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FLASH-INDUCED CAROTENOID RADICAL CATION FORMATION IN PHOTOSYSTEM II

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Light excitation of chloroplasts at low temperature produces absorption changes (ΔA) with a large positive peak at 990 nm and a bleaching around 480 nm. ΔA at 990 nm rises with $t_{1/2} = 0.6$ ms at 20–77 K and remains largely stable. This signal is not observed when Photosystem II (PS II) photochemistry is blocked by reduction of the primary plastoquinone. It is observed also in purified PS II particles, in which case it could be shown that during a sequence of short flashes, the absorption at 990 nm rises in parallel with plastoquinone reduction measured at 320 nm. In chloroplasts the light-induced 990-nm ΔA at 77 K is increased under oxidizing conditions (addition of ferricyanide) and upon addition of 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (ANT2p). At 21°C, flash excitation of chloroplasts or of PS II particles induces only a very small ΔA at 990 nm, even when this is measured with a 100-ns time resolution or when the material is preilluminated. In both materials, however, a large flash-induced ΔA takes place when various lipophilic anions are added. After a flash the signal rises in less than 100 μs and its decay varies with experimental conditions; the decay is strongly accelerated by benzidine. The difference spectrum measured in PS II particles includes a broad peak around 990 nm and a bleaching around 490 nm. These absorption changes are attributed to a carotenoid radical cation formed at the PS II reaction center. It is estimated that in the presence of lipophilic anions at room temperature, one cation can be formed by a single flash in 80% of the reaction centers. At cryogenic temperature approx. 8% of the PS II reaction centers can oxidize a carotenoid after a single flash.

Introduction

Carotenoids are ubiquitous in oxygen-evolving photosynthetic organisms and these molecules have often been hypothesized to play a direct role in oxygenic photosynthetic electron transport [1]. Early studies in vitro showed that strong oxidants such as iodine could form with carotenoids complexes that had some charge-transfer character [2–4]. More recently, it has been shown that carotenoids in vitro can be oxidized by a flash of light in the presence of a dye sensitizer and that both anion and cation radicals of carotenoids can be prepared by pulse radiolysis [5–7]. The quenching of chlorophyll fluorescence in vitro by β-carotene has supported the involvement of carotenoids in electron transfer by photosynthetic model systems [8]. Strong evidence supporting carotenoid-mediated redox reactions in vivo has been more difficult to obtain. Steady-state photobleaching experiments have shown that the ground state absorp-

Abbreviations: PS, photosystem; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ANT2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TPB, tetraphenylboron; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 4-morpholineethanesulfonic acid

tion bands of carotenoids in vivo can undergo light-induced changes that have been interpreted as evidence for redox reactions [9–11]. It has been shown that antibodies to carotenoids appear to inhibit electron transfer, probably near the PS II reaction center [12]. Extraction-reconstitution studies [13–15] have revealed that carotenoids do not appear to be necessary for primary photochemical activity in PS II but that these polyenes are necessary for the C-550 absorption shift which is an indirect indicator of the reduction of the primary plastoquinone. Carotenoids also appear to be necessary for the low-temperature oxidation of cytochrome *b*-559 [14]. None of these experiments, however, leads to a precisely defined participation of carotenoids in electron transfer.

In earlier studies on the high-potential electron donors of PS II, measurements of flash-induced absorption changes near 820 nm revealed that the oxidized primary electron donor of PS II (P-680$^+$) is reduced by a one-electron donor D_1 [16–18]. In Tris-treated chloroplasts or purified subchloroplast PS II fractions, D_1 has a high redox potential (greater than +555 mV) and reduces P-680$^+$ in approx. 10 μs. D_1 is of unknown chemical nature and the present work as begun in an attempt to characterize spectroscopically D_1 by measuring flash-induced ΔA at long wavelengths (900–1100 nm) where P-680$^+$ has little or no absorption. In the present work, a species absorbing maximally around 990 nm in both chloroplasts and purified PS II preparations is observed and shown to be the radical cation of a carotenoid. The spectrum of this species is in agreement with the previously observed spectrum for carotenoid cations in vitro [5,6]. We show that the absorbance changes attributed to the formation of the radical cation are enhanced at cryogenic temperature, in agreement with an observation reported previously by Lozier and Butler [19]. We also show that at room temperature certain lipophilic anions are capable of greatly stimulating the light-induced formation of the carotenoid cation radical, confirming and extending a recent report by Velthuys [20]. Our study indicates that one carotenoid is tightly associated with the donor side of PS II and can be oxidized with high efficiency. This carotenoid, however, is not D_1 and its physiological role remains obscure.

