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Genetic Evidence for Coenzyme Q Requirement in Plasma Membrane Electron Transport

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Plasma membranes isolated from wild-type *Saccharomyces cerevisiae* crude membrane fractions catalyzed NADH oxidation using a variety of electron acceptors, such as ferricyanide, cytochrome *c*, and ascorbate free radical. Plasma membranes from the deletion mutant strain *coq3*Δ, defective in coenzyme Q (ubiquinone) biosynthesis, were completely devoid of coenzyme Q_6 and contained greatly diminished levels of NADH–ascorbate free radical reductase activity (about 10% of wild-type yeasts). In contrast, the lack of coenzyme Q_6 in these membranes resulted in only a partial inhibition of either the ferricyanide or cytochrome-*c* reductase. Coenzyme Q dependence of ferricyanide and cytochrome-*c* reductases was based mainly on superoxide generation by one-electron reduction of quinones to semiquinones. Ascorbate free radical reductase was unique because it was highly dependent on coenzyme Q and did not involve superoxide since it was not affected by superoxide dismutase (SOD). Both coenzyme Q_6 and NADH–ascorbate free radical reductase were rescued in plasma membranes derived from a strain obtained by transformation of the *coq3*Δ strain with a single-copy plasmid bearing the wild type *COQ3* gene and in plasma membranes isolated form the *coq3*Δ strain grown in the presence of coenzyme Q_6. The enzyme activity was inhibited by the quinone antagonists chloroquine and dicumarol, and after membrane solubilization with the nondenaturing detergent Zwittergent 3–14. The various inhibitors used did not affect residual ascorbate free radical reductase of the *coq3*Δ strain. Ascorbate free radical reductase was not altered significantly in mutants *atp2*Δ and *cor1*Δ which are also respiration-deficient but not defective in ubiquinone biosynthesis, demonstrating that the lack of ascorbate free radical reductase in *coq3*Δ mutants is related solely to the inability to synthesize ubiquinone and not to the respiratory-defective phenotype. For the first time, our results provide genetic evidence for the participation of ubiquinone in NADH–ascorbate free radical reductase, as a source of electrons for transmembrane ascorbate stabilization.

KEY WORDS: Coenzyme Q; plasma membranes; electron transport; ascorbate stabilization.

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INTRODUCTION

Coenzyme Q (CoQ, ubiquinone) is a lipophilic electron transport molecule that participates as intermediate in the inner mitochondrial membrane, driving electrons from the NADH and succinate dehydrogenases to the cytochrome bc_1 complex. In addition to this well-characterized function, it is apparent that CoQ in its reduced form ($CoQH_2$, ubiquinol) also participates in the antioxidant protection of membrane lipids and serum lipoproteins, either by direct scavenging of

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lipid peroxyl radicals, or mediating the regeneration of α-tocopherol (Kagan *et al.*, 1996; Littarru *et al.*, 1996). Furthermore, a role in protection of proteins and DNA from oxidative damage has been also documented (Forsmark-Andrée *et al.*, 1995).

During the last several years, evidence has accumulated for the requirement of CoQ in transplasma membrane redox activity (Sun *et al.*, 1992). Solvent extraction of lyophilized plasma membranes results in an inactivation of transmembrane NADH–ascorbate free radical (AFR) reductase, whereas NADH–cytochrome-*c* reductase, a marker for *cis* electron transport on the cytoplasmic side of the plasma membrane, remains unchanged (Villalba *et al.*, 1995). Addition of CoQ to cultured HL-60 cells increases their ability to stabilize extracellular ascorbate (Gómez-Díaz *et al.*, 1997a). In addition, depletion of mitochondrial DNA by prolonged incubation of cell cultures with ethidium bromide, results in both an increase of CoQ in the plasma membrane and augmented ascorbate stabilization (Gómez-Díaz *et al.*, 1997b), a measurement for transplasma membrane redox activity that is accounted partially by the AFR reductase (Navas *et al.*, 1994; Rodríguez-Aguilera and Navas, 1994).

Yeasts provide a powerful tool for the genetic study of cell processes because of the ease in obtaining mutants. This approach has been used successfully in the molecular dissection of the system involved in iron reduction and high-affinity uptake in yeast cells (Stearman *et al.*, 1996; Kaplan and O'Halloran, 1996) and has enabled functional analogies between the yeast and mammalian systems to be established (Kaplan and O'Halloran, 1996). CoQ_6 is the ubiquinone present in *Saccharomyces cerevisiae* (Umizawa and Kishi, 1989). Eight complementation groups (*coq1–coq8*) of *S. cerevisiae* mutants, deficient in CoQ_6 biosynthesis, have been described (Tzagoloff *et al.*, 1975a, b; Tzagoloff and Dieckmann, 1990). These mutants lack CoQ and, hence, are respiratory defective and unable to grow on nonfermentable carbon sources. The *COQ3* gene of *S. cerevisiae* encodes the 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase (Clarke *et al.*, 1991), an enzyme conserved among eukaryotes (Marbois *et al.*, 1994). Yeasts harboring a *COQ3* gene deletion (*coq3*Δ) do not synthesize CoQ (Clarke *et al.*, 1991) and have been used very recently to prove the role played by CoQ in antioxidant protection of yeast cells (Do *et al.*, 1996) and in ascorbate stabilization (Santos–Ocaña *et al.*, 1998).

