# Effect of $b$–$c_1$-site inhibitors on the midpoint potentials of mitochondrial cytochromes $b$

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Anaerobic potentiometric titrations of $b$ cytochromes have been carried out in beef heart submitochondrial particles in the presence of several specific inhibitors of electron transfer through the $b$–$c_1$-site of the respiratory chain. Whereas antimycin shows no significant effect on the titration curve of cytochrome $b$-562, NoHOQnO is found to shift the $E_m$ of $b$-562 by 20–30 mV to the positive. Funiculosin raises the $E_m$ of $b$-562 by >100 mV and also appears to bring about a minor shift of $b$-566 midpoint potential. In the presence of myxothiazol, both $b$ cytochromes titrate with $E_m$ values 15–30 mV more positive than in the control.

*Respiratory chain* *Cytochrome* b *Redox titration* *2-Alkyl-4-hydroxyquinoline* N-*oxide* *Funiculosin* *Myxothiazol*

## 1. INTRODUCTION

Under certain conditions antimycin can greatly increase the reducibility of mitochondrial cytochromes $b$-566 and $b$-562 by succinate and some other electron donors [1,2]. The mechanism of this effect had been studied in considerable detail. It was first proposed that the midpoint potentials of $b$ cytochromes are raised by the action of the inhibitor [1], but subsequent experiments showed that the reducing effect of antimycin is absolutely dependent on the presence of an oxidant, such as $O_2$ or ferricyanide, and is accounted for by a purely kinetic mechanism (reviewed [3]). In particular, anaerobic redox titrations did not reveal any positive shift of $E_m$ of either cytochrome $b$-566 or $b$-562 in the presence of antimycin [4,5].

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*Abbreviations:* HpHOQnO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; NoHOQnO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide; SMP, submitochondrial particles

A number of other $b$–$c_1$-site specific inhibitors including HpOHQnO [6,7], funiculosin [8], diuron [9,10], mucidin [9–11] and myxothiazol (in preparation) have been observed to bring about 'extra-reduction' of a variable size of one or both mitochondrial $b$ cytochromes. However, in neither case had the mechanism of the effect been scrutinized to the same extent as with antimycin.

According to [7], the additional reduction of cytochromes $b$ observed in the presence of HpHOQnO is fully analogous to the extra-reduction induced by antimycin. However, we have found that NoHOQnO can stimulate the reduction of cytochrome $b$-562 in submitochondrial particles under the conditions in which antimycin does not exert such an effect; e.g., in the presence of ascorbate and redox mediators such as *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, dichlorophenolindophenol, phenazine methosulfate [12]. A similar difference between the effects of funiculosin and antimycin was noticed in [8].

Here, NoHOQnO, funiculosin and myxothiazol, in contrast to antimycin, increased reducibility

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of cytochromes *b* under the conditions of anaerobic potentiometric titration.

## 2. METHODS

Antimycin and NoHOQnO were purchased from Serva. Funiculosin was a generous gift from Dr P. Bollinger (Sandoz, Basel) obtained through the courtesy of Professor P. Walter (University of Basel). Myxothiazol was kindly donated by Dr W. Trowitzsch (Gesellschaft für Biotechnologische Forschung, Braunschweig). $Ru(NH_3)_6Cl_3$ was from Alfa Products (Denver CO) and 1,4-naphthoquinone-2-sulfonate was from Eastman-Kodak. Other reagents were commercial products mainly from Sigma, Serva and Fluka of the purest grade available.

Beef heart SMP were prepared according to [13] with minor modifications. Anaerobic potentiometric titrations were carried out as detailed in [14] in a magnetically stirred 1 cm standard spectrophotometer cell in an Aminco DW $2^a_{TM}$ spectrophotometer. Redox potential was measured with a platinum wire electrode *vs* a calomel semimicro reference electrode (K 141, Radiometer) fed into an MV-870 digital pH/mV meter (DDR) with a readability of 0.1 mV. The electrode system was calibrated with a ferricyanide/ferrocyanide buffer as in [15]. Argon, purified by passing through an alkaline pyrogallol solution, was flushed continuously over the reaction mixture during the titrations. In each titration, stepwise reduction of *b* cytochromes was first carried out by small additions of freshly prepared ascorbate, NADH and dithionite solutions and, after the completion of the reduction, oxidative titration by ferricyanide was performed. The detailed titrations were carried out at a fixed wavelength pair (562 nm minus 575 nm), but in control experiments spectra were also scanned at various characteristic $E_h$ values, allowing the mixture to equilibrate for 3–5 min at each redox potential.

