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# BYPASSES OF THE ANTIMYCIN A BLOCK OF MITOCHONDRIAL ELECTRON TRANSPORT IN RELATION TO UBISEMIQUINONE FUNCTION *

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**Two different bypasses around the antimycin block of electron transport from succinate to cytochrome *c* via the ubiquinol-cytochrome *c* oxidoreductase of intact rat liver mitochondria were analyzed, one promoted by *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and the other by 2,6-dichlorophenolindophenol (DCIP). Both bypasses are inhibited by myxothiazol, which blocks electron flow from ubiquinol to the Rieske iron-sulfur center, and by 2-hydroxy-3-undecyl-1,4-naphthoquinone, which inhibits electron flow from the iron-sulfur center to cytochrome $c_1$. In the bypass promoted by TMPD its oxidized form (Wurster's blue) acts as an electron acceptor from some reduced component prior to the antimycin block, which by exclusion of other possibilities is ubisemiquinone. In the DCIP bypass its reduced form acts as an electron donor, by reducing ubisemiquinone to ubiquinol; reduced DCIP is regenerated again at the expense of either succinate or ascorbate. The observations described are consistent with and support current models of the Q cycle. Bypasses promoted by artificial electron carriers provide an independent approach to analysis of electron flow through ubiquinol-cytochrome *c* oxidoreductase.**

## Introduction

A large body of evidence supports the view that the sequence of electron-transfer reactions in the mitochondrial ubiquinol-cytochrome-*c* oxidoreductase system (complex III), as well as the analogous system of photosynthetic bacteria, is nonlinear and that oxidation of ubiquinol proceeds by two consecutive one-electron transfers to different acceptors, with the intermediate formation of ubisemiquinone ($QH^{\cdot}$). Much evidence suggests that in mitochondria the first electron from ubiquinol ($QH_2$) passes to the Rieske FeS center and thence to cytochrome $c_1$; the second electron passes from ubisemiquinone to cytochrome *b*. The reduced cytochrome *b* is then reoxidized by ubiquinone. This sequence of electron-transfer reactions is the basis of the Q cycle [1], which can also account for $H^+$ translocation in this segment of the mitochondrial respiratory chain. The cycle explains and is supported by such observations as the oxidant-induced reduction of cytochrome *b*

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Abbreviations: BAL, British antilewisite; DCCD, *N,n'*-dicyclohexylcarbodiimide; DCIP, 2,6-dichlorophenolindophenol; $DCIPH_2$, reduced 2,6-dichlorophenolindophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; UHNQ, 2-hydroxy-3-undecyl-1,4-naphthoquinone; $WB^+$, Wurster's blue; cyt *b*, cytochrome *b*.

[2,3], the effect of depletion of the FeS center [4–6], the action of BAL [7], as well as the effects of the newly described inhibitors myxothiazol [8–13], UHDBT and UHNQ [14–17]. Although different variants of the Q cycle have arisen from these experimental observations [6,18,19], there is little doubt that ubisemiquinone plays a key role in electron flow through this segment of the electron-transport chain.

In this paper we describe further analysis of the sequence of electron-transfer reactions in complex III with the use of two different artificial electron carriers to create bypasses around the antimycin A block of electron flow, an approach introduced in the important early study of Wikström and Berden [2]. The first such bypass analyzed here is that promoted by the redox couple tetramethylphenylenediamine/Wurster's blue (TMPD/$WB^+$). This was first described by Ernster and colleagues [20,21], but the precise pathway by which it directs electron flow around the site of antimycin inhibition has not been elucidated. We have recently employed the TMPD bypass to show [22] that the translocation of 4 $H^+$ coupled to transport of $2e^-$ through energy-conserving site 2 occurs in two steps, 2 $H^+$ being translocated prior to the antimycin block, probably via the oxidation of ubiquinol (site 2A), and the other 2 $H^+$ translocated in a subsequent antimycin-inhibited electron transfer reaction (site 2B). This conclusion is supported by more recent observations that DCCD prevents translocation of 2 of the 4 $H^+$ in site 2 [23–25], presumably those translocated in site 2B.

In this paper we show that the antimycin block of electron flow in rat-liver mitochondria can also be bypassed by 2,6-dichlorophenolindophenol (DCIP), but in this case the bypass proceeds by a different pathway than that promoted by TMPD. Evidence is given that in the bypass promoted by TMPD its oxidized form, Wurster's blue ($WB^+$), acts as the electron acceptor from some component of the $QH_2$-cytochrome-*c* oxidoreductase prior to the point of action of antimycin A, whereas in the case of the DCIP bypass, its reduced form $DCIPH_2$ acts as an electron donor by reducing some component, presumably ubisemiquinone, whose reduction is necessary to complete the electron transfer cycle in the antimycin-inhibited $QH_2$-cytochrome-*c* oxidoreductase. Moreover, both bypasses are inhibited by myxothiazol and by ubiquinone analogs [8–17]. The observations described here, which are consistent with most earlier evidence on the role of ubisemiquinone. provide an independent and relatively simple approach to examination of the pathway of electron flow in site 2, which is capable of further exploitation with other artificial mediators.

## Experimental details

Rat liver mitochondria were prepared from 0.25 M sucrose homogenates and washed three times in 0.25 M sucrose. $O_2$ uptake rates were determined with a calibrated Clark electrode. Optical changes were monitored in an Aminco DW-2 spectrophotometer.