Materials and Methods

Biological material. Chloroplasts were isolated from spinach leaves, in an isotonic buffer (0.4 M sucrose/0.01 M NaCl/0.02 M Tricine, pH 7.8) and centrifuged. In most cases they were fresh. A pellet was suspended in a hypotonic buffer (0.01 M NaCl/0.05 M Tricine, pH 7.0) and homogenized; the suspension was kept in ice and further diluted before each experiment. In some experiments (Figs. 2 and 5) the homogenized chloroplasts were mixed with 2 vol. glycerol and then stored in small tubes in liquid N_2. A newly thawed tube was used for each experiment. Purified PS II particles were prepared from spinach (Ref. 21; termed F II particles in the following) or from the alga *Chlamydomonas* (Ref 22; termed PS II particles). The latter preparation was completely devoid of PS I activity, since it was prepared from a mutant strain lacking PS I polypeptides. The particles were stored at low tempreature and diluted in an appropriate buffer just before use. The buffers and added chemicals are indicated in the figure legends.

Chemicals. Most chemicals were reagent grade. TPB was purchased from Sigma. ANT2p was obtained from Dr. J. Garnier. FCCP was obtained from Dr J. Galmiche. Stock solutions of lipophilic anions were prepared in H_2O or ethanol and were sufficiently concentrated to keep the solvent concentration at less than 1% of the sample volume during an experiment.

Spectroscopic measurements. For measurements at low temperature the sample was introduced into a 1 mm thick plexiglas cuvette which was then inserted into a cryostat cooled by helium gas at a controlled temperature. The cuvette was held at 45° to the mutually perpendicular measuring and exciting beams and the optical path of the measuring beam was 1.1 mm. The excitation was provided by a dye laser (590 nm) pumped by a xenon flash (duration 800 ns) or by a frequency-doubled pulse from a YAG laser (duration 30 ns). In some cases the excitation as provided by a 600 W tungsten lamp whose output was filtered with 2 cm of water and a Corning CS 4-96 filter. This light beam passed through an electromechanical shutter. The measuring light was obtained from a pulsed 800 W tungsten lamp and its frequency was selected with interference filters. The absorption changes were

measured with a PIN-10 photodiode using an amplifier with 1 or 5 MHz bandwidth, as described in Ref. 23. For the slower measurements, a d.c. amplifier with 1 kHz bandwidth was used. For the measurements in the ultraviolet, we used the same apparatus as that described in Ref. 24. For the recording of the stable absorption difference spectra, the helium cryostat was placed in a Cary 17 spectrophotometer. The absorption spectrum was measured and stored in a memory before and after illumination of the sample and the difference spectrum was obtained by subtraction. In the figure legends, the absorption of the material in the cuvette is measured along the same optical path as the measurement of the light-induced absorbance changes.

For the measurements at room temperature, the material was placed in a 10×10 mm cuvette. For most experiments we used the same kinetic spectrophotometer as for the low-temperature experiments. Some experiments (Figs. 8, 9 and 12) were performed with another kinetic spectrophotometer [25] in which the measuring light was delivered in the form of short flashes at a given time after a 1 μs actinic xenon flash.

