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Antimycin inhibition of the cytochrome *bd* complex from *Azotobacter vinelandii* indicates the presence of a branched electron transfer pathway for the oxidation of ubiquinol

Susanne Jünemann, John M. Wrigglesworth*

Metals in Biology and Medicine Centre, Division of Life Sciences, King's College London, Campden Hill Road, London, W8 7AH, UK

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Abstract

Antimycin A and UHBDT inhibit the activity of the purified cytochrome *bd* complex from *Azotobacter vinelandii*. Inhibition of activity is non-competitive and antimycin A binding induces a shift to the red in the spectrum of a *b*-type haem. No inhibitory effects were seen with myxothiazol. Steady-state experiments indicate that the site of inhibition for antimycin A lies on the low-potential side of haem b_{558}. In the presence of antimycin A at concentrations sufficient to inhibit respiration, some direct electron transfer from ubiquinol-1 to haem b_{595} and haem *d* still occurs. The results are consistent with a branched electron transfer pathway from ubiquinol to the oxygen reduction site.

Key words: Cytochrome *bd*; Antimycin A; *Azotobacter vinelandii*

1. Introduction

The cytochrome *bd* complex is one of two terminal oxidases of *Azotobacter vinelandii* [1] which are thought to play an essential role in aerotolerant nitrogen fixation [2]. The complex from *A. vinelandii* shows spectral, immunological [3,4] and genetic [5] similarities to the *E. coli bd* oxidase. It comprises two subunits [6] and functions as a quinol oxidase with the quinol binding site, in the *E. coli* enzyme, located on a hydrophilic loop of subunit I at the periplasmic side of the bilayer [7,8]. Subunit I also contains the hexacoordinate haem b_{558} whilst the other two redox centres (haem b_{595} and haem *d*) appear to be located in subunit II [9], although ligation to both subunits cannot be excluded.

The use of specific inhibitors has long been a productive method for probing structure–function relationships in the active centres of redox proteins. Monoclonal antibodies have been used on the cytochrome *bd* complex of *E. coli* to localize the ubiquinol oxidation site to between the fifth and sixth putative membrane spanning segments of subunit I [8]. Trypsin digestion of the *E. coli* enzyme has been shown to cleave subunit I with a corresponding loss of ubiquinol oxidase activity while TMPD oxidase activity was unaffected [7]. Electron flow from ubiquinol to molecular oxygen in *E. coli* is reported to be inhibited by antimycin A [10], and the oxygen reduction site itself has been probed using cyanide [11], CO [12–14] and nitrite [15,16]. Recently, interest has been raised in the mechanism of ubiquinol oxidation in the copper-containing quinol oxidases by speculation about a Q-loop mechanism for electron and proton transfer in these enzymes [17]. The *bd* oxidases are non-homologous in sequence with the copper-containing oxidases but do share the common feature of a quinol oxidation site close to a hexacoordinate haem *b*. Antimycin A, myxothiazol and UHDBT are classical inhibitors of the Q-cycle in the mitochondrial bc_1 complex [18] and we report here their effects on the purified cytochrome *bd* complex. We have found that antimycin A and UHDBT inhibit ubiquinol, but not TMPD, oxidation by the *bd* oxidase. Myxothiazol has no significant inhibitory effect with either substrate. The site of action of antimycin A has been localised to the low potential side of haem b_{558} and a branched electron transfer pathway appears to be involved in the oxidation of ubiquinol.

