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The interaction of quinone analogues with wild-type and ubiquinone-deficient yeast mitochondria

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The interaction of the exogenous quinones, duroquinone (DQ) and the decyl analogue of ubiquinone (DB) with the mitochondrial respiratory chain was studied in both wild-type and a ubiquinone-deficient mutant of yeast. DQ can be reduced directly by NADH dehydrogenase, but cannot be reduced by succinate dehydrogenase in the absence of endogenous ubiquinone. The succinate-driven reduction of DQ can be stimulated by DB in a reaction inhibited 50% by antimycin and 70–80% by the combined use of antimycin and myxothiazol, suggesting that electron transfer occurs via the cytochrome *b*-c_1 complex. Both DQ and DB can effectively mediate the reduction of cytochrome *b* by the primary dehydrogenases through center o, but their ability to mediate the reduction of cytochrome *b* through center i is negligible. Two reaction sites for ubiquinol seem to be present at center o: one is independent of endogenous Q_6 with a high reaction rate and a high K_m; the other is affected by endogenous Q_6 and has a low reaction rate and a low K_m. By contrast, only one ubiquinol reaction site was observed at center i, where DB appears to compete with endogenous Q_6. DB can oxidize most of the pre-reduced cytochrome *b*, while DQ can oxidize only 50%. On the basis of these data, the possible binding patterns of DB on different Q-reaction sites and the requirement for ubiquinone in the continuous oxidation of DQH are discussed.

Introduction

The mechanism by which various quinones, including ubiquinone, plastoquinone and menaquinone, as well as their homologues and analogues, interact with the energy-conserving electron transfer chains of mitochondria, chloroplasts and bacteria has been the subject of several recent symposia [1]. Soon after the discovery of the natural quinones, many homologues and analogues were synthesized and tested for their catalytic activity [2]. In a recent study [3], some analogues, such as ubiquinone-2 (Q_2), the decyl analogue of ubiquinone (DB) and plastoquinone-2 were shown to be excellent substitutes for the natural quinones in restoring electron transfer activities, while other homologues and analogues with long saturated side-chains or with a larger number of conjugated double bonds were shown to be poor substrates [3]. Many analogues, especially those with a hydroxy group at position 6 of the ring, have also been shown to act as inhibitors of enzyme activity [4]. The quinone-like inhibitors of electron trans-

Abbreviations: DB and DBH_2, oxidized and reduced forms of 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; DQ and DQH_2, oxidized and reduced forms of duroquinone; Q_2 and Q_6, ubiquinones containing side-chains with 2 and 6 isoprene units, respectively.

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fer include compounds such as rotenone [5], piericidin [6], 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole [7], and 2-alkyl-3-hydroxynaphthoquinone [8]. Despite the interest in the ability of a given quinone to support electron flow or to restore phosphorylative activity [1], little attention has been directed to the effect of substitutions on the ring or changes in the side-chain on the mechanism by which quinones react with the different specific quinone-reacting sites in the electron transport chain.

Von Jagow and Bohrer [9] first reported that the reduction of duroquinone (DQ) by succinate in beef heart mitochondria was antimycin-sensitive, indicating the involvement of the cytochrome b-c_1 complex in DQ reduction. This observation was confirmed by Chen et al. [10], who suggested that the reduction of DQ by succinate might occur through protein–protein interactions between succinate: ubiquinone oxidoreductase and the cytochrome b-c_1 complex. Subsequently, we [11] extended these studies in yeast mitochondria and concluded that succinate: ubiquinone oxidoreductase interacts preferentially with center i of the cytochrome b-c_1 complex, while NADH: ubiquinone oxidoreductase interacts preferentially with center o using the terminology of the Q cycle [1]. Furthermore, endogenous Q_6 was shown to be necessary for the reduction of DQ by succinate, but not required for the reduction of DQ by NADH.

In the current study, we have used a ubiquinone-deficient strain of yeast [12] to demonstrate that the reduction of DQ by succinate in this strain can be restored by the addition of the decyl analogue of ubiquinone, DB. A kinetic analysis of the DB-'catalyzed' reaction under various experimental conditions has permitted a description of the binding of DB to the different ubiquinone-reactive sites in the electron transfer chain. In addition, the continuous oxidation of DQH_2, previously shown to require endogenous ubiquinone [13,14], has been studied in these ubiquinone-deficient yeast mitochondria. It was observed that DQH_2 can be rapidly oxidized at center o in the absence of endogenous quinone, but that DQ cannot effectively oxidize cytochrome b through center i in the absence of ubiquinone.

Experimental procedures

Yeast strains and cell growth. The prototrophic strain, D273-10B and the coenzyme Q-deficient strain, E3-24 [15], were obtained from Dr. Alexander Tzagoloff. The cells were grown aerobically at 30 °C in semi-synthetic medium [16] 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 [17], which generally can reveal the existence of less than 1% of the normal Q content. In addition, succinate: cytochrome-*c* reductase was completely absent in these mutants. The rest of the respiratory chain was unaffected in this mutant, as addition of certain Q analogues restored cytochrome-*c* reductase activity to that of the wild type if grown to late log-phase, and hence completely derepressed.