Results and Discussion

Effect of illumination at low temperature

Fig. 1 shows kinetic traces of absorption changes induced by a photochemically saturating flash in broken spinach chloroplasts at 20 K. Ferricyanide was added to oxidize chemically *P*-700. At 820 nm (top trace), the flash elicits a rapid absorption increase followed by a largely monophasic decay with a half-life of approx. 1.6 ms. This signal is attributed to the primary donor *P*-680 which is photooxidized by the flash and then rereduced principally by charge recombination [16,26,27]. If one measures absorbance changes at 980 nm, one observes a different kinetic behavior (Fig. 1, middle trace). There is a small rapid rise, but the majority of the signal grows with a half-life of 0.6 ms and the decay is too slow to be measured with a 5 ms sweep time. The lower trace in Fig. 1 shows that if the primary plastoquinone is photochemically reduced prior to the flash, ΔA at 980 nm is modified; the 0.6 ms rise-time absorption increase disappears and only a small rapidly rising, slowly

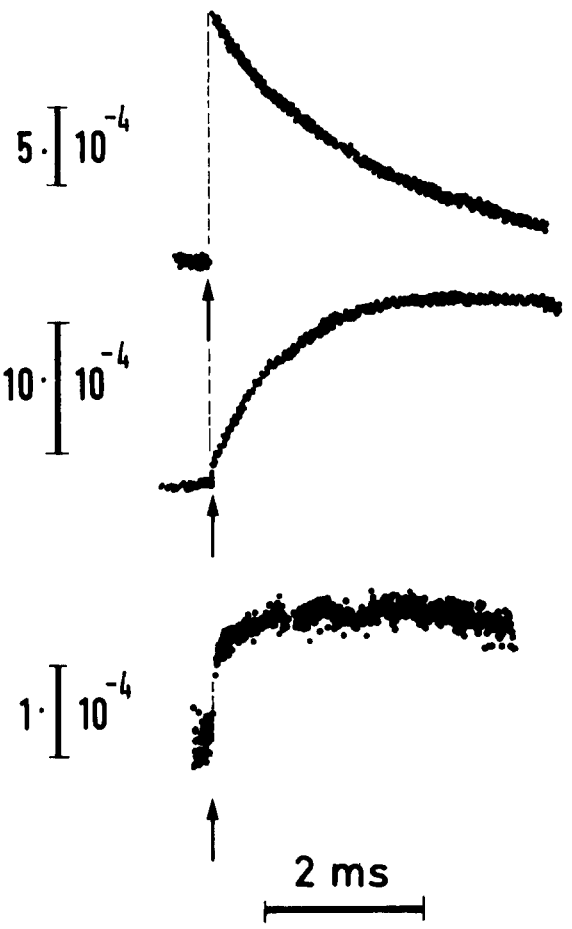

Fig. 1. Kinetics of flash-induced ΔA measured with chloroplasts at 20 K (1 part 50 mM Tricine, pH 7, 10 mM NaCl, plus 2 parts glycerol). $A_{678} = 2.2$. Upper trace: 820 nm, sum of third and fourth flashes. Middle trace: 980 nm, first flash (the same cuvette was used for the upper and the middle traces). Lower trace: 980 nm, sum of first and second flashes. Upper and middle traces: 5 M $K_3Fe(CN)_6$ present. Lower trace: 20 μM DCMU and 100 μM hydroxylamine present, illuminated while cooling. Electrical bandwidth: 100 Hz–0.3 MHz. Actinic light source: 30-ns saturating flashes.

decaying component remains. This may correlate with the small rapidly rising component seen when the primary plastoquinone is unreduced before the flash (Fig. 1, middle trace). The amplitude of the ΔA measured at 820 or 980 nm is a function of the flash number in a multiflash sequence. After 14 photochemically saturating flashes the initial amplitude measured at 820 nm decreased by 25%, while at the same time the amplitude measured at

980 nm approx. 5 ms after a flash decreased by 80%. The kinetics of formation measured at 980 nm did not appear to be strongly dependent on flash number. ΔA at 980 nm was unchanged in Tris-treated chloroplasts (prepared as in Ref. 17). In chloroplasts that had been treated at pH 4.0 (as in Ref. 28), resuspended at pH 7 and then sonicated to disperse the aggregate, ΔA at 980 nm increased 2.7-fold.

