Reduction of Cytochrome *b* in Mitochondria from Yeast Lacking Coenzyme Q†

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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 but had comparable amounts of cytochromes *b* and c_1 as wild-type mitochondria. Addition of succinate to the mutant mitochondria resulted in a slight reduction of cytochrome *b*; however, the subsequent addition of antimycin resulted in a biphasic reduction of cytochrome *b*, leading to reduction of 68% of the total dithionite-reducible cytochrome *b*. No "red" shift in the absorption maximum was observed, and no cytochrome c_1 was reduced. The addition of either myxothiazol or alkylhydroxynaphthoquinone blocked the reduction of cytochrome *b* observed with succinate and antimycin, suggesting that the reduction of cytochrome *b*-562 in the mitochondria lacking coenzyme Q may proceed by a pathway involving cytochrome *b* at center *o* where these inhibitors block. Cyanide did not prevent the reduction of cytochrome *b* by succinate and antimycin in the mutant mitochondria. These results suggest that the succinate dehydrogenase complex can transfer electrons directly to cytochrome *b* in the absence of coenzyme Q in a reaction that is enhanced by antimycin. Reduced dichlorophenolindophenol (DCIP) acted as an effective bypass of the antimycin block in complex III, resulting in oxygen uptake with succinate in antimycin-treated mitochondria. By contrast, reduced DCIP did not restore oxygen uptake in the mutant mitochondria, suggesting that coenzyme Q is necessary for the bypass. The addition of low concentrations of DCIP to both wild-type and mutant mitochondria reduced with succinate in the presence of antimycin resulted in a rapid oxidation of cytochrome *b* perhaps by the pathway involving center *o*, which does not require coenzyme Q.

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 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). 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.

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 (Berden et al., 1982; 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, 1985; Yu & Yu, 1981) 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 (Mitchell, 1976; Trumpower, 1981; 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 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). In a recent study, we suggested 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 wild-type mitochondria to the level of coenzyme QH_2:cytochrome *c* reductase (Beattie & Clejan, 1986).

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 such as Q_3 or Q_6 (Desantis et al., 1982). Furthermore, electrogenic proton pumping at site 2 can be restored in the mutant mitochondria by the addition of the decyl analogue of coenzyme Q (Beattie & Clejan, 1986).

Spectral studies of mitochondria from the coenzyme Q deficient yeast cells revealed that several of the coenzyme Q analogues can reconstitute the extrareduction of cytochrome *b* observed in the presence of antimycin but without the typical "red" shift observed in the wild-type mitochondria. In addition, no red shift was observed when antimycin was added to dithionite-reduced mutant mitochondria in the presence or absence of coenzyme Q analogues.

In this study, we have further investigated the spectral properties of the mitochondria lacking coenzyme Q. The

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results indicate that succinate can reduce significantly cytochrome *b* in these mitochondria in the presence of antimycin in a myxothiazol- and alkylhydroxynaphthoquinone-sensitive pathway.

Experimental Procedures

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). 2-Hydroxy-3-nonyl-1,4-naphthoquinone (HNQ) was purchased from Aldrich and dissolved in dimethyl sulfoxide prior to addition. Myxothiazol was a generous gift of Dr. W. Trowitzch. All other reagents were of the highest purity commercially available.

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). Each preparation of mitochondria was tested to show that no succinate:cytochrome *c* reductase activity was present.

Preparation of Mitochondria and Submitochondrial Particles. The cells were broken with glass beads (0.45–0.50-mm diameter) in a Bronwill shaker and mitochondria prepared as described by Brown and Beattie (1977) 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). 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. 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). For the experiments in which oxygen uptake was studied, mitochondria were prepared by the procedure described by Villalobo et al. (1981), except Zymolyase 5000 (3 mg/g wet weight of cells) was used to obtain spheroplasts. The final mitochondrial pellet was resuspended by gentle homogenization at a protein concentration of 60–80 mg/mL. From this suspension a 4 mg/mL final concentration was used in the reaction chamber.

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

Oxygen Uptake. Yeast mitochondria (4.0 mg/mL) were incubated in 3.0 mL of a medium containing 0.60 M mannitol, 10 mM imidazole hydrochloride (pH 6.4), and 1.0 μM CCCP. Oxygen uptake rates were measured with a Clarke oxygen electrode.

Results

Spectral Analysis of Cytochrome b in Coenzyme Q Deficient Yeast Mitochondria. Previously, we reported that addition of exogenous coenzyme Q analogues restored both succinate:cytochrome *c* reductase and NADH:cytochrome *c* reductase activities as well as electrogenic proton pumping in mitochondria isolated from coenzyme Q deficient yeast cells (Beattie & Clejan, 1986). The oxidant-induced extrareduction of cytochrome *b* was observed in these mitochondria incubated with succinate and coenzyme Q analogues; however, the red shift observed in the wild-type mitochondria upon the addition of antimycin was not observed in the mutant mitochondria.

