THE EFFECTS OF DIBROMOTHYMOQUINONE ON FLUORESCENCE AND ELECTRON TRANSPORT OF SPINACH CHLOROPLASTS

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1. Introduction

Trebs et al. [1] reported that dibromothymoquinone, i.e., 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), inhibited electron transport between Photosystem II and Photosystem I in chloroplasts by acting as an antagonist of plastoquinone. The inhibitor blocked electron transport so that the two photosystems could not function in series but each could function separately. Water could serve as an electron donor to Photosystem II acceptors and reduced DCPIP** could serve as an electron donor to Photosystem I acceptors but water could not supply electrons to Photosystem I acceptors. Böhme et al. [2] in a continuation of that study concluded that the inhibitor blocked electron transport on the Photosystem I side of plastoquinone and Böhme and Cramer [3] presented further supportive evidence by showing that DBMIB blocked the reduction of cytochrome *f* by Photosystem II.

The experiments reported here were undertaken to obtain additional evidence for the site of inhibition by DBMIB. The fluorescence induction curve can be interpreted to indicate the size of the pool of reducible electron carriers [4], largely plastoquinone [5], that equilibrates with Q, the primary electron acceptor and fluorescence quencher of Photosystem II. The known electron carriers between plastoquinone and Photosystem I, i.e., cyt *f* and P_{700}, are normally in the reduced state in the dark and do not contribute to the fluorescence induction kinetics. If electron transport were blocked by an inhibitor so that the pool of secondary electron acceptors for Photosystem II were decreased, the area above the fluorescence induction curve should decrease proportionately [4], and the site of inhibition would be localized. The interpretation of the experiments reported was complicated by the finding that the oxidized form of DBMIB quenched fluorescence and that it also functioned as an electron acceptor for Photosystem II. However, the reduced form of DBMIB, which does not quench fluorescence, also inhibits electron transport to Photosystem I. In the latter case, the reduced form of DBMIB has essentially no influence on the fluorescence induction kinetics, thus confirming that the site of inhibition is on the Photosystem I side of plastoquinone.

2. Materials and methods

Spinach chloroplasts were prepared as described previously [6] and suspended in a medium containing 15 mM Tris-HCl buffer (pH 7.8), 20 mM NaCl, and 4 mM $MgCl_2$. Stock solutions of DBMIB and DCMU (gifts from Professor Achim Trebst and Dr. P.G. Heytler, respectively) in methanol were prepared at appropriate concentrations such that the final methanol concentration never exceeded 1% in the chloroplasts.

Fluorescence yield measurements (with a weak chopped measuring beam) were made at room temperature by methods described previously [6]. Fluorescence induction experiments were conducted at room temperature with the chloroplast suspension in a 1 × 1 cm cuvette. The exciting light source consisted of a tungsten lamp with 4 cm 5% $CuSO_4 \cdot 5H_2O$, a Corning 3-69 filter and a Rohm and Haas green plastic

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** Abbreviations: DCPIP, 2,6-dichlorophenol-indophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

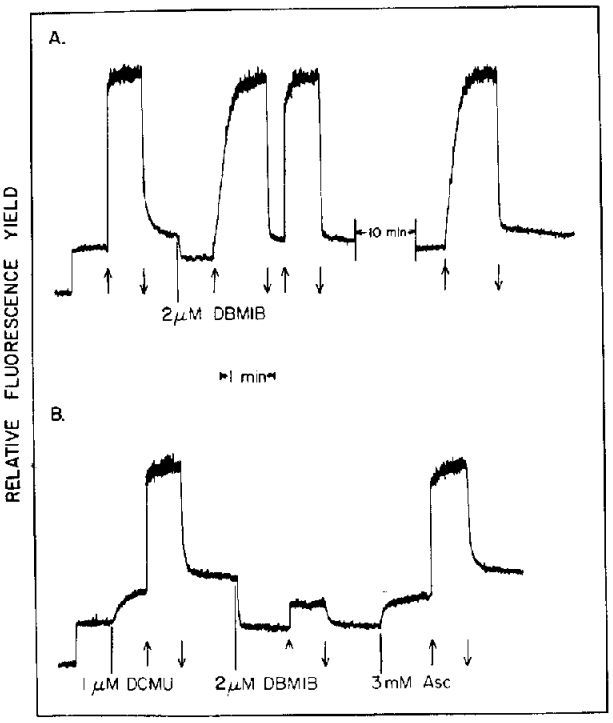

Fig. 1. Fluorescence yield changes of chloroplasts (6 μg chlorophyll per ml) induced by actinic light (645 nm, 7×10^4 ergs cm^{-2} sec^{-1}; on at upward arrows and off at downward arrows) or inhibitors as indicated. Fluorescence was excited by a weak chopped measuring beam (659 nm, 14 ergs cm^{-2} sec^{-1}, 300 Hz). A) Effect of DBMIB and its reversal by light. B) Effect of DBMIB in the presence of DCMU and its reversal by ascorbate.