2. Materials and methods

The cytochrome *bd*-overproducing *A. vinelandii* strain, MK8, was grown essentially as previously described [5] with the modification of increased aeration. All steps were carried out at 4°C. Membrane vesicles [5] were first washed with 25 mM Tris-HCl, pH 8.0, containing 10 mM EDTA and 0.5 M KCl, then suspended in 0.1 M potasium phosphate buffer, pH 7.2, containing 2% (w/v) sodium cholate. After at least 4 h stirring, the mixture was centrifuged at 45,000 × *g* for 20 min and the pellet again extracted twice with 2% cholate in phosphate buffer. The final pellet was suspended in 0.1 M phosphate buffer, pH 7.2, containing 1% (w/v) lauryl maltoside and, after 1 h stirring, centrifuged at 45,000 × *g* for 15 min. Cytochrome *bd* was purified from the supernatant by an ammonium sulphate cut between 50% and 80% saturation. The final preparation was suspended to a final concentration of 10–15 mg protein · ml^{-1} in 0.1 M potassium phosphate buffer, pH 7.2, and stored under liquid nitrogen. The pyridine haemochrome spectrum of the preparation showed the presence of haem *b* and haem *d*. No *c*-type haem was detected.

Ubiquinone-1 was a gift from R. Cammack (King's College London, UK) and was purified before use by thin-layer chromatography on silica gel plates using chloroform:hexane:diethylether (10:10:1). Cy-

*Corresponding author.

Abbreviations: UHDBT, undecylhydroxydioxobenzothiazole; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DTT, dithiothreitol.

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tochrome *bd* activity was assayed at room temperature using an oxygen electrode. A 2:1 (w/w) mixture of cytochrome *bd* complex and soybean phosphatidylcholine (Type IV-S; Sigma) was pre-incubated for 5 min in 0.1 M phosphate buffer, pH 7.9. The mixture was then injected into the oxygen electrode to an assay medium of 0.1 M phosphate buffer, pH 7.9, containing up to 150 μM ubiquinol-1 plus 1 mM DTT (or 0.5 mM TMPD plus 2 mM sodium ascorbate). Inhibitors were added from stock solutions in ethanol. Their concentrations were determined from optical spectra using the extinction coefficients given by von Jagow and Link [18]. The concentration of antimycin A used in the spectral experiments was raised to maintain an approximate antimycin/protein ratio as was used in the oxygen electrode experiments. Optical spectra were recorded on a Varian Cary 210 spectrophotometer at room temperature, using a spectral bandwidth of 1 nm and a scan rate of 2 nm · s^{-1}. Kinetic spectra were recorded using a Hewlett Packard 8452A diode-array spectrophotometer. All spectra were stored in digital form.

3. Results and discussion

CIt can be seen from Fig. 1 that oxygen reduction by the purified cytochrome *bd* complex is inhibited by antimycin A and UHDBT when ubiquinol-1 is used as the respiratory substrate. No inhibitory effect could be detected with myxothiazol. When the substrate TMPD (plus ascorbate) was used in place of ubiquinol-1 (plus DTT), none of the inhibitor compounds showed any inhibitory effect on the oxygen reduction rate (results not shown). Using ubiquinol-1 as the respiratory substrate, inhibition values were measured at various inhibitor concentrations for different concentrations of substrate. Cornish–Bowden and Dixon plots [19] for antimycin A inhibition were typical of a non-competitive interaction, with $K_i = K_i' = 11 \pm 3$ μM. Similar non-competitive inhibition was found for UHDBT with $K_i = K_i' = 20 \pm 5$ μM.

In the mitochondrial bc_1 complex, antimycin A binding induces a spectral shift in both the α and γ bands of the high potential reduced haem [18]. We have found a similar red shift in the Soret region around 430 nm on binding antimycin A to the reduced form of the purified *bd* complex of *A. vinelandii* (Fig. 2). Some perturbation around 560 nm also takes place. The spectral effects indicate that antimycin A binding affects one of the *b*-type haems, probably haem b_{558}. No spectral changes could be detected when UHBDT was added to the purified complex.