Although reported data support the participation of CoQ-shuttling electrons for the reduction of extracellular acceptors (as it is the case for the plasma membrane AFR reductase), a genetic approach demonstrating unequivocally the role played by CoQ in different plasma membrane-associated redox activities has not yet been presented. In this study we have measured several redox activities of plasma membranes obtained from wild-type yeast, isogenic mutant strain *coq3*Δ and the *coq3*Δ mutant rescued for CoQ_6 either by growing in the presence of the quinone or by transformation with a single-copy plasmid bearing the wild-type *COQ3* gene. Our results demonstrate that the NADH–AFR reductase is due to a system fully dependent on the presence of CoQ in yeast plasma membranes. The dependence of this system on CoQ correlates with the CoQ requirements for ascorbate stabilization by whole yeast cells (Santos-Ocaña *et al.*, 1998). On the other hand, a portion of NADH–ferricyanide and cytochrome-*c* reductases is mediated by the CoQ semiquinone, which generates the formation of superoxide by one-electron reduction of O_2.

MATERIALS AND METHODS

Plasmid Constructions

The plasmid pCC-COQ3 was constructed by ligating the 2.2-kb *Sma*I fragment containing the *COQ3* gene from pRS12A (Clarke *et al.*, 1991) into the *Sma*I site of pRS313 (Sikorski and Hieter, 1989).

Yeast Strains and Culture Conditions

Mutant strains of *S. cerevisiae* used in this work are listed in Table I. CC303.1 (*coq3*Δ) and CC304.1 (*atp2*Δ) strains were constructed by one-step gene replacement as described (Clarke *et al.*, 1991; Do *et al.*, 1996). As previously reported, gene disruptions were verified by Southern blot analysis of yeast genomic DNA. *ATP2*, *COR1*, and *COQ3* null mutants failed to grow on a nonfermentable carbon source. Yeast cells were grown in YPD media (1% yeast extract, 2% peptone, 2% dextrose) at 30°C with shaking. Yeast strains harboring plasmid pRS313 or pCC-COQ3 were grown in synthetic medium without histidine to select cells containing the plasmid.

Isolation of Plasma Membrane Fractions

Unless otherwise specified, all steps for plasma membrane isolation were performed at 4°C. Yeasts

Table I. Genotype and Sources of *Saccharomyces cerevisiae* Strains Used in this Work

Strain	Genotype	Source
W303.1B	α *ade2-1 his3-11, 15 leu2-3,112 trp1-1 ura3-1*	Repetto and Tzagoloff (1989)
CC303.1	*W303.1B-coq3Δ::LEU2*	Do *et al.* (1996)
CC304.1	*W303.1B-atp2Δ::LEU2*	Do *et al.* (1996)
W303-Δ*cor1*	α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 COR1::HIS3*	Tzagoloff *et al.* (1986)

were harvested at the end of the logarithmic phase (A_{600nm} = 2–3) and washed twice with cold distilled water. Cells were homogenized by shaking vigorously with glass beads (Serrano, 1988). The homogenate was centrifuged for 10 min at 700 × *g* to remove debris, and the resulting supernatant was centrifuged for 60 min at 20,000 × *g* to obtain a crude membrane fraction. For plasma membrane purification, total membranes from about 10 g of cells were resuspended in 6 ml of 20% (w/w) sucrose, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 1 mM DTT (sucrose buffer), and applied to a discontinuous sucrose gradient made of 4 ml 43% (w/w) sucrose and 2 ml 53% (w/w) sucrose in the same buffer. After centrifugation for 4 h at 100,000 × *g*, plasma membranes were recovered at the 43/53 interphase. Plasma membranes were then diluted with sucrose buffer, washed by centrifugation, and resuspended in 1 ml of sucrose buffer. Membranes were stored at −80°C until use. To avoid interference, plasma membranes were washed twice and resuspended in sucrose buffer without DTT before determinations of oxidoreductase activities and superoxide measurements (see below).

Purity of plasma membrane fractions was checked by marker enzyme analysis. The following marker activities were carried out: diethylstilbestrol (DES)-sensitive ATPase for plasma membrane (Serrano, 1988), NADPH–cytochrome-*c* oxidoreductase for endoplasmic reticulum (Storrie and Madden, 1990), cytochrome-*c* oxidase for mitochondria (Storrie and Madden, 1990), and latent IDPase for Golgi apparatus (Asard *et al.*, 1987). Protein determinations were carried out by the dye-binding method adapted for samples containing membranes (Stoscheck, 1990).

Quantification of CoQ_6

For lipid extraction, plasma membranes were disrupted with 1% SDS and then, 2 vol of 95% ethanol–5% isopropanol were added. Lipids were separated from SDS–ethanolic solutions by extraction with 5 vol of hexane. The extraction step was repeated twice and all hexane phases were combined and evaporated at reduced pressure. Lipid extracts were resuspended in 100 μl of ethanol and 20 μl were used for determination of the natural yeast ubiquinone homologue CoQ_6 by reverse-phase high-performance liquid chromatography (HPLC). Chromatography was performed at 1 ml/min with an Ultrasphere C-18 5 μm 0.46 × 5 cm precolumn fitted at the top of a 25-cm C-18 analytical column (Beckman, USA). Mobile phase was 10% ethanol–90% methanol, and eluates were monitored at 275 nm. The CoQ_6 peak was identified by both its retention time and by automatic recording of absorption spectra of substances eluted from the column. Using our conditions, CoQ_6 eluted at about 17 min (Fig. 1). CoQ_6 amounts were quantified by integration of peaks and comparison with external standards (Sigma, Spain) and were then referred to plasma membrane protein.

