COMPLETE INHIBITION OF ELECTRON TRANSFER FROM UBIQUINOL TO CYTOCHROME *b* BY THE COMBINED ACTION OF ANTIMYCIN AND MYXOTHIAZOL

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1. Introduction

Oxidative phosphorylation is established by a link of proton translocation (across a membrane) to the flow of redox equivalents (vectorially from the substrates to finally oxygen) [1]. This flow consists of an alternating transfer of hydrogen atoms and electrons. The hydrogen atoms are transported by two electron carriers, e.g., flavoenzymes and ubiquinone; the electrons are transported by one-electron carriers, e.g., iron–sulfur proteins and cytochromes. Such a switch from a two-redox-equivalent hydrogen atom carrier, namely ubiquinone, to a one-redox-equivalent electron carrier, namely cytochrome *b*, takes place at complex III, the ubiquinol:cytochrome *c* reductase [2].

This study was performed to obtain further insight into the arrangement of the electron carriers in the cytochrome bc_1 segment of the respiratory chain. The experiments were carried out with submitochondrial particles and with the isolated complex III from beef heart. The action of antimycin and of a new antifungal antibiotic, myxothiazol [3,4] was investigated. Myxothiazol has been shown to belong to the novel moa-inhibitors, i.e., inhibitors containing E-β-methoxyacrylate as an essential structural segment [5]. Myxothiazol was found to bind to a site different from the antimycin binding site [3,4]. These experiments are restricted to the mechanism of electron transfer, while the proton transfer was not taken into consideration. It was tested whether the 2 inhibitors antimycin and myxothiazol, binding to different sites of cytochrome *b*, combine in their inhibitory action on the electron transfer from ubiquinol onto cytochrome *b*.

2. Materials and methods

Myxothiazol was a generous gift from Dr H. Reichenbach of the Gesellschaft für Biotechnologische Forschung (Braunschweig). Antimycin was purchased from Sigma (St Louis) all other biochemical reagents from Boehringer (Mannheim).

The inhibitors were dissolved in ethanol. The absorbance coefficients used were 10.5 $mM^{-1} . cm^{-1}$ at 313 nm for myxothiazol and 4.8 $mM^{-1} . cm^{-1}$ at 320 nm for antimycin [5].

Submitochondrial particles from beef heart were prepared as in [4] and stored in liquid nitrogen. Complex III was isolated according to [6], the antimycin–bc_1 complex devoid of iron–sulfur protein according to [7]. Both preparations were stored in the presence of 50% glycerol at −20°C at 50 μM cytochrome *b* in 0.05% Triton X-100, 100 mM NaCl, 20 mM 2(*N*-morpholino)ethane–sulfonic acid (Mops), 1 mM NaN_3 (pH 7.2). The 2 complexes and the submitochondrial particles were diluted by a buffer containing 0.25 M sucrose, 50 mM KP_i, 0.2 mM ethylenglycol–bis-(aminoethyl)-tetraacetic acid (EGTA) and 2 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). In the case of the complexes 0.05% Triton X-100 was added to the buffer medium.

2.1. *Spectroscopy*

The absorption spectra were measured in a Shimadzu UV 300 spectrophotometer using a 1 nm bandwidth. Addition or subtraction of the spectra was performed with a Shimadzu Sapcom 1 spectral data processor. The absorbance coefficients used were 28.5 $mM^{-1} . cm^{-1}$ at 562–575 nm for cytochrome *b*, and 19.0 $mM^{-1} . cm^{-1}$ at 553–540 nm for cytochrome c_1 [7]. The reduction kinetics were measured at 562–575 nm

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with a Perkin Elmer model 156 spectrophotometer using a 2 nm bandwidth.

3. Results

3.1. *The binding of antimycin and myxothiazol*

The two different red shifts of the absorbance spectrum of reduced cytochrome *b* induced by antimycin and myxothiazol can serve for tracing the binding of the 2 inhibitors [5]. The following binding experiments were performed in order to determine which heme center(s) is (are) influenced by the respective inhibitor and whether the iron–sulfur protein is involved in the binding of myxothiazol. The study material used for this purpose consisted of a complex III preparation containing all subunits, isolated in Triton X-100 [6] and a depleted complex III species devoid of iron–sulfur protein due to its preparation in an antimycin-loaded state with high Triton X-100 concentrations [7]. Fig.1A shows the binding of the inhibitors to the paternal complex. The myxothiazol red shift with a maximum at 568 nm was induced first; then a new base line was established by adding the same amount of myxothiazol to the reference cuvette (not shown). Subsequently the anti-