TMPD oxidation in the *E. coli* enzyme has been shown by Lorence et al. [7] to be unaffected by trypsin digestion of subunit I, a procedure that abolishes ubiquinol oxidation. Similarly, in the present experiments, TMPD oxidation is unaffected by antimycin A and UHDBT whilst ubiquinol oxidation is inhibited. The inhibitors therefore appear to be exerting their effect prior to the electron entry site of TMPD. We have further localised the site of inhibition by following spectral changes of the complex during reduction by ubiquinol-1 in the presence and absence of antimycin A. Spectra (with an integration time of 0.5 s) were taken every 10 s following the addition of ubiquinol-1 (plus DTT) to a solution of purified *bd*

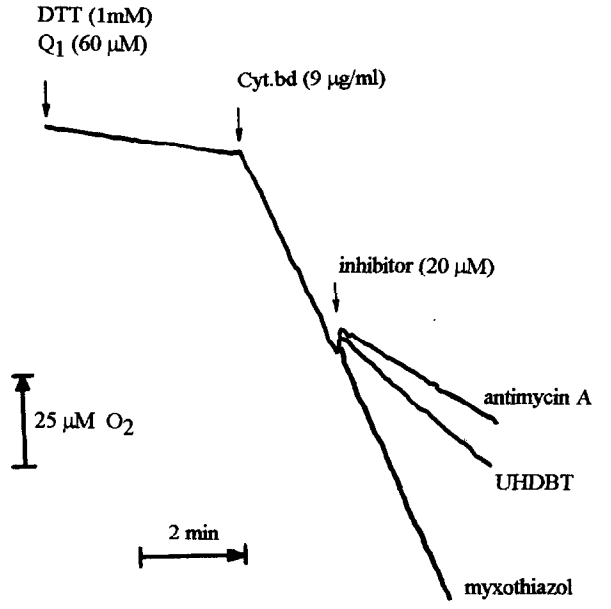

Fig. 1. Effect of antimycin A, UHBDT and myxothiazol on oxygen reduction by cytochrome *bd* with ubiquinol-1 as substrate. The inhibitors were added, in separate experiments, to a 1 ml suspension of cytochrome *bd* (9 μg protein/ml) in an oxygen electrode system as described in section 2. The separate traces are combined for the figure.

complex under aerobic conditions. The digitalised spectra were analysed to extract absorbance changes with time at the wavelength pairs 560–578 nm, 595–604 nm, 628–604 nm and 604–650 nm (see Fig. 2). Changes at 560–578 nm were used to monitor the redox state of both *b*-type haems. It is known for the *E. coli bd* complex that haem b_{595} has a strong β-band around 560 nm which overlaps the haem b_{558} α-band, with relative contributions at 560 nm of approximately 50:50 in the reduced minus air-oxidised spectrum [12]. The extinction changes at 595–604 nm were used to monitor haem b_{595} alone. Ferrous haem *d* was monitored by changes in the 628 nm band (628–604 nm). The trough at 650 nm is attributed to the oxygenated haem *d* [20] and the wavelength pair 604–650 nm was used to monitor the changes in the haem *d*–oxy complex.

It can be seen from Fig. 3 that the steady-state level of reduction of the *b*-type haems (560–578 nm) during respiration with ubiquinol-1 as substrate is higher in the presence of antimycin A compared to its absence. This correlates with a slower respiration rate as measured by the increased time taken to anaerobiosis. When the system becomes anaerobic, the absorbance at 560 nm increases rapidly in the control sample, to around 85% of the final fully reduced spectrum (Fig. 3A). In the presence of antimycin A, the amount of rapidly reduced *b*-type haem decreases. Full reduction only occurs after several minutes, at a much slower rate of around 0.002 s^{-1} (Fig. 3B). At first sight, the simplest explanation might be that antimycin A is acting to block electron

