An Inhibitor of Mitochondrial Respiration Which Binds to Cytochrome b and Displaces Quinone from the Iron-Sulfur Protein of the Cytochrome bc₁ Complex*

(Received for publication, December 9, 1983)

Gebhard von Jagow‡§, Per O. Ljungdahl‡, Paul Graff, Tomoko Ohnishii, and Bernard L. Trumpower†

From the ‡Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03756, the §Institute of Physical Biochemistry, University of Munich, Goethestrasse 83, 8000 Munich 2, West Germany, and the †Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Myxothiazol, an antibiotic from Myxococcus fulvus, which inhibits mitochondrial respiration in the bc₁ complex of the respiratory chain, has effects on the redox components of isolated succinate-cytochrome c reductase complex which suggest that it interacts with both cytochrome b and the iron-sulfur protein of the bc₁ complex. The inhibitor appears to increase the midpoint potentials of cytochromes b-562 and b-566, as indicated by an increase in their reducibility by the succinate/fumarate couple. It also causes a red shift in the optical spectrum of ferrocytochrome b-566, as reported previously (Becker, W. F., Von Jagow, G., Anke, T., Steglich, W. (1981) FEBS Lett. 132, 329–333). This red shift is enhanced by Triton X-100, and there is no shift in the spectrum of b-566. These results are consistent with evidence that mutations conferring myxothiazol resistance in yeast map to the mitochondrial gene for cytochrome b (Thierbach, G., and Michaelis, G. (1982) Mol. Gen. Genet. 186, 501–506).

In addition, myxothiazol has effects on reduction of the cytochromes b and c₁ by succinate or ubiquinol which are identical to those caused by removal of the iron-sulfur protein from the bc₁ complex. It blocks reduction of cytochrome c₁ during single and multiple turnovers of the bc₁ complex, but does not block reduction of the b cytochromes. In the presence of antimycin, it blocks reduction of both cytochromes b and c₁. In contrast to antimycin, myxothiazol inhibits oxidant-induced reduction of both b cytochromes and does not inhibit their oxidation by fumarate.

Myxothiazol also inhibits reduction of the iron-sulfur protein by ubiquinol and shifts the g₇ resonance in the EPR spectrum of the iron-sulfur protein from g = 1.79 to 1.76. It does not affect the midpoint potential of the iron-sulfur protein, but does eliminate the increase in midpoint potential which is caused by inhibitory hydroxyquinones which bind to the iron-sulfur protein.

The effects of myxothiazol are consistent with a protonmotive Q cycle pathway of electron transfer in which myxothiazol binds to cytochrome b and displaces quinone from the iron-sulfur protein of the bc₁ complex. These results suggest either that a myxothiazol-induced conformational change in cytochrome b is transmitted to a quinone binding site on the iron-sulfur protein, or that there is a quinone binding site which consists of peptide domains from both cytochrome b and iron-sulfur protein.

Myxothiazol is an antifungal antibiotic isolated from the myxobacterium Myxococcus fulvus (1). It is the most potent of a family of antibiotics (2, 3), including oudemansin (4) and the strobilurins (5), which contain a β-methoxyacrylate system as a common structural feature (2, 5), and which appear to act at a common site in the respiratory chain of eukaryotes (3).

Myxothiazol is cytotoxic to eukaryotic cells, but generally ineffective at inhibiting growth of Gram negative or Gram positive bacteria (6). It blocks O₂ consumption in yeast and fungi when these are grown aerobically (6). This growth inhibition is prevented and reversed by glucose, which allows fermentative growth.

Myxothiazol blocks mitochondrial respiration in the cytochrome bc₁ segment of the respiratory chain (7). Addition of myxothiazol to beef heart mitochondria respiring on endogenous substrate(s) or yeast respiring on ethanol resulted in complete oxidation of cytochromes c₁, c, and cytochrome oxidase, while the b cytochromes remained reduced. This "crossover" between the b and c cytochromes was different from that caused by antimycin, in that myxothiazol did not cause the "extra reduction" not the shift in the optical spectrum of the b cytochromes which are caused by antimycin (7).

When NADH was added to submitochondrial particles treated with myxothiazol, cytochrome b-562 was reduced but b-566 was not (8, 9). This was also in contrast to the effect of antimycin, which enabled extensive reduction of b-566 in addition to b-562. When myxothiazol was added in combination with antimycin, the two inhibitors completely abolished reduction of both b cytochromes by NADH (8, 9).

