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A benzopyrylium dye as a novel effective cationic uncoupler of oxidative phosphorylation

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Benzopyrylium dye BDBP was found to be an effective uncoupler of oxidative phosphorylation It stimulates state 4 respiration of mitochondria 2-fold at about 2 μM, reveals a maximum stimulation (8-fold) at 30 μM and inhibits the respiration at higher concentrations. BDBP is also an effective proton (hydroxyl) carrier through bilayer lipid membranes (BLM) as is seen from the electrical properties of BLM with BDBP. Picrate enhances the effects of BDBP on the mitochondrial respiration and BLM conductance. The BDBP activities are accounted for by formation of dimers and complexes with picrate.

Cation uncoupler; Proton (hydroxyl) carrier; Benzopyrylium dye; Rat liver mitochondrion; Bilayer lipid membrane

1. INTRODUCTION

The first hydrophobic bases which were reported to cause uncoupling of oxidative phosphorylation by a protonophoric (hydroxylophoric) mechanism in the absence of lipophilic anions were decylamine and tributylamine [1,2], which stimulate state 4 respiration 2-fold at 2–5 mM and as such are far less effective than most of the weak-acidic uncouplers [2]. None of the basic uncouplers described later [3–9] were shown to transport proton (hydroxyl) across bilayer lipid membranes (BLM) in the absence of lipophilic anions.

BDBP (Fig. 1) is one of a series of benzopyrylium derivatives synthesized recently [10]. An acido-basic equilibrium in BDBP ethanol–aqueous (1:1) solution [10] demonstrates the presence of the positively charged and electroneutral species of BDBP in similar concentrations at physiological pH ($pK_a = 8.31$). Hence, BDBP was expected to be a carrier of protons or hydroxyls and an uncoupler of oxidative phosphorylation. This paper reports the experimental verification of these expectations.

2. MATERIALS AND METHODS

Liver mitochondria were isolated from male Wistar rats. The experimental medium (1.5 ml) consisted of 50 mM KCl, 150 mM sucrose, 3 mM KH_2PO_4, 4 mM succinate, 2 μg/ml rotenone 10 mM Tris-HCl, pH 7.5, and 2–3 mg of mitochondrial protein. Other additives are indicated in the legends to figures. Mitochondrial respiration was measured with an oxygen electrode at 25°C.

BLMs were formed at 20°C with a solution of bovine brain lipids in *n*-decane (20 mg/ml) at an orifice of about 1 mm diameter in the vertical wall of a Teflon cell. The BLM zero voltage conductance was evaluated by conventional methods [11]. The bathing solutions and additives are described in the legends to figures.

Chemicals used were: succinate from Sigma; rotenone, Tris from Serva; sucrose recrystallized and purified with Dowex-50 from Serva. BDBP was synthesized as described earlier [10], and was added to the aqueous medium as aliquots of concentrated ethanol–aqueous (1:1) or dimethylsulphoxidic solutions.

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Abbreviations: BDBP, 2-benzylamino-4-methyl-7-diethylaminobenzopyrylium tetrafluoroborate; BLM, bilayer lipid membrane(s).

3. RESULTS

Fig. 2 shows the effects of BDBP on state 4 respiration in the absence and presence of 10 μM sodium picrate. The respiration rate increases with dye concentration, reaching a peak at about 30 μM in the absence of picrate and at 4 μM with picrate. Further increases in dye concentration represses respiration, as is typical of classical uncouplers. It was also verified that picrate alone does not influence the state 4 respiration at the concentration used.

In 10 mM Tris-HCl, pH 7.5, the increase of the zero voltage conductance of BLM by BDBP is observed at about 10^{-7} M of dye. The slope of the concentration conductance curve in a double logarithmic plot changed from about 1 at concentrations up to 10 μM to approximately 2 at above 30 μM (Fig. 3, curve 1).

BDBP-treated BLM behaves as an $H^+(OH^-)$-specific electrode giving 55–58 mV per unity of transmembrane pH gradient at neutral pH. Thus, BDBP is a carrier of protons (hydroxyls) across lipid membranes. At sufficiently low pH values the substitution of $BDBP^+$ specificity for the $H^+(OH^-)$ one in BDBP-treated BLM oc-