UHNQ (2-hydroxy-3-undecyl-1,4-naphthoquinone) was obtained from Aldrich Chemical Co., Milwaukee, WI. Myxothiazol was a gift from Drs. Reichenbach, Tierbach and Trowitzsch.

## Results

### *Bypass of the antimycin block by reduced dichlorophenolindophenol*

The following experiments show that reduced DCIP makes possible electron flow around the antimycin block of complex III. Fig. 1 shows, in accordance with Ref. 2, that reduced DCIP is not readily oxidized via mitochondrial cytochrome *c* and cytochrome oxidase in the presence of antimycin A, rotenone and FCCP, as indicated by the low rate of $O_2$ uptake observed upon supplementing rat-liver mitochondria with DCIP and its nonenzymatic reductant ascorbate. However, when succinate is also added to the system a large increase in the rate of $O_2$ uptake occurs, which is inhibited by cyanide. The increased rate of $O_2$ uptake is due to oxidation of succinate, since it is inhibited by 2-thenoyltrifluoroacetone, which specifically blocks electron flow from succinate to ubiquinone [26]. In similar experiments in which succinate was replaced with NAD-linked substrates, DCIP + ascorbate again were capable of bypassing the antimycin block (data not shown). As expected, rotenone inhibited oxidation of NAD-linked substrates, but 2-thenoyltrifluoroacetone did not. In other experiments (not shown) it

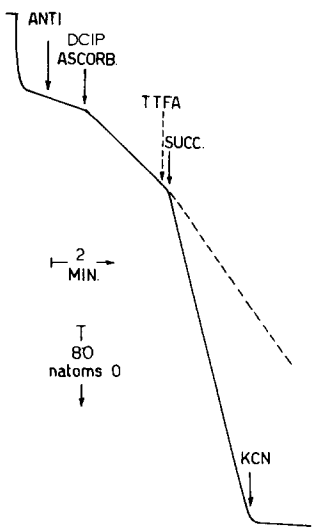


Fig. 1. By-pass of the antimycin block by reduced DCIP. Electron transfer from succinate (SUCC.) to $O_2$. Rat-liver mitochondria (8.0 mg) were incubated in 250 mM sucrose, 10 mM potassium Hepes (pH 7.15), 1.0 μM FCCP and 4.0 μM rotenone. Where indicated antimycin A (0.30 nmol/mg), 3.0 mM ascorbate (ASCORB.), 25 μM DCIP, 3.0 mM succinate, 50 μM 2-thenoyltrifluoroacetone (TTFA), and 2.0 mM KCN were added. Volume, 1.75 ml; temperature, 25 °C.

was found that electron transfer from succinate to mitochondrial cytochrome *c*, monitored spectrophotometrically in the presence of cyanide, proceeds at a high rate in the presence of succinate, antimycin A and DCPIP + ascorbate, but when DCPIP + ascorbate or succinate are absent, cytochrome *c* is only slowly reduced in the presence of antimycin.

These experiments therefore show that the presence of reduced DCIP can effectively bypass the antimycin block between succinate and cytochrome *c*. Since in these experiments DCIP was always over 98% reduced because of the presence of ascorbate (and succinate), it appears that the reduced form of DCIP is required for electrons to pass around the antimycin block of the pathway from $QH_2$ to cytochrome *c*. This was supported by control experiments in which low concentrations of the oxidized form of DCIP in the absence of ascorbate were found to yield very low initial rates of $O_2$ uptake.

*Inhibition of the DCIP bypass by myxothiazol and ubiquinone analogs*

The bypass of the antimycin block promoted by reduced DCIP is sensitive to other inhibitors of electron transport through complex III, in particular, myxothiazol [8–13] and the ubiquinone analog UHNQ [14–17]. These inhibitors almost completely abolished the extra $O_2$ uptake due to succinate oxidation in the presence of reduced DCIP and antimycin A (Fig. 2A). Both myxothiazol and UHNQ also inhibited slightly the slow $O_2$ uptake in the presence of DCIP and ascorbate alone, before addition of succinate, which probably arises from the oxidation of residual endogenous substrated mediated by DCIP. Succinate oxidation via the DCIP bypass of the antimycin block proceeds with a $V_{max}$ of about 120 nmol $O \cdot min^{-1} \cdot mg^{-1}$ (Fig. 2B), comparable to the rate of succinate oxidation in the absence of antimycin A and other mediators and inhibitors. The double-reciprocal plot in Fig. 2B also yields $K_m$ for reduced DCIP, about 250 μM. The points plotted in Fig. 2B represent the difference between the rates of $O_2$ uptake in the absence and presence of myxothiazol at each concentration of $DCIPH_2$. The non-enzymatic reductant ascorbate is not an absolute requirement in experiments such as those in Figs. 1 and 2, since succinate alone can reduce DCIP in a myxothiazol-insensitive reaction.

