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The interaction of yeast Complex III with some respiratory inhibitors

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We have examined the effects of eight inhibitors of the bovine-heart mitochondrial Complex III on the catalytic activity of the analogous complex from yeast mitochondria. All eight compounds were inhibitory, with potent inhibition being obtained with antimycin, myxothiazol and UHDBT (5-*N*-undecyl-6-hydroxy-4,7-dioxobenzothiazole). These three inhibitors, and also funiculosin, have been further studied by characterizing their effects on the visible absorbance, magnetic circular dichroism and EPR spectra of the complex and also on the potentiometric properties of the individual metal centers present in the complex. All four inhibitors had little or no effect on either the absorbance or magnetic circular dichroism spectra. Funiculosin produced a change in the EPR lineshape of the iron-sulfur cluster; EPR spectra recorded at 12 K also revealed complete reduction of cytochrome *b*-562 by ascorbate. UHDBT also changed the lineshape of the iron-sulfur cluster and this change could be partially reversed by myxothiazol. Neither antimycin nor myxothiazol affected the iron-sulfur cluster and produced only small changes in the EPR absorption envelope of the *b* cytochromes. Both funiculosin and UHDBT raised the midpoint potential of the iron-sulfur cluster, by about 150 and 70 mV, respectively. Only UHDBT changed the potential of c_1, lowering it by about 30 mV. Funiculosin raised the potential of *b*-562 by about 30 mV, while myxothiazol had no effect; the other two compounds produced only small changes. All four compounds had only small effects on the midpoint potential of *b*-566. The relative contributions of the two *b* cytochromes to the magnetic circular dichroism amplitudes could be changed by the addition of inhibitors, even though the absolute magnetic circular dichroism spectra of oxidized and reduced complex were unaffected.

Introduction

As part of our program to characterize the properties of Complex III from the respiratory chain of yeast mitochondria [1–4] we have studied the consequences of reacting this enzyme system with several compounds known to inhibit the catalytic activity of this segment of the electron-transfer chain. This study had two principal objectives: (i) to provide a comparison of the behavior of the yeast complex with that found with the heart enzyme and (ii) as a preparation for a study of the effects of these compounds on the kinetics of oxidation and reduction of the yeast enzyme system.

To that end, we have examined the effects of these inhibitors on the catalytic activity, optical, magnetic CD and EPR spectra and the potentiometric properties of the iron-sulfur and heme-containing redox centers present in our preparation of this enzyme complex.

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Abbreviations: UHDBT, 5-*N*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; CD, circular dichroism; PBQ, 2,3-dimethoxy-5-methyl-6-pentylbenzohydroquinone; OHNQ, 2-OH-3-octyl-1,4-naphthoquinone; UHNQ, 2-hydroxy-3-undecyl-1,4-naphthoquinone; DBMIB, 2,5-dibromo-3-methyl-5-isopropylbenzoquinone; DMSO, dimethyl sulfoxide; HOQNO, 2-heptyl-(4-hydroxyquinoline-*N*-oxide).

Materials and Methods

Yeast Complex III was prepared by our published procedure [1]. A typical preparation contained 4 nmol c_1 per mg of protein and a catalytic activity of 70 mol cytochrome *c* reduced per s per mol c_1 in the standard assay [1] which consists of measuring the reduction of 50 μM ferricytochrome *c* by 25 μM 2,3-dimethoxy-5-methyl-6-pentylbenzohydroquinol ($PBQH_2$) (pH = 7.4, $T = 25°C$).

Antimycin A and 2-heptyl-(4-hydroxy-quinoline-*N*-oxide) (HOQNO) were obtained from Sigma and 2-OH-3-octyl-1,4-naphthoquinone (OHNQ) from the Aldrich Company. 5-*N*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) and 2-hydroxy-3-undecyl-1,4-naphthoquinone (UHNQ) were gifts of Dr. B. Trumpower (Dartmouth). Myxothiazol was donated by Dr. H. Reichenbach (Braunschweig) and 2,5-dibromo-3-methyl-5-isopropylbenzoquinone (DBMIB) was a gift of Dr. R. Malkin (Berkeley, CA). Funiculosin was provided by Dr. G. Lenaz (Bologna). Inhibitors were prepared as 1 or 10 mM solutions in DMSO. PBQ was a gift from Dr. K. Folkers (Austin).