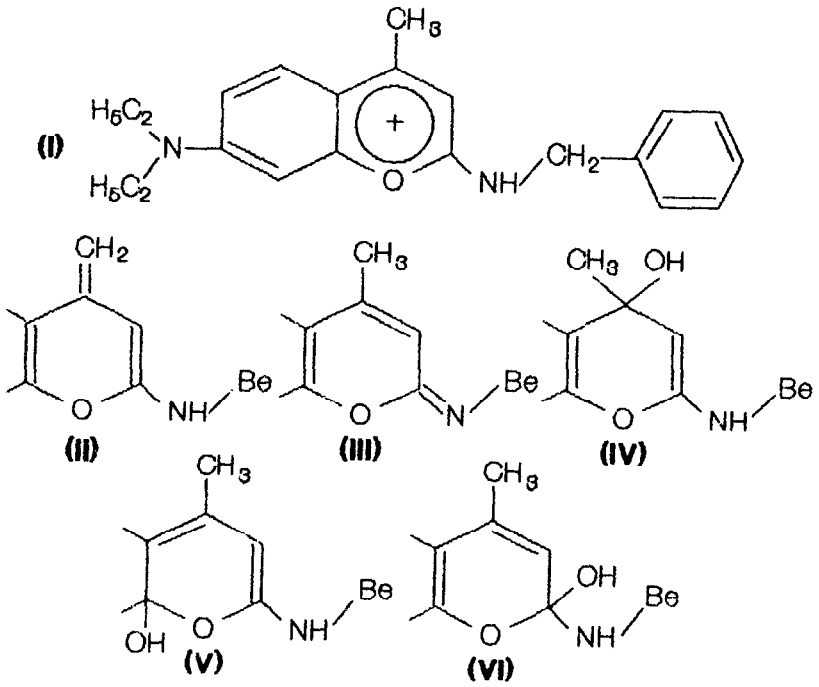

Fig 1. Formula of the BDBP charged form (I) and possible structures of its neutral form (II–VI) Be, benzyl.

curs. For example, at pH 3 the membrane potential under the transmembrane concentration gradient of BDBP is equal to about 30 mV per decade.

In the presence of 10 μM picrate (Fig. 3, curve 2) the effective concentration range of BDBP may be divided into two regions: below 20 μM BDBP (enhanced effect of dye with a slight increase of the curve slope compared with curve 1) and above 30 μM BDBP (no influence of picrate). The transient narrow zone between these two regions seems to be caused by precipitation of a BDBP–picrate complex (the sediment becomes visible above 100 μM of each component).

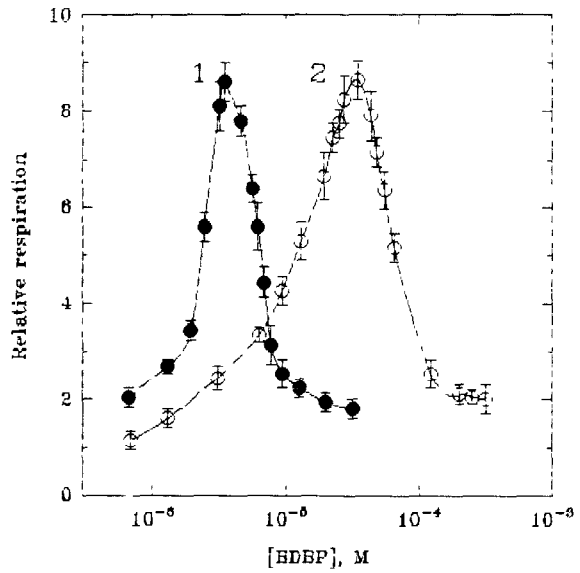

Fig. 2. Dependence of the mitochondrial respiration rate on BDBP concentration in the presence of 10 μM sodium picrate (curve 1) and without picrate (curve 2). The incubation medium is detailed in section 2. The respiration rate is normalized to that in state 4 (about 20 ng atom O/min/mg of protein). Each curve is the average of three experiments

In the presence of 100 μM picrate the BLM conductance increment is a linear function of BDBP concentration up to 2 μM of the dye (Fig. 3, curve 3), being in this range considerably greater than at lower picrate concentrations (Fig. 3, curves 1 and 2). The lack of a BLM conductance increase above 2 μM is due to mutual sedimentation of BDBP and picrate.

4. DISCUSSION

Liberman et al. [12] carried out a comparative investigation of uncoupling and protonophoric efficiencies of a series of weak acids. They plotted the concentrations of uncouplers inducing 2-fold stimulation of succinate oxidation in mitochondria against the concentrations of the same agents increasing the BLM proton conductance by 5×10^{-9} S/cm^2 and obtained in a double logarithmic plot a straight line with a slope of unity passing through the origin of coordinate axes. Figs. 2 (curve 2) and 3 (curve 1) show that BDBP activities correspond to this linear correlation. This fact is indicative of a common protonic (hydroxylic) pathway induced by acidic and basic uncoupling agents.