There is one high affinity binding site (Kₐ ≤ 1 × 10⁻⁹ M) for myxothiazol in the bc₁ complex of mitochondria from beef heart (8, 9) and yeast (7). This site is distinct from the antimycin site, as shown by the effects of the inhibitors on the optical spectra of the b cytochromes. Antimycin shifts the absorption maximum of the α band of ferrocytochrome b-562 to 564 nm (10, 11). Myxothiazol shifts the absorption maximum of ferrocytochrome b-566 to a longer wavelength, and

* This investigation was supported by National Institutes of Health Grant GM 23379 and National Science Foundation Grant PCM 81-17284. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 One exception to this generality is that myxothiazol blocks respiration in Pseudomonas denitrificans, a Gram negative bacteria which contains a respiratory chain very similar to that of mitochondria (E. Berry, D. Munt, and B. L. Trumpower, manuscript in preparation).
the bathochromic shifts caused by antimycin and myxothiazol are additive (8, 9).

Although it is clear that antimycin and myxothiazol act at different sites, the site of myxothiazol binding and its effects on electron transfer reactions within the bc1 complex are not adequately established. Many of the effects cited above indicate that myxothiazol binds to cytochrome b. This is further supported by the findings that in yeast (12) and cultured mouse cells (13) resistance to myxothiazol results from a mutation in the mitochondrial gene for cytochrome b.

On the other hand, some of the effects of myxothiazol, such as the inhibition of cytochrome c1 reduction, suggest an effect on the iron-sulfur protein of the bc1 complex (14). In photosynthetic bacteria, myxothiazol relieves the inhibitory effect of hydroxyquinones on re-reduction of cytochromes c1 + c during flash-induced turnover (15), as if the latter inhibitors are displaced from their binding site, which is on the iron-sulfur protein of the bc1 complex (16, 17).

Although there is agreement that myxothiazol inhibits cytochrome b reduction when added in the presence of antimycin (8, 9, 15, 18), there are conflicting reports regarding the effect of myxothiazol on reduction of cytochrome b in the absence of antimycin. In contrast to earlier reports (8, 9), De Vries and coworkers (18) reported that myxothiazol blocks reduction of a portion of b-562. This apparent contradiction is especially significant, since inhibition of a portion of b-562 reduction is a critical piece of evidence in support of a "dimeric Q cycle" mechanism of electron transfer in the bc1 complex (18), and which distinguishes such a mechanism from earlier versions of the Q cycle (20–23).

In order to better understand how myxothiazol acts in the bc1 complex, and to clarify some of the above mentioned discrepancies, we have examined the effects of myxothiazol on oxidation-reduction reactions and spectral and thermodynamic properties of the cytochromes b, c1, and iron-sulfur protein in isolated succinate-cytochrome c reductase complex.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antimycin, ascorbic acid, cytochrome c (Type III), and succinic acid were obtained from Sigma. Myxothiazol was a gift of Drs. H. Reichenbach and W. Trowitzsch of the Gesellschaft für Biotechnologische Forschung (Braunschweig, West Germany). Myxothiazol and antimycin were dissolved in ethanol and dimethyl sulfoxide for optical and EPR spectroscopy experiments, respectively, and their concentrations determined spectrophotometrically (1, 24).

**Preparation of Succinate-Cytochrome c Reductase**—Succinate-cytochrome c reductase was isolated from beef heart mitochondria. Using our earlier method (25), we recently encountered a 25 to 50% loss of cytochrome c reductase activity after removing cholate by dialysis and freezing at ~70 °C. Part, but not all, of this activity could be restored by incubating the thawed reductase with phospholipid plus ubiquinone-10. To avoid this problem, we modified our preparation as follows.

After allowing the partially purified reductase to stand overnight at 4 °C and removing the aggregated cytochrome oxidase by centrifugation, the resulting dark red reductase was diluted with an equal volume of glycerol, rather than dialyzed (see Ref. 25). The reductase was then stored at ~10 °C in 50 mM sodium phosphate, 0.25 mM EDTA, 0.02% cholate, 50% glycerol, pH 7.4. The reductase remained fully active when stored in this manner for at least 6 months. In fact, during the first 10 days of storage there was a 15 to 25% increase of cytochrome c reductase activity. This increase was possibly due to dissociation of bound cholate, since during this period the initially transmembrane reductase became slightly turbid.

**Optical Spectroscopy**—Rates of oxidation-reduction and absorption spectra of the cytochromes were measured at ambient temperature in an open-stirred cuvette (mixing time < 1 s) on an Aminco DW 2a dual wavelength spectrophotometer with a 2-nm band pass (26). Data was collected on an Explorer III digital storage oscilloscope equipped with a floppy disk memory (Nicolet Instrument Co.). The spectrophotometer was interfaced to the oscilloscope through an optical encoder attached to the monochromer drive such that data points were collected at 0.1-nm intervals.2 The wavelength was calibrated by the emission peak of the deuterium lamp at 486 nm.