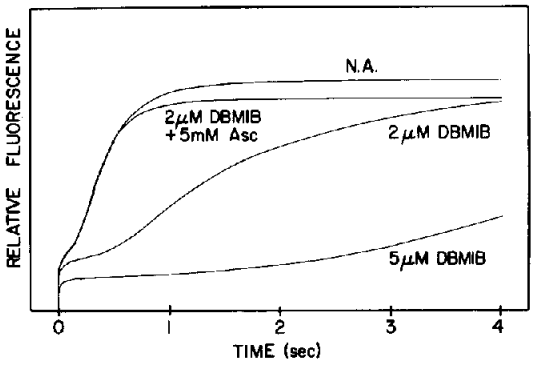

Fig. 2. Fluorescence induction of chloroplasts (33 μg chlorophyll/ml) with no additions (NA), 2 μM DBMIB and 5 mM ascorbate, 2 μM DBMIB or 5 μM DBMIB as indicated. Green exciting light (5×10^4 ergs $cm^{-2}sec^{-1}$) admitted at time 0.

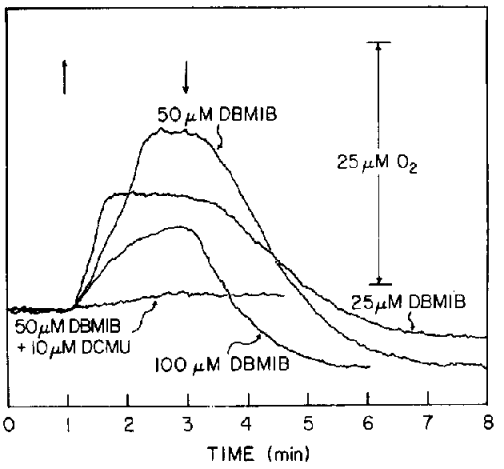

Fig. 3. Oxygen exchange of chloroplasts (30 μg chlorophyll/ml) with DBMIB and DCMU as indicated. White actinic light (5×10^5 ergs $cm^{-2}sec^{-1}$) was turned on at upward arrow and off at downward arrow.

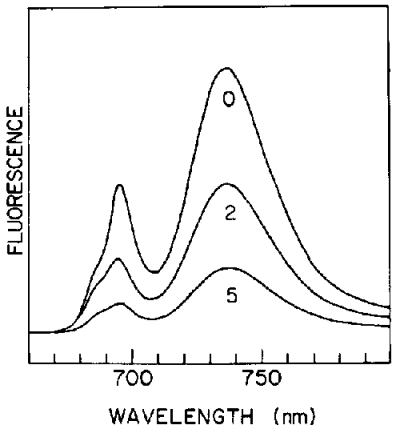

Fig. 4. Fluorescence emission spectra of chloroplasts (32 μg chlorophyll/ml) at 77°K. Samples were frozen with liquid nitrogen in the dark in the presence of DBMIB concentrations (μM) indicated under each curve. Green exciting light, intensity was 3×10^4 ergs $cm^{-2}sec^{-1}$.

filter. The filter combination had maximum transmission at 534 nm and a half band width of 46 nm. Fluorescence was detected perpendicular to the exciting beam with an EMI 9558C photomultiplier blocked with a Corning 2-64 filter. The photocurrent was amplified, then recorded by a Fabri-Tek 1072 computer. The time base scan of the computer was triggered by a photocell so that it started at the same time that the shutter on the exciting light source opened. For fluorescence emission spectra, the chloroplast suspension in a 4 × 10 mm cuvette was cooled to 77°K in the dark before admitting the exciting beam. The fluorescence was dispersed with a 500 mm Bausch and Lomb monochromator (half band width 5 nm) and detected with a Fairchild 6911 photomultiplier blocked with a Corning 2-58 filter. The photocurrent was amplified and plotted versus wavelength on an X-Y recorder. The spectra were not corrected for the spectral response of the instrument.

