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The Site of Inhibition of Dibromothymoquinone in Mitochondrial Respiration

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Abstract

Dibromothymoquinone (2,5-dibromo, 6-isopropyl, 3-methyl benzoquinone, DBMIB) is a quinone analogue recently introduced as a specific inhibitor of chloroplast photosynthesis at the level of plastoquinone.

In beef heart mitochondria DBMIB inhibits the oxidation of both succinate and NAD linked substrates; the apparent K_I is 6 μM for βhydroxybutyrate oxidation and 61 μM for succinate oxidation respectively. In sonic fragments NADH oxidation is also inhibited; however, the rotenone block of respiration can be partially bypassed by the autooxidation of reduced DBMIB. Under the same conditions succinoxidase of ETP is inhibited, as in intact mitochondria; autoxidation of DBMIB reduced by succinate can however be obtained in presence of detergents. Hexahydrocoenzyme Q_4 reverses the DBMIB inhibition of succinate in sonic fragments. The site of inhibition by DBMIB is the oxygen side of CoQ, since DBMIB can function as electron acceptor in the NADH-CoQ assay for site I energization in submitochondrial particles, studied by measuring the quenching of atebrin fluorescence.

Introduction

The role and localization of Coenzyme Q (ubiquinone) in the mitochondrial respiratory chain are not yet fully understood [1]; moreover the steric arrangement and possible compartmentation [2, 3] of this quinone in the mitochondrial membrane represent now an

important subject of investigation, in view of the sidedness of the membrane and of the Mitchell hypothesis of coupling in oxidative phosphorylation [4]. By extraction and reinsertion studies, ubiquinone has been found to be required for activity of both NADH oxidase and succino-oxidase [5]. Because of its lipid solubility, it was considered a mobile carrier which pools reducing equivalents from the two dehydrogenases to the cytochrome b-c_1 segment of the chain [6]; such function has been confirmed [1] on the basis of the sigmoidal curve of electron transfer inhibition by Antimycin A [7].

Specific inhibitors can be extremely useful in the study of respiratory carriers. Trebst *et al* [8] have reported that 2,5-dibromo, 3-methyl 6-isopropyl, p-benzoquinone (DBMIB or dibromothymoquinone) inhibits electron transport between photo-systems II and I in spinach chloroplasts, and that the inhibition is completely reversed by plastoquinone [9]. This effect on photosynthesis raises the problem whether DBMIB may be a competitive inhibitor also of mitochondrial ubiquinone. We have therefore investigated the action of DBMIB on the respiratory activities of beef heart mitochondria and submitochondrial particles.

Materials and Methods

DBMIB was a kind gift of Dr. A. Trebst, Ruhr-Universität, Bochum.

Hexahydrocoenzyme Q_4 was generously supplied by Dr. K. Folkers, Institute for Biomedical Research, Austin, Texas.

Beef heart mitochondria (BHM) were prepared by a small scale procedure [10] and were sonicated in order to obtain submitochondrial particles (ETP). Phosphorylating particles ETP_H were obtained by sonicating Heavy beef heart mitochondria in presence of ATP, Mg^{2+} and Mn^{2+} according to Hansen and Smith [11].

Respiratory activity was recorded with a Y.S.I. oxygen electrode, Mod. 5400, in a total volume of 7 ml and at the temperature of 30°C. The experimental details of the different assays are given in the legends of the Figures describing the individual experiments.

Energization of ETP_H in Site I [12] was assayed monitoring the quenching of atebrin fluorescence [13] using the filter fluorimeter previously described [14].

