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MYXOTHIAZOL, A NEW INHIBITOR OF THE CYTOCHROME b-c_1 SEGMENT OF THE RESPIRATORY CHAIN *

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Myxothiazol inhibited oxygen consumption of beef heart mitochondria in the presence and absence of 2,4-dinitrophenol, as well as NADH oxidation by submitochondrial particles. The doses required for 50% inhibition were 0.58 mol myxothiazol/mol cytochrome *b* for oxygen consumption of beef heart mitochondria, and 0.45 mol/mol cytochrome *b* for NADH oxidation by submitochondrial particles. Difference spectra with beef heart mitochondria and with cell suspensions of *Saccharomyces cerevisiae* revealed that myxothiazol blocked the electron transport within the cytochrome b-c_1 segment of the respiratory chain. Myxothiazol induced a spectral change in cytochrome *b* which was different from and independent of the shift induced by antimycin. Myxothiazol did not give the extra reduction of cytochrome *b* typical for antimycin. Studies on the effect of mixtures of myxothiazol and antimycin on the inhibition of NADH oxidation indicated that the binding sites of the two inhibitors are not identical.

Introduction

Myxothiazol (Fig. 1) is a new antibiotic produced by the myxobacterium *Myxococcus fulvus*. The production and isolation of the compound, some of its biological and physicochemical properties, and its chemical structure have been described previously [2,3]. First experiments on the mode of action of myxothiazol indicated that the antibiotic interferes with respiration [4]. In this paper, we wish to report on studies with mitochondria and submitochondrial particles which allow the determination of the site of interference more precisely.

* Part of this work has been presented at the 33rd conference of the Gesellschaft für Biologische Chemie in Reisensburg, September 19–21, 190 [1].

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

Materials and Methods

Myxothiazol and its derivatives were kindly supplied by Dr. W. Trowitzsch of this institute. Antimycin A was purchased from Serva (Heidelberg, F.R.G.). The inhibitors were dissolved in methanol, and their concentrations determined with a spectrophotometer. The absorption coefficients used were 4.8 $mM^{-1} \cdot cm^{-1}$ at 320 nm for antimycin [5], 10.5 $mM^{-1} \cdot cm^{-1}$ at 313 nm for myxothiazol [2], 10.5 $mM^{-1} \cdot cm^{-1}$ at 313 nm for demethylmyxothiazol [3], and 10.1 $mM^{-1} \cdot cm^{-1}$ at 296 nm for hexahydromyxothiazol [3]. The methanol concentration in the tests did not exceed 2% (v/v) and had no inhibitory effect. The absorption coefficients for NADH and DCIP were 6.2 $mM^{-1} \cdot cm^{-1}$ at 340 nm, and 21.0 $mM^{-1} \cdot cm^{-1}$ at 600, respectively [6].

Protein was estimated by the procedure of Lowry et al. [7], with bovine serum albumin as a standard. Cytochrome contents were measured according to the

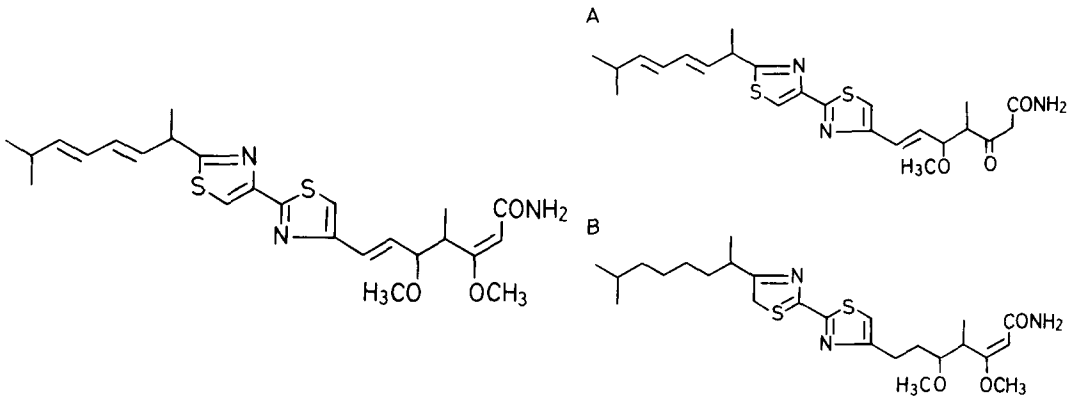

Fig. 1. Chemical structure of myxothiazol (left) and its derivatives, demethylmyxothiazol (A) and hexahydromyxothiazol (B)

method of Azzone et al. [8]. The ADP/O ratio and the respiratory control ratio were determined accroding to the method of Estabrook [9].

