CAROTENOID AND CYTOCHROME *b* 559 REACTIONS IN PHOTOSYSTEM II IN THE PRESENCE OF TETRAPHENYLBORON

B. R. VELTHUYS

Martin Marietta Laboratories, 1450 South Rolling Road, Baltimore, MD 21227, USA

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1. Introduction

The reagent tetraphenylboron (ϕ_4B^-) is known to affect chloroplast reactions in at least three ways (e.g., [1–3]).

(1) It causes the photogenerated transmembrane electrical field to collapse within microseconds, presumably by the ready diffusion of its anion through the membrane ([2], cf. [4]);

(2) It acts as an artificial electron donor to PS II [1,2,5], an effect that is rather poorly understood; it has yet to be reconciled with the electrochemical and spectroscopic evidence indicating that ϕ_4B^- is a 2-electron donor, which is transformed according to the relation [5–7]:

$$\phi_4B^- \rightarrow \phi_2 + \phi_2B^+ + 2e^-$$

presumably followed by:

$$2\,H_2O + \phi_2B^+ \rightarrow \phi_2BO^- + 2H^+$$

(3) It stimulates cytochrome *b* oxidation [3].

Here, I describe measurements of flash-illuminated chloroplasts that indicate that the donor side of PS II acts, as required, as a 2-electron acceptor in the oxidation of ϕ_4B^- (oxidation does not occur with every flash). The reactions involve cytochrome *b* 559, which appears to be alternately oxidized and reduced by PS II in the presence of ϕ_4B^-, and a carotenoid. The carotenoid is appreciably oxidized during the reduction of cytochrome *b* 559.

2. Materials and methods

Techniques and procedures were described in [8,9]. ϕ_4B^- was added as a 1 mM solution of the sodium salt in water to 40 μM final conc., the saturating concentration for our investigations. The accelerated decay of electrochromic absorbance changes due to ϕ_4B^- [2] interferes with some of the measurements. Where significant, the degree of interference is indicated (cf. fig.6). (Interference in the measurements for fig.1 and 4 was prevented by the use of gramicidin D (plus Na^+).) The extent of the cytochrome *b* 559 redox changes in the experiments of fig.1 and 3 was calculated by assuming a reduced-minus-oxidized extinction coefficient of 15 $mM^{-1} \cdot cm^{-1}$ for the wavelength pair 559–570 nm [10].

3. Results and interpretation

3.1. *Cytochrome* b *559 oxidation by PS II*

As noted, ϕ_4B^- stimulates the oxidation of cytochrome *b* 559 by PS II [3]. This effect is quite dramatic (fig.1). Dark-adapted chloroplasts were incubated for a short time with ϕ_4B^-, followed by an illumination with a single-turnover flash. The flash-induced absorbance changes were measured in a narrow range around 555 nm and corrected for absorbance changes observed in parallel measurements without ϕ_4B^-. The bottom of the curve, close to 559 nm, indicates bleaching due to the oxidation of cytochrome *b* 559. The amplitude of the change (40 ms after the flash; squares) corresponds to ~0.75 molecules of cytochrome *b* 559 oxidized per 400 molecules of chlorophyll. At 2 ms after the flash (○), the oxidation is more than half complete (cf. fig.1, □). [More detailed measurements (not shown) indicate the kinetics to be biphasic, with about equal contributions of phases with half-times of ~30 μs and ~4 ms.]

