DIBROMOTHYMOQUINONE INTERACTION WITH THE CoQ-CYTOCHROME *b* LOCUS OF THE MITOCHONDRIAL RESPIRATORY CHAIN

Further evidence for the Q cycle

Sergey A. SURKOV and Alexander KONSTANTINOV

A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117234, USSR

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1. Introduction

Dibromothymoquinone (2,5-dibromo-6-isopropyl-3-methyl-benzoquinone, DBMIB) is a well known inhibitor of photosynthetic electron transfer in chloroplasts [1–7] and bacteria [8]. The effect of this compound on the mitochondrial [9,10] and bacterial [11–13] respiratory chains has also been described. It was reported that, in mitochondria, DBMIB:

(i) Inhibits respiration with succinate- or NAD-linked substrates [9,10]

(ii) Brings about oxidation of *b* cytochromes when added in the presence of substrates and cyanide [9].

These two effects were ascribed [9] to DBMIB specifically inhibiting the reduction of cytochromes *b* by the $QH_2/QH^{\cdot}$ redox couple in keeping with Wikström and Berden's model [14] of electron flow in the QH_2-cytochrome *c*-reductase span of the respiratory chain. Contrary to [9] and in agreement with [11] we have found [15] that oxidation of *b* cytochromes is largely due to DBMIB being an autooxidizable electron acceptor for *b* cytochromes and/or CoQ.

An unusual steady-state electron flow occurs in aerobic KCN-inhibited submitochondrial particles (SMP) supplemented with DBMIB and succinate or NADH:

$$\text{substrate} \rightarrow \text{CoQ} \overset{\text{antimycin}}{-\!\!\|\!\rightarrow} \text{cytochromes } b \rightarrow \text{DBMIB} \rightarrow O_2$$

Abbreviations: DBMIB, dibromothymoquinone; QH_2, ubiquinol; $QH^{\cdot}$, ubisemiquinone; Q, ubiquinone; CoQ, ubiquinone irrespective of its redox state; HOQNO, 2-alkyl-4-hydroxyquinoline-*N*-oxide; SMP, submitochondrial particles

It is shown that in this system the antimycin inhibition site is localized on the reducing side of *b* cytochromes, which is another piece of evidence for the Mitchell Q cycle scheme [16,17].

2. Methods

Beef heart SMP were obtained according to [18]. Oxygen uptake was measured with a teflon-covered oxygen electrode. Spectrophotometric measurements were made in 1 cm thermostatted cuvettes in an Aminco $DW2^{a}_{TM}$ instrument. DBMIB was a generous gift of Dr M. Baltscheffsky and was used as a freshly prepared solution in twice-distilled ethanol.

3. Results

3.1. *DBMIB as an autooxidizable electron acceptor for a CoQ-cytochromes* b *locus*

As shown in fig.1, the addition of DBMIB to KCN-inhibited SMP in the presence of either succinate or NADH restores a high rate of oxygen uptake. This effect is evidently due to DBMIB autooxidation [10,11,15]. It is not clear why the DBMIB-mediated O_2 uptake was not observed in mung bean mitochondria [19].

With succinate, the KCN-insensitive DBMIB-induced 'respiration' is not inhibited by antimycin but is completely suppressed by α-thenoyltrifluoroacetone (fig.1, *1–3*). This indicates that DBMIB accepts electrons at the level of CoQ and/or *b* cytochromes [11] rather than from succinate dehydro-