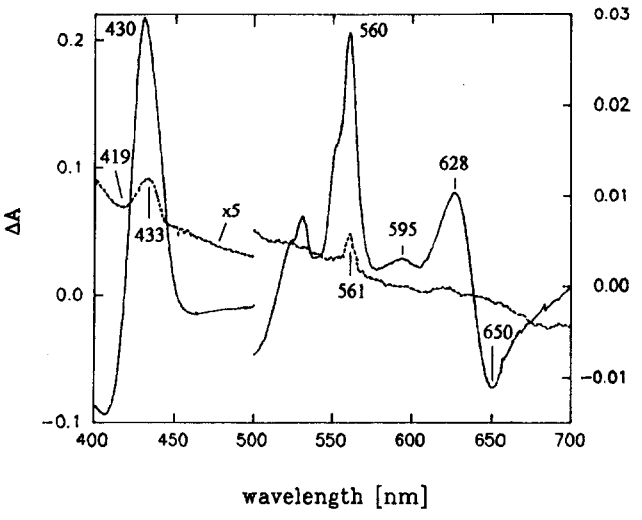

Fig. 2. Perturbations in the spectrum of dithionite reduced cytochrome *bd* complex induced by the binding of antimycin A. Antimycin A was added to a final concentration of 350 μM to a suspension of cytochrome *bd* (1.7 μM) in 0.1 M HEPES buffer, pH 7.0, plus 4 mM dithionite. The dotted line shows the antimycin/reduced minus reduced spectrum (Soret region enlarged by a factor of 5). Also shown, for comparison, is the dithionite reduced minus air-oxidised spectrum of the complex (solid line), with wavelength indicated for monitoring the spectral changes of the individual haems. See main text for details.

flow between haems b_{558} and b_{595}. Thus, when the system becomes anaerobic, b_{558} shows fast reduction by ubiquinol but electron flow to b_{595} is much slower. However, further observations complicate this interpretation. The first is that the slow rate of reduction in the inhibited enzyme after anaerobiosis (0.002 s^{-1}) is much less than the apparent electron flow rate through the same enzyme before the system goes anaerobic. This steady-state respiration rate can be calculated from the time taken for the system to go anaerobic and is approximately 2 s^{-1}, which agrees with separate experiments using the oxygen electrode. If b_{595} was part of a linear electron transfer pathway from quinol to oxygen, then its rate of reduction on anaerobiosis should match the rate of electron flow through the haem under steady-state (inhibited) conditions. This is clearly not the case. A more reasonable explanation for the findings, suggested by the non-competitive form of the inhibition, is that antimycin A binding to a *bd* complex renders it kinetically inactive. The inhibited sample therefore would contain a mixture of fully active and fully inhibited enzyme, the proportion depending on the concentration of antimycin A. Some proportion of the fast phase of *b* haem reduction at anaerobiosis is then due to active enzyme molecules that have not bound antimycin A, and the slow phase is due to the reduction of inactive, antimycin A-inhibited enzyme. A similar interpretation for the non-competitive inhibition of cytochrome oxidase by psychosine has been presented [21]. One prediction of this model is that increasing antimycin A concentrations should decrease the proportion of the fast phase of reduction but not significantly affect the rate of slow phase reduction. This is found to be the case, but it should be noted that the amount of fast phase never fell to below 50% at this wavelength (Fig. 4) despite the high inhibition of turnover. It would appear that the reduction of one of the *b* haems is insensitive to antimycin A binding. The spectral changes at 595 nm show this to be haem b_{595}. At all the levels of antimycin used, there was always a fast electron transfer to haem b_{595} when the system became anaerobic. We conclude that the slow spectral change at 560 nm in