As in previous experiments (26), the oxidation-reduction status of the cytochromes was checked before and after each reaction. Base lines and spectra were stored on the floppy disk and, where indicated, difference spectra were obtained by subtraction on the oscilloscope.

**Redox Potentiometry and Electron Paramagnetic Resonance Spectroscopy**—EPR spectra were obtained with a Varian E-109 X-band spectrometer as described previously (17). Details of the spectrometer settings are described in the figure legends. Potentiometric titrations of reductase complex and collection of samples for EPR spectroscopy were carried out with the apparatus described by Dutton (27). Measurements used to equilibrate the reductase to the potentiometric electrode were also as described previously (17).

**RESULTS**

**Effects of Myxothiazol on Reduction of the Cytochromes by Succinate and Ubiquinol**—Myxothiazol blocks reduction of cytochrome c1, but does not block reduction of cytochrome b, when succinate is added to isolated succinate-cytochrome c reductase. The traces in Fig. 1, a and c, show reduction of the cytochromes b and c1, respectively, when succinate is added to the reductase in the absence of inhibitor. As reported previously (14, 17, 26), approximately 70% of dithionite reducible b and all of c1 are reduced in less than 1 s.

When myxothiazol is added before succinate, it blocks reduction of c1 (Fig. 1d), but does not block reduction of cytochrome b (Fig. 1b). Under these conditions the cytochrome b which is reduced in the absence of inhibitor consists primarily of b-562 and a small portion of the low potential b-566 (28). As can be seen by comparing the traces in Fig. 1, a and b, addition of myxothiazol before succinate causes an increase in the amount of b which is rapidly reduced. If the inhibitor is added after reduction with succinate, there is a slow increase in b reduction (Fig. 1a), such that the amount of b finally reduced is identical when myxothiazol is added before or after succinate.

This increase in b reduction is due to an increase in the amount of b-566 which is reducible by succinate in the presence of myxothiazol, as shown by the absorption spectra in Fig. 2. The spectrum of the succinate reduced cytochromes, in the absence of inhibitor, is shown in Fig. 2a. When myxothiazol was added before succinate (Fig. 1b), the spectrum at the completion of the reaction (Fig. 2b) shows lack of c1 reduction and increased b reduction. When myxothiazol was added after succinate (Fig. 1c), the spectrum (Fig. 2c) again shows increased b reduction, and also confirms that c1 was reduced under these conditions. A difference spectrum of the "extra" b reduced after addition of myxothiazol is shown in Fig. 2d. The absorption maximum (565.5 nm) and the shoulder at approximately 558 nm are typical of the low potential b-566 (29).

We also tested the effects of myxothiazol on reduction of the cytochromes by a synthetic analogue of ubiquinol-2 (14). In the absence of inhibitor, ubiquinol rapidly reduced cytochrome c1 and approximately 40% of the dithionite reducible b. This b consists solely of b-562, owing to the relatively high potential of the quinone/quinol couple. Myxothiazol had the same effects as when succinate was used as substrate; it blocked reduction of c1, but did not block reduction of b (results not shown). Again, myxothiazol caused an increase in b reduction when added either before or after ubiquinol. In this instance the difference spectrum of the extra b reduced had a maximum at 562 nm and no shoulder (results not shown). These results suggest the inhibitor enables increased

---

2 The encoder was designed and constructed by Dr. Edward Berry, for whose assistance we are appreciative.
Mechanism of Myxothiazol Inhibition of Respiration

**FIG. 1. Effects of myxothiazol on reduction of cytochromes b and c₁ by succinate.** The traces in a and b show reduction of cytochrome b, monitored at 563 versus 579 nm. The traces in c and d show reduction of cytochrome c₁, monitored at 553 versus 559 nm. Reductase complex was suspended at 0.69 mM cytochrome c₁ in 100 mM sodium phosphate, 0.5 mM EDTA, pH 7.5, containing 0.5% sodium cholate. The oxidation-reduction status of the cytochromes was checked prior to each reaction and, if necessary, a minimal amount (≤2 μM) of ferricyanide was added to oxidize the cytochromes. Reactions were started by adding 5 mM succinate. Where indicated, 2 μM myxothiazol was added. Discontinuities in the kinetics traces indicate discontinuities in the time scales.

Reduction of b-562 by ubiquinol, analogous to its effect on reduction of b-566 by succinate.