Preparation of mitochondria and submitochondrial particles. The cells were broken with glass beads (0.45–0.50 mm diameter) in a Dynomill, and mitochondria were prepared as described by Brown and Beattie [17] in a medium containing 0.65 M sorbitol/0.1 mM EDTA/10 mM Tris-HCl (pH 6.5). The mitochondria were resuspended in the same medium to a concentration of 2–3 mg/ml and stored at −20 °C prior to use. Submitochondrial particles were prepared in 0.1 M potassium phosphate buffer (pH 7.5)/0.1 mM EDTA according to Clejan et al. [18].

Unless otherwise stated, all enzymatic reactions were performed in 50 mM Tris-HCl buffer (pH 7.4)/250 mM sucrose/1 mM EDTA using an Aminco-DW2C double-beam, dual-wavelength spectrophotometer thermostated at 25 °C. The reduction of DQ and DB was monitored using the wavelength pair 272–290 nm. Cytochrome b reduction in the presence of antimycin or myxothiazol was monitored with the wavelength pair 431–443 nm for greater sensitivity. Under the latter conditions, the c-type and a-type cytochromes remain oxidized, and therefore do not interfere with the measurement of cytochrome b reduction. Under the conditions, the contribution due to flavin reduction was negligible. The rates of cytochrome b reduction are presented as $T_{1/2}$, which is the time required to reduce 50% of the

reducible cytochrome *b* under saturating concentrations of substrate. Semi-logarithmic plots of the data were used for these calculations.

Antimycin and DQ were purchased from Sigma, myxothiazol from Boehringer Mannheim, and DQH_2 from Aldrich. Q_2 (ubiquinone-2) was a generous gift from Hoffman LaRoche. All other chemicals were of the highest purity available. DB was prepared according to Margolis [19].

Results

The reduction of duroquinone by succinate in Q-deficient mutant mitochondria stimulated by DB

Previous studies have shown that the continuous oxidation of DQH_2 requires endogenous ubiquinone or the additon of ubiquinone homologues or analogues [13,14,20]. In order to investigate further the effect of changes in the ring structure and side-chain of ubiquinol on its interaction with the b-c_1 complex, the reduction of DQ by succinate was studied in Q-deficient mitochondria. In these mitochondria, the reduction of DQ by succinate occurred very slowly unless Q_2 or DB was added. The reduction of DQ 'catalyzed' by DB was inhibited approx. 50% by antimycin, was insensitive to myxothiazol, and was inhibited 70–80% by antimycin plus myxothiazol, as was previously reported in pig heart mitochondria [10]. The antimycin- and myxothiazol-insensitive activity increased with increasing concentrations of DB, suggesting that DB also reacts directly with succinate dehydrogenase to catalyze DQ reduction. Under these conditions, DB was reduced before DQ as indicated in Fig. 1A by the two straight lines representing the rate of reduction recorded at 272–290 nm. The initial rate is faster than the subsequent rate and the extent of reduction in the first phase is equivalent to the absorbance change observed during the reduction of DB alone (not shown). The traces in the second phase are straight for most of the reaction and the extent of reduction reached in this phase is proportional to the amount of DQ added (data not shown).

Since antimycin and myxothiazol are inhibitors of the b-c_1 complex with extremely high affinity, their binding to the respective center, in fact, eliminates that center from further enzymatic activity. If the antimycin- and myxothiazol-sensitive reaction can be attributed to the catalytic DB bound to the corresponding centers, then the apparent K_m and the V_{max} can be determined separately under the following conditions: (1) DB alone (which is not affected by the inhibitors of the b-c_1 complex); (2) the DB catalyzed reduction of DQ by succinate dehydrogenase (in the presence of antimycin and myxothiazol); and (3) the reduction of DQ occurring through the antimycin- and myxothiazol-sensitive sites (i.e., center i and center o of the b-c_1 complex). These reactions all follow Michaelis-Menten kinetics (Fig. 1B and 1C), with the calculated values for K_m and V_{max} summarized in Table I. Identical results were obtained when these data were examined in Eadie-Hefstee plots.

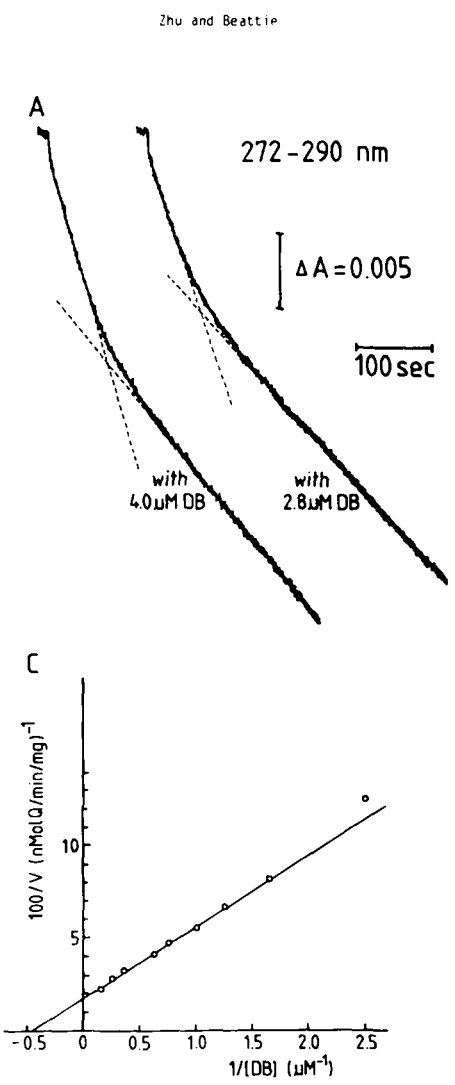
The data obtained suggest that the K_m of DB at center i, the antimycin-sensitive site, is the lowest (0.15 μM), while the K_m for DB at center o, the myxothiazol-sensitive site, is higher (0.76