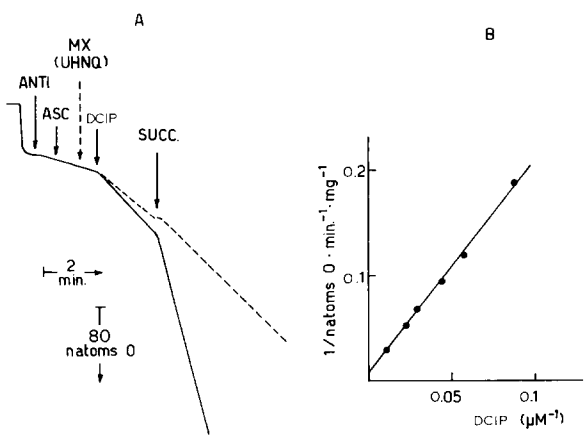


Fig. 2. Inhibition of the DCIP-promoted bypass of the antimycin block. (A) Inhibition by myxothiazol and UHNQ. The incubation system was as in Fig. 1. Where indicated myxothiazol (MX) (0.10 nmol/mg) and UHNQ (7.0 μM) were added. (B) Kinetics of the DCIP-promoted bypass. The system was as in Fig. 1A, with 6.3 mg of mitochondrial protein. the points represent the difference between the rates of $O_2$ consumption in the absence and presence of myxothiazol at each concentration of reduced DCIP, ASC, ascorbate, SUCC., succinate.

Fig. 3A shows the effect of myxothiazol, an inhibitor of electron transfer fro ubiquinol to the FeS center of complex III [12,13], on normal succinate oxidation and on the electron transfer mediated by the DCIP bypass of the antimycin block. Maximal inhibition of both oxidative reactions was observed at the same myxothiazol concentration. The shape of the two curves is, however, significantly different. The inhibition of the DCIP bypass was linear with the concentration of myxothiazol, while the inhibition of unmediated succinate oxidation was strongly sigmoidal; in the latter case little inhibition occurred at low myxothiazol but increased sharply at the highest concentrations (Fig. 3A). The sigmoidicity of the myxothiazol titration of succinate oxidation in the absence of antimycin A may be a consequence of the relatively large pool of mitochondrial

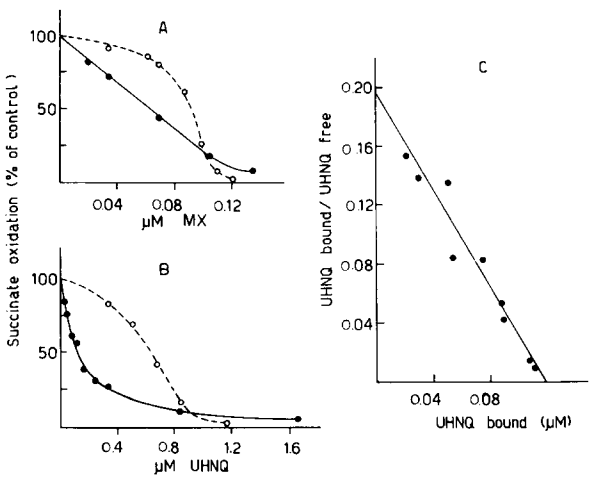


Fig. 3. Inhibitor titration of normal succinate oxidation and of succinate oxidation via the DCIP-promoted bypass. (A) Titration with myxothiazol. The incubation system was as in Fig. 1. ○——○, Myxothiazol titration of succinate oxidation, with antimycin A, ascorbate, and DCIP omitted. The maximum or 100% velocity was 150 natoms $O \cdot min^{-1} \cdot mg^{-1}$. ●——●, Myxothiazol titration of DCIP by-pass in the presence of antimycin A (0.30 nmol/mg), 3.0 mM ascorbate and 25 μM DCIP. 100% velocity was 20 natoms $O \cdot min^{-1} \cdot mg^{-1}$. DCIP, ascorbate and succinate were added 4 min after myxothiazol. The blank rate at saturating myxothiazol in the absence of succinate was subtracted from all the points. (B) Titration with UHNQ. ○——○, antimycin, ascorbate and DCIP omitted; ●——● antimycin, ascorbate and DCIP present. DCIP, ascorbate and succinate were added 4 min after UHNQ. The blank rate at saturating UHNQ in the absence of succinate was subtracted from all the points. (C) Scatchard plot for the binding of UHNQ. See text for details.

ubiquinone and the probability that the succinate-ubiquinone reductase step is the rate-limiting reaction in uninhibited complex III. Under these conditions the overall rate of succinate oxidation will be largely insensitive to the inhibition of a relatively large fraction of the numerous catalytic units of complex III, the decrease in the number of active catalytic units being compensated for by the increasing rate of ubiquinol oxidation via the unaffected systems. In this respect the action of myxothiazol resembles that of antimycin A [18]. On the other hand, the linear myxothiazol-sensitivity of the DCIP-mediated bypass in the presence of antimycin indicates that some other reaction is rate-limiting, i.e., the interaction between the mediator and the antimycin-inhibited complex III, so that the overall rate of electron transfer becomes proportional to the number of uninhibited catalytic units. Furthermore, the linearity of the myxothiazol titration of the DCIP bypass (Fig. 3A) also indicates that the affinity of complex III for myxothiazol is very high [cf. 9], so that at any concentration of myxothiazol in the experiment of Fig. 3A it is virtually completely bound. The results of Fig. 3A also show, in accordance with Refs. 10 and 12, that antimycin does not interfere with the binding of myxothiazol.