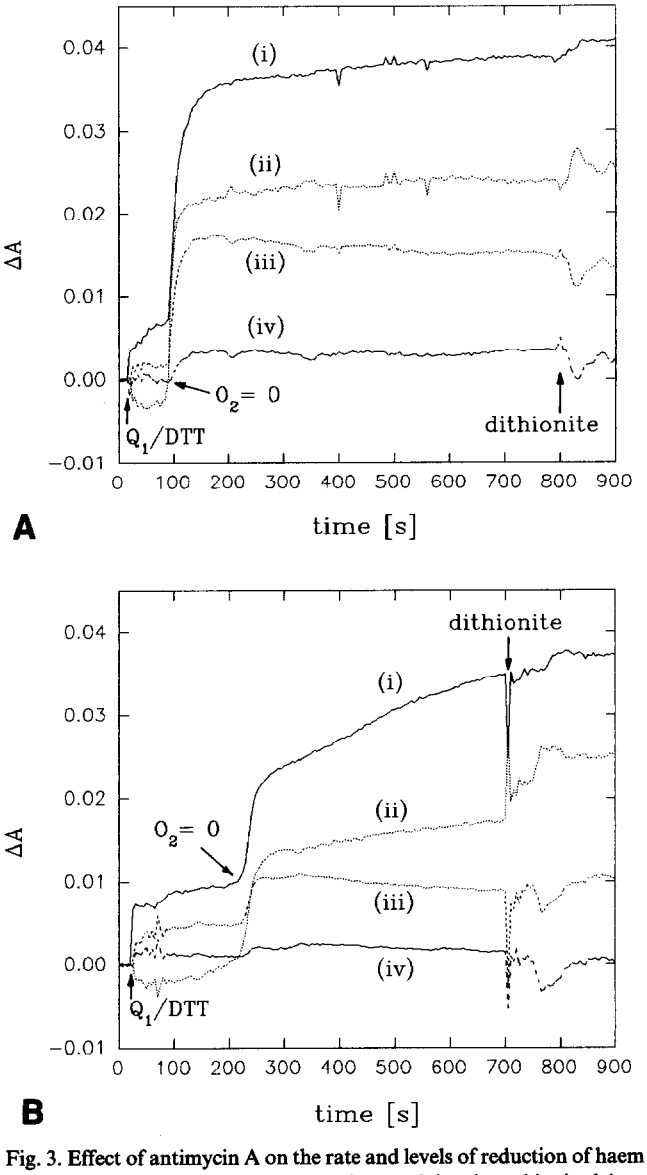

Fig. 3. Effect of antimycin A on the rate and levels of reduction of haem b_{558}, haem b_{595} and haem *d* in cytochrome *bd*, using ubiquinol-1 as respiratory substrate. Ubiquinol-1 (30 μM) plus DTT (3 mM) was added to a solution of cytochrome *bd* (3 μM) in 0.1 M potassium phosphate buffer, pH 7.9, and spectra taken every 10 s by a diode array spectrophotometer (scan time 0.1 s) over the wavelength range 400 to 700 nm. Changes in absorbance with time were then calculated at the following wavelength pairs (see Fig. 2): (i) 560–578 nm; (ii) 604–650 nm; (iii) 628–604 nm; (iv) 595–604 nm. (A) Control, (B) plus antimycin A (328 μM).

the presence of antimycin A must be due to a slow reduction of haem b_{558}. This was confirmed by taking the difference spectrum of dithionite reduced minus 100 s after anaerobiosis. The resulting spectrum was typical of a *b*-type haem with features at 560 nm but none at 595 nm. Similarly, antimycin A did not affect the rate of formation of ferrous haem *d*, monitored at 628 nm. However, when the oxy-form of haem *d* was monitored at 650 nm in the presence of antimycin A, the maximum absorbance change did not occur until dithionite was added. It would appear that some oxy-form of haem *d* is trapped when the system becomes anaerobic and full reduction of the bound oxygen is inhibited.

The lack of effect of antimycin A on the reduction of haem b_{595} and haem *d* whilst inhibiting the reduction of haem b_{558}, as well as the activity of the enzyme, strongly suggests a branched electron transfer pathway for the oxidation of ubiquinol. As pointed out recently by Musser et al. [17], the difficulties of oxidizing a two electron donor by a one electron acceptor can be solved in a split electron transfer pathway. The present results provide experimental evidence for such a pathway in cytochrome *bd* (Fig. 5). We propose that under non-inhibited conditions, the two electron oxidation of ubiquinol involves two one-electron transfer reactions, one directly to haem b_{558} and the other to haem b_{595} (or haem *d*). Antimycin binding to the low potential side of haem b_{558} prevents electron transfer to one of the branches of