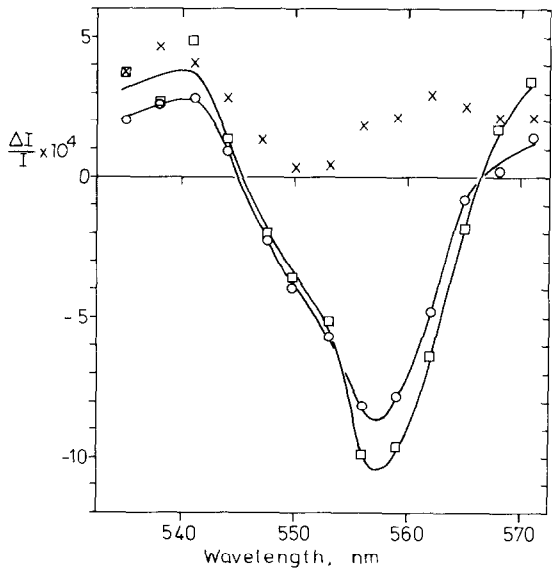


Fig.1. Time-resolved spectra of the flash-induced absorbance change at 2 ms (○) and 40 ms (□), in the presence of ϕ_4B^-. The ϕ_4B^- (40 μM) was added to dark-adapted chloroplasts (100 μg chl/ml) 30 s before the flash. The flash-induced absorbance change in a parallel measurement without ϕ_4B^- has been subtracted. The background change at 2 ms is shown separately (×). The reaction mixture contained 10 mM NaCl, 4 mM Hepes–NaOH (pH 7.5), and 2 μM gramicidin D; measuring beam half-bandwidth, 3.2 nm; optical pathlength, 2 mm.

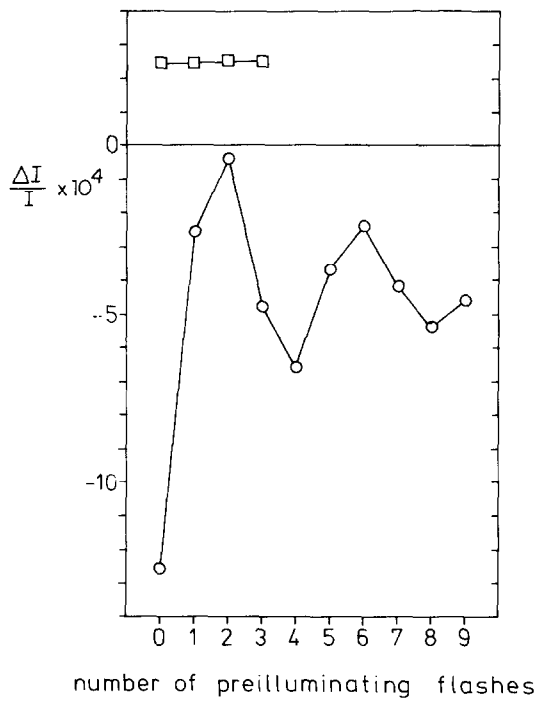


Fig.2. Pre-illumination dependence of the flash-induced absorbance change in the presence of ϕ_4B^-. The ϕ_4B^- was added 10 s after pre-illumination with a series of 0–9 flashes (intervals, 1 s). The reaction mixture was as for fig.1, but contained no gramicidin D. The absorbance change was measured 10 ms after the flash at 559 nm (○). Blank measurements (without ϕ_4B^-) were not subtracted, and are presented separately (□).

The role of PS II in the reactions is indicated in fig.2. In this experiment, the chloroplasts were pre-illuminated with a variable number of flashes just before the addition of ϕ_4B^-. Another flash was then given, and its effect upon cytochrome *b* was monitored. Fig.2 shows that the flash-induced oxidation diminishes after pre-illumination, and that the extent of the decrease depends on the number of pre-flashes with a periodicity of 4. This results is readily explained in terms of the well-known 'S-state' oscillation of PS II [11]. Presumably, cytochrome *b* 559 is immediately oxidized upon additions of ϕ_4B^- when the donor side of PS II is in the oxidation states S_2 or S_3 (predominant after 1 or 2 flashes), but not when PS II is in states S_0 or S_1 (predominant after dark-adaption or after 3 or 4 flashes). The dark oxidation precludes oxidation of cytochrome *b* 559 by the subsequent flash.

3.2. *Oxidant-induced cytochrome* b *559 reduction*

Since cytochrome *b* 559 can be pre-oxidized by incubation with ferricyanide in the dark (e.g., [12]) we should be able to use this means of thwarting the oxidizing effect of a flash. In fact, we found an additional phenomenon. Fig.3 shows the effect of ferricyanide on the flash-induced change in the cytochrome *b* 559 redox state in the presence of ϕ_4B^-. At increasing incubation time and concentration, the flash-induced oxidation of cytochrome *b* 559 was not simply eliminated, but replaced by a flash-induced reduction. The spectrum of the flash-induced absorbance change (fig.4) confirms this observation. The amplitude of the reduction is similar to that of the oxidation shown in fig.1, i.e., ~0.8 molecules of cytochrome *b* 559 per 400 molecules of chlorophyll. In addition, the reduction is also about as rapid as the oxidation, i.e., half complete within 2 ms. (More detailed measurements (not shown) indicate biphasic reduction kinetics. The slow phase and the second phase of the oxidation have similar half-times, i.e., 4 or 5 ms. The half-time of the first phase is of the order of 0.3 ms.)

