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EFFECTS OF DIBROMOTHYMOQUINONE ON THE STRUCTURE AND FUNCTION OF THE MITOCHONDRIAL bc_1 COMPLEX

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We have investigated in detail the effects of dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, DBMIB) on the ubiquinol-cytochrome *c* reductase (cytochrome bc_1 complex) from bovine heart mitochondria. The inhibitory action of DBMIB on the steady-state activity of the bc_1 complex is related to the specific binding of the quinone to the purified enzymatic complex. At concentrations higher than 10 mol per mol of the enzyme, DBMIB is able to stimulate an antimycin-insensitive reduction of cytochrome *c* catalyzed by the bc_1 complex. In accordance with kinetic data showing a competition by endogenous ubiquinone in the inhibitory action, DBMIB can be considered as a product-like inhibitor of the ubiquinol-cytochrome *c* reductase activity. The site of specific binding of dibromothymoquinone in the bc_1 complex enables it to interact with the iron-sulphur center of the enzyme, as indicated by changes induced in the EPR spectrum of the center. However, the inhibitor also directly interacts with cytochrome *b*, promoting a fast chemical oxidation of the reduced heme center. In spite of these effects, DBMIB has been found not to exert significant effects on the first turnover of the fully oxidized bc_1 complex, as monitored by the rapid reduction of both cytochromes *b* and c_1 by ubiquinol-1. In the presence of antimycin, only a stimulation of cytochrome c_1 reduction, in parallel to an enhanced cytochrome *b* reoxidation, is observed. Moreover, DBMIB does not affect the oxidant-induced extra cytochrome *b* reduction in the presence of antimycin. On the basis of the evidences suggesting a competition with the endogenous ubiquinone in the redox cycle of the bc_1 complex, a model is proposed for the mechanism of DBMIB inhibition. Such model can also explain at the molecular level the redox bypass induced by dibromothymoquinone in the whole respiratory chain (Degli Esposti, M., Rugolo, M. and Lenaz, G. (1983) FEBS Lett. 156, 15–19).

Introduction

In the last three years there has been an increasing interest on the quinol-acceptor oxidoreductases, an ubiquitous class of redox enzymes catalyzing the oxidation of quinols by a high-potential, hydrophilic acceptor (cytochrome *c* or plastocyanin) [1]. Such enzymes, constituted by various polypeptides to form a multiprotein complex, play a central role in the electron-transfer chains of all aerobic organisms, and form one of the main sites of energy conservation [1,2].

Two electron routes have been postulated for the redox mechanism of such complexes [1–4]. One, the most important under physiological con-

Abbreviations: BAL, 2,3-dimercaptopropanol (British Anti Lewisite); DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); HNQ, 3-alkyl-2-hydroxynaphthoquinones; Q_1, ubiquinone-1; Q_1H_2, ubiquinol-1; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HQNO, 2-nonyl-4-hydroxy-quinoline-*N*-oxide.

ditions, involves ubiquinol oxidation at center o in a Q-cycle scheme [3], by means of a concerted reaction with the low-potential form of cytochrome *b* (usually called *b*-566) and the iron-sulphur center (usually denominated Rieske protein) [4]. A second one, catalyzed by the high-potential form of cytochrome *b* (usually called *b*-562), should result from a reversal of the normal pathway of cytochrome *b* oxidation by ubiquinone at center i in a Q-cycle scheme [2–4]. A number of specific inhibitors of the quinol-acceptor oxidoreductase activity have contributed to the identification of such two-electron routes. They can be classified into two main classes: inhibitors acting at center i in a Q-cycle scheme [2–4], and inhibitors acting at center o in a Q-cycle scheme [2,4]. The former class includes antimycin and antimycin-like compounds such as funiculosin and HQNO [5,6], inhibitors capable to induce the oxidant-induced extra cytochrome *b* reduction [1,2]. The latter class includes a large variety of compounds which modify ubiquinol oxidation in different manners, but do not induce the oxidant-induced extra-*b* reduction. They are able to block cytochrome *b* reduction by substrates in the presence of antimycin, and prevent the oxidant-induced extra *b* reduction [2–4]. The most powerful of such inhibitors are of quinoid structure, such as myxothiazol [7,8] and its related antibiotics [9], 5-undecyl-6-hydroxy-4,7-dioxobenzothiazol (UHDBT) [10–12], mucidin [13], *n*-heptadecylmercapto-6-hydroxy-4,7-quinolinequinone (HMHQQ) [14], and 3-alkyl-2-hydroxy-naphthoquinones (HNQ) [15]. Other less specific sulphydryl reagents like 2,3-dimercapto-propanol (BAL) and DTNB are generally classified as center o inhibitors, since they directly react with the Rieske center [2,16,17]. All of the above-mentioned quinoid inhibitors have been also shown to affect this center, as judged by EPR spectroscopy [2,4,12,14,15].

Another quinoid compound which appears to alter the Rieske center is dibromothymoquinone (DBMIB), discovered as a powerful inhibitor of the the b_6f complex in chloroplasts [1,18]. This enzyme contains an iron-sulphur center very similar in its physico-chemical and functional properties to that present in the mitochondrial bc_1 complex [4,18,19]. Its EPR signal after ascorbate reduction is almost identical to that of the mitochondrial Rieske center, and is dramatically affected by treatment with DBMIB [18,20,21]. The effect is parallel to the inactivation of the redox function of the complex [1,18]. We have recently found that DBMIB is able to inhibit also the redox activity of the bc_1 complex at low concentrations [22]. This has prompted us to investigate the effects of such quinoid inhibitor, in order to understand its mechanism of action. To this purpose, both spectrophotometric fast kinetics and EPR spectroscopy have been employed.