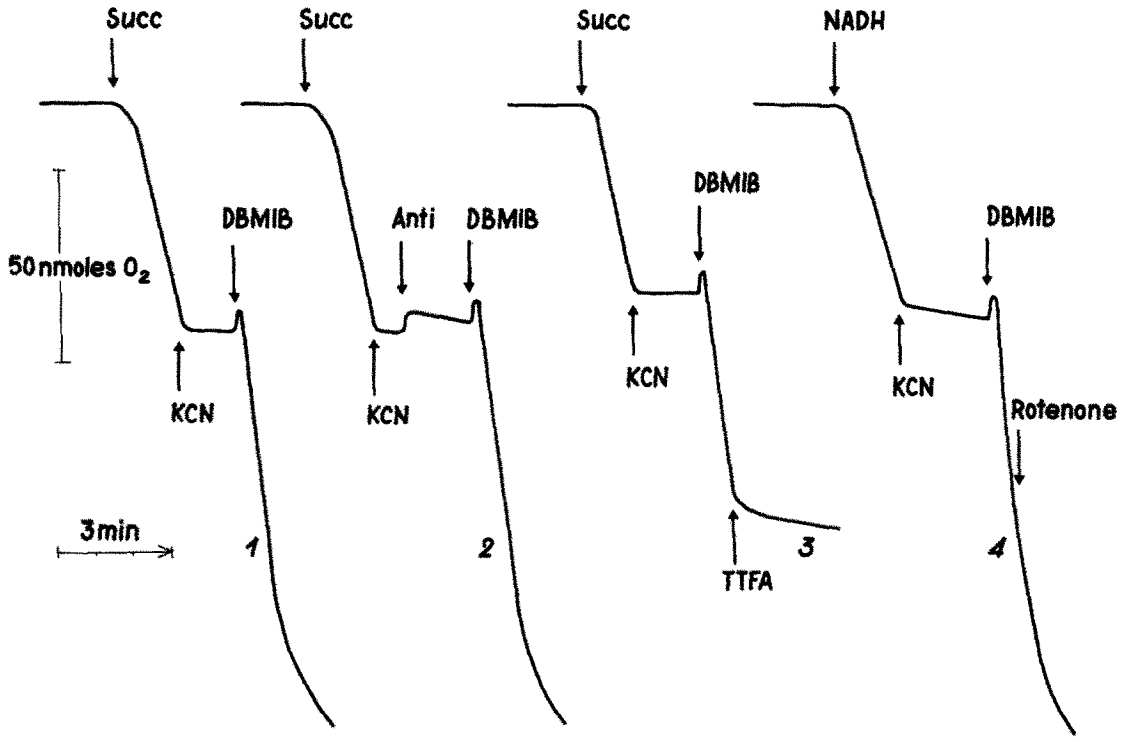


Fig.1. DBMIB-induced oxygen uptake by terminally inhibited SMP. A basic reaction mixture contained 0.25 M sucrose, 30 mM Hepes-buffer (pH 7.5), 1 μM carbonyl cyanide *m*-chlorophenyl hydrazone and (except for trace *4*) 3 μM rotenone. Other additions: SMP, 0.7 mg protein/ml; succinate, 5 mM; NADH, 2 mM; KCN, 4 mM; α-thenoyltrifluoroacetone (TTFA), 1 mM; antimycin, 0.5 μg/mg protein; DBMIB, 114 nmol/mg protein.

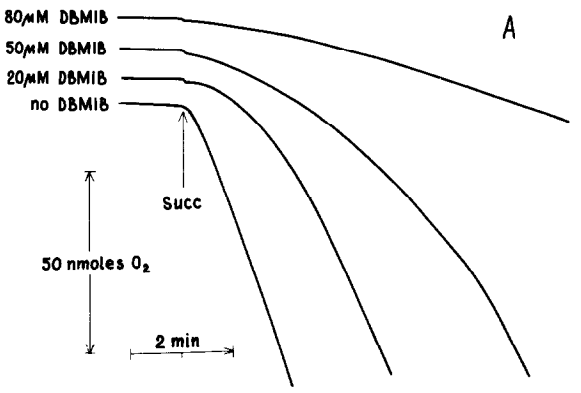
genase as suggested in [10]. With NADH, the DBMIB-mediated oxygen consumption was only slightly sensitive to rotenone (fig.1,*4*); thus there is an additional site of DBMIB reduction by the respiratory chain on the substrate side of the rotenone block (cf. [10]).