Fig. 3B shows the results of a similar experiment performed with UHNQ instead of myxothiazol. UHNQ is a less potent inhibitor than myxothiazol and maximal inhibition of both normal and DCIP-mediated oxidation of succinate is observed at comparatively much higher concentrations. Here again, the titration curves are different. The UHNQ titration of succinate oxidation in the absence of antimycin A is sigmoidal, similar to the myxothiazol titration in Fig. 3A. However, the titration of the DCIP bypassed system shows the highest increments of inhibition at low UHNQ concentrations, which become progressively less at higher levels of UHNQ. If it is assumed that at any given percent inhibition of the DCIP bypass equal amounts of UHNQ or myxothiazol are bound to the inhibited sites in complex III (i.e., if the same fraction of the FeS-$c_1$ complexes are inhibited) and that the affinity of myxothiazol for its binding site is so high that it is essentially completely bound, it is possible to construct a

Scatchard plot for UHNQ binding. This is shown in Fig. 3C, in which the points of Fig. 3B have been replotted after calculating bound UHNQ, assuming that all myxothiazol is bound, as in Fig. 3A. The linearity of the resulting Scatchard plot (Fig. 3C) strongly indicates that UHNQ binds to a single site in the $b$-$c_1$ complex, with a $K_D$ of 0.6 $\mu$M.

Taken together, the results of Figs. 1–3 show that in the presence of antimycin A some intermediate accumulates in complex III whose reduction by DCIP makes possible the transfer of electrons from $QH_2$ to the FeS center and thence to cytochromes $c_1$ and $c$. This is shown by the inhibition of the DCIP-activated electron flow by myxothiazol, which prevents electron transfer to the FeS center, and by UHNQ, which inhibits transfer of electrons from the FeS center to cytochrome $c_1$ [14–7]. Interestingly, no significant DCIP-promoted $O_2$ uptake was observed in experiments in which myxothiazol (or UHNQ) was the sole inhibitor, in the absence of antimycin.

*Inhibition of the TMPD bypass of the antimycin block*

The TMPD/Wurster's blue couple has long been known to provide a bypass of the antimycin A block of electron flow through site 2 [20,21]. In an earlier communication [22] we showed that the TMPD bypass is 2-thenoyltrifluoroacetone-sensitive when succinate is the substrate. We report here that the TMPD bypass of the antimycin block, with succinate as electron donor, is also highly sensitive to myxothiazol (over 90%) and to a lesser extent (about 70%) to UHNQ (Fig. 4). The low rate of $O_2$ uptake insensitive to myxothiazol and UHNQ observed before addition of succinate is probably due to non-specific interaction of Wurster's blue with mitochondrial NADH. Unlike the case of DCIP, which initiates a bypass by acting as a reductant of some oxidized component of complex III, as shown above, the TMPD bypass is initiated by transfer of electrons from some reduced component of complex III to Wurster's blue ($WB^+$), which thus is acting as an electron acceptor. This is shown in the experiment in Fig. 5, in which electron transfer to and from Wurster's blue was monitored optically at 650–690 nm. TMPD was first oxidized to $WB^+$ by transfer of electrons

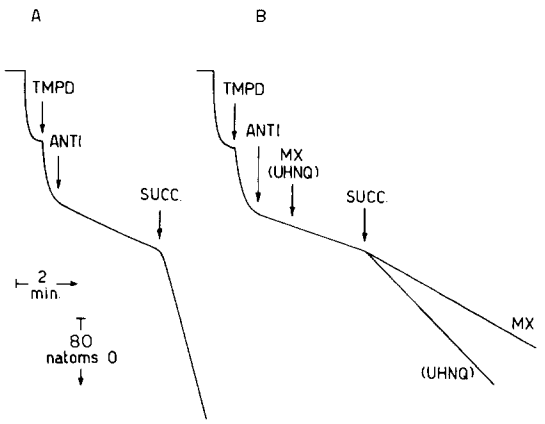


Fig. 4. The TMPD-promoted bypass of the antimycin block and its sensitivity to myxothiazol and UHNQ. Rat-liver mitochondria (8.0 mg) were incubated in a medium of 120 mM sucrose, 60 mM KCl, 20 mM potassium HEPES (pH 7.15), 1.0 $\mu$M FCCP and 4.0 $\mu$M rotenone. Where indicated TMPD (57 $\mu$M), antimycin A (0.30 nmol/mg), 3.0 mM succinate (SUCC.), myxothiazol (MX) (0.10 nmol/mg) and UHNQ (7.0 $\mu$M) were added. Volume, 1.75 ml; temperature, 25 °C. ASC, ascorbate.

to cytochrome $c$ and cytochrome oxidase of mitochondria, a process that does not involve any component of complex III, since it is insensitive to antimycin A and myxothiazol. Cytochrome oxidase was then inhibited with KCN and the reduction of $WB^+$ was initiated by addition of succinate in the presence of antimycin (trace A), of antimycin +

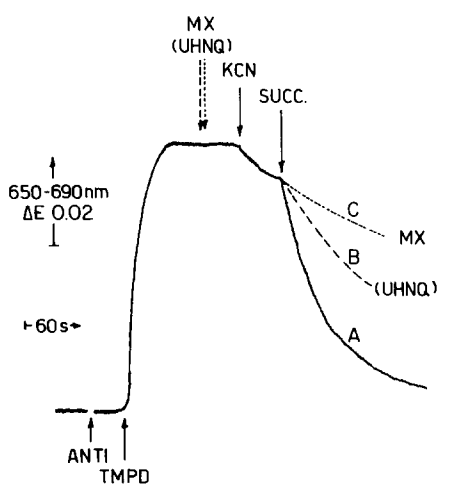


Fig. 5. The TMPD-promoted bypass of the antimycin block. Experimental conditions as in Fig. 4. TMPD was 40 $\mu$M, UHNQ 5.0 $\mu$M and KCN 2.0 mM. The volume was 2.5 ml. SUCC., succinate; MX, myxothiazol.