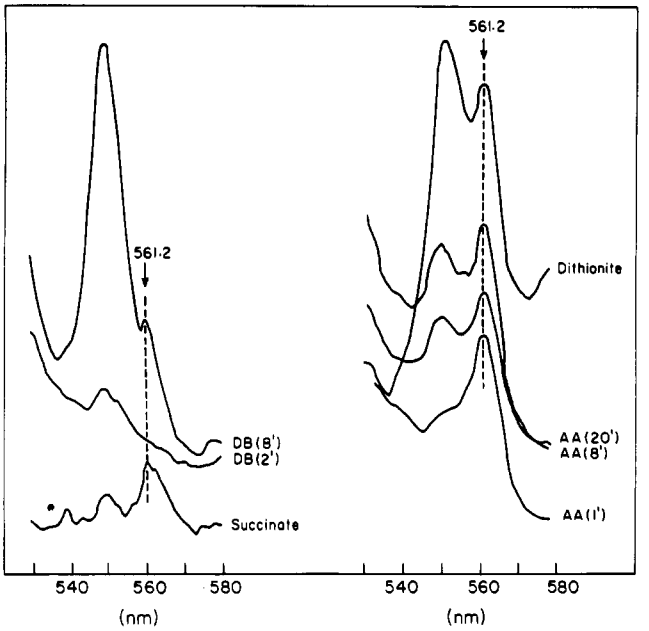

FIGURE 1: Reduction of cytochromes *b* and c_1 in DB-reconstituted mitochondria from Q-deficient yeast. Mitochondria were prepared from strain E3-24 (Beattie et al., 1981) and suspended at 2.5 mg/mL in 800 μL of the assay medium described by Meunier-Lemesle et al. (1980). After base line adjustment, 30 mM sodium succinate and 150 nmol of DB were added. Difference spectra were recorded after 2 and 8 min (left panel). Subsequently, 22 nmol of antimycin were added and spectra recorded at 1, 8, and 20 min. A few grains of dithionite completely reduced cytochrome *b* (right panel).

In the present study, the reduction of the cytochromes of mitochondria from the yeast cells lacking coenzyme Q was examined upon addition of succinate and the decyl analogue of coenzyme Q (DB). As seen in Figure 1, the addition of succinate to the mitochondria from the coenzyme Q deficient yeast cells resulted in the reduction of less than 20% of the total dithionite-reducible cytochrome *b*. Two minutes after the addition of DB, an initial oxidation of cytochrome *b* was observed with the concomitant reduction of cytochrome c_1. After further incubation for 8 min, cytochrome c_1 was completely reduced while cytochrome *b* again became partially reduced. This triphasic reduction of cytochrome *b* has been explained by classical shemes for the Q cycle (Hatefi, 1985) and was initially reported by Jin et al. (1981). The kinetics of reduction of cytochrome *b* were reported in our earlier publication (Beattie & Clejan, 1986).

The addition of antimycin to these succinate- and DB-treated mitochondria resulted in the extrareduction of cytochrome *b* with the simultaneous oxidation of cytochrome c_1; however, as mentioned above, the red shift in the absorption maximum of cytochrome *b* was not observed in the mitochondria from the Q-deficient yeast. With increasing times of incubation, cytochrome c_1 gradually became more reduced, suggesting that the superoxide produced by exogenous quinols present in the incubation mixture may act to reduce cytochrome c_1 in the presence of antimycin.

We were interested in exploring further the observation that the addition of succinate appeared to result in a partial reduction of cytochrome *b* in these Q-deficient mutants without the addition of exogenous quinones. As seen in Figure 2, the

[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.

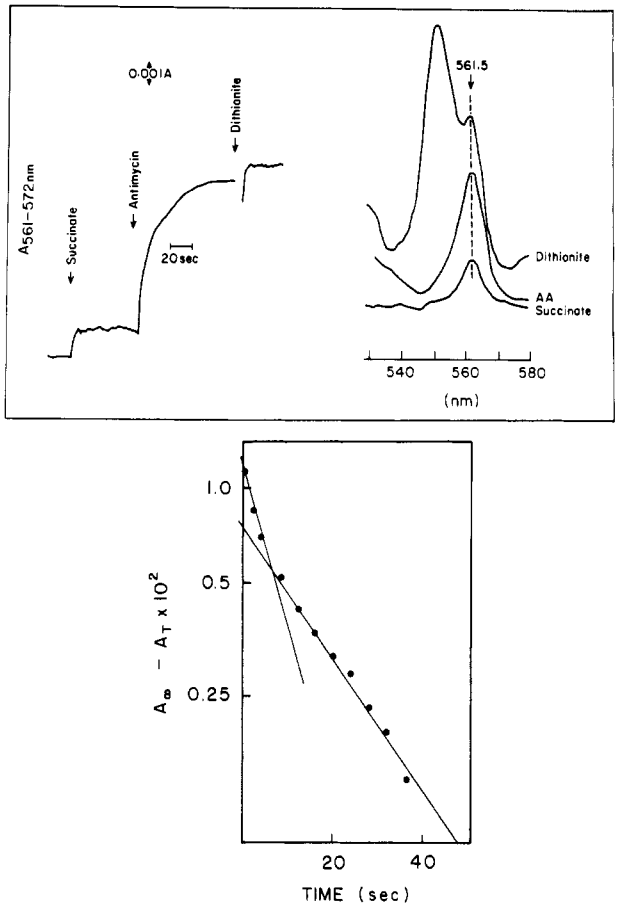

FIGURE 2: Reduction of cytochrome *b* in Q-deficient mitochondria by succinate in the presence of antimycin. Mitochondria were prepared as described in the legend to Figure 1. Upper panel: The following additions were made after base line adjustment: succinate (30 mM), antimycin (22 nmol), and dithionite. Spectra were recorded in both double-wavelength (left panel) and double-beam (right panel) modes. Lower panel: The time course of the reduction of cytochrome *b* was analyzed to demonstrate the biphasic nature of the reaction.