3.2. *The inhibitory action of DBMIB on the respiratory chain*

In view of DBMIB being able to by-pass the cytochrome chain, the use of this compound as a respiration inhibitor looks somewhat dubious. But then, we found in keeping with [9,10], that relatively low concentrations of DBMIB added to SMP prior to succinate were indeed inhibitory. The inhibition however was markedly released with time (fig.2A). Thus $[I]_{0.5}$ were ~8 μM and 55 μM when measured in 1 and 8 min, respectively, after succinate addition (fig.2B). The steady-state difference spectra [15] indicated the DBMIB inhibition to be localized on the reducing side of both *b* and *c* cytochromes.

At higher concentrations of DBMIB (>60 μM) the release of inhibition became less pronounced and the resulting rate of O_2 uptake appeared to be much lower than in the experiments where the same concentrations of DBMIB had been added to KCN-blocked SMP prereduced with succinate (cf. fig.1 and 2), which is in agreement with [11]. A tentative explanation of this complicated mode of inhibitory action could be that: (1) DBMIB is inhibitory in the oxidized form [9] so that inhibition is released upon its reduction by the respiratory chain; and (2) the DBMIB inhibition occurs on the reducing side of the respiratory component (presumably the CoQ pool) that reduces DBMIB (e.g., at the dehydrogenase level [13], cf. [9]).

Anyhow, whatever the mechanism, DBMIB is a poor succinate oxidase inhibitor at ≲20 μM (fig.2B, *2*; [9,10,15,19]); this is the only point essential for the discussion below.



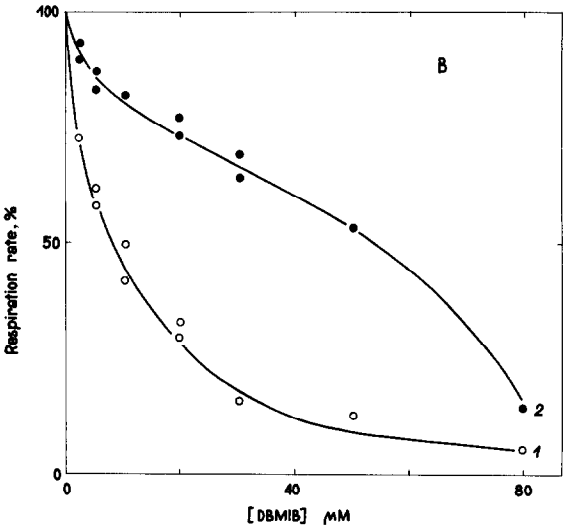


Fig.2. Inhibition of the succinate oxidase activity by DBMIB. SMP (0.4 mg protein/ml) were preincubated with the indicated concentrations of DBMIB for 3 min and respiration was initiated with 5 mM succinate. The basic reaction mixture. (A) Representative curves. (B) The inhibited respiration rate as measured in 1 min (*1*) or in 8 min (*2*) after the succinate addition.

3.3. *The DBMIB-induced oxidation of* b *cytochromes in the KCN-inhibited SMP; potentiating effect of antimycin and HOQNO*

We confirmed [15] the observation [9] that high concentrations of DBMIB brought about specific oxidation of *b* cytochromes in KCN-inhibited SMP. This effect appears to be entirely dependent on oxygen present in the reaction mixture. One can see that when DBMIB is added to SMP supplemented with KCN after O_2 has been consumed (fig.3,*1*), there is only a transient oxidation of *b* cytochromes followed by instantaneous re-reduction. Transient oxidations of *b* cytochromes could be subsequently induced under these conditions simply by stirring the sample, just as if there were no cyanide in the mixture.

If, on the other hand, cyanide is added to SMP before succinate (fig.3,*2*), the DBMIB-induced partial oxidation of *b* cytochromes is stable for several minutes, finally followed by re-reduction upon exhaustion of O_2 via the DBMIB by-pass (not shown).