Beef heart mitochondria were isolated following the proteinase method of Smith [10] with minor modifications. For homogenization, the following medium was used: 250 mM saccharose, 10 mM KH_2PO_4, 10 mM Tris, 2 mM EGTA, 2 mM $MgCl_2$, pH 7.4. All subsequent procedures were carried out in the same medium without EGTA. The mitochondria thus obtained had the following characteristics. With 3 mM pyruvate + 3 mM malate, the oxygen uptake rate was 120 nmol/mg protein per min, the ADP/O ratio 2.9, and the respiratory control ratio 4.8. The specific cytochrome contents, in nmol/mg protein, were 0.55 for cytochromes aa_3, b and c, and 0.21 for cytochrome c_1 (average of six determinations).

For the preparation of submitochondrial particles, the mitochondria were isolated as described above with the exception that a blender (Ultra Turrax TP 18-10 from Janke and Kunkel, Staufen im Breisgau, F.R.G.) was used for homogenization. The mitochondria were diluted with isolation medium to a protein concentration of approx. 10 mg/ml. Batches of approx. 20 ml were treated 10 times for 15 s each with ultrasound at maximum energy (MSE/PG 100, from MSE Scientific Instruments, Crawley, U.K.). The sonicated suspension was centrifuged at 12000 $\times g$ for 10 min, and the supernatant then at 100000 $\times g$ for 45 min. The pellet was suspended in 75 mM sodium phosphate buffer, pH 7.4, plus 1 mM EDTA plus 1 mM $MgCl_2$ (buffer 1). The resulting submitochondrial particles had the following characteristics. The rate of NADH oxidation (measured as NADH decrease) was 1.7 μmol/mg protein per min; the specific cytochrome contents, in nmol/mg protein, were 0.64 for cytochromes aa_3 and b, 0.41 for cytochrome c, and 0.28 for cytochrome c_1 (average of three determinations).

Oxygen consumption was measured with an oxygen electrode (E 5046 from Radiometer, Copenhagen, Denmark) at 30°C in a closed cell containing the biological material suspended in 3.4 ml of buffer 2: 250 mM saccharose, 10 mM K_2HPO_4, 10 mM Tris, and 5 mM $MgCl_2$; pH 7.4.

The rate of NADH oxidation by submitochondrial particles was measured with a PMQ 3 spectrophotometer (Zeiss, Oberkochen, F.R.G.) at 340 nm, with an optical pathway of 1 cm, at 30°C. The reaction was carried out in buffer 1 saturated with air. The test volume was 2.5 ml. The reaction was started by the addition of NADH (Merck, Darmstadt, F.R.G.) to give a final concentration of 0.157 mM.

The succinate-ubiquinone reductase activity of submitochondrial particles was assayed in buffer 1 with DCIP according to the method of Ziegler and Rieske [11]. The rate of DCIP reduction was measured at 600 nm with an optical pathway of 1 cm, at 30°C. The test mixture had the following composition: 47.6 μM DCIP, 3 mM KCN, 5 mM succinate, 2% (v/v) methanol, and inhibitors as indicated in Table

IV. The test volume was 2.5 ml. The reaction was started by the addition of succinate.

Difference spectra were recorded with an Aminco DW 2A spectrophotometer (American Instruments, Silver Springs, MD, U.S.A.) with a bandwidth of 1–2 nm and an optical pathway of 1 cm. The baseline was first adjusted to give a horizontal line, before the difference spectrum was recorded.