Oxidoreductase Assays

NADH–ferricyanide reductase activity was assayed spectrophotometrically at 30°C by measuring the decrease in absorbance at 340 nm in a medium containing 50 mM Tris-HCl, pH 7.6, 0.2 mM potassium ferricyanide, 0.2 mM NADH and 50–100 μg plasma membrane protein. The reaction was initiated with the addition of NADH. Rates obtained were corrected for both nonenzymatic reaction between NADH and ferricyanide and the rate of NADH oxidation in the absence of added acceptor. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used in calculations of specific activities. NADH–AFR reductase was assayed by measuring the decrease in absorbance at 340 nm upon addition of 60 mU ascorbate oxidase to a reaction mixture containing 50 mM Tris-HCl, pH 7.6, 0.2 mM NADH, 1 mM ascorbate, and about 100 μg protein. NADH–cytochrome-*c* reductase was assayed by mea-

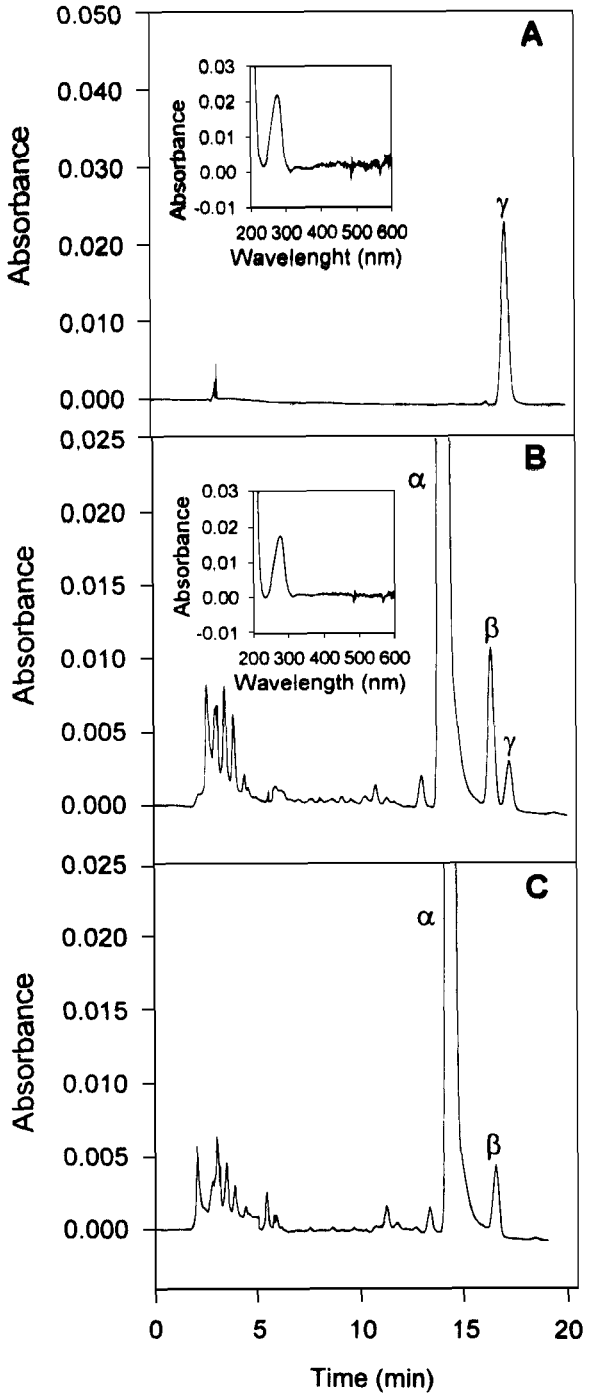

Fig. 1. HPLC chromatograms of commercial purified CoQ_6 (100 pmol) (A), lipid extracts from plasma membranes isolated from wild-type (B), and *coq3*Δ strains (C). Insets in A and B, absorption spectrum of the compound eluting at 17 min (CoQ_6, peak γ).

suring the increase in absorbance at 550 nm (reduction of the cytochrome) in a medium containing 0.2 mM NADH, 80 μM cytochrome *c*, 1 mM KCN, and 75–100 μg plasma membrane protein. An extinction coefficient of 29.5 mM^{-1} cm^{-1} was used.

Superoxide Measurements

The effect of SOD addition to the reaction mixtures (80 units/ml) was studied to estimate the participation of superoxide anions in redox activities. Superoxide generation by one-electron reduction of quinones was demonstrated by recording the absorbance increases at 550 nm, induced by addition of 0.2 mM NADH to a reaction medium containing 50 mM Tris-HCl, pH 7.6, 0.1 mM CoQ_0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone), 20 μM acetylated cytochrome *c*, and 50 μg plasma membrane protein. Assays were carried at 30°C with gentle stirring.