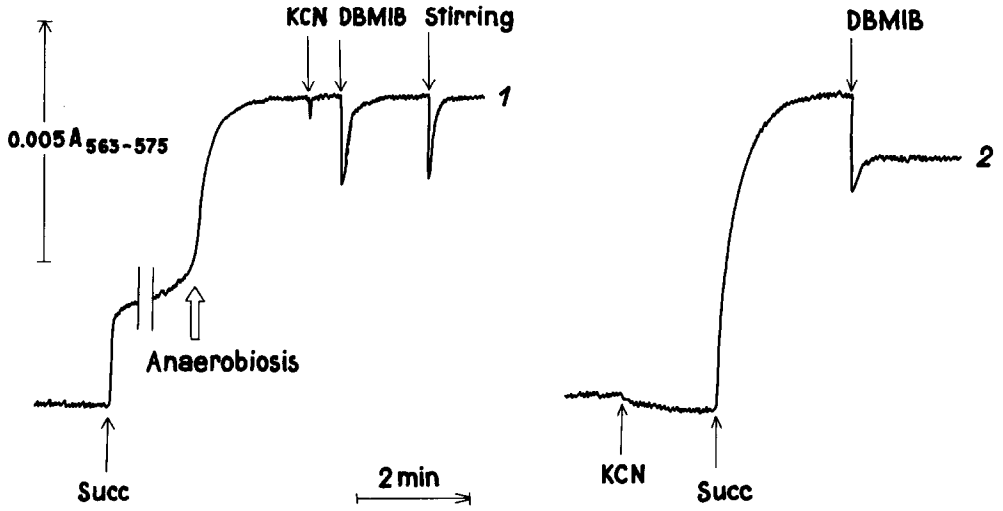


Fig.3. Effect of DBMIB on the redox state of *b* cytochromes in KCN-inhibited SMP. The basic reaction mixture. Other additions: SMP, 0.5 mg protein/ml; succinate, 5 mM; KCN, 4 mM; DBMIB, 50 nmol/mg protein.

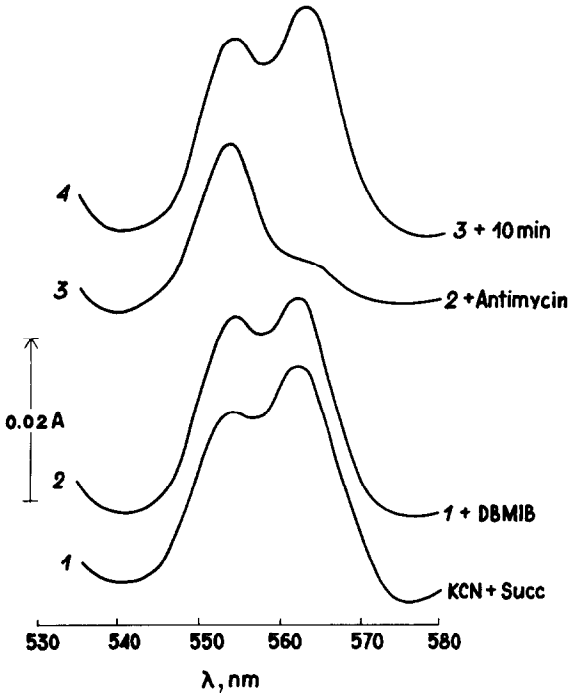


Fig.4. Antimycin-induced oxidation of *b* cytochromes in the KCN + DBMIB-treated SMP. The basic reaction mixture. Other additions: SMP, 2.5 mg protein/ml; KCN, 4 mM; succinate, 5 mM; DBMIB, 32 nmol/mg protein; antimycin, 0.5 μg/mg protein. The difference spectra are recorded versus the oxidized SMP in the reference cell.