Fig. 2 shows several spectra of light-induced ΔA in broken chloroplasts at 77 K. These spectra were taken from raw data similar to those of Fig. 1, however, the time resolution was lower (approx. 100 μs). The filled circles show ΔA measured at 5 ms after a single photochemically saturating flash. After a 2 min interval, the cuvette was illuminated with a 0.15 s pulse of blue light and ΔA was simultaneously measured (open circles in Fig. 2). Only a small fraction of ΔA measured in the infrared spectral region decays in less than 10 s (approx. 15%, $t_{1/2} \simeq 1$ s). The results show a large positive absorbance peak near 1000 nm with a width of approx. 80 nm at half-maximum (right-hand scale). Light-induced absorption changes also occur in the blue spectral region. These absorbance changes were small and difficult to measure with our apparatus. We therefore used chloroplasts that had been treated at pH 4 and sonicated. The triangles in Fig. 2 show the sum of ΔA measured at 5 ms after each of the first five saturating flashes (left-hand scale). The results show a bleaching centered near 490 nm. The scatter in the data obscures possible fine structure. In these experiments an actinic effect of the measuring light may have artifactually decreased ΔA in the blue spectral region. Thus, the relative amplitudes of ΔA in the blue and in the infrared cannot be meaningfully compared.

Absorption changes around 820 and 980 nm were also observed upon flash excitation of PS II particles at low temperature. The kinetic properties were nearly the same as in chloroplasts (data not shown). The spectrum of stable light-induced absorption changes was measured with PS II particles at 50 K during a period of several minutes following saturating illumination by continuous white light. In the near-infrared region a clear peak appears around 990 nm (Fig. 3) that shows a strong resemblance to the spectrum of light-induced ΔA measured in chloroplasts (Fig. 2). The

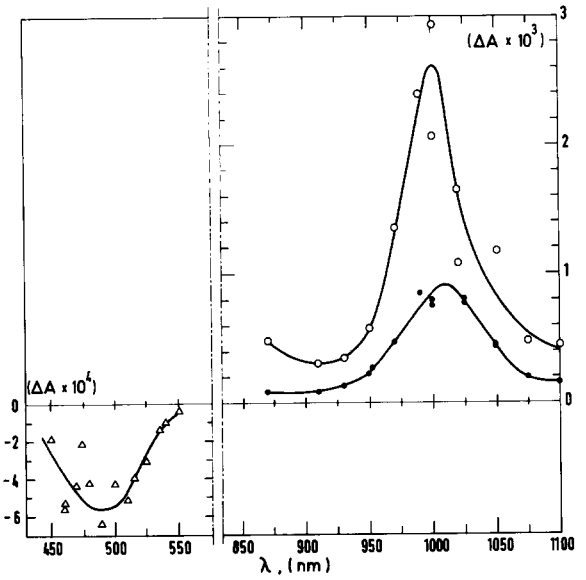

Fig. 2. Visible and near-infrared spectra of light-induced ΔA measured with chloroplasts at 77 K (one part 50 mM Tricine, pH 7, 10 mM NaCl, 5 mM $K_3Fe(CN)_6$ plus 2 parts glycerol). Infrared measurements: right hand scale, $A_{678} = 0.77$; visible spectrum: left-hand scale, $A_{678} = 0.20$ (chloroplasts were treated at pH 4.0 and then sonicated). Actinic light source: (●) single 30 ns flash; (△) sum of ΔA induced by five 30-ns flashes; (○) 0.15 s blue-light pulse, given 2 min after the 30-ns flash.