Oxygen exchange was measured with a Clark oxygen electrode (Yellow Springs Instrument Co.). The actinic light source consisted of a tungsten lamp and 7 cm of 1% $CuCO_4 \cdot 5H_2O$. Light intensities were measured with a Kettering radiometer.

3. Results and discussion

The effect of DBMIB on the fluorescence yield of chloroplasts is shown in fig. 1. As nomenclature we shall refer to the minimum fluorescence yield of dark adapted chloroplasts as F_0, the yield in the weak measuring light as F_m, and the maximal yield obtained in strong actinic light (in the absence of an electron acceptor) as F_M. Normal fluorescence yield changes are shown in the first part of curve A. Addition of 2 μM DBMIB causes a lowering of the fluorescence yield in the measuring light due to a quenching of the F_0 level. On turning on the actinic light the fluorescence yield increases and finally reaches the same F_M level as the control but the rate of increase from F_m to F_M is much slower in the presence of DBMIB. On turning off the actinic light the fluorescence yield returns to approximately the same F_m level obtained in the absence of DBMIB. On turning on the actinic light again after a short dark period (1 min or less) the fluorescence yield rises rapidly to the F_M level. After a 10 min dark period, however, the F_m level is lower and the rate of increase to F_M is again slow.

The effect of adding DBMIB to the chloroplasts in the presence of DCMU is shown in curve B of fig. 1. The normal light-induced fluorescence yield changes in the presence of DCMU are shown in the first of that curve. Addition of 1 μM DCMU causes F_m to increase (reduction of Q by the weak measuring light is facilitated when electron transport out of Q is clocked by DCMU) to a level which depends on the intensity of the measuring beam. Irradiation with actinic light causes a further increase to the F_M level. Subsequent addition of 2 μM DBMIB markedly quenches both of the F_m and F_M levels. That quenching effect is eliminated by ascorbate.

The kinetics of the fluorescence induction curves are shown in fig. 2. The fluorescence induction period is prolonged in the presence of DBMIB as if the inhibitor also functioned as an electron acceptor. The effect of DBMIB on the fluorescence induction curve is largely eliminated by adding ascorbate which reduces DBMIB and thus prevents its action as an electron acceptor. Only the oxidized form of DBMIB quenches fluorescence and that quenching is relieved when the inhibitor is reduced, either photochemically in a Hill reaction or chemically by adding ascorbate.

The activity of DBMIB as an electron acceptor is confirmed by oxygen measurements (fig. 3). Addition of 25 μM DBMIB supports the evolution of 12 μM oxygen, the expected stoichiometry for a two-electron reductant. Addition of larger concentrations of DBMIB appear to inhibit the Hill reaction. The oxygen measurements also show that the photoreduced DBMIB is slowly autooxidized in the dark, which explains the observation in fig. 1A that an appreciable dark period was required between two irradiation periods to obtain the prolonged fluorescence induction at the second irradiation.

The autooxidation of DBMIB suggests that the compound, acting as an autooxidizable electron acceptor, might shunt electrons to oxygen, thereby preventing electron transport to Photosystem I. However, the rate of autooxidation of photoreduced DBMIB is too slow to account for such a mechanism of inhibition. Such a mechanism was also precluded by the observation that the reduced form of DBMIB inhibits photoreduction of Photosystem I acceptors as well as the oxidized form [2]. We also found that the photoreduction of NADP under anaerobic conditions was

inhibited by 2 μM DBMIB in the presence or absence of excess ascorbate. The observations that reduced DBMIB inhibits electron transport between Photosystem II and I and that it does not alter the fluorescence induction kinetics confirms that the inhibitor (at least in its reduced form) acts on the Photosystem I side of plastoquinone.

The quenching of chlorophyll fluorescence by the oxidized forms of various quinones has been reported previously [7]. DBMIB, however, is a much more effective quencher than any of the quinones studied previously. The observation that DBMIB quenched the F_0 level as well as the F_m level suggested that both Photosystem I and Photosystem II fluorescence were quenched. This is confirmed in low temperature fluorescence spectra (fig. 4) which show that low concentrations of DBMIB quench the 730 nm emission band of Photosystem I as well as the 685 and 695 nm emission bands of Photosystem II. The fluorescence quenching imposed by DBMIB at low temperature is not relieved by irradiation. (The quenching by DBMIB is similar to that observed previously [8] with hexane extracted chloroplasts that had been reconstituted with plastoquinone A).

Acknowledgements

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