Optical spectra were obtained using a Cary 17 recording spectrophotometer. Magnetic CD spectra were recorded with a Jasco J500C spectropolarimeter equipped with a 1.3 Tesla electromagnet and EPR spectra with a Varian E6 X-band spectrometer. All instruments were connected to the laboratory data system for the storage, manipulation and presentation of the data. Only absolute spectra were recorded; difference spectra were obtained by calculation.

Magnetic CD potentiometric titrations of the cytochromes and low-temperature potentiometric titration of the iron-sulfur center were performed as described previously, except that the buffer (0.1 M potassium phosphate, pH 7.4) contained 0.1% octyl-β-D-glucoside (Calbiochem), 0.1% cholate and 1 mM EDTA. Unless noted otherwise, the potentiometric response of the *b* cytochromes could be resolved as two $n = 1$ components of arbitrary amplitude; the relative amounts of the two components is quantified by the ratio b_H/b_L which simply measures the contribution to the total magnetic CD intensity of the two components.

Results and Discussion

Catalytic activity

Our initial step was to survey the effect of a number of established inhibitors of the bovine heart Complex III on the ability of yeast Complex III to catalyze the reduction of cytochrome *c* using reduced PBQ as electron donor (Table I). Of the eight compounds tested, potent inhibition was obtained only with antimycin A, myxothiazol and UHDBT. These compounds were extremely effective inhibitors at close to 1 : 1 stoichiometry implying the formation of extremely tight complexes with the relevant binding site on the enzyme. By comparison, the compounds DBMIB, OHNQ, UHNQ, HOQNO and funiculosin were somewhat less potent although their inhibitory efficacies were still substantial. Because of this quantitative difference in potency, we elected to study the properties of antimycin, myxothiazol and UHDBT in more detail. Although a weaker inhibitor, funiculosin has striking effects on both spectroscopic and potentiometric properties of the complex, and therefore funiculosin was studied further.

The dependence of the catalytic activity on the ratio of inhibitor to enzyme complex (expressed in terms of cytochrome c_1) is shown in Fig. 1.

As we reported previously [3], inhibition by antimycin A is stoichiometric with maximum in-

TABLE I

EFFECT OF MISCELLANEOUS INHIBITORS ON THE CATALYTIC ACTIVITY OF YEAST COMPLEX III

Catalytic activity was measured at pH 7.4 unless indicated otherwise, using the reaction of reduced PBQ with cytochrome *c*. I_{50} is the ratio of inhibitor to c_1 required to produce 50% inhibition of catalytic activity. The absolute concentration of c_1 was about 5 μM.

Compound	I_{50}
AntimycinA	0.5
Myxothiazol	0.8
UHDBT (pH = 6.7)	1.0
UHDBT (pH = 7.4)	> 8
DBMIB	> 4
OHNQ	> 4
UHNQ	> 4
HOQNO	> 16
Funiculosin	5

hibition obtained with 1 : 1 ratio (Fig. 1) of antimycin to cytochrome c_1. This 1 : 1 stoichiometry was also demonstrated by fluorescence quenching and direct binding experiments. In separate experiments in which we employed much higher concentrations of enzyme to facilitate measurement of the residual activity, we found that at least 98% of the activity is eliminated by preincubation with a 12 : 1 molar ratio of antimycin. Direct addition of the antimycin to the assay resulted in 4% residual activity. This residual (possibly uninhibitable) activity is somewhat smaller than the range of 4–8% found with heart-submitochondrial particles [5].

UHDBT is slightly less potent. At pH 6.7, the pH at which the data shown in Fig. 1 were obtained, one equivalent of UHDBT produces 50% inhibition. However, complete inhibition is difficult to achieve with 15% activity remaining even at quite high ratios of inhibitor to complex. At higher pH values, the concentration of UHDBT must be increased to obtain maximum inhibition.