Materials and Methods

We have routinely used the isolated bc_1 complex purified according to the method of Rieske [23] and a cruder mitochondrial subfraction, fraction S_1 [23], which can be considered a crude succinate-cytochrome *c* reductase preparation [24]. Reconstitution of the isolated complex into soya bean vesicles has been accomplished as previously described [25]. Ubiquinols were prepared as in Ref. 23.

Spectra of cytochromes were performed in a Perkin-Elmer 559 spectrophotometer in the presence of 1% deoxycholate. Cytochrome c_1 concentration was determined after reduction with dithionite at 554–541 nm using an extinction coefficient of 28 mM^{-1}, a value higher than that reported for the purified cytochrome [26] in order to account for the overlapping of cytochrome *b* spectrum at the above wavelengths [27].

EPR spectra were performed in a Varian E9 spectrometer at either liquid nitrogen or liquid helium temperature. The isolated bc_1 complex, at about 30–45 μM, was previously reduced with solid sodium ascorbate or dithionite. For further details see the legend of Fig. 4.

The rapid spectrophotometric traces were monitored in a Sigma Biochem ZWS II dual wavelength spectrophotometer equipped either with a rapid mixing apparatus of our own design (mixing time, 270 ms) or with a stopped-flow system [24]. In the latter, one syringe contained the mitochondrial fraction suspended in 0.1 M potassium phosphate, 1 mM EDTA (pH 7.4) at about 1–3 μM cytochrome c_1; the other syringe contained the desired reactant dissolved in the same buffer. All the

experiments were carried out at 20 °C. The reduction of cytochrome *b* was followed both at 562–575 and 430–410 nm, whereas that of cytochrome c_1 at 553–541 nm. The time response of the phototube channels was set at 5–10 ms, and the wavelength bandwidth at 1–3 nm. The traces were routinely stored in the digital memory of the instrument, and then transferred at the desired amplification in an *x–y* recorder [24].

To obtain a stable fully-oxidized preparation, the subfraction was previously homogenized in the phosphate buffer containing 2 mM ferricyanide, and then centrifuged at 100 000 × *g* to remove the excess of oxidant. Since this procedure partially washed away some detergent usually bound to the bc_1 complex, occasionally it was necessary to add 0.1% deoxycholate to the subfraction suspension in order to better solubilize it.

The ubiquinol- and succinate-cytochrome *c* reductase activities were assayed as previously described [28].

DBMIB was a generous gift from Prof. A. Trebst, University of Bochum, F.R.G., and was stored in ethanol at −16 °C. Its concentration was evaluated at 289 nm in ethanol using an extinction coefficient of 14.5 mM^{-1} [22].

All the reagents were the purest commercially available.

Results

DBMIB inhibition of the steady-state activity of the bc_1 complex

Dibromothymoquinone is a rather specific inhibitor of the redox activity of the bc_1 complex, but its mode of inhibition is affected by a number of experimental parameters, which can be summarized in the following points.

(i) The higher the turnover of the bc_1 complex under the assay conditions, the lower the apparent titer for DBMIB inhibition [22].

(ii) Although ubiquinol-2 appears to be the most efficient substrate for the bc_1 complex (Table I) [1], the most potent action of DBMIB has been found with ubiquinol-1 (Table I). The latter quinol has been shown to be quite physiological [29], not to equilibrate with the endogenous ubiquinone [25,30], and to display a well-characterized steady-state kinetics [25]. We have therefore used Q_1H_2 as the selected substrate for studying the effects of DBMIB. When exogenous ubiquinones are added to the isolated bc_1 complex or the crude succinate-cytochrome *c* reductase at low ratios to cytochrome c_1, the apparent half-inhibition titer of DBMIB generally increases (Table I). In intact mitochondrial particles, the titers of inhibition are higher than in subfractions containing less endoge-

TABLE I

EFFICIENCY OF DBMIB INHIBITION OF THE REDOX ACTIVITY OF THE bc_1 COMPLEX WITH VARIOUS SUBSTRATES

The cytochrome *c* reductase rates of isolated bc_1 complex or cruder mitochondrial subfractions were measured at a ratio of exogenous cytochrome *c* to cytochrome c_1 ranging from 100 to 2000. The reaction was usually started with the reducing substrate.

Substrate	Apparent $K_{50\%}$ inhibition		Turnover range in absence of DBMIB (s^{-1})
	(mol/mol cytochrome c_1)	(μM range)	
Succinate	70 ± 30	1.5 –2	5– 15
Ubiquinol-1	3 ± 2	0.03–0.15	60–200
Ubiquinol-2	8 ± 3	0.05–0.2	300–500
Ubiquinol-3	7 ± 2	0.06–0.1	70–150
Ubiquinol-3 [a]	40	0.36	100

[a] Crude succinate-cytochrome *c* reductase reconstituted with about 20 mol per mol cytochrome c_1 of exogenous ubiquinone-3 prior to the addition of DBMIB.

nous ubiquinone, such as the isolated bc_1 complex. Extraction of most of the endogenous quinone complement in mitochondria facilitates the inhibitory action of DBMIB (Fig. 1A).

