Differential inhibition by plastoquinone analogues of photoreduction of cytochrome *b*-559 in chloroplasts

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The high-potential form of cytochrome *b*-559 (*b*-559 HP) is closely linked to the oxygenic photosystem (photosystem II) but its relation to other redox components of the photosynthetic apparatus, including plastoquinone, is still obscure. We investigated the photoreduction of cytochrome *b*-559 HP by isolated chloroplasts in the presence of 3 antagonists of plastoquinone, of which, DBMIB (dibromothymoquinone) and DNP-INT (dinitrophenyl ether of iodonitrothymol) are known to inhibit the oxidation of the plastoquinone pool (PQ) by the FeS–cytochrome f/b_6 complex and one, UHDBT (5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole) is known to inhibit the reduction of PQ by Q_B. Q_B is a protein-bound plastoquinone that serves as a two-electron gate for the reduction of PQ. We found that DBMIB and DNP-INT did not inhibit but low concentrations of UHDBT severely inhibited the photoreduction of cytochrome *b*-559 HP. These results suggest that the electron donor for the reduction of cytochrome *b*-559 HP was either Q_B or a portion of the PQ pool that was oxidized by a new pathway free of binding sites for DBMIB and DNP-INT.

Photosynthesis *Plastoquinone* *Electron transport* *Photosystem II*

1. INTRODUCTION

Plastoquinone antagonists are among the most useful tools for the study of the role of plastoquinone (PQ) in photosynthetic electron transport [1]. They inhibit both oxidation and reduction of PQ, the most abundant redox component of chloroplasts [2,3]. Low concentrations of UHDBT ($< 1\ \mu M$) inhibit at the site of PQ reduction [4–6], whereas DBMIB and DNP-INT inhibit at the site of PQ oxidation [1,7,8]. At higher concentrations, these effects are reversed: UHDBT inhibits the oxidation of PQ [4,6–8] and DBMIB inhibits the reduction of PQ [1].

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Abbreviations: PS, photosystem; PQ, plastoquinone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4-7-dioxobenzothiazole; diuron (DCMU), 3-(3,4-dichlorophenyl)-1,1-dimethylurea

These 3 antagonists were used here to probe the interaction of PQ with the high-potential form of cytochrome *b*-559 (*b*-559 HP) ($E_{m7} \sim +350$ mV [9,10]) under conditions when no electron donor other than water was provided. Numerous investigations have amply documented the close association of cytochrome *b*-559 HP with the oxygenic (water-oxidizing) photosystem (PS II) but the role of this cytochrome and its interaction with PQ are still not settled (reviews, [11,12]).

Different kinds of plastoquinone are associated with the oxygenic photosystem. Q_A (Q) is a special plastoquinone bound to the oxygenic photocenter where it undergoes only one-electron reduction to plastosemiquinone anion which in turn reduces Q_B. Q_B is a protein-bound plastoquinone that serves as a two-electron gate for the reduction of the PQ pool which is the carrier of reducing equivalents generated by PS II (review [13]). An alternative view is that Q_B is not bound but represents diffusible PQ molecules from the pool that undergo reduction and oxidation by alternatively attaching themselves to a PQ-reducing site

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on the Q_B protein and a PQH_2-oxidizing site at the FeS–cytochrome f/b_6 complex [14].

We report here that DBMIB and DNP-INT did not inhibit, but low concentrations of UHDBT severely inhibited the photoreduction of cytochrome *b*-559 HP in a manner similar to that of diuron (DCMU). These results suggest that the light-induced reduction of cytochrome *b*-559 HP did not involve the recognized pathway of plastoquinol oxidation via the Rieske iron-sulfur center in the cytochrome f/b_6 complex [7,8]. It appears that the electron donor for the reduction of cytochrome *b*-559 HP was either Q_B or a portion of the plastoquinone pool that was being oxidized by a new pathway free of binding sites for DBMIB and DNP-INT.

2. MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves (*Spinacia oleracea*, var. Marathon) grown in a greenhouse in nutrient solution culture [15] and freshly harvested before each experiment. The isolated chloroplasts were osmotically shocked ('broken') to give a preparation that retained the integrity of the thylakoid membrane structure needed for complete electron transport from water to $NADP^+$ [16]. Chlorophyll was estimated as in [15]. DBMIB, DNP-INT and UHDBT were dissolved in dimethyl sulfoxide; the final solvent concentration did not exceed 1%.

