Coenzyme Q Analogues Reconstitute Electron Transport and Proton Ejection but Not the Antimycin-Induced "Red Shift" in Mitochondria from Coenzyme Q Deficient Mutants of the Yeast *Saccharomyces cerevisiae*†

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ABSTRACT: Mitochondria isolated from coenzyme Q deficient yeast cells had no detectable NADH:cytochrome *c* reductase or succinate:cytochrome *c* reductase activity but contained normal amounts of cytochromes *b* and c_1 by spectral analysis. Addition of the exogenous coenzyme Q derivatives including Q_2, Q_6, and the decyl analogue (DB) restored the rate of antimycin- and myxothiazole-sensitive cytochrome *c* reductase with both substrates to that observed with reduced DBH_2. Similarly, addition of these coenzyme Q analogues increased 2–3-fold the rate of cytochrome *c* reduction in mitochondria from wild-type cells, suggesting that the pool of coenzyme Q in the membrane is limiting for electron transport in the respiratory chain. Preincubation of mitochondria from the Q-deficient yeast cells with DBH_2 at 25 °C restored electrogenic proton ejection, resulting in a $H^+/2e^-$ ratio of 3.35 as compared to a ratio of 3.22 observed in mitochondria from the wild-type cell. Addition of succinate and either coenzyme Q_6 or DB to mitochondria from the Q-deficient yeast cells resulted in the initial reduction of cytochrome *b* followed by a slow reduction of cytochrome c_1 with a reoxidation of cytochrome *b*. The subsequent addition of antimycin resulted in the oxidant-induced extrareduction of cytochrome *b* and concomitant oxidation of cytochrome c_1 without the "red" shift observed in the wild-type mitochondria. Similarly, addition of antimycin to dithionite-reduced mitochondria from the mutant cells did not result in a red shift in the absorption maximum of cytochrome *b* as was observed in the wild-type mitochondria in the presence or absence of exogenous coenzyme Q analogues. These results suggest that exogenous coenzyme Q analogues may reconstitute electron flow and electrogenic proton pumping in mitochondria from Q-deficient yeast cells but may not be reconstituted into the membrane exactly as is the tightly bound coenzyme Q reported to be an integral part of the cytochrome b–c_1 complex.

The mitochondrial respiratory chain is considered to be composed of discrete lipid–protein enzyme complexes, each of which catalyzes a distinct part of the overall oxidation reaction (Hatefi et al., 1962, 1975). The role of coenzyme Q in this scheme, although not well understood, is apparently to act as a mobile electron carrier between complex I (NADH:cytochrome *c* reductase), complex II (succinate:cytochrome *c* reductase), and complex III (coenzyme QH_2:cytochrome *c* reductase) (Hauska & Hurt, 1982). Recent studies in Hackenbrock's laboratory (Schneider et al., 1982; Gupte et al., 1984) have indicated that the respiratory chain complexes as well as coenzyme Q are free to diffuse laterally and independently of each other in the plane of the mitochondrial membrane. The results of studies in which the diffusion coefficients of the three respiratory chain complexes and coenzyme Q were measured suggest that mitochondrial electron transport is diffusion-coupled, since the observed rate of electron transport is slower than the theoretical limit set by the lateral diffusion of the redox components (Gupte et al., 1984).

While the concept that coenzyme Q may act as a mobile carrier in the respiratory chain has gained acceptance, the exact mechanism by which it functions within the cytochrome b–c_1 complex is not clear. The possible interaction of the free quinones in the "pool", which is in great excess compared to the other membrane-bound components of the respiratory chain (Hauska & Hurt, 1982), with the postulated protein-bound specialized quinone molecules within the complex is not understood (Matsuura et al., 1983). The isolation of coenzyme Q binding proteins (King, 1982) and the detection of different semiquinones in the respiratory chain (Ohnishi & Trumpower, 1980; DeVries et al., 1981) have also suggested specific roles for coenzyme Q in energy transduction and electron transport. Furthermore, the possible role of coenzyme Q in the mechanism of proton ejection by either a Q-cycle or a *b*-cycle mechanism is still under active investigation (Wikstrom et al., 1981).

The isolation of mutants of yeast lacking coenzyme Q (Tzagoloff et al., 1975) has provided an excellent tool for the further investigations of the role of coenzyme Q in the respiratory chain (Sidhu & Beattie, 1985). These mutants lack any detectable quinone and have no cytochrome *c* reductase activity or respiratory chain activity. These activities can be restored by the addition of exogenous coenzyme Q analogues, suggesting that the cytochromes and primary dehydrogenases are fully expressed in the absence of coenzyme Q (Brown & Beattie, 1977). These Q-deficient mutants have also been used to demonstrate that endogenous coenzyme Q is not required for the oxidation of exogenous coenzyme Q by complex III (Pasquali et al., 1981) and that ATP synthesis coupled to succinate oxidation can be restored by the addition of exogenous analogues of coenzyme Q (Desantis et al., 1982).

In the current study, the role of the pool of coenzyme Q in electron transfer in the NADH:cytochrome *c* reductase and

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succinate:cytochrome *c* reductase span of the respiratory chain has been investigated. The results obtained suggest that the "pool" of coenzyme Q is rate-limiting for electron transfer, since the addition of exogenous coenzyme Q analogues stimulated the rate of NADH:cytochrome *c* reductase and succinate:cytochrome *c* reductase activities in both the mutant and the wild-type mitochondria to the level of coenzyme QH_2:cytochrome *c* reductase. Furthermore, spectral studies indicated that the analogues reconstitute the extrareduction of cytochrome *b* in the presence of antimycin without the typical "red" shift observed in the wild-type mitochondria.

Experimental Procedures

Yeast Strains and Cell Growth. The prototrophic strain D273-10B and the coenzyme Q deficient strain E3-24 (Tzagoloff et al., 1975) were obtained from Dr. Alexander Tzagoloff. The cells were grown aerobically at 30 °C in semisynthetic medium (Beattie et al., 1981) containing 3% galactose as carbon source and harvested at the late logarithmic phase of growth. Mitochondria obtained from strain E3-24 were shown to have no detectable coenzyme Q in spectra of ether–methanol extracts (Brown & Beattie, 1977).