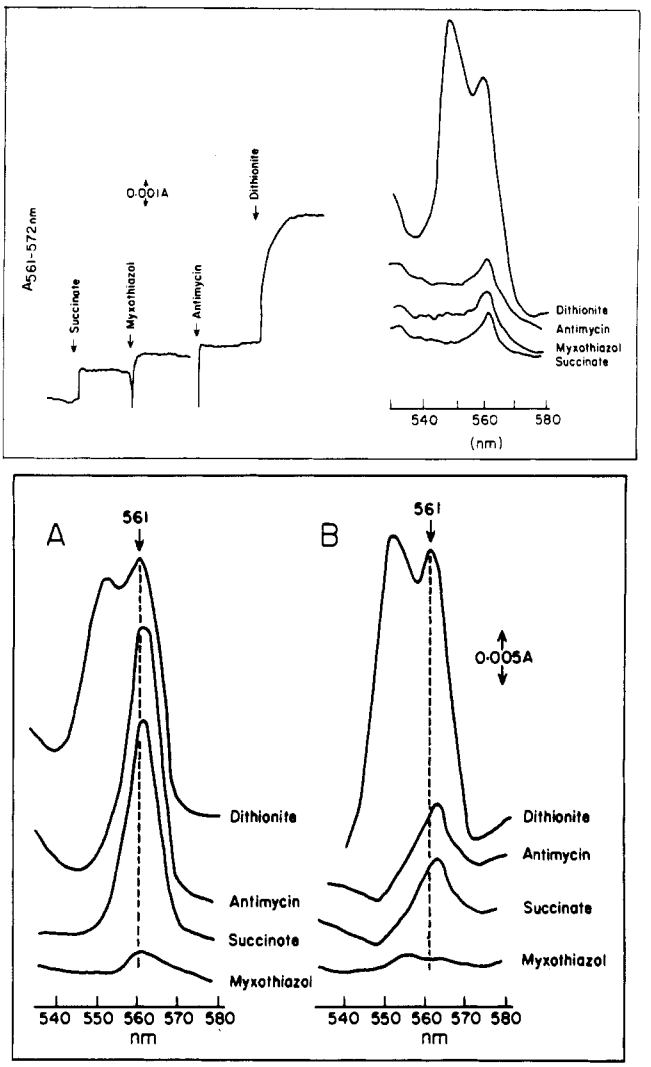

FIGURE 3: Myxothiazol inhibits reduction of cytochrome *b* by succinate and antimycin in the Q-deficient mutant. Mitochondria were prepared in the medium described by Meunier-Lemesle et al. (1980) and solubilized in 0.5% Triton X-100. Upper panel: After the base line was adjusted, succinate (30 mM), myxothiazol (25 μM), antimycin (22 nmol), and dithionite were added to the sample cuvette. Spectra were scanned in either the double-wavelength (lef panel) or double-beam (right panel) mode. Lower panel: (A) D273-10B mitochondria at 2.5 mg/mL or (B) E3-24 mitochondria at 3.0 mg/mL were treated as described for the upper panel except that the myxothiazol was added to the sample before the succinate.

addition of succinate to these mitochondria resulted in a slight reduction of cytochrome *b* but not of cytochrome c_1. The subsequent addition of antimycin resulted in a biphasic reduction of cytochrome *b* with both a rapid (within 5 sec) and a slow phase, eventually leading to reduction of 68% of the total dithionite-reducible cytochrome *b*. Again, it should be noted that no red shift in the absorption maximum of cytochrome *b* was observed after the addition of antimycin in either the succinate- or dithionite-reduced mitochondria. Furthermore, cytochrome c_1 was not reduced by succinate in the presence of antimycin in these mitochondria.

In an attempt to understand the mechanism by which succinate reduced cytochrome *b* in the absence of coenzyme Q, different well-characterized inhibitors of electron transfer in the cytochrome b–c_1 complex were studied. Myxothiazol has been shown previously to bind to cytochrome *b*-566 and prevent the flow of electrons to the iron–sulfur protein of complex III (Von Jagow et al., 1984). The addition of myxothiazol to succinate-reduced mutant mitochondria did not result in an appreciable reduction of cytochrome *b*. Moreover, the presence of myxothiazol completely blocked any further reduction of cytochrme *b* upon addition of antimycin (Figure 3, upper panel).

The second approach involved the initial addition of myxothiazol to mitochondria prior to the addition of succinate and antimycin. In mitochondria from wild-type yeast cells, the addition of myxothiazol had no effect on the absorption of cytochrome *b*; however, addition of succinate resulted in the reduction of 90% of the dithionite-reducible cytochrome *b* with no reduction of cytochrome c_1 (Figure 3, lower panel). Under these conditions, the absorption maximum of the succinate-reduced cytochrome *b* in the presence of myxothiazol was shifted to a longer wavelength. By contrast, addition of succinate to the mutant mitochondria previously treated with myxothiazol resulted again in a slight reduction of cytochrome *b* (Figure 3, lower panel B), but the subsequent addition of antimycin did not result in any reduction of cytochrome *b*. Again, in the presence of myxothiazol, the absorption maximum of the small amount of reduced cytochrome *b* observed after the addition of succinate was shifted to a longer wavelength even in the mitochondria lacking coenzyme Q. Treatment of both control and mutant mitochondria with dithionite after this experimental protocol caused the absorption maximum of cytochrome *b* to shift to 561 nm. The addition of succinate to the mutant mitochondria previously treated with both myxothiazol and antimycin did not result