To decide which of the two ions, proton or hydroxyl,

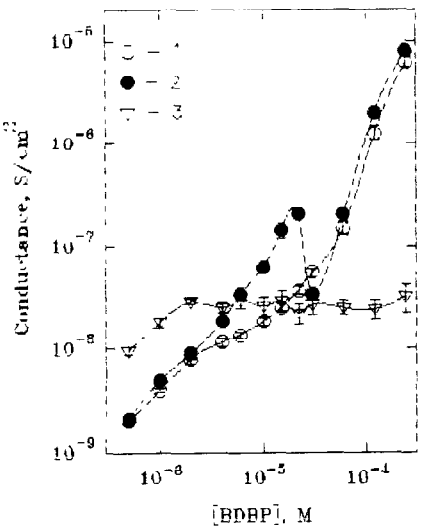

Fig. 3. The BDBP-induced zero voltage conductance of BLM as a function of BDBP concentration in 10 mM Tris-HCl, pH 7.5, without (curve 1) and with 10 μM (2) or 100 μM (3) sodium picrate. Every line is the average of 2–3 original curves obtained each with a single membrane. In the absence of BDBP the average conductances of the membranes used were $(8 \pm 3) \cdot 10^{-10}$ (2 BLM), $(3.7 \pm 0.2) \cdot 10^{-9}$ (3) and $(3.4 \pm 0.2) \cdot 10^{-8}$ (2) S/cm^2 at 0, 10 and 100 μM sodium picrate, respectively. Standard errors usually did not exceed the size of symbols.

is transported by BDBP through membranes, i.e. whether BDBP is a protonophore or a hydroxylophore, it is enough to know the formula of the BDBP neutral form implicated in the acid–base equilibrium. Indeed, of all its possible structures (see Fig. 1) II and III can be obtained by chipping the proton off the charged form I, and IV–VI can be formed by joining the hydroxyl to I. Available data permit the exclusion of some of these structures. For instance, II is possible only for benzopyryliums with an alkyl radical at C_4, however, the removal of a methyl group from BDBP decreases its pK_a, i.e. increases the stability of its neutral form [10]. Hence, we may exclude structure II. Then, the absorption spectra of I and III at the same pH are different [10], and this permits the exclusion of structure III. The substitution of alkyl, piperidyl or morpholyl (but not phenyl) for benzyl at the C_2 amino group sharply increases the pK_a [10], i.e. destabilizes the neutral form. This allows us to conclude that structure VI is the more preferable formula of the BDBP neutral form because VI is the single molecule stabilized by an intramolecular Hπ- bond between the OH group and an aromatic cycle in the above position [13,14]. Hence, we incline to the opinion that the acid–base equilibrium in a BDBP aqueous solution can be presented schematically as $B^+ + OH^- \rightleftarrows BOH$, i.e. that BDBP is a hydroxylophore.

The simplest way to realize the hydroxylophoric function of BDBP in membranes is shown in Fig. 4a. The comparison of the $BDBP^+$ and tetraphenylphosphonium cation, however, allows us to conclude that this mechanism can explain only the BDBP-induced conductance of BLM, which is considerably smaller than that observed in the experiment.

In order to account for the high conductance of BLM with BDBP one should consider the formation of BDBP-containing charged complexes which, because of their larger size and, maybe, greater hydrophobicity, are more permeant ions compared with $BDBP^+$. For example, a suitable particle is the complex B_2OH^+ (but not the dimer B_2^{2+}), and its formation (presumably in the membrane with participation of hydrogen bonds) at BDBP concentrations above 20 μM is manifested in the slope 2 of the curves 1 and 2 in Fig. 3. In mitochondria, B_2OH^+ enters the matrix along the electric field, binds one hydroxyl and leaves the matrix as 2 molecules of BOH (Fig. 4b). As a result, the respiratory chain introduces into the matrix one hydroxyl per B_2OH^+ entered, and enhances the substrate consumption. As to membrane conductance at BDBP concentrations smaller than 20 μM, it is determined by univalent complexes containing only one BDBP particle (Fig. 3, curve 1) but not by $BDBP^+$ alone as we noted above. We have stated that these are complexes with Tris molecules present in the medium as buffer (data not shown).