Curve fitting was performed in a Nova 3D computer using a program developed by Dr Alexander L. Drachev in this laboratory.

## 3. RESULTS

Fig.1 compares the effects of NoHOQnO and antimycin on the high-potential half of *b* cytochrome potentiometric titration curve, known to belong to cytochrome *b*-562 [2–5]. (The low-potential part of the curve was not assayed in detail in the antimycin- or NoHOQnO-inhibited SMP in this experiment.) It can be seen that in the presence of NoHOQnO (curve 2), the high-potential cytochrome *b* titrates at $E_h$ values ~30 mV more positive compared to the control (curve 1).

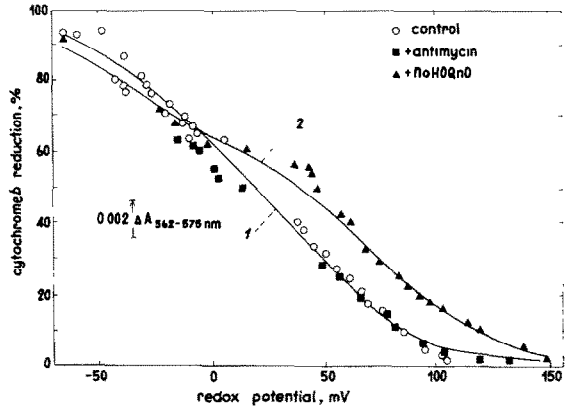


Fig.1. NoHOQnO-induced shift of cytochrome *b*-562 midpoint potential. Beef heart SMP (1 mg protein/ml) in the medium containing 0.29 M sucrose, 20 mM morpholinopropane sulfonate (pH 6.8), 0.5 mM EDTA and, where indicated, 1.5 $\mu$M antimycin or 6.5 $\mu$M NoHOQnO. Redox mediators: 100 $\mu$M of each diaminodurol, phenazine methosulfate and duroquinone, 50 $\mu$M 1,4-naphthoquinone-2-sulfonate; (■,▲) 100 $\mu$M phenazine ethosulfate. Theoretical Nernst curves for 2 one-electron components are drawn through the points with $E_m$ values: (1) +45 mV and −25 mV; (2) +75 mV and −40 mV; the contributions of the high- and low-potential components to $\Delta A^{red-ox}_{562-575}$ are 0.6 and 0.4, respectively. In this experiment cytochrome *b*-566 was not titrated in detail in the NoHOQnO- or antimycin-inhibited SMP; therefore, the characteristics of the low-potential component of curve 2 are rather approximate and have been concerned solely to allow for simulation of the complete titration curve. The 100% reduction level was measured in each case upon addition of a small excess of dithionite.

Control spectra recordings showed clearly, in agreement with [12], that it was increased reduction of cytochrome *b*-562 brought about by NoHOQnO under anaerobic conditions over $E_h$ 0–120 mV (not shown). At lower redox potentials, a small spectral shift of *b*-562 [16] was the only effect of NoHOQnO resolved by the difference spectra; no increased reduction of *b*-566 could be detected (not shown, [12]).

In contrast to NoHOQnO, antimycin exerts no significant effect on the redox titration of *b* cytochromes in the high-potential region (see (○) and (■) in curve 1 of fig.1), which corroborates the data in [4] (but see [5]). Accordingly, antimycin has been found to reverse the additional reduction of cytochrome *b*-562 induced by NoHOQnO under anaerobic conditions [12].

The effect of funiculosin on the potentiometric titration curve of *b* cytochromes has been found to be more dramatic than that of NoHOQnO (fig.2). One can see that, in the presence of this antibiotic the high-potential cytochrome *b*, identified as *b*-562 by appropriate spectral recordings (not shown), titrates at $E_h$ ~120 mV more positive compared to the control. There seems to be also a minor shift to the positive of the low-potential component of the titration curve (cytochrome *b*-566).