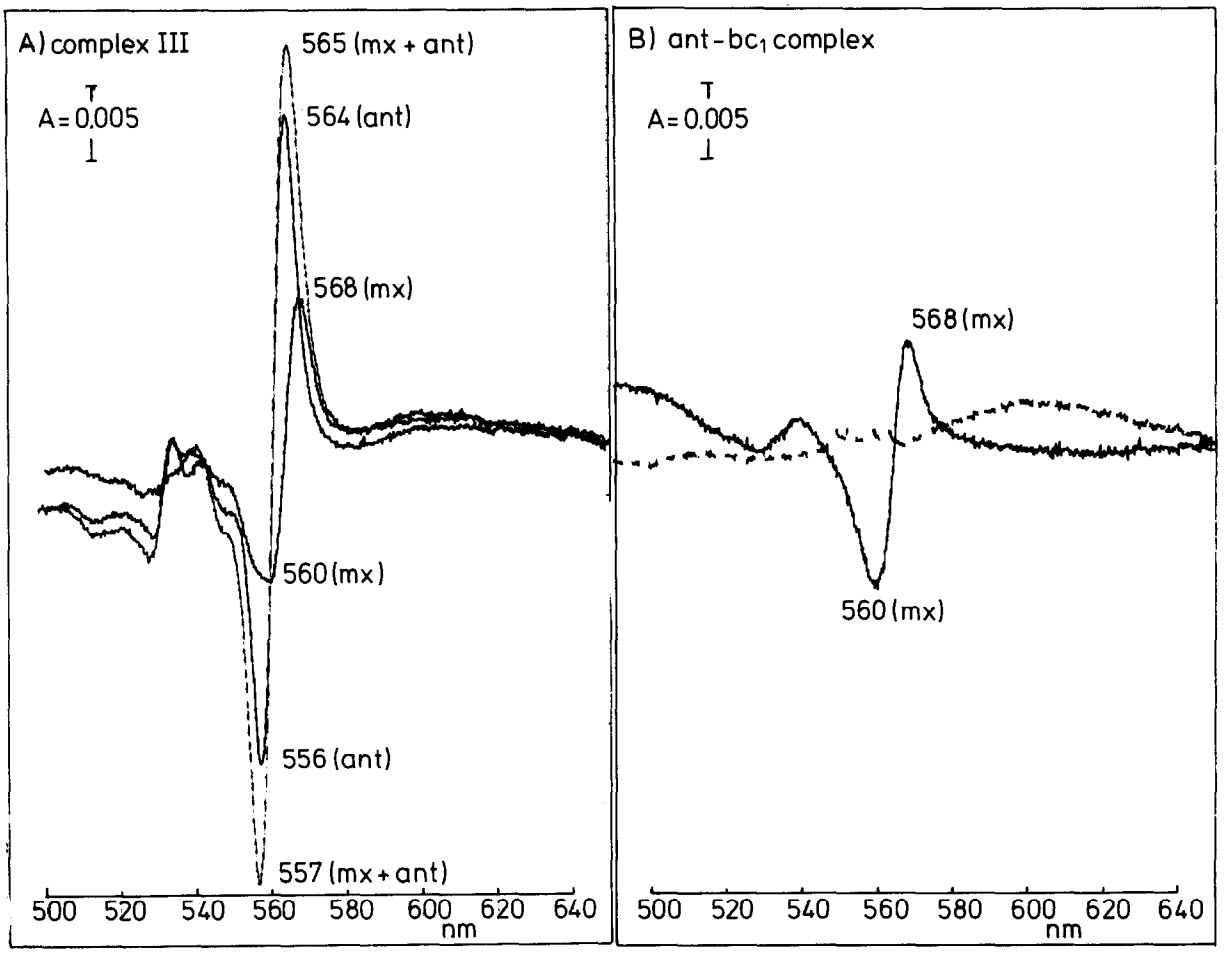


Fig.1. The red shifts of the ferrocytochrome *b* spectra induced by the binding of myxothiazol and antimycin: (A) red shift spectra of complex III containing all subunits [6]; (B) myxothiazol-induced red shift of antimycin–bc_1 complex, loaded with antimycin during preparation and lacking the iron–sulfur protein [7]. The antibiotics were added at saturating levels: 20 μM myxothiazol and 10 μM antimycin; 4 μM (in c_1) of complex III and 3.6 μM (in c_1) of antimycin–bc_1 complex were present. The complex was reduced by dithionite.

