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Coenzyme Q_6 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 Q_6 (CoQ_6). Strains with defects in CoQ_6 synthesis exhibit decreased capacity for ascorbate stabilization compared with wild type or with *atp2* or *cor1* respiratory-deficient mutant strains. Both CoQ_6 content in plasma membranes and ascorbate stabilization were increased during log phase growth. The addition of exogenous CoQ_6 to whole cells resulted in its incorporation in the plasma membrane, produced levels of CoQ_6 in the *coq3* 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 CoQ_6-independent reduction of ascorbate free radical was not due to copper uptake, pH changes or to the presence of CoQ_6 biosynthetic intermediates, but decreased to undetectable levels when *coq3* mutant strains were cultured in media supplemented with ferric iron. Plasma membrane CoQ_6 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 CoQ_6 is the central electron carrier, whereas the remainder is independent of CoQ_6 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 deshydrogenase 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 (CoQ_6), and yeast mutants with defects in the *COQ* genes are being used to characterize the enzymes involved in CoQ_6 synthesis pathway (12–15). Recently, its importance as an antioxidant was illustrated by the hypersensitivity of CoQ_6-deficient yeast mutants to oxidative stress induced by treatment with polyunsaturated fatty acids (16). The present work employs yeast mutants deficient in CoQ_6 synthesis to study the relationship between the extracellular ascorbate stabilization and CoQ_6. The results of this study suggest that part of the ascor-

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[1] The abbreviations used are: ROS, reactive oxygen species; CoQ_6, coenzyme Q_6.

TABLE I
Genotype and sources of S. cerevisiae strains

Strain	Genotype	Ref.
W303.1B	MAT a *ade2–1, his3–11, 15, leu2–3, 112, trp1–1, ura3–1*	42
CC303.1	W303.1B-coq3Δ::LEU2	16
W303ΔCOQ7	W303.1B-*coq7*Δ::*LEU2*	14
W303ΔCOQ2	W303.1B-*coq2*Δ::*HIS3*	12
CC304.1	W303.1B-*atp2Δ2*::*LEU2*	16
W303ΔCOR1	W303.1B-*cor1*::*HIS3*	43
CM3262	*MAT a ino1–13 leu2–3,112 gcn4–101 his3–609 ura3–52*	25
FTRUNB1	CM3262Δctr1::URA3	25

bate stabilization by whole cells depends on the CoQ_6 content of the plasma membrane and can be increased by the external addition of CoQ_6. Both ascorbate stabilization and CoQ_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_6 content are regulated as a function of the growth phase. The CoQ_6-independent ascorbate stabilization is not due to CoQ_6 biosynthetic intermediates or other antioxidants but is apparently due to electron transport by the plasma membrane ferric reductase complex. The CoQ_6-independent ascorbate stabilization is suppressed when the *coq3* 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_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 pRS12A2–2.5SB (13) and pNMQ71 (14) restored both CoQ synthesis and growth on nonfermentable carbon sources in strains harboring deletions in the *COQ3* and *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 pRS12A2–2.5SB and pNMQ71 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 $A_{660\,nm}$, and the cultures were collected in late log phase ($A_{660\,nm}$ = 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 $mM^{-1}\cdot cm^{-1}$ 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 μM $CuSO_4$. The addition of ascorbate (final concentration, 0.15 mM) to the cell suspension initiated the ascorbate oxidation due to the presence of Cu^{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 $mM^{-1}\cdot cm^{-1}$.

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_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 nitrocelulose 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_6 Extraction—CoQ_6 extraction of whole cells was initiated with a saponification of cell pellets. Yeast samples (about 0.5 g of wet weight) were weighed out 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_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_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_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 V810 software. The reverse phase column (Ultrasphere C-18, 5 μm, 4.6 × 250 mm) was equilibrated in 90% methanol and 10% ethanol at 1 ml/min, and after 10 min, 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_6 was made by injection of external standard of known amounts of commercial CoQ_6 (Sigma). The concentration of standard was determined using a extinction coefficient measured using the method described above, and showed a value of 15.33 $mM^{-1}\cdot cm^{-1}$, in agreement with previous work (14).