TABLE I

K_m AND V_{max} FOR DB IN DB-STIMULATED REDUCTION OF DQ AND REDUCTION OF DB BY SUCCINATE

Data were taken from Fig. 1B and C.

Electron Acceptor	Conditions	K_m (μM)	V_{max} (nmol Q/min per mg)
DQ	No inhibitors	0.29	11.4
	Antimycin-sensitive portion	0.15	5.6
	Myxothiazol-sensitive portion	0.76	3.1
	Antimycin and myxothiazol insensitive portion	1.14	4.9
DB	No inhibitors	2.1	57.7



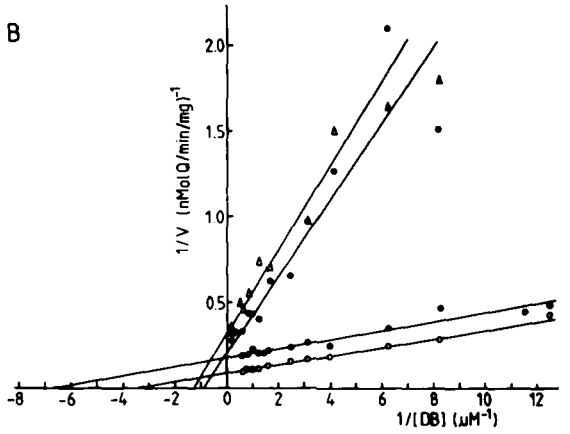


Fig. 1. Determination of K_m values for DB in DB-stimulated reduction of DQ by succinate. Submitochondrial particles from the Q-deficient mutant were suspended at a cytochrome c_1 concentration of 0.019 μM in the standard buffer. After 5 min incubation with 40 μM DQ and variable amounts of DB in the presence of 1.6 mM KCN, 2 mM succinate was added to start the reaction. (A) The reduction of DQ by succinate in the presence of 2.8 μM and 4 μM DB. (B) Determination of K_m for DB under the following conditions: ○, control, no additions; ●, the antimycin-sensitive portion; △, the myxothiazol-sensitive portion; ⊕, the antimycin- and myxothiazol-insensitive portion (since myxothiazol alone has no inhibitory effect, this K_m was calculated as the additional inhibition in the presence of antimycin). The reaction rate was taken from the second (slower) phase of the reduction traces illustrated in (A), which represents the reduction of DQ. (C) Determination of K_m for the reduction of DB itself. Data were taken from the first (faster) phase of the reduction traces illustrated in (A), which represents the reduction of DB. In calculating the specific activities, it was assumed that both DB and DQ have an absorption coefficient of 7 mM^{-1} for their reduction at the wavelength pair 272–290 nm.

μM). The K_m for DB on succinate dehydrogenase for the reduction of DQ, determined in the presence of myxothiazol and antimycin, is still greater (1.14 μM). The K_m for the reduction of DB itself by succinate dehydrogenase is the highest value measured (2.1 μM). In addition, the reduction of DB by succinate was much faster than the DB-stimulated reduction of DQ by succinate, possibly due to the higher mid-point potential of DB. In agreement with previously published results [9], antimycin inhibited 50% of the rate of DQ reduction with respect to V_{max} (Table I). It should also be noted that, since the above parameters were determined in the presence of 40 μM DQ, the actual K_m values should be even lower (see below).

On the other hand, exogenous NADH effectively reduced DQ without the addition of DB [14]. The K_m for DQ reduction by NADH was determined to be 105 μM (data not shown).

Quinone-mediated cytochrome b reduction through center o in Q-deficient mitochondria

Without endogenous ubiquinone, electron transfer from the dehydrogenases to cytochrome *b* is very slow (Fig. 2A). In the presence of antimycin, the half-time of reduction was 190 s (see Fig. 4). Since no ubiquinone is present, electron

