Inhibition of the mitochondrial bc_1 complex by dibromothymoquinone

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We have studied the effects of dibromothymoquinone (DBMIB) in various redox activities of the succinate–cytochrome *c* span of the mitochondrial respiratory chain. At concentrations higher than 50 mol/mol of cytochrome c_1 the inhibitor produces a bypass of electron transfer on the substrate side of the bc_1 complex, because of its autooxidation capability. This induces an artifactual overestimation of the real inhibition titer of the redox activity of this enzyme, which has been found to be 3–6 mol/mol of cytochrome c_1 by following the ubiquinol–cytochrome *c* reductase activity. This action is reversed by addition of excess of sulphydryl compounds like cysteine.

Dibromothymoquinone *Ubiquinol–cytochrome c oxidoreductase (bc_1 complex)* *Mitochondria* *Respiratory chain* *Iron–sulphur protein*

1. INTRODUCTION

The use of specific inhibitors is one of the main tools for investigating the mechanism of action of the mitochondrial bc_1 complex (complex III) [1,2]. A number of analogs of ubiquinone have been found to inhibit the function of this enzyme by binding to specific sites close to the iron–sulphur center ('Rieske' protein) of the complex [2]. Among them, 5-*n*-undecyl-4,7-dioxobenzothiazole (UHDBT) [3–5], and *n*-heptadecyl-mercapto-6-hydroxy-5,8-quinoline quinone (HQMHQQ) [6] are the most powerful, and have been extensively studied.

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; DCIP, dichlorophenolindophenol; EDTA, ethylendiaminotetraacetate; EPR, electron paramagnetic resonance; Q_o, 2,3-dimethoxy-5-methylbenzoquinone; Q_1, ubiquinone-1; Q_1H_2, Q_3H_2, ubiquinol-1 and ubiquinol-3; TTFA, thenoyltrifluoroacetone; UHDBT, 5-*n*-undecyl-4,7-dioxobenzothiazole

Another ubiquinone analog which blocks electron transfer in the bc_1 region is dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropylbenzoquinone, DBMIB), discovered as a potent inhibitor of plastoquinol oxidation in chloroplasts [7,8] acting at the level of the b_6f complex [9]. Earlier works in the mitochondrial respiratory chain [10–12] have shown that DBMIB inhibits electron transfer at concentrations about one order of magnitude higher than those required to block the b_6f complex from chloroplasts [1,9]. Owing to its autooxidation properties, it has been considered more as a redox mediator than a specific inhibitor in the mitochondrial bc_1 region [12]. Nevertheless, the recent findings [13–15] that DBMIB is able to stoichiometrically bind and modify the iron–sulphur center of the chloroplast b_6f complex [13–15], which appears very similar in its physicochemical and functional properties to the analogous Fe–S center of the mitochondrial bc_1 complex [2,9], have outlined the usefulness of dibromothymoquinone for clarifying the function of this redox center [2]. It has been reported, in fact, that DBMIB induces a similar alteration also of the

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iron–sulphur cluster in the mitochondrial succinate–cytochrome *c* reductase [2]. We have investigated the effects of DBMIB in the mitochondrial bc_1 complex, and found it to be a powerful inhibitor of the enzyme when tested under conditions avoiding it to bypass electron transfer in the complex.

2. MATERIALS AND METHODS

Beef heart mitochondria were a generous gift from Professor D.E. Green (Enzyme Institute, Madison). Crude succinate–cytochrome *c* reductase (fraction S_1) was obtained from mitochondria by the method in [16] as described in [17]. The bc_1 complex was purified from fraction S_1 by the method in [18]. Ubiquinols were prepared as in [18]. The electron transfer reaction was assayed at room temperature (20°C) as in [19] for the succinate–ubiquinone-1–dichlorophenolindophenol (DCIP) reductase activity, and as in [20] for the succinate and ubiquinol–cytochrome *c* reductase activity. The reduction of DCIP was followed at 600–700 nm, and that of cytochrome *c* at 550–540 nm in a Sigma Biochem ZWS II dual wavelength spectrophotometer equipped with a stopped-flow apparatus. Absorbance spectra were performed in a Perkin-Elmer 559 spectrophotometer. The concentration of cytochrome c_1 was measured by the absorbance at 553–540 nm in the dithionite-reduced spectra of the mitochondrial fractions, with an extinction coefficient of 20 mM^{-1}.