Results and Discussion

The effect of DBMIB on the oxidation of succinate and β-hydroxybutyrate in intact freshly prepared BHM is described in Fig. 1. Both activities are inhibited by the quinone in a sigmoidal fashion; 50%

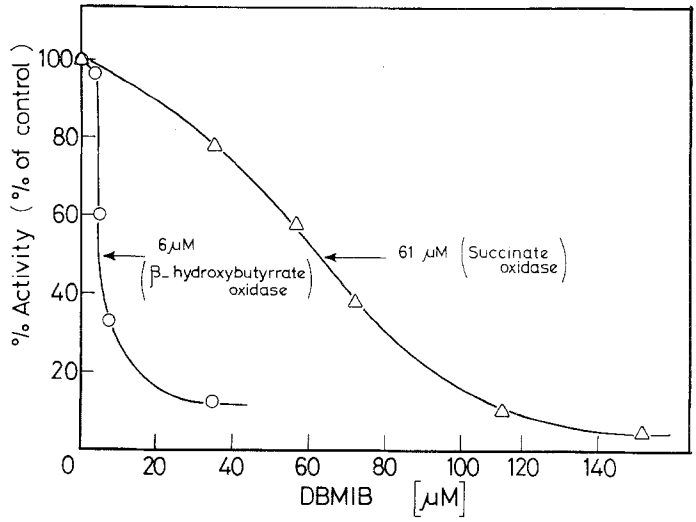

Figure 1. Effect of DBMIB on succinate oxidase and β-hydroxybutyrate oxidase activities in intact beef heart mitochondria. The assay medium for measuring β-hydroxybutyrate and succinate oxidase activities contained in a final volume of 7 ml: phosphate buffer, pH 7.4, 28 μmoles; sucrose, 105 μmoles; mannitol, 315 μmoles; EDTA, 140 μmoles; KCl, 280 μmoles; $MgCl_2$, 140 μmoles; ADP, 10 μmoles and particles corresponding to 3.58 mg of protein were finally added. Before addition of the substrates (Na-succinate, 40 μmoles and β-hydroxybutyrate, 20 μmoles) the mitochondria were preincubated with the inhibitor for 10 min at 30°C. β-Hydroxybutyrate activity was 54 ngatoms 0/min × mg of protein. Succinate oxidase activity was 142 ngatoms 0/min × mg of protein.

inhibition of succinoxidase occurs at 6.1×10^{-5} M DBMIB, whereas half inhibition of NAD-linked β-hydroxybutyrate oxidation is observed at lower inhibitor concentration (6×10^{-6} M). Similar effects are obtained if NAD-linked respiration is determined using pyruvate plus malate as substrates. In order to obtain such inhibition constants, BHM must be preincubated with DBMIB for 10 minutes, otherwise the apparent K_Is are one order of magnitude higher.

The inhibitory effect of DBMIB is also evident for succinate oxidation in ETP (Fig. 2). At 2.5×10^{-5} M, DBMIB half inhibits succinoxidase. Addition of exogenous quinone (8 mM hexahydro CoQ_4) restores succinoxidase activity partially, increasing the apparent K_I to an approximate value of 7×10^{-5} M DBMIB. At 5×10^{-5} M DBMIB, succinoxidase activity is about 20% of the control, but is brought up to 55% in the presence of the Coenzyme Q homologue.

The choice of hexahydro CoQ_4 as an antagonist to DBMIB has been dictated by its higher hydrophilicity in comparison with the natural CoQ_{10} of BHM, by its full activity in restoring succinoxidase in

ubiquinone-depleted mitochondria [3], and by its wide use in biochemical and biomedical studies [15].

Inhibition activity, immediately after addition of DBMIB, can be observed in ETP also for NADH oxidase (Fig. 3); the apparent K_I for this effect is around 6×10^{-6} M. This inhibitory effect is variable in different preparations of ETP: paradoxically a stimulation NADH oxidase was occasionally observed. However, in all cases, when NADH oxidase was inhibited by rotenone (6 μM), DBMIB was able to sustain a considerable NADH oxidase activity, insensitive, in addition to rotenone (Fig. 4). This activity was insensitive also to other electron transport inhibitors such as antimycin A (6 μM) and KCN (10^{-3}M). Obviously a by-pass of the respiratory chain at the level of NADH dehydrogenase may be operative, DBMIB being enzymatically reduced by NADH through the specific dehydrogenase and reoxidized by molecular oxygen. This interpretation agrees with the observation that reduced DBMIB autooxidizes, when dissolved in an aqueous medium at pH 8.0. DBMIB when reduced by NADH in ETP must therefore be situated in a site which is available to oxygen attack in a hydrophilic superficial area in NADH dehydrogenase,