Preparation of Mitochondria and Submitochondrial Particles. For enzymatic and spectral assays, the cells were broken with glass beads (0.45–0.50-mm diameter) in a Bronwill shaker in a medium containing 0.65 M sorbitol, 0.1 mM EDTA,[1] and 10 mM Tris-HCl, pH 6.5 (Meunier-Lemesle et al., 1980). Mitochondria were prepared in this medium by the method described by Brown and Beattie (1977). The mitochondrial suspension was diluted with water to a final concentration of 1 mg/mL prior to enzymatic assay. The mitochondria were resuspended in the assay medium of Meunier-Lemesle et al. (1980) to a concentration of 2–3 mg/mL prior to spectral assays.

For the proton ejection studies and for some spectral determinations, mitochondria were prepared by the procedure described by Villalobo et al. (1981) with zymolyase 5000 (4 mg/g wet weight of cells) for digestion of the cell wall. Submitochondrial particles were prepared in 0.1 M potassium phosphate buffer, pH 7.5, and 0.1 mM EDTA according to Clejan et al. (1983).

Enzymatic Assays and Spectral Determinations. Succinate:cytochrome *c* reductase, coenzyme QH_2:cytochrome *c* reductase, and NADH:cytochrome *c* reductase activities were assayed by the procedure of Brown and Beattie (1977). The preincubation and reaction medium contained 25 mM sodium phosphate, pH 7.6, 0.2 mM EDTA, 5 mM sodium azide, and 20–40 μg of mitochondrial protein in 1 mL with or without 10 mM succinate. Coenzyme Q analogues were added in the concentrations indicated in the tables and figures. The reaction was started by the addition of 50 μM cytochrome *c*. Alternatively, the coenzyme Q analogues with or without cytochrome *c* were added to the reaction mixture minus succinate, and the reaction was initiated with succinate. All incubations were at 22 °C except those in which coenzyme Q_6 was used when a temperature of 30 °C was used.

Proton ejection measurements were performed at 20–30 °C in a 2-mL chamber equipped with magnetic stirring. Proton movements were measured by a combination pH glass electrode (Corning) and recorded on a Sargent-Welch Model DSR6-2 recorder (Clejan et al., 1984). In each experiment, known amounts of HCl and KOH were added to calibrate the pH electrode. The $H^+/2e^-$ ratios were calculated according to Price and Brand (1983).

[1] Abbreviations: DB and DBH_2, oxidized and reduced forms of 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Table I: Succinate:Cytochrome *c* Reductase Activity in Wild-Type and Q-Deficient Mutants[a]

analogue (nmol)	wild type	E3-24
DB		
0.0	0.285	0.007
1.0		0.385
2.5	0.634	0.444
5.0	0.795	0.549
10	0.769	0.513
20	0.645	0.513
DBH_2		
5	0.507	0.334
10	0.597	0.527
15	0.633	0.691

[a] Mitochondria were suspended at 6 mg/mL in a medium containing 0.65 M sorbitol, 0.1 mM EDTA, and 10 mM Tris-HCl, pH 6.5. Prior to enzymatic assay, a 1.6 dilution with water was made. Aliquots of 20–40 μg of mitochondrial protein were preincubated for 8 min at 22 °C in 1 mL of the reaction medium (25 mM sodium phosphate, pH 7.6, 0.2 mM EDTA, 5 mM sodium azide, 10 mM succinate) containing the indicated quantities of DB or DBH_2. The reaction was started with 50 μM cytochrome *c* and followed at 550 nm. Activities are expressed as μmol of cytochrome *c* min^{-1} (mg of protein)$^{-1}$ and represent a mean of three determinations.

The spectral assays were performed at room temperature with a dual-beam–dual-wavelength Perkin-Elmer Model 557 spectrometer as described by Clejan et al. (1983). The same preparation was assayed in both the double-beam and double-wavelength modes.

Materials. The decyl analogue of coenzyme Q (DB, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone) was synthesized from Q_0 obtained from Fluka according to the method of Margolis (1976). Coenzymes Q_1 and Q_2 were generous gifts from Hoffmann-La Roche. Coenzyme Q_6 was purchased from Sigma. Myxothiazol was a generous gift of Dr. W. Trowitzch. All other reagents were of the highest purity commercially available.

Results

Reconstitution of Cytochrome c Reductase Activity with Analogues of Coenzyme Q. In our initial attempts to reconstitute succinate:cytochrome *c* reductase activity in the coenzyme Q deficient yeast strain E-3-24, the decyl analogue of coenzyme Q (DB) was used. Mitochondria from either the wild-type or the mutant strain were preincubated at 22 °C with increasing concentrations of DB for 6–8 min in the assay medium containing succinate but lacking cytochrome *c*. Table I indicates that with increasing amounts of the analogue the activity of succinate:cytochrome *c* reductase in both wild-type and mutant mitochondria increased, reaching a maximum at 5 nmol of DB/800 μL of assay medium.

It also should be noted that at each concentration of DB the succinate:cytochrome *c* reductase activity of mitochondria from the mutant strain was restored to approximately 70% of that of the wild type (Table I). Furthermore, with the optimal concentration of DB, the activity of succinate:cytochrome *c* reductase was equivalent to the activity of coenzyme QH_2:cytochrome *c* reductase assayed with increasing amounts of the reduced coenzyme Q analogue, DBH_2 (Table I). Again, the cytochrome *c* reductase activity with coenzyme QH_2 as substrate was almost identical in the mitochondria of mutant and wild-type yeast cells, suggesting that the cytochrome b–c_1 region of the respiratory chain is functional in the coenzyme Q lacking mutants after reconstitution with the exogenous coenzyme Q.