Ubiquinones were kindly offered by Eisai (Tokyo). DBMIB was a generous gift from Professor A. Trebst, University of Bochum; it was dissolved in ethanol and stored in concentrated solution at −16°C; its concentration was evaluated by the absorbance at 289 nm in ethanol, using an extinction coefficient of 14.5 mM^{-1}.

3. RESULTS AND DISCUSSION

The concentration of dibromothymoquinone required for half-inhibition of the succinate or NADH oxidation in intact mitochondrial particles is around 25 μM [11], corresponding to about 100 mol/mol of cytochrome c_1. In order to pinpoint the action of DBMIB, we have titrated its effects on the following redox activities in a mitochondrial fraction enriched in the bc_1 complex [17]: succinate–Q_1–DCIP reductase, succinate–cytochrome *c* reductase, and ubiquinol–cytochrome *c* reductase. As shown in table 1, DBMIB induces half-inhibition of Q_3H_2–cytochrome *c* reductase activity at very low concentrations, and of succinate–cytochrome *c* reductase activity at concentrations about one order of magnitude higher.

Moreover the latter activity cannot be completely inhibited, and is rather stimulated by high DBMIB concentrations in an antimycin-insensitive fashion. On the other hand, the rate of DCIP reduction is markedly stimulated, and results less sensitive to the specific inhibitor TTFA (not shown). This effect may be due to the capability of DBMIB to accept electrons from the succinate–ubiquinone reductase complex, and rapidly transfer them to the dye and/or to molecular oxygen because of its autooxidation properties [12]. Such possibility is supported by two experimental evidences. Firstly, in detergent-solubilized submitochondrial particles, DBMIB can function by itself as a substrate replacing exogenous ubiquinone in the succinate–ubiquinone–DCIP reductase activity (see also [10]). Secondly, the rate of cytochrome *c* reduction by succinate becomes practically antimycin-insensitive above 100 mol DBMIB/mol of cytochrome c_1 (cf. table 1). This non-enzymatic cytochrome *c* reduction is, however, sensitive to superoxide dismutase, and hence can be due to a chemical reduction promoted by superoxide radicals generated by DBMIB autooxidation. We have, in fact, observed that dibromothymoquinone markedly stimulates oxygen consumption in the presence of antimycin and KCN in fraction S_1. Thus DBMIB, at concentrations higher than 50 mol/mol of cytochrome c_1, may produce a redox bypass at the level of the junction between the dehydrogenases and the bc_1 complex, deviating the electrons that flow from substrates (and/or from endogenous ubiquinol) to oxygen instead of complex III, which is already inhibited. The mechanism of such bypass can be described as shown in fig.1. This scheme accounts for the stimulation of succinate oxidation at high levels of DBMIB [10] and for the KCN-insensitive oxygen consumption induced by the inhibitor [12].

For a correct evaluation of the specific effects of DBMIB on the bc_1 complex we have to directly

Table 1

Effect of DBMIB on various redox activities of the succinate–cytochrome *c* span in the mitochondrial fraction S_1[a]

Addition	% Activity					
	Succinate–Q_1–DCIP (1 mM) (50 μM)	(26 μM)	Succinate–cytochrome *c* (1 mM)	(20 μM)	Q_3H_2–cytochrome *c* (22 μM)	(20 μM)
	Turnover no. = 2.4 s^{-1}		Turnover no. = 4.5 s^{-1}		Turnover no. = 70 s^{-1}	
None	100		100		100	
7 mol DBMIB/mol of bc_1	117		87		48	
72 mol DBMIB/mol bc_1	260		50		9	
330 mol DBMIB/mol bc_1	494		83[b]		0	