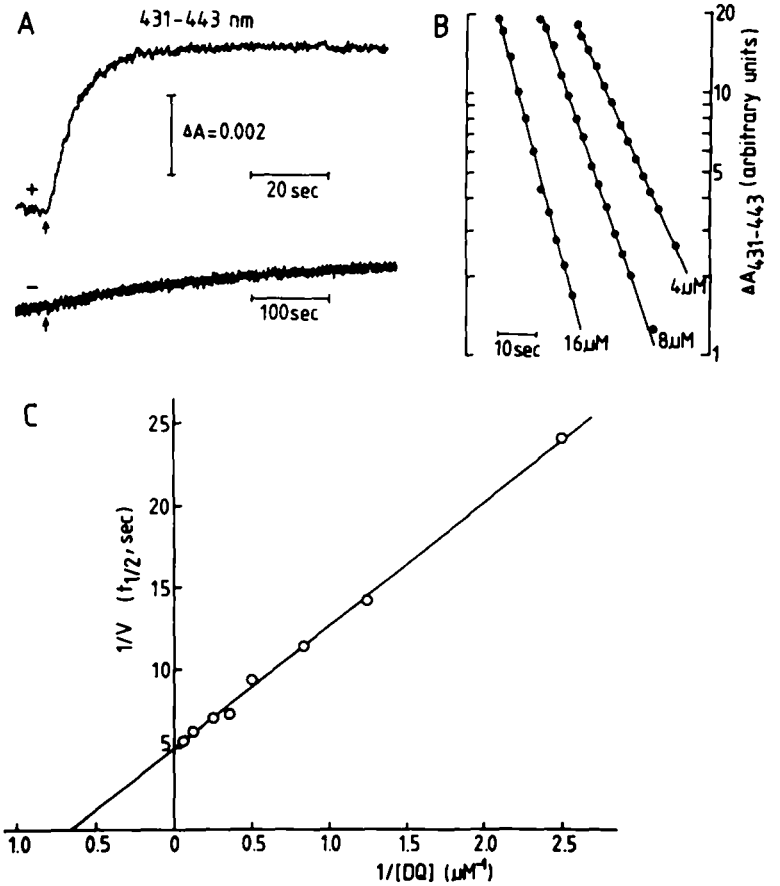


Fig. 2. DQ-mediated reduction of cytochrome *b* in the presence of antimycn. Q-deficient mitochondria (cytochrome c_1 concentration, 0.013 μM) were incubated with various concentrations of DQ for 2 min, and then 2 μM antimycin was added to induce the reduction of cytochrome *b* by endogenous substrate. No cytochrome *b* reduction was observed before the addition of antimycin. (A) Cytochrome *b* reduction in the presence of 2 μM anticymin with (+) or without (−) 8 μM DQ. (B) Semi-log plot of DQ reduction in the presence of three different concentrations of DQ, indicated on the figure. (C) Determination of K_m for DQ. The reaction rate was obtained from the slope of the straight lines illustrated in (B).

transfer must either occur via a direct interaction between the dehydrogenase and the *b*-c_1 complex [21], or perhaps via a pathway involving superoxide.

The addition of DQ greatly increased the rate of cytochrome *b* reduction by endogenous substrate in the presence of antimycin (Fig. 2A). The semi-log plot of these data indicates that these reactions follow first-order kinetics (Fig. 2B) A plot of $1/V$ against $1/(DQ)$ produced a straight line from which an apparent K_m of 1.5 μM and a $T_{1/2}$ of 4.9 s were calculated (Fig. 2C). Diffusion of DQ or antimycin into the membrane does not appear to be the rate-limiting step (longer incubation with DQ before antimycin addition does not increase the rate; when DB is used, addition of antimycin leads to a higher rate of cytochrome *b* reduction).

The DB-mediated reduction of cytochrome *b* was also studied in the Q-deficient mitochondria in the presence of succinate (Fig. 3A). The K_m, determined for DB under these conditions is 0.2 μM (Fig. 3B). In the previous section, we reported that the K_m for DB at center o mediating DQ reduction was 0.76 μM. After correction for the competition of 40 μM DQ present during these experiments, according to the formula $K_m - K_m^o$ $(1 + S_2/K_{m_2})$ where S_2 and K_{m_2} stand for the