Table II: Effect of Different Coenzyme Q Analogues of Succinate:Cytochrome *c* Reductase Activity in Wild-Type and Q-Deficient Yeast Mutants[a]

		wild type		E3-24	
analogue	nmol	expt 1	expt 2	expt 1	expt 2
none		0.248	0.101	0.011	0.001
DB	5	0.562	0.252	0.217	0.189
Q_2	10	0.749	0.488	0.527	0.379
Q_6	10	0.240	0.151	0.164	0.230[1]
					0.041[2]
Q_1	10	0.538		0.238	

[a] Aliquots of 20–40 μg of mitochondrial protein were added to 1 mL of a medium containing 25 mM sodium phosphate, pH 7.6, 0.2 mM EDTA, 5 mM sodium azide, and 50 μM cytochrome *c*. The reaction was started by the addition of 10 mM succinate. Units are μmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$. Experiment 1, Q_6 incubated at 30 °C; experiment 2, Q_6 incubated at 22 °C.

Various coenzyme Q analogues with side chains of lengths ranging from Q_1 with one isoprene unit to Q_6 with six isoprene units were compared for their ability to reconstitute succinate:cytochrome *c* reductase activity. In these experiments, the coenzyme Q analogue was added to the incubation medium containing cytochrome *c* and the reaction started by addition of succinate. The data of Table II from both the wild-type and the mutant cells represent activities obtained with two different mitochondrial preparations from yeast cultures grown to different cell densities. Often the yeast cultures were not grown to late logarithmic phase, and hence, the respiratory chain was partially repressed, resulting in lower activities of cytochrome *c* reductase activity. Despite the differences in the reductase activity of the wild-type mitochondria, addition of optimal concentrations of DB resulted in a 2.5-fold increase in activity. The results of Table II indicate that the analogue containing the two isoprene unit side chain, coenzyme Q_2, results in the highest cytochrome *c* reductase activity in both wild-type and mutant mitochondria. Similar activities were obtained when either DB, which has a decyl side chain, or Q_1 with a single isoprene side chain was used to reconstitute cytochrome *c* reductase activity in the wild-type or mutant mitochondria. For each analogue, the optimal concentration was determined and shown to be very similar as indicated by the values in Table II. In addition, the inhibition by both antimycin and myxothiazol (Theirbach & Reichenbach, 1981; Von Jagow et al., 1984) on the electron transport activity in this assay was greater than 85% in mitochondria reconstituted with each analogue with the exception of Q_1. Addition of antimycin to the assays using Q_1 for reconstitution lead to variable inhibitory effects ranging from 25 to 60%, and hence, this analogue was not used in further experiments.

Reconstitution with Q_6, the more hydrophobic but naturally occurring coenzyme Q in yeast, was also examined. Addition of this analogue did not lead to any increase in succinate:cytochrome *c* reductase activity in mitochondria from the wild-type cells; however, it increased the cytochrome *c* reductase activity of the mutant mitochondria to the same extent as DB in most experiments (Table II). To obtain the maximum reconstitution with coenzyme Q_6, it was necessary to perform the preincubation of 30 °C, suggesting that this higher temperature may facilitate the insertion of Coenzyme Q into the mitochondrial membrane.

A stimulation of cytochrome *c* reductase activity by the addition of analogues of coenzyme Q was also observed with NADH as substrate. As seen in Table III, addition of all three analogues resulted in a similar NADH:cytochrome *c* reductase activity in the wild-type and mutant mitochondria. As was observed with succinate as substrate, the greatest stimulation

Table III: Reconstitution of NADH:Cytochrome *c* Reductase with Different Coenzyme Q Analogues[a]

analogue	wild type	E3-24
none	0.513	0.001
DB	0.691	0.628
Q_2	1.050	0.967
Q_6	0.488	0.728

[a] The enzymatic assay was performed in the reaction medium described in the legend to Table I with 0.5 mM NADH as substrate and 5 nmol of the respective Q analogue. Activities are expressed in μmol of cytochrome *c* min^{-1} (mg of protein)$^{-1}$.

Table IV: Proton Ejection in Wild-Type and Q-Deficient Mitochondria Reconstituted with DBH_2[a]

	wild type	E3-24
$H^+/2e^-$	3.22 ± 0.58 (17)	3.35 ± 0.54 (5)
$H^+/2e^-$ plus CCCP	1.92 ± 0.13 (10)	1.90 ± 0.31 (4)

[a] Mitochondria were prepared as described by Villalobo et al. (1981) with zymolyase 5000 for cell wall digestion and resuspended in medium containing 0.52 M mannitol, 2 mM imidazole, 10 mM KCl, and 5 mM $MgCl_2$ at 2–3 mg/mL. The following additions were made to the chamber: 32 mM *N*-ethylmaleimide, 3.3 μM NaCN, 0.42 mM sodium succinate, and 2 μg of valinomycin. The reaction was started by the addition of 50–100 nmol of $K_3Fe(CN)_6$. The Q-deficient mitochondria were preincubated for 5 min with 150 nmol of DBH_2 prior to addition of $K_3Fe(CN)_6$. The $H^+/2e^-$ ratios were calculated as described by Beattie and Villalobo (1982). The numbers in parentheses represent the number of experiments.

was observed with Q_2 in both mitochondrial preparations while Q_6 had little or no stimulatory effect in the wild-type mitochondria.

Reconstitution of Electrogenic Proton Pumping in Coenzyme Q Deficient Yeast. Villalobo et al. (1981) recently studied proton ejection coupled to electron flow in the succinate:cytochrome *c* region of the respiratory chain in tightly coupled yeast mitochondria. They observed that the $H^+/2e^-$ ratio approached an average value of 3 when K^+ (in the presence of valinomycin) was used as the charge-compensating cation. In the presence of the proton-conducting agent CCCP, the $H^+/2e^-$ ratio was equal to 2, representing the two scalar protons derived from the substrate. Using their experimental conditions, we have observed an average $H^+/2e^-$ ratio of 3.3 (17 determinations on 4 mitochondrial preparations) using succinate as electron donor and the impermeable ferricyanide as electron acceptor (Table IV). The $H^+/2e^-$ ratio in the presence of CCCP was 1.92.