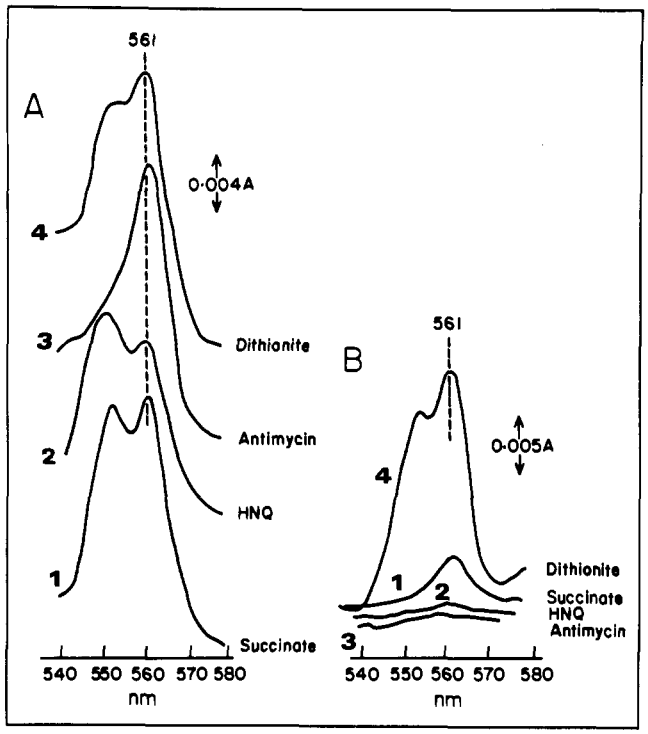

FIGURE 4: HNQ inhibits reduction of cytochrome *b* by succinate in the presence of antimycin in the Q-deficient mutant. Mitochondria from wild-type (A) and Q-deficient mutants (B) were resuspended at 3 mg/mL in the medium described by Meunier-Lemesle et al. (1980). After base line adjustment, difference spectra were recorded following the sequential addition of 30 mM succinate, 200 nmol of HNQ, 33 nmol of antimycin, and dithionite. The same sequence of additions was performed in the mutants as indicated by the numbers on the traces.

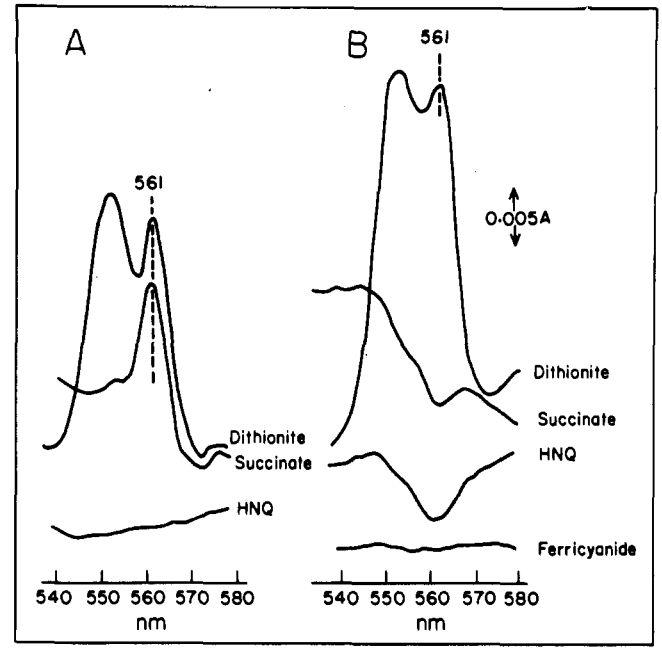

FIGURE 5: Effect of HNQ on reduction of cytochrome *b* in the Q-deficient mutant. Mitochondria were prepared at 2.5 mg/mL as described in the legend to Figure 1. After the base line correction, the following additions were made to the cuvette: 200 nmol of HNQ, 30 mM succinate, and dithionite. (A) Wild-type mitochondria; (B) mutant mitochondria.

in any reduction of cytochrome *b*.

The alkylhydroxynaphthoquinone inhibitor, HNQ, which is considered to bind to a coenzyme Q binding site closely associated with the iron–sulfur protein and thus block electron transfer (Matsuura et al., 1983), was also examined. The addition of HNQ to succinate-reduced wild-type mitochondria resulted in a slight oxidation of cytochrome *b* (Figure 4). The subsequent addition of antimycin resulted in a further reduction of cytochrome *b* while shifting the absorption maximum to a longer wavelength with the concomitant oxidation of cytochrome c_1. By contrast, in the mutant mitochondria, the addition of HNQ after succinate caused the complete oxidation of cytochrome *b* and completely blocked any further reduction upon addition of antimycin (Figure 4B).

The effects of addition of HNQ to mitochondria prior to the substrate were also studied in both types of mitochondria (Figure 5). In the wild-type mitochondria treated with HNQ, addition of succinate resulted in the reduction of 80% of the total dithionite-reduced cytochrome *b* without any reduction of cytochrome c_1. By contrast, the initial addition of HNQ to the mutant mitochondria (in which cytochrome *b* was slightly reduced) resulted in the oxidation of cytochrome *b*, which remained oxidized after the subsequent addition of succinate (Figure 5B). The addition of antimycin after succinate did not result in any change in the spectrum (data not shown). In a separate experiment, it was observed that the addition of HNQ to mutant mitochondria previously treated with succinate and antimycin did not result in the oxidation of cytochrome *b*.

The results of the experiments with both HNQ and myxothiazol suggest that the reduction of cytochrome *b* observed in the presence of antimycin may involve center *o*, which is sensitive to both of these inhibitors (Von Jagow et al., 1984; Matsuura et al., 1983).