mycin red shift with a maximum at 564 nm was induced. The red shift signal caused by antimycin has its maximum at a lower wave length than that caused by myxothiazol, indicating that antimycin influences predominantly the ligand field of the b_K center (b-562), while myxothiazol influences that of the b_T center (b-566). An addition of both spectra is obtained when the two inhibitors are added simultaneously to the sample cuvette. This spectrum is identical to that obtained when the two individual red shift spectra are accumulated by the storage device of the spectrophotometer. Fig.1B shows the myxothiazol red shift spectrum of the depleted species lacking the iron–sulfur protein and still carrying antimycin. This spectrum is somewhat smaller than that of the complete complex species, but the maxima and minima are identical. From this we conclude that cytochrome b is, but iron–sulfur protein is not involved in the binding of myxothiazol.

3.2. *The influence of antimycin and myxothiazol on the kinetics of cytochrome* b *reduction*

The experiments were performed with beef heart submitochondrial particles. Any possible influence by energy-transforming reactions on the electron transfer was eliminated by adding uncoupler, causing a collapse of the membrane proton gradient. The particles were preincubated for 5 min with saturating concentrations of the respective inhibitor(s), the reaction was started by adding NADH or succinate as electron donor. Fig.2A shows the complete cytochrome spectrum when the heme centers are 100% reduced. The 605 nm band represents the cytochrome a,a_3 spectrum, the 552 nm band a mixed spectrum of cytochrome c_1 and that part of cyt. c which was not extracted during the preparation of the submitochondrial particles. The 563 nm band belongs to cytochrome b. This band is caused by 2 different heme b centers, probably residing within 1 cytochrome b molecule [8]. Fig.2B shows a mixed spectrum of cytochrome b_K and b_T which are reduced by NADH in the antimycin-inhibited steady state, when oxygen is present. Under these conditions cytochrome c_1 and c are fully oxidized and cytochrome a,a_3 is only minimally reduced. Fig.2C shows those heme centers which are not reduced by NADH in the antimycin-inhibited state (cf. legend to fig.2C). These are the cytochromes a,a_3 with their band at 605 nm and cytochrome c_1 plus cytochrome c with a band at 552 nm. The shoulder in the long wavelength flank

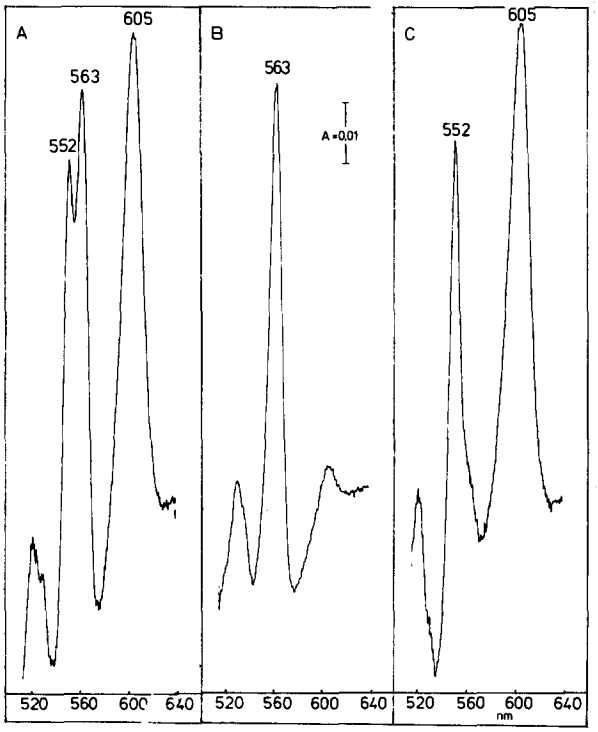


Fig.2. Reduction of cytochrome b_K (b-562) and b_T (b-566) of submitochondrial particles in the antimycin-inhibited steady state. (A) 100% reduction control: difference (red–ox) spectrum of the cytochromes. The sample side was reduced by dithionite, the reference side oxidized by 15 μM ferricyanide. (B) Reduction of cytochrome b by NADH in the inhibited steady state. The particles on the sample side were preincubated for 5 min with 10 μM antimycin, then the reaction was started by addition of 2 mM NADH. The reference side was in oxidized state. The submitochondrial particles were present at 3 μM in cytochrome b. (C) Difference spectrum of the cytochromes still oxidized in the antimycin-inhibited state. The spectrum was obtained by subtracting spectrum 2B from spectrum 2A.

of the latter may be caused by the heme center of cytochrome b of complex II.