RESULTS

Measurement of Ascorbate Stabilization in Respiratory-deficient, CoQ_6-deficient, and Wild Type Yeast Strains—The stabilization of extracellular ascorbate was determined to be about 32 nmol/10^7 cells/h in the wild type strain W303.1B (Fig. 1). Mutant strains harboring a deletion in either *coq3*, *coq7*, or *coq2*, and hence unable to synthesize CoQ_6, showed an ascorbate stabilization activity that was about 65% that of wild type.

Because *coq3*, *coq2*, and *coq7* 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 *ATP2* gene (encoding the β-subunit of the mitochondrial F1 ATPase) and a strain carrying a deletion of the *COR1* gene (encoding a protein subunit of *bc1* mitochondrial complex) were studied. As shown in Fig. 1, the ascorbate stabilization activity in the *atp2* null and *cor1* mutant strains was not impaired and, in fact, was slightly higher than in the wild type. Transformation of the *coq3* and *coq7* mutants with single copy plasmids containing the *COQ3*

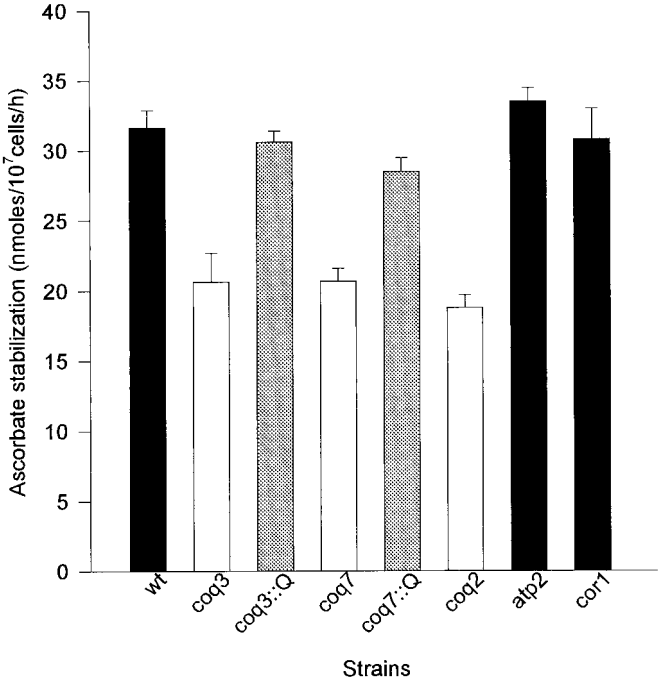

FIG. 1. **Ascorbate stabilization is reduced in yeast mutants that lack CoQ_6.** Cells were harvested during final log phase to measure the ascorbate stabilization as described under "Experimental Procedures." Specific activity is shown as the mean ± S.E. of three separate experiments. *coq3::Q* and *coq7::Q* are strains coq3 and coq7 transformed with plasmids that carry the wild type genes *COQ3* and *COQ7*, respectively. *wt*, wild type.