The effects of cyanide on the reduction of the cytochromes of the electron transport chain was also compared in the coenzyme Q deficient yeast cells. The addition of cyanide after the initial reduction with succinate resulted in the reduction of cytochrome c_1, amounting to approximately 50% of the total dithionite-reducible cytochrome c_1 (Figure 6). The subsequent addition of antimycin resulted in a slow reduction of cytochrome *b* with the kinetics of the slow phase observed when antimycin is added to succinate-treated mutant mitochondria (Figure 2), suggesting that cyanide abolishes the rapid phase of cytochrome *b* reduction. The complete reaction resulted in an almost 80% reduction of the total dithionite-reducible cytochrome *b*. Again, under these experimental conditions, no red shift in the maximum absorption of cytochrome *b* was observed.

Bypass of the Antimycin Block by Reduced Dichlorophenolindophenol (DCIP). Recently, Alexandre and Lehninger (1984) reported that reduced DCIP makes possible electron flow around the antimycin block of complex III. As seen in Figure 7, DCIP maintained in a reduced state by ascorbate was not readily oxidized via the respiratory chain in the presence of antimycin, as indicated by the extremely low rate of oxygen uptake in the wild-type mitochondria (D273-10B). The subsequent addition of succinate, however, resulted in a large increase in the rate of oxygen uptake that is sensitive to cyanide. These results obtained with yeast mitochondria confirm those of Alexandre and Lehninger (1984) obtained with rat liver mitochondria and suggest that reduced DCIP can effectively bypass the antimycin block between succinate and cytochrome *c*.

In similar studies with the coenzyme Q deficient yeast mitochondria (E3-24), a slightly greater rate of oxygen uptake was observed in the presence of DCIP and ascorbate than was observed with the wild-type mitochondria. The subsequent addition of succinate, however, did not result in any increase in the rate of oxygen uptake, suggesting that the bypass of the

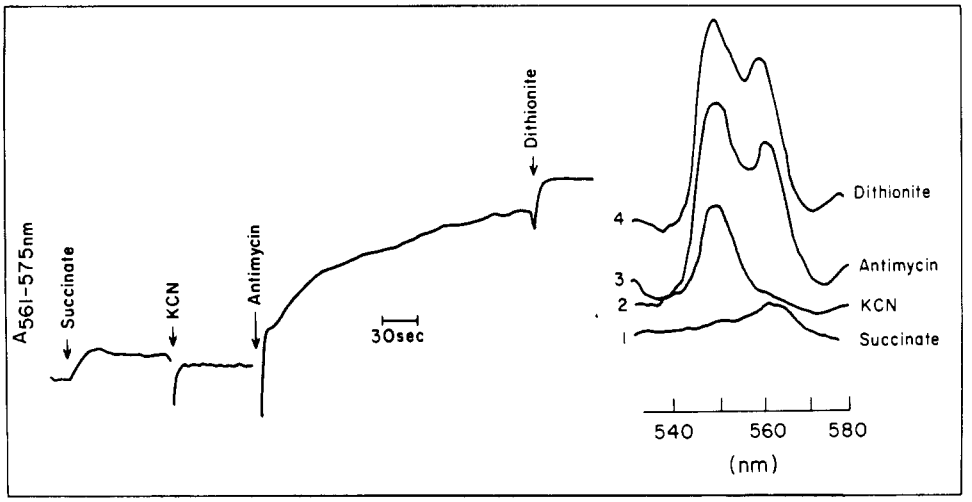

FIGURE 6: Effect of cyanide on reduction of cytochrome *b* by succinate in the presence of antimycin in the Q-deficient mutant. Mitochondria from the mutant were suspended at 3 mg/mL in the medium described by Meunier-Lemesle et al. (1980). After a base line correction, double-wavelength scans (left) and double-beam difference spectra were recorded after the addition of 30 mM succinate, 1 mM KCN, 33 nmol of antimycin, and dithionite.

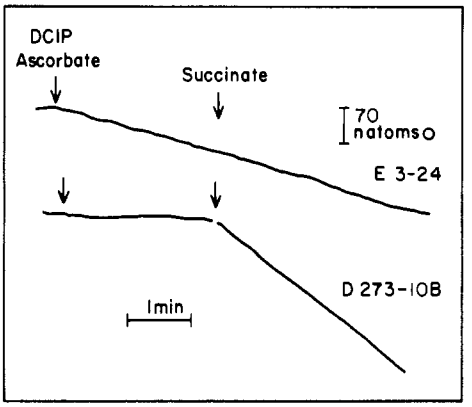

FIGURE 7: Bypass of the antimycin block by DCIP in wild-type mitochondria but not in Q-deficient mitochondria. Mitochondria were obtained by the procedure of Villalobo et al. (1981) and resuspended in the medium described under Experimental Procedures. The initial rates of oxygen uptake were determined following the addition of 1 μM CCCP, 10 ng of antimycin, 3 mM ascorbate, 25 μM DCIP, and 3 mM sodium succinate by using a Clarke oxygen electrode and a Sargeant-Welsh recorder at 28 °C. Upper trace, E3-24 mutant; lower trace, D273-10B.