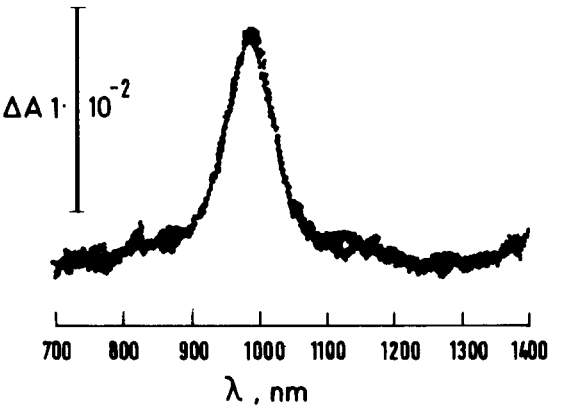

Fig. 3. Light-induced absorption spectrum measured with PS II particles at 50 K (1 part 50 mM Mes, pH 5.9, 0.5 mM $K_3Fe(CN)_6$ plus 2 parts glycerol). $A_{674} = 4.1$. The sample was cooled in darkness and its absorption spectrum recorded. The sample was then illuminated for 30 s with strong white light, the absorption spectrum recorded again and the first spectrum subtracted from the second one.

position of the baseline in Fig. 3 is uncertain, but it is probably near the flat part of the spectrum on either side of the peak. In the visible spectral region we detected absorption changes attributable to C-550 and apparent chlorophyll bandshifts in the red associated with the reduction of the primary plastoquinone acceptor [19,29]. Parallel experiments with dithionite-reduced PS II particles and hydroxylamine-treated particles that had been illuminated during cooling to 50 K revealed that at least 75% of the C-550 became stabilized in its reduced form under conditions described in the legend to Fig. 3.

Fig. 4 shows flash-induced ΔA in PS II particles at 50 K. In response to a train of saturating flashes triggered at 1 Hz, we observed a simultaneous rise of the absorption at 320 and 980 nm. In this experiment fast transients were not recorded and only the stable ΔA was measured. The absorption increase at 320 nm is presumably due to the reduction of the primary plastoquinone of PS II [30] (the particles are devoid of PS I, and cytochrome *b*-559 is kept oxidized by added ferricyanide). The species absorbing at 980 nm thus accumulates in parallel with the plastoquinone anion.

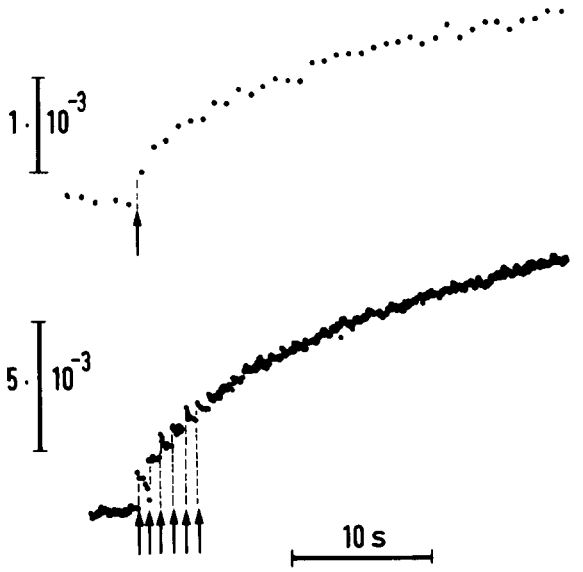

Fig. 4. Absorbance changes at 320 and 990 nm with PS II particles (same conditions as for Fig. 3) induced by a train of saturating 30-ns flashes spaced 1 s apart. Electrical bandwidth: d.c. to 1 Hz. In the upper trace (320 nm) the signal was averaged and digitized every second. Lower trace: 990 nm.