Results

Myxothiazol inhibited oxygen uptake by beef heart mitochondria with the same efficiency as antimycin (Fig. 2). The titration curves of both inhibitors were strongly sigmoidal and identical. The doses of myxothiazol required for 50 and 90% inhibition of oxygen uptake were 0.32 and 0.40 nmol/mg protein, respectively. With cytochrome *b* as reference, the corresponding ratios were 0.58 and 0.73 mol/mol cytochrome *b*. The NADH oxidation by submitochondrial particles was inhibited by antimycin and by myxothiazol with similar stoichiometry (Figs. 5 and 6). The dose required for 50% inhibition was in this system 0.44 mol/mol cytochrome *b* for both inhibitors. For 90% inhibition, the ratios were 0.68 mol myxothiazol/mol cytochrome *b*, and 0.60 mol antimycin/mol cytochrome *b*. In contrast to oligomycin, myxothiazol inhibited coupled respiration as well as 2,4-dinitrophenol-uncoupled respiration of beef heart mitochondria (Table I). This indicated that myxothiazol did not interfere with oxidative phosphorylation.

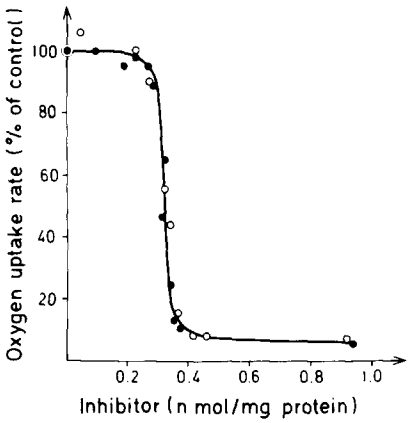

Fig. 2. Effect of myxothiazol and of antimycin on the rate of oxygen uptake of beef heart mitochondria. Air-saturated buffer 2 plus 3 mM pyruvate and 3 mM malate was placed into the reaction vessel. Mitochondria were injected into the reaction cell at a concentration of 0.372 mg protein/ml. Then myxothiazol (●———●) or antimycin (○———○) was added. After 4 min, the reaction was started by the addition of ADP (final concentration 600 μM). The rate of oxygen uptake in the control without inhibitor was 114 nmol O_2/mg protein per min.

Fig. 3 shows the difference spectra of beef heart mitochondria (reduced minus oxidized) in the presence of myxothiazol or antimycin. Cytochrome *b*, absorbing at 563–564 nm (α band), 532 nm (β band) and 431–432 nm (γ band), was reduced in the presence of either myxothiazol or antimycin. Cytochromes aa_3 (α band at 605 nm, γ band at 444 nm) and cytochromes $c + c_1$ (α band at 552 nm, β band at 522 nm, γ band at 421 nm) remained in the oxidized state. This established that myxothiazol interacted with the cytochrome b-c_1 segment of the respiratory chain. The same type of difference spectrum was obtained with suspensions of starved *Saccharomyces cerevisiae* cells, reduced with ethanol in the presence of myxothiazol or antimycin.

The difference spectra in Fig. 3 further show that in the presence of antimycin, the reduction of cytochrome *b* goes far beyond the level obtainable with the substrate alone. The amount of cytochrome *b*

TABLE I

EFFECT OF DIFFERENT INHIBITORS ON COUPLED AND UNCOUPLED RESPIRATION OF BEEF HEART MITOCHONDRIA

The experimental conditions were as described in Fig. 2. The inhibitors were added at a concentration of 1 μM. Coupled respiration was initiated by the addition of ADP (600 μM), and uncoupled respiration by the addition of 2,4-dinitrophenol (10 μM). The rates of oxygen uptake in the controls without inhibitor were: 109 nmol O_2/mg protein per min for coupled respiration, and 70 nmol O_2/mg protein per min for uncoupled respiration.