To further resolve the effects of myxothiazol on the two b cytochromes, we used mixtures of succinate plus fumarate in varying ratios to adjust the oxidation-reduction poise of the cytochromes and then examined the effects of the inhibitor on the absorption spectra and oxidation-reduction status of the b cytochromes at different applied potentials. Succinate plus fumarate in a ratio of 1:200 ($E_\text{a} = +100 \text{ mV}$) reduced approximately 20% of the dithionite reducible b. As expected, at this relatively high potential only b-562 was reduced. Addition of myxothiazol then caused a slow increase in b reduction, analogous to that in Fig. 1a, until a new equilibrium was reached in which approximately 25% of the b was reduced. Absorption spectra before and after addition of myxothiazol, and a calculated difference spectrum, showed this extra b to be b-562. There was no shift in the absorption maximum of the ferrocytochrome b-562 coincident with this increased reduction (results not shown).

When the cytochromes were equilibrated to succinate plus fumarate in a ratio of 10,000:1 ($E_\text{a} = -87 \text{ mV}$), myxothiazol caused increased reduction of b-566 identical to that in Fig. 1a. The increased reduction of b-566 was accompanied by a bathochromic shift in the optical spectrum of this cytochrome. However, in contrast to the marked bathochromic shift which was observed with complex III (3, 8, 9), myxothiazol caused only a small shift in the optical spectrum of b-566 in succinate-cytochrome c reductase, such that this shift was barely perceptible in a calculated difference spectrum (Fig. 3, b-d).

One difference between these two preparations is that the complex III is prepared in Triton X-100 (32), while the reductase is prepared in cholate (25). We thus tested and found that the bathochromic shift induced by myxothiazol is significantly enhanced by addition of Triton to the reductase, as can be seen in the calculated difference spectrum in Fig. 3, d-c.

We also tested the effects of myxothiazol on reduction of the cytochromes when added in combination with antimycin. In agreement with previous reports (8, 9), myxothiazol in combination with antimycin completely blocked reduction of both cytochromes b and c₁ by succinate and by ubiquinol. If the two inhibitors were added after reduction of the cytochromes, their addition did not cause reoxidation of the cytochromes (see Fig. 5c). Likewise, if myxothiazol was added in combination with 2-nonyl-4-hydroxyquinoline-N-oxide, the combination of these two inhibitors completely blocked reduction of both cytochromes b and c₁ (results not shown).

**FIG. 2. Absorption spectra showing effects of myxothiazol on reduction of cytochromes b and c₁ by succinate.** The spectrum in a was taken after reduction by succinate, in the reaction shown in Fig. 1a, and before addition of myxothiazol to the succinate-reduced complex. The spectrum in b was taken at the end of the reaction shown in Fig. 1b, in which myxothiazol was added before reduction by succinate. The spectrum in c was taken at the end of the reaction shown in Fig. 1c, after myxothiazol was added to the succinate-reduced complex. The spectrum in d is a calculated difference spectrum, in which the spectrum in a was subtracted from that in c, showing increased reduction of b-566 which results when myxothiazol is added to the succinate-reduced complex.

**FIG. 3. Effect of myxothiazol on the optical spectrum of cytochrome b-566 in the absence and presence of Triton X-100.** Reductase complex was suspended at 1.5 μM cytochrome c₁ and reduced by addition of dithionite. Spectra a and b are of reductase suspended in 100 mM sodium phosphate, 0.5 mM EDTA, 0.5% cholate, pH 7.2, before and after addition of 5 μM myxothiazol, respectively. Spectra c and d are of reductase suspended in 20 mM MOPS, 100 mM sodium chloride, 0.5% Triton X-100, pH 7.2, before and after addition of 3 μM myxothiazol, respectively. Difference spectra of the myxothiazol treated minus untreated samples are shown beneath the spectra.
Mechanism of Myxothiazol Inhibition of Respiration

FIG. 4. Effect of myxothiazol on oxidant-induced reduction of cytochrome b-562. The trace in a is a control, showing reduction of cytochrome b by succinate, when cytochrome c1 is previously reduced by ascorbate. The trace in b shows that after adding antimycin and reducing cytochrome c with ascorbate, succinate fails to reduce cytochrome b, which then is reduced by oxidant-induced reduction when ferricyanide is added. The trace in c shows that myxothiazol does not block reduction of cytochrome b by succinate when c1 is previously reduced by ascorbate. The trace in d shows that myxothiazol blocks the ferricyanide-induced reduction of cytochrome b which is otherwise observed in the presence of antimycin when c1 is reduced by ascorbate. Reduction of cytochrome b is rapidly reduced by succinate (Fig. 4c). Comparison of the trace in Fig. 4d with that in b shows that myxothiazol blocks the oxidant-induced reduction of b-562.