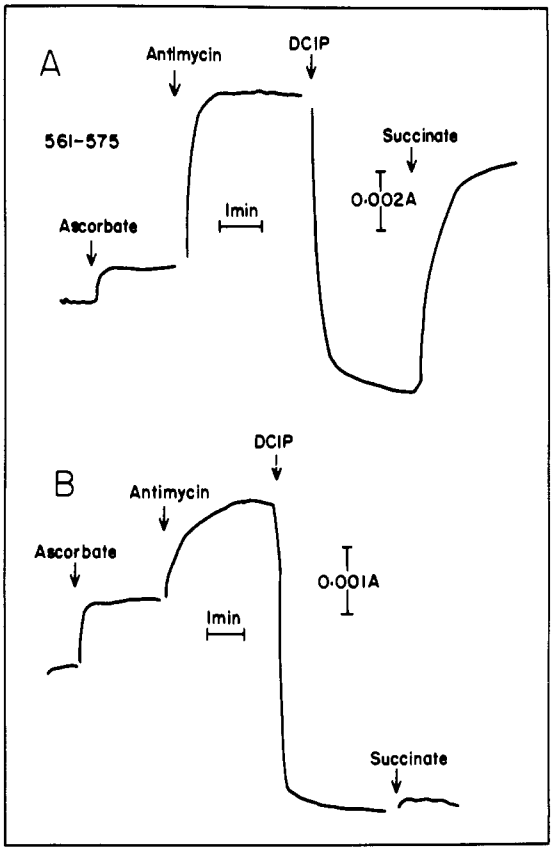

FIGURE 8: DCIP bypass of the antimycin block and the redox state of cytochrome *b* in the wild-type and Q-deficient mitochondria. Mitochondria were prepared by Zymolyase digestion as described under Experimental Procedures and suspended in a medium containing 0.6 M mannitol, 10 mM imidazole, pH 6.4, and 1.0 μM CCCP. The double-wavelength mode was set at 561–575 nm, and the base line was adjusted and scanned after the addition of 3 mM sodium ascorbate, 40 μmol of antimycin, 6 μM DCIP, and 3 mM sodium succinate. (A) Wild-type mitochondria; (B) Q-deficient mitochondria.

antimycin block by DCIP and ascorbate requires the presence of coenzyme Q.

The addition of antimycin to ascorbate-treated wild-type mitochondria resulted in the reduction of cytochrome *b* by endogenous substrates to a level approximately 75% of the maximum reduction observed after addition of succinate and antimycin (Figure 8). The addition of very low concentrations of DCIP led to a very rapid and complete oxidation of cytochrome *b* which was completely reversible by addition of succinate.

In similar experiments with mitochondria from the coenzyme Q deficient yeast (Figure 8B), the addition of antimycin led to reduction of 40% of the total cytochrome *b* with the biphasic kinetics noted previously in the presence of succinate and antimycin (Figure 2). The addition of DCIP resulted in a rapid and complete oxidation of cytochrome *b*; however, the subsequent addition of succinate did not result in any reduction of cytochrome *b* in the mutant mitochondria (Figure 8).

DISCUSSION

The results of the current study demonstrate that cytochrome *b* can be reduced by succinate in mitochondria isolated from mutants of yeast lacking any coenzyme Q (Brown & Beattie, 1977). The reduction was enhanced by the addition of antimycin, resulting in approximately 70% of the total dithionite-reducible cytochrome *b*. The rate of reduction in the mutant mitochondria was slower than that of the wild type and occurred in two phases, a rapid phase complete within 5

s and a slower phase lasting 60 s or more (Figure 2). No cytochrome c_1 was reduced in the mutants supplemented with succinate or with succinate plus antimycin, confirming previous studies that succinate and NADH:cytochrome *c* reductase activities do not occur in the mutant lacking any coenzyme Q (Beattie & Clejan, 1986). It should be noted that mutant E3-24 has been shown to accumulate 3,4-dihydroxy-5-hexaprenylbenzoate (Goewert et al., 1981), a compound unlikely to substitute for ubiquinone.

The reduction of cytochrome *b* by succinate in the antimycin-treated mitochondria without any detectable coenzyme Q does not appear to be nonspecific reaction, as the addition of myxothiazol inhibits significantly, while HNQ completely blocks it. Previous studies in several laboratories (Von Jagow et al., 1984; Von Jagow & Engel, 1981) have indicated that myxothiazol binds to cytochrome *b*-566, resulting in conformational changes that may act to displace the quinone from the iron–sulfur protein. By contrast, Kamensky et al. (1985) have suggested that myxothiazol and the related antibiotic mucidin, a compound identical with strobilurin (Von Jagow et al., 1986), may bind to cytochrome *b*-562 as well as cytochrome *b*-566 since these inhibitors affect the spectra and redox potentials of both cytochromes *b*. As a consequence of the binding of myxothiazol to the *b* cytochromes, QH_2 oxidation by the iron–sulfur protein at center *o* of the Q cycle may be blocked.

It should be noted that cytochrome *b*, however, was reduced in the presence of myxothiazol in wild-type mitochondria by the antimycin-sensitive pathway (Von Jagow et al., 1984). The >70% inhibition of cytochrome *b* reduction by myxothiazol in the mutants lacking coenzyme Q suggests that the transfer of electrons from succinate dehydrogenase to complex III may occur via the myxothiazol-sensitive center *o* pathway without coenzyme Q acting as an intermediate electron transport carrier. The inhibition of cytochrome *b* reduction in the presence of both myxothiazol and antimycin suggests that this cytochrome *b* is not that suggested to be present in complex II (Hatefi, 1985). Furthermore, the physiological relevance of this reduction of cytochrome *b* is unclear; however, in the absence of coenzyme Q no electron transport to oxygen is possible.