Stimulation of base-induced effects in mitochondria by lipophilic anions, such as that shown in Fig. 2, is well known [4,5,7,9]. The most well-established mechanism of this stimulation proposed by Garlid and Nakashima [5] is presented by the left part of Fig. 4c in which A^- is the lipophilic anion, in our case picrate. Anion enters the matrix as an electroneutral complex with B^+, and leaves it as a free charged species. If anion is more permeating than B^+ this makes the cyclic mechanism more effective (the efficiency of the system is limited by the transport of the charged species because molecule BOH is many orders of magnitude more permeating than B^+, B_2OH^+ and anions such as picrate) at low concentrations of the dimers B_2OH^+. It seems, however, that this mechanism cannot explain the observable stimulation of BDBP uncoupling and BLM conductance by picrate.

These effects can be accounted for by supposing, together with a neutral complex, BA, entering the matrix, the existence of some negatively charged complexes of picrate and BDBP ejected from the matrix and/or positively charged complexes entering the matrix. For instance, the complex, $A(BOH)_2^-$ (A^-, picrate) or $A(BOH)_3^-$ are perfectly compatible with our data. Thus, the mechanism of Fig. 4c can explain, quantitatively, the picrate stimulation of mitochondrial respiration observed (Fig. 2), the abrupt increase of this stimulation with the BDBP concentration (see the difference of curves 1 and 2 in Fig. 2) as well as the cooperativity of the BDBP and picrate in BLM shown in Fig. 3 (the BLM conductance in the presence of BDBP and picrate is larger than the sum of the conductances induced by BDBP and picrate

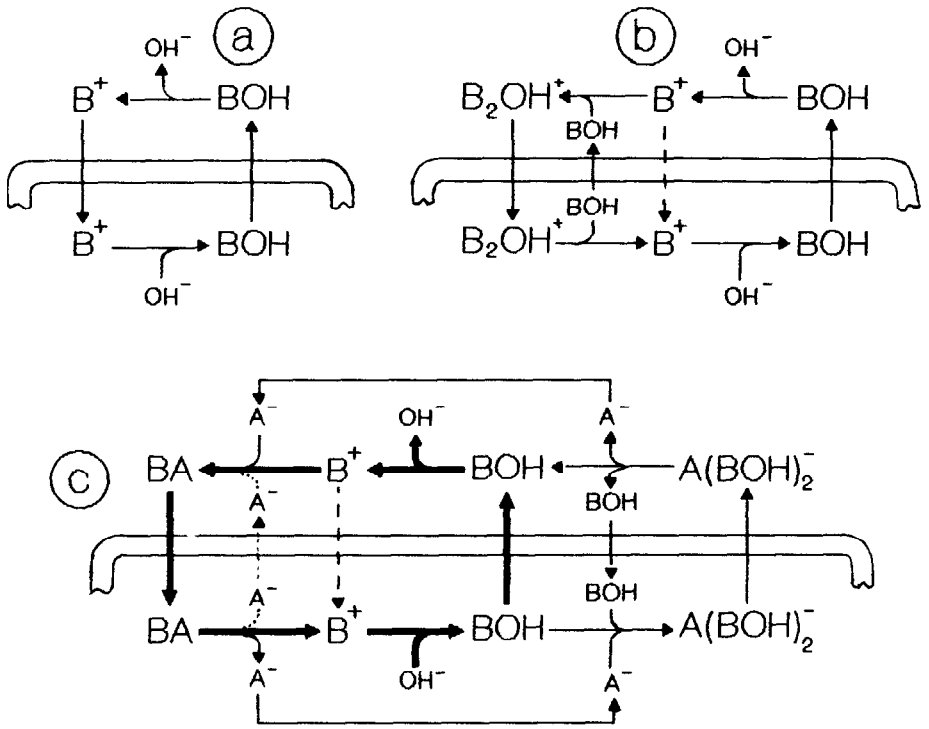

Fig. 4 Different mechanisms of hydroxyl transport across membrane in the presence of BDBP. The dye is supposed to have two monomeric species, B^+ and BOH, in equilibrium, $BOH \rightleftarrows B^+ + OH^-$ The bottom of each diagram represents the matrix of energized mitochondria or the aqueous medium with negative potential in the BLM system. (a) A simple carrier mechanism. (b) A dimer mechanism The dashed line indicates an ineffective route of transport. (c) A carrier mechanism with participation of an hydrophobic anion as proposed by Garlid and Nakashima [5] (bold, dotted and dashed lines, the last being an ineffective transport pathway) and modified according to our data (the entire diagram where dashed and dotted lines are ineffective transport routes) See text for details.

separately). All these phenomena can also be explained by assuming the existence of complexes AB_2^+ entering the matrix, but cannot be accounted for within the framework of the original mechanism of Garlid and Nakashima.

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