myxothiazol (trace C), and of antimycin + UHNQ (trace B). $WB^+$ was quickly reduced by succinate in the presence of antimycin A. However, myxothiazol almost completely prevented the reduction of $WB^+$ by succinate (trace C); UHNQ also inhibited reduction of $WB^+$, although less completely (trace B), thus providing a close parallel to the pattern of Fig 4, in which the electrons from complex III were allowed to reduce oxygen. These observations show that these inhibitors prevent the reduction of $WB^+$ and thus it may be concluded that $WB^+$ functions as a specific electron acceptor from some reduced intermediate site(s) within the antimycin-inhibited complex III. Myxothiazol and UHNQ titrations of succinate oxidation and of the TMPD-mediated succinate oxidation in the presence of antimycin have also been carried out (not shown). As was observed with the DCIP bypass (Fig. 3A), inhibition of the TMPD bypass by myxothiazol was found to be linear. Similarly, inhibition of the TMPD bypass by UHNQ resembled the titration of the DCIP bypass (Fig. 3B) and was limited to about 70% total inhibition. As in the case of the DCIP bypass, no significant $WB^+$-promoted $O_2$ uptake was observed in experiments in which myxothiazol or UHNQ was the sole inhibitor, in the absence of antimycin.

Addition of succinate to antimycin-blocked mitochondria oxidizing TMPD (+ascorbate) via cytochrome *c* and cytochrome oxidase does not give extra $O_2$ uptake (not shown). This observation indicates that the reduced species TMPD per se is incapable of providing significant bypass of the antimycin block. Thus the oxidant species $WB^+$ is requipred as an electron acceptor in the TMPD bypass, whereas reduced $DCIPH_2$ is required as an electron donor in the DCIP bypass.

*Effect of DCIP and TMPD on the reduction of cytochrome b*

When antimycin is added to FCCP-uncoupled, rotenone-inhibited mitochondria, cytochrome *b* is partially reduced by residual endogenous substrates, giving about 80% of the maximal reduction afforded by succinate + antimycin. As already noted [2], very low concentrations of reduced DCIP promote a fast and complete oxidation of cytochrome *b*. This is readily reversed by succinate

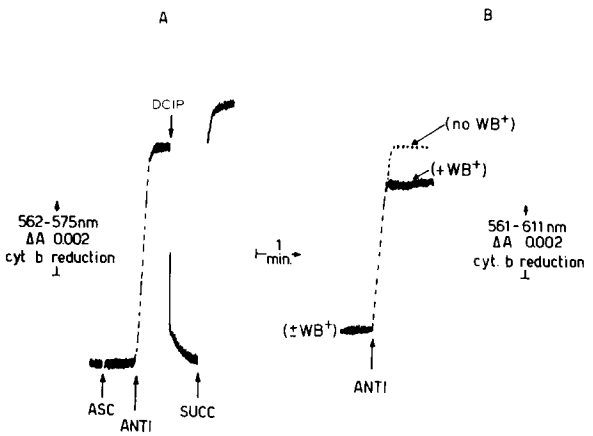


Fig. 6. Redox transitions of cytochrome *b* in the presence of DCIP + ascorbate (trace A) and TMPD (trance B). The incubation medium was as in Fig. 5. Trace (A): where indicated, 6 μM DCIP prereduced with traces of ascorbate was added. Trace (B): where indicated TMPD (100 nmol) was present from the beginning of the experiment; ferricyanide (150 nmol) was added after 50 s of incubation. SUCC., succinate.

(Fig 6A). Similarly, reduced DCIP added prior to antimycin completely abolishes the antimycin-promoted reduction of cytochrome *b* in the absence of added substrate.

At an appropriate wavelength pair (561–611 nm), and provided the redox changes of $WB^+$ are minimized during the experiment, it is possible to monitor the redox state of cytochrome *b* in the presence of $WB^+$. Fig. 6B reports such an experiment, where rotenone + FCCP-treated mitochondria are first allowed to oxidize TMPD to $WB^+$; a slight excess of ferricyanide is then added to maintain $WB^+$ in the fully oxidized state and antimycin A is added last. Under these conditions a significant reduction of cytochrome b took place, which was, however, 20–30% less than observed in the control experiment without $WB^+$. Although it is not clear why the $WB^+$-induced oxidation of cytochrome *b* is less complete than the oxidation induced by reduced DCIP (Fig. 6B), it can be concluded that both the reductant (reduced DCIP) and the oxidant ($WB^+$) affect cytochrome *b* in a similar way, at least qualitatively.

The effect of myxothiazol and UHNQ on the oxidation of cytochrome *b* induced by reduced DCIP is shown in Fig. 7. Fig. 7A shows the results of an experiment similar to that of Fig. 6A, but where myxothiazol was added prior to DCIP.

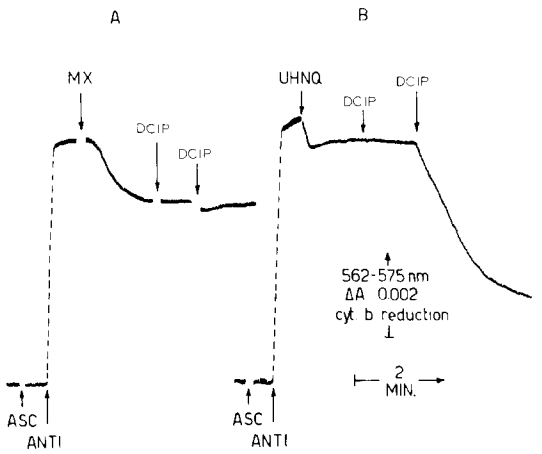


Fig. 7. Effect of myxothiazol (MX) (trace A) and UHNQ (trace B) on the DCIP-induced redox transitions of cytochrome *b*. Experimental conditions as in Fig. 6. DCIP was 5.0 μM, myxothiazol 0.10 nmol per mg, UHNQ 7.0 μM. Temperature, 16 °C. Asc, ascorbate.