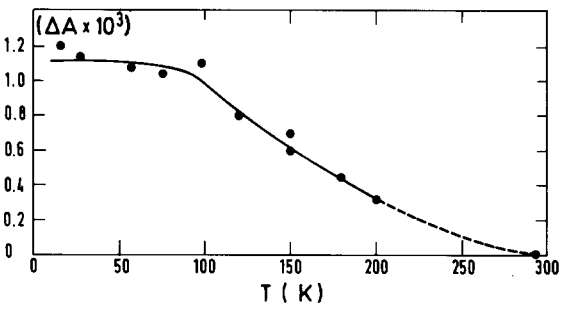

Fig. 5. Effect of temperature on the maximum flash-induced ΔA at 980 nm in chloroplasts (1 part 50 mM Tricine, pH 7, 10 mM NaCl, 5 mM $K_3Fe(CN)_6$ plus 2 parts glycerol). $A_{678} = 1.1$. Sum of the effects of the first four saturating 800-ns flashes given to dark-adapted chloroplasts.

Fig. 5 shows the effect of temperature on the flash-induced ΔA at 980 nm in chloroplasts with 5 mM ferricyanide. Between 15 and 100 K the signal is maximal and does not vary with temperature. Above 100 K, ΔA becomes smaller until at room temperature the remaining ΔA is less than 5% of its maximum value. The signal size is also increased at high redox potential with chloroplasts at 100 K, as shown in Fig. 6. A single flash elicits a

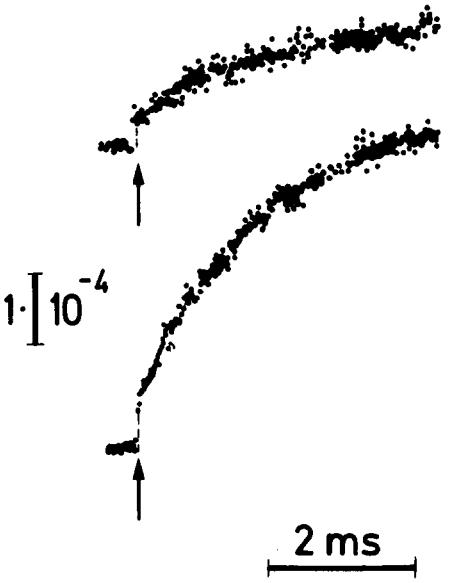

Fig. 6. Absorption change at 990 nm induced by the first saturating 800-ns flash in chloroplasts at 100 K (1 part 50 mM Tricine, pH 7, 10 mM NaCl plus 2 parts glycerol) with no further addition (upper) or with 1 mM $K_3Fe(CN)_6$ (lower). $A_{678} = 2.1$.

several-fold greater ΔA at 990 nm when ferricyanide is present.

From all the preceding experiments we conclude that most of the ΔA at 990 nm in chloroplasts is associated with PS II photochemistry. It is present in PS II particles and the 990-nm ΔA nearly disappears when PS II photochemistry is blocked. This ΔA is due neither to $P\text{-}680^+$ (see Fig. 1) nor to the first plastoquinone electron acceptor which is reduced immediately and the anion of which decays like $P\text{-}680^+$ at low temperature [31]. Among the chemical species present in chloroplasts and in PS II particles, carotenoids are the only ones which are known to give rise to species presenting a well defined absorption peak around 1000 nm. This is true for their cation radicals [5–7] and their anion radicals [6,7] as well as for their protonated state [32]. The involvement of a carotenoid would be in agreement with the bleaching observed around 490 nm. The spectral data do not permit one to choose between the three carotenoid derivatives, all of which have similar absorption spectra. However, it seems unlikely that the species formed in vivo could be a carotenoid anion; the strongest reductant generated in PS II cannot have a reducing potential lower than -1.0 V whereas E_m for the one-electron reduction of β-carotene in organic solvents is near -1.5 V [33]. Our results are better explained if a carotenoid C becomes oxidized at the donor side of PS II, most probably directly by $P\text{-}680^+$. As one possible model, we propose that $P\text{-}680^+$ can be reduced by three donors (in addition to the back-reaction with plastoquinone, which is dominant below 100 K): D_1, cytochrome *b*-559 and C. D_1 becomes disconnected when the temperature is lowered, and cytochrome *b*-559 and C are then in competition. This would explain the higher formation of C^+ when cytochrome *b*-559 is chemically oxidized. In this model, C should be oxidized at the same rate with which $P\text{-}680^+$ becomes reduced. This is not exactly the case; Fig. 1 shows that the oxidation of C is actually faster than the reduction of $P\text{-}680^+$. This discrepancy is rather puzzling. It should be realized, however, that the extent of carotenoid oxidation after a single flash at low temperature is probably low. The extinction coefficient of the carotenoid cation radicals in the near infrared is not well known; values of 130000 and 218000 $M^{-1} \cdot cm^{-1}$ have been reported [5,6]. Accepting an intermediate value of 175000 $M^{-1} \cdot cm^{-1}$, we find that a single flash oxidizes one C per $5 \cdot 10^3$ chlorophylls in chloroplasts, i.e., in about 8% of the PS II reaction centers. The kinetic discrepancy can be rationalized by hypothesizing that the population of PS II reaction centers is inhomogeneous with respect to the microscopic rate constants for electron transfer from C to $P\text{-}680^+$ as well as for the back-reaction. The kinetics of decay of $P\text{-}680^+$ would then actually be multiphasic.

