The site and mechanism of action of myxothiazol as an inhibitor of electron transfer in *Rhodopseudomonas sphaeroides*

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1. INTRODUCTION

A number of antibiotics have been used as inhibitors in the study of electron transport through the ubiquinone:cyt. *c* oxidoreductase (*b*–c_1 complex) of mitochondria and photosynthetic bacteria. Myxothiazol, a new antibiotic inhibitor, has been introduced to the study of the mitochondrial chain [1–4]. Myxothiazol has been shown to inhibit electron transport through the *b*–c_1 complex of mitochondria or submitochondrial particles, by blocking the reduction of cytochromes *c* and c_1 by succinate or NADH. In the presence of antimycin, but not in its absence, myxothiazol also inhibited reduction of the *b*-type cytochromes by succinate or NADH [3,4]. This pattern of inhibition was quite distinct from that of antimycin, and suggested a different site of action. In confirmation of this, the binding of myxothiazol and of antimycin, as assayed by tritration, were independent [4], and myxothiazol induced a red shift in a *b* cytochrome which was independent of and additive to the antimycin-induced red shift [3]. The structure and physical properties of myxothiazol have also been determined and are given in [2,3].

Cells or chromatophores from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* contain a ubiquinol:cyt. c_2 oxidoreductase which functions in photosynthetic cyclic electron-transport chain. The bacterial *b*–c_1 complex appears to be completely analogous to the mitochondrial *b*–c_1 complex in both structure and function [5–7] and might therefore be expected to be sensitive to the same antibiotics. Here, we present an initial report on the effects of myxothiazol on the bacterial *b*–c_1 complex, and show the site and mechanisms of inhibition in the electron-transfer chain.

2. MATERIALS AND METHODS

The kinetics of flash-induced changes in redox state of components of the chain, and the dependence of these on ambient potential were measured using a computer-linked kinetic spectrophotometer and redox titration assembly as in [8,9]. Redox poising was done as in [10]. Spectra were obtained using a computer-linked scanning spectrophotometer [9]. Antimycin was obtained from Sigma and 2-hydroxy-3-undecyl-1,4-naphthoquinone (UHNQ) was obtained from Aldrich. 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) was a gift from Dr B.L. Trumpower and myxothiazol was a kind gift from Drs Reichenbach, G. Thierbach and W. Trowitzsch.

3. RESULTS AND DISCUSSION

The effect of myxothiazol on the kinetic changes of cyt. $c_1 + c_2$, and cyt. b_{561} following a series of flashes are shown in fig. 1. At an ambient redox potential (E_h) of 100 mV, flash illumination of uncoupled chromatophores induced a rapid oxidation cyt. c_t (cyt. c_2 plus cyt. c_1, measured at

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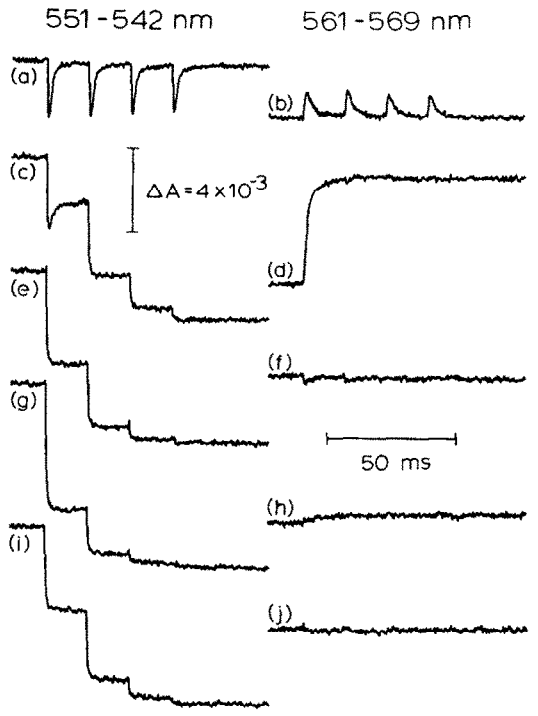