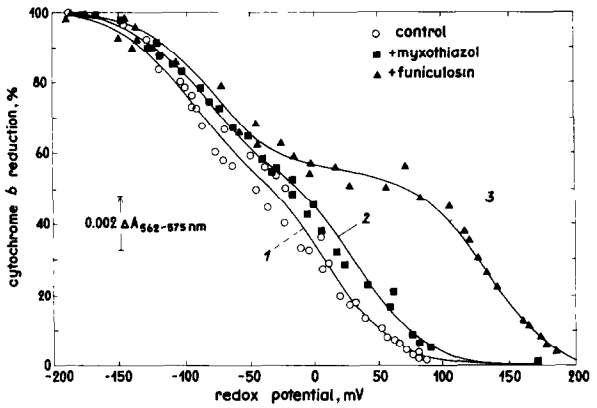


Fig.2. Effects of funiculosin and myxothiazol on the redox titration of cytochromes *b*. Beef heart SMP (0.8 mg protein/ml) in the medium containing 0.3 M sucrose, 20 mM *N*-2-hydroxyethylpiperazine–*N'*-2-ethane sulfonate (pH 7.7), 5 mM $MgSO_4$ and, where indicated, myxothiazol (1.2 μg/ml) or funiculosin (2 μg/ml); $T$ = 30°C. Redox mediators: 50 μM diaminodurene, 50 μM 1,4-naphthoquinone-2-sulfonate, 40 μM $Ru(NH_3)_6Cl_3$, 25 μM phenazine methosulfate, 25 μM phenazine ethosulfate, 50 μM duroquinone and 50 μM menadione. The two-component Nernst curves drawn through the points assume the following $E_m$ values for the high- and low-potential cytochromes: (1) +10 mV and −100 mV; (2) +35 mV and −85 mV; (3) +130 mV and −80 mV. The contributions of the high- and low-potential components to $\Delta A^{red-ox}_{562-575}$ are 0.55 and 0.45, respectively.

In fig.2, redox titration of *b* cytochromes in the presence of myxothiazol is shown. It can be seen that the inhibitor brings about a slight but discernible dislocation of the entire titration curve to higher $E_h$ values, which can be accounted for by the positive shifts of $E_m$ of *b*-566 and *b*-562 by ~20 mV.

## 4. DISCUSSION

These results indicate that the increased reducibility of *b* cytochromes induced by NoHOQnO [6,7,12] and funiculosin [8] may in fact comprise two different effects.

(1) In the presence of succinate these inhibitors are likely to bring about the oxidant-dependent extra-reduction of both cytochromes *b*-566 and *b*-562 [6,8,12] similarly to antimycin [2,3]. This non-equilibrium effect will be consistent with NoHOQnO and funiculosin inhibiting the electron flow at the same site as antimycin [7,8,17]; i.e., between *b* cytochromes and ubiquinone in centre *i* of the Q cycle [18]. Accordingly, all 3 antibiotics stimulate $H_2O_2$ and $O_2^{\cdot-}$ generation in *b*–$c_1$-site [19,20].

(2) NoHOQnO and funiculosin stimulate the reduction of cytochrome *b*-562 as a consequence of the positive shift of this haemoprotein midpoint potential; this effect accounts for the differences in redox behaviour of *b* cytochromes in the presence of antimycin, and NoHOQnO [12] or funiculosin [8].

The preferential influence of funiculosin and NoHOQnO on $E_m$ of the high-potential of the two *b* cytochromes agrees well with the hypothesis that it is the reaction of *b*-562 with CoQ which is blocked by centre *i* inhibitors [18]. Accordingly, both inhibitors perturb the optical absorption spectrum of cytochrome *b*-562 in yeast [9,20] and beef heart [16,22] mitochondrial respiratory chain. It may seem surprising that antimycin, which is considered to be a typical centre *i* inhibitor [18] and is known to alter optical and EPR spectra of cytochrome *b*-562 [1], does not modulate the $E_m$ of this redox centre. Although in the presence of antimycin the high-potential part of *b*-562 redox titration curve shifted to lower $E_h$ [5], such an effect has not been observed by others ([4]; this

paper). However, the effect of antimycin on the $E_m$ of cytochromes *b* may depend critically on pH since this inhibitor was shown to modify the protonic function ($E_m$/pH-dependence) of *b* cytochromes [23,24].

In contrast to NoHOQnO and funiculosin, myxothiazol [25,26] behaves as a typical centre *o* inhibitor [27] and suppresses the antimycin-induced extra-reduction of *b* cytochromes (in preparation). Therefore, we were somewhat perplexed by an observation that myxothiazol itself brings about a small additional reduction of *b*-562 and *b*-566 in the KCN-inhibited succinate/fumarate-poised SMP. The finding that myxothiazol slightly raises the $E_m$ of both cytochromes *b* can resolve this discrepancy. The same explanation applies possibly to a very small additional reduction of cytochromes *b*-566 and *b*-562 induced by mucidin [11].

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