animal cells (8). The redox chemistry of CoQ is crucial for its role in the plasma membrane electron transport system, where the ubiquinone acts as a carrier between an internal NADH-dehydrogenase and an external side final acceptor (9). This NADH dehydrogenase activity is attributed to a NADH-ubiquinone reductase in the plasma membrane of pig liver hepatocytes (10, 11). The ubiquinone present in S. cerevisiae is ubiquinone-30 (CoQ₆), and yeast mutants with defects in the COQ genes are being used to characterize the enzymes involved in CoQ₆ synthesis pathway (12–15). Recently, its importance as an antioxidant was illustrated by the hypersensitivity of CoQ₆-deficient yeast mutants to oxidative stress induced by treatment with polyunsaturated fatty acids (16). The present work employs yeast mutants deficient in CoQ₆ synthesis to study the relationship between the extracellular ascorbate stabilization and CoQ₆. The results of this study suggest that part of the ascor-
biate stabilization by whole cells depends on the CoQ\textsubscript{6} content of the plasma membrane and can be increased by the external addition of CoQ\textsubscript{6}. Both ascorbate stabilization and CoQ\textsubscript{6} content in plasma membrane can be also restored by transformation with plasmids containing the COQ3 or COQ7 genes. Ascorbate stabilization activity and plasma membrane CoQ\textsubscript{6} content are regulated as a function of the growth phase. The CoQ\textsubscript{6}-dependent ascorbate stabilization is not due to CoQ\textsubscript{6} biosynthetic intermediates or other antioxidants but is apparently due to electron transport by the plasma membrane ferric reductase complex. The CoQ\textsubscript{6}-independent ascorbate stabilization is suppressed when the \textit{coq}3 mutant strain is cultured in media supplemented with ferric iron. The results indicate that ascorbate stabilization is due to two electron transport systems in the yeast plasma membrane, one dependent on CoQ\textsubscript{6} and the other dependent on the iron-regulated ferric reductase complex.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—The yeast strains used in this study are described in Table I. Plasmids pRS121A2–2.2SB (13) and pNM7Q1 (14) restored both CoQ\textsubscript{6} synthesis and growth on nonfermentable carbon sources in strains harboring deletions in the \textit{COQ3} and \textit{COQ7} genes, respectively. Cells were grown on YPD medium (2% peptone, 1% yeast extract, and 2% glucose) incubated at 30 °C with shaking (17). Yeast harboring the plasmids pRS121A2–2.2SB and pNM7Q1 were grown in synthetic complete medium (16). In experiments with iron, 2 mM Fe-EDTA was added to YPD, and the YPD minus iron was made removing the iron with several washings with 5% hydroxyquinoline in chloroform, pure chloroform, and ether (18).

**In Vivo Assays**—The ascorbate stabilization assay was described previously (7). Growth was monitored by determining the A\textsubscript{660 nm} and the cultures were collected in late log phase (A\textsubscript{660 nm} = 3–3.5). Cells were washed once in 5 mM EDTA, pH 8, and twice in cold water. Ascorbate oxidation was followed by the direct reading at 265 nm, with an extinction coefficient of 14.5 mM\textsuperscript{-1} cm\textsuperscript{-1} at pH 7.4 (5). Cells were resuspended at \(10^7\) cells/ml in 0.1 M Tris-HCl buffer, pH 7.4, with 0.06 mM CuSO\textsubscript{4}. The addition of ascorbate (final concentration, 0.15 mM) to the cell suspension initiated the ascorbate oxidation due to the presence of Cu\textsuperscript{2+}. Cells were removed by centrifugation, and 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 without cells.

Iron reduction was measured using the methods described in Ref. 19. Cells (0.5 mg of dry weight/ml) were resuspended in reaction buffer (50 mM sodium citrate, pH 6.5, and 5% glucose). After incubation for 10 min at 30 °C with magnetic shaking, 1 mM bathophenanthroline disulfonic acid and 1 mM ferric chloride were added. Iron reduction was assayed by the formation of the complex bathophenanthroline disulfonic acid–Fe(II), as monitored by absorbance readings at 535 nm with an extinction coefficient of 17.5 mM\textsuperscript{-1} cm\textsuperscript{-1}.