Inhibitor	Oxygen uptake rate (%)	
	Coupled respiration	Uncoupled respiration
Control	100	100
Oligomycin	21	86
Antimycin	6	10
Myxothiazol	2	9

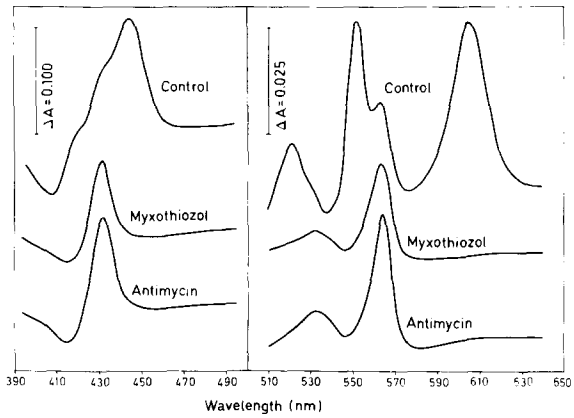

Fig. 3. Difference spectra of beef heart mitochondria (reduced minus oxidized) in the presence of myxothiazol or antimycin. Mitochondria were suspended in prewarmed (25°C) buffer 2 saturated with air and containing 2 mM ADP. After the baseline had been recorded, the inhibitor was added to the sample cuvette to give a concentration of 2 μM. Methanol was added to the reference cuvette. After an incubation time of 2 min, 4 mM pyruvate plus 4 mM malate was added to the sample cuvette. When the reduction was at its maximum, the difference spectrum was recorded at a bandwith of 1 nm. In the control, no inhibitor was added to the sample cuvette. The protein concentration was 1.32 mg/ml between 390 and 500 nm and 2.57 mg/ml between 500 and 650 nm.

reducible with substrate alone was 61–68% of the reduction level obtained with dithionite. In the presence of antimycin this proportion rose to 80–88%, giving an extra reduction of approx. 20%. In the presence of myxothiazol, a very slight extra reduction could be seen at best. An analysis of difference spectra of beef heart mitochondria which were reduced with different substrates in the presence of myxothiazol or antimycin is given in Table II.

In the presence of antimycin, the absorption maximum of dithionite-reduced cytochrome *b* is shifted by 2–3 nm to longer wavelengths [12]. To see whether myxothiazol gives a similar effect, submitochondrial particles were first reduced by dithionite, then the inhibitor was added to the samples cuvette, and the resulting difference spectrum was recorded between 540 and 580 nm (Fig. 4). Antimycin gave the typical 'red shift' signal, with a maximum at 565.5 nm, and a minimum at 558.5 nm. Myxothiazol gave a shift signal with a maximum at 565.0 nm, but without a minimum. The titration curves for the spec-

TABLE II

EFFECT OF ANTIMYCIN AND MYXOTHIAZOL ON CYTOCHROME *b* REDUCTION IN BEEF HEART MITOCHONDRIA

The experimental conditions were as described in Fig. 3. The substrate concentration was 4 mM. Cytochrome *b* reduction was calculated from $\Delta A_{563-577}$. The 100% reduction was achieved by the addition of solid dithionite to the sample cuvette and corresponds to an absorbance difference of 0.029. The protein concentration was 1.87 mg/ml.

Substrate	Cytochrome *b* reduction (%)		
	Control	Antimycin	Myxothiazol
Pyruvate + malate	61	80	65
Succinate	68	87	68
Malate	65	88	66

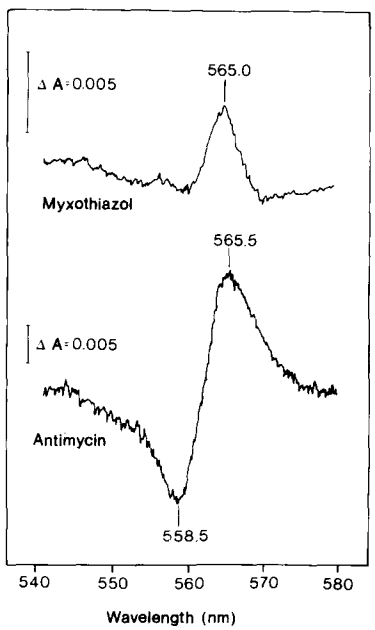

Fig. 4. The spectral change induced by myxothiazol and by antimycin, in dithionite-reduced submitochondrial particles. Submitochondrial particles (26.6 mg/ml = 16.8 μM cytochrome *b*) were placed into the cuvettes and reduced by the addition of solid dithionite. After the baseline had been adjusted, either myxothiazol or antimycin was added to the sample cuvette. The concentrations of the inhibitors were 11.0 μM for antimycin, and 12.5 μM for myxothiazol.