Fig. 1. The effects of inhibitors on the absorption changes of cytochromes c_t and b_{566}. Chromatophores from *Rps. sphaeroides* strain Ga were suspended to 0.56μM [reaction center] in buffer (50mM MOPS, 100mM KCl) at pH 7. Traces are an average of 4, with 100ms sweep full scale, and the instrument response time was 200μs. The potential was adjusted to 113 ± 3mV by small additions of solutions of concentrated potassium ferricyanide or sodium dithionite. The mediators present were as follows: 10μM 1,4-naphthoquinone, 1,2-napthoquinone, duroquinone and *p*-benzoquinone; 2μM DAD; 1μM PMS, PES and pyocyanin. Also present, at 2μM each, were valinomycin and nigericin: (a–f) from one sample; (g–h) from a second sample; (a,b) in the absence of inhibitors; (c,d) obtained from the same sample after the addition of 10μM antimycin; (e,f) cytochrome kinetics after 3μM myxothiazol was added to the sample used for traces (c,d); (g,h) cytochrome changes in the presence of the mediators above and 10μM antimycin and 80μM UHNQ; (i,j) cytochrome changes after the addition of 12μM myxothiazol of the sample used for (g,h).

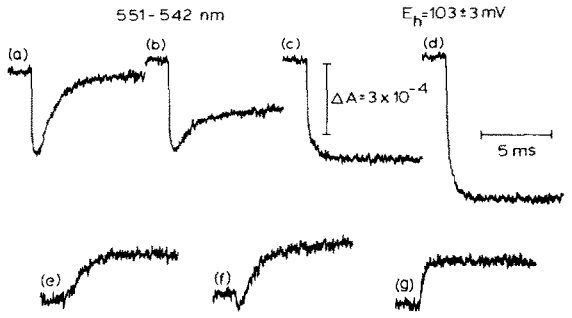

Fig. 2. Kinetics of cytochrome c_t in the presence of several different inhibitors. Chromatophores (0.42μM reaction center) were suspended in the same buffer and with the same mediators as in fig. 1. Traces are an average of 16 (10ms sweep time, instrument response 20μs): (a) absence of inhibitors; (b) presence of 10μM antimycin; (c) presence of myxothiazol (12μM); (d) presence of UHNQ (80μM); (e) difference (a–b) shows the antimycin-sensitive cyt. *c* mediation; (f) difference (b–c) showing the myxothiazol-sensitive phase of cyt. c_t re-reduction which is antimycin-insensitive; (g) difference (c–d) showing the UHNQ-sensitive phase of cyt. c_t re-reduction which is insensitive to both myxothiazol and antimycin.

551–542 nm), which was followed by a complete re-reduction with $t_{1/2}$ 1.5 ms, and a transient reduction and reoxidation of cyt. b_{561} (traces a,b). Addition of antimycin inhibited the oxidation of cyt. b_{561} and partially inhibited the re-reduction of cyt. c_t ($c_1 + c_2$) (c,d). Addition of myxothiazol to antimycin inhibited chromatophores caused a complete inhibition of the reduction of cyt. b_{561} and a greater inhibition of cyt. c_t re-reduction (e,f). UHDBT or UHNQ, added instead of myxothiazol, had a similar effect on the kinetics of the *b*- and *c*-type cytochromes (g,h), but comparison of the effects of UHNQ and myxothiazol on cyt. c_t re-reduction following the first flash, shows that in the presence of myxothiazol a partial re-reduction of the *c*-type cytochromes had occurred which was eliminated by UHNQ, leaving cyt. c_t more oxidized in the presence of UHNQ. The additional re-reduction of cyt. c_t on the first flash seen in the presence of myxothiazol, but not with UHNQ, suggests that a component was able to donate electrons to the *c*-type cytochromes, in a reaction which was insensitive to myxothiazol, but sensitive to UHNQ.