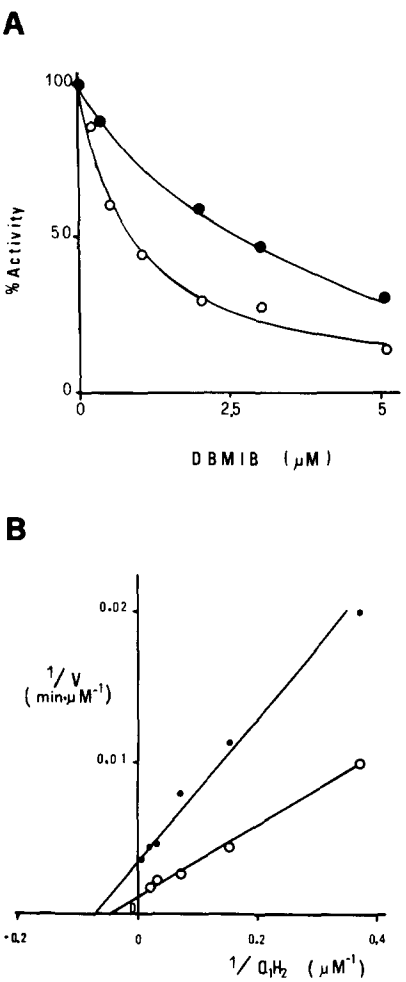

Fig. 1. (A) Effect of the extraction of the endogenous ubiquinone on the DBMIB inhibition of the Q_1H_2 (40 μM)-cytochrome c (20 μM) reductase in liophylized bovine heart mitochondria. ●———●, pentane extracted, and then reconstituted with the extract as described in Ref. 30, containing 29 mol of ubiquinone per mol of cytochrome c_1. ○———○, pentane extracted, containing a residual amount of ubiquinone around 2 mol per mol cytochrome c_1. DBMIB was directly added to the assay mixture. The unhinibited rates were around 60 s^{-1} in both preparations. (B) Q_1H_2 titration at a saturating cytochrome c concentration (13 μM) in a crude succinate cytochrome c reductase preparation, in the absence (○———○) and in the presence (●———●) of 4 mol DBMIB per mol of cytochrome c_1. The inhibitor was incubated with the concentrated solution of the fraction, whose maximal activity was 65 s^{-1}.

These evidences outline that probably dibromothymoquinone acts on competition with the oxidized form of ubiquinone, which actually is one of the products of the ubiquinol-cytochrome c reductase catalysis. Earlier results in both mitochondria [31] and chloroplasts]32] support such idea. The finding that under any experimental conditions DBMIB behaves as a non-competitive inhibitor with respect to ubiquinol (Fig. 1B) is also in accordance with the above possibility. DBMIB, therefore, can be considered as a product-like inhibitor of the redox activity of the bc_1 complex.

(iii) If the ubiquinol-cytochrome c reductase assays were started by the oxidizing substrate cytochrome c, after ubiquinol has been already equilibrated with the enzyme, the inhibition of DBMIB results clearly higher (Fig. 2A). The time-course of cytochrome c reduction when the starting substrate is ubiquinol, however, is comparatively more inhibited than the initial rate. The titer for half-in-

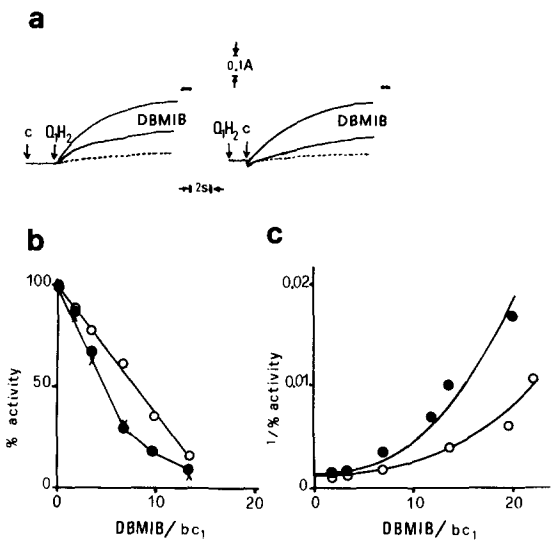

Fig. 2. (A) Effect of the order of substrate addition on the inhibition by DBMIB (5.5 mol per mol of cytochrome c_1) of the Q_1H_2 (60 μM)/cytochrome c (22 μM) reductase activity of the isolated bc_1 complex at 150 nM cytochrome c_1. The dashed lines represent the traces obtained in the presence of antimycin (10 μM) with or without DBMIB. (B) Concentration dependence of the inhibition by DBMIB of the initial rates of cytochrome c reduction starting the reaction with Q_1H_2 (○———○) or cytochrome c (●———●); (×———×) refers to the time-courses of cytochrome c reduction initiated by ubiquinol-1. (C) Dixon plots of the initial rates in B.

hibition of the rate-constants results in close correspondence with that obtained starting the reaction with cytochrome *c* (Fig. 2B). Such phenomenon probably reflects the fact that the inhibition progressively increases with the number of turnovers made during the enzymatic reaction (cf. point i). The Dixon plots of DBMIB inhibition are non-linear, of parabolic shape, independently of the order of substrate addition (Fig. 2C).

We have found that dibromothymoquinone also inhibits the proton-translocating activity of the bc_1 complex reconstituted into phospholipid vesicles, almost in parallel to its redox activity. The apparent half-inhibition is reached at around 2 mol per mol of cytochrome c_1.

Binding of DBMIB to the isolated bc_1 complex and effects on its EPR spectrum

Since DBMIB is soluble and quite stable in water, it has been possible to quantitatively determine its binding to the bc_1 complex by centrifugation [6]. The Scatchard plot obtained evaluating the absorbance at 292 nm (the λ_{max} of DBMIB in water) of the supernatants is clearly biphasic (Fig. 3A and B). Graphical computation of the results according to Ref. 33 as in Ref. 6 reveals two populations of binding sites, with largely different affinity for the inhibitor. The most specific binding sites have a maximal value of 1.4 mol per mol of cytochrome c_1, and display a dissociation constant K_D of 73 nM. The latter value is within the