Fig.3A,B reflect the same sort of spectra, but with myxothiazol as inhibitor instead of antimycin. The spectrum of fig.2A can again be used as the 100% reduction control. Fig.3A shows that with the inhibitor myxothiazol only 1 heme center of cytochrome b is reduced. The maximum of the band at 562 nm indicates that it is the b_K center. In spectrum 3B which shows those heme centers which are not reduced in the myxothiazol-inhibited state, cytochromes a,a_3 and $c_1 + c$ become visible at 605 and 552 nm, respec-

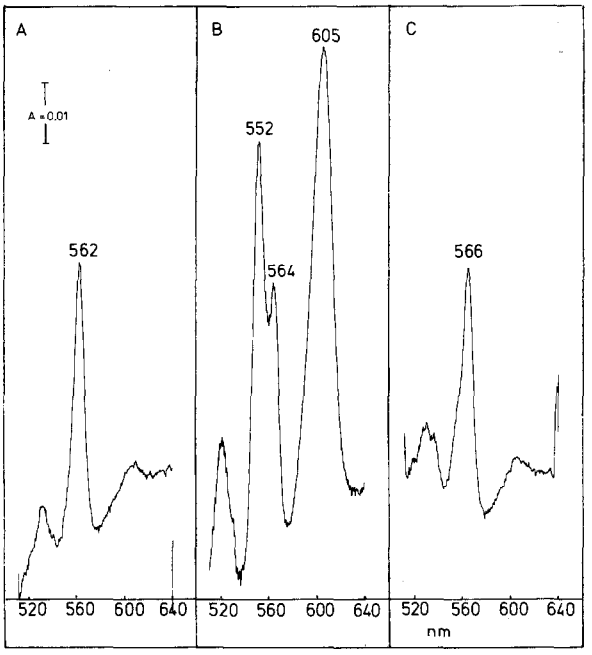


Fig.3. Reduction of cytochrome b_K (b-562) of submitochondrial particles in the myxothiazol-inhibited steady state. (A) Reduction of cytochrome b by NADH in the inhibited steady state. The particles were preincubated with 20 μM myxothiazol. Further conditions as in fig.2. (B) Difference spectrum of the cytochromes still oxidized in the myxothiazol-inhibited state. The spectrum was obtained by subtracting spectrum 3A from spectrum 2A. (C) Spectrum of cytochrome b_T (b-566). The spectrum was obtained by subtracting spectrum 3A from spectrum 2B.

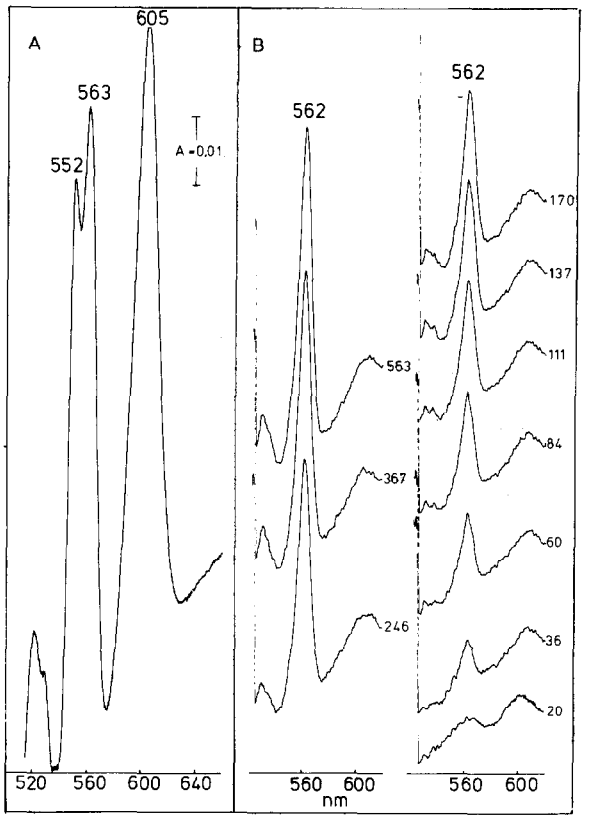


Fig.4. Inhibition of cytochrome b reduction of submitochondrial particles by the combined action of myxothiazol and antimycin. (A) 100% reduction control: difference (red–ox) spectrum of the cytochromes. (B) Kinetics of the cytochrome b_K (b-562) recuction. The kinetics were traced by repetitive scanning as described in the text (please read fig.4B from right to left), the particles preincubated with myxothiazol plus antimycin as before. Experimental conditions as described for fig.2, incubation temperature 22°C.