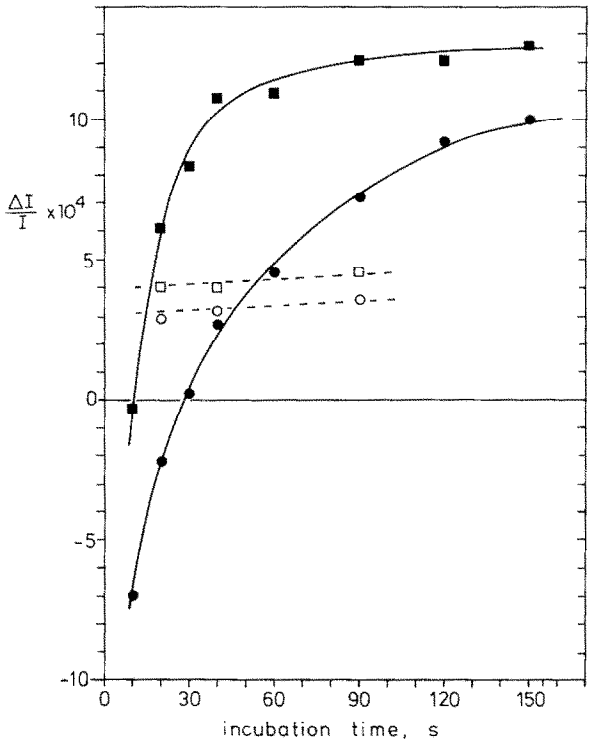
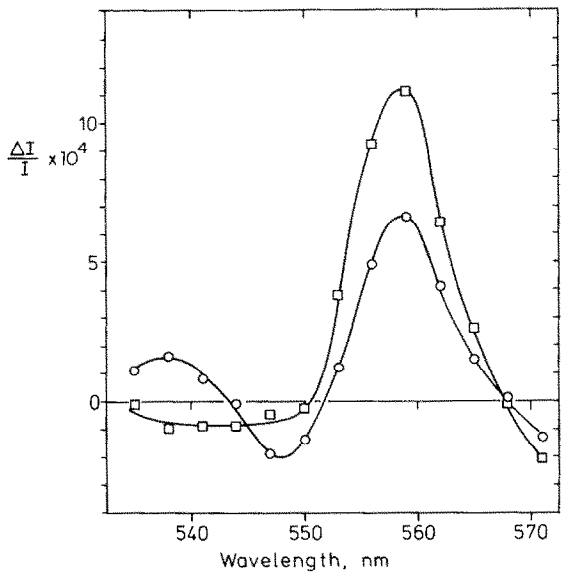


Fig.3. Ferricyanide effect upon the flash-induced absorbance change in the presence of ϕ_4B^-. Ferricyanide, 50 μM (•) or 200 μM (▪), was added together with 40 μM ϕ_4B^- to dark-adapted chloroplasts (80 μg chl/ml). The flash-induced absorbance change was measured after various times. The reaction mixture contained 200 mM sucrose; detecting time, 20 ms; wavelength, 559 nm; (○,□) blank measurements (without ϕ_4B^-).



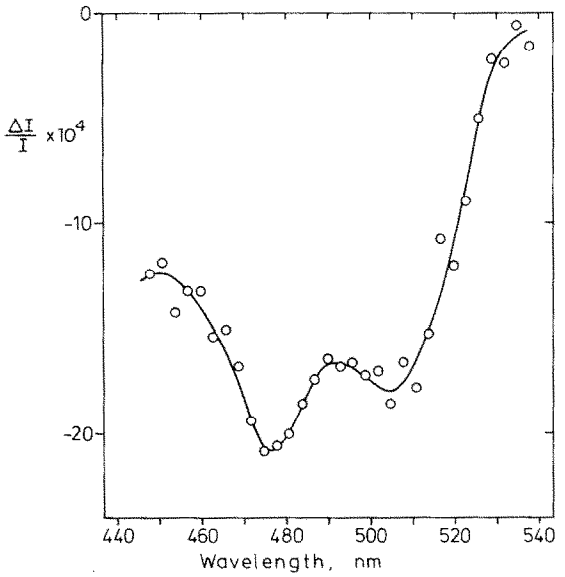


Fig.5. Spectrum of a transient flash-induced absorbance change in the presence of 40 μM ϕ_4B^- and 0.5 mM ferricyanide. The data points represent the absorbance change at 200 μs after the flash minus the absorbance change at 2 ms. Conditions were similar to those in fig.4.