The traces in Fig. 5 show oxidant-induced reduction of cytochrome b-566 and inhibition of this reaction by myxothiazol. The trace in Fig. 5a is a control, in which the high potential portion of the b cytochromes is reduced by succinate, after which antimycin is added. Subsequent addition of a high potential oxidant, in this instance cytochrome c1, then elicits oxidant-induced reduction of cytochrome b-566 (31).

Absorption spectra of the reduced cytochromes before and after addition of cytochrome c, and a calculated difference spectrum, are shown to the right of the kinetics trace (Fig. 5a). The difference spectrum shows a peak at approximately 566 nm, characteristic of the low potential b (28, 29), and a second peak at approximately 550 nm. The latter is due to reduction of cytochrome c, which occurs at a slow rate during the interval required to scan the spectrum after addition of cytochrome c. If ferricyanide is used to elicit oxidant-induced reduction of b-566, the resulting difference spectrum has a maximum at 566 nm and a shoulder at approximately 558 nm (see Fig. 2 in Ref. 31).

If myxothiazol is added after reduction with succinate, subsequent addition of cytochrome c does not elicit oxidant-induced reduction of b-566 (Fig. 5b). If myxothiazol is added in combination with antimycin after reduction with succinate, the former inhibitor blocks the oxidant-induced reduction of b-566 which is otherwise elicited by addition of cytochrome c (Fig. 5c).

Effects of Cytochrome b-566 on Oxidation of Cytochrome c1 by Ferri cyanide and Re-reduction by Succinate—Inhibitors may have different effects on electron transfer reactions in the bc1 complex under conditions of single and multiple turnovers. For instance, antimycin does not block reduction of cytochrome c1 during the first turnover of the complex, but does inhibit during subsequent turnovers (26). In order to test the effect of myxothiazol on electron transfer from succinate to cytochrome c1 during multiple turnovers of the bc1 complex, we examined its effect on re-reduction of the cytochrome following oxidation by ferricyanide.

Myxothiazol blocked re-reduction of cytochrome c1. In the presence of the inhibitor, ferricyanide caused complete oxidation of the cytochrome and the slow re-reduction was retarded such that c1 was only 50% reduced after approximately 5 min (Fig. 6b).

Effects of Antimycin and Myxothiazol on Oxidation of Cytochrome b by Fumarate—When cytochrome b is reduced by an appropriately low concentration of succinate, subsequent addition of fumarate raises the potential of the succinate/fumarate couple and oxidizes the cytochrome b (Fig. 7a). The range of potentials which can be spanned by the succinate/fumarate couple is such that approximately 60% of the succinate reducible b can be oxidized by fumarate. As shown in Fig. 7b, antimycin inhibits oxidation of cytochrome b by fumarate. Myxothiazol, however, does not inhibit oxidation of cytochrome b by fumarate (Fig. 7c).

Effects of Myxothiazol on the Iron-Sulfur Protein of the Cytochrome bc1 Complex—The effects of myxothiazol on the optical and thermodynamic properties of the b cytochromes suggest that the inhibitor binds to cytochrome b. However,
Mechanism of Myxothiazol Inhibition of Respiration

The effects of the inhibitor on the oxidation-reduction reactions of the cytochromes, both in the absence and presence of antimycin, are identical to those which result from removal of the iron-sulfur protein from the bc, complex (For a review see Ref. 23). It was thus of interest to test more directly how the inhibitor might interact with the iron-sulfur protein.

In agreement with the findings of De Vries and coworkers (18) in submitochondrial particles, myxothiazol inhibited the rate of reduction of the iron-sulfur protein in isolated succinate-cytochrome c reductase. When ubiquinol was added to isolated reductase in the absence of inhibitor, the iron-sulfur protein was 100% reduced in \( \pm 15 \) s at 4 °C before freezing the sample for EPR spectroscopy. In the presence of the inhibitor, the iron-sulfur protein was less than 50% reduced in this same interval, as judged by the intensity of the \( g = 1.90 \) signal (results not shown).

Myxothiazol also shifted the \( g_s \) resonance in the EPR spectrum of the iron-sulfur protein from 1.79 to 1.76 (Fig. 8). This shift is similar to that caused by UHDBT. However, close examination of the spectra reveals that the \( g_s \) resonance is somewhat broader in the presence of myxothiazol than in the presence of UHDBT (Fig. 8).

The shift in \( g_s \) resonance caused by UHDBT (17) and analogous hydroxyanthraquinones (33) is thought to result from a displacement of ubiquinone from a binding site on the iron-sulfur protein (23). Since myxothiazol bears no such obvious structural similarity to ubiquinone, it was of interest to test whether the resonance shift caused by this inhibitor also results from displacement of quinone from the iron-sulfur protein.