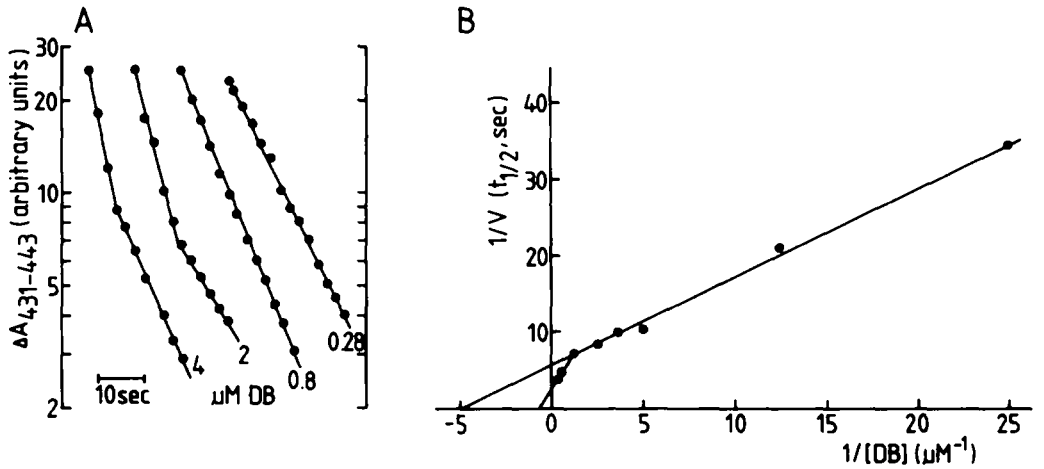


Fig. 3. DB-mediated reduction of cytochrome *b* by succinate in the presence of antimycin. Q-deficient mitochondria were suspended at a cytochrome c_1 concentration of 0.013 μM. After the addition of 2 mM succinate and various amounts of DB, antimycin (2 μM) was added to achieve the complete reduction of cytochrome *b*. In the absence of antimycin, the reduction level of cytochrome *b* increased very slowly. (A) Semi-log plot of cytochrome *b* reduction in the presence of four representative concentrations of DB. (B) Determination of K_m for DB. The reduction rate was taken from the slope of the first phase of the biphasic reaction in the semi-log illustrated in (A).

total concentration of the competing substrates and the K_m for that substrate, respectively, the same value of K_m, 0.20 μM, is obtained for DB. At low concentrations of DB, the reduction of cytochrome *b* was a single first-order reaction; however, increasing the DB concentration to 0.8 μM resulted in a biphasic reduction plus a reaction with a larger K_m and higher V_{max} (Fig 3B).

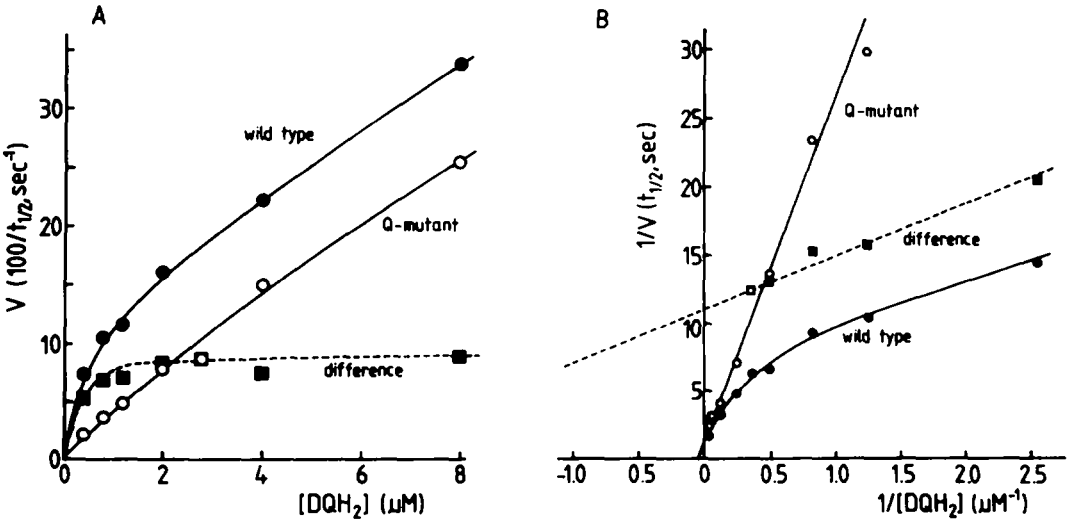


Fig. 4. Cytochrome *b* reduction by DQH_2 in the presence of antimycin. Both Q_6-containing (wild-type) and Q-deficient mitochondria were suspended at a cytochrome c_1 concentration of 0.013 μM. 20 s after the addition of 2 μM antimycin, various amounts of DQH_2 were added to reduce cytochrome *b*. (A) Rate of cytochrome *b* reduction expressed as $T_{1/2}$ as a function of DQH_2 concentration in wild type and Q-deficient mitochondria. In all cases, the reduction rate was taken from the slope of the first phase of the biphasic rate. (B) Lineweaver-Burk plot of the data in (A).

Quinone-mediated cytochrome b reduction through center i in Q-deficient mitochondria

The preceding results indicate that DQ and DB can effectively mediate the reduction of cytochrome *b* through center o; however, their ability to mediate cytochrome *b* reduction through center i is limited. In the presence of myxothiazol, no DQ-dependent cytochrome *b* reduction above the background rate was observed in the absence of exogenous Q analogues. After addition of DB or Q_2, 20–30% of the total cytochrome *b* was reduced. The rate of reduction, however, was still only 2–3% of that observed in the wild-type mitochondria. This result may reflect a very low DBH_2/DB ratio, since DBH_2 can reduce cytochrome *b* through center i as discussed later.

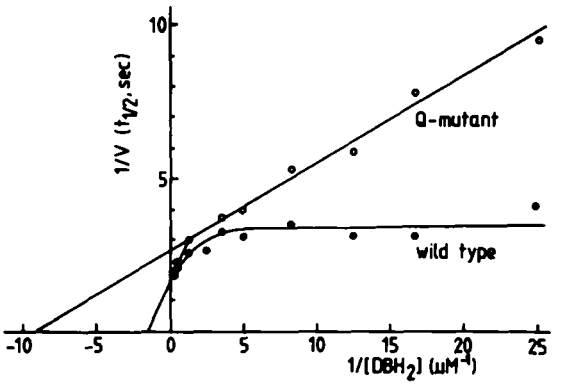


Fig. 5. Lineweaver-Burk plot of cytochrome *b* reduction by DBH_2 in the presence of antimycin. The experimental conditions were the same as in Fig. 4, except that DBH_2 replaced DQH_2.