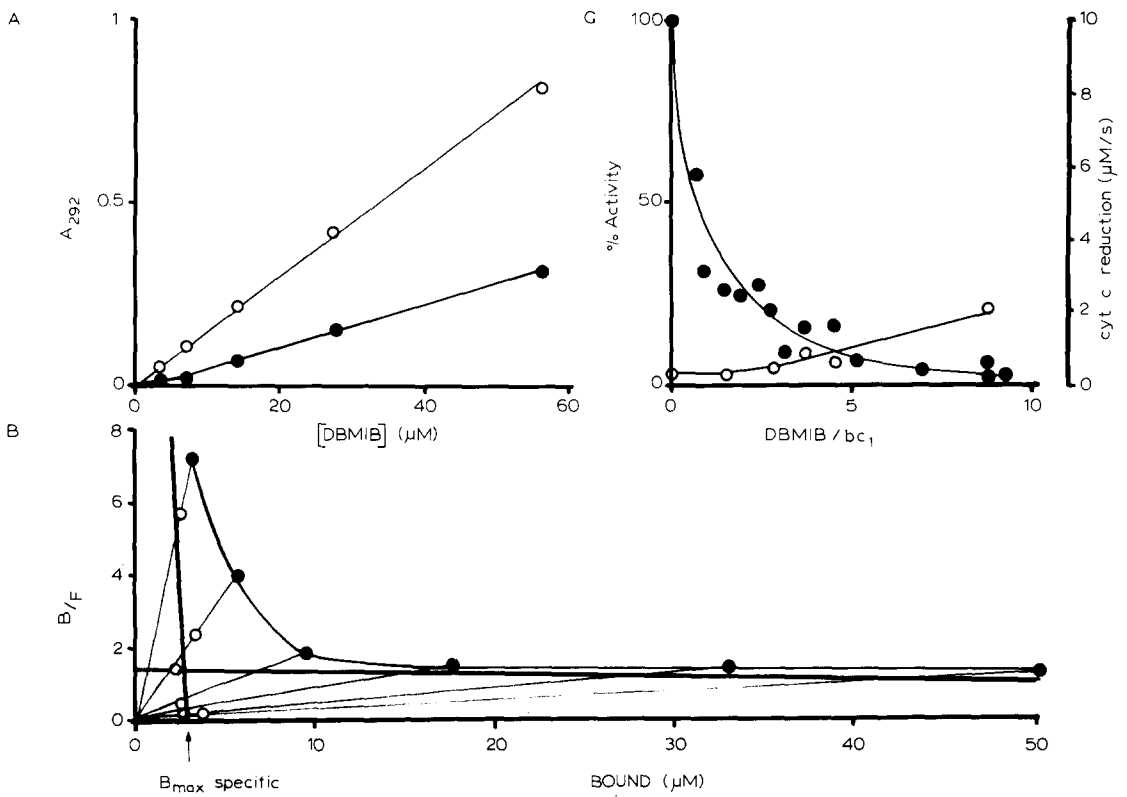

Fig. 3. Binding of DBMIB to the isolated bc_1 complex suspended at 2 μM in the same buffer used for the enzymatic assays. (A) Absorbance at 292 nm of the supernatants after centrifugation at 100000 × *g* for 30 min to pellet the enzyme (●———●), and in the absence of the complex (○———○). (B) Scatchard plot of the data in A. The graphical computation of the two linear curves was carried out according to Ref. 33. (C) Correlation between the total DBMIB bound to the bc_1 complex and the antimycin-sensitive (●———●) and the antimycin-insensitive (○———○) rates of cytochrome *c* reduction (28 μM) by 60 μM Q_1H_2 preincubated with 0.3 μM bc_1 complex. The relative proportion of the rates is respected in the graph.

range of the apparent half-inhibition constants determined in inhibition studies (cf. Table I). The less specific binding sites have a maximal binding and a K_D three and four orders of magnitude higher, respectively.

The correlation between the specific binding and the inhibition of the antimycin-sensitive reductase rates appears quite good (Fig. 3C). The antimycin-insensitive cytochrome *c* reduction starts to be stimulated after the most specific sites have been already occupied by the inhibitor (Fig. 3C). We have verified that the previously quoted factors affecting the mode of DBMIB inhibition are related to the inhibitor bound to the complex, and not to its concentration in the medium.

We have studied the EPR spectra of the isolated bc_1 complex reduced with different reagents to monitor the effects of DBMIB on the paramagnetic spectrum of the Rieske center, which is visible also at liquid nitrogen temperatures [2,34–36]. Treatment with DBMIB concentrations leading to inhibition of the steady-state redox activity of the bc_1 complex induces a remarkable modification of such a spectrum (Fig. 4). In particular, the g_y signal of the Rieske iron-sulphur center at $g = 1.90$ is decreased, and a resonance around $g = 1.94$ appears or dramatically increases in intensity. Even the purest preparations of the bc_1 complex contain large contaminations of EPR signals centered around $g = 1.94$ of ferredoxin-type iron-sulphur centers [2,34]. Although they normally are not reducible by ascorbate [34], one may argue that the observed EPR changes induced by DBMIB could be caused by an enhanced reduction of such centers promoted by the quinone, acting as a redox mediator. Therefore, it is questionable to interpret the effect of DBMIB as a real shift of the EPR spectrum of the Rieske center, as it has been proposed in the b_6f complex [18,21]. However, the following experimental evidences outline a direct interaction of DBMIB with the iron-sulphur center of the bc_1 complex, which can be mainly responsible for the observed EPR changes.