Hydroxyquinones increase the midpoint potential of the iron-sulfur protein by preferentially binding to the reduced form of the protein (16, 17, 33). This effect of UHDBT is shown in Fig. 9, in which the binding of the hydroxyquinone caused an increase in midpoint potential of approximately 100 mV. Myxothiazol does not change the midpoint potential of the iron-sulfur protein (Fig. 9). However, when myxothiazol was added in combination with UHDBT, there was also no significant change in midpoint potential compared to the control (+260 mV, Fig. 9), indicating that the former inhibitor eliminates the change in potential caused by the latter.

---

Footnote:
2 The abbreviations used are: UHDBT, 5-n-undecyl-6-hydroxy-4,7-dioxobenzotheiazole; MOPS, 4-morpholinepropanesulfonic acid.
FIG. 6. Effects of myxothiazol on oxidation of cytochrome c1 by ferricyanide and re-reduction by succinate. The kinetics traces in a and b show reduction of c1 by succinate and the effect of subsequent addition of 10 μM ferricyanide. Discontinuities in the kinetics traces indicate discontinuities in the time scale. The black bars under the kinetics traces indicate the time intervals during which absorption spectra were scanned. The spectra to the right of the kinetics traces are difference spectra calculated by subtracting the spectra of the reoxidized complex, after addition of ferricyanide, from that of the succinate-reduced complex, before addition of ferricyanide. Concentrations of reductase complex, succinate, and inhibitors were as described in the legend to Fig. 4.

**DISCUSSION**

The property of myxothiazol which is uniquely characteristic of this antibiotic is that it binds with high affinity to a single site (7–9), and yet its effects on the redox components of the bc1 complex suggest that it interacts with at least two polypeptides of the complex. One polypeptide with which myxothiazol appears to interact is cytochrome b. On the basis of the heme content of the purified protein (34) and its molecular weight as deduced from the sequence of the mitochondrial gene (35–37), it is likely that cytochrome b is a single polypeptide containing the hemes of both b-562 and b-566 (38). Thus, the simultaneous increased reducibility of both of these hemes (Figs. 1 and 2) can be attributed to an increase in their midpoint potentials caused by binding of the inhibitor to the cytochrome b polypeptide, the binding being tighter when the hemes are reduced. Indeed, since these experiments were carried out, Kunz and Konstantinov (39) have reported that myxothiazol causes a small (15 to 30 mV) increase in the midpoint potentials of the b cytochromes in potentiometric titrations.

The bathochromic shift in the optical spectrum of b-566 also indicates binding of the inhibitor to the cytochrome. This shift, which is not seen in the spectrum of b-562, is subtle and apparently depends on exposure of the bc1 complex to Triton X-100. This may explain why a different shift was observed in the optical spectrum of the b cytochromes in photosynthetic bacteria (15), and why the change in the optical spectrum of submitochondrial particles appears to be due to increased reduction of b-566, rather than a true spectral shift (compare Fig. 4 in Ref. 7 with Fig. 3 above). How Triton X-100 enables myxothiazol to change the heme ligand environment of b-566 remains to be investigated.

The effects of myxothiazol on the thermodynamic and optical properties of the cytochrome b hemes are most simply explained by binding of the inhibitor to this protein. A myxothiazol binding site on cytochrome b would also account for the fact that resistance to the inhibitor maps on the mitochondrial gene for this protein (12, 13).

However, our results indicate that myxothiazol also appears to interact with the Rieske iron-sulfur protein, in that the inhibitor affects the oxidation-reduction reactions of the cytochromes in the same manner as does removal of this protein from the bc1 complex (14, 23). Myxothiazol inhibits reduction of cytochrome c1 during single (Fig. 1) and multiple (Fig. 6) turnovers of the bc1 complex. It does not inhibit b reduction, either by succinate or by ubiquinol, when added in the absence of antimycin (Fig. 1), but does inhibit b reduction in the presence of antimycin (results not shown). These effects of myxothiazol on b reduction are generally confirmatory of previously reported results (8, 9, 15, 40), except for those of De Vries and coworkers (18), who reported that myxothiazol inhibits a portion of b-562 reduction. An explanation for this difference in results is discussed below.

We have also shown that myxothiazol inhibits oxidant-induced reduction of both b-562 (Fig. 4) and b-566 (Fig. 5). It does not inhibit reduction of cytochrome b when c1 is pre-reduced by ascorbate (Fig. 4), and does not inhibit reoxidation...
Mechanism of Myxothiazol Inhibition of Respiration

Fig. 8. Effect of myxothiazol on the g＊ resonance absorbance in the EPR spectrum of the iron-sulfur protein. Reduc- tase complex was suspended at 33.7 μM cytochrome c1 and poised potentiometrically at +200 mV. Where indicated, the spectra were obtained by adding UHDBT or myxothiazol at 100 μM. The spectra were obtained at a microwave frequency of 91.13 GHz, modulation frequency of 100 kHz, modulation amplitude of 1.25 milliTesla, microwave power of 5 milliwatts, time constant of 0.128 s, scan rate of 0.05 Tesla/min, and a sample temperature of 15 K.

