Benzofuroxan as Electron Acceptor at Photosystem I

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The midpoint potential of BFO, the sensitivity of its photoreduction to DCMU, DBMIB and KCN, and the photosystem I activity, suggest that the photoreduction of BFO in the chloroplast is at the primary electron acceptor × of photosystem I, and is irreversible.

Rates of electron transport are similar in basal phosphorylating or uncoupled conditions although electron transport is coupled to photophosphorylation.

Introduction

Bipyridilium salts and quinones are reduced by photosystem I in chloroplasts and are widely used as electron acceptors [1, 2]. These compounds are autooxidizable and they catalyze a Mehler reaction. In this communication benzofuroxan (BFO) is described as an electron acceptor at the reducing side of primary electron acceptor X; it was discovered in the course of the search for compounds with inhibiting effects on PS II.

Materials and Methods

Benzofuroxan from Sigma was recrystallized from methanol until a constant melting point was obtained. DBMIB (2,5-dibromo-3-methyl-6-*iso*-propyl-*p*-benzo quinone) was synthetized as reported by Trebst [3].

Chloroplast thylakoids were isolated from market spinach leaves (*Spinacea oleracea* L.) as described earlier [4], and suspended, unless otherwise indicated, in 100 mM sorbitol, 5 mM MgCl, 240 mM KCl, 30 mM tricine buffer at pH 7.6 with either 3 mM ferricyanide or 0.05 mM Methylviologen or benzofuroxan were added as electron acceptors. KCN (1 mM) was added to inhibit catalase activity. Benzofuroxan photoreduction was monitored spectrophotometrically at 350 nm (the absorption maximum of benzofluroxan; $E350 = 4300$ liter mol^{-1} cm^{-1} [6] in the supernatant of illuminated chloroplasts. Chlorophyll [6], non-cyclic electron transport from water to methylviologen [4], photosystem I electron transport from DCIP/ascorbate to MV [4] or ATP synthesis [7], chloroplast's plastocyanin inhibition by KCN [8] were determined as described in literature. BFO photoreduction was determined polarographically or spectrophotometrically. All reaction mixtures were illuminated by actinic light as reported previously [4].

The cyclic voltammograms of BFO were performed with a Bioanalytical Systems Inc model CV-1B cyclic voltammetry unit. The voltammograms were registered in a Hewlett-Packard 7004B X-Y recorder. A mercury electrode was used as cathode [9]. A glassy carbon electrode was used as counter electrode and saturated calomel as reference.

Results

The basal electron transport rate from water to BFO was measured spectrophotometrically at 350 nm Table I at the saturation concentration of BFO (25 μM). The same type of results can be obtained if oxygen evolution is measured polarographycally (data not shown). The rate of BFO reduction as shown Table I is very low compared with that of ferricyanide [10], methylviologen, or class III electron acceptors [11, 12]. From these data, it can be concluded that BFO accepts electrons from the electron transport chain in chloroplasts. Table I also shows that the phosphorylating electron transport rate and the uncoupled electron transport rate in the presence of BFO are not accelerated by ADP plus Pi or an uncoupler, when compared with basal electron transport, as occurs with ferrycianide [10, 12] or methylviologen [10, 12]. Chloroplasts used in this project are coupled, since control experiments using ammonium chloride (2.5 mM) increased 2- or 3-fold

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Table I. Noncyclic electron transport and photosystem II activity. Reaction mixture as described in Materials and Methods. Concentrations of BFO 25 μM, MV 50 μM, 100 μM DCIP, DCMU 10 μM, DBMIB 1 μM, ADP 1 mM, Pi 3 mM.

Conditions	Electron donor	Electron acceptor	Rate of electron transport (per cent of control) [%]
None control	H_2O	FBO	100
None	H_2O	MV	474
None	H_2O	DCIP	671
ADP + Pi	H_2O	BFO	60
NH_4Cl	H_2O	BFO	78
DCMU	H_2O	BFO	10
DCMU + ADP + P_i	H_2O	BFO	28
DCMU + NH_4Cl	H_2O	BFO	38
DBMIB	H_2O	BFO	21
DBMIB + ADP + Pi	H_2O	BFO	42
DBMIB + NH_4Cl	H_2O	BFO	45

the electron transport rate from water to methylviologen (data not shown).

Further experiments were performed in order to determine the site where BFO accepts electrons. DCMU and DBMIB inhibit basal, coupled or uncoupled electron transport rates from water to BFO (Table I). These results indicate that BFO accepts electrons after PQ.

To clarify the zone of electron acceptance further, PSI activity was measured with DCIP/ascorbate as electron donor and the results are shown in Table II. It is observed that BFO photoreduction by chloroplasts is inhibited by DCMU and it is re-established by the addition of DCIPH. The results are similar to those in which DCIPH is added to DCMU inhibited cloroplasts (Table II) in the presence of MV, and suggest that BFO accepts electrons in photosystem I or at least in or after cyt *f*. Since the photoreduction of BFO can be also inhibited by DBMIB (Table I) chloroplasts were inhibited by KCN which inhibits electron transport at the plastocyanin level [8] DCIP/ascorbate was used as the electron donor. In this system BFO is still photoreduced (Table III), which suggests that BFO accepts electrons in/or after P-700.

Table II. Photosystem I activity of BFO compared with MV. Reaction mixture as described in Materials and Methods. Concentrations of BFO 30 μM, MV 50 μM, DCMU 10 μM and $DCIPH_2$ 10 μM, DBMIB 1 μM.

