# On the inhibition of the $b$—$c_1$ segment on the mitochondrial respiratory chain by quinone analogues and hydroxyquinoline derivatives

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b—c$_1$ *segment* *Mitochondria* *Quinone analogues* *Hydroxyquinoline derivatives* *Antimycin*

## 1. INTRODUCTION

The cytochrome $b$—$c_1$ [1,2] complex corresponds to the second coupling side of the mitochondrial respiratory chain and functions as a redox proton pump [2–7]. The mechanism by which reducing equivalents enter the complex and are transferred to cytochrome $c$ is, as yet, not fully understood [1,6–12]. Definite contributions towards these problems come from the use of specific inhibitors. Antimycin inhibits electron flow between the $b$-cytochromes and cytochrome $c_1$ [13], and probably binds to the apoprotein of $b$-cytochromes [2,14] close to the heme $b_{562}$ [13]. It promotes also oxidant-induced reduction of $b$-cytochromes [15] and, under particular conditions, inhibits reduction of $b$-cytochromes by substrates [10,12,16]. These and related observations have led to the view that electron flow in the $b$—$c_1$ complex takes place through branched [8,15] or cyclic pathways [6,7,10–12] rather than by linear sequence.

Alkyl hydroxyquinoline derivatives and quinone analogues are other interesting inhibitors of the $b$—$c_1$ complex. Hydroxyquinoline derivatives exert a twofold action on the complex. These compounds, besides displaying an inhibitory action similar to that of antimycin [1,13,17,18], appear to exert a second inhibitory effect on redox component(s) of the $b$—$c_1$ complex [19,20].

*Abbreviations:* 2-Heptyl-4-hydroxyquinoline-*N*-oxide, HQNO; 5-(*n*-undecyl)-6-hydroxyl-4,7-dioxobenzothiazole, UHDBT.

It has been proposed that quinone analogues like DBMIB (2,5-dibromo-3-methyl-6-isopropylbenzoquinone) and UHDBT inhibit the $b$—$c_1$ complex by disturbing the redox activity of the FeS-protein $g$ = 1.90 of the complex [16,21].

Here we describe a stopped-flow kinetic analysis of the action of the hydroxyquinoline derivative HQNO and of UHDBT on redox as well as protonmotive activity of the $b$—$c_1$ complex. The results provide information on the sites of action of these inhibitors and the role of the complex in redox-linked proton translocation by the cytochrome chain.

## 2. MATERIALS AND METHODS

Valinomycin, oligomycin, antimycin A and HQNO were obtained from Sigma Chemical Co. UHDBT was prepared as described in [21]. All the other reagents were of the highest purity grade available.

Heavy beef-heart mitochondria were prepared as in [22]. Oxidoreduction of cytochromes was monitored with a Johnson Foundation dual wavelength spectrophotometer equipped with regenerative stopped flow apparatus. The mixing ratio was 1:62, the reaction time during the continuous-flow phase was 12 ms (for details see [4]). Proton translocation was measured with a Roughton-type, repetitive, continuous-flow pH-meter (mixing ratio 1:60). For details see [4,23].

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## 3. RESULTS AND DISCUSSION

In fig.1 a stopped-flow analysis is presented of the effect of inhibitors of the $b$–$c_1$ complex on redox transitions of $b$ and $c$ cytochromes elicited by rapid oxygenation of anaerobic beef-heart mitochondria whose respiratory carriers had been reduced by succinate. Malonate was added to inhibit re-reduction of respiratory carriers oxidized by oxygen. Oxygenation caused, after a lag of 12 ms, net oxidation of $b$-cytochromes (fig.1a) [9,24]. Antimycin (fig.1c) changed the response of $b$-cytochromes to oxygenation from net oxidation to net extra-reduction [9,24]. HQNO (fig.1d) mimicked in this respect antimycin. It should, however, be noted that the $t_{1/2}$ for the HQNO promoted reduction of $b$-cytochromes was significantly higher than the $t_{1/2}$ for the antimycin-promoted reduction. UHDBT (fig.1b) behaved differently from the other two inhibitors. In fact, it simply depressed the rate of aerobic oxidation of $b$-cytochromes without causing net extra-reduction. 50% inhibition by UHDBT of cytochrome $b$ oxidation was caused by 0.5 nmol/mg protein (fig.2). All the three inhibitors enhanced the net rate of aerobic oxidation of $c$-cytochromes.