**Isolation of Plasma Membranes**—Yeast plasma membranes were purified by disruption of cells with glass beads followed by a step sucrose gradient (20). These preparations were used for CoQ\textsubscript{6} determinations. Protein was determined by the dye-binding method modified for membrane samples with γ-globulin as standard (21).

**Biochemical Markers**—Plasma membrane ATPase was measured as the liberation of inorganic phosphate (22). Cytochrome c oxidase activity (inner mitochondrial membrane marker) and NADPH-cytochrome c reductase activity (endoplasmic reticulum marker) were determined as described (23). IDPase activity (Golgi marker) was measured as described (24). Outer mitochondrial membrane contamination was determined measuring the presence of porin in plasma membrane fractions by means Western blotting. Fractions were analyzed by SDS-PAGE and subsequent transfer into nitrocellulose membrane (Millipore). Membranes were blocked in 50 mM Tris-HCl buffer, pH 7.0, containing 200 mM NaCl, 0.05% Tween 20, and 2% skim milk for 1 h and then incubated for 1 h with anti-porin (polyclonal antibody, developed in rabbit and kindly provided by V. Haucke, Biozentrum, University of Basel, Basel, Switzerland). Membranes were incubated with alkaline-phosphatase-conjugated anti-rabbit secondary antibody.

**Coenzyme Q\textsubscript{6} Determination**—Coenzyme Q\textsubscript{6} extraction of whole cells was initiated with a saponification of cell pellets. Yeast samples (about 0.5 g of wet weight) were washed once with and added to 10 ml of a methanolic potassium hydroxide solution (65 g of potassium hydroxide in 650 ml of 90% methanol in water) containing 0.81 g of pyrogallol in a 40-ml saponification flask. The mixture was heated under reflux in a water bath for 30 min and cooled to 25 °C after leaving the flask on ice. The dark saponified sample was filtered through a syringe with glass wool and was extracted three times with hexane (10 ml/2 min with shaking). The upper phase of hexane was recovered, pooled, and then evaporated under vacuum in a Rotavapor (Büchi, Flawil, Switzerland). The residue was dissolved in 500 µl of ethanol.

Extraction of CoQ\textsubscript{6} from plasma membrane samples (500 µl, 0.5–1 mg of protein) was carried out by adding an equal volume of 2% SDS and vortexing for 1 min; then, 1 ml of 5% isopropanol in ethanol was added, and samples were vortexed for 1 min. To recover CoQ\textsubscript{6}, 5 ml of hexane were added, and the mixture was vortexed at top speed for 1 min and centrifuged at 1000 × g for 5 min. The upper phase was recovered, dried, and dissolved in 200 µl of ethanol.

**Coenzyme Q\textsubscript{6} Determination**—Chromatography was performed with a Beckman high performance liquid chromatography system composed of two 126–2 pumps and a 168–4 detector. Data were collected with a System Gold V910 software. The reverse phase column (Ultraphase C-18, 5 µm, 4.6 × 250 mm) was equilibrated in 90% methanol and 10% ethanol at 1 ml/min, and the sample was injected. After data collection (20 min) the percentage of ethanol was increased to 100% in 5 min, and then the mobile phase was returned to the initial composition. Quantitation of CoQ\textsubscript{6} was made by injection of external standard of known amounts of commercial CoQ\textsubscript{6} (Sigma). The concentration of standard was determined using an extinction coefficient measured using the method described above, and showed a value of 15.33 mM\textsuperscript{-1} cm\textsuperscript{-1}, in agreement with previous work (14).

**RESULTS**

**Measurement of Ascorbate Stabilization in Respiratory-deficient, CoQ\textsubscript{6}-deficient, and Wild Type Yeast Strains**—The stabilization of extracellular ascorbate was determined to be about 32 nmol/10\textsuperscript{7} cells/h in the wild type strain W303.1B (Fig. 1). Mutant strains harboring a deletion in either \textit{coq}3, \textit{coq}7, or \textit{coq}2, and hence unable to synthesize CoQ\textsubscript{6}, showed an ascorbate stabilization activity that was about 65% that of wild type.