Addition of myxothiazol in the absence or presence of antimycin led to kinetic traces similar to those of fig. 1 (e,f) with a titration showing half-maximal effect at 0.45μM (1.7 myxothiazol/cyt. b_{561}) and complete inhibition at 3μM, independent of the presence of antimycin. In the presence of 80μM UHNQ or 12μM UHDBT giving maximal

inhibition, addition of myxothiazol had little effect at concentrations which produced half-maximal inhibition in the absence of UHNQ or UHDBT. However, as the concentration of myxothiazol was increased, the extra oxidation induced by addition of UHNQ or UHDBT was lost, so that at $>12\mu M$ myxothiazol the kinetic traces in the presence of UHNQ were the same as in its absence (trace (i)). The ability of myxothiazol to reverse the effects of UHNQ or UHDBT suggests that it is able to displace UHNQ and UHDBT from their binding site, and that these inhibitors have a common binding site with myxothiazol.

In fig. 2 kinetic traces are shown on a faster time scale of the cyt. c_t and cyt. b_{561} changes induced by single turnover flash, 90% saturating, in the presence of different inhibitors. From these traces three different phases of cyt. c_t re-reduction can be determined:

(i) The antimycin-sensitive phase (e), shown by the difference between the kinetics with no addition (a), and with antimycin present (b), has a half-time of 0.6 ms, after a lag of 0.5 ms.

(ii) The antimycin-insensitive but myxothiazol-sensitive phase (f) (traces (c–b)), has a similar half-time;

(iii) The UHNQ-sensitive but myxothiazol-insensitive phase of cyt. c_t re-reduction (g), (traces (d–e)) has a half-time of 200–250 μs.

In fig. 3 titrations are shown of the extent of cyt. $c_2 + c_1$ oxidation in the presence of myxothiazol and UHNQ, and the difference, showing the myxothiazol-insensitive, UHNQ-sensitive cyt. c_t reduction following a single flash, as a function of E_h. The titration of the UHNQ-sensitive but myxothiazol-insensitive cyt. c_t re-reduction should show the midpoint(s) of the component(s) providing the extra electrons for the re-reduction of cyt. c_t. This titration curve can be well fitted by a single one-electron component with an E_{m7} of 286 mV, characteristics similar to those of the Rieske iron–sulfur center ($E_{m7} = 290$ mV, $n = 1$).

To discuss the differential effects of the three types of inhibitor used here, we must introduce a model for the mechanism of ubiquinol–cyt. c_2 oxidoreductase. We have suggested a modified Q-cycle model with the following features [11,12]:

(1) The complex acts to oxidize ubiquinol from the pool, and reduce cyt. c_2 in reactions which are kinetically independent of the photochemical reaction center.

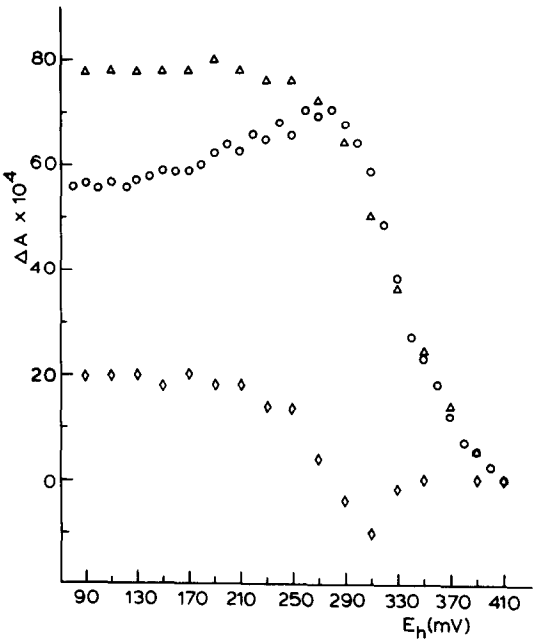

Fig. 3. A redox titration of the extent of cytochrome c_t oxidation in the presence of UHNQ or myxothiazol. Chromatophores (0.43 μM reaction center) were suspended in the same buffer and with the same mediators as in fig. 1. Antimycin A (10 μM) was also added. The redox potential was adjusted by small additions of solutions of concentrated sodium dithionite or potassium ferricyanide. The extent of oxidation of cyt. c was measured 32 ms after a single 90% saturating flash from traces (av. 2, sweep = 100 ms, 200 μs instrument response time) obtained at each potential shown. Cytochrome c_t kinetics were measured at 551–542 nm: (△) oxidation in the presence of UHNQ (80 μM); (○) oxidation in the presence of myxothiazol (12 μM); (◇) difference in the extent of oxidation between UHNQ and myxothiazol.