tively. So far the spectrum resembles that of the antimycin-inhibited state, but additionally the signal of the second heme center, the b_T center, becomes visible at 564 nm. To determine whether the cytochrome b band in the antimycin-inhibited steady state really comprises the signals of b_K and b_T, the myxothiazol-induced band (fig.3A) was subtracted from the antimycin-induced band (fig.2B). As shown by fig.3C, the spectrum of cytochrome b_T with a maximum at 566 nm appears.

When the 2 inhibitors are bound simultaneously, the reduction of cytochrome b is severely inhibited. Cytochrome b_K is reduced, but now within a minute range. Fig.4A again exhibits the 100% reduction control, fig.4B shows the slow kinetic of b_K reduction in the myxothiazol + antimycin-inhibited steady state. The numbers on the right hand side of the traces indicate the seconds needed to reach the 562 nm maximum of the respective spectrum (read fig.4B from right to left). Fig.5 gives a comparison of the reduction kinetics obtained with dithionite and the 2 inhibitors when the kinetics are traced in the dual wavelength mode. For the sake of control all heme centers of the submitochondrial particles were reduced by dithionite (first trace from the left). The kinetic is not resolved, since the reaction occurs within milliseconds. The second trace from the left shows the cytochrome b reduction by NADH in the antimycin-inhibited steady state. A time resolution is again not possible since this catalytic reaction occurs in a millisecond range too [9]. Both heme b centers are reduced in this steady state, as shown before (fig.2B). The