Because \textit{coq}3, \textit{coq}2, and \textit{coq}7 yeast are unable to respire, the decrease in ascorbate stabilization activity might result from the respiration-defective phenotype. To test this possibility, a strain carrying a deletion of the \textit{ATP2} gene (encoding the β-subunit of the mitochondrial F\textsubscript{1} ATPase) and a strain carrying a deletion of the \textit{COR1} gene (encoding a protein subunit of \textit{bc}1 mitochondrial complex) were studied. As shown in Fig. 1, the ascorbate stabilization activity in the \textit{atp2} null and \textit{cor1} mutant strains was not impaired and, in fact, was slightly higher than in the wild type. Transformation of the \textit{coq}3 and \textit{coq}7 mutants with single copy plasmids containing the COQ3
and COQ7 yeast genes, respectively, restored the ascorbate stabilization activity to that of wild type cells.

All strains displayed a CoQ₆-independent ascorbate stabilization activity. Because decreases in either the pH of the buffer or the copper concentration could decrease the rate of ascorbate oxidation, these parameters were investigated. The pH was unchanged throughout the assays when run for 4 h. The property to oxidize ascorbate by buffer was abolished when copper was not added (Fig. 2). The incubation of cells for 4 h in buffer (here named conditioned buffer) did not change its property to oxidize ascorbate. This conditioned buffer still contained copper and did not contain any protein released from the cells during incubation.

When cells were present, ascorbate oxidation rates were decreased (Fig. 2) as a consequence of ascorbate stabilization at the plasma membrane (7). Boiled cells lost the ability to prevent ascorbate oxidation. Because copper is required to oxidize ascorbate and yeast have a high affinity copper transporter, we checked this activity in the FTRUNB1 strain lacking copper transporter at the plasma membrane (25). This strain showed the same ascorbate stabilization as the wild type parental strain (CM3262). These results rule out copper uptake as responsible for the CoQ₆-independent ascorbate stabilization.

Neither superoxide dismutase nor catalase modified the ascorbate oxidation rates observed in the presence of cells, indicating that the ascorbate stabilization by yeast was not due to the production of ROS during the oxidation of ascorbate.

**Determination of CoQ₆ Content in Yeast Cells and Plasma Membrane Fractions**—The concentration of CoQ₆ was measured in both whole cells and plasma membrane purified fractions of all yeast strains harvested during the final log phase of growth. Yeast lipid extracts were separated by high performance liquid chromatography, and CoQ₆ was identified based on its retention time of about 17 min at 20 °C and by the characteristic spectrum of the quinone. Wild type contained about 18 pmol of CoQ₆/mg of dry weight whole cells (Table II). This level of CoQ₆ was 25% higher than present in the atp2 strain and 33% lower than in the cor1 strain. CoQ₆ was not detected in the coq3, coq7, or coq2 mutant strain, but CoQ₆ synthesis was restored when these strains harbored the respective COQ3 or COQ7 genes on a single copy plasmid.

The CoQ₆ concentration was also determined in yeast plasma membrane fractions. Wild type yeast atp2 and cor1 mutant strains contained about 150, 195, and 236 pmol CoQ₆/mg protein, respectively. Again, CoQ₆ was not detected in the plasma membrane fraction isolated from the coq3, coq7, or coq2 mutant.

Different membrane markers were used to check the purity of plasma membrane fractions (Table III). The plasma membrane marker ATPase was highly enriched in plasma membrane fractions compared with total membranes isolated by the sucrose gradient method. However, endomembrane markers were greatly decreased in these fractions. Thus, CoQ₆ concentrations determined here represent those extracted from the plasma membrane. We did not detect porin (a marker of the mitochondria outer membrane) by Western blotting of plasma membrane fractions with a polyclonal antibody against yeast porin (data not shown).

**Measurement of Ascorbate Stabilization and CoQ₆ Content at Different Growth Stages**—Ascorbate stabilization by both wild type and coq3 mutant strains was determined during log and stationary phases of growth. Both strains reached stationary phase between 9 and 12 h, although the wild type culture attained a higher density than the coq3 mutant (Fig. 3A). Ascorbate stabilization in wild type cells was increased during log phase and reached a plateau at the end of log phase (Fig. 3B). Ascorbate stabilization in the coq3 strain showed a slight increase during the first 6 h but then decayed to the initial level (Fig. 3B).