RESULTS

Purity of Plasma Membranes and CoQ_6 Analysis

Plasma membranes isolated from preparations of yeast crude membranes were of a very high level of purity, showing a fourfold enrichment of the plasma membrane marker DES-sensitive ATPase compared to the fraction of total membranes (Table II), a value which is in accordance with those previously reported (Serrano, 1988; Serrano *et al.*, 1991). On the other hand, both NADPH–cytochrome-*c* oxidoreductase as measurement of contamination with membranes

Table II. Marker Enzyme Analysis of *Saccharomyces cerevisiae* Plasma Membranes[a]

Enzyme marker	Crude membranes	Plasma membranes	Enrichment
DES–ATPase	186 ± 12	810 ± 28	4.35
Latent IDPase	353 ± 10	16 ± 3	0.045
NADPH cytochrome *c*	36 ± 5	2 ± 0.1	0.055
Cytochrome-*c* oxidase	895 ± 26	7 ± 0.3	0.0078

[a] Data listed on the table were obtained from the wild-type W303.1B strain. Specific activities of marker enzymes are expressed as nmol/min/mg. Enrichment values were calculated as the ratios of activities measured with plasma membranes to those measured with total membranes. Data are mean ± S.D. (n = 4).

derived from the endoplasmic reticulum and latent IDPase as marker for Golgi apparatus membranes, were about twentyfold lower in the plasma membrane compared to crude fractions. Further, the mitochondrial marker cytochrome-*c* oxidase was about 130 times lower in plasma membrane fractions compared to starting crude membranes. No significant differences in purity were observed among plasma membranes prepared from the different strains used in this work (not shown). CoQ_6 concentrations of plasma membranes derived from different yeast strains were studied by HPLC analysis of lipid extracts (Fig. 1). Plasma membranes from wild-type yeasts contained about 150 pmol CoQ_6/mg protein. CoQ_6 concentrations in plasma membranes isolated from the *atp2*Δ and *cor1*Δ strains were 30–40% higher and, as expected, no CoQ_6 could be detected in the *coq3*Δ mutant (Fig. 1; Table III).

Role of CoQ_6 on Ferricyanide and Cytochrome-*c* Reductases

Wild-type yeast plasma membranes displayed NADH-dependent redox activities with a variety of electron acceptors (Table III). Both NADH–ferricyanide and –cytochrome-*c* oxidoreductases were decreased in the *coq3*Δ mutant to about 60% of the activity observed with wild-type membranes, indicating that part of these activities is mediated by CoQ, but a significant part can be considered as CoQ independent. The CoQ_6 increase in the *atp2*Δ and *cor1*Δ mutants did not result in a similar increase in ferricyanide and cytochrome-*c* reductases, no significant differences being obtained between wild-type yeasts and these mutants.

CoQ_6-Mediated Reduction of Ferricyanide and Cytochrome *c* Involves Superoxide

Generation of superoxide by NADH-driven redox cycling of semiquinones was demonstrated by measuring the reduction of acetylated cytochrome *c* in a medium containing plasma membranes, NADH, and CoQ_0. Addition of the quinone caused a rapid reduction of the cytochrome that was detected by an increase of absorbance at 550 nm. The reduction of acetylated cytochrome *c* was mediated by superoxide since it was abolished when assays were carried out in the presence of SOD (Fig. 2).

To test whether or not superoxide anions might play a role in CoQ-mediated ferricyanide and cytochrome-*c* reductases, assays were carried out with CoQ_6-supplemented plasma membranes and/or in the presence of 80 mU/ml SOD (Fig. 3). This amount of SOD was enough to eliminate superoxide produced by reduction of endogenous CoQ_6 (not shown). Addition of extra CoQ_6 to the plasma membranes from all yeast strains resulted in the activation of both activities, the degree of stimulation being much higher for the cytochrome-*c* reductase. Including SOD in the reaction mixture abolished most CoQ_6-activated redox activity, demonstrating the involvement of superoxide anions in the stimulated activity. SOD itself was inhibitory in the absence of added CoQ_6, showing that superoxide generation by reduction of the endogenous quinone mediates a portion of the redox activities. It is noteworthy that some inhibition by SOD was also observed in the *coq3*Δ mutants in the absence of added CoQ_6, indicating that superoxide could be also generated by quinone-independent mechanisms.

Table III. CoQ_6 Concentrations and Redox Activities of Plasma Membranes Isolated from Different Yeast Strains[a]

Strain	CoQ_6	Acceptor		
		Ferricyanide	Cytochrome *c*	AFR
Wild-type	150 ± 8	54.2 ± 3.5	15.9 ± 1.3	22.1 ± 2
*coq3*Δ	ND	32.3 ± 2.9	10.5 ± 0.2	2.6 ± 0.2
*atp2*Δ	195 ± 10	51.0 ± 0.9	21.6 ± 1.5	21.2 ± 0.6
Δ*cor1*	209 ± 11	45.5 ± 1.5	16.9 ± 0.8	15.8 ± 0.3
*coq3*Δ-pCC-COQ3	280 ± 5	46.5 ± 6	13.9 ± 1	21.2 ± 0.4
*coq3*Δ + CoQ_6^b	185 ± 3	79.8 ± 3	17.1 ± 1	23.9 ± 0.05

[a] CoQ_6 concentrations are pmol/mg plasma membrane protein. CoQ_6 restoration was achieved by culturing cells in the presence of 0.7 μM CoQ_6. Redox activities are nmoles/min/mg. ND, not detected. Data are mean ± S.D. (n = 5).