[a] The enzyme concentrations were 25 nM cytochrome c_1 for the succinate–DCIP and the succinate–cytochrome *c* reductase activities; and 10 nM for the ubiquinol–cytochrome *c* reductase activity. The buffer was 50 mM potassium-phosphate (pH 7.4) containing 0.5 mM EDTA and 1 mM KCN. The reaction was started by addition of DCIP, cytochrome *c*, and ubiquinol-3 for succinate–ubiquinone reductase, succinate–cytochrome *c* reductase and ubiquinol–cytochrome *c* reductase activity, respectively

[b] The reduction rate of cytochrome *c* was almost completely antimycin-insensitive, but was markedly depressed by addition of purified superoxide dismutase, to about 40% of the control

study its ubiquinol–cytochrome *c* reductase activity. The titer for half-inhibition of such activity ranges between 3 and 6 mol DBMIB/mol of the enzyme in all mitochondrial preparations tested (submitochondrial particles, fraction S_1, and isolated bc_1 complex) and the inhibition shape tend to be biphasic (fig.2). These values are comparable to that found in the analogous b_6f complex from chloroplasts [9]. The extent of inhibition does not depend on the absolute concentration of DBMIB, but on its ratio to the bc_1 complex (table 2). The potency of the inhibitor increases by increasing the number of turnovers made by the enzyme under the assay conditions; i.e., the higher the substrates concentration, the lower the titer. Furthermore, the effect of DBMIB is more pronounced if the bc_1 complex is reduced first, as for instance when the assay is started by the oxidizing substrate

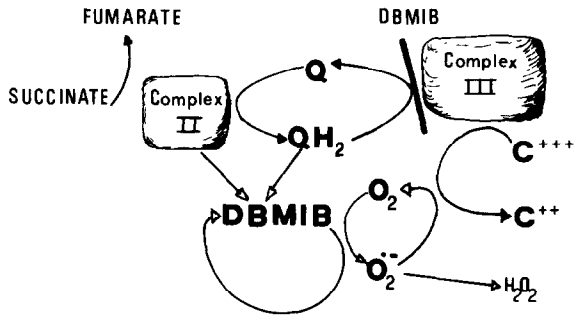

Fig.1. Scheme for the possible mechanism of the redox bypass operated by DBMIB in the succinate–cytochrome *c* span of the mitochondrial respiratory chain. For the sake of clarity, the reduction routes of DCIP are omitted from the drawing. The dye is normally reduced by ubiquinol, but it could also be reduced by either DBMIB or by superoxide radicals, or by both.

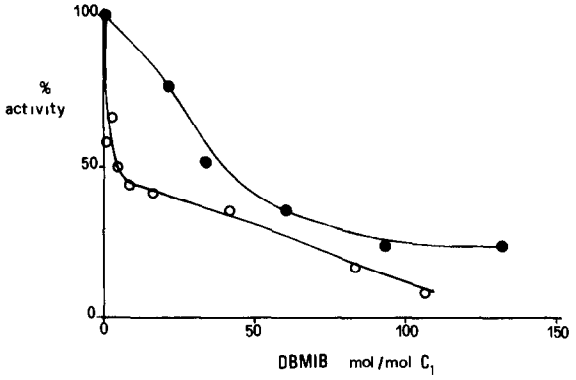

Fig.2. Inhibition by DBMIB of the ubiquinol-1 (36 μM)–cytochrome *c* reductase (○---○) and the succinate (5 mM)–cytochrome *c* reductase activity (●---●) in fraction S_1. The experimental conditions were those employed in table 1, except that in both cases the reduction was started with the reducing substrate. The uninhibited antimycin-sensitive rates were 126 s^{-1} and 15 s^{-1} for the two redox activities, respectively.