The effects of myxothiazol on the g＊ resonance of cytochrome b by fumarate (Fig. 7). These effects of myxothiazol are also identical to those which result from removal of the iron-sulfur protein (14, 23).

It appears that myxothiazol mimics removal of the iron-sulfur protein by interfering with the interaction of ubiquinone and/or ubiquinol with a site on the iron-sulfur protein. Myxothiazol eliminates the change in iron-sulfur protein midpoint potential (Fig. 9) and the inhibition of electron transfer from iron-sulfur protein in fumarate to cytochrome c1 in the bc1 complex of photosynthetic bacteria (15) which are caused by UHDBT. These effects can be explained by displacement of this hydroxquinone from a binding site on the iron-sulfur protein. The shift in g＊ resonance in the EPR spectrum of the iron-sulfur protein (Fig. 8) is also similar to that which coincides with displacement of ubiquinone from a site on this protein (23).

The effects of myxothiazol on oxidation-reduction reactions of the cytochromes are consistent with a protonmotive Q cycle pathway of electron transfer in the bc1 complex as shown in Fig. 10. In this scheme, myxothiazol blocks oxidation of ubiquinol (or ubiquinol anion, see Ref. 41) by iron-sulfur protein. By blocking reduction of iron-sulfur protein, it also blocks reduction of c1.

The different effects of myxothiazol and antimycin on oxidation-reduction reactions of the cytochromes are further evidence for two pathways for reduction of cytochrome b (14, 33) as in the Q cycle (Fig. 10). One of these is sensitive to antimycin and insensitive to myxothiazol; the other is sensitive to myxothiazol and insensitive to antimycin. Only when both inhibitors are present is reduction of b blocked (8, 9, 15, 18). In the absence of antimycin, myxothiazol does not inhibit reduction of the b cytochromes, which can proceed through the anti-

myxothiazol sensitive reduction of b-562. In the presence of antimycin, myxothiazol blocks reduction of the b cytochromes, since it prevents formation of the ubisemiquinone anion (Qc) which is their reductant when the antimycin sensitive pathway through b-562 is blocked (Fig. 10). In similar fashion, myxothiazol inhibits oxidant-induced reduction of the b cytochromes by blocking formation of this ubisemiquinone anion (Qc) coincident with oxidation of c1 and iron-sulfur protein.

It is interesting to note that, while there are two pathways for reduction of the b cytochromes by succinate, there appears to be only one pathway for oxidation of the succinate reducible b by fumarate, and this is inhibited by antimycin and not by myxothiazol (Fig. 7). That there is no myxothiazol sensitive pathway for oxidation of the succinate reducible b by fumarate is consistent with a Q cycle configuration (Fig. 10) in which the potential of cytochrome b-562 is not sufficiently negative to couple oxidation of iron-sulfur protein (and/or cytochrome c1) to the reduction of the fumarate/succinate couple.

We have not measured ubisemiquinone and thus can only infer that myxothiazol inhibits reduction of the b cytochromes by a species corresponding to Qc in the Q cycle. However, a ubisemiquinone having properties expected of Qc is formed during oxidant-induced reduction of cytochrome b (42). This ubisemiquinone is distinct from the ubisemiquinone (43) which corresponds to Qc (Fig. 10). The ubisemiquinone which is detected during oxidant-induced reduction is thought to be responsible for production of superoxide anion in the bc1 complex (44, 45), since this semiquinone is strongly reducing and formed in the presence of antimycin, which also promotes production of the oxygen radical. If production of superoxide anion is dependent on Qc, our inference that myxothiazol blocks formation of Qc is supported by the finding that it blocks production of superoxide anion in the bc1 complex (46).

As noted above, De Vries and coworkers (18) reported that
Mechanism of Myxothiazol Inhibition of Respiration

myxothiazol inhibits reduction of a portion of $b$-562, and cited this as evidence of a dimeric Q cycle, which includes two species of $b$-562 (19). However, their results show that myxothiazol appears not to inhibit $b$ reduction, especially when compared to its effect on $c_1$ reduction, but rather to convert the reduction from a biphasic to a monophasic reaction (Fig. 7 in Ref. 18). In addition, in their experiments myxothiazol caused the same increase in amount of $b$ reduced by duroquinol as we observed (Fig. 1).