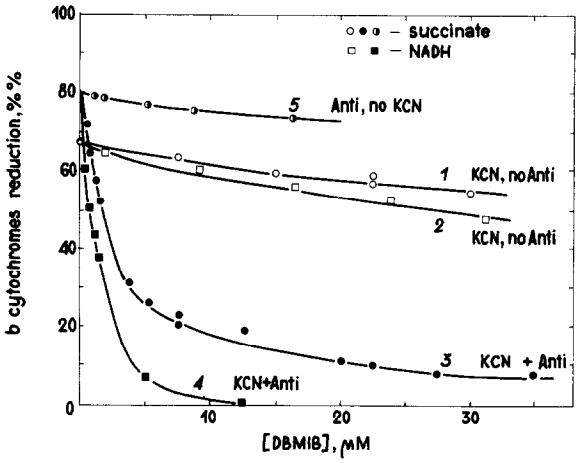


Fig.5. Potentiating effect of antimycin on the DBMIB-induced oxidation of *b* cytochromes. The basic reaction mixture; rotenone was omitted in curves *2,4*. SMP (0.5 mg protein/ml) were supplemented with 4 mM KCN (open symbols), 0.5 μg/mg protein of antimycin (half-filled circles) or KCN + antimycin (filled symbols), and *b* cytochromes were reduced aerobically with either 5 mM succinate (circles) or 2 mM NADH (squares). Subsequently DBMIB was added, and the resulting reduction level of *b* cytochromes (cf. fig.3, *2*) is plotted versus [DBMIB]. Reduction of *b* cytochromes was monitored at 563 nm minus 575 nm; the dithionite induced absorption level was taken as 100%.

It seems therefore likely (see also [15]) that DBMIB brings about oxidation of *b* cytochromes largely due to its being an autooxidizable electron acceptor [5,11] rather than merely an inhibitor [9].

Figure 4 shows that antimycin added to KCN-inhibited SMP reduced aerobically by succinate and supplemented with DBMIB, induces drastic oxidation of *b* cytochromes (cf. spectra *3,2*) which is reversed upon anaerobiosis (spectrum *4*); this effect of antimycin is in contrast with the usual extra-reduction of cytochromes *b* [14] observed under identical conditions but omitting DBMIB (not shown). Reciprocally, DBMIB proved a much more effective oxidant of *b* cytochromes in KCN-inhibited SMP when added in the presence of antimycin with either succinate or NADH as reductant (fig.5). It is to be emphasized that the DBMIB concentrations sufficient to bring about almost complete oxidation of *b* cytochromes in the presence of antimycin, could inhibit the respiratory chain only insignificantly (cf. fig.5 and 2).

Essentially the same results were obtained with 2-nonyl-4-hydroxyquinoline-*N*-oxide (HOQNO) used instead of antimycin (data not shown, [15]).

4. Discussion

Considering the effects of the substituents in the *p*-benzoquinone ring [20] one could evaluate E_{m7} of DBMIB to be in the range of +200 mV. It is not therefore surprising that this compound serves as an effective electron acceptor for the CoQ-cytochromes *b* locus of the mitochondrial redox chain as it does in chloroplasts [4,5] and bacteria [11]. As DBMIB is also autooxidizable [5,9,10], an unusual steady-state electron flow is maintained in cyanide-inhibited SMP, so that CoQ and *b* cytochromes reduced by dehydrogenases are oxidized by O_2 via DBMIB.

Under these conditions, antimycin (or HOQNO [15]) greatly enhances the DBMIB-induced oxidation of *b* cytochromes. This effect could be due to antimycin either (i) stimulating the rate of cytochrome *b* oxidation by O_2 via DBMIB, or (ii) inhibiting cytochrome *b* reduction by substrates.