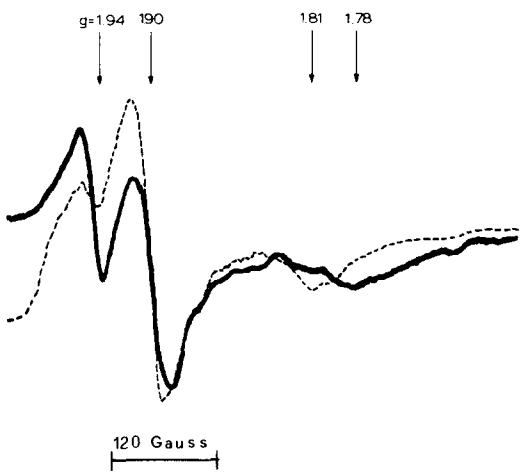

Fig. 4. EPR spectrum of the ascorbate-reduced bc_1 complex at liquid helium temperature (12K). The dashed line is the control, and the solid line represents the spectrum after the addition of 10 mol DBMIB per mol cytochrome c_1. The EPR conditions were: microwave frequency, 9.247 GHz; microwave power, 0.2 mW; time constant, 1 s; scan rate, 1 gauss/s. The experiment was performed in collaboration with Prof. G. Palmer and Dr. A.L. Tsai, Dept. of Biochemistry, Rice University, Houston, TX, U.S.A.

(1) After complete reduction with dithonite, the overall EPR spectrum in the presence of DBMIB is different than that of the control, still presenting a clearly higher signal at around $g = 1.94$–1.95. (2) A decrease of the $g = 1.90$ and the formation of a new resonance at $g = 1.95$ has been found in the presence of DBMIB after specific reduction of the Rieske center by a fumarate/succinate redox poise, performed according to Ref. 2. (3) At liquid helium temperatures also an upfield shift of the g_x resonance (from $g = 1.81$ to $g = 1.78$, Fig. 4) is observed upon DBMIB addition. The latter effect appears very similar to that induced by UHDBT [12] or other quinoid inhibitors [15], but apparently has not been detected in the b_6f complex [21]. Complexively, the modification of the EPR spectrum of the Rieske center produced by dibromothymoquinone in the bc_1 complex is close, but not identical to that found in chloroplasts [21].

Effects of DBMIB on the pre-steady-state kinetics of the bc_1 complex

Under conditions leading to both the inhibition of the steady-state reductase activity and the modification of the EPR signal of the Rieske center, the first redox turnover of the bc_1 complex is not significantly altered by DBMIB (Fig. 5). The first turnover can be considered the reduction of either cytochrome *b* or cytochrome c_1 in the fully-oxidized

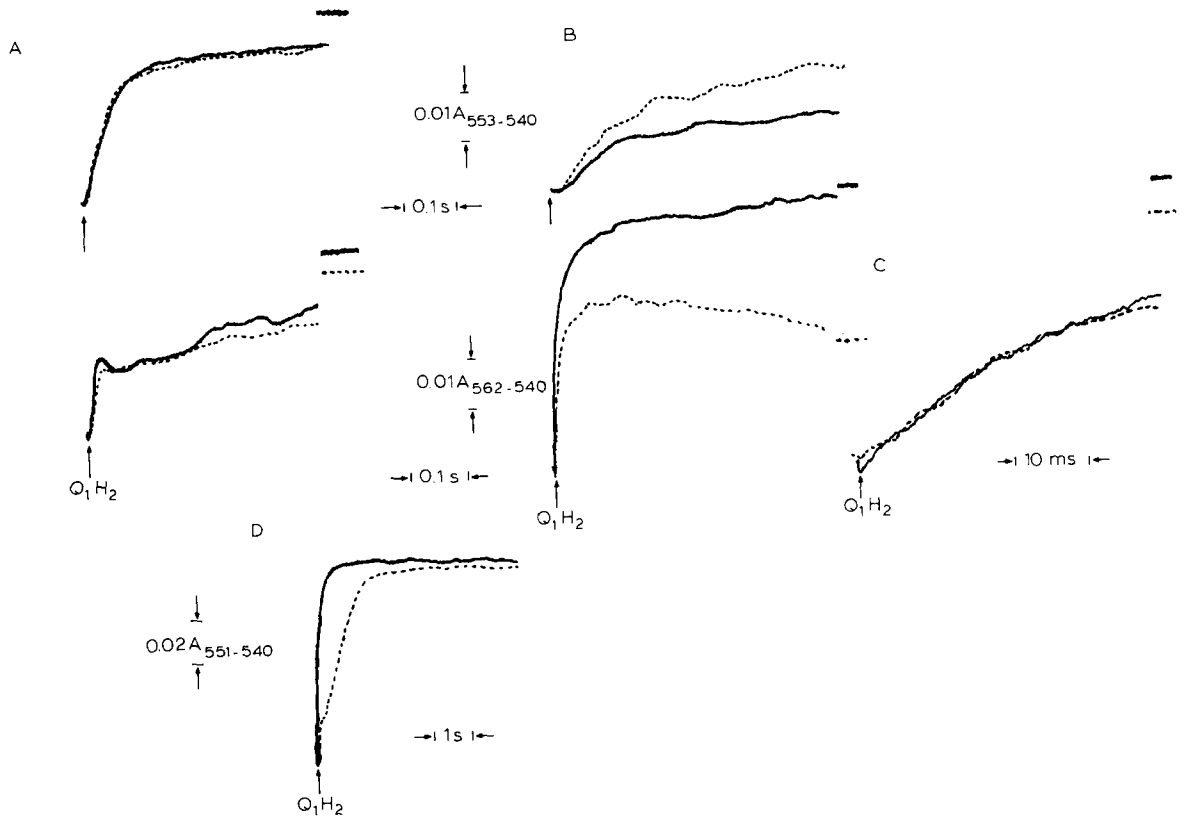

Fig. 5. Stopped-flow traces of cytochrome c_1 (upper lines in A and B) and cytochrome *b* (lower lines in A, B and C) reduction by 30 μM Q_1H_2. In the experiment was employed a crude succinate-cytochrome *c* reductase preparation at 1.6 μM cytochrome c_1 final concentration. The solid lines represent the control traces, and the dashed lines those obtained in the presence of DBMIB at 8 mol per mol of cytochrome c_1 (in A and B) or at 4 mol per mol of cytochrome c_1 (in C and D). (A) Without antimycin. (B) In the presence of 10 μM antimycin; note the stimulation of cytochrome c_1 reduction concomitant to the enhanced cytochrome *b* reoxidation. (C) As in A and B, but the cytochrome *b* reduction trace is represented in a more expanded time-scale, to better resolve the kinetics of reduction. (D) Cytochrome c_1 plus cytochrome *c* (3.6 μM) reduction. Under such conditions the bc_1 complex performed about three turnovers.