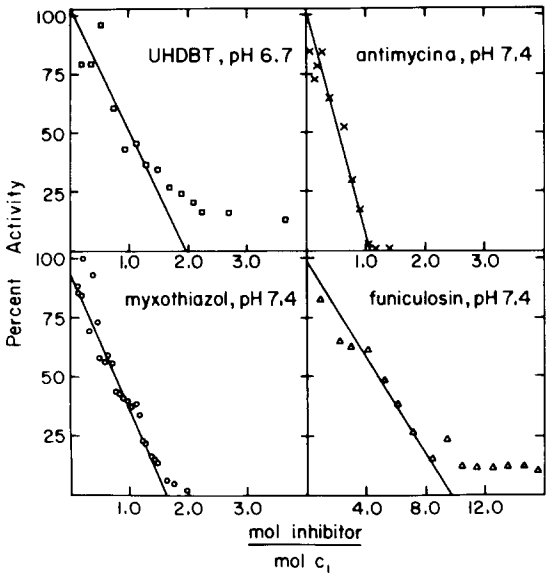

Fig. 1. The effect of several inhibitors on the catalytic activity of yeast complex III. 15 nmole Complex III was gradually titrated with 1 mM or 10 mM solutions of the appropriate inhibitor, and samples were removed for enzyme assay as described in Materials and Methods. Each datum is an average of 2 or 3 measurements. The buffer was 0.1 M potassium phosphate (pH 7.4 or 6.7) containing 0.1% deoxycholate, 0.1% Triton QS-30, 1 mM EDTA. The temperature was 25°C.

For example, at pH 7.4 an 8-times molar excess of inhibitor is necessary to obtain 50% inhibition. The decreased effectiveness of UHDBT at higher pH has previously been noted by Trumpower [6] for the bovine heart complex and correlated by him with a pK of 6.5 present in UHDBT.

Under our experimental conditions, maximum inhibition with myxothiazol requires a 2 : 1 ratio of myxothiazol to c_1 (Fig. 1), although its efficacy at low ratios may be comparable to that obtained with antimycin. The residual activity at a 2-fold excess of myxothiazol is less than 3%. With funiculosin approx. 5% of the activity remained at high inhibitor ratios (Fig. 1).

Optical and magnetic CD spectra

Cytochrome *b* of yeast Complex III is now believed to consist of a single polypeptide [7]; the two component chromophores alternatively referred to as the high potential heme (or b_K or *b*-562) and the low potential heme (or *b*-566 or b_T) are consequently rationalized as heme *b* coordinated in two different domains of this polypeptide. As gauged by EPR spectra, each of these domains appear to be subject to structural strain [8,9].

It has been known for more than twenty years that antimycin affects the optical properties of the *b* cytochromes in heart mitochondria [10], submitochondrial particles [11,12] and succinate cytochrome *c* reductase [13]. A similar effect has recently been observed for myxothiazol with submitochondrial particles, heart Complex III [14,15] and a Complex III from *Rhodopseudomonas sphaeroides* [16]. Both compounds produce a small, about 2 nm, red-shift in the maximum of the alpha-band of the reduced *b* component of the bovine-heart enzyme: a blue shift has been observed in a bacterial enzyme in the presence of myxothiazol. It has also been reported that the two compounds together cause a red-shift which is the sum of that observed separately [14,17].

Addition of two equivalents of antimycin A to reduced yeast Complex III produces only a slight change in the position of the absorption maximum of the composite alpha band of the two cytochromes *b* (Fig. 2a); the computed difference spectrum has a derivative profile and, in the rigid-shift approximation, the extremum at 564 nm (Fig. 2b)