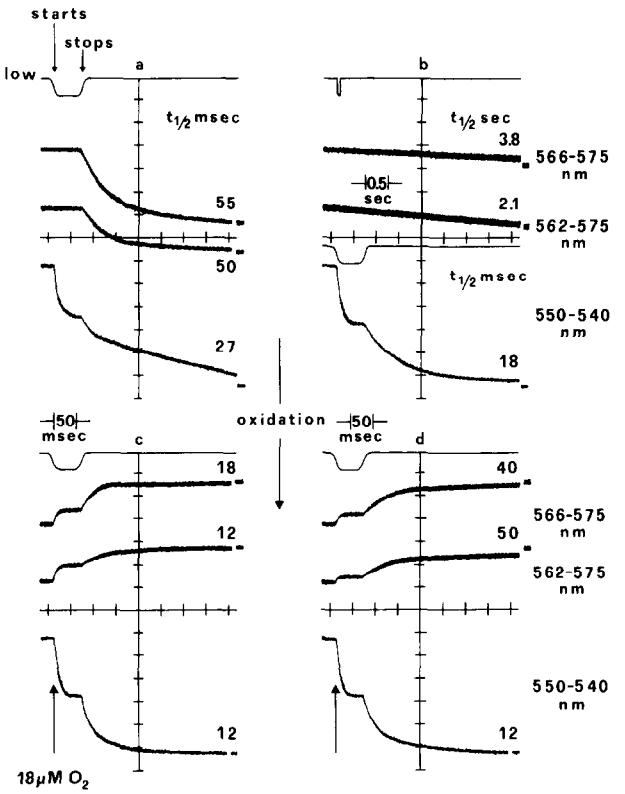


Fig.1. Effect of UHDBT, antimycin A and HQNO on the kinetics of aerobic redox transitions of cytochromes in beef-heart mitochondria. Mitochondria (2 mg protein . $ml^{-1}$) were incubated in the main-syringe of the flow-apparatus at 25°C in: 200 mM sucrose, 20 mM KCl, 3.5 mM succinate, 0.5 μg valinomycin . mg protein$^{-1}$, 0.5 μg rotenone . mg protein$^{-1}$ and 2 μg oligomycin . mg protein$^{-1}$. pH 7.2. The system became anaerobic due to succinate reduction of respiratory carriers. Then 3.5 mM K-malonate was added. Oxygen was delivered from the side-syringe of the flow-apparatus as: 200 mM sucrose and 30 mM KCl. (a), control; (b), 5 nmol UHDBT . mg protein$^{-1}$; (c) 0.5 nmol antimycin . mg protein$^{-1}$; (d) 5 nmol HQNO . mg protein$^{-1}$. The number on the traces refer to the $t_{1/2}$ of $b$-cytochrome oxidation or reduction and $c$-cytochromes oxidation. All the $t_{1/2}$ are given in ms except those of oxidation of $b$-cytochromes in presence of UHDBT, which are given in seconds.

