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SITE SPECIFIC INHIBITION BY α-BENZYL-α-BROMOMALODINITRILE (BBMD) OF ELECTRON TRANSPORT IN SPINACH CHLOROPLASTS

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SUMMARY

The addition of α-benzyl-α-bromomalodinitrile to different controlled states (non-phosphorylating [2]. phosphorylating [3], ATP-inhibited [4] and uncoupled) of photosynthetic electron transport to ferricyanide or benzoquinone demonstrate a significant inhibition in isolated spinach chloroplasts. α-Benzyl-α-bromomalodinitrile pretreatement of isolated chloroplasts or addition of α-benzyl-α-bromomalodinitrile at the onset of illumination completely abolished the O_2 evolving reaction. The level of the steady state fluorescence in intact chloroplasts showed a α-benzyl-α-bromomalodinitrile concentration-dependent increase. The gradual decrease in the reoxidation capacity of the reduced quencher, Q with increasing α-benzyl-α-bromomalodinitrile concentrations provides evidence for an additional inhibitory site for α-benzyl-α-bromomalodinitrile between the two photosystems.

INTRODUCTION

Considerable information regarding the nature of the photosynthetic electron transport chain has been obtained through the study of various partial reactions using different electron transport inhibitors. α-Benzyl-α-bromomalodinitrile (BBMD) was introduced recently by Brandon and Elgersma [1] as an efficient electron acceptor for photosystem II reactions. They have shown (i) on addition of 50 μM BBMD to chloroplasts a partial deflection of electron flow from acceptors like 2,6-dichlorophenolindophenol to oxygen, which is CCCP sensitive but DCMU insensitive; (ii) on pretreatment of chloroplasts with BBMD complete inhibition of electron transport to 2,6-dichlorophenolindophenol, ferricyanide or NADP. No recovery of the Photosystem II reaction with the artificial electron donor was shown. Recently Wolff et al.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; DBMIB, 2,5-dibromo-3-methyl-6-isoprophyl-*p*-benzoquinone; DCMU, 3 (3,4-dichlorophenyl)-1,1-dimethylurea; DPIP, 2,6-dichlorophenolindophenol. BBMD, α-benzyl-α-bromomalodinitrile.

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[2] also reported, on the basis of the flash-induced absorbance changes at 515 nm in spinach chloroplasts, an inhibitory action of BBMD in Photosystem II-mediated reaction. In addition, based on the changes in the kinetics of *P* 700 photooxidation they concluded that BBMD is possibly an electron acceptor at Photosystem I.

This paper describes further elaboration and critical analysis on the controversial action of BBMD in spinach chloroplasts isolated as "Type A, complete" [3].

MATERIALS AND METHODS

Complete Type A [3] spinach chloroplasts were isolated from the greenhouse grown plants as described earlier [4].

Photosynthetic O_2 evolution was continuously monitored on a Rank Bros., Cambridge, O_2 electrode. Reaction mixtures with a 2.0 ml volume contained: *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulphonic acid (HEPES), pH 7.5, 50 mM; NaCl, 10 mM; $MgCl_2$, 2 mM; $MnCl_2$, 1 mM; EDTA, 2 mM; sorbitol, 100 mM and chloroplasts equivalent to 100 μg chlorophyll. Illumination was provided by two slide projectors. Light was passed through a 3-cm water heat filter and an orange-red filter "Cinemoid" (Rank Strand Electric, London, WC2) transmitting light between 540 and 740 nm. Light intensity at the surface of the reaction vessel was $8.8 \cdot 10^4$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}. The temperature of the reaction mixture was maintained at 15 °C.

Concentration of chlorophyll was determined by the method of Arnon [5].

For fluorescence measurements chloroplasts were suspended at a final chlorophyll concentration of 3 μg per ml in the reaction mixture used for photosynthetic measurements, except that the concentration of sorbitol was increased to 300 mM to maintain the integrity of the chloroplasts. Chlorophyll fluorescence was excited by broad band blue light (400–620 nm, Corning 9788). The intensity of the excitation light was $5 \cdot 10^4$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}. Fluorescence was collected through an interference filter (Schott, Mainz; λ maximum 687 nm). The signal from the photomultiplier (R 562) was displayed on an oscilloscope (Tektronix 5B 12N) and photographed.