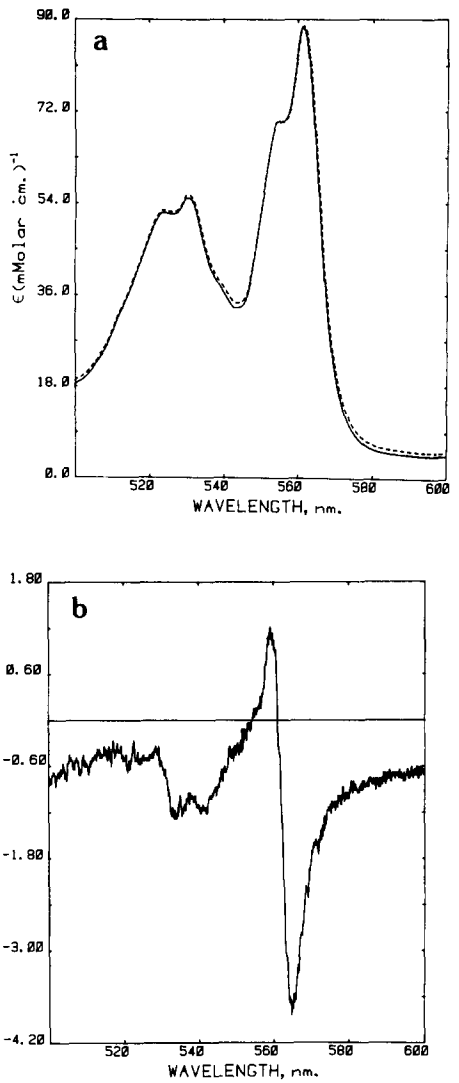

Fig. 2. Absolute and difference absorbance spectra of dithionite-reduced Complex III in the absence and presence of antimycin A. (a) The absolute absorbance spectra of 7.5 μM Complex III reduced with a few grains of dithionite. Subsequently, 15 nmol antimycin A was added and the spectrum rerecorded. (———) Minus antimycin; (- - - - -) plus antimycin. The data were obtained at 23°C. (b) Difference spectrum (control minus antimycin-treated) obtained using the spectral subtraction routine of the laboratory data system.

represents the wavelength of maximum slope in the original absorbtion band. On occasion a slightly larger (smaller than 0.4 nm) red-shift was observed but this was deemed to be within the reproducibility of the system. This absence of a significant spectral effect was confirmed on a second computer-controlled spectrophotometer (IBM 9430) which showed even smaller shifts in the absorption maximum.

The magnetic CD spectra of the same two samples are essentially identical, with the observed changes in wavelength maxima being less than 0.1 nm, the nominal limit in resolution under the conditions of the experiment. This difference is less than the variation observed in repeated runs on the same sample. Again, the computed difference spectrum shows only the slightest changes in the spectra of the sample before and after addition of the inhibitor. It should be stressed that we have observed much larger effects in the absorption and magnetic CD spectra of bovine-heart Complex III upon treatment with antimycin A (unpublished data obtained in collaboration with M. Degli Esposti and G. Lenaz).

Similar small effects on the absorption spectra are found using four equivalents of myxothiazol; in this case, the optical spectrum shows a red shift of about 0.2 nm with the difference spectrum peaking at about 564 nm. Neither antimycin nor myxothiazol produces a discernible effect on the optical spectrum of the oxidized complex between 500–600 nm.

When UHDBT is added to oxidized Complex III small increases in absorbance are observed between 500 and 530 nm with no change in shape; however, no changes are seen between 550 and 560 nm. Addition of this inhibitor to reduced Complex III produces an extremely small increase in the intensity at 562 nm; the computed difference spectra gave no evidence for a spectral shift.

Funiculosin, when added in 10-times excess to ascorbate-reduced Complex III, causes a reduction of approx. 7% of cytochrome *b*, whereas very little change was observed when this amount of inhibitor was added in the dithionite-reduced complex. A slight decrease (less than 1%) of the absorbance at 558 and 561.5 nm was observed in the optical spectrum with a corresponding decrease of the 562 nm signal of the magnetic spectrum. No wavelength shifts were apparent.

EPR spectra

Trumpower and co-workers [6] have reported that UHDBT produces several changes in the line-shape of the Rieske iron-sulfur center with the

peak associated with g_z sharpening and shifting slightly to lower field, while the trough at g_x shifts to higher fields.

A similar pattern of behavior can be demonstrated with the yeast complex. The EPR spectrum of the iron-sulfur center reduced with a minimal amount of ascorbate has g-values of 2.029, 1.898 and 1.81 (Fig. 3, bottom), while that reduced with dithionite (or a level of ascorbate adequate for reduction of cytochrome c_1 but insufficient for reduction of cytochrome b_H) is somewhat different (Fig. 3, top); the most noticeable change being a pronounced broadening and upfield shift to 1.76 of the trough associated with g_x.

Addition of UHDBT produces changes in the EPR lineshape which are independent of the reductant used, and results in a third spectral form (Fig. 3, middle). The trough at g_x is at an intermediate position ($g_x = 1.78$) and has a linewidth comparable to that obtained on reduction with minimal ascorbate; g_y has moved upfield to 1.89, while the peak at g_z moves downfield slightly ($g_z = 2.031$) and increases in amplitude. These effects of UHDBT are very similar to those found by Trumpower with bovine succinate cytochrome *c* reductase [6]. Addition of myxothiazol partially reversed the EPR line shape change caused by UHDBT (Fig. 4). This may be related to the observation of Von Jagow et al. [18] who found that the midpoint potential shift induced by UHDBT can be totally reversed by myxothiazol. This effect of myxothiazol on the EPR appeared to be complete with a 5-fold excess; higher levels did not restore the original lineshape of the untreated enzyme.