The above experiments show that, whilst HQNO exerts an antimycin-like effect on the $b$–$c_1$ complex, UHDBT acts differently. It may be recalled

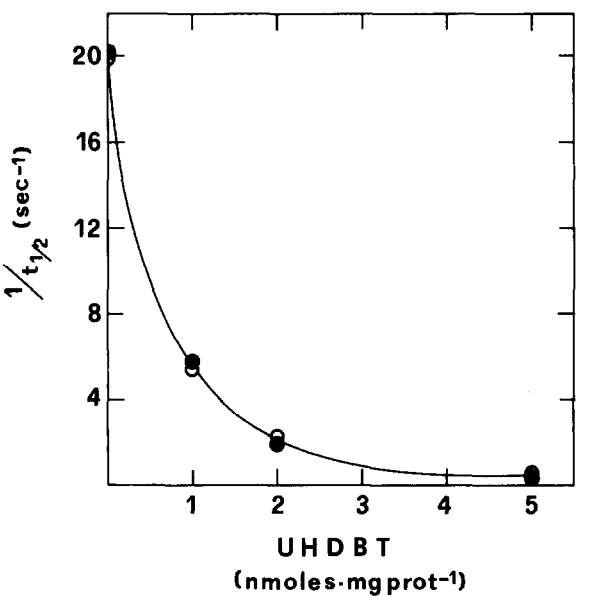


Fig.2. Titration of the inhibition by UHDBT of aerobic oxidation of $b$-cytochromes in beef-heart mitochondria. For experimental conditions see legend to fig.1. Mitochondria were incubated with UHDBT 5 min in anaerobiosis.

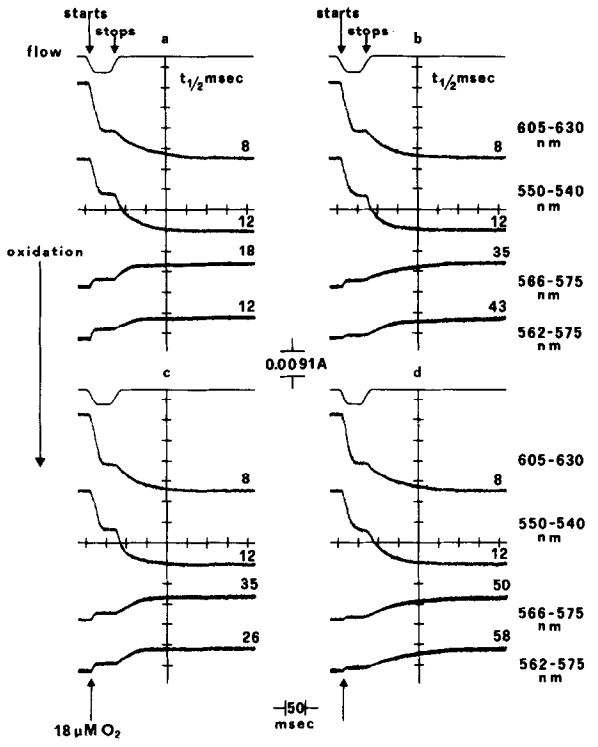


Fig.3. Effect of UHDBT and HQNO on redox transitions of cytochromes induced by oxygen pulses of anaerobic antimycin A treated beef-heart mitochondria. The experimental conditions were those described in the legend to fig.1 except that 0.5 nmol antimycin A . mg protein$^{-1}$ was present. Further additions: (a) none; (b) 1 nmol UHDBT . mg protein$^{-1}$; (c) 2 nmol HQNO . mg protein$^{-1}$; (d) 1 nmol UHDBT . mg protein$^{-1}$ + 2 nmol HQNO . mg protein$^{-1}$.

that the concentrations of the three inhibitors used in the experiments of fig.1 depressed to the same extent (95%) the respiratory activity of mitochondria with succinate as substrate (see [13,18,21]).

Figure 3 illustrates the effect of HQNO and UHDBT on the kinetics of antimycin-promoted oxygen-induced reduction of *b*-cytochromes. Either HQNO or UHDBT caused a significant depression of the rate of cytochrome *b*-reduction. Furthermore the inhibitory effects of UHDBT and HQNO were additive. These experiments show that HQNO, in addition to the antimycin like inhibitory effect documented in fig.1, exerts a second distinct inhibitory effect. This latter inhibitory effect is analogous to that caused by UHDBT and is apparently exerted, as shown by the additivity of inhibition, at the same site of action of UHDBT.