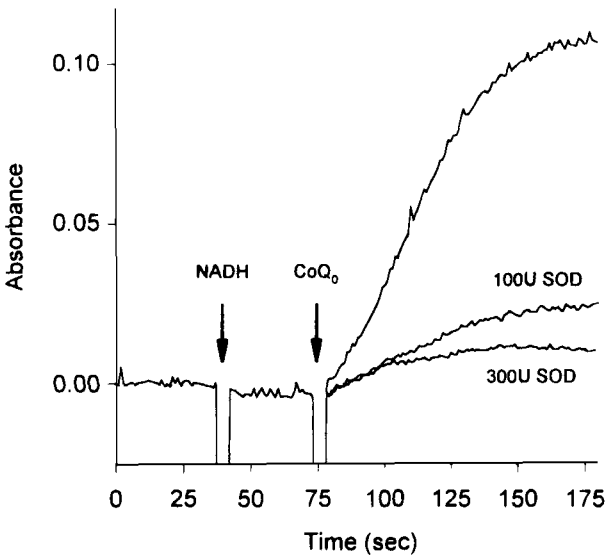

Fig. 2. Superoxide generation by plasma membrane CoQ reductase. The reaction was initiated by addition of CoQ_0 at the point indicated in the figure to a medium containing 0.2 mM NADH, 20 μM acetylated cytochrome *c*, and 50 μg wild-type yeast plasma membrane. Assays were carried out in the absence or in the presence of indicated amounts of SOD.

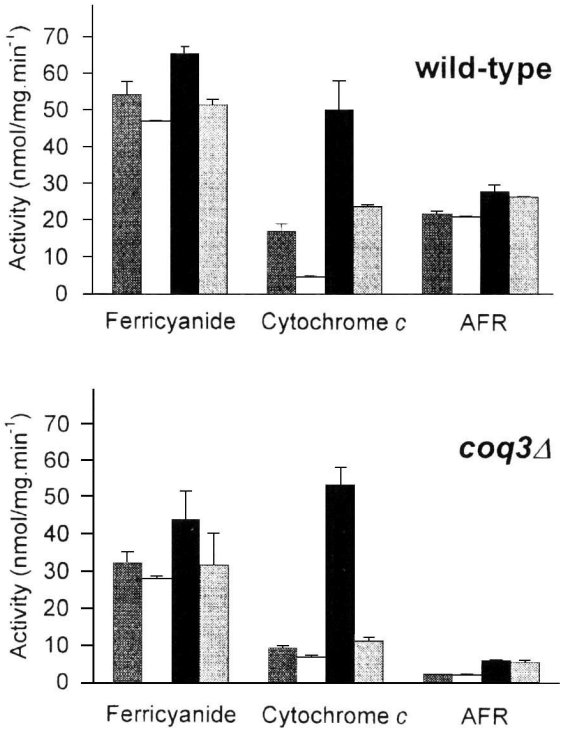

Fig. 3. Effect of SOD addition and/or CoQ_6 supplementation on plasma membrane redox activities. Plasma membranes were isolated from wild-type and *coq3*Δ yeast strains. SOD was used at 80 U/ml. CoQ_6 in ethanol was added to the plasma membranes in assay buffer to a final concentration of 50 μM. Membranes were then preincubated for 3 min at 30°C to allow for incorporation of the quinone, before assaying for ferricyanide, cytochrome *c*, and AFR reductases. (n = 5). ▨, no addition; □, SOD; ■, CoQ_6; ▩, SOD plus CoQ_6.

The AFR Reductase Requires CoQ but Does Not Involve Superoxide

The NADH–AFR reductase was different from the activities described above and highly dependent on the presence of CoQ_6 in the plasma membrane. Only a residual activity was detected in plasma membranes from the *coq3*Δ mutant (Table III). Addition of SOD to the reaction mixture had no significant effect on the AFR reductase (Fig. 3) indicating that superoxide anions do not mediate the reduction of the free radical. Supplementing plasma membranes with 50 μM CoQ_6 resulted in the activation of AFR reductase of wild-type plasma membranes, but only in a partial restoration of the activity in plasma membranes from the *coq3*Δ mutant strain. Both activated and restored activity were not mediated by superoxide, since they were unaffected by SOD (Fig. 3). Unlike the result obtained with the *coq3*Δ mutant, the lack of mitochondrial function in *atp2*Δ and *cor1*Δ mutants did not result in a significant loss of plasma membrane AFR reductase. However, CoQ_6 increases in the latter mutant strains did not correlate with similar increases of the NADH–AFR reductase (Table III).

NADH–AFR Reductase Can Be Rescued by CoQ Restoration

To confirm that the lack in NADH–AFR reductase in the *coq3*Δ strain was due to the *coq3* gene deletion, the *COQ3* gene on a single-copy plasmid (pCC-COQ3), or the vector plasmid alone (pRS313) were introduced into the CC303.1 strain (*coq3*Δ). In addition, in a separate set of experiments, the deletion mutant strain was cultured in the presence of 0.7 μM CoQ_6. Both procedures led to the recovery of CoQ_6 at the plasma membrane (Table III). Although direct supplementation of mutant plasma membranes with CoQ_6 had little effect on the AFR reductase (see above), full restoration of the redox activity was achieved in *coq3*Δ yeasts, both after transformation with the plasmid bearing the wild-type *COQ3* gene and after culturing in the presence of CoQ_6 (Table III;

Fig. 4). The vector plasmid alone was not sufficient (not shown). As described for the wild-type strain, the AFR reductase rescued by either of the two methods, was unaffected by SOD (Fig. 4). These results indicate that the lack of AFR reductase in *coq3*Δ mutants results solely from their inability to produce CoQ_6 and is not related to the respiratory-deficient phenotype.