Neither antimycin, DBMIB nor myxothiazol alone had any effect on the EPR spectrum of the iron-sulfur center. By contrast, the effect of myxothiazol on bovine heart Complex III is similar to that of UHDBT [18,19] while DBMIB has been shown to have a marked effect on the EPR of both the cytochrome b_6-*f* complex from spinach [20] and on Complex III from bovine-heart mitochondria (Malkin, R. and Trumpower, B.L., quoted in Ref. 21).

Addition of funiculosin (10-times excess) to the complex reduced with a small amount of ascorbate produced a change in EPR lineshape similar to that obtained on addition of dithionite to complex

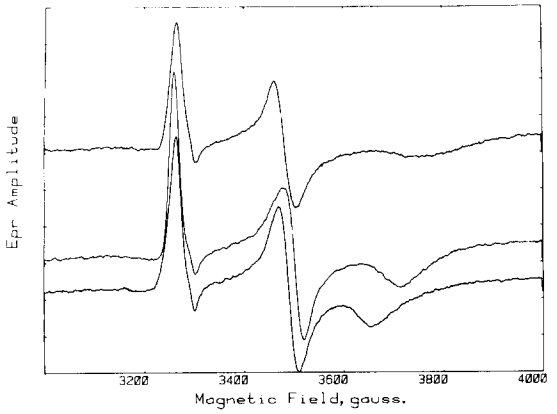

Fig. 3. Comparison of the EPR on the iron-sulfur center of Complex III in the presence and absence of UHDBT. Top: large excess of ascorbate in absence of UHDBT; middle: large excess of ascorbate plus UHDBT; bottom: minimal ascorbate, no UHDBT. 0.3 ml 60 μM Complex III was transferred to calibrated EPR tubes, a minimal or large excess of solid ascorbate was added and mixed. Four equivalents of UHDBT were present in a third sample which was then reduced with an excess of ascorbate. EPR spectra were recorded between 3000 and 4000 gauss under the following conditions: microwave power, 0.1 mW; modulation amplitude, 10 G; microwave frequency, 9.241 GHz; temperature, 12 K.

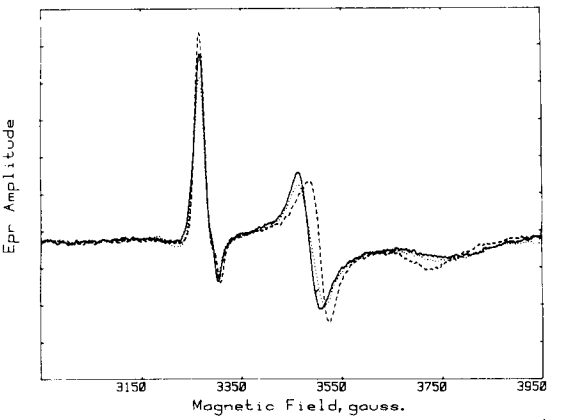

Fig. 4. The ability of myxothiazol to reverse (partially) the change in EPR lineshape produced by UHDBT. ———, Ascorbate-reduced; - - - - - -, plus UHDBT; ·····, plus UHDBT and myxothiazol. 0.3 ml 57 μM Complex III was reduced with an excess of ascorbate and the EPR spectrum recorded. Then, four equivalents of UHDBT were added and the spectrum was rerecorded. Finally, 1–5 equivalents of myxothiazol were added to this sample and spectra were recorded a third time. The EPR conditions are the same as described in Fig. 3.

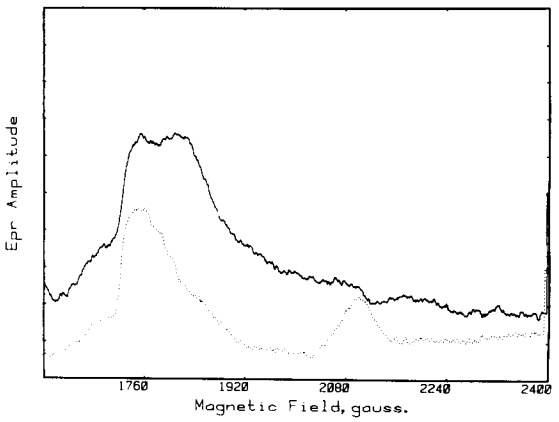

Fig. 5. The effect of funiculosin on the EPR spectrum of cytochrome *b*. ———, Ascorbate-reduced; - - - - -, plus funiculosin. The samples described in Fig. 5 were examined between 1400 and 2400 gauss. The EPR conditions are: microwave power, 40 mW; modulation amplitude, 10 G; microwave frequency, 9.246 GHz; temperature, 9.4 K.

in the absence of inhibitor (see Fig. 3, top vs. bottom). (By analogy to UHDBT, this lead us to suspect that funiculosin might increase the midpoint potential of the iron-sulfur protein; this was later confirmed by potentiometric titration; see below.)