It has been reported that rapid aerobic oxidation of terminal respiratory carriers in antimycin-inhibited mitochondria supplemented with inhibitors of dehydrogenases and valinomycin (plus $K^+$) results in synchronous proton release [3,8,25,26]. It is disputed whether this proton ejection results simply from antimycin-insensitive residual oxidation of hydrogenated carriers on the substrate side of cytochrome *c* [8,9,25,26] or if it reflects also a proton pumping activity of cytochrome *c* oxidase [27].

Figure 4 shows that the rapid proton ejection accompanying the aerobic oxidation of *c* cytochromes and reduction of *b* cytochromes was inhibited by both HQNO and UHDBT. Also these inhibitory effects of UHDBT and HQNO were additive.

The $H^+/e^-$ stoicheiometry for experiments as those presented in figs. 3 and 4 are reported in table 1. The total amount of electron flow to oxygen was obtained by summing up the nmoles of hemes *a* and $a_3$ oxidized and the equivalent amount of copper atoms, the nmoles of cytochromes $c+c_1$ and of the FeS-protein of the $b-c_1$

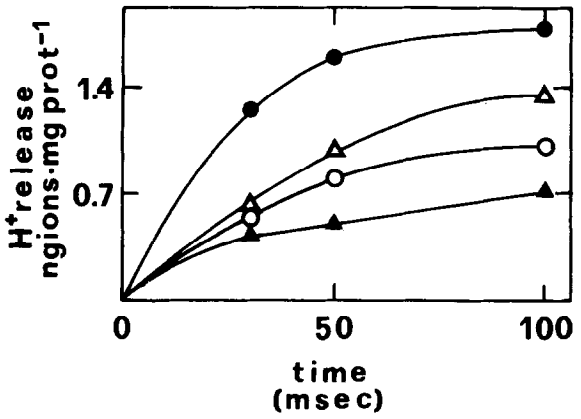


Fig.4. Effect of UHDBT and HQNO on $H^+$ release induced by oxygen pulses of anaerobic antimycin treated beef-heart mitochondria. For experimental procedure see Materials and Methods. For experimental conditions see legend to fig.3. Symbols: (•——•) control; (△——△) 1 nmol UHDBT . mg protein$^{-1}$; (○——○) 2 nmol HQNO . mg protein$^{-1}$; (▲——▲) 1 nmol UHDBT . mg protein$^{-1}$ + 2 nmol HQNO . mg protein$^{-1}$.

Table 1

Stoicheiometry of oxidation of redox carriers and $H^+$ release caused by oxygen pulses of anaerobic antimycin-inhibited beef-heart mitochondria. Effect of UHDBT and HQNO

| | | 50 ms | | | |
|---|---|---|---|---|---|
| | | (a) | (b) | (c) | (d) |
| $H^+$ released (ng-ions . mg protein$^{-1}$) | | 1.60 | 1.02 | 0.82 | 0.60 |
| Respiratory carriers oxidized (nmol . mg protein$^{-1}$) | $a+a_3$ | 0.80 | 0.81 | 0.81 | 0.80 |
| | Cu | 0.80 | 0.81 | 0.81 | 0.80 |
| | $c+c_1$ | 0.56 | 0.60 | 0.60 | 0.57 |
| | FeS | 0.28 | 0.30 | 0.30 | 0.29 |
| $e^-$ equivalent to cyt. *b* reduced | | 0.16 | 0.10 | 0.15 | 0.09 |
| $\Sigma e^-$ flow | | 2.60 | 2.62 | 2.67 | 2.55 |
| $H^+/\Sigma e^-$ | | 0.61 | 0.39 | 0.31 | 0.23 |