The results of De Vries and coworkers (18), and the difference between their results and those of ourselves and others (8, 9, 15, 40), are probably due to the triphasic nature of $b$ reduction. Tang et al. (47) and Jin et al. (48) have shown that when electrons are donated into the $bc_1$ complex slowly, such as at low temperature or low substrate concentration, reduction of cytochrome $b$ is a triphasic reaction consisting of an initial rapid partial reduction followed by rapid oxidation and then finally by a slow reduction. Alteration of the relative rates of the initial reduction and reoxidation would convert this triphasic reaction to a biphasic reaction as observed by De Vries and coworkers (18). When high concentrations of substrate are used to reduce the $bc_1$ complex, as in our experiments, the slow reduction phase becomes sufficiently fast, relative to the initial reduction and reoxidation phases, that reduction of the $b$'s becomes apparently monophasic.4

Triphasic reduction of cytochrome $b$ is accounted for by a conventional Q cycle and a single species of $b$-562 as shown in Fig. 10. When added to the fully oxidized complex, ubiquinol or duroquinol initially reduces iron-sulfur protein (plus $c_1$) and cytochrome $b$ in a myxothiazol sensitive reaction at center $o$ (Fig. 10). As quinone (or Q1) is formed, the $b$ cytochromes are then reoxidized in an antimycin sensitive reaction. Iron-sulfur protein and $c_1$ remain reduced, preventing further formation of $Q_0$; consequently, subsequent reduction of the $b$ cytochromes proceeds by reversal of the antimycin sensitive reaction. By eliminating the initial reduction of $b$ through center $o$, myxothiazol converts the reduction of cytochrome $b$ from a triphasic (or biphasic) to a monophasic reaction. Under the conditions of our experiments, the rate of this monophasic reaction approaches the mixing time of the spectrophotometer, and this obscures the difference in the myxothiazol sensitive and antimycin sensitive rates of $b$ reduction.

Since the biphasic reduction of cytochrome $b$ is accounted for without postulating two species of $b$-562, it is unnecessary to postulate a dimeric Q cycle. The dimeric Q cycle (19) is also subject to criticism on two points. First, there are now three classes of inhibitors of the $bc_1$ complex, exemplified by antimycin, myxothiazol, and UHDBT, which inhibit with a stoichiometry of one per cytochrome $c_1$. Unless one postulates a high degree of positive cooperativity at all three of these sites, the dimeric Q cycle would require less than one inhibitor per $c_1$. A second objection to the dimeric Q cycle is that it does not account for oxidant-induced reduction of $b$-562 and $b$-566, nor the inhibition of these reactions by myxothiazol.

The Q cycle shown in Fig. 10 is not subject to these criticisms and operates with a single species of $b$-562. In our view, this Q cycle, which is essentially identical to that originally proposed (20–23), is a preferable hypothesis for the electron transfer and proton translocation reactions in the $bc_1$ complex.

REFERENCES


Figure 10. Protonmotive Q cycle scheme for electron transfer through the cytochrome $bc_1$ complex, showing the postulated site of inhibition by myxothiazol. The scheme depicts electron transfer in the forward direction, from a dehydrogenase (deH) to cytochrome $c_1$, under steady state conditions. Under pre-steady state conditions, as in the experiments described here, when succinate is added to the fully oxidized complex, ubiquinone is reduced by the dehydrogenase and then further reduced by center $o$ in an antimycin insensitive reaction. Ubiquinol then reduces the $b$ cytochromes through one of two possible pathways. One pathway of $b$ reduction is inhibited by myxothiazol, which blocks oxidation of ubiquinol by iron-sulfur protein and thus blocks formation of the ubisemiquinone reductant of $b$-566. If oxidation of ubiquinol by iron-sulfur protein is blocked, ubiquinol can reduce the $b$ cytochromes by an antimycin sensitive reduction of $b$-562. UHDBT blocks electron transfer from iron-sulfur protein (ISP) to cytochrome $c_1$ by binding to reduced iron-sulfur protein. For reviews of the Q cycle, see Refs. 21 and 23.

H. L. Tang and B. L. Trumpower, unpublished results.
Mechanism of Myxothiazol Inhibition of Respiration

An inhibitor of mitochondrial respiration which binds to cytochrome b and displaces quinone from the iron-sulfur protein of the cytochrome bc1 complex.
G von Jagow, P O Ljungdahl, P Graf, T Ohnishi and B L Trumpower


Access the most updated version of this article at [http://www.jbc.org/content/259/10/6318](http://www.jbc.org/content/259/10/6318)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/259/10/6318.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/259/10/6318.full.html#ref-list-1](http://www.jbc.org/content/259/10/6318.full.html#ref-list-1)