enzyme by a pulse of ubiquinol [24]. In both cases, the inhibitor has little effect on the reduction pattern. Only a slight decrease of the extent of cytochrome *b* reduction is observed (Fig. 5A). In the presence of antimycin, which leads to a higher cytochrome *b* reduction, and to a slower cytochrome c_1 reduction [2,24,37], the former cytochrome is reduced at the same rate, but faster reoxidized after the addition of DBMIB (Fig. 5B). Such effect is more relevant at DBMIB concentrations capable to stimulate the antimycin-insensitive cytochrome *c* reduction at steady-state (cf. Fig. 3C). This faster oxidation is accompanied by a higher reduction of cytochrome c_1 (cf. Fig. 5B). If exogenous cytochrome *c* is added in the presence of DBMIB, a clear inhibition of the *c* cytochromes reduction is seen, even if the first portion of the trace, mainly due to cytochrome c_1, remains unaffected (Fig. 5D). Thus, DBMIB starts to inhibit the bc_1 complex after the first turnover.

We have verified that the chemical reaction between Q_1H_2 and DBMIB under our conditions can not interfere with the observed kinetics of the cytochromes, since it is three orders of magnitude slower ($1.7 \cdot 10^3\ M^{-1} \cdot s^{-1}$).

The effects of dibromothymoquinone on the pre-steady-state reactions of the bc_1 complex are in contrast to those reported by other quinoid inhibitors such as UHDBT or HNQ, that inhibit the first turnover of the bc_1 complex [12,15]. UHDBT

and DBMIB, however, are mutually exclusive in their binding to the bc_1 complex, as indicated by kinetic studies of their inhibition according to the method in Ref. 38. Similar conclusions have been reached in the b_6f complex by the EPR studies of Malkin [20,21]. Nevertheless, contrary to UHDBT, DBMIB does not affect the oxidant-induced extra *b* reduction in the presence of antimycin. In the crude succinate-cytochrome *c* reductase prereduced with either succinate or ascorbate, no kinetic effect on the extra-*b* reduction has been observed using both ferricyanide and cytochrome *c* as the oxidant. In the isolated bc_1 complex reduced by ubiquinol-1, the extra-*b* reduction is less rapid, allowing us to measure it even by our rapid mixing apparatus, without relevant underestimations of the reaction rate. As shown in Fig. 6, no change in the rate and in the extent of cytochrome *b* extra reduction has been observed in the presence of DBMIB concentrations almost completely inhibiting the steady-state redox activity of the enzyme. Only the subsequent reoxidation is accelerated by the inhibitor (see Fig. 6, and cf. Fig. 5B).

Mechanism of DBMIB interaction with the bc_1 complex

Rich and Bendall [39] have recently proposed a hypothetical model for the mechanism of action of DBMIB in both *bf* and bc_1 complexes. The model is based on the lower p*K* of the reduced form of the inhibitor, that could be able to displace endogenous (or exogenous) ubiquinol from its binding site at the Rieske center, and then undergo a series of proton-electron transfer reactions, which can even explain the observed higher rates of cytochrome c_1 (or *f*) reduction induced by DBMIB [39]. We have not confirmed such a stimulation using low concentrations of DBMIB (cf. Fig. 5A), and the model fails to adequately explain many of the data presented here. In particular, we have found that DBMIB competes with the oxidized form of ubiquinone (i.e., the product of the bc_1 complex activity) rather than with ubiquinol, the substrate of the enzyme (cf. Fig. 1 and Table I). Thus, the mechanism of DBMIB inhibition may derive from its quinoid structure, conferring the capability to displace ubiquinone from its reaction site in the bc_1 complex. According to Mitchell's Q-cycle [3] and its most recent versions [2,4], such