Quinol-mediated cytochrome b reduction through center o in Q-deficient and wild-type mitochondria

When DQH_2 was added to the Q-deficient mitochondria, in the presence of antimycin, cytochrome *b* was reduced at a rate faster than that of the DQ-mediated reduction in the presence of endogenous substrates (Fig. 2A). A K_m of 19 μM for DQH_2 at center o and a $T_{1/2}$ of 1.2 s were calculated (Fig. 4).

On the other hand, the reduction of cytochrome *b* by DQH_2 in wild-type mitochondria does not follow a single-saturation curve (Fig. 4). The overall rate-concentration curves for wild type mitochondria consist of two single-saturation curves: one is identical to that observed in the Q-deficient mitochondria, while the other has a much higher affinity (0.36 μM) but a much lower rate ($T_{1/2}$ = 11 s). These results suggest that there are possibly two reaction sites for DQH_2 at center o: one is the same in both Q-deficient and wild-type mitochondria and is not affected by endogenous Q_6, with a low affinity but high reaction velocity; the other is found only in wild-type mitochondria and thus is 'created' by endogenous Q_6, and has a high affinity for DQH_2 but a high $T_{1/2}$ (low reaction rate). In both wild-type and Q-deficient mitochondria, cytochrome *b* was reduced in a single phase only when the concentration of QH_2 was low.

When DBH_2 was used as the substrate in the presence of antimycin, two values of K_m and V_{max} were detected in Q-deficient mitochondria: 0.11 μM, which is similar to the value 0.2 μM determined for DB, and 0.66 μM (Fig. 5). The $T_{1/2}$ was 2.7 s for the high-affinity site and 1.6 s for the low-affinity site. The latter value is close to the $T_{1/2}$ for the reduction of cytochrome *b* by DQH_2.

In wild-type mitochondria, however, there appears to be a site with a very high affinity for DBH_2, the K_m of which cannot be detected. The $T_{1/2}$ at this site was close to that of the high-affinity site in the Q-deficient mitochondria. At relatively high concentrations of DBH_2, the site with a K_m of 0.66 μM and $T_{1/2}$ of 1.6 sec appeared, suggesting that the presence of endogenous Q_6 further increased the affinity of the low K_m site, but had no effect on the high K_m site.

Quinol-mediated cytochrome b reduction through center i in Q-deficient and wild-type mitochondria

When DQH_2 was used to reduce cytochrome *b* in the Q-deficient mitochondria in the presence of myxothiazol, the rate of cytochrome *b* reduction increased linearly with DQH_2 concentration in a second-order reaction within the limits of detection, and no binding was detected. (Fig. 6A). The rate constant was calculated to be $1.3 \cdot 10^3$ $M^{-1} \cdot S^{-1}$ if the initial rate of cytochrome *b* reduction was taken as the reaction rate. In the wild-type mitochondria, the reaction was still largely second-order (Fig. 6B). At low concentrations of DQH_2, (less than 40 μM) some binding apparently occurred. The rate constant calculated by the same method was $1.8 \cdot 10^1$ $M^{-1} \cdot S^{-1}$, 72-times

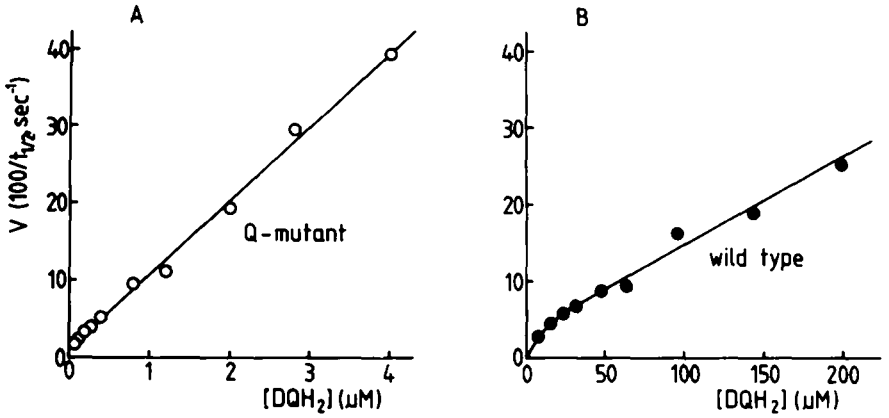


Fig. 6. Cytochrome *b* reduction by DQH_2 in the presence of myxothiazol. The experimental conditions were the same as in Fig. 4, except that 2 μM myxothiazol replaced antimycin. Reduction rate, in terms of $T_{1/2}$, was obtained from semi-log plot (not shown). (A) Q-deficient mitochondria. (B) Q_6-containing (wild-type) mitochondria.

slower than that calculated for the Q-deficient mutant mitochondria. This difference can be largely explained by the presence of a large pool of Q_6 in the wild-type mitochondria (Q/c_1 ratio of 32) which is in rapid redox equilibrium with cytochrome *b* through center i [22]. (Any electron transferred to cytochrome *b* through center o will be shared between *b* and the Q pool.) Since the apparent K_m of cytochrome *b* 562 is very close to that of the Q pool [23], these two components are reduced together on a single pool. Therefore, the apparent rate of cytochrome *b* reduction would be 64-times slower if the rate of electron transfer from DQH_2 to cytochrome *b* were the same in wild-type and Q-deficient mitochondria.