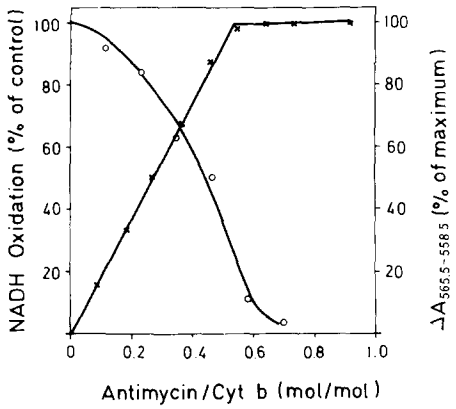

Fig. 5. Titration curves of the antimycin-induced spectral shift (X———X) and of the NADH oxidation (o———o) by submitochondrial particles. The titration curve of the antimycin-induced spectral shift was obtained with dithionite-reduced submitochindrial particles at a protein concentration of 17.4 mg protein/ml (=11.0 μM cytochrome *b*). A difference in absorbance of 100% corresponds to 0.0245. The titration curve of the NADH oxidation was obtained with submitochondrial particles suspended in buffer 1 at a protein concentration of 0.103 mg/ml (=0.067 μM cytochrome *b*). The suspension was preincubated with antimycin for 2 min before NADH was added. Cyt, cytochrome.

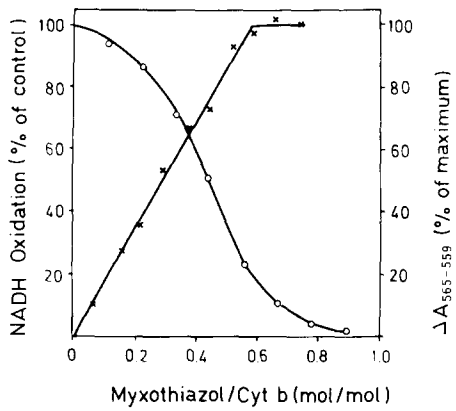

Fig. 6. Titration curves of the myxothiazol-induced spectral change (X———X) and of the NADH oxidation (o———o) by submitochondrial particles. The titration curve of the myxothiazol-induced spectral change was obtained with dithionite-reduced submitochondrial particles at a protein concentration of 26.59 mg/ml (=16.8 μM cytochrome *b*). A difference in absorbance of 100% corresponds to 0.0055. The experimental conditions for the titration of the NADH oxidation were as described in Fig. 5. Cyt, cytochrome.

tral shifts as well as for the inhibition of the NADH oxidation are shown in Figs. 5 and 6. The signal height of the spectral shifts increased linearly with the concentration, up to a ratio of 0.54 and 0.58

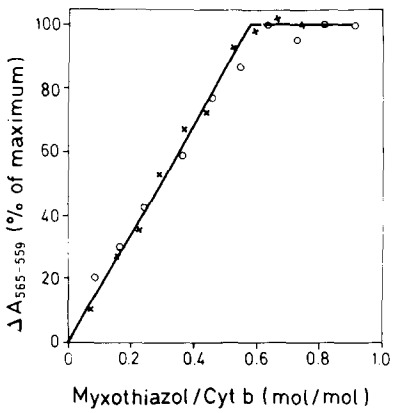

Fig. 7. Titration curve of the myxothiazol-induced spectral change in the presence of antimycin. Dithionite-reduced submitochondrial particles (17.4 mg protein/ml = 11.0 μM cytochrome *b*) were pretreated with antimycin (0.92 mol/mol cytochrome *b*). Then the particles were titrated with myxothiazol (o———o). A difference in absorbance of 100% corresponds to 0.0067. The titration curve of myxothiazol without antimycin (X———X) is included for comparison. Cyt, cytochrome.