(2) The complex has two catalytic sites at which quinone and quinol from the pool can react:

Site (i) is a ubiquinol:FeS, ferricyt. b_{566} oxidoreductase site, catalyzing a concerted reaction in which one electron from QH_2 is passed to a high potential chain of electron carriers in series consisting of FeS, cyt. c_1, cyt. c_2 (or cyt. c) and P (or cytochrome oxidase), and a second electron is passed to a low potential chain consisting of cyt. b_{566} and cyt. b_{561};

Site (ii) catalyzes a ferricyt. *b*: ubiquinone oxidoreductase reaction, in which two electrons stored on the *b*-type cytochromes can reduce ubiquinone to the quinol.

We have suggested that antimycin completely blocks all reaction at site (ii), possibly by displacing the quinone and quinol species from their binding site [7].

(3) To turn over completely, the quinol oxidase site has to turn over twice (introducing 2 electrons into the *b*-cytochrome chain), and the quinone reductase site turns over once, to give a net oxidation of 1 quinone/complex.

(4) In the presence of antimycin, electrons cannot leave the *b*-cytochrome chain, and the quinol oxidase site is constrained to <2 turnovers for thermodynamic reasons.

Based on the above results, we suggest that myxothiazol binds to the ubiquinol oxidase site of the b–c_1 complex, as do UHNQ and UHDBT. Unlike the quinone analogues, myxothiazol does not effect the redox properties, or inhibit oxidation, of the Rieske FeS center, but does inhibit its reduction by ubiquinol from the pool. We further suggest that the component providing electrons for the re-reduction of cyt. c_1 in the presence of myxothiazol is the Rieske FeS center, since both UHDBT and UHNQ inhibit the oxidation of the Rieske FeS center, but have no effect on the oxidation of the *c* cytochromes [13,14]. From these facts we conclude that the Rieske FeS center can be oxidized in the presence of myxothiazol. In terms of a Q-cycle model, this proposed site of action would also explain the inhibition of cyt. b_{561} reduction, since binding of myxothiazol to the ubiquinol oxidase site would prevent the binding of QH_2, and its oxidation, which are necessary for the reduction of cyt. b_{561}. If our proposal is correct, then the rate of oxidation of the Rieske FeS center can be measured by the difference between the cyt. c_1 kinetics measured in the presence of UHNQ, which prevents the oxidation of the FeS center, and the kinetics measured in the presence of myxothiazol, which inhibits the reduction of the FeS center. This difference is shown in fig. 2g and gives a half-time of 200 μs for the oxidation of the Rieske FeS center, after a lag of ~50 μs. This half-time fits nicely with a scheme in which the FeS center, and cytochromes c_1 and c_2 form a linear chain, and with the measured half-times of oxidation of cytochrome c_2 (4–10 μs), and cytochrome c_1 (150 μs) [5].

In fig. 4 difference spectra are shown induced by addition of the antimycin or myxothiazol to chromatophores with cytochrome *b* reduced. In the presence of excess dithionite the addition of antimycin induced a shift in the absorption of the *b* cytochrome(s) as reported in [15] (a). Further addition of myxothiazol to this sample produced a narrowing of the shift (b). The difference (c) shows a blue shift with a maximum and minimum at 562 nm and 568 nm, respectively, and a crossover point at 565 nm. The myxothiazol-induced shift was independent of the presence of antimycin and the antimycin-induced shift was independent of myxothiazol as is shown in (d–f). In (d) the addition of myxothiazol induced a blue shift. The