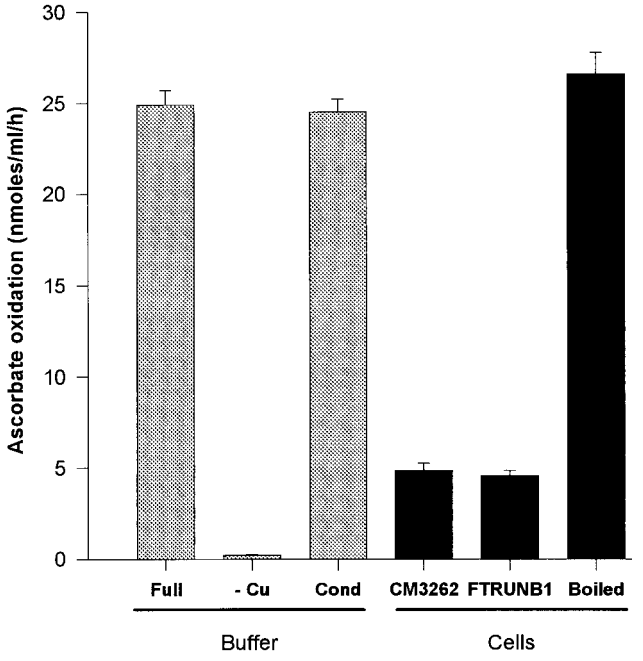

FIG. 2. **Effect of copper on ascorbate oxidation rates.** Ascorbate oxidation was followed in assay buffer with or without copper. Conditioned buffer was established after the incubation of 10^7 cells/ml for 4 h after the cells were discarded. Also, ascorbate oxidation was stimulated in buffer containing the CM3262 strain (wild type) and the FTRUNB1 strain, which lack the copper transporter. Specific activity is shown as the mean ± S.E. of three separate experiments. *Full*, buffer with copper; *Cond*, conditioned buffer.

and *COQ7* yeast genes, respectively, restored the ascorbate stabilization activity to that of wild type cells.

All strains displayed a CoQ_6-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 CoQ_6-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 CoQ_6 Content in Yeast Cells and Plasma Membrane Fractions—The concentration of CoQ_6 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 CoQ_6 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 CoQ_6/mg of dry weight whole cells (Table II). This level of CoQ_6 was 25% higher than present in the *atp2* strain and 33% lower than in the *cor1* strain. CoQ_6 was not detected in the *coq3*, *coq7*, or *coq2* mutant strain, but CoQ_6 synthesis was restored when these strains harbored the respective *COQ3* or *COQ7* genes on a single copy plasmid.

The CoQ_6 concentration was also determined in yeast plasma membrane fractions. Wild type yeast *atp2* and *cor1* mutant strains contained about 150, 195, and 236 pmol CoQ_6/mg protein, respectively. Again, CoQ_6 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, CoQ_6 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 CoQ_6 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. 3*A*). Ascorbate stabilization in wild type cells was increased during log phase and reached a plateau at the end of log phase (Fig. 3*B*). Ascorbate stabilization in the *coq3* strain showed a slight increase during the first 6 h but then decayed to the initial level (Fig. 3*B*).

CoQ_6 content in both total and plasma membrane fractions

TABLE II
Concentration of coenzyme Q_6 in plasma membrane fraction and whole cells

All strains were grown in the appropriate medium, harvested in final log phase, and processed to extract and determine CoQ_6 concentration. Data (mean ± S.E. from two separate experiments) are expressed in pmol/mg of protein in plasma membrane and pmol/mg of dry weight in whole cells.

Strain	CoQ_6 concentration	
	Plasma membrane	Whole cells
Wild type	150 ± 8	18 ± 1
coq3	ND[a]	ND
coq3:pRS12A2–2.5SB[b]	280 ± 5	15 ± 0.2
coq7	ND	ND
coq7:pNMQ7[b]	286 ± 4	17 ± 0.3
coq2	ND	ND
cor1	236 ± 9	27 ± 0.9
atp2	195 ± 10	14 ± 1

[a] ND, not detected.
[b] The medium used was SD to avoid the loss of plasmid.

TABLE III
Biochemical markers of membranes to check the plasma membrane cross-contamination with other endomembranes

The samples used were obtained using glass beads disruption and a sucrose step gradient. All activities were measured with 20–30 μg protein/ml of reaction volume and each assay was performed at 30 °C. Activity data (mean ± S.E. from two or three experiments) are expressed in nmols/mg/min. PM, plasma membrane; MF, total cell membranes; DES, diethylstibestrol-sensitive; GA, Golgi apparatus; ER, endoplasmic reticulum; IM, mitochondrial inner membrane.

Marker	MF	PM
ATPase DES-sensitive (PM)	186 ± 12	810 ± 28 (435)[a]
IDPase (GA)	353 ± 10	16 ± 3 (4.5)
NADPH cytochrome *c* oxidoreductase (ER)	36 ± 5	2 ± 0.1 (5.5)
Cytochrome *c* oxidase (IM)	895 ± 26	7 ± 0.3 (1)

[a] Numbers in parentheses indicate the percentage *versus* MF.

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. 3*B*).