The effect of these four inhibitors on the EPR absorption of the *b*-cytochrome between $g = 3.4$ and 3.8 in ascorbate-reduced Complex III was also examined.

In the presence of funiculosin, ascorbate appears to reduce cytochrome *b*-562, and the resultant EPR spectrum exhibits the characteristically assymetric EPR of cytochrome *b*-566 (Fig. 5). This result was most unexpected because the room temperature optical spectrum of ascorbate-reduced, funiculosin-treated enzyme shows only a small reduction of cytochrome *b*. However, potentiometric data reveal that the midpoint potential of the higher potential cytochrome *b* is raised by this inhibitor (see below). In addition, funiculosin induces an increase in signal at $g = 2.9$; this cannot be accounted for by the amount of DMSO added to the sample and suggests that this inhibtor either partially relaxes the strain at the heme [9] or denatures a portion of the sample.

Both antimycin and UHDBT produce only small changes in the EPR lineshape in this region. Antimycin produced an increase in the relative amplitude of the component with a *g*-value of 3.6 (cytochrome *b*-562); UHDBT led to a decrease in the resolution of the two *b* components, with only a single absorption maximum replacing the split peak observable in the absence of this reagent. Dervartanian [22] has reported that, in bovine Complex III, antimycin causes the absorption maximum of the higher field *b* cytochrome to shift to lower field; this has been confirmed by De Vries et al. [19] who also find that myxothiazol shifts the same absorption to higher field. Both reagents are reported to sharpen the absorption peak of the low-field cytochrome. No effect of myxothiazol was observed with yeast Complex III.

Potentiometric measurements

Fig. 6 shows the potentiometric results obtained in the presence of antimycin, myxothiazol and UHDBT (data obtained with funiculosin are shown in Fig. 7). Data on the cytochromes were obtained by the following changes in the intensity of the magnetic CD spectra in the neighborhood of the alpha-band; such measurements are essentially unaffected by the strong color of the mediators which exhibit no magnetic CD. The data on the iron-sulfur cluster were obtained by low-temperature EPR. The values for the midpoint potentials deduced from the data are summarized in Table II. In this table, the data on the *b* cytochromes in the untreated complex and in the presence of antimycin are somewhat different than we reported earlier [2]. The present data were obtained using octyl glucoside as detergent; this detergent has no effect on the catalytic activity. Our earlier data were acquired in Triton QS30-deoxycholate, a combination of detergents which reduces the catalytic activity by a factor of about 5.

Of the four inhibitors examined, only UHDBT affects the midpoint potential of cytochrome c_1, lowering the potential of this center by 40 mV. At the same time, this inhibitor raises the midpoint potential of the Rieske iron-sulfur center by about 75 mV. A similar increase in potential with UHDBT was observed previously by Trumpower in bovine succinate cytochrome *c* reductase [6] and interpreted, reasonably enough, as indicating that this compound binds about 15-times more strongly to the reduced iron-sulfur cluster than it does to