CoQ_6 restoration by either of the two methods also increased ferricyanide and cytochrome-*c* reductases in the mutant strain. As described for wild-type yeasts, a considerable part of the activity was mediated by superoxide (Table III; Fig.4).

CoQ-Independent AFR Reductase Is Mediated by a Separate Enzyme System

To distinguish between CoQ-dependent and -independent AFR reductases, activities from wild-type and *coq3*Δ yeasts plasma membranes were compared on the basis of their sensitivity to effectors and inhibitors known to have an effect on redox enzymes. Plasma membranes isolated for the mutant strain grown in the presence of CoQ_6 were also included in these experiments as an additional control for CoQ_6 dependency (Table IV). Preincubation with 50 μM CoQ_6 stimulated the AFR reductase in wild-type plasma membranes, but resulted in only a partial recovery of the activity in mutant plasma membranes (Fig. 3; Table IV). A total recovery of the activity was observed in plasma membranes from the *coq3*Δ strain grown in the presence of 0.7 μM CoQ_6. While solubilization of wild-type yeast plasma membranes with the nondenaturing detergent Zwittergent 3–14 produced a 77% inactivation the AFR reductase, no effect was observed on the CoQ-independent activity measured with *coq3*Δ yeast plasma membranes (Table IV). On the other hand, less inactivation by Zwittergent 3–14 was observed for the NADH–cytochrome-*c* reductase, whereas the ferricyanide reductase was even stimulated (not shown).

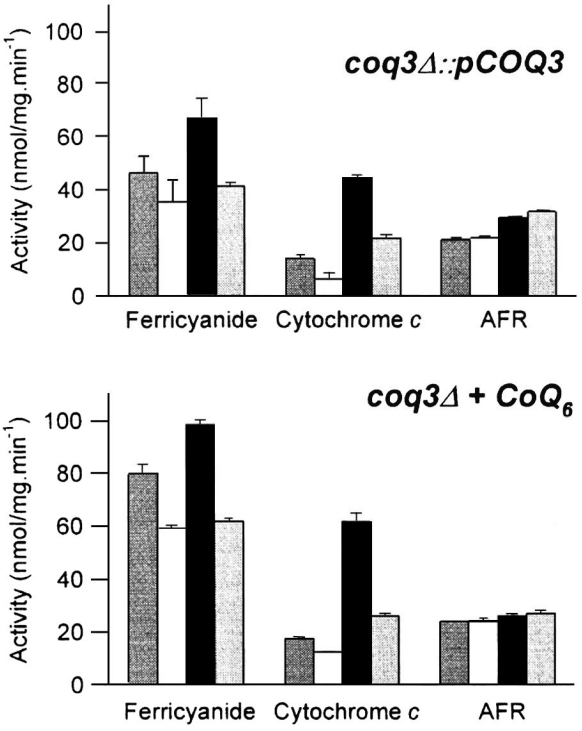

Fig. 4. Restoration of redox activities by CoQ_6 Plasma membranes were isolated from the *coq3*Δ strain either transformed with plasmid pCC-COQ3 containing the *COQ3* gene, or grown in the presence of 0.7 μM CoQ_6 Cells were harvested and plasma membranes isolated as detailed in the section on material and methods. Incorporation of the quinone was verified by HPLC analysis. The effect of SOD addition and/or CoQ_6 supplementation was also tested as described in Fig. 3 (n = 5). ▨, no addition; □, SOD; ■, CoQ_6; ▩, plus CoQ_6.

The AFR reductase activity from wild-type yeast plasma membranes was inhibited 90% by the thiol reagent *p*-hydroxymercuribenzoate (PHMB). Thenoyltrifluoroacetone (TTFA) produced a similar degree of inhibition. The AFR reductase was moderately inhibited by the quinone antagonist chloroquine, and only slightly inhibited by the DT-diaphorase inhibitor dicumarol. No significant inhibition was observed with KCN. None of the compounds tested had a significant effect on the AFR reductase measured with plasma membranes isolated from the *coq3*Δ strain. It is noteworthy that, except in the case of the quinone antagonist chloroquine and in CoQ_6 supplementation, a response similar to that of wild-type plasma membranes was observed with plasma membranes isolated form the *coq3*Δ strain grown in the presence of CoQ_6. Thus, our results support the proposal that the residual CoQ-independent AFR reductase is due to a separate system as yet unidentified.

DISCUSSION

Biochemical characterization of redox activities associated with the animal plasma membrane supports the participation of CoQ as an intermediate electron carrier (Sun *et al.*, 1992). CoQ requirement distinguishes activities related to *trans*plasma membrane

Table IV. Effect of Different Compounds on CoQ-Dependent and -Independent NADH–AFR Reductase from Yeast Plasma Membrane[a]