For experimental conditions see legends to figs. 3 and 4. The values reported above refer to 50 ms after oxygenation. The nmoles of hemes $a+a_3$ were calculated with a $\Delta\varepsilon_{mM}$ of 14 [28]. The atoms of copper oxidized were taken as equivalent to nmoles of hemes $a+a_3$ oxidized [29]. The nmoles of *c*-cytochromes were calcualted with a $\Delta\varepsilon_{mM}$ of 19.1 from the absorbance changes at 550–540 nm [30]. The nmoles of FeS-protein $g = 1.90$ were taken as equivalent to ½ the amount of *c*-cytochromes oxidized. The nmoles of *b*-cytochromes reduced were calculated with a $\Delta\varepsilon_{mM}$ of 20 [31] from the absorbance changes at 562–575 nm (see text). Further additions: Expt. (a), none; expt. (b), 1 nmol UHDBT . mg protein$^{-1}$; expt. (c), 2 nmol HQNO . mg protein$^{-1}$; expt. (d), 1 nmol UHDBT . mg protein$^{-1}$ and 2 nmol HQNO . mg protein$^{-1}$.

complex oxidized, the latter is taken as equivalent to half the amount of *c*-cytochromes oxidized. Since the aerobic reduction of *b*-cytochromes is generally thought as being produced by antimycin-insensitive oxidation of quinol to semiquinone [6–8,16] the sum of electron flow included also an amount of electrons equivalent to the nmoles of *b*-cytochromes reduced. This may, however, represent a minimum estimate of the amount of quinol actually oxidized (see [24]).

The $H^+/e^-$ ratio in the absence of other inhibitors but in the presence of antimycin amounted to 0.6 which is practically half the value expected in the case where oxidase pumped $H^+$ in the medium at a stoicheiometry of 1 $H^+$ per $e^-$ [7]. HQNO or UHDBT decreased the $H^+/e^-$ ratio. In the presence of UHDBT and HQNO added together, the $H^+/e^-$ ratio was lower than that found with either of the two inhibitors added alone; it amounted only to 0.2.

It should be noted that UHDBT and HQNO at the concentrations used in the experiments of table 1 do not exert any significant protonophoric activity [21,32,33]. It has, in fact, been shown that even FCCP, at concentrations displaying maximal protonophoric activity in coupling membranes, is ineffective on rapid $H^+$ transfer reactions associated to fast redox transitions of respiratory carriers which take place in a millisecond time scale [34].

On the basis of the results presented, which are consistent either with a branched [8,15] or a cyclic mechanism of electron transfer by the $b$–$c_1$ complex [6,7,10–12], we draw the following conclusions (see also [8]).

1. Antimycin and UHDBT exert their inhibitory effects at two different sites of the $b$–$c_1$ complex:

the former inhibits electron transfer from cytochrome $b_{562}$ [6,8] to a protein bound quinone (semiquinone) system [35,36] probably associated to the FeS-protein of the complex [16]. UHDBT acts by blocking the redox activity of the FeS-protein, possibly inhibiting oxidation of bound ubiquinol by the FeS-protein [16].

2. HQNO exerts a twofold inhibitory effect on the complex [19,20]. A predominant antimycin-like inhibitory effect at the same inhibition site [13,17,18,20] and an apparently weaker inhibitory effect [20] similar and at the same inhibition site of UHDBT. Evidence has in fact been presented that UHDBT and nonyl-4-hydroxyquinoline-*N*-oxide compete for a common binding site in the $b$–$c_1$ complex [37].

3. The rapid ejection of protons observed upon aerobic oxidation of the reduced cytochrome chain in antimycin (and valinomycin) treated mitochondria derives from electron flow in the $b$–$c_1$ complex [25,26]. In fact inhibition of antimycin-insensitive electron flow in the $b$–$c_1$ complex by UHDBT and HQNO suppresses almost completely the proton release. The residual $H^+$ release is not higher than the scalar proton release which may arise from redox Bohr effects in cytochrome *c* oxidase and the FeS-protein which can amount, at pH 7.2, to 0.9 and 0.8 equivalent $H^+$ per hemes $a+a_3$ and FeS protein undergoing oxidation [38]. It seems that under the conditions of a single turnover there is no sign of a proton pumping activity of cytochrome *c* oxidase (compare [8,25,26,33,39]; contrast [7,27]).

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