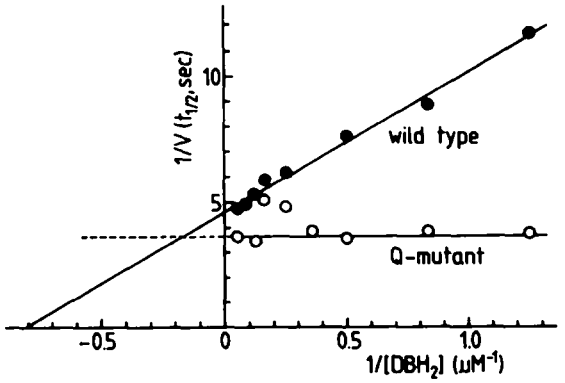


Fig. 7. Cytochrome *b* reduction by DBH_2 in the presence of myxothiazol. The experimental conditions were the same as in Fig. 6, except that DBH_2 replaced DQH_2.

The reduction of cytochrome *b* in wild type mitochondria with different concentrations of DBH_2 follows saturation kinetics, with a calculated K_m of 1.2 μM (Fig. 7). In the Q-mutant mitochondria, however, the K_m is too low to measure. At any DBH_2 concentration producing a measurable reduction of cytochrome *b*, $T_{1/2}$ (3.7 s) was constant and slightly lower than the $T_{1/2}$ in the wild type (4.6 s) (Fig. 7a). This difference in K_m indicates that Q_6 acts as a competitor of DBH_2, i.e., DBH_2 and Q_6 bind at the same reaction site.

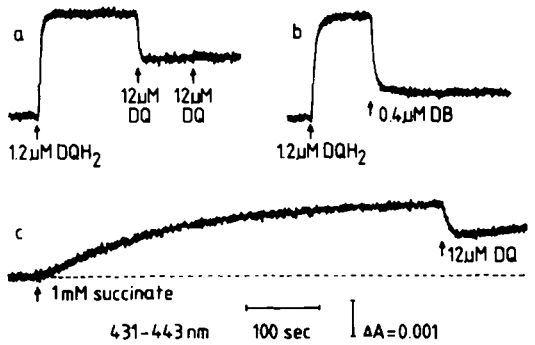


Fig. 8. The oxidation of pre-reduced cytochrome *b* by DB and DQ. Q-deficient mitochondria were suspended at a cytochrome c_1 concentration of 0.013 μM. The mitochondria were first reduced with 1.2 μM DQH_2 and then oxidized by the addition of 12 μM DQ (a) or 0.4 μM of DB (b). (c) Kinetics of cytochrome *b* reduction by succinate in the presence of 2 μM myxothiazol. DQ (12 μM) was added to oxidize the previously reduced cytochrome *b*.

The addition of DBH_2 or Q_2H_2 resulted in the rapid reduction of only about 50% of the total DQH_2-reducible cytochrome *b* in both wild-type and Q-deficient mitochondria in the presence or absence of myxothiazol (data not shown). Benzohydroquinone, however, cannot reduce any cytochrome *b*. An interesting observation relevant to this phenomenon was that DB could oxidize most of the pre-reduced cytochrome *b*, whereas DQ could rapidly oxidize only 50% of the pre-reduced cytochrome *b* (Fig. 8).

Discussion

Mitochondria isolated from the ubiquinone-deficient strain of yeast provide an excellent system to define the requirements and kinetic parameters for quinone in the mitochondrial respiratory chain. The data presented in this paper clearly show that not only the continuous oxidation of DQH_2 by the respiratory chain required ubiquinone, but also the reduction of DQ by succinate. Furthermore, the data also suggest that the requirement for ubiquinone in this reaction depends largely on the involvement of the Q-reacting sites on the cytochrome *b*-c_1 complex. For example, the substrate-dependent inhibitor sensitivity [11] of DQ reduction (antimycin strongly inhibits succinate: DQ reductase, while myxothiazol inhibits NADH : DQ reductase) suggests that the free DQH_2 formed by either NADH or succinate dehydrogenase cannot be an effective electron donor to DQ on the *b*-c_1 complex, or there would be no substrate-dependent sensitivity to the antibiotics. It would thus appear that ubiquinone bound either to succinate: oxidoreductase or to the cytochrome *b*-c_1 complex at either center i or center o acts as the major electron donor from the primary dehydrogenases to the *b*-c_1 complex.