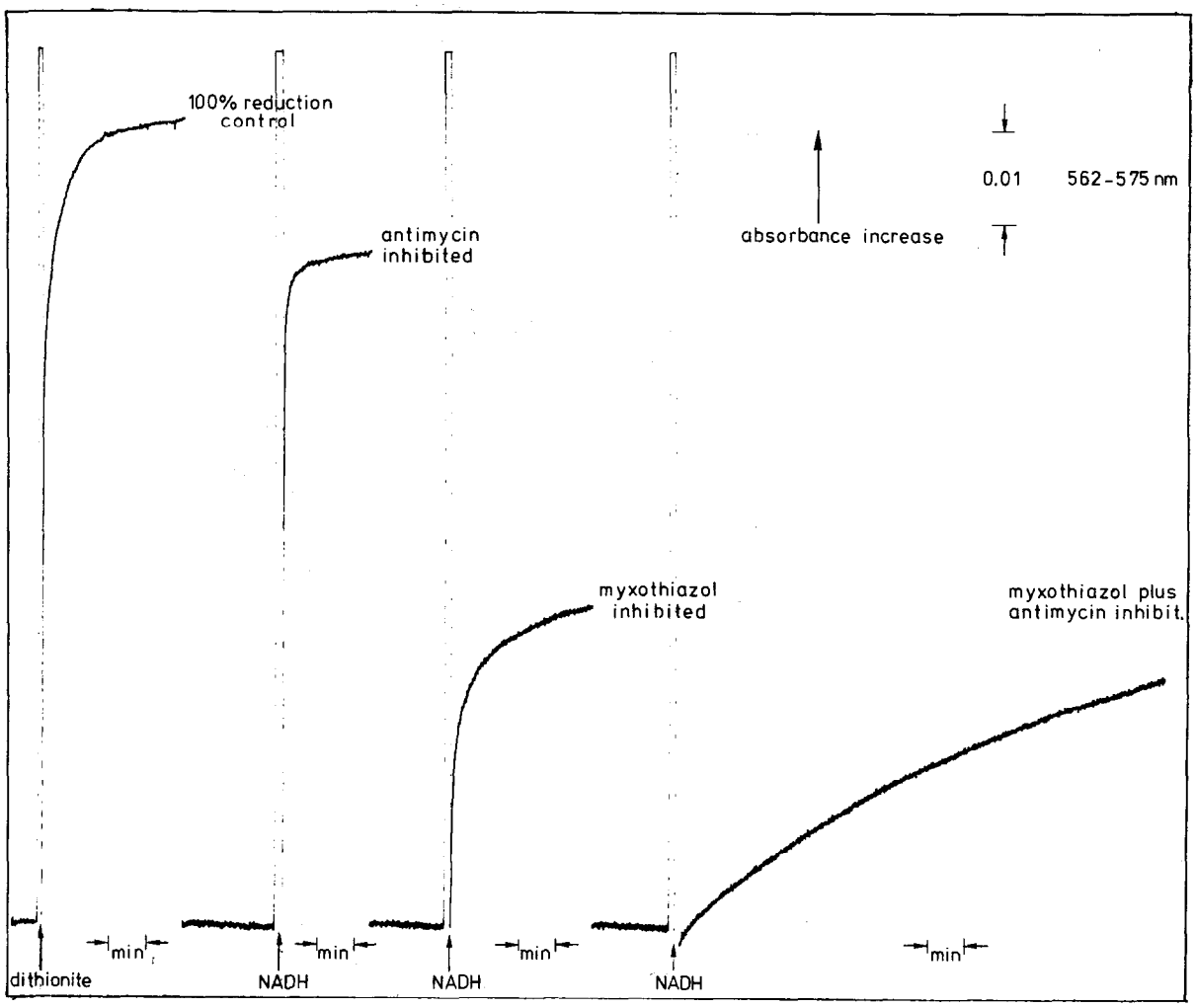


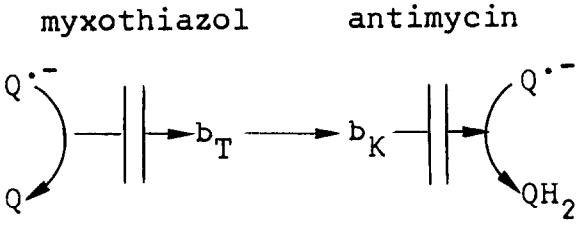
Fig.5. Reduction kinetics of cytochrome *b* of submitochondrial particles in the various inhibited steady states. The reactions were started by addition of dithionite or NADH. Experimental conditions as in fig.2, incubation temp. 27°C.

difference in the signal height of the dithionite and the NADH/antimycin spectrum has to be attributed to cytochrome *b* of complex II. In the presence of myxothiazol (third trace from the left) only the b_K center is reduced (cf. fig.3A). The reduction kinetic seems to be slower than that of antimycin; it is also not resolved here, but this is under process. However, when myxothiazol and antimycin act together (right-hand trace) the reduction of cytochrome *b* takes a number of minutes, even for only the b_K center (cf. fig.4B). Compared to the physiological electron transfer, the rate is slowed down to 1/10 000 of the normal rate, indicating an unspecific reaction, due maybe to a radical mechanism.

4. Summary and conclusion

The moa-inhibitors, represented in this study by myxothiazol, open up a new line of approach for a closer study of the bc_1 segment of the respiratory chain. The red shift maximum of myxothiazol lies at 568 nm, that of antimycin at 564 nm. The red shift signals are on top of each other. Myxothiazol seems to bind directly or in the close vicinity of the b_T-center (*b*-566), whereas antimycin binds directly or near to the b_K-center (*b*-562). The 2 inhibitors do not influence each other while interacting with the ligand fields of the 2 heme *b* centers. When both inhibitors are bound, electrons can no longer be trans-

ferred in a physiological way, the 2 heme centers now being completely shielded against electrons. This finding seems to match the assumption of a linear sequence of the 2 heme *b* centers in the path of electron flow with two ubiquinone binding sites, one for each heme center:



This sequence and functioning of the heme *b* centers was assumed in the conception of the ubiquinone cycle [10] which gained further experimental from [11,12]. According to this working hypothesis, one electron is transferred at the first ubiquinone binding site probably from a semiquinone anion onto b_T; at the second ubiquinone binding site this electron is transferred from the b_K center, presumably again onto a semiquinone anion. This reaction sequence may also bring about a proton transport across the mitochondrial membrane. However, the results obtained are also reconcilable with a different arrangement and functioning of the respiratory components [13]. Therefore further experimental results are required to elucidate this system in more detail.

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