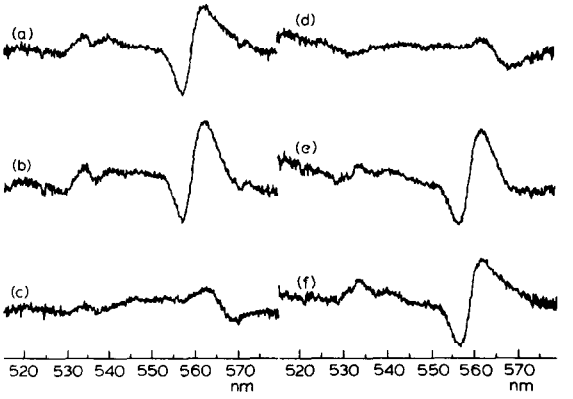

Fig. 4. Difference spectra of changes in *b*-type cytochromes induced by addition of inhibitors. Chromatophores were suspended to ~3 μM reaction center in 50 mM MES, 100 mM KCl (pH 6) in an open cuvette, a crystal of sodium dithionite was added, and the pH adjusted to 5.75 ± 0.03: (a–c) represent one sample; (d–f) a second sample; (a) difference between the spectrum with antimycin (10 μM) and that without inhibitor; (b) difference between the spectrum with antimycin and myxothiazol (12 μM), and that without inhibitors; (c) is difference (a–b); (d) difference between the spectrum with myxothiazol (12 μM) and that without inhibitor; (e) is the same difference as shown in (b), but after the addition of antimycin (10 μM) to the myxothiazol-treated sample; (f) difference (d–e).

subsequent addition of antimycin (e) gave an apparently narrowed antimycin shift, the difference (f) gives the normal antimycin shift. Preliminary results from redox titrations suggest that the shift induced by myxothiazol is a blue shift in the 566 nm peak of cytochrome b_{566} (double α band at 559 nm and 566 nm, $E_{m7} = -90$ mV), and that myxothiazol may also change the value of E_m for cyt. b_{566} by 30–60 mV (from -90 to -45 ± 15 mV). Myxothiazol has no effect on the spectra or midpoints of the other *b* cytochromes.

These results provide several clues as to the relative positions and reactions of the *b*-type cytochromes and the Rieske FeS center. Since myxothiazol reacts with cyt. b_{566}, and displaces UHNQ or UHDBT from the Rieske FeS–center, it appears that these redox components must be relatively close to each other, and that their proteins may form the reaction site at which ubiquinol from the pool is oxidized [11–13]. The main effect of antimycin is to induce a red shift in cyt. b_{561}, although the broader red lobe of the antimycin-induced difference spectrum suggests that the spectral change may contain a small contribution from cyt. b_{566}. The independence of the effects of myxothiazol and antimycin, both with respect to titration, and to induced band shift, show that the two inhibitors bind at different sites. We may conclude that the heme of cyt. b_{561} is closely associated with the antimycin-sensitive site, identified as the ferrocyt. *b*:ubiquinone oxidoreductase site of the complex. This distribution of cyt. *b* hemes is consistent with our earlier suggestion that cyt. b_{566} and cyt. b_{561} form a serial electron-transfer chain across the membrane connecting the ubiquinol oxidase and ubiquinone reductase sites of the chromatophore complex [11,12], as has also been suggested for the mitochondrial complex [6,16,18].

4. SUMMARY

Myxothiazol will provide a useful tool in the study of electron transport through the bacterial cyt. b–c_1 complex. Its mode of inhibition is unlike that of the other classes of inhibitor so far characterized. It allows the oxidation of the Rieske FeS center while blocking the ubiquinol oxidase reaction site and so inhibits the reduction both of the *b*-type cytochromes and of the Rieske FeS center. It also alters the spectral properties of a *b* cytochrome, but is independent of antimycin. Finally myxothiazol appears to have a specific binding site of high affinity and completely inhibits electron transport at 3 myxothiazol/oxidoreductase complex. The suggested mode of action explains the effects of myxothiazol observed on mitochondrial electron transfer, and on the kinetics of the isolated cyt. b–c_1 complex, and our model is compatible with suggestions for the site of action suggested from work with mitochondrial systems [3,4].

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