The observed reduction and oxidation of cytochrome *b* 559 are two alternative results of the reaction of ϕ_4B^- with PS II. Since the oxidation is induced, as demonstrated, by the oxidizing side of PS II, we may expect the same to be true for the reduction. Indeed, the reduction, as the oxidation, is not inhibited by diuron, an inhibitor of secondary electron flow at the reducing side of PS II (not shown). We conclude that 'oxidant-induced' reduction occurs here (a phenomenon not uncommon for *b* cytochromes (e.g., [8])) i.e., a pair of 1-equivalent acceptors (cytochrome *b* 559 and a photogenerated oxidant) is reduced in parallel by a 2-equivalent donor (ϕ_4B^-).

3.3. *Carotenoid oxidation and reduction with* ϕ_4B^-

Another novel reaction observed in chloroplasts in the presence of ϕ_4B^- is a short-lived bleaching with a spectrum (fig.5) characteristic of carotenoid (e.g.,

Fig.4. Time-resolved spectra of the flash-induced absorbance change at 2 ms (○) and 40 ms (□), in the presence of ϕ_4B^- and ferricyanide. Same conditions (also same day, same chloroplast suspension) as in fig.1, except that 1 mM ferricyanide was added 30 s before the flash.

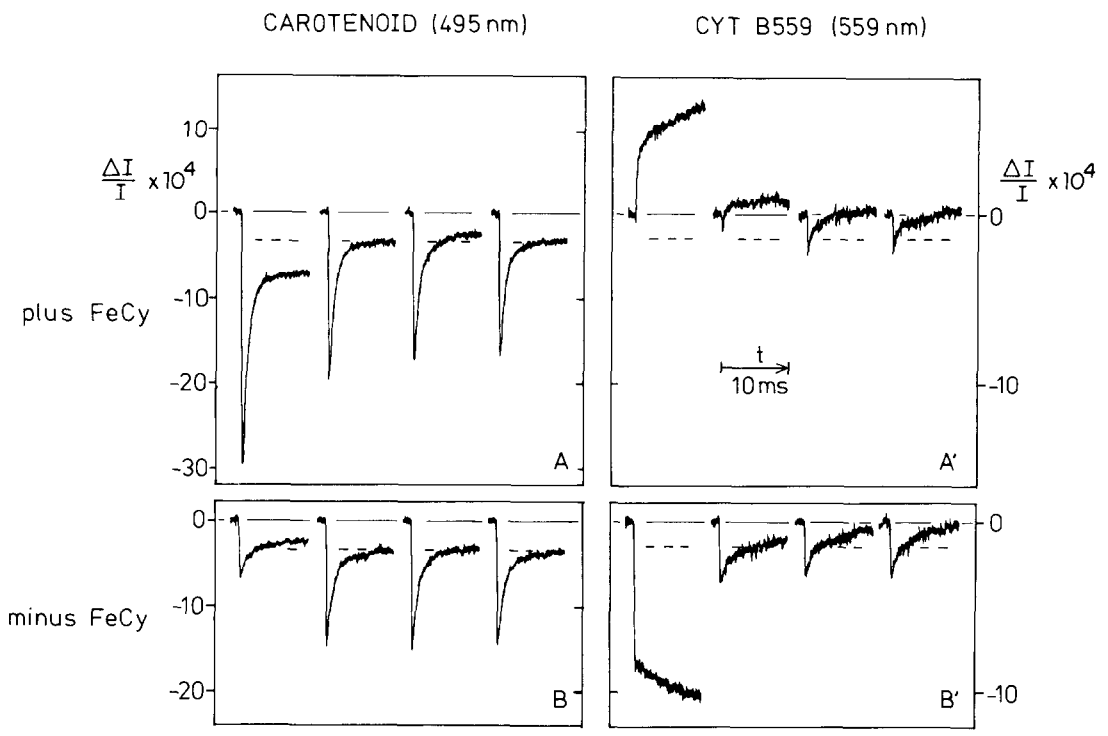


Fig.6. Parallel measurements of the carotenoid bleaching (A,B) and the cytochrome *b* 559 redox changes (A',B') during the first 10 ms after each of a series of 4 flashes in the presence of ϕ_4B^-. Incubation time was 120 s for measurements A and A' with 100 μM ferricyanide. Incubation time was 20 s for measurements B and B' (no ferricyanide). Carotenoid was measured at 495 nm, cytochrome *b* 559 at 559 nm. Traces measured without ϕ_4B^- have been subtracted; flash intervals, 1.2 s; electronic time response, 0.1 ms. The expected post-flash baseline is displaced because of the electrochromic changes abolished by ϕ_4B^-, and is indicated by a broken line.

[13]). The kinetics (not illustrated in detail; see fig.6) indicate generation and decay half-times of ~15 μs and ~1 ms, respectively. The maximal absorbance change observed (fig.6A, first flash) corresponds to a bleaching of ~0.15 molecules of carotenoid per 400 molecules of chlorophyll (assuming a carotenoid extinction coefficient at 495 nm of ~250 $mM^{-1} . cm^{-1}$ [13] and a differential flattening factor at 495 nm of 1.85 [14]).

The carotenoid and cytochrome *b* 559 reactions are compared in fig.6. Fig.6A,B shows measurements of the carotenoid bleaching and fig.6A',B' shows the cytochrome *b* 559 redox changes after a short series of flashes, with (fig.6A,A') and without (fig.6B,B') preincubation with ferricyanide. Comparing the results under both conditions, especially the effect of the first flash, we note that a large carotenoid transient is associated with the reduction of cytochrome *b* 559, but not with its oxidation. Another significant feature of the figure is the similarity of each reaction under both conditions from the second flash on: the (apparent) absence of cytochrome *b* 559 redox changes, and the presence of carotenoid transients, which are about half as large as that for the first flash after ferricyanide preincubation. It thus seems likely that, with each flash, ~50% of the centers reduce cytochrome *b* 559 and ~50% oxidize the cytochrome under 'steady-state' conditions of ϕ_4B^- oxidation.