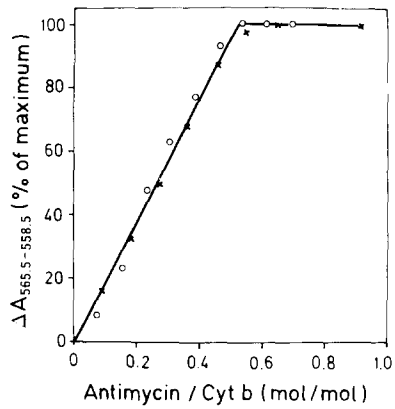

Fig. 8. Titration curve of the antimycin-induced spectral shift in the presence of myxothiazol. Dithionite-reduced submitochondrial particles (26.59 mg protein/ml = 16.8 μM cytochrome *b*) were pretreated with myxothiazol (0.74 mol/mol cytochrome *b*). Then the particles were titrated with antimycin (o———o). A difference in absorbance of 100% corresponds to 0.0290. The titration curve of antimycin without myxothiazol (X———X) is included for comparison. Cyt, cytochrome.

mol/mol cytochrome *b* for antimycin and myxothiazol, respectively. In a parallel experiment the ratios were 0.45 for antimycin, and 0.53 for myxothiazol. At these ratios the heights of the shift signals had reached their maximum, and the NADH oxidation was inhibited by approx. 75%.

The antimycin-induced spectral shift was also obtained with particles pretreated with an excess of myxothiazol, and vice versa. Dithionite-reduced submitochondrial particles were treated with 0.92 mol antimycin/mol cytochrome *b* in sample and reference cuvettes, and subsequently the sample was titrated with myxothiazol. As can be seen from Fig. 7, the presence of antimycin did not affect the titration curve of the myxothiazol-induced spectral change. Fig. 8 shows the titration curve of antimycin in the presence of 0.74 mol myxothiazol/mol cytochrome *b*. Again, the presence of myxothiazol did not affect the titration curve of the antimycin-induced spectral shift.

At the same inhibitor/cytochrome *b* ratio, a mixture of myxothiazol and antimycin gave a lower inhibition than did myxothiazol or antimycin alone (Table III). Myxothiazol did not inhibit the succinate-ubiquinone reductase of submitochondrial particles. This enzyme is blocked by thenoyltrifluoroacetone [11] (Table IV).

Fig. 9 shows the titration curves of the NADH oxidation by submitochondrial particles with demeth-

TABLE III

INHIBITION OF THE NADH OXIDATION BY SUBMITOCHONDRIAL PARTICLES WITH ANTIMYCIN, MYXOTHIAZOL, AND MIXTURES OF THE TWO AT DIFFERENT INHIBITOR/CYTOCHROME *b* RATIOS

The protein concentration in the test was 0.103 mg/ml (=0.067 μM cytochrome *b*). The NADH oxidation of the control without inhibitor was 1.43 μmol NADH/mg protein per min.

Myxothiazol/ cytochrome *b* (mol/mol)	Antimycin/ cytochrome *b* (mol/mol)	Total inhibitor/ cytochrome *b* (mol/mol)	Inhibition (%)
0.45	0.00	0.45	51
0.00	0.45	0.45	51
0.22	0.23	0.45	35
0.10	0.35	0.45	40
0.58	0.00	0.58	82
0.00	0.58	0.58	82
0.35	0.23	0.58	57
0.23	0.35	0.58	56
0.70	0.00	0.70	93
0.00	0.70	0.70	93
0.47	0.23	0.70	82
0.35	0.35	0.70	80

TABLE IV

THE EFFECTS OF MYXOTHIAZOL AND OF THENOYLTRIFLUOROACETONE ON THE SUCCINATE-UBIQUINONE REDUCTASE OF SUBMITOCHONDRIAL PARTICLES

The protein concentration in the test was 0.113 mg/ml (=0.082 nmol cytochrome *b*/ml). Before the reaction was started the particles were preincubated for 2 min with the inhibitor.

Inhibitor	Specific activity	
	nmol DCIP/ min per mg protein	% of control
Control	184.6	100
Thenoyltrifluoroacetone (200 μM)	34.6	19
Myxothiazol (5.4 μM)	183.3	99