Since the rate of the KCN-insensitive DBMIB-

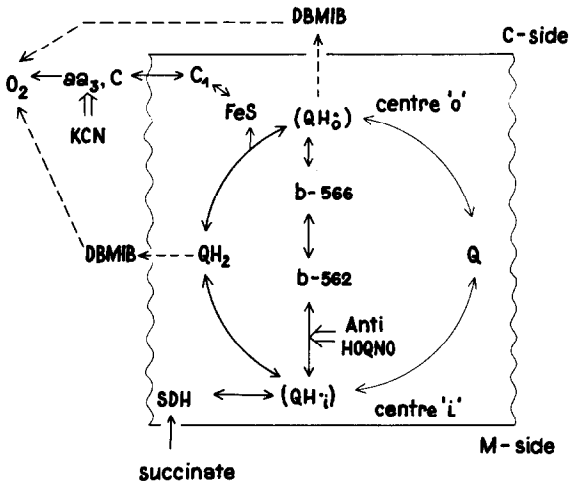


Fig.6. A diagram of electron flow in coupling site 2 according to the Q cycle scheme. Electron transfer between the respiratory chain components is shown by thick lines and diffusion of oxidized CoQ is indicated by thin lines. Dashed lines, the DBMIB-mediated electron flow. Double arrows, the action of inhibitors. The specific oxidation of *b* cytochromes by the low concentrations of DBMIB in the presence of KCN and antimycin is due to DBMIB accepting electrons in the centre '*o*' whereas it is the DBMIB reduction by QH_2 pool which is largely responsible for KCN insensitive O_2 uptake.

mediated oxygen uptake was not affected appreciably by antimycin, possibility (i) is unlikely. We therefore conclude that antimycin (and HOQNO) inhibits electron transfer from dehydrogenases to *b* cytochromes.

Such an unorthodox effect of this classical inhibitor considered traditionally to block electron transfer chain on the oxidizing side of *b* cytochromes, is in agreement with the Q cycle scheme in [16,17] (fig.6). A similar effect of antimycin has been observed in SMP inhibited with British Anti-Lewisite [21] and in succinate–cytochrome *c*-reductase complex depleted of the FeS_{Rieske} protein [22,23].

According to the Q cycle, cytochromes *b* can be reduced via either of the 2 CoQ-reactive centres designated '*o*' and '*i*' [17] (fig.6). When QH_2 oxidation in the centre *o* is sluggish due to cytochrome c_1 (and, presumably, FeS_{Rieske}) being highly reduced in the presence of cyanide and substrate, it is mainly the reversed electron transfer via the centre *i* that counteracts the oxidizing effect of DBMIB + O_2 and keeps *b* cytochromes reduced. Antimycin blocks the electron flow via the centre *i* and, consequently, promotes oxidation of cytochromes *b*.

Indeed, in SMP inhibited by antimycin in the absence of KCN (cytochrome c_1 and FeS_{Rieske} oxidized, centre *o* kinetically competent), DBMIB has been found virtually ineffective in oxidizing *b* cytochromes (fig.5, *5*) as compared to the antimycin + KCN-inhibited state (fig.5,*3*,*4*).

Similar explanation applies to the relevant data on the 'controlled reduction' of *b* cytochromes [24–26] as discussed in [17].

Finally, we would like to emphasize that although QH_2 is probably the major electron donor to DBMIB, the resulting decrease of the $[QH_2]/[Q]$ steady-state ratio cannot be a trivial reason for the DBMIB-induced oxidation of *b* cytochromes in the presence of antimycin. In fact, from the succinate/fumarate titrations of aerobic KCN + antimycin-inhibited SMP it follows that in order to account for cytochrome *b*-562 50% oxidation ($E_{m_7}^{app} \approx +125$ mV [14,27]), ubiquinone ($E_{m^7} = +65$ mV, $n = 2$ [22]) should be ≈99% oxidized. DBMIB concentrations sufficient to bring about half-oxidation of cytochrome *b*-562 (≈12 μM with succinate and ≈4 μM with NADH as substrates, fig.5) released the KCN- or KCN + antimycin-inhibited O_2 uptake to at most 20% of the normal uninhibited respiration rate [15]; consequently the $[QH_2]/[Q]$ ratio of 0.01 is not realistic under these conditions.

However, even a slight leakage of electrons to O_2 from the respiratory carriers clamped between the two extremely slow reaction steps (viz. QH_2 oxidation to $QH^{\cdot}$ in the centre *o* and a leak through the antimycin block in the centre *i*) would be sufficient to keep *b* cytochromes oxidized. We therefore assume that DBMIB, in addition to its being reduced by CoQ pool, can also accept electrons in the centre *o* from ubisemiquinone or directly from *b* cytochromes (fig.6).

Acknowledgements

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