Similarly, the alkylhydroxynaphthoquinones such as HNQ also block electron transfer to the iron–sulfur protein, presumably acting as competitive inhibitors with coenzyme Q (Matsuura et al., 1983). These compounds, which act in a similar manner as 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) (Bowyer et al., 1982), change the redox potential of the iron–sulfur protein and may share a common binding site on the iron–sulfur protein with coenzyme Q. Interestingly, the addition of HNQ to mitochondria from the coenzyme Q deficient yeast mitochondria caused a pronounced oxidation of cytochrome *b* and completely prevented any further reduction upon addition of succinate or antimycin. The actual oxidant of cytochrome *b* under these conditions is not known.

The inhibition of cytochrome *b* reduction in the mutant mitochondria by HNQ provides further evidence for the close association of cytochrome *b*-566 and the iron–sulfur protein. If HNQ indeed binds as a competitive inhibitor with coenzyme Q on the iron–sulfur protein, it must occupy this site in the coenzyme Q deficient mitochondria. The resulting conformational changes in the *b* cytochromes may thus act to prevent any subsequent reduction by succinate dehydrogenase by the pathway not involving coenzyme Q.

Another recently described inhibitor of electron transport in complex III is stigmatellin, which has been shown to bind to both cytochrome *b*-566 and the iron–sulfur protein while shifting the midpoint potential of the iron–sulfur protein (Von Jagow & Ohnishi, 1985). The conclusion that these inhibitors interact with both cytochrome *b*-566 and the iron–sulfur protein suggests that these two proteins may be tightly associated functionally. Previously, we had suggested a close physical association between these two proteins on the basis of the immunoprecipitation of both proteins from Triton X-100 solubilized mitochondria with the specific antiserum against the iron–sulfur protein (Sidhu et al., 1983).

The antimycin-enhanced reduction of cytochrome *b* by succinate was also observed in the presence of cyanide, providing further evidence for the lack of ubisemiquinone, which might act to produce superoxide which can reduce cytochrome *b*. Recently, Tarrens et al. (1985), using complex III from beef heart mitochondria, reported that ubisemiquinone can generate superoxide in a reaction that is inhibited by cyanide. Addition of cyanide did result in a slower rate of cytochrome *b* reduction when antimycin was added to succinate-treated mutant mitochondria. The observed reduction by endogenous substrates of cytochrome c_1 (and presumably the iron–sulfur protein with its similar redox potential) after addition of cyanide suggests that the altered conformation of the complex known to occur upon reduction may prevent the rapid transfer of electrons from the primary dehydrogenase to its acceptor in complex III in the absence of coenzyme Q.

In a recent preliminary communication, DeSantis et al. (1985) reported that NADH and succinate caused a reduction of cytochrome *b* in submitochondrial particles from the coenzyme Q lacking yeast strain used in the present study. These workers suggested that a connection between the dehydrogenases and complex III occurred in the native mitochondrial membrane. Similar results were obtained in ubiquinone-depleted submitochondrial particles by Ernster et al. (1969), who reported that succinate partially reduced cytochrome *b* in the presence or absence of cyanide; however, the addition of antimycin caused a rapid and complete reduction of cytochrome *b*.

The addition of antimycin to mitochondria from the yeast cells lacking coenzyme Q did not result in a red shift in the absorption maximum of cytochrome *b*. Antimycin failed to cause a change in the absorption spectrum of cytochrome *b* whether or not the mutant mitochondria were supplemented with exogenous coenzyme Q analogues. Previously, we had suggested that the presence of endogenous coenzyme Q was necessary for the antimycin-induced red shift in the spectrum in mitochondria reduced with either succinate or dithionite (Beattie & Clejan, 1986). The lack of this response in the mitochondria lacking any coenzyme Q further supports this conclusion.

Myxothiazol, however, caused a shift in the absorption maximum of cytochrome *b*, to a longer wavelength in the mutant mitochondria lacking coenzyme Q. Previously, Von Jagow and Engel (1981) had reported a myxothiazol-induced shift in the absorption of cytochrome *b*-566 in complex III from which the iron–sulfur protein had been removed. These results suggest that myxothiazol binds to cytochrome *b*, causing a conformational change independently of the presence of these other redox components of the complex.

Finally, the data presented in the current study confirm the suggestion of Alexandre and Lehninger (1984) that the DCIP bypass of the antimycin site in complex III feeds in at coenzyme Q prior to the reduction of the iron–sulfur protein.

Their conclusion was based on the sensitivity of the DCIP bypass to both myxothiazol and HNQ. While the DCIP bypass worked as effectively in the wild-type yeast mitochondria as in the rat liver mitochondria, no increased rate of oxygen uptake was observed in the antimycin-treated mutant mitochondria after the addition of ascorbate, DCIP, and succinate. Hence, the presence of coenzyme Q is necessary for the DCIP bypass to function in antimycin-treated mitochondria.

Under these same experimental conditions, very low concentrations of DCIP resulted in a rapid and complete oxidation of cytochrome *b* in both the wild-type and mutant mitochondria in the presence of antimycin but in the absence of exogenous substrate. Alexandre and Lehninger (1984) have attributed the oxidation of cytochrome *b* by DCIP (reduced by the presence of ascorbate) to the reduction of the bound ubisemiquinone at center *o* to yield bound QH_2. The reduced quinone thus formed might then exchange with a molecule of free oxidized Q, which subsequently might accept electrons from cytochrome *b* in the reverse direction. This suggestion cannot explain the equally rapid oxidation of cytochrome *b* by DCIP in the mitochondria lacking any coenzyme Q. Alternatively, we would suggest that DCIP may directly oxidize cytochrome *b* at center *o* by the same pathway by which electrons are donated to the complex from the primary dehydrogenases in the absence of coenzyme Q. Recently, Pasquali et al. (1985) have reported that ferricyanide can oxidize cytochrome *b* in coenzyme Q depleted mitochondria treated with antimycin, also suggesting that cytochrome *b* can be oxidized independently of coenzyme Q.