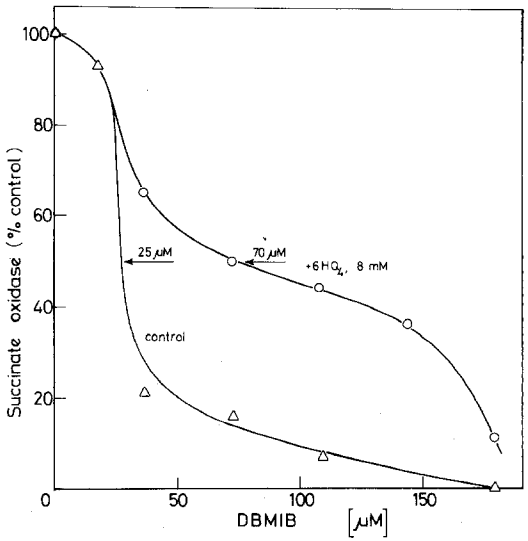

Figure 2. Inhibition by DBMIB of succinate oxidase and partial restoration of the activity by addition of hexahydro Q_4 in ETP. The assay medium contained in a final volume of 7 ml: Tris-Cl, pH 8.0, 350 μmoles; sucrose, 3.5 mmoles and ETP corresponding to 2.1 mg of protein. For reconstitution experiments ETP were preincubated in presence of a mixture containing, in addition to the normal components of the assay, Asolectin (about 200 μg phosphorus) and hexahydro Q_4, 56 μmoles. After 4 minutes DBMIB was added and preincubation was carried on for additional 10 min. The reaction was started by addition of Na-succinate, 40 μmoles. 100% activity was 251 ngatoms 0/min x mg of protein.

since no preincubation appears to be required for autooxidation. The extent of rotenone-insensitive, DBMIB-sustained NADH oxidase will depend on the degree of accessibility of reduced DBMIB to oxygen and therefore from the degree of "opening" of the site of reduction of DBMIB, which is apparently variable in different ETP preparations. This relatively high probability of damaging the site of DBMIB reduction by NADH during ETP preparation is consistent with the concept of a rather superficial location of this site, and is also in agreement with the lack of requirement for preincubation in order to obtain inhibition in undamaged particles. On the other hand DBMIB reduced by succinate in ETP is situated in an inaccessible, lipophilic area, since inhibition of succinoxidase requires preincubation of the particles with the inhibitor. Such a suggestion is supported by the fact that when the particles are treated with 0.02% (v/v) Triton X-100, DBMIB becomes autooxidizable in presence of succinate without preincubation (Fig. 4). Triton, at this concentration, inhibits succinoxidase activity of ETP to 45% of the initial activity, and a maximal fivefold stimulation occurs at 3×10^{-5} M DBMIB.

The competitive inhibition of mitochondrial respiration by DBMIB when it is bound in the natural hydrophobic CoQ site could be caused by