As anticipated, no proton ejection was observed when succinate was added to the coenzyme Q deficient yeast mitochondria, reflecting the lack of succinate:cytochrome *c* reductase determined spectrophotometrically. DBH_2, the reduced coenzyme Q analogue, however, was used successfully to restore electrogenic proton ejection activity in the mutant mitochondria (Table IV). Preincubation with 150 nmol of DBH_2 for 5 min in the proton ejection medium resulted in $H^+/2e^-$ ratios, both the electrogenic and that observed in the presence of CCCP, approaching that observed in the wild type. Furthermore, the rate of H^+ ejection, a measure of DBH_2 oxidation, was identical in the wild-type and mutant mitochondria, and the reaction was 90% inhibited by antimycin. These results provide additional evidence that the cytochrome b–c_1 region of the respiratory chain has not been significantly affected in the mutants lacking coenzyme Q and that oxidative phosphorylation can be reconstituted by addition of exogenous coenzyme Q analogues.

Attempts to reconstitute proton ejection by addition of either DB or coenzyme Q_6 to these mitochondria in the presence of

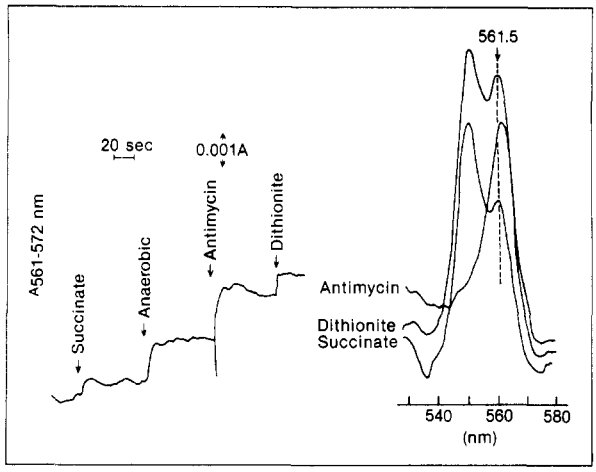

FIGURE 1: Extrareduction and red shift of cytochrome *b* after antimycin addition to wild-type mitochondria. Mitochondria from strain D273-10B were resuspended at 2 mg/mL in the medium described by Meunier-Lemesle et al. (1980). (Left) Kinetics of reduction of cytochrome *b* after the sequential addition of 30 mM succinate, 22 nmol of antimycin, and a few crystals of dithionite at the times indicated. Wavelength pair was 561–572 nm. (Right) Difference spectra of the succinate minus ferricyanide-oxidized sample and after addition of antimycin and dithionite. Slit width was 1.3 nm.

succinate were unsuccessful. The optimal concentrations of the oxidized analogues as determined from the earlier studies with cytochrome *c* reductase (Table II) required such a large quantity of analogue with the higher concentration of protein needed for the proton ejection assay that the analogue appeared as an oil on top of the chamber.

Spectral Analysis of Cytochrome b in Reconstituted Coenzyme Q Deficient Yeast. In the wild-type mitochondria, addition of succinate resulted in reduction of 50% of the total dithionite-reducible cytochrome upon anaerobiosis. Addition of antimycin resulted in the extrareduction of 78% of the total cytochrome *b* with the concomitant red-shift, which in yeast mitochondria is from 561.5 to 564.0 nm (Figure 1). The effects of antimycin on the spectral response of cytochrome *b* were also examined in mitochondria or submitochondrial particles from the coenzyme Q deficient yeast cells. The submitochondrial particles were incubated sequentially with succinate and then DB, simultaneously incubated with both succinate and DB, and incubated with DBH_2 either alone or with succinate. Several novel observations were made whether the coenzyme Q analogue used for reconstitution was oxidized or reduced. First, addition of either the combination of succinate and DB or DBH_2 alone did not result in the reduction of cytochrome *b* after steady state had been achieved (Figures 2 and 3A). In the first 30 s after addition of DB in the presence of succinate, a slight reduction of both cytochromes *b* and c_1 was observed; however, progressively more cytochrome c_1 was reduced with time, while the cytochrome *b* was slightly oxidized (Figure 2). The reduction and subsequent reoxidation of cytochrome *b* is similar to the triphasic reduction reported by Jin et al. (1981). In addition, the inability of the exogenous quinones to cause a significant reduction of cytochrome *b* in the mutant compared to wild-type mitochondria may result because of the redox potential of the QH_2/Q pair.

By contrast, when succinate was added just prior to the DBH_2, initially cytochrome *b* was reduced to 60% of the total dithionite-reducible cytochrome *b* (Figure 3B). Within 3 min, however, a gradual increase in the reduction of cytochrome c_1 was observed. Under these condiitons, the cytochrome *b* remained reduced and was not oxidized as when succinate plus DB or DBH_2 alone was used for reconstitution, suggesting that