Effect of External CoQ_6 Addition on Ascorbate Stabilization and CoQ_6 Content—Our results suggest that plasma membrane CoQ_6 participates in ascorbate stabilization. To determine the effect of CoQ_6 supplementation on ascorbate stabilization, both wild type and *coq3* mutant yeast were incubated with exogenous CoQ_6 (Table IV). Both wild type and *coq3* strains were cultured and harvested in mid log phase, resuspended in buffer (10^8 cells/ml), and incubated 1 h at 30 °C with or without 50 μM CoQ_6. After the incubation, cells were used to determine the ascorbate stabilization and to measure the CoQ_6 content in plasma membrane purified by sucrose step gradient. Exogenous CoQ_6 significantly increased the content of CoQ_6 in the plasma membrane of the wild type strain and also increased the rates of ascorbate stabilization (Table IV). Exogenous CoQ_6 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 CoQ_6 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. 5*A*). 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. 5*B*) and was similarly modulated by the pres-

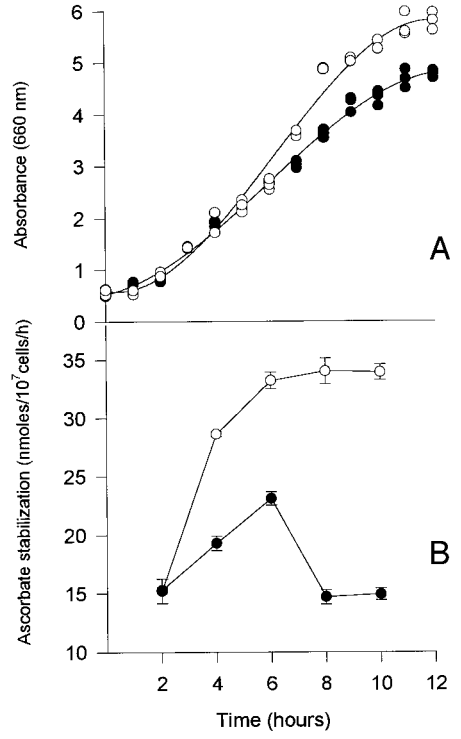

FIG. 3. **Ascorbate stabilization by wild type and *coq3* strains during different stages of growth.** YPD cultures (500 ml) were inoculated with both strains (10^6 cells/ml), and the growth was monitored by absorbance at 660 nm (*A*). Every 2 h, an appropriate cell culture volume was taken to measure the ascorbate stabilization (*B*). Ascorbate stabilization activity is shown as the mean ± S.E. of three determinations. *Open circles*, wild type strain; *closed circles*, *coq3* strain.

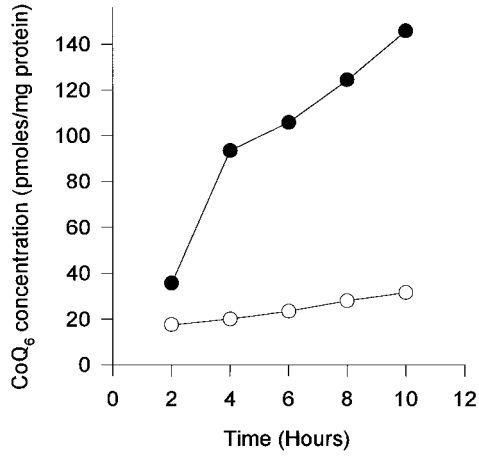

FIG. 4. **CoQ_6 content in whole cells and plasma membrane during different phases of growth.** CoQ_6 concentration is shown as the mean of two determinations (S.E. ≤ 5%) and is expressed as pmol/mg of protein in plasma membrane fractions and as pmol/mg of dry weight in whole cells. *Open circles*, wild type whole cells; *closed circles*, wild type plasma membrane fractions. CoQ_6 was not detected in *coq3* cells.

TABLE IV

Effects of exogenus CoQ_6 incubation on the ascorbate stabilization activity and CoQ_6 content of plasma membrane

Cells of both strains were cultured and harvested in final log phase, resuspended in buffer (10^8 cells/ml) and incubated for 1 h at 30 °C with or without 50 μM CoQ_6 50 μM benzoquinone, and 30 μM α-tocopherol. After the incubation, cells were used to measure the ascorbate stabilization and to determinate the CoQ_6 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 nmol/10^7 cells/h, and CoQ_6 content data (mean ± S.E. from two separate experiments) were expressed in pmol/mg protein.