RESULTS

Changes in the rate of O_2 evolution upon the addition of BBMD at different levels of the electron transport are shown in Fig. 1. Freshly prepared spinach chloroplasts showed a low basal State 2 (non-phosphorylating) rate of electron transport in the presence of ferricyanide, and a high State 3 (phosphorylating) rate upon the addition of ADP, finally leading to an ADP-depleted State 4 (see ref. 6 for details). Addition of NH_4Cl in State 4 caused a greatly increased rate of uncoupled electron transport.

Addition of BBMD during State 3 reduced the rate to about 38 % of the previous rate; however, the NH_4Cl-uncoupled electron transport was found to be still high. BBMD addition at an early stage i.e., in State 2, showed a concentration-dependent inhibition (Fig. 1B and 2). More than 50 % inhibition was observed even at 10 μM. This inhibition was also evident in the subsequent states of electron transport. Parallel decreases in both State 3 and uncoupled electron transport were observed with increasing BBMD concentrations. Even at 100 μM BBMD the net elec-

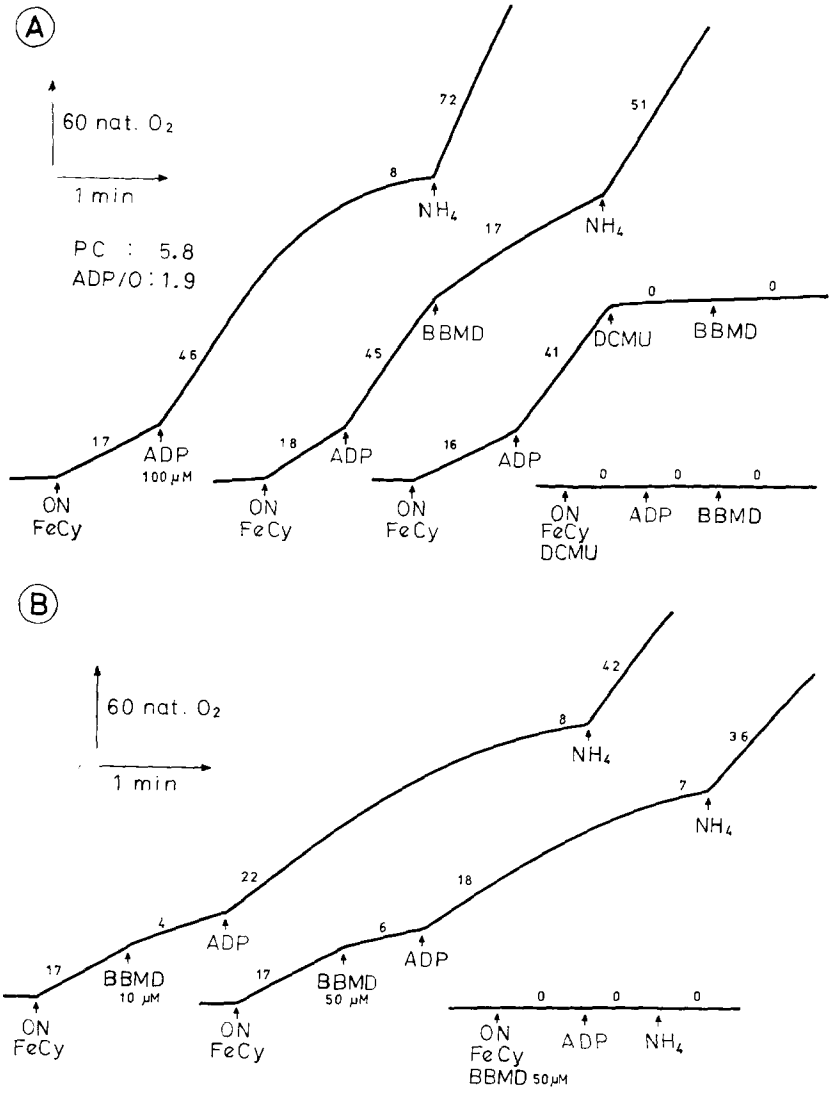

Fig. 1. Changes in the rate of O_2 evolution in spinach chloroplasts upon the addition of BBMD at different states of electron transport. Unless otherwise mentioned concentrations of ADP and BBMD were 200 and 50 μM, respectively. The numbers along the tracings indicate the O_2 evolution rate in μmol · mg chlorophyll^{-1} · h^{-1}. For other details see Materials and Methods.

tron transport rate was found to be about 15–20 %. However, the same sample when exposed to light after a period of darkness (30 s) showed complete loss of photosynthetic activity (data not shown). Pretreatment of chloroplasts with 50 μM BBMD completely abolished the O_2 evolving reaction and no activation was observed upon the addition of either ADP or NH_4Cl. No recovery of the photosynthetic O_2 evolution was observed in the chloroplasts treated with DCMU in the presence of BBMD. Fig. 1B also shows that adding BBMD simultaneously with ferricyanide when the