Addition	Wild-type	*coq3*Δ	*coq3*Δ + CoQ_6[b]
None	15 ± 0.6 (100)	2.6 ± 0.1 (100)	23.9 ± 0.1 (100)
CoQ_6[c] (50 μM)	19.3 ± 1.7 (129)	6.6 ± 0.2 (248)	26.3 ± 0.6 (110)
Zwittergent 3–14 (2 mM)	3.5 ± 0.1 (23)	2.2 ± 0.1 (81)	5.0 ± 0.5 (21)
100 μM PHMB (100 μM)	1.6 ± 0.1 (10)	2.6 ± 0.1 (96)	5.3 ± 0.8 (22)
Chloroquine (50 μM)	5.6 ± 0.3 (37)	2.7 ± 0.3 (101)	14.1 ± 0.3 (59)
TTFA (1 mM)	2.0 ± 0.03 (13)	2.8 ± 0.5 (104)	2.7 ± 0.1 (11)
Dicumarol (100 μM)	11.7 ± 0.1 (78)	2.5 ± 0.3 (92)	17.1 ± 0.8 (71)
KCN (50 μM)	14 ± 0.25 (94)	2.3 ± 0.25 (87)	24.1 ± 0.7 (101)

[a] Assays for total AFR reductase were carried out with plasma membranes isolated from the wild-type strain. Assays for CoQ-independent AFR reductase were performed with plasma membranes from the *coq3*Δ strain. Activities are nmoles/min/mg. Data are mean ± S.D. (n = 3). Parentheses, percentage of activities relative to no addition.

[b] CoQ_6 was added to culture media to a final concentration of 0.7 μM.

[c] CoQ_6 in ethanol was added to isolated plasma membranes in assay buffer. Determination of redox activities was carried out after preincubation for 3 min at 30°C to allow for incorporation of the quinone.

electron transport from activities related to *cis* electron transport on the cytosolic face of the plasma membrane (Villalba *et al.*, 1995). Since genetic manipulation is greatly facilitated in yeasts, they provide an excellent tool for further definition of *trans*membrane electron transport and CoQ dependency.

Plasma membranes were isolated from both wild-type yeasts and the deletion mutant *coq3*δ strain, which completely lacks CoQ_6 in membranes, thus being respiration-defective (Clarke *et al.*, 1991). To demonstrate that possible differences in redox activities were due to contents of ubiquinone, and not to the repiratory-deficient phenotype, we included as controls two yeast mutants also deficient in respiration, but containing CoQ_6: *atp2*Δ, a null mutant in the *ATP2* gene, which encodes the β subunit of the mitochondrial ATPase (Do *et al.*, 1996), and *cor1*Δ, a null mutation in the *COR1* gene, which encodes the 44 kDa core protein of mitochondrial ubiquinol–cytochrome-*c* reductase (Tzagoloff *et al.*, 1986).

We have studied three redox activities that represent different levels of electron transport at the plasma membrane (Villalba *et al.*, 1993a): NADH–ferricyanide reductase, catalyzed mainly by the cytochrome b_5 reductase (Kim *et al.*, 1995); NADH–cytochrome-*c* reductase, that requires both the reductase and the cytochrome b_5 on the cytosolic side of the plasma membrane (Steck and Kant, 1974); and NADH–AFR reductase, that apparently involves the reductase, CoQ, and one as yet unidentified component located on the external surface of the plasma membrane (Navas *et al.*, 1988; Villalba *et al.*, 1993a).

NADH–ferricyanide and–cytochrome-*c* reductases were partially dependent on the presence of CoQ_6, although substantial activity was still present in the mutant *coq3*Δ. The cytochrome b_5 reductase–cytochrome b_5 system can account for CoQ-independent ferricyanide and cytochrome-*c* reductases. Solubilization of the ferricyanide reductase with Zwittergent 3–14 supports the participation of a single polypeptide in the catalysis (Kim *et al.*, 1995; Villalba *et al.*, 1995) and partial inhibition of the cytochrome-*c* reductase by the detergent may represent the separation of the cytochrome b_5 and its reductase (Steck and Kant, 1974). Decrease of plasma membrane redox activities in the *coq3*Δ strain was not due to the loss of mitochondrial function, since plasma membrane redox activities were unchanged or even stimulated *atp2*Δ and *cor1*Δ mutant strains. The latter two strains contained elevated amounts of CoQ_6 in their plasma membranes compared to wild-type yeasts. Elevation of plasma membrane-associated CoQ has been also found during the establishment of a mitochondria-deficient HL-60 cell line (Gómez-Díaz *et al.*, 1997b). Thus, this adaptation could be considered as a general response to impaired mitochondrial function in order to regulate cytosolic NADH/NAD^+ levels (Larm *et al.*, 1994).

Here we have shown that the yeast plasma membrane contains an intrinsic reductase that catalyzes the one-electron reduction of ubiquinones to ubisemiquinones and thus could provide reducing equivalent from cytosolic NADH to intramembrane CoQ_6, which might play a role either as intermediate electron carrier (Sun *et al.*, 1992) or as antioxidant (Do *et al.*, 1996). Simi-

larly, the NADH–cytochrome-b_5 reductase of animal plasma membranes has been demonstrated to act as a CoQ reductase displaying a one-electron reaction mechanism (Nakamura and Hayashi, 1994; Navarro *et al.*, 1995). Since superoxide may be generated by reaction of ubisemiquinones with oxygen, we tested the involvement of this free radical in CoQ-dependent ferricyanide and cytochrome-*c* reductases. Addition of SOD to the reaction mixture inhibited these redox activities, demonstrating the participation of superoxide generated by endogenous semiquinones. The slight inhibition by SOD in *coq3*Δ plasma membranes supports the fact that superoxide may be also generated by quinone-independent mechanisms. Incorporation of an extra amount of CoQ_6 into the plasma membranes resulted in a substantial activation of the reductases, especially when cytochrome *c* was used as acceptor. This activation was mediated by superoxide since it was reversed by SOD.