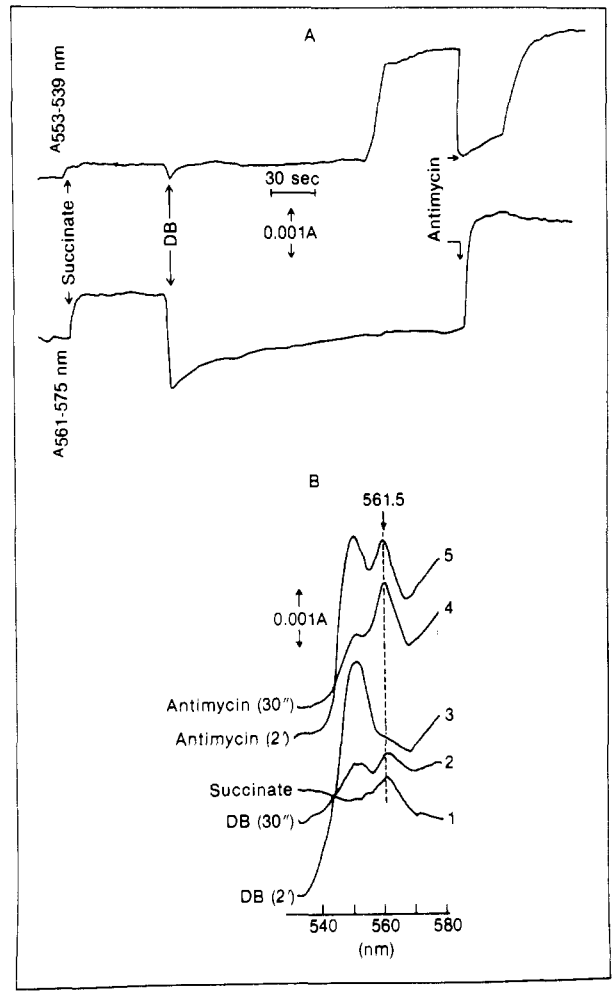

FIGURE 2: Effect of succinate and DB plus antimycin on the cytochrome spectrum in submitochondrial particles from Q-deficient yeast cells. The submitochondrial particles were suspended at a concentration of 2 mg/mL in the medium described by Meunier-Lemesle et al. (1980). (A) Kinetics of reduction of cytochrome *b* (lower trace) and cytochrome c_1 (upper trace) in the dual wavelength mode. Succinate (30 mM), DB (150 μM), and antimycin (11 nmol) were added where indicated. (B) Complete spectra after addition of succinate (trace 1), DB (traces 2 and 3), and antimycin (traces 4 and 5). The slit was 1.5 nm and the scanning speed 60 nm/min.

a more negative redox potential is achieved with both reducing substrates present.

After addition of antimycin to the submitochondrial particles reduced by any of the three experimental conditions described above, the typical extrareduction of cytochrome *b* was observed with the simultaneous oxidation of cytochrome c_1 (Figures 2 and 3); however, no red shift in the absorption peak of cytochrome *b* was observed as with the wild-type mitochondria. Furthermore, with time, cytochrome c_1 gradually was reduced within 2 min almost to the level observed after complete reduction with dithionite (Figures 2 and 3).

The naturally occurring form of coenzyme Q (Q_6) in yeast mitochondria was also used for these reconstitution experiments. The optimal concentration of coenzyme Q_6 was determined from the enzymatic reconstitution experiments while a preincubation at 30 °C was performed prior to addition of succinate. As seen in Figure 4, in the presence of succinate and coenzyme Q_6 only a slight reduction of both cytochrome *b* and c_1 was observed after anaerobiosis was reached. The subsequent addition of antimycin resulted in the extrareduction of cytochrome *b*, reaching 80% of the total dithionite reducible; however, even in the presence of the naturally occurring co-

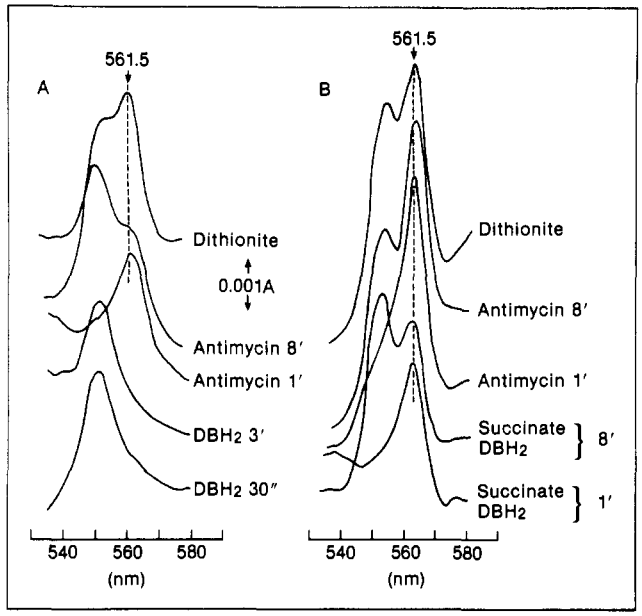

FIGURE 3: Absence of an antimycin-induced red shift in the spectrum of cytochrome *b* in Q-deficient mitochondria reconstituted with DBH_2 and succinate or DBH_2 alone. Mitochondria were resuspended at 2 mg/mL in the medium described by Meunier-Lemesle et al. (1980). (A) After the base line was traced, 150 nmol of DBH_2, 22 nmol of antimycin, and a few crystals of dithionite were added sequentially to the sample cuvette and the spectra scanned at the times indicated. (B) Difference spectra were recorded at the times indicated after the addition to the sample cuvette of 30 mM succinate, 150 nmol of DBH_2, 22 nmol of antimycin, and dithionite.

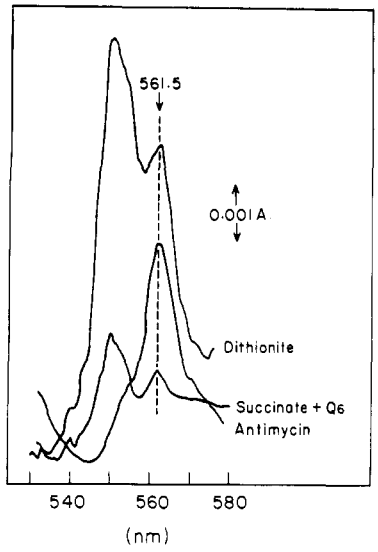

FIGURE 4: Difference spectra upon reconstitution of the Q-deficient mitochondria with succinate and Q_6. Mitochondria (2 mg/mL) were incubated with 100 μM Q_6 for 5 min at 30 °C. After the base line was traced, 30 mM succinate was added to the sample cuvette, and the difference spectrum was traced against the ferricyanide-oxidized reference cuvette. Spectra were subsequently recorded after addition of 22 nmol of antimycin and a few crystals of dithionite.

enzyme Q in yeast mitochondria, no red-shift in the absorption maximum of cytochrome *b* was observed. With Q_6, however, cytochrome c_1 remained oxidized.

One explanation for the reversibility of the oxidation of cytochrome c_1 observed in the presence of antimycin in the mutants reconstituted with exogenous coenzyme Q analogues is that these compounds produce superoxide, which subsequently reduces cytochrome c_1 in a slow step. To test this possibility, either DBH_2 or DB was added with the substrate succinate to wild-type mitochondria prior to addition of antimycin (Figure 5). In the quinone-supplemented mitochondria, the extrareduction of cytochrome *b* was observed with an identical red shift of 1.8 nm as was observed in the control mitochondria; however, cytochrome c_1 was only partially oxidized in the supplemented mitochondria, suggesting that the exogenous quinones may act to reduce cytochrome c_1 under these conditions.