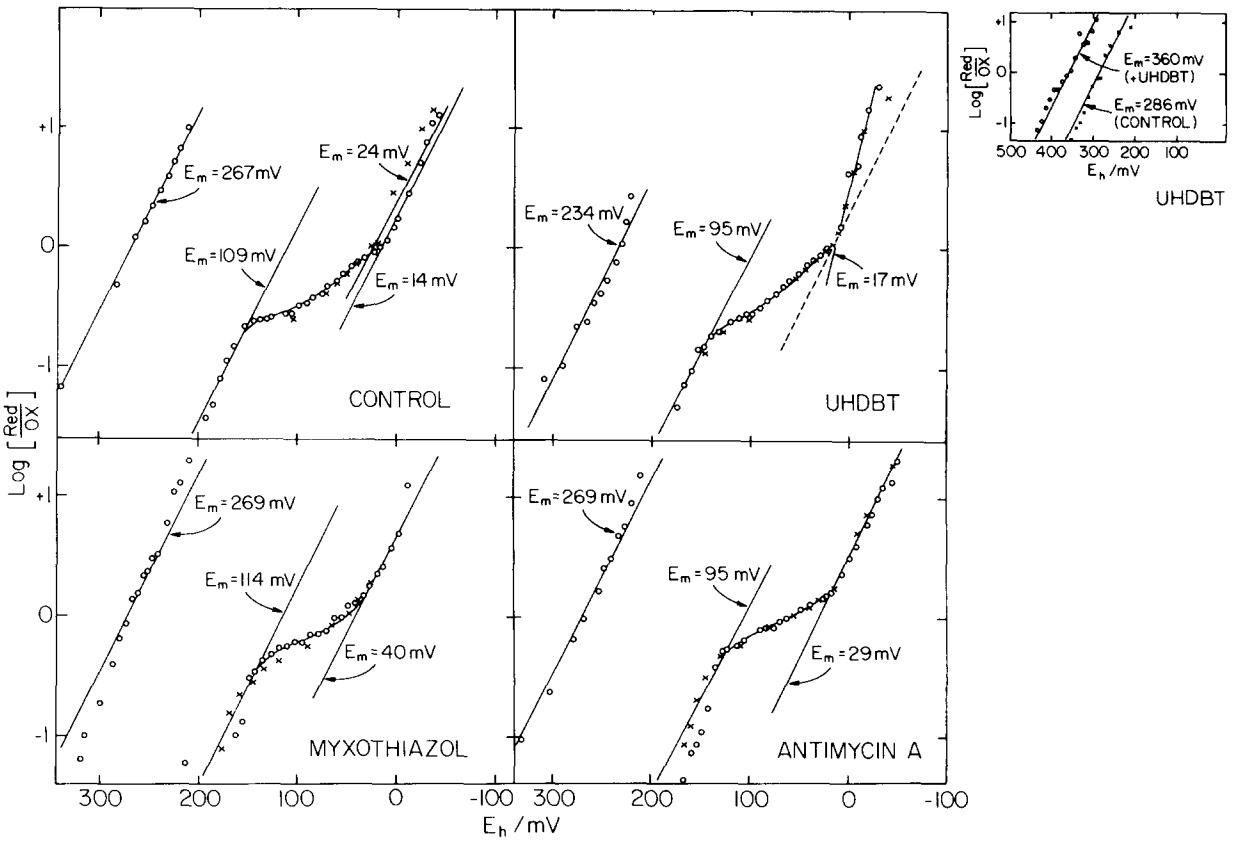

Fig. 6. Effect of inhibitors on the midpoint potentials of redox centers of native Complex III. ○, Reduction; ×, reoxidation. The midpoint potentials of *b* and c_1 cytochromes are determined by room temperature magnetic CD potentiometric titration as described in Ref. 2. Titrations were carried out at 23°C in 0.1 M potassium inorganic phosphate (pH 7.4), containing 1 mM EDTA, 0.1% cholate and 0.1% octyl-β-D-glucoside and 5 μM Complex III. 20 μM of the following dyes were included in the reaction mixture: *p*-aminophenol sulfate, TMPD, DCIP, 1,2-naphthoquinone, phenazin methosulfate, methylene blue, indigo-tetrasulfonic acid. UHDBT (4×), myxothiazol (2×) and antimycin-A (2×) are included where indicated. Upper right: The midpoint potentials of iron-sulfur center with or without UHDBT were obtained using an EPR-potentiometric titration technique as detailed in Ref. 2. Thirty micromolar Complex III and 4-fold excess of UHDBT was added in the UHDBT-treated sample. Titrations were performed at 23°C and the observations of the $g = 1.89$ EPR signal of the iron-sulfur center were carried out by liquid helium EPR as described in Fig. 3.

TABLE II

THE EFFECT OF INHIBITORS ON THE MIDPOINT POTENTIALS FOR THE REDOX CENTERS OF YEAST COMPLEX III

Addition	Midpoint potential E_m (mV)				
	c_1	b_H	b_L	b_H/b_L	FeS
None	267	109	19	0.36	286
Antimycin A (2×)	269	95	29	0.91	–
UHDBT (4×)	234	95	17 [a]	0.36	360
Myxothiazol (2×)	269	114	40	0.58	–
Funiculosin (3×)	270	142	23	1.07	442

[a] The $n = 2$ behavior was observed.