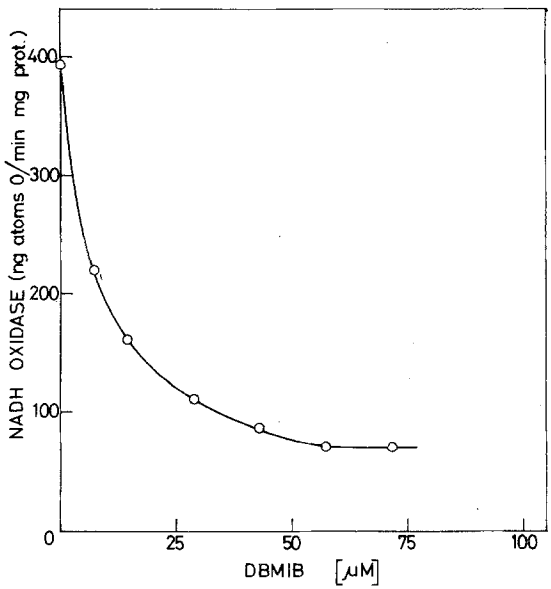

Figure 3. Inhibition of NADH oxidase by DBMIB in ETP. The assay medium for NADH oxidase contained in a final volume of 7 ml: Tris-Cl, pH 8.0, 350 μmoles; sucrose, 3.5 mmoles and ETP corresponding to 2.3 mg of protein. The reaction was started by addition of NADH, 6 μmoles.

two possible phenomena: either (a) DBMIB displaces endogenous CoQ and is not reduced, or (b) it displaces CoQ, becomes reduced but is not reoxidized by the subsequent electron acceptor.

In order to discriminate between these two hypotheses we have tried to demonstrate whether DBMIB may function as an electron acceptor substituting for CoQ_1 in a NADH-CoQ_1 assay for the first coupling site of the respiratory chain. When phosphorylating ETP_H are supplied with NADH and CoQ_1, the latter becomes reduced through the first site with

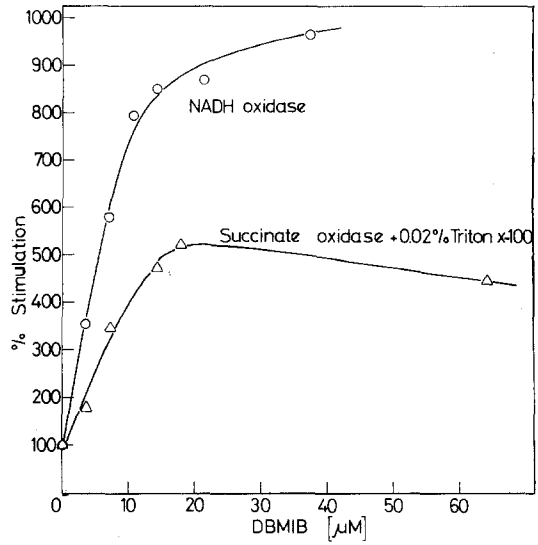

Figure 4. Stimulatory effect by DBMIB of rotenone inhibited NADH oxidase (curve a) and of succinate oxidase (curve b) in presence of Triton X-100. Succinate oxidase and NADH oxidase activities were measured as described in Figure 2 and Figure 3. Before addition of succinate, ETP were preincubated with Triton X-100 (0.02% v/v) for 3 min at 30°C. The effect of DBMIB was immediate. NADH oxidase activity in absence or presence of rotenone (6 μmolar) was: 190 ngatoms 0/min x mg of protein and 22 ngatoms 0/min x mg of protein, respectively. Succinate oxidase activity in absence or presence of Triton X-100 was 212 and 117 ngatoms 0/min x mg of protein, respectively.

a $P/2e^-$ ratio approaching 1 [12]. Energization may be conveniently demonstrated measuring the quenching of the fluorescence of atebrine. When CoQ_1 is added to ETP previously reduced by NADH in the presence of KCN, energization takes place and results in a quenching which is maintained as long as oxidized CoQ_1 is available (Fig. 5). If DBMIB is used in place of CoQ_1 the same phenomenon is observed. Obviously DBMIB acts as an electron acceptor for NADH dehydrogenase, and electrons must flow through the first phosphorylation site, as also indicated by the fact that rotenone inhibits quenching in presence of DBMIB.

It is therefore evident that even in submitochondrial particles, although an aliquot of the inhibitor may be reduced (and immediately autooxidizes) at a level before the first coupling site and before the rotenone block, another aliquot may accept electrons flowing through the energizing pathway.