Table 2

Dependence of DBMIB inhibition on the ratio with the bc_1 complex[a]

DBMIB (μM)	bc_1 (nM)	DBMIB/bc_1 (mol/mol)	% residual antimycin-sensitive activity
0.26	2.9	92	5
0.26	5.7	46	11
0.26	9.5	27	20

[a] Different concentrations of isolated bc_1 complex were assayed for the ubiquinol-2-(15 μM)–cytochrome *c* (21 μM) reductase activity in 25 mM potassium-phosphate buffer, containing 0.5 mM EDTA, pH 7.4. The uninhibited reductase rate was 225 s^{-1}

cytochrome *c*, after the reducing substrate ubiquinol has been already equilibrated with the enzyme. This behaviour is analogous to that exhibited by other quinoid inhibitors, such as UHDBT [5].

The ubiquinol–cytochrome *c* reductase rates lowered by DBMIB still remain antimycin-sensitive. On the other hand, the slow cytochrome *c* reduction by succinate in the presence of saturating concentration of antimycin, which is about 5–8% of the overall rate, is abolished by low concentrations of DBMIB (75% decrease with 8 mol/mol of cytochrome c_1). Thus the effect of DBMIB appears to be synergistic to that of antimycin in blocking the bc_1 complex.

The inhibitory action of DBMIB appears very similar to that previously reported for Br-Q_0 [21] which can be reversed by addition of sulphydryl compounds like cysteine. A similar reversal of the DBMIB effects by thiol compounds has been found in chloroplasts [22], and could be related to the removal of the modification of the iron–sulphur cluster induced by the inhibitor in the b_6f complex [15]. We have therefore thought of interest to test the effect of thiol compounds on the DBMIB inhibition of ubiquinol–cytochrome *c* reductase in the mitochondrial bc_1 complex. As shown in fig.3, the addition of cysteine substantially removes the inhibition induced even by high concentrations of DBMIB, leaving the reductase rate equally antimycin-sensitive.

The observed effects of dibromothymoquinone appear to be related to specific modifications of

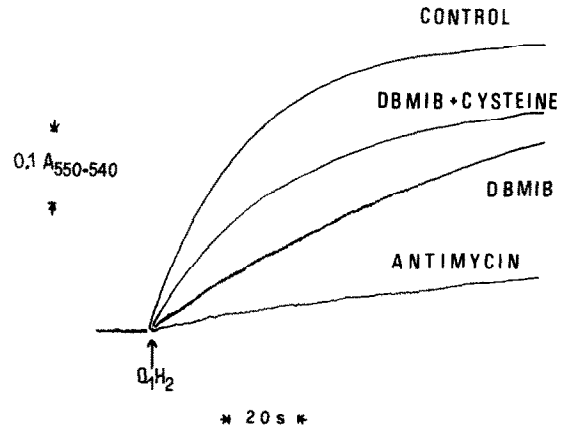

Fig.3. Effect of cysteine on the inhibition of DBMIB. The assay conditions were those of fig.2 for the ubiquinol-1–cytochrome *c* reductase activity. DBMIB was 83 mol/mol of cytochrome c_1, giving a 83.3% inhibition of the reductase rate. Cysteine was 2.5 mM and did not stimulate per se the reductase activity, but rather produced slightly lower rates with respect to the control without any addition. Presented at the bottom of the figure is the trace obtained in the presence of 1 μM antimycin A, which was identical with or without cysteine.

the mitochondrial bc_1 complex, as judged by relevant changes of its absorption spectra in the 300–400 nm spectral range (M. Degli Esposti, unpublished). Moreover, in collaboration with Professor G. Rotilio of the University of Rome, we have verified that in fraction S_1 the EPR signal at $g_y = 1.90$ of the iron–sulphur cluster of the bc_1 complex is dramatically modified by DBMIB concentrations corresponding to those inhibiting the redox activity of the enzyme (3–8 mol/mol of cytochrome c_1) with a spectral shift of the g_y value of 1.94, resulting almost identical to that reported in [15,23] in the b_6f complex. Thus, we can now consider dibromothymoquinone as one of the few specific and universal inhibitors of the bc_1- and *bf*-type redox complexes.

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