Strain	Additions	Ascorbate stabilization	CoQ_6 content
Wild type	None	24.9 ± 0.2 (100)[a]	339 ± 15 (100)
	CoQ_6	30.8 ± 1.3 (123)	492 ± 18 (145)
	Benzoquinone	26.87 ± 1.3 (108)	NM[b]
	α-Tocopherol	25.1 ± 0.5 (101)	NM
coq3	None	19.5 ± 0.7 (100)	ND
	50 μM CoQ_6	30.90 ± 0.31 (158)	710 ± 45
	Benzoquinone	21.3 ± 0.31 (109)	NM
	α-Tocopherol	21.6 ± 0.63 (110)	NM

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

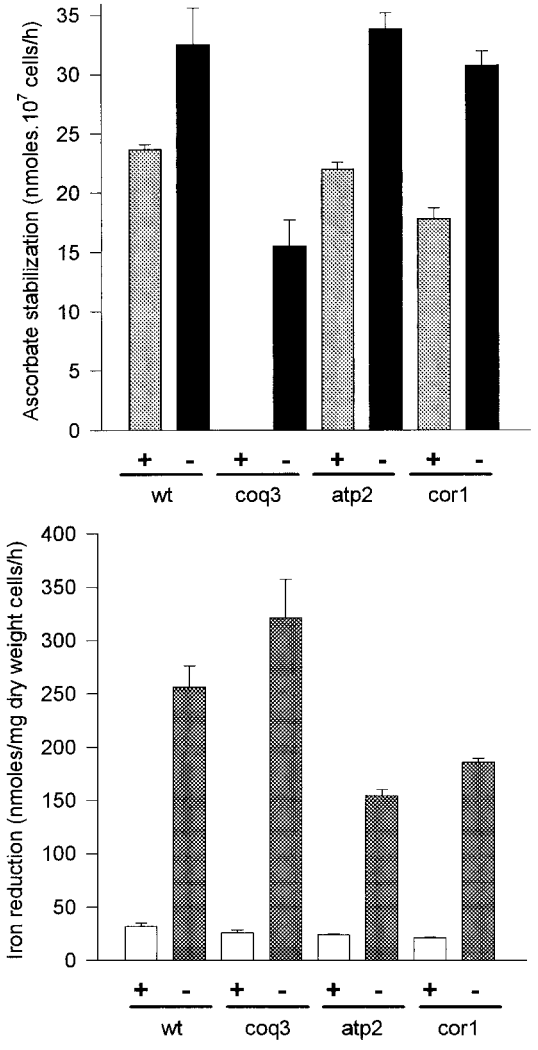

FIG. 5. **Ascorbate stabilization and ferric reduction in several strains cultured in iron presence or absence.** *A*, ascorbate stabilization. Cells were harvested during final log phase, and the activity is shown as the mean ± S.E. of three separate experiments. *B*, ferric reduction. Cells were harvested during final log phase, and the activity is shown as the mean ± S.E. of three separate experiments. In both experiments, + indicates cells cultured in YPD with the addition of 2 mM FeEDTA, and − indicates cells cultured in YPD without iron (iron was extracted using a chemical method described under "Experimental Procedures").

ence or absence of iron. The *coq3* strain displayed a higher ability to reduce ferric iron, twice that of the other respiratory-deficient mutant strains. The presence or absence of iron in the culture did not significantly change the CoQ_6 content in these strains (Table V).

TABLE V

CoQ_6 contents in several strains cultured in the presence or absence of iron

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 CoQ_6. Concentration data (mean ± S.E. from two separate experiments) are expressed in pmol/mg of protein of plasma membrane.

Strain	Plasma membrane CoQ_6 concentration	
	YPD + 2mM Fe-EDTA	YPD without Fe
Wild type	158 ± 5	151 ± 6
coq3	ND[a]	ND
cor1	209 ± 11	220 ± 9
atp2	200 ± 5	206 ± 7

[a] ND, not detected.

DISCUSSION

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 CoQ_6 (7).