Myxothiazol induced some oxidation of cytochrome *b*, but the new steady state so produced is unaffected by further additions of DCIP. The myxothiazol inhibition of DCIP-dependent oxidation of cytochrome *b* is also observed at high DCIP concentrations. In contrast, the effect of UHNQ on the oxidation of cytochrome *b* promoted by reduced DCIP is variable. UHNQ behaves similarly to myxothiazol if the concentration of DCIP is kept relatively low and the temperature does not exceed 12–14 °C (Fig. 7B); the inhibitory effect disappears at higher DCIP and higher temperatures. As described above (Fig. 6B), $WB^+$ inhibition of the antimycin-promoted reduction of cytochrome *b* is incomplete. A full oxidation of cytochrome *b* is, however, induced in the conditions of Fig. 7B by further addition of myxothiazol or of UHNQ (not shown).

## Discussion

The results reported in this paper show that antimycin-inhibited electron flow from succinate to oxygen can be reactivated by an artificial electron donor (reduced DCIP) as well as by an artificial electron acceptor ($WB^+$). Both mediators interact specifically with center(s) within complex III, as indicated by the sensitivity of both mediated systems to specific inhibitors of terminal steps in the reduction of cytochrome $c_1$. The concentrations of these inhibitors required for maximum inhibition of succinate oxidation in the absence of antimycin A vs. the mediator-promoted bypasses of the antimycin block are identical, although the apparent affinities for the two inhibitors are different as developed by the experiments in Fig. 3. Most of the experiments in this paper were performed with the commercially available UHNQ, although control experiments with the analog UHDBT gave identical results. This class of inhibitors is believed to interact with the *b*-$c_1$ complex in such a way that upon binding at or close to the FeS center electron transfer from the FeS center to cytochrome *c* is blocked. Myxothiazol, on the other hand, shifts to the red the absorption peak of reduced cytochrome *b*-566, an effect also observed in the *b*-$c_1$ complex depleted of the FeS center [10]; myxothiazol thus prevents electron transfer from reduced ubiquinone to the FeS center [12,13].

The apparently puzzling finding that electron transfer to cytochrome $c_1$ in the presence of succinate + antimycin can be restored either by an oxidant or a reductant is explained by and supports Q cycle pathways of electron transfer, particularly as proposed for the $center_{out}$ portion. Such schemes propose that oxidation of $QH_2$ on the cytosolic side (i.e., $center_{out}$) proceeds by two sequential one-electron steps, in which protein-bound $QH_2$ releases its first electron to the Rieske FeS center, thus generating bound ubisemiquinone, which in turn donates the second electron to the *b* cytochromes. It is also widely accepted that reoxidation of the *b* cytochromes, which is antimycin-sensitive, involves electron transfer to ubiquinone. This sequence of reactions explains the phenomenon of oxidant-induced reduction of the *b* cytochromes as observed in the presence of antimycin, and is supported by the recent discovery of an EPR signal attributable to ubisemiquinone, which accumulates in the presence of antimycin + oxygen [27].

In accordance with this pathway, it is proposed that reduced DCIP can activate electron transfer from succinate to cytochrome *c* in the presence of antimycin by reducing the only reducible species present under these conditions, i.e., ubise-

miquinone at $center_{out}$, which is thus reduced to $QH_2$, as is shown in Fig. 8A. One electron passes from $QH_2$ to the FeS center and thence to cytochrome $c_1$, thus forming ubisemiquinone, which can be reduced to $QH_2$ again by reduced DCIP. Consecutive one-electron transfers from $QH_2$ to FeS would result in continuous oxidation of reduced DCIP via the $QH_2/Q^-$ couple, thus providing a bypass around the antimycin block. Although reduced DCIP is consumed in this series of reactions it is constantly regenerated through non-enzymatic reduction by ascorbate or enzymatic reduction by succinate. These conclusions are supported by the finding that inhibition of electron transfer to the FeS center with myxothiazol or from the FeS center to cytochrome $c_1$ with UHNQ (or UHDBT) blocks the antimycin-insensitive electron transfer promoted by reduced DCIP. Also consistent with this explanation are the observations that low concentrations of reduced DCIP completely oxidize the reduced cytochrome *b* formed in the presence of antimycin A and absence of added substrate (see also Ref. 2) and that the DCIP-induced oxidation of cytochrome *b* is readily reversed upon addition of succinate (Fig. 6A). In the absence of added substrate, i.e., in conditions where the mitochondrial Q is largely in its oxidized form, antimycin can still cause accumulation of the semiquinone and extensive reduction of the *b* cytochromes, because of the relatively high redox potential of the $QH_2/Q^-$ couple and the relatively low potential of the $Q^-/Q$ couple. Under these circumstances, reduced DCIP reduces bound ubisemiquinone at $center_{out}$ to yield bound $QH_2$. The latter may possibly exchange with a molecule of free oxidized Q, which can then, in bound form, accept electrons from cytochrome *b*, i.e., in the opposite direction of normal electron flow. However, when succinate is present, ubisemiquinone bound to $center_{out}$ is also reduced by reduced DCIP to $QH_2$, but in this case $QH_2$ is not displaced by oxidized Q, which is unavailable, but is reoxidized to ubisemiquinone by the FeS center, thus forming a bypass (Fig. 8A). The increased steady-state concentration of ubisemiquinone in the presence of antimycin A promotes the reduction of the *b* cytochromes (Fig. 6A).