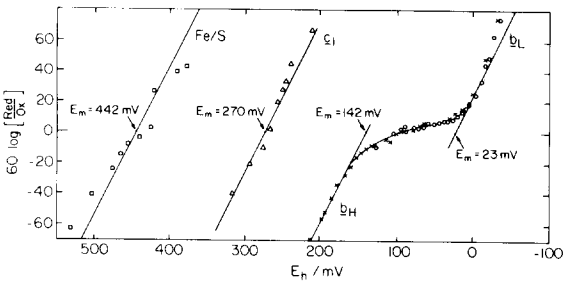

Fig. 7. The effect of funiculosin on the midpoint potentials of each redox center of Complex III. (○) Reduction; (×) re-oxidation. The midpoint potentials were determined by the same procedure as described in Fig. 6 with ten equivalents of funiculosin present in the reaction mxiture. 10 mM ferricyanide was gradually added in order to obtain data at potentials greater than 300 mV.

the oxidized cluster. More striking is our finding that funiculosin raises the midpoint potential of the Fe/S center to 442 mV, an increase of about 160 mV, suggesting that this inhibitor binds to the reduced cluster some 500-times more avidly than to the oxidized. This is the largest inhibitor-induced change in potential of any center in the yeast complex and, as far as we know, in the bc_1 complex from any source.

The changes produced by antimycin A and myxothiazol are much less dramatic, as was also found with bovine heart mitochondria [23]. Antimycin and UHDBT produce a 15 mV decrease in the midpoint potential of the high-potential *b* component, while myxothiazol has essentially no effect. By contrast, UHDBT has no effect on the low-potential *b* component, while antimycin and myxothiazol produced increases of 10 and 20 mV, respectively.

It should be noted that the titration data on the *b* components in the presence of UHDBT could not be resolved into two $n = 1$ components; the high-potential limb of the data did exhibit $n = 1$ behavior, but the low-potential component had $n = 2$ character.

Room temperature magnetic CD potentiometry in the presence of funiculosin reveals that the potential of the high-potential *b* is raised by about 30 mV in the presence of this inhibitor (Fig. 7); this finding is similar to that reported for the bovine heart enzyme [23]. This increase, together with an additional increase apparently induced by lowering the temperature, leads to the complete reduction of *b*-562 at 12 K, as judged by EPR (see above).

Antimycin, myxothiazol and funiculosin produce changes in the relative contributions of the two heme *b* centers to the high- and low-potential phases of the changes in magnetic CD intensity with the largest differentiation observed with funiculosin. In all cases, the total magnetic CD intensity is unaffected by the presence of an inhibitor.

The simplest interpretation of the observed variations in intensity is that these inhibitors produce compensating changes in the magnetic CD contributions of the two *b* components such that their individual contributions change, but with the sum of their contributions remaining constant. In view of the different nature of the compounds producing this effect, this 'sum rule' seems somewhat unlikely. A more plausible interpretation requires that the optical properties of the fully oxidized and fully reduced complex be insensitive to the reagent added but that the absorbance and MCD characteristics of the complex in which the *b* cytochromes are only 50% reduced be variable. This hypothesis attributes a special malleability to the half-reduced *b* cytochrome, a requirement consistent with our proposal [8,9] that the hemes in the oxidized cytochrome may be subject to structural strain.

Conclusions

The response of yeast Complex III to the three inhibitors antimycin A, myxothiazol and UHDBT generally conforms to expectations derived from earlier work with the bovine heart system.

The most striking difference in the data on the two systems are the very small effects that antimycin and myxothiazol exert on the spectroscopic properties of the cytochromes *b* of the yeast complex. This insensitivity is seen in the absorbance, magnetic CD and EPR data. The latter parameter is particularly telling, for the magnitude of the low-field *g*-value of low-spin hemes is extremely sensitive to the geometry of the ligand field at the heme iron [9], and one can reasonably conclude from these results that these inhibitors do not significantly perturb the heme geometry. There is thus the inference that the binding sites for these

reagents are distant from either heme *b*. It should be remembered, however, that there is a body of data, predominantly genetic, locating the binding site for both antimycin and myxothiazol in the cytochrome *b* polypeptide [15,24]. The simplest conclusion is that these inhibitors bind to the polypeptide at a site remote from the heme and have a variable effect on the heme itself. However, it should be remembered that we failed to demonstrate the binding of antimycin to isolated cytochrome *b* even though the solubilized cytochrome appears to maintain its strained characteristics [3].

Acknowledgements

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