A dual-wavelength spectrophotometer (Aminco, model DW-2) was used in the split-beam mode to measure chemically induced absorbance changes of cytochromes and in the dual-wavelength mode to measure light-induced absorbance changes. Samples were illuminated with a red light beam of 663 nm (1.3×10^4 erg $\cdot$ cm^{-2} $\cdot$ s^{-1}) isolated from a 150 W tungsten-halogen lamp. Type FCS with an interference filter (Baird Atomic) while two blue filters (Corning, 4–96) were used to shield the phototube.

3. RESULTS

In freshly isolated chloroplasts both of their high potential (hydroquinone-reducible) cytochromes, cytochrome *b*-559 HP and cytochrome *f* (λ_{max} = 554 nm) are already in the reduced state [11]. In order to measure the photoreduction of cytochrome *b*-559 HP, the high potential cytochromes were preoxidized chemically in the dark by low concentrations of potassium ferricyanide. The design was to oxidize the cytochromes without leaving in the reaction mixture sufficient excess of ferricyanide to serve as an effective acceptor for a noncyclic electron flow from water.

When freshly isolated chloroplasts (100 μg chl/ml) were titrated with potassium ferricyanide (FeCy) a concentration of about 10 μM FeCy was usually needed to oxidize fully the reduced cytochromes (fig.1, upper trace). That both cyt *b*-559 HP and cyt *f* were fully reduced in the chloroplasts as isolated and were subsequently fully oxidized by ferricyanide is evident from the complete reduction by hydroquinone (HQ) of the ferricyanide-oxidized cytochromes (fig.1, lower trace) and by the similarity of the absorbance and peak wavelengths in the upper and lower spectra.

Fig.2 shows the effect of the 3 plastoquinone analogues and of diuron the light-induced increase in absorbance at 559 nm which denotes reduction of cytochrome *b*-559 HP. Each of the inhibitors

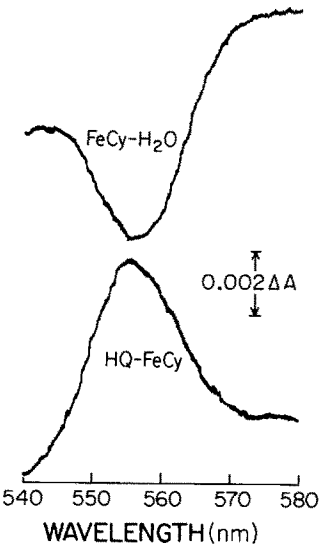


Fig.1. Oxidation-reduction of hydroquinone reducible cytochromes. The reaction mixtures in cuvettes (1.0 cm light path) containined spinach thylakoids equivalent to 100 μg chlorophyll/ml, 50 mM Tricine-KOH, pH 8.2, 10 mM $MgCl_2$ and where indicated, 10 μM potassium ferricyanide (FeCy) and 400 μM hydroquinone (HQ).

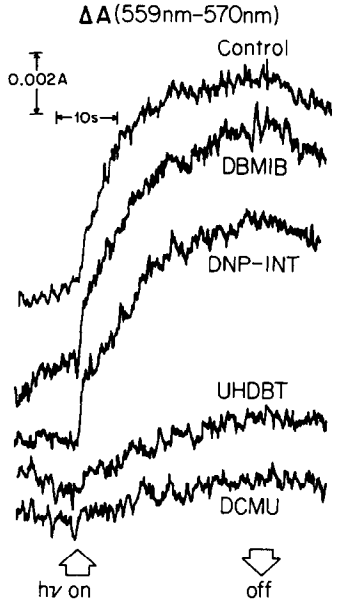


Fig.2. Effect of inhibitors on photoinduced absorbance changes at 559 nm. Reference wavelength, 570 nm. The reaction mixtures in cuvettes (1.0 cm light path) contained spinach thylakoids equivalent to 100 μg chlorophyll/ml, 50 mM Tricine-KOH, pH 8.2, 10 mM $MgCl_2$, 5 mM ADP, 5 mM K_2HPO_4, 10.0 μM potassium ferricyanide and where indicated, 1.0 μM DBMIB, 10.0 μM DNP-INT, 0.5 μM UHDBT or 1.0 μM DCMU.

was added at a concentration that effectively inhibited the oxygenic photoreduction of $NADP^+$.