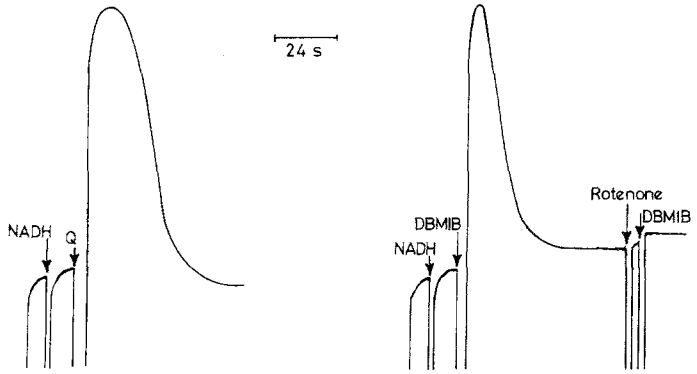

Figure 5. Quenching of the fluorescence of atebrine induced by oxidation of NADH by Q_1 or DBMIB. The assay contained in a final volume of 2.5 ml: glycylglycine, pH 8.0, 100 μmoles; sucrose, 1.25 mmoles; $MgCl_2$, 12.5 μmoles; KCl, 250 μmoles; EDTA, 2.5 μmoles; valinomycin, 4 μg; atebrine, 10 nmoles and ETP_H corresponding to 480 μg of protein. The following additions were made: KCN, 1 μmole; NADH, 0.5 μmole; Q_1, 0.1 μmole; DBMIB, 0.1 μmole and rotenone, 20 μmoles.

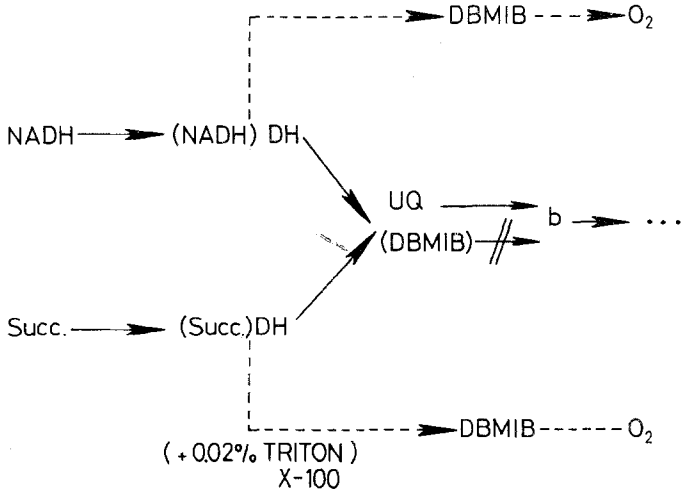

Figure 6. Scheme indicating the possible action of DBMIB on the respiratory chain of beef heart mitochondria.

Such energization experiments give therefore a hint on the mechanism of inhibition: since DBMIB supports energization of site I, it must be reduced by NADH dehydrogenase, but it is not reoxidized by the chain segment which is normally reduced by natural ubiquinone. The inhibitory activity is higher in NADH oxidation, indicating that it has higher affinity for the reducing site in NADH dehydrogenase than for the reducing site in succinate dehydrogenase. DBMIB therefore acts in the electron transport chain of beef heart mitochondria in a way extremely similar to that operating in spinach chloroplasts [8]; in this system DBMIB has shown to behave as a plastoquinone analog which can be competitively reduced in place of plastoquinone in a reaction specific for Photosystem 2 [9] and coupled to active proton translocation [16, 17] and which blocks the photosynthetic electron transport chain after the plastoquinone pool [17]. Fig. 6 schematically indicates the actions of DBMIB on the respiratory chain.

DBMIB is a novel addition to the number of inhibitors acting at site I of oxidative phosphorylation [18], but unlike rotenone and piericidin A, it allows energy conservation at site I. In contrast to piericidin A [19], DBMIB is also a good inhibitor of succinoxidase (100 fold more effective than piericidin A). It may be of interest that DBMIB is an effective inhibitor of succinoxidase at the CoQ level: there is a well-known lack of inhibitors for succinate oxidation before cytochromes and the antimycin block if we exclude certain short-side chain ubiquinone 5-hydroxy analogs [20].

Acknowledgements

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