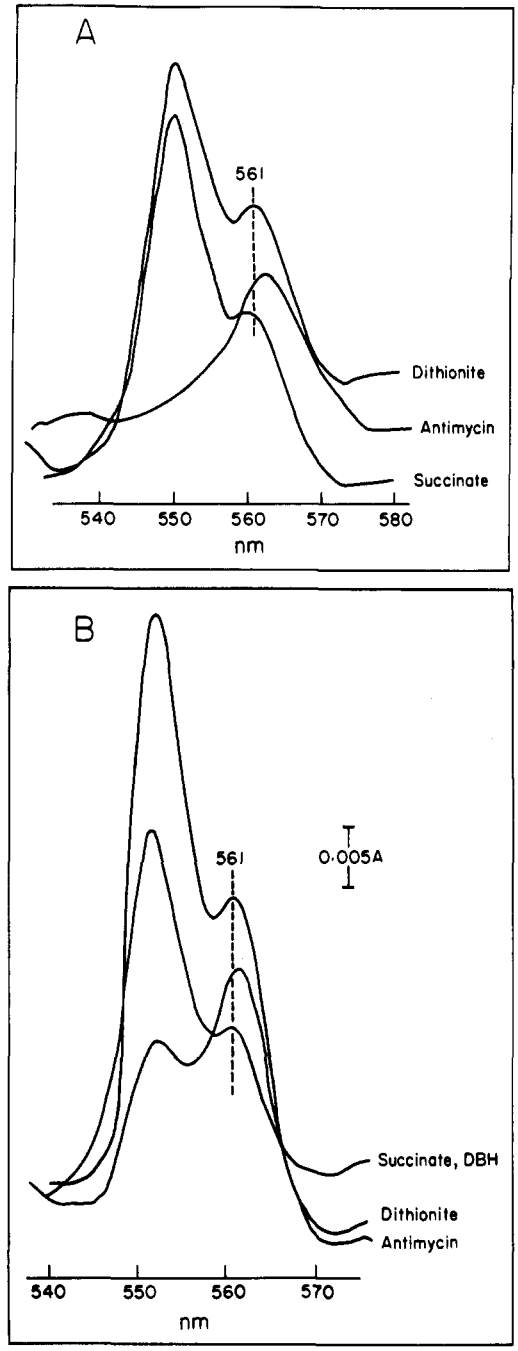

FIGURE 5: Difference spectra upon addition of antimycin to succinate and DBH_2-reduced mitochondria from wild-type yeast mitochondria. Mitochondria (2 mg/mL) of wild-type yeast were suspended in 1 mL of the medium described by Meunier-Lemesle et al. (1980). After the base line was traced, spectra were recorded after the addition to the sample cuvette of 30 mM succinate (A) or 30 mM succinate and 100 μM DBH_2 (B), 22 nmol of antimycin, and a few grains of dithionite. Slit width was 1 nm.

The antimycin-induced red shift was also compared in the mutant and wild-type mitochondria after an initial reduction with dithionite (Figure 6). A 1-nm shift was observed in the reduced wild-type mitochondria after addition of antimycin even when supplemented with exogenous DB or DBH_2 (data

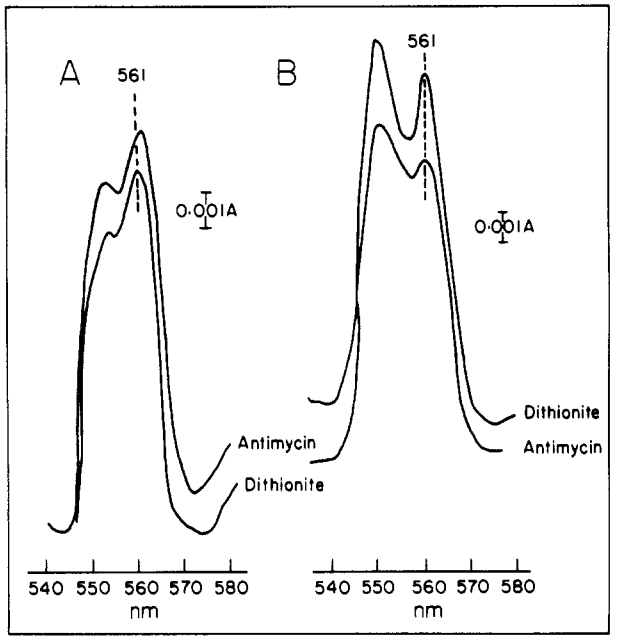

FIGURE 6: Difference spectra upon addition of antimycin to dithionite-reduced mitochondria from wild-type and Q-deficient yeast mitochondria. Mitochondria (2.5 mg/mL) of the wild type (A) or mutant (B) were suspended in 1 mL of the medium described by Meunier-Lemesle et al. (1980). After the base line was traced, sodium dithionite was added to the sample cuvette, ferricyanide was added to the reference cuvette, and the spectrum recorded. Subsequently, a methanolic solution of antimycin (11 nmol/mg of protein) was added to the sample and an equal volume of methanol to the reference cuvette, and the spectrum was recorded. Slit width was 1 nm.

not shown). By contrast, no shift in the absorption maximum of cytochrome *b* was observed in the mutant mitochondria after addition of antimycin.

DISCUSSION

The results of the current study suggest that the coenzyme Q pool in yeast mitochondria is rate-limiting for electron transport from both succinate and NADH to cytochrome *c*. Addition of exogenous coenzyme Q analogues with side chains ranging in length from one to six isoprene units to mitochondria obtained from both wild-type and coenzyme Q deficient yeast cells resulted in parallel increases in cytochrome *c* reductase activity. For example, preincubation with the decyl analogue of coenzyme Q DB generally lead to a 2.5–3-fold increase in enzymatic activity of the wild-type mitochondria and increased the activity in the mutant mitochondria, which have no detectable cytochrome *c* reductase activity in the absence of exogenous Q analogues, to approximately the same level as that of the wild type.