CoQ₆ content in both total and plasma membrane fractions
increased with culture density in wild type yeast (Fig. 4). The increase in plasma membrane content was particularly marked and followed apparently the same pattern as the observed ascorbate stabilization activity (Fig. 3B).

**Effect of External CoQ6 Addition on Ascorbate Stabilization and CoQ6 Content**—Our results suggest that plasma membrane CoQ6 participates in ascorbate stabilization. To determine the effect of CoQ6 supplementation on ascorbate stabilization, both wild type and coq3 mutant yeast were incubated with exogenous CoQ6 (Table IV). Both wild type and coq3 strains were cultured and harvested in mid log phase, resuspended in buffer (10^6 cells/ml), and incubated 1 h at 30°C with or without 50 μM CoQ6. After the incubation, cells were used to determine the ascorbate stabilization and to measure the CoQ6 content in plasma membrane purified by sucrose step gradient. Exogenous CoQ6 significantly increased the content of CoQ6 in the plasma membrane of the wild type strain and also increased the rates of ascorbate stabilization (Table IV). Exogenous CoQ6 was incorporated in coq3 cells and attained a concentration at the plasma membrane that was almost twice that of wild type (Table IV). Such treatment resulted in a 58% increase in ascorbate stabilization activity in the coq3 strain.

The same incubation experiments were carried out with two well known antioxidants, benzoquinone and α-tocopherol. Neither of the two compounds showed a significant effect on ascorbate stabilization (Table IV).

**Ascorbate Stabilization and CoQ6 Contents in Cells Cultured in the Presence or Absence of Iron**—Ascorbate stabilization in several strains cultured in media with or without 2 mM iron was measured (Fig. 5A). Wild type, atp2 and cor1 strains displayed high ascorbate stabilization in iron-deprived media, and this activity was decreased when iron was present. The ascorbate stabilization in the coq3 strain also showed an iron-regulated ascorbate stabilization that was almost abolished in the presence of iron. The ferric iron reductase, measured under the same conditions as the ascorbate stabilization, was similar in all strains (Fig. 5B) and was similarly modulated by the pres-
Effects of exogenous CoQ₆ incubation on the ascorbate stabilization activity and CoQ₆ content of plasma membrane

Cells of both strains were cultured and harvested in final log phase, resuspended in buffer (10⁶ cells/ml) and incubated for 1 h at 30 °C with or without 50 μM CoQ₆, 50 μM benzoquinone, and 30 μM α-tocopherol. After the incubation, cells were used to measure the ascorbate stabilization and to determine the CoQ₆ content previous plasma membrane purification. The method was described under “Experimental Procedures.” Ascorbate stabilization data (mean ± S.E. from three separate experiments) were expressed in pmol/10⁶ cells/h, and CoQ₆ content data (mean ± S.E. from two separate experiments) were expressed in pmol/mg protein.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Additions</th>
<th>Ascorbate stabilization</th>
<th>CoQ₆ content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>None</td>
<td>24.9 ± 0.2 (100)*</td>
<td>339 ± 15 (100)</td>
</tr>
<tr>
<td></td>
<td>CoQ₆</td>
<td>30.8 ± 1.3 (123)</td>
<td>492 ± 18 (145)</td>
</tr>
<tr>
<td></td>
<td>α-Tocopherol</td>
<td>28.87 ± 1.3 (108)</td>
<td>NM*</td>
</tr>
<tr>
<td>coq3</td>
<td>None</td>
<td>25.1 ± 0.5 (101)</td>
<td>NM</td>
</tr>
<tr>
<td></td>
<td>CoQ₆</td>
<td>19.5 ± 0.7 (100)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>α-Tocopherol</td>
<td>30.90 ± 0.31 (105)</td>
<td>710 ± 45</td>
</tr>
<tr>
<td></td>
<td>Benzoquinone</td>
<td>21.3 ± 0.31 (109)</td>
<td>NM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21.6 ± 0.63 (110)</td>
<td>NM</td>
</tr>
</tbody>
</table>

a Numbers in parentheses show the percentage versus control (no addition).
b NM, not measured; ND, not detected.