To determine the functional requirement of CoQ_6 in ascorbate stabilization, we have studied mutant strains with defects in CoQ_6 synthesis. No CoQ_6 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 CoQ_6, although its distribution inside the cell was different. Thus, although wild type cells had a higher content of CoQ_6 than did the *atp2* mutant, the latter contained more CoQ_6 at the plasma membrane. However, both the plasma membrane and whole cell CoQ_6 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 of 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 CoQ content and the ascorbate sta-

bilization activity (33). These results are all consistent with the idea that the higher CoQ_6 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 CoQ_6 constitutes 8–16% of the total CoQ_6 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 CoQ_6 (Table IV).

Yeast CoQ_6 synthesis and CoQ_6 content is increased during log phase growth and reaches a maximum at stationary phase (15). Similarly, CoQ_6 content in both plasma membrane and whole cells increased during log phase growth, but the accumulation of CoQ_6 in plasma membrane increased dramatically as compared with whole cells. Ascorbate stabilization showed a similar increase but reached a plateau at the stationary phase. CoQ exerts its antioxidant function when it is reduced and requires an appropriate equilibrium with its reductase, such as cytochrome b_5 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 CoQ_6 was incorporated in the plasma membrane of both wild type and *coq3* strains, although the latter showed a very high capacity to incorporate CoQ_6. As a consequence of incubation with exogenous CoQ_6, ascorbate stabilization was stimulated in wild type and was restored to wild type levels in the *coq3* mutant. Exogenous CoQ stimulates the trans-plasma membrane electron transport (9) and significantly increases the ascorbate stabilization in animal cells (35). CoQ also acts through the plasma membrane redox system to replace pyruvate as an essential medium component required for the growth of mitochondria-deficient ρ° Namalwa cells (32).

The data presented here show that ascorbate stabilization was not absent in the CoQ_6 mutant yeast strains and thus cannot depend exclusively on CoQ_6. This CoQ_6-independent activity is not due to changes in pH or copper uptake, because FTRUNB1 strain lacking the copper transporter (25) shows the same ascorbate stabilization activity as the wild type strain. The contribution of CoQ_6 biosynthetic intermediates to the CoQ_6-independent ascorbate stabilization can be excluded by the examination of the *coq2* yeast mutant strain. Such mutants are defective in transferring the polyprenyl group to *p*-hydroxybenzoic acid (the aromatic ring precursor of CoQ) and hence are incapable of generating any prenylated CoQ biosynthetic intermediates (12). Other phenolic compounds display antioxidant properties and may be able to reduce peroxidation damage (37, 38). For example, 1,4-benzoquinone is a plasma membrane redox system component thought to provide a defense against free radicals produced by the mycelial fungus *Phanerochaete chrysosporium* during lignin peroxidase-mediated mineralization (39). But the absence of an effect produced by either 1,4-benzoquinone or the antioxidant α-tocopherol tend to argue against the role of other antioxidants in the ascorbate stabilization.

It seems more likely that the remaining activity is due to another plasma membrane electron transport system in which CoQ_6 is not involved. A possible candidate is the system responsible for the reduction of ferric iron, a necessary step in iron assimilation (40, 41). In agreement with previous studies (4), this system is modulated by the presence or absence of iron in the culture medium (Fig. 5*B*). The ascorbate stabilization is also modulated by iron in all strains examined (Fig. 5*A*) in a manner that is independent of the CoQ_6 content (Table V). The most pronounced effect was displayed in the *coq3* strain, in which the ascorbate stabilization was abolished when cells were cultured in iron-supplemented media. In this situation, the lack of CoQ_6 at the plasma membrane, combined with the repression of iron reduction, results in the absence of ascorbate stabilization. The data indicate that ascorbate stabilization depends on two redox systems at the plasma membrane, one that is CoQ_6-dependent and the ferrireductase complex. The contribution of the ferrireductase complex accounts for the increase in ascorbate stabilization observed in the *coq3* stain during log phase growth, because iron reduction was also increased during this phase. Thus, the high level of iron reduction in the *coq3* mutant strain accounts for the stabilization of ascorbate despite the absence of the CoQ_6-dependent system.

In conclusion, ascorbate stabilization at the plasma membrane of *S. cerevisiae* is in part dependent on CoQ_6, whereas the remaining activity is due to the action of the plasma membrane iron reductase system. Both of these systems cooperate to maintain a reduced environment, based on ascorbate, at the apoplast.

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