Additions	Electron donor	Electron acceptor	Rate of electron transport (per cent of control) [%]
none control	H_2O	FBO	100
DCMU	H_2O	BFO	5
DCMU	$DICIPH_2$	BFO	83
DCMU	$DICIPH_2$	MV	90
none	H_2O	MV	100
DCMU	H_2O	MV	4
DBMIB	H_2O	MV	5

Table III. Electron transport from P700 to BFO in KCN treated chloroplasts. Reaction mixture as described in Materials and Methods. Concentrations of BFO 30 μM, MV 50 μM and $DCIPH_2$ 100 μM.

Condition	Electron donor	Electron acceptor	Rate of electron transport (per cent of control) [%]
No treatment control	H_2O	BFO	100
KCN treated	H_2O	BFO	9
KCN treated	$DCIPH_2$	BFO	80
KCN treated	$DCIPH_2$	MV	85
No treatment control	H_2O	MV	100

To determine the site of electron acceptance for BFO, the midpoint reduction potential of BFO alone was measured by cyclic voltammetry. Fig. 1 shows two electrochemical reduction peaks for BFO, one at −25 mV and the other at −320 mV, *vs.* the saturated calomel electrode which corresponds to +219 mV and 75.6 mV, respectively, *vs.* the normal hydrogen electrode. The first peak was considered the midpoint potential for BFO. These values are in close agreement with those of Thompson [13]. The pattern of the voltammogram (Fig. 1) suggests an irreversibly reduced compound. No anodic peaks were observed, which is contrary to what is observed in the case of methylviologen, which in the reduced stated presents two catodic peaks and two reversible peaks in the oxidized state. The values of the redox potential suggest that BFO in the chloroplast is photoreduced by the primary acceptor: Xred + BFOox → Xox + BFOred. Although BFO with an Eo value of +219 mV could accept electrons be-

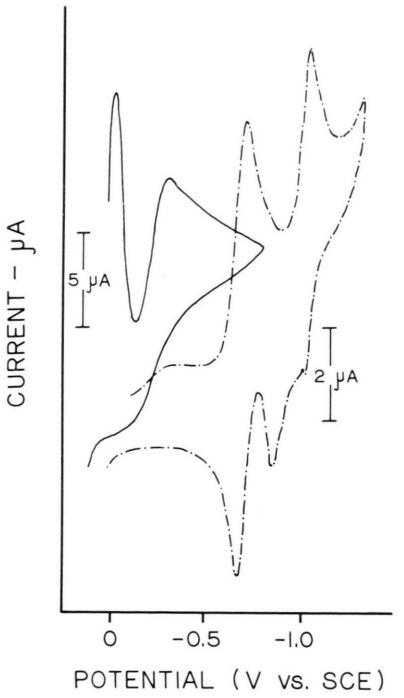

Fig. 1. A comparison of the cyclic voltammogram of (BFO (———) and methylviologen (–·–·–). Experimental conditions were as described at supra in the section "Materials and Methods". BFO and paraquat solutions were prepared at a concentration of 1 mM in the electrolyte solution 5 mM $MgCl_2$, 100 mM KCl, 30 mM, KH_2PO_4 (pH 6.0) was used. The sweep rate was 100 mv/s.

tween PS I and PS II, our experiments with the electron flow inhibitor showed this not to be the case, since KCN still inhibits BFO activity. This behavior might be explained by the inaccessibility of BFO to the electron chain flow.

To find out whether the electron transport from water to BFO is coupled to ATP formation. ATP synthesis was measured following the reduction of NADP in the presence of glucose, hexokinase and glucose-6-phosphate dehydrogenase. The rate of ATP synthesis is directly proportional to the BFO concentration (Fig. 2); saturation of the system is reached at approximately 20 μM of BFO. These data indicate that ATP formation is coupled to electron transport. The P/2e ratio calculated from the rate of ATP synthesis and reduction of BFO was 0.6. BFO photoreduction was measured in similar conditions to those used for phosphorylation measurements except that NADP, hexokinase and glucose-6-phosphate dehydrogenase were omitted.

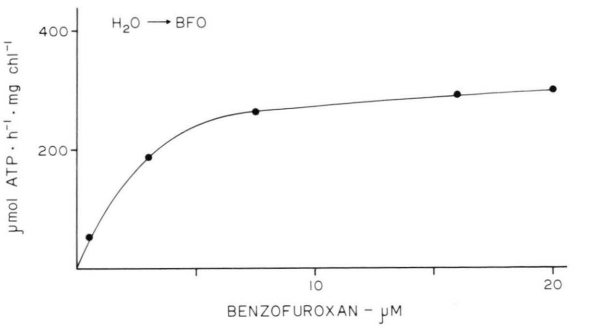

Fig. 2. Photophosphorylation as a function of BFO concentration. Reaction mixture as described at supra (Materials and Methods); each cuvete contained 40 μg of chlorophyll per ml; reaction time one minute aerobic conditions; saturating white light.

Discussion

It has been well established that the basal electron flow rate from water to NADP, MV or ferricyanide is increased several fold by the addition of phosphorylation cofactors or uncouplers [14]; however, the rate of electron transport from water to benzofuroxan is the same in basal, phosphorylating and uncoupled electron transport (Table I), which makes BFO significantly different from other known acceptors. In addition, benzofuroxan photoreduction in the chloroplasts has the following characteristics: it is a high potential compound with lipophyllic properties; it allows a lower rate of basal electron transport as compared with other Hill electron acceptors; in spite of the fact that phosphorylating electron transport is not accelerated by ADP and Pi (Table I), it is still coupled to photophosphorylation (Fig. 2) (ATP/2e = 0.6 suggests that ATP formation is limited by the low rate of BFO reduction); benzofuroxan is subject to an irreversible reduction and does not act as an uncoupler, since it has a similar electron transport rate curve shaped as methylviologen when pH was varied from 6.0 to 8.4 (data not shown) and does not behave as DCIP [10].

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