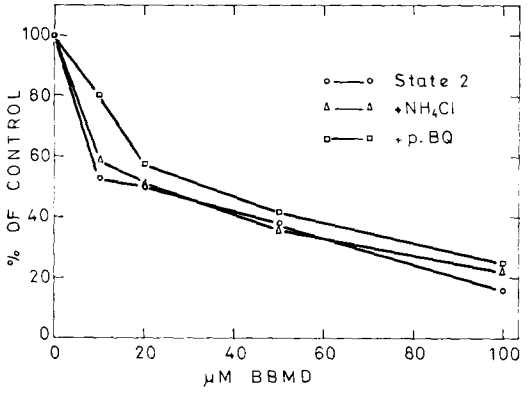

Fig. 2. Effect of BBMD at different concentrations on the level of State 2, NH_4Cl uncoupled electron transport, and the benzoquinone catalyzed Hill reaction in spinach chloroplasts. Concentration of ferricyanide and benzoquinone was 3 mM. The 100 % values were: state 2, 17; uncoupled electron transport, 72 and benzoquinone Hill reaction, 63 μmol $O_2 \cdot$ mg chlorophyll$^{-1} \cdot h^{-1}$.

light is turned on completely inhibits O_2 evolution even upon the subsequent addition of ADP and NH_4Cl; this is in contrast to adding BBMD in States 2 or 3, 1 min or longer after the light has been turned on.

The decrease in the rate of electron transport due to ferricyanide was found to be not mainly due to an inhibitory action of BBMD at the level of phosphorylation since the Hill reaction mediated by benzoquinone which accepts electrons directly at the oxidizing site of the quencher in Photosytem II (based on fluorescence [7] and photosynthetic studies [8–10] in DBMIB-inhibited cells), also followed the same inhibition pattern as that observed with ferricyanide (Fig. 2).

Since all the electron transport rates showed parellel concentration dependent inhibition one could expect an inhibitory site for BBMD either in the water oxidizing reaction or in the reaction center of Photosystem II. To study this in detail, fluorescence induction kinetics were followed. Changes in the kinetics of fluorescence induction in chloroplasts upon treatment with different concentrations of BBMD are shown in Fig. 3A. In normal active chloroplasts excitation with blue light brought about a rapid increase in the fluorescence level (F_0) followed by a slower one which reached the maximum (F_α) level after about 1 s, which in turn quenches to a steady state (F_s) level; the quenching being an indication of the presence of an efficient oxidizing Photosystem I. Blocking the electron transport at the oxidizing site of quencher with DCMU resulted in a very rapid increase in the fluorescence level (Fig. 3A). Treatment of intact chloroplasts with BBMD showed a concentration dependent increase in the F_s level. Quenching of the fluorescence was completely abolished even after treatment with 5 μM BBMD. Maximum F_s level was reached with 50 μM and further increasing the concentration to 100 μm did not influence this level. Simultaneously a gradual reduction in the half rise time ($t_{\frac{1}{2}}$) for the sigmoidal O to P rise, due to a decrease in the level of reoxidation of the reduced quencher by Photosystem I, was also observed. Even after treatment with higher BBMD concentrations the fluorescence induction curve showed a sigmodial rise.