The TMPD bypass of the antimycin block, in which $WB^+$ functions as an oxidant, is also readily

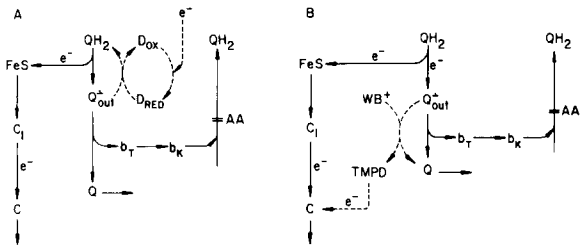


Fig. 8. Pathways of electron flow in (A) the DCIP and (B) the TMPD bypasses of the antimycin (AA) block. In the DCIP bypass $Q^-_{out}$ is constantly rereduced to $QH_2$ by reduced DCIP (abbreviated $D_{red}$), as shown by the dashed arrows. Electron flow from $QH_2$ to cytochrome *c* thus takes place entirely via the FeS center and cytochrome $c_1$. Reduced DCIP must be constantly regenerated by ascorbate or succinate. In the TMPD bypass one electron from $QH_2$ passes to the FeS center and the other passes from $Q^-$ to $WB^+$, both ultimately reducing cytochrome *c*.

explained by an supports the Q cycle scheme, since the $WB^+$ must accept electrons from some reduced species that accumulates in the presence of antimycin, i.e., either ubisemiquinone at $center_{out}$ or the reduced *b* cytochromes. Thus $WB^+$ provides a continuous escape for electrons from ubisemiquinone or reduced cytochrome *b*, which would otherwise constitute a dead end. There is no net consumption of $WB^+$, since it is regenerated by the oxidation of TMPD by cytochrome *c*; thus the TMPD/$WB^+$ couple acts catalytically (Fig. 8B). In the TMPD bypass, one electron from $QH_2$ passes to the FeS center, the second electron (from $Q^-$) passes via $WB^+$ to cytochrome *c*, the ultimate acceptor of both electrons (Fig. 8B). Here again, inhibition of electron transfer to the FeS center (by myxothiazol) or from the FeS center to cytochrome $c_1$ (by UHNQ or UHDBT) inhibits the overall TMPD bypass reaction. $QH_2$ is regenerated again by reduction of Q by succinate. In the experiments reported in this paper reduced DCIP was found to act as a reductant and $Wb^+$ as a suitable oxidant. The two mediators could in principle also operate in the opposite manner (i.e., DCPIP as oxidant and TMPD as reductant) without changing our conclusions. However, we have been unable to observe a significant bypass of the antimycin block in the presence of oxidized DCIP and of TMPD + ascorbate. In the case of TMPD + ascorbate, no significant oxidation of cy-

tochrome *b* was observed in the absence of succinate, in accordance with Fig. 6 of Ref. 2. As described in the same figure of Ref. 2, TMPD + ascorbate induce oxidation of cytochrome *b* at anaerobiosis. This effect was ascribed to the more negative redox potential attained by the TMPD/$WB^+$ couple in this situation. Thus a bypass of the antimycin block might in principle take place if TMPD could be kept in a more highly reduced state in the presence of $O_2$.

Some secondary observations require comment. It was found that the extent of UHNQ inhibition of the TMPD bypass is significantly less than that of myxothiazol inhibition. This effect can be explained on the basis that $WB^+$ is not completely specific as an oxidant of ubisemiquinone or reduced cytochrome *b*; it may also be capable of slow reoxidation of the reduced FeS center when the latter is blocked in its reduced form in the presence of UHNQ. This explanation suggests the interesting possibility that the inhibitory effect of UHNQ on electron flow through complex III may be abolished if some other artificial acceptor can be found to provide an escape for electrons from the reduced FeS center. Another point of interest is that $WB^+$ promotes oxidation of reduced cytochrome b in the presence of antimycin A, but this oxidation is only partial even at the highest testable concentrations of $WB^+$. This result seems puzzling, but it might indicate that the accessibility of $WB^+$ to its reaction center is decreased when ubiquinone is poised at a relatively high redox potential. Further experiments on these points are under way with other redox mediators.

Finally, the effect of myxothiazol and UHNQ on the redox behavior of the *b* cytochromes in the presence of TMPD and $WB^+$ requires comment. As shown in Fig. 7A myxothiazol abolished the oxidation of cytochrome *b* induced by reduced DCIP. This effect is expected if the binding of myxothiazol prevents the formation of ubisemiquinone at $center_{out}$, similar to the situation observed after destruction of the FeS center by BAL [27]. In this case no DCIP-reducible redox center would be available. Also, UHNQ interferes with the oxidation of cytochrome *b* by reduced DCIP and might indicate that UHNQ interferes with the formation of ubisemiquinone. However, this effect is observed only at very low DCIP concentrations and generally at temperatures below 14–15 °C. This is probably a reflection of the poor binding of UHNQ when the chain is highly oxidized [17].