The NADH–AFR reductase has been proposed to play a role in the *trans*membrane flux of electrons that results in extracellular ascorbate stabilization by living cells (Navas *et al.*, 1994). Both AFR reductase in plasma membrane and the ascorbate stabilization by animal cells require CoQ (Villalba *et al.*, 1995; Gómez-Díaz *et al.*, 1997a, b). Yeast cells also stabilize extracellular ascorbate as a result of transplasma membrane electron transfer to provide a reducing environment based on ascorbate in the apoplast (Santos–Ocaña *et al.*, 1995). A portion of the ascorbate stabilization process in yeast cells requires CoQ_6 (Santos-Ocaña *et al.*, 1998). However, the demonstration of an AFR reductase in the yeast plasma membrane and the possible participation of CoQ in the catalysis had not been presented.

Unlike ferricyanide and cytochrome-*c* reductases, the bulk of NADH–AFR reductase required CoQ_6 and only a minor portion of the activity was conserved in the *coq3* strain. The AFR reductase did not involve superoxide, since SOD was without effect on the activity. AFR reductases measured with plasma membranes from the deletion mutants *atp2* and *cor1* (which contained elevated CoQ_6, see above) did not differ significantly from the activity measured with plasma membranes from the wild-type strain. In addition, these three strains show similar levels of extracellular ascorbate stabilization (Santos–Ocaña *et al.*, 1998). These results may indicate that the substrate AFR, generated by reaction of ascorbate and ascorbate oxidase, can become limiting for accepting electrons when membranes have increased CoQ_6 levels. According to this idea, AFR reduction by liver plasma membranes did not follow saturation kinetics with respect to the steady-state concentration of AFR generated by ascorbate plus ascorbate oxidase (J. M. Villalba, personal communication).

Almost 90% of the NADH–AFR reductase activity in isolated plasma membranes was found to be dependent on CoQ_6. However, only about 35% of ascorbate stabilization by whole yeast cells requires the quinone, the remainder activity being explained by the ferrireductase system (Santos–Ocaña *et al.*, 1998). These results may indicate that NADH is not an efficient electron donor for the ferrireductase, which has been proposed to use mainly NADPH (Lessuise and Labbe, 1992). In addition, isolation of plasma membranes could produce the lack of some regulatory proteins that may be necessary for electron transfer to the *FRE1/FRE2* gene products from a putative common NAD(P)H dehydrogenase (Lesuisse *et al.*, 1996). Inactivation of the yeast plasma membrane AFR reductase by Zwittergent 3–14 is in accordance with a similar inhibition of rat liver AFR reductase by the nondenaturing detergent CHAPS and supports the participation of more than one component in the electron transfer from NADH to the AFR (Villalba *et al.*, 1993a).

Interestingly, little recovery of AFR reductase was achieved by direct supplementation of *coq3*Δ plasma membranes with CoQ_6, but full restoration of the activity was obtained by either transformation with a single-copy plasmid containing the wild-type *COQ3* gene, or by culturing the cells in the presence of CoQ_6. These results may indicate that an additional cellular component, as yet unidentified, may be required for correct integration of the quinone in the plasma membrane in order to function as a transmembrane intermediate electron carrier. The presence of CoQ_6 might also be necessary for the correct assembly of the functional AFR reductase enzyme system.

The AFR reductase was strongly inhibited by TTFA, which has been reported as an effective inhibitor of the AFR reductase from animal cells (Schweinzer and Goldenberg, 1993). Inhibition of the NADH–AFR reductase by PHMB indicates the participation of thiol groups, as reported previously for the AFR reductase of liver plasma membrane (Villalba *et al.*, 1993b). It has been proposed that electrons for CoQ-mediated AFR reduction are delivered by a cytochrome-b_5 reductase in liver plasma membrane (Villalba *et al.*, 1995; Navarro *et al.*, 1995), an enzyme that contains essential thiol groups in its active site (Shirabe *et al.*, 1991). The one-electron reaction mech-

anism of the NADH–cytochrome-b_5 reductase and the CoQ reductase detected in yeast plasma membrane (see above), also supports the participation of a similar enzyme. The lack of substantial inhibition by dicumarol at 100 μM makes unlikely the participation of the two-electron quinone reductase DT-diaphorase (Preusch *et al.*, 1991). Inhibition of the yeast plasma membrane AFR reductase by chloroquine is in accordance with similar inhibitions by quinone antagonists in animal plasma membrane AFR reductase (Villalba *et al.* 1995) and ascorbate stabilization by animal (Gómez-Díaz *et al.*, 1997a) and yeast cells (Santos-Ocaña *et al.* 1995). Less sensitivity to chloroquine and less degree of activation by CoQ_6 supplementation was observed for plasma membranes isolated from the *coq3*Δ strain grown in the presence of CoQ_6. These results may be easily explained on the basis on a higher CoQ_6 content in these membranes, since inhibition by chloroquine can be reversed by CoQ (Sun *et al.*, 1992). A higher initial concentration of CoQ_6 in the membranes will also lead to less activation by added quinone, if the acceptor substrate AFR is limiting (see above).

In conclusion, the genetic approach has allowed us to demonstrate unequivocally the participation of CoQ in plasma membrane redox activities. Since redox systems in yeast and animal plasma membranes appear to function in a very similar way, the yeast model will help to elucidate the molecular architecture of plasma membrane CoQ-dependent electron transport in the near future.

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