As indicated by the traces, UHDBT severely inhibited the photoreduction of cytochrome *b*-559 HP whereas DBMIB and DNP-INT had hardly any effect (fig.2). The severe inhibition by UHDBT was similar to that by diuron which is also known to inhibit at the Q_B protein site [1]. In the treatments in which the cytochrome was reduced in the light, it remained in the reduced state after the light was turned off (fig.2), thereby confirming that no excess ferricyanide was present in the reaction mixture.

The spectra of the light-induced absorbance changes in the presence and absence of UHDBT are shown in fig.3. UHDBT gave virtually a total inhibition of the photoreduction of cytochrome *b*-559 HP. By blocking the outflow of electrons from Q_B, UHDBT also inhibited the photoreduction of other redox components, including that of cytochrome *f*.

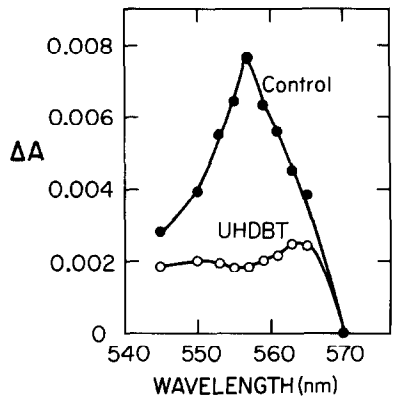


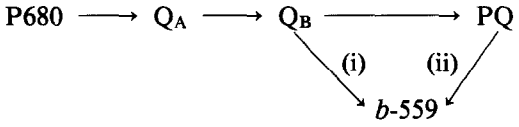
Fig.3. Effect of UHDBT on oxygenic photoreduction of cytochromes. Experimental conditions were as in fig.2 except that no inhibitors were added to the control and 0.5 μM UHDBT was present where indicated. Reference wavelength, 570 nm.

4. DISCUSSION

The 3 plastoquinone antagonists used here, DBMIB, DNP-INT and UHDBT have, in other investigations, yielded support for the conclusion that the oxidation of plastohydroquinone is catalyzed by the FeS-cytochrome f/b_6 complex via its Rieske iron-sulfur center [7,8,13,17]. There is now extensive evidence that the FeS-cytochrome f/b_6 complex of chloroplasts is analogous to membrane-bound redox centers in mitochondria and bacteria that comprise cytochromes *b* and c_1 and a high-potential (Rieske) FeS protein and function as ubiquinol-cytochrome *c* or plastoquinol-plastocyanin oxidoreductases (reviews [8,13].

Here, the inhibition of photoreduction of cytochrome *b*-559 HP by low concentrations of UHDBT (figs 2 and 3) suggests that plastohydroquinone was the electron donor, in apparent agreement with earlier conclusions [18,19] that cytochrome *b*-559 HP (in competition with cytochrome *f*) is reduced by electrons from the plastoquinone pool. However, the reduction of cytochrome *b*-559 HP was not inhibited by DBMIB and DNP-INT (fig.2), the inhibitors that block the oxidation of the plastoquinone pool by the FeS-cytochrome-f/b_6 complex [8]. It appears therefore that the reduction of cytochrome *b*-559 involved a pathway different from that of

cytochrome *f* reduction. Since UHDBT at low concentrations interacts with the Q_B protein [4–6], it is possible that the Q_B plastohydroquinone was the reductant. Alternatively, cytochrome *b*-559 could have been reduced by a portion of the plastohydroquinone pool that was being oxidized by a totally new pathway lacking binding sites for DBMIB and DNP-INT. Further evidence bearing on these possibilities represented schematically below as (i) and (ii), will be sought in other studies.



Attempts to assign within the conceptual framework of the Z scheme a role for cytochrome *b*-559 HP in the noncyclic electron flow from water to ferredoxin-$NADP^+$ or on a branch pathway tied to PS II have not been successful (reviews 11,12). We have postulated [20] that cytochrome *b*-559 HP is a redox carrier in a PS II-linked cyclic electron transport pathway, which functions to facilitate the transport of protons liberated inside the thylakoid membrane by photooxidation of water. The possible relevance of the present findings to this postulation will be considered separately.

ACKNOWLEDGEMENTS

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