The partial reduction of cytochrome *b* which is still observed in the presence of antimycin + $WB^+$ is slowly abolished by further addition of myxothiazol. It can therefore be argued that myxothiazol, by preventing electron flow to the FeS center also prevents electrons from entering the $Q^-/b$ pathway, thus making possible slow escape of electrons from cytochrome *b* to the artificial oxidant $WB^+$. It is also to be emphasized that myxothiazol and UHNQ inhibit the mediator-activated succinate oxidation about as well as in the absence of antimycin. This observation indicates that electrons entering complex III in the presence of myxothiazol or UHNQ, but in the absence of antimycin, are not available for interaction with the mediators. This can be explained if the reactive center for both reduced DCPIP and $WB^+$ is $Q^-$at $center_{out}$, whose formation might be prevented by myxothiazol and UHNQ.

Altogether most of the data reported in this paper can be explained by and support a non-linear sequence of electron transfer reactions in the mitochondrial ubiquinol-cytochrome *c* reductase system as proposed in the original Q cycle [1]. Some of our observations, however, e.g., the failure of cytochrome *b* to be fully oxidized in the presence of antimycin + $WB^+$, are not easily explained by the original Q cycle. Similarly, recent reports on the kinetics of reduction of cytochrome $c_1$ and the FeS center in the presence of antimycin A are not accounted for by a simple Q cycle and have led to more complex variants [13,18]. Furthermore, it has been reported that DCCD can inhibit translocation of 2 of the 4 $H^+$ that accompany passage of an electron pair through complex III, without inhibiting the overall rate of electron flow and without producing uncoupling [23–25], observations that suggest our understanding of the sequence and mechanism of electron flow through complex III is still incomplete.

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## References


1 Mitchell, P. (1976) J. Theor, Biol. 62, 327–367
2 Wikström, M.K.F. and Berden, J.A. (1972) Biochim. Biophys. Acta 283, 403–420
3 Rieske, J.S. (1976) Biochim. Biophys. Acta 456, 195–247
4 Edwards, C.A., Bowyer, J.R. and Trumpower, B.L. (1982) J. Biol. Chem. 257, 3705–3713
5 Bowyer, J.R., Edwards, C.A. and Trumpower, B.L. (1981) FEBS Lett. 126, 93–97
6 Trumpower, B.L. (1981) Biochim. Biophys. Acta 639, 129–155
7 Slater, E.C. (1981) in Chemiosmotic Proton Circuits in Biological Membranes (Skulachev, V.P. and Hinkle, P., eds.), pp. 69–104, Addison-Wesley Publishing Co., Reading, MA
8 Becker, W.F., Von Jagow, G., Tierbach, G. and Reichenbach, H. (1980) Hoppe-Seyler's Zeit. Physiol. Chem. 361, 1467
9 Becker, W.F., Von Jagow, G., Anke, T. and Steglich, W. (1981) FEBS Lett. 132, 329–333
10 Von Jagow, G. and Engel, W.D. (1981) FEBS Lett. 136, 19–24
11 Tierbach, G. and Reichenbach, H. (1981) Biochim. Biophys. Acta 638, 282–289
12 Meinhardt, S.W. and Crofts, A.R. (1982) FEBS Lett. 149, 217–222
13 De Vries, S., Albracht, S.P.J., Berden, J.A., Marres, C.A.M. and Slater, E.C. (1983) Biochim. Biophys. Acta 723, 91–103
14 Bowyer, J.R., Dutton, P.L., Prince, R.L. and Crofts, A.R. (1980) Biochim. Biophys. Acta 592, 445–460
15 Bowyer, J.R., Matsuura, K., Dutton, P.L. and Ohnishi, T. (1982) in Second European Bioenergetics Conference, Short Reports, pp. 201–202, LBTM-CNRS Ed., Lyon
16 Matsuura, K., O'Keefe, D.P. and Dutton, P.L. (1983) Biochim. Biophys. Acta 722, 12–22
17 Bowyer, J.R., Edwards, C.A., Ohnishi, T. and Trumpower, B.L. (1982) J. Biol. Chem. 257, 8321–8330
18 De Vries, S., Albracht, S.P.J., Berden, J.A. and Slater, E.C. (1982) Biochim. Biophys. Acta 681, 41–53
19 Crofts, A.R. and Wraight, C.A. (1983) Biochim. Biophys. Acta 726, 149–185
20 Lee, C.P., Sottocasa, G.L. and Ernster, L. (1967) Methods. Enzymol. 10, 33–37
21 Norling, B., Nelson, B.D., Nordenbrand, K. and Ernster, L. (1972) Biochim. Biophys. Acta 275, 18–32
22 Alexandre, A., Galiazzo, F. and Lehninger, A.L. (1980) J. Biol. CHem. 255, 10721–10730
23 Price, B.D. and Brand, M.D. (1982) Biochem. J. 205, 419–421
24 Beattie, D.S. and Villalobo, A. (1982) J. Biol. CHem. 257, 14745–14752
25 Degli Esposti, M., Saus, J.B., Timoneda, J., Bertoli, E. and Lenaz, G. (1982) FEBS Lett. 147, 101–105
26 Ackrell, B.A.C., Kearney, E.B., Coles, C.J., Singer, T.P., Beinert, H., Wan, Y.-P. and Folkers, K. (1977) Arch. Biochem. Biophys. 182, 107–117
27 De Vries, S., Albracht, S.P.J., Berden, J.A. and Slater, E.C. (1981) J. Biol. Chem. 256, 11996–11998