It should also be noted that after addition of optimal amounts of exogenous quinones, the cytochrome *c* reductase activities obtained with either succinate or NADH as electron donor were equal to or greater than those obtained with the reduced coenzyme Q analogue DBH_2 as substrate. These results contrast with previous reports indicating that the primary dehydrogenases are rate-limiting for the rate of electron flow through the respiratory chain (Brown & Beattie, 1977; Berden et al., 1982) and suggest that the pool of apparently excess coenzyme Q does play an important role in electron transfer. That this observation was made with yeast mitochondria is even more striking, since the pool of coenzyme Q is much larger in yeast mitochondria than that of mammalian mitochondria (Hauska & Hurt, 1982).

These conclusions are also in agreement with one of the current concepts of the mitochondrial respiratory chain suggesting that electron transfer between the flavoprotein dehydrogenases and the cytochrome b–c_1 complex occurs by lateral diffusion of coenzyme Q and the protein complexes within the plane of the membrane (Schneider et al., 1982). According to this model, the rate of electron transfer between the various membrane redox components may be diffusion-coupled. Calculations of the diffusion coefficients of the oxidation–reduction components of the mitochondrial inner membrane by fluorescence recovery after photobleaching revealed that coenzyme Q and complex III had the lowest collision efficiency of all the redox pairs studied and were much lower than the collision efficiency between coenzyme Q and either complex I or complex II (Gupte et al., 1984). Perhaps, the low efficiency of collision between these redox partners explains the observation that the rate of electron flow in the wild-type mitochondria can be stimulated by exogenous coenzyme Q.

Recently, Fato et al. (1985) have measured the lateral diffusion coefficients of coenzyme Q_{10} in lipid vesicles by fluorescence quenching of 12-(9-anthroyl)stearate. The diffusion constants measured were several orders of magnitude higher than those calculated by other techniques for membrane lipids that should also include coenzyme Q (Hochman et al., 1985) and much higher than the rates of electron transfer. These workers concluded that the rate of diffusion of coenzyme Q in the membrane is not rate limiting for electron transfer.

The results of the present study also indicate that exogenous coenzyme Q analogues can restore electrogenic proton pumping in mitochondria from the coenzyme Q deficient yeast. The addition of DBH_2, the reduced decyl analogue of coenzyme Q, was capable of supporting proton ejection with an average $H^+/2e^-$ ratio of 3.35, a value slightly higher than that of 2.77 reported by Villalobo et al. (1981) for yeast mitochondria and considerably less than 4.0 reported for rat liver mitochondria in most laboratories including ours (Clejan et al., 1984). Hence, in the absence of endogenous coenzyme Q_6 in the mitochondrial membrane, short-chain analogues of the quinone can effectively restore both electron transport and energy transduction. It should also be noted that these results provide further evidence that the mitochondrial respiratory chain is unchanged in these mutants, which are blocked in one step of the coenzyme Q biosynthetic pathway (Sidhu & Beattie, 1985).

Previously, it was reported that ATP formation could be restored to pentane-extracted chromatophores from *Rhodopseudomonas capsulata* with coenzyme Q analogues containing a minimum side chain of three isoprene units (Baccarini-Melandri et al., 1980). Similarly, DeSantis et al. (1982) reported that ATP synthesis could be restored with Q_3 but not Q_1 in mitochondria obtained from the same Q-deficient yeast mutant used in the current study; however, this group did not report the ability of Q_2 to restore ATP synthesis in this system.

The results of the current study also suggest that exogenous coenzyme Q analogues may not interact with cytochrome *b* and/or other proteins in the b–c_1 complex in the same manner as does endogenous coenzyme Q. This conclusion is based on the observation that the extrareduction of cytochrome *b* that occurs after addition of antimycin to the mutant mitochondria does not induce a red shift in the spectrum even after addition of any of the exogenous coenzyme Q analogues, including the naturally occurring Q_6. The analogues tested were all capable of reconstituting antimycin- and myxothiazol-sensitive NADH:cytochrome *c* reductase and succinate:cytochrome *c* reductase activities to the same level as observed in the wild-type mitochondria (Tables I and II). Furthermore, an-

timycin did not cause a red shift in the spectrum of cytochrome *b* in dithionite-reduced mitochondria from the coenzyme Q deficient yeast in the absence or presence of exogenous coenzyme Q analogues.

The absence of a spectral shift after addition of antimycin suggests that without the endogenous tightly bound coenzyme Q in complex III antimycin does not cause a conformational change in cytochrome *b* leading to a shift in the absorption maximum. The suggestion that there may be a coenzyme Q tightly bound to cytochrome *b* has been strengthened by the recent reports that arylazido derivatives of coenzyme Q were bound to both cytochrome *b* and to a 14-kDa protein of complex III (Yu & Y, 1982; Yu et al., 1985). The latter protein, also termed the Q-binding protein of complex III, has been recently purified and sequenced (Wakabayashi et al., 1985). Furthermore, this protein was previously shown to reconstitute ubiquinol:cytochrome *c* reductase activity in a cytochrome b–c_1 complex from which the Q-binding protein had been previously removed (Wang & King, 1982).