The further addition of succinate to the DCIP-oxidized wild-type mitochondria resulted in a rapid reduction of cytochrome *b*. Succinate would be expected in wild-type mitochondria treated with low amounts of reduced DCIP to reduce the ubisemiquinone at center *o* to QH_2, which would then reduce the iron–sulfur protein and cytochrome *b* in the presence of antimycin. The lack of reduction of cytochrome *b* in the coenzyme Q deficient mitochondria after addition of succinate is consistent with this suggestion.

The results of this study confirm previous observations that the coenzyme Q deficient mutants of yeast provide an excellent model system to investigate the pathways of electron transfer and proton pumping in the cytochrome b–c_1 region of the respiratory chain. Currently, we are planning to study these reactions in complex III isolated from these mutants.

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Location of Lysine-β162 in Mitochondrial F_1-Adenosinetriphosphatase†

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ABSTRACT: The quenching of the fluorescence of bovine heart F_1-adenosinetriphosphatase labeled specifically at its essential Lys-β162 with 7-chloro-4-nitro-2,1,3-benzoxadiazole (*N*-NBD-F_1) by 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-triphosphate (TNP-ATP) has been studied. Analysis of the fluorescence data in the presence of 1 mM ATP shows that the dissociation constant of TNP-ATP from its first binding site in the covalently labeled enzyme is 250-fold lower than that of ATP, which it replaces in pH 7.0 buffer containing 25% glycerol, and that this binding causes a 54% quenching of the fluorescence of the *N*-NBD label due to energy transfer to the weakly fluorescent TNP-ATP molecule. Computation based on the observed quenching gives a distance of 25.6 ± 0.4 Å between the NBD label and the chromophore of the bound TNP-ATP molecule. Since the distance between the chromophore and the farthest O atom of the bound TNP-ATP is about 16 Å, it seems quite likely that the ϵ-amino group of Lys-β162 is near the γ-phosphate group of the TNP-ATP bound at the catalytic site. Similar measurements in the presence of 1 mM ADP show that the replacement of ADP at the catalytic site by TNP-ATP causes a 49% quenching of the fluorescence of the *N*-NBD label, which gives a distance of 26.5 ± 0.4 Å between the label and the chromophore of the bound TNP-ATP molecule.

Since the determination of the amino acid sequence of the β subunit of a number of F_1-ATPases[1] (Sarate et al., 1981; Kanazawa et al., 1982; Runswick et al., 1983; Kurawski et al., 1982; Krebbers et al., 1982), several essential functional groups have been identified with specific labeling reagents. In the case of F_1-ATPase from bovine heart mitochondria, the amino acid residues with essential functional groups identified in this way include Glu-β199 (Yoshida et al., 1982), Tyr-β197 (Ho & Wang, 1983), Tyr-β311 (Andrews et al., 1984a), Lys-β162 (Andrews et al., 1984b), and Lys-β401 (Sutton & Ferguson, 1985). The available data seem to show that the essential Lys labeled by NBD-Cl is Lys-β162. Although the probable secondary structure of the β subunit has been predicted (Kanazawa et al., 1982), we have little direct information on the locations of the above essential functional groups relative to the substrate bound at the catalytic site.

Labeling one of the three Lys-β162 residues in bovine heart mitochondrial F_1-ATPase with NBD-Cl completely inactivates the enzyme. Is this because the labeled single Lys-β162 is at the catalytic site or because the labeling induces long-range conformation change that inactivates the protein? The question could be answered by determining the location of the *N*-NBD label relative to the bound substrate. In the present work, the quenching of fluorescence of the *N*-NBD label on Lys-β162 of F_1 in the presence of 1 mM ATP by added TNP-ATP has been determined as a function of TNP-ATP concentration. Since the TNP-ATP is bound to the catalytic site (Grubmeyer & Penefsky, 1981) and since the molar ratios of *N*-NBD label to F_1 and bound TNP-ATP to F_1 are both less than 1, it is possible to calculate the distance between the *N*-NBD label and the chromophore of the TNP-ATP in a very simple way directly from the observed efficiency of electronic excitation energy transfer from the single donor group to the single acceptor group in the labeled enzyme.

Experimental Procedures

Materials

2′,3′-*O*-(2,4,6-Trinitrocyclohexadienylidene)adenosine 5′-triphosphate (TNP-ATP) was purchased from Molecular Probes and further purified by chromatography on Sephadex LH-20-100 (Hiratsuka & Uchida, 1973). [^{14}C]NBD-Cl was supplied by Research Products International Corp. It was found to have a specific radioactivity of 77 mCi/mmol (Wang et al., 1986).

Mitochondria were prepared from fresh bovine heart (Low et al., 1963). F_1-ATPase was prepared from frozen mitochondria by Betty Stone in our laboratory and stored as described by Knowles and Penefsky (1972). *N*-[^{14}C]NBD-F_1

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; F_1 or F_1-ATPase, F_1-adenosinetriphosphatase; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; *N*-NBD-F_1, F_1 labeled by NBD-Cl at its essential Lys-β162; *O*-NBD-F_1, F_1 labeled by NBD-Cl at its essential Tyr in its catalytic β subunit; qs, quinine sulfate; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate, also called 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-triphosphate.

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