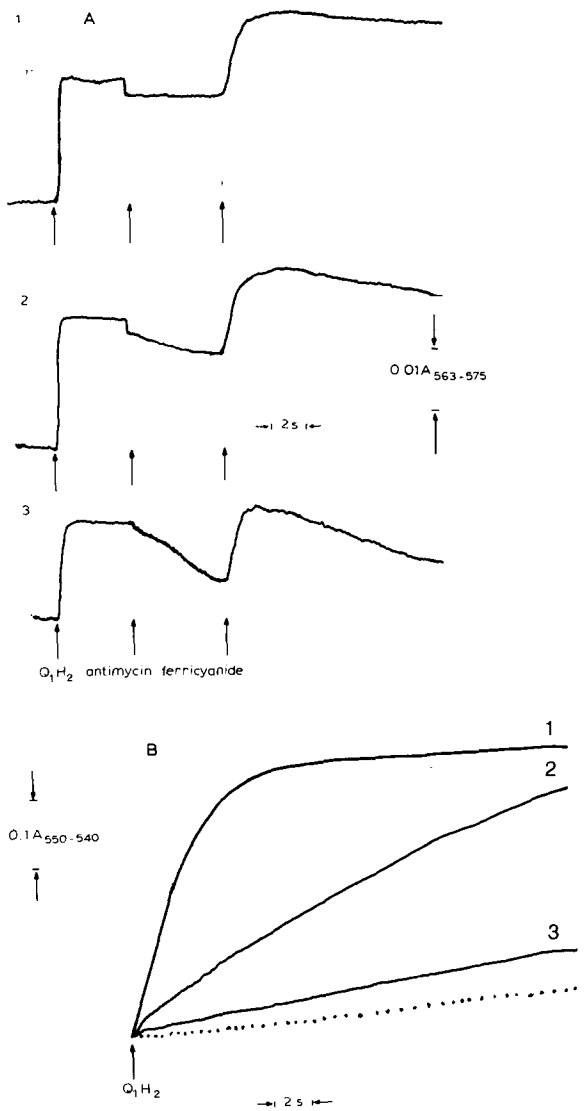

Fig. 6. (A) Oxidant-induced extra cytochrome *b* reduction in isolated bc_1 complex (0.7 μM cytochrome c_1) reduced with 110 μM Q_1H_2 in the presence of 12 μM antimycin. Ferricyanide was 20 μM, and DBMIB was absent in trace 1 (control), was 5.4 and 24 mol per mol of cytochrome c_1 in trace 2 and 3, respectively. (B) Q_1H_2-cytochrome *c* reductase with 70 nM bc_1 complex and 19 μM cytochrome *c*. The dotted line represents the traces obtained in the presence of 10 μM antimycin in 1 and 2; in 3 the reaction was somewhat faster. The numbers refer to the same samples as in A. The experiment was obtained with the rapid-mixing apparatus.

site is constituted by the antimycin-sensitive oxidation of cytochrome *b* at center i, which allows the recycling of electrons flowing from ubiquinol oxidation at center o. DBMIB may displace

ubiquinone in this reaction, and then deviate the electron flux out from the physiological pathway.

It is well known that DBMIB rapidly oxidizes reduced cytochrome *b* [40,41]. By stopped-flow experiments, we have found that such reaction is faster than that produced by even higher concentrations of Q_1 (Fig. 7). DBMIB oxidation is a direct chemical reaction with the cytochrome. On the contrary, the oxidation induced by Q_1 reaches saturation as a function of the quinone, and, at least in mitochondrial subfractions, results more sensitive to antimycin than that of DBMIB (Fig. 7, dashed lines). The higher midpoint potential of the inhibitor (+160–180 mV [1,39] vs. +120 mV for Q_1 [1]) could account for such different sensitivity. This evidence may be very important in the elucidation of the mechanism of the redox bypass to oxygen produced by DBMIB in the whole respiratory chain [22,41], which is antimycin-insensitive [22]. The semiquinone of DBMIB formed at cytochrome *b* oxidation could directly donate electrons to cytochrome *c* or c_1 even in the presence of antimycin. This explains the stimulation of cytochrome c_1 reduction in parallel to that of cytochrome *b* oxidation in the presence of antimycin (cf. Fig. 5B). When the high-potential components of the respiratory chain are already reduced, the semiquinone of DBMIB would mainly autooxidize via molecular oxygen. Such reaction accounts for the antimycin and KCN-insensitive oxygen consumption of the respiratory chain, particularly evident at high concentrations of the inhibitor [22,41]. Therefore, the DBMIB redox by-pass could be due to an altered electron-transfer pathway induced within the redox interconversions of the bc_1 complex. The previously proposed mechanism to explain the phenomenon [22] should be adapted accordingly, as shown in the scheme presented in Fig. 8.

It still remains unclear how DBMIB is not able to modify the first turnover of the bc_1 complex, although it apparently interacts with both cytochrome *b* and the iron-sulphur center of the enzyme (cf. Figs. 7 and 4). To explain such behaviour in view of a simple Q-cycle scheme, it is sufficient to postulate that the real inhibitory form of dibromothymoquinone is its semiquinone (anion), formed by the direct oxidation of reduced cytochrome *b*. Such a reaction occurs after two electrons have entered the bc_1 complex at center o in the ubiquinol oxidation of the first turnover. Therefore, the requirements for inhibition could be met only at the end of the first turnover, when cytochrome *b* is reduced. Also the oxidant-induced extra-*b* reduction can be considered a redox event occurring in the first turnover of the enzyme [4]. It is likely that the rapid reoxidation of the high potential components of the bc_1 complex by ferricyanide, and the consequent extra-*b* reduction, precedes the formation of the semiquinone of DBMIB, the real inhibitory form.

The ascorbate-reduced bc_1 complex is not sig-

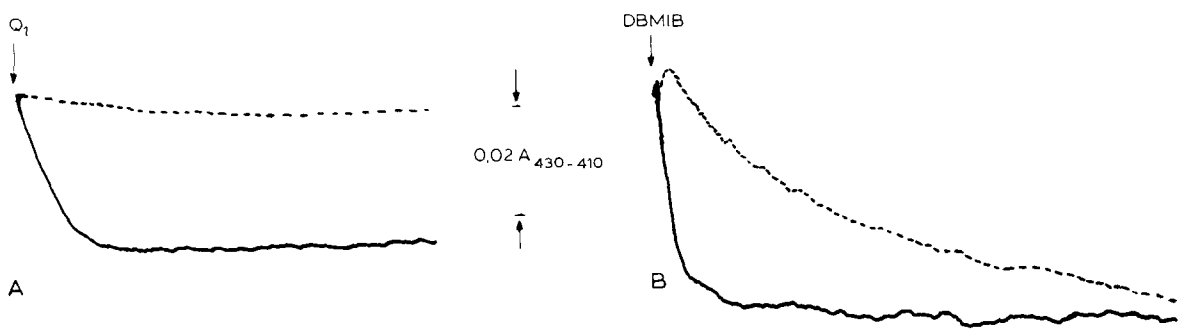

Fig. 7. Stopped-flow traces of cytochrome *b* oxidation by 31 μM Q_1 (A) and by 3 μM DBMIB (B) in a crude succinate-cytochrome *c* reductase preparation previously reduced with 1 mM succinate in the presence of 2 mM KCN. After 10 min of incubation, 2 mM of malonate was added to the mixture to block the electron input into the bc_1 complex. Cytochrome *b* was partly oxidized for a transient period of some seconds with both quinones, although DBMIB resulted more efficient also in the extent of oxidation. The dashed lines are the traces obtained in the presence of 10 μM antimycin. In this case DBMIB induced a complete reoxidation of cytochrome *b* (not shown in the figure).