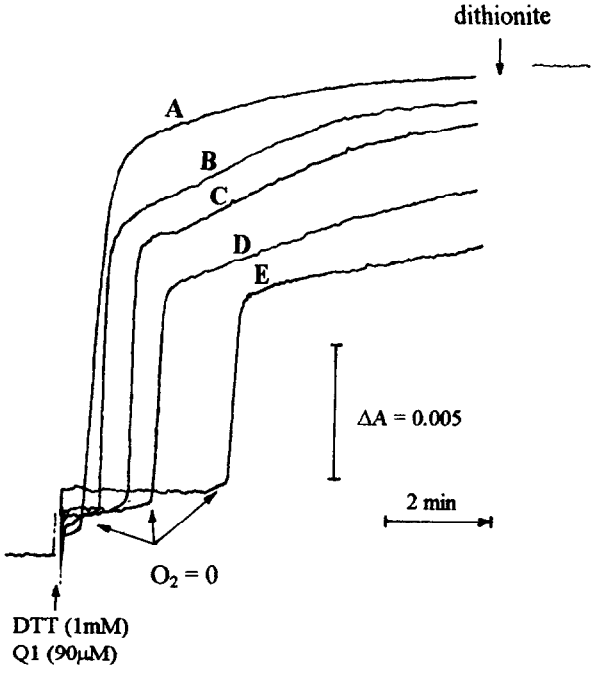

Fig. 4. Effect of antimycin A concentration on the absorbance changes of the *b*-type haems, monitored at 560–578 nm, during the reduction of the cytochrome *bd* complex by ubiquinol-1. Cytochrome *bd* (1.2 μM) was suspended at room temperature in potassium phosphate buffer (0.1 M, pH 7.9) with ubiquinol-1 (90 μM) plus DTT (1 mM) in the absence (A) and presence (B–E) of antimycin A (B, 40 μM; C, 100 μM; D, 200 μM; E, 300 μM).

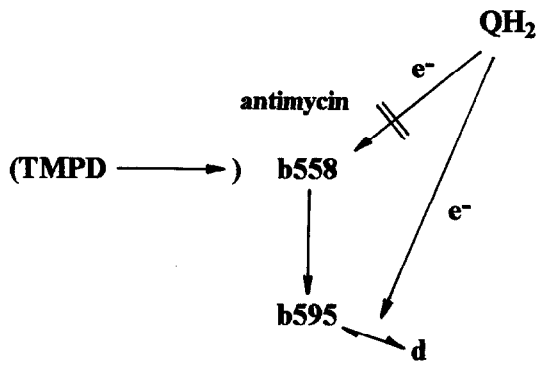

Fig. 5. Proposed branched electron transfer pathway for the oxidation of ubiquinol by cytochrome *bd*. Ubiquinol oxidation involves direct reduction of both b_{558} and the haem b_{595}/haem *d* centre. Antimycin A binds at the low-potential side of haem b_{558}, preventing reduction of b_{558} but not b_{595}. The interruption of the branched pathway by antimycin A can trap partially reduced oxygen intermediates on haem *d*.

the pathway but not to haem b_{595} (and haem *d*). Activity is then inhibited by the inability of the semi-quinone to deliver further electrons to the oxygen reduction centre. Any intermediate forms of oxygen reduction will be trapped on haem *d* and only become reduced on the addition of dithionite.

No inhibitory effect of myxothiazol could be detected on the *bd* oxidase but UHBDT was found to inhibit activity. Spectral experiments with UHBDT were complicated by the absorbance of the reduced form of the inhibitor but essentially similar results to those using antimycin A were observed on anaerobiosis. In cytochrome bc_1, the primary effect of UHBDT binding takes place at the iron-sulphur level, which is not present in cytochrome *bd*. However, UHBDT has also been shown to bind to the bc_1 complex, with lower affinity, at or near the same site as antimycin A [22]. This may be the case for cytochrome *bd*. It is unlikely that a branched electron transfer pathway for the oxidation of ubiquinol in the *bd* oxidase would be involved in any Q-loop mechanism since proton translocation by the *bd* oxidase (in *E. coli*) has not been detected [23]. However, it would be interesting to compare the effects of these inhibitors on the copper- containing quinol oxidases where putative Q-loop mechanisms have been considered [17].

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