4. Discussion

ϕ_4B^- is unusual in that it is a lipid-soluble anion. Thus, ϕ_4B^- is likely to have electrophilic or even simply electrostatic interactions with the chain of endogenous donors of PS II, when this chain contains a positive charge as a result of the photoreaction. Due to the interaction with the charged chain, ϕ_4B^- will cause

some redistribution of the positive charge and favor the oxidation of those chain components that it can approach most closely or with which it can complex most strongly. This effect (a charge redistribution within the donor chain, supported by complexation with ϕ_4B^-) may be the 'primary' effect of ϕ_4B^- upon PS II and precede the oxidation as well as the reduction of cytochrome *b* 559.

In the resulting condition, the chain with its associated cytochrome *b* 559 is apparently chemically unstable. The reactions which follow depend on the redox state of the cytochrome *b* 559. Reduced cytochrome *b* 559 is oxidized by the chain, which should release the ϕ_4B^- unchanged, but also prepares the centers for the oxidation of ϕ_4B^- by the next flash. When both the donor chain and the cytochrome are oxidized, they are able to accept a pair of electrons from the ϕ_4B^-, thereby irreversibly oxidizing it. In all, the system seems to work as a primitive charge-accumulating device to oxidize the 2-electron donor.

If a PS II center contains 1 molecule of cytochrome *b* 559, the amplitude of the apparent carotenoid oxidation, or the sign of the (net) cytochrome *b* 559 redox change, would be expected to oscillate with a periodicity of 2 under flash illumination in the presence of ϕ_4B^-. But, as we have seen (fig.6), no such oscillation is observed. In fact, each center probably contains 2 molecules of cytochrome *b* 559. The 2 molecules differ somewhat in their characteristics, as evidenced by the apparent inhomogeneity of cytochrome *b* 559 in measurements of its mid-point potential [10], but may very well behave similarly photochemically [15]. The 'steady-state' behaviour immediately after the first flash indicates that when 1 of the 2 cytochromes is reduced and the other oxidized, the center is equally likely to perform cytochrome oxidation as cytochrome reduction. These considerations suggest a pair of unconnected, parallel, cytochromes *b* 559 (which is very similar to the proposed organization of another pair of *b* cytochromes, i.e., cytochromes b_6 [8]).

Acknowledgements

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