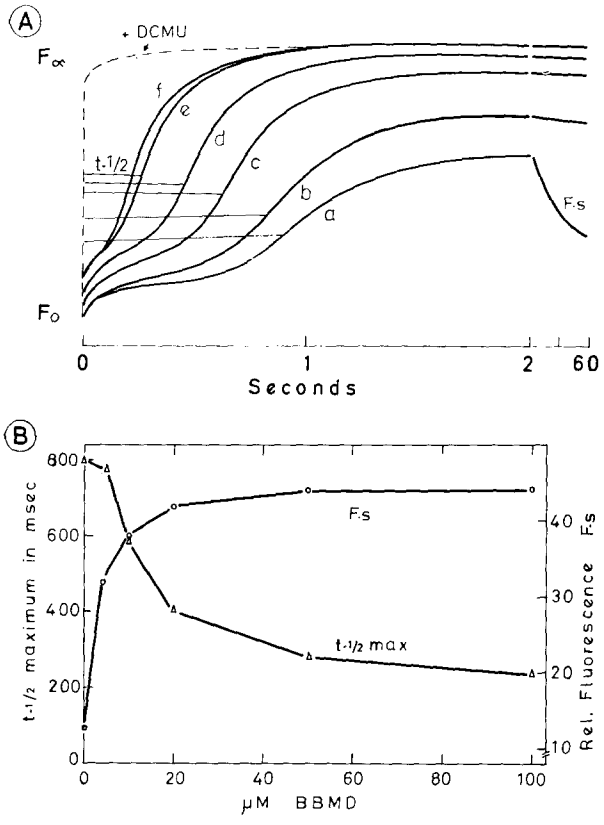

Fig. 3.A. Changes in the kinetics of the fluorescence induction in intact spinach chloroplasts on treatment with different concentrations of BBMD. a, control; b, c, d, e and f, respectively with 5, 10, 20, 50 and 100 μM BBMD. All additions were made just prior to the measurements. For other details see Materials and Methods. B. Changes in the level of steady state fluorescence (F_s) and half rise time ($t_{\frac{1}{2}}$ max) for the sigmoidal rise in fluorescence in intact spinach chloroplasts on treatment with different concentrations of BBMD. Levels of F_s were measured 60 s after onset of illumination and $t_{\frac{1}{2}}$ max as shown in Fig. 3A.

DISCUSSION

When BBMD is added to State 2 a subsequent phosphorylating State 3 could be induced by the addition of ADP (followed by a State 4) and also an NH_4Cl-uncoupled electron transport could be induced in the presence of BBMD, thus resembling in some respects the unusual action of phloridzin [11]. Such a process could be explained as a result of inhibition of the water oxidizing system by BBMD, thereby decreasing the net electron transport to the added acceptor. The previously proposed action of BBMD as a Photosystem II acceptor (at the oxidizing site of the quencher) may not be possible as is evidenced by the measurements of the benzoquinone-mediated reaction which itself accept electrons at the oxidizing site of the quencher [7, 8–10]. Also, no recovery of the O_2 evolving reaction upon the addition of BBMD was observed in DCMU-treated chloroplasts. Preincubation of the chloroplasts with

BBMD or addition of BBMD in the dark just before the onset of the illumination completely inactivated the photosynthetic O_2 evolution, which was not recovered by the addition of any electron acceptors. On the other hand, addition of BBMD at State 2 or 3 showed only partial inhibition. This could possibly be due to competitive behaviour of the photochemical processes to the added inhibitors. However, the same sample when exposed to the light after a short dark interval of about 30 s resulted in complete inactivation of the reaction. Similar partial and complete inhibition of the photosynthetic O_2 evolution upon the addition of 10^{-7} M DCMU, respectively, in the light and dark period was also observed under in vivo conditions in *Scenedesmus* cells (personal observation).

Further support for the action of BBMD as an inhibitor of the electron transport was obtained from the kinetic studies of fluorescence induction. BBMD treatment caused a concentration dependent increase in F_s level. Changes in the fluorescence kinetics from a sigmoidal to a non-sigmoidal pattern was reported to occur either when the electron transport between the two photosystems was blocked by inhibitors like DCMU and DBMIB [12–15] or under strong light intensities [16]. In both cases the steady state fluorescence would remain at a higher level. Since BBMD treatment completely abolished the fluorescence quenching it is possible to assume the presence of an additional inhibition site in the electron transport chain apart from the well demonstrated inhibitory site in the water oxidizing system. This secondary inhibitory site must be after the plastoquinone pool as evidenced by the observations of the sigmoidal fluorescence increase even after treatment with 100 μM BBMD, indicating the presence of a functional oxidized plastoquinone pool.

Based on the *P*-700 photooxidation reaction it was concluded [2] that BBMD acts an electron acceptor of Photosystem I. This may not be true under the condition of our measurements, since no quenching as is seen in the presence of methyl viologen [12] was observed with BBMD. The gradual decrease in the half rise time ($t_{\frac{1}{2}}$max) of the sigmoidal increase could be due to different levels of inhibition between the two photosystems.

This present work thus may provide a useful (albeit modified) method for the study of Photosystem II electron transport by eliminating Photosystem I and the water oxidizing system upon treatment with BBMD. This conclusion was drawn from (i) photosynthetic electron transport measurements and (ii) changes in the kinetics of the fluorescence induction. It also provides cautionary data on the use BBMD when it is added at different stages during the illumination of the chloroplast reaction mixtures. It also substantiates the cautionary note of Wolff et al. [2] that BBMD is not a specific modifier. This restricts the applicability of BBMD in photosynthesis research.

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