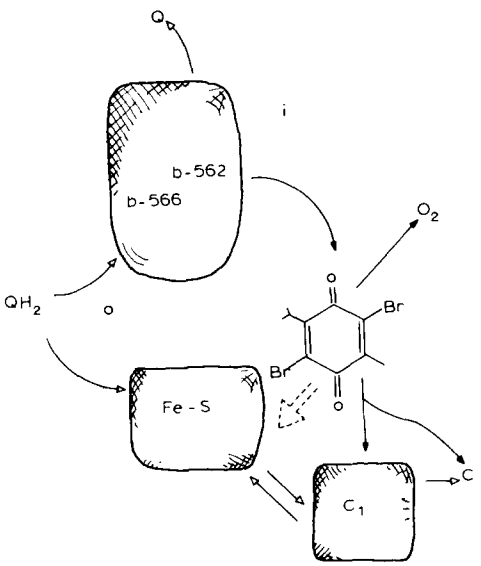

Fig. 8. Schematic representation of the proposed model for the mechanism of action of DBMIB in the bc_1 complex in view of a Q-cycle system. The open arrows indicate the physiological electron routes, and the black arrows the reactions mediated by the inhibitor, whose main action is to functionally displace ubiquinone in the reoxidation of ctyochrome *b*. The semiquinone of DBMIB thus formed can transfer electrons to oxygen and/or to cytochrome *c* and c_1, particularly at high concentrations. At concentrations approaching that of the enzyme, the semiquinone form may directly react with the Rieske center, possibly involving conformational changes of the whole multiprotein complex (dashed arrow). This interaction is responsible for the inhibition of the redox transfer of the bc_1 complex, but can occur only after one turnover of the enzyme has been performed at center o. When DBMIB concentration largely exceeds the specific binding sites in the complex, other molecules of the oxdized inhibitor can oxidize reduced cytochrome *b* in a partially antimycin-insensitive fashion (cf. Fig. 7B), deviating the electron flux to molecular oxygen. This accounts for the antimycin and KCN-insensitive bypass to oxygen in the whole respiratory chain, which is induced by relatively high DBMIB concentrations [22,41]. For further explanations, see the text. The symbols o and i indicate the two catalytic centers of the bc_1 complex. Fe-S is the Rieske iron-sulphur protein. Note that the site of DBMIB insertion has been located between cytochrome *b* and the Rieske protein, since may not physically correspond to the center i.

nificantly oxidized by DBMIB, excluding that its semiquinone can be formed by either cytochrome c_1 or the iron-sulphur center. On the other hand, in a bc_1 subcomplex essentially devoid of the Rieske protein, DBMIB is still capable to rapidly oxidize reduced cytochrome *b* (results not shown). These observations, together with the effects on the pre-steady-state kinetics of the cytochromes (cf. Fig. 5), suggest that the inhibitor could bind to a site located between cytochrome *b* and the Rieske protein. The latter subunit should not be necessary, however, for the insertion of the quinone in the bc_1 complex. The binding site may also not physically correspond to the physiological center i, and oxidized DBMIB could be only loosely attached until its semiquinone is formed. Then the binding could become tighter, allowing a more direct interaction with the Rieske iron-sulphur cluster. This leads to the inhibition of further electron transfer through the complex. Such an interaction may be accompanied by conformational changes of the enzyme. In fact, DBMIB also induces changes in the absorbance spectrum (mostly in the 300–400 nm region) and in the magnetic circular dichroism spectrum (mainly in the alpha region of cytochrome *b*) of the reduced or partially reduced complex (Degli Esposti, M., unpublished data). Similar effects have not been detected in the oxidized enzyme. Such spectroscopic alterations, and even the perturbation of the EPR spectra (cf. Fig. 4), could merely reflect conformational changes occurring when the complex is in its reduced state, involving its overall quaternary structure. The lack of effect of DBMIB on the EPR signal of the isolated Rieske protein from the b_6f complex [42] is in accordance with this view. The finding of an antiferromagnetic coupling between the radical of DBMIB and the Rieske cluster [18] supports the idea of a stronger interaction of the inhibitor once reduced to its semiquinone form. Such an idea can also explain the higher potency of DBMIB when the bc_1 complex is previously reduced by ubiquinol, and during the progress of the steady-state reaction (cf. Fig. 2). In both cases, the formation of the inhibitory form is facilitated by the higher reduction level of the enzyme.

The scheme of Fig. 8 also accounts for the above mode of action of dibromothymoquinone in the structure of the bc_1 complex.

Alternatively, other explanations are possible, but apparently more intriguing, since they should require a reaction scheme for the bc_1 complex substantially different from the most accepted versions of the Q-cycle.

In conclusion, the present work outlines that DBMIB can not be considered as a simple redox

mediator [41], nor as a center o inhibitor (though it interacts with the Rieske center) competing with ubiquinol oxidation, as it is commonly assumed [1,2,4,21]. The mechanism of action of dibromothymoquinone may essentially be constituted by its antagonism with ubiquinone in the reaction sequence of the bc_1 complex, which can also explain the redox bypass of the respiratory chain induced by the inhibitor (cf. Fig. 8).

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