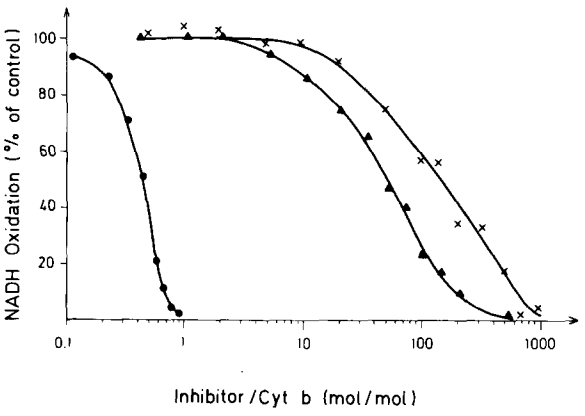

Fig. 9. Titration curves of the NADH oxidation by submitochondrial particles with demethylmyxothiazol (X———X) and hexahydromyxothiazol (▲———▲). The protein concentration in the test was 0.085 mg/ml (=0.062 μM cytochrome *b*). The NADH oxidation of the control was 2.02 μmol NADH/mg protein per min. The titration curve with myxothiazol (●———●) is included for comparison.

ylmyxothiazol and hexahydromyxothiazol (Fig. 1A and B). The inhibition exerted by these two derivatives was strikingly different from that of myxothiazol. The doses required for 50% inhibition were 145 mol/mol cytochrome *b* for demethylmyxothiazol, and 52 mol/mol cytochrome *b* for hexahydromyxothiazol.

Discussion

Our data clearly demonstrate that myxothiazol is an inhibitor of the cytochrome *b*-c_1 segment of the respiratory chain of beef heart mitochondria. In this respect, myxothiazol acts in the same general area as other inhibitors of the respiratory chain, viz., antimycin [13], 2-heptyl-4-hydroxyquinoline *N*-oxide [14], diuron [15], mucidin [16], funiculosin [17] and tridemorph [18].

With respect to the efficiency of inhibition and the sigmoidal shape of the inhibition curve of oxygen uptake (Fig. 2), myxothiazol closely resembles the classical inhibitor antimycin. On the other hand, there are two effects which are different from those produced by antimycin. Firstly, myxothiazol does not yield an extra reduction of cytochrome *b* like antimycin. In this respect, it resembles the inhibitor mucidin [16]. Secondly, myxothiazol induces a spectral change in cytochrome *b* in dithionite-reduced submitochondrial particles, which is not identical with the antimycin-induced shift and cannot be explained as a simple red shift (Fig. 4). Although we are not able to interpret the signal produced by myxothiazol, the appearance of the signal still shows that the antibiotic somehow interacts with cytochrome *b*.

Assuming that binding of myxothiazol is responsible for the observed spectral change, we are able to comment on the binding behavior of the antibiotic. The binding shows a linear concentration dependence up to a ratio of 0.53–0.58 mol myxothiazol/mol cytochrome *b* (Fig. 6). Almost identical results have been obtained with antimycin (Fig. 5). Von Jagow et al. [19], Berden and Slater [20,21] and Rieske [22] have reported a stoichiometry of 0.5 mol antimycin/mol cytochrome *b* for the binding of antimycin to submitochondrial particles, to succinate-cytochrome *c* reductase, and to Complex III from beef heart mitochondria. We therefore assume that myxothiazol has the same number of binding sites as antimycin and a similar binding constant. Because of the spectral change induced by myxothiazol (Fig. 4), we may speculate that the binding site of myxothiazol is probably cytochrome *b* in the cytochrome *b*-c_1 segment. Cytochrome *b* in the succinate-ubiquinone segment [23] cannot be a candidate for the binding site because myxothiazol does not inhibit the succinate-ubiquinone reductase of submitochondrial particles (Table IV). While we do not really know the binding site of myxothiazol, we can provide some evidence that it is at least not identical with the antimycin-binding site. This is suggested by the fact that antimycin does not affect the titration curve of the myxothiazol-induced spectral change, and vice versa (Figs. 7 and 8). Furthermore, the results of the inhibition of NADH oxidation with mixtures of myxothiazol and antimycin (Table III) can only be explained by different binding sites.

Our results with two derivatives of myxothiazol (Figs. 1 and 9) give some indication about the chemical groups which are important for the action of the antibiotic. Loss of the enol methyl ether group (demethylmyxothiazol) resulted in a 330-fold, and partial reduction of the double bonds of the side chains of myxothiazol (hexahydromyxothiazol) in a 120-fold decrease in the inhibitory activity. We therefore assume that these structural elements are essential for the action of the antibiotic.

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