The first possibility is that only succinate : ubiquinone oxidoreductase requires ubiquinone to effectively reduce DQ on the *b*-c_1 complex. If a dehydrogenase with a bound quinone were active, then an identical K_m for DB would have been determined with or without the specific inhibitors. Apparently, this was not the case, as different K_m values were observed. Perhaps the ubiquinone bound to center i or center o makes the difference. The concentration of DQ at these two centers might be the rate-limiting factor, resulting in a faster and more effective collision between these centers and succinate : ubiquinone oxidoreductase. Thus, the measured K_m values for DB in the presence of antimycin or myxothiazol would reflect the binding parameters of DB to these two centers. Alternatively, perhaps succinate : ubiquinone oxidoreductase alone requires ubiquinone, and perhaps the interaction between this dehydrogenase with center o or center i has changed the conformation of the Q reaction site, resulting in changes in the apparent K_m values in the presence of the inhibitors.

A second major possibility is that only center o or i require ubiquinone, and that succinate dehydrogenase itself does not require ubiquinone for the reduction of the DQ bound to the *b*-c_1 complex. Under these circumstances, the measured K_m for DB on these two centers would represent the binding properties of DB to these sites. The fact that NADH : ubiquinone oxidoreductase can reduce DQ on the *b*-c_1 complex without ubiquinone, however, makes this suggestion appear less likely.

The third possibility is that the interaction between succinate : ubiquinone oxidoreductase with center o or i requires only one ubiquinone molecule to transfer electrons to DQ. It is irrelevant whether this ubiquinone molecule was originally bound to the Q reductase or to one of the sites on the *b*-c_1 complex. In any event, the measured K_m values would reflect the binding of DB to these two centers but with the added involvement of succinate : ubiquinone oxidoreductase. It should be noted that all three possibilities discussed above for interpreting the role of ubiquinone in succinate : DQ reductase involve the determination of a K_m for DB at the two quinone binding sites on the cytochrome *b*-c_1 complex.

The results of the current study also clearly show that DQH_2, like DBH_2, can be oxidized directly at center o. The reduction of cytochrome *b* through center o by DQH_2 in the presence of antimycin no longer required a direct interaction between the dehydrogenases and the *b*-c_1 complex, as indicated by the higher K_m for the DQH_2 reduction of cytochrome *b* (19 μM as compared to the K_m of 1.5 μM for DQ). In addition, two different apparent binding sites for ubiquinol were

observed. One site with a K_m of 19 μM for DQH_2 and 0.66 μM for DBH_2 was observed in mitochondria obtained from coenzyme Q-deficient and wild-type yeast. A second K_m for DBH_2 with a value of 0.11 μM was also observed in the Q-deficient mitochondria, but this site in the wild-type mitochondria was apparently decreased to a value too low to be measured. A second K_m, however, for DQH_2 was observed in wild-type mitochondria. These results suggest that only one K_m (or apparent binding site) at center o is affected by the endogenous Q_6 present in the b-c_1 complex. Thus, exogenous ubiquinol analogues can interact at center o independently of the endogenous Q_6 with a high reaction rate and a high K_m at one site, and may also interact with the endogenous ubiquinone with a low reaction rate and a low K_m through the other site. Since this endogenous Q_6 mediates electron flow from DQH_2 to cytochrome b, it must interact directly with cytochrome b at center o (since antimycin is present).

Several groups have suggested that the cytochrome b-c_1 complex functions as a dimer [23]. Hence, the two observed K_m values may represent the ubiquinone-reacting site on each monomer. Nalecz and Azzi [24] have reported the biphasic dependence on QH_2 concentration of ubiquinol–cytochrome-c reductase activity in the dimer of the b-c_1 complex but a linear dependence of QH_2 in the monomer. Perhaps the formation of a dimer results in a change in the conformation of each monomer leading to a change in the ubiquinol binding site. The existence of two different reaction sites for DQH_2 at center o also agrees with the double-cycle model of De Vries et al. [25] and their observation that in the presence of antimycin only half of the cytochrome c_1 and iron sulfur clusters are rapidly reduced by DQH_2 [26].

On the other hand, no such multiplicity of reaction sites for QH_2 was observed at center i. This result could mean that at center i, there is either one single or two identical ubiquinone-reacting sites.

The results of the current study also indicate that DQ itself cannot be effectively reduced by the cytochrome b-c_1 complex, as exogenous DQ was able to oxidize only 50% of pre-reduced cytochrome b. Further, this observation provides an explanation for previous observations of DQH_2 that could not be continually oxidized by the cytochrome b-c_1 complex in the absence of ubiquinone. Under these circumstances, DQH_2 can be oxidized at both center o and center i during the first turnover in the absence of ubiquinone, and DQ itself can oxidize some cytochrome b. According to both the double-cycle model of DeVries et al. [25] or the single-cycle model of Crofts and Meinhardt [26], cytochrome b acts as a two-electron gate at center o. Reducing equivalents can leave center i only when two electrons are passed from cytochrome b to ubiquinone to form ubiquinol. DQ, either due to its lower mid-point potential or due to its inability to form a stable semiquinone at center i, or both, cannot operate at this center. Thus, DQ cannot sustain the continuous turnover of the cytochrome b-c_1 complex necessary for the Q cycle.

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