In conclusion, the different coenzyme Q analogues may reconstitute electron flow and under the proper experimental conditions electrogenic proton ejection and ATP synthesis (DeSantis et al., 1982) but may not be reconstituted into the membrane in the same manner as the tightly bound coenzyme Q reported to be an integral part of cytochrome b–c_1 complexes of photosynthetic bacteria and mitochondria (Rich, 1984). In an earlier study, Cabrini et al. (1981) had reported that endogenous coenzyme Q was not necessary for coenzyme Q_1 oxidase activity of Q-depleted mitochondria and suggested that the quinones observed in the purified cytochrome b–c_1 complexes may not play an essential role as a permanently associated prosthetic group but instead may be trapped in the complex during purification (Pasquali et al., 1981). The presence of two different binding sites for coenzyme Q in the cytochrome b–c_1 complex III has been implicated by several different experimental approaches. One coenzyme Q is proposed to be near the Fe–S cluster of the iron–sulfur protein where it acts as an oxidant/reductant, while the second coenzyme Q binding site is similar to the antimycin-binding site (Zhu et al., 1982). Similarly, two different species of semiquinone anion have been detected in coenzyme QH_2:cytochrome *c* reductase in submitochondrial particles with different sensitivities to inhibitors (DeVries et al., 1981; Ohnishi & Trumpwer, 1980). The detection of functionally different types of coenzyme Q in the two different sites in the complex does not mean that these molecules are necessarily permanently fixed in these positions but may result from the properties of a pool quinone distorted by its occupation of a reaction site (Rich, 1984).

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Registry No. Q_1, 727-81-1; Q_2, 606-06-4; Q_6, 1065-31-2; DB, 55486-00-5; DBH_2, 87742-20-9; H^+, 12408-02-5; cytochrome *b*, 9035-37-4; cytochrome c_1, 9035-42-1; succinate:cytochrome *c* reductase, 9028-10-8; NADH:cytochrome *c* reductase, 9027-14-9.

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Isolation of a Manganese-Containing Protein Complex from Photosystem II Preparations of Spinach†

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ABSTRACT: Purified ^{125}I-labeled 33-kDa protein binds to calcium-washed photosystem II preparations at high-affinity and low-affinity binding sites. Filling 70% of the high-affinity site with 33-kDa protein induces 63% of the maximum achievable reconstitution of O_2-evolving activity. When *N*-succinimidyl [(4-azidophenyl)dithio]propionate modified 33-kDa protein was reconstituted into Ca(II)-washed membranes under conditions that primarily filled the high-affinity site and then cross-linked to adjacent proteins by illumination of the photoaffinity label, a cross-linked protein complex was formed that could be solubilized from the membranes with sodium dodecyl sulfate. The protein complex consisted of 22-, 24-, 26-, 28-, 29-, and 31-kDa proteins cross-linked to the 33-kDa protein and contained about 3–4 mol of Mn/mol of protein.

The light reactions of photosystem II create strong oxidants that are ultimately reduced by the oxidation of water to molecular oxygen, a reaction catalyzed by the oxygen-evolving complex (OEC).[1] The OEC contains four manganese (Cheniae & Martin, 1970), which can be released by any of several treatments that inactivate O_2-evolving activity. These treatments include, among others, incubation of the membranes at high pH (Briantais et al., 1977) or in Tris (Yamashita & Butler, 1968). However, cross-linking the proteins in the thylakoid membranes with low concentrations of glutaraldehyde reduces the extent of flash-induced inactivation of oxygen-evolving activity by Tris to one-fourth of the extent in un-cross-linked membranes (Frasch & Cheniae, 1980). This resistance to inactivation apparently results from an increased stabilization of the manganese that is associated with the proteins of the OEC caused by the cross-linked proteins.

The polypeptide composition of this manganoenzyme has not been elucidated though progress has been made in recent years. Purified membrane particles (Bertold et al., 1981; Kuwabara & Murata, 1982) and PS II core complexes (Tang & Satoh, 1985; Ikeuchi et al., 1985), which catalyze the electron transport reactions of PS II, have led to the characterization of many of the proteins that mediate these reactions. In the former preparations, light-harvesting complex proteins (25–29 kDa) are the proteins found in greatest abundance (Arntzen, 1978) but are absent in the core complexes. Two proteins run as diffusely staining bands in SDS–PAGE. First is the herbicide-binding protein called D_1 (32 kDa), which has been identified to contain the binding site for the second quinone acceptor, Q_B (Pfister et al., 1981). Second is the D_2 protein (34 kDa), which has been hypothesized to be on the oxidizing side of PS II. Cytochrome *b*-559, associated with PS II, has been purified as a 9-kDa protein (Metz et al., 1983; Widger et al., 1984). The chlorophyll proteins CPa-1 (47 kDa) and CPa-2 (43 kDa) have been suggested to be the reaction center and subantenna for PS II, respectively (Delepelaire & Chua, 1979; Green & Cam, 1983; Nakatani et al., 1984), although recent evidence suggests a close correlation between the reaction center proteins of *Rhodopseudomonas viridis* with D_1 and D_2 of PS II (R. Sayre, personal communication; A. Trebs, personal communication).

Three extrinsic membrane proteins (17, 23, and 33 kDa) are found on the inside of the thylakoid membrane and serve functional roles on the oxidizing side of PS II. Akerlund et al. (1982) found that when the 17- and 23-kDa proteins were removed from inside-out thylakoid vesicles by washing in dilute salt, the ability to evolve oxygen was lost. Oxygen-evolving activity was partially restored when the 23-kDa protein was reconstituted with the membranes (Akerlund et al., 1982). Yamamoto et al. (1981) observed that Tris causes the release of the three extrinsic proteins as well as the manganese from the membrane, which results in an irreversible loss of O_2-evolving activity. However, Ono and Inoue (1983) recently found that washing the membranes in high concentrations of calcium removes the three extrinsic proteins from PS II preparations without substantial loss of manganese. A significant amount of O_2-evolving capacity can be restored to Ca-washed membranes by reconstitution with the 33-kDa protein, and activity can be enhanced further by the addition of the 23-kDa protein or Ca(II) (Kuwabara et al., 1985).

Because the 23-kDa protein (Yamamoto et al., 1983; Kuwabara & Murata, 1983) as well as the 33-kDa protein can

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[1] Abbreviations: PS II, photosystem II; PAGE, polyacrylamide gel electrophoresis; OEC, oxygen-evolving complex; SDS, sodium dodecyl sulfate; SADP, *N*-succinimidyl [(4-azidophenyl)dithio]propionate; SMN, 400 mM sucrose, 50 mM MES, 5 mM $MgCl_2$, and 10 mM NaCl, pH 6.0; MES, 4-morpholineethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; PMSF, phenylmethanesulfonyl fluoride.

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