All strains were grown in YPD plus 2 mM FeEDTA or YPD with iron extracted, harvested in final log phase, and processed to extract and determine CoQ₆. Concentration data (mean ± S.E. from two separate experiments) are expressed in pmol/mg of protein of plasma membrane.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasma membrane CoQ₆ concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>YPD + 2mM Fe-EDTA</td>
</tr>
<tr>
<td></td>
<td>YPD without Fe</td>
</tr>
<tr>
<td>coq3</td>
<td>158 ± 5</td>
</tr>
<tr>
<td></td>
<td>ND*</td>
</tr>
<tr>
<td>cor1</td>
<td>209 ± 11</td>
</tr>
<tr>
<td>atp2</td>
<td>200 ± 5</td>
</tr>
</tbody>
</table>

a ND, not detected.

Discussions

Extracellular ascorbate stabilization is an activity present not only in yeast but in other animal and plant cells (26, 27). In animal cells, the function is clearly directed to the maintenance of an optimal redox state and may be related to effects on cell growth and differentiation (28, 29). In plants, extracellular ascorbate stabilization plays an important role in cell elongation through ascorbate peroxidases (30). In yeast, we recently showed that a plasma membrane electron transport system, which depends on the viability of intact cells or protoplasts, is responsible for ascorbate stabilization, indicating the possible participation of plasma membrane CoQ₆ (7).

To determine the functional requirement of CoQ₆ in ascorbate stabilization, we have studied mutant strains with defects in CoQ₆ synthesis. No CoQ₆ was detected in the plasma membrane or whole cells of these strains, which also showed a lower activity of ascorbate stabilization. Wild type yeast atp2 and cor1 (respiratory-deficient strains) contained detectable CoQ₆, although its distribution inside the cell was different. Thus, although wild type cells had a higher content of CoQ₆ than did the atp2 mutant, the latter contained more CoQ₆ at the plasma membrane. However, both the plasma membrane and whole cell CoQ₆ content is higher in cor1 mutant strains than in wild type cells. These findings may account for the higher ascorbate stabilization activity in the atp2 and cor1 strains and indicate that ascorbate stabilization is not dependent on mitochondrial respiratory function. An explanation for this behavior derives from the observed increase of trans-plasma membrane electron transport in mitochondrial-deficient animal cells, which probably functions to regulate the ratio of cytosolic NAD+/NADH levels (31, 32). Previous work has shown that the establishment of a mitochondrial-deficient cell line produced increases in both plasma membrane CoQ₆ content and the ascorbate sta-
bilization activity (33). These results are all consistent with the idea that the higher CoQ₆ content in the cor1 and atp2 strains may result from the imposed respiratory deficiency.

In S. cerevisiae, plasma membrane protein represents 1–2% of total cell protein (34). Considering this percentage, plasma membrane CoQ₆ constitutes 8–16% of the total CoQ₆ in the cell. This value was increased in both wild type and respiratory defective yeast strains after the incubation of cells in buffer with exogenous CoQ₆ (Table IV).

Yeast CoQ₆ synthesis and CoQ₆ content is increased during log phase growth and reaches a maximum at stationary phase (15). Similarly, CoQ₆ content in both plasma membrane and whole cells increased during log phase growth, but the accumulation of CoQ₆ in plasma membrane increased dramatically as compared with whole cells. Ascorbate stabilization showed a similar increase but reached a plateau at the stationary phase. CoQ₆ exerts its antioxidant function when it is reduced and requires an appropriate equilibrium with its reductase, such as cytochrome b₅ reductase, at the plasma membrane (35). This behavior during growth is similar to that observed for other plasma membrane redox activities in yeast (36). The coq₃ mutant strain also showed a slight increase in ascorbate stabilization during the first hours of log phase growth, but instead of reaching a plateau, the ascorbate stabilization quickly returned to basal levels of activity. Thus, this activity may be due to another component that was increased during the growth.

Exogenous CoQ₆ was incorporated in the plasma membrane of both wild type and coq₃ strains, although the latter showed a very high capacity to incorporate CoQ₆. As a consequence of incubation with exogenous CoQ₆, ascorbate stabilization was abolished to another component that was increased during the growth (9). The coq₃ mutant strain also showed a slight increase in ascorbate stabilization during the first hours of log phase growth, but instead of reaching a plateau, the ascorbate stabilization quickly returned to basal levels of activity. Thus, this activity may be due to another component that was increased during the growth.

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