The near-infrared spectrum of C^+ has a very large extinction coefficient and extends toward 800 nm. It is quite possible that absorbance changes which have a flat spectrum above 800 nm and which appear in chloroplasts under oxidizing conditions [16] are not due to a chlorophyll cation but rather to C^+. This species may also participate significantly in the light-induced free radical ESR signals observed at low temperature in chloroplasts and PS II particles [34–37]. Several authors have already suggested that donors other than cytochrome *b*-559 were functioning in PS II at low temperature (e.g., see Ref. 27) and that the kinetic properties were heterogeneous (Ref. 38 and references therein).

Flash-induced absorption changes at ambient temperatures

Flash excitation of chloroplasts at 21°C leads to only very small ΔA at 980 nm, even when the measurement is done with 100 ns time resolution (lower right-hand trace in Fig. 7). No significant stimulation of the flash-induced ΔA was obtained by flash preillumination, addition of ferricyanide or a prior treatment of the chloroplasts with Tris or at low pH. If one adds the lipophilic anion TPB to the chloroplasts, then the flash-induced ΔA at 980 nm is enhanced dramatically (remaining traces in Fig. 7). ΔA reaches its maximum extent at approx. 150 μs after the flash and the rise is clearly multiphasic. The faster component, which accounts for approx. 15% of the total ΔA, has an apparent $t_{1/2}$ of 250 ns. The true $t_{1/2}$ may be even shorter, since the measuring apparatus has a 5 MHz bandwidth. The slower component of the rise has an approximate $t_{1/2}$ of 10 μs. Similar observations were made with chloroplasts in the presence of the

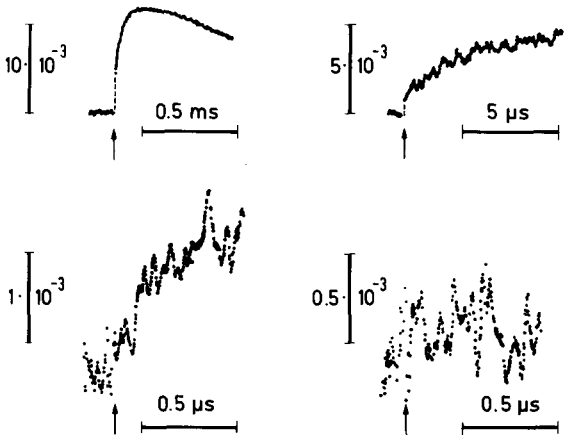

Fig. 7. Kinetics of ΔA at 980 nm induced by 30-ns saturating flashes in chloroplasts at 21°C (50 mM Tricine, pH 7.6, 10 mM NaCl). $A_6 8 d 7_8 = 4.6$. Addition of 9 μM tetraphenylboron (TPB), except for the lower right-hand trace. Electrical bandwidth: 100 Hz to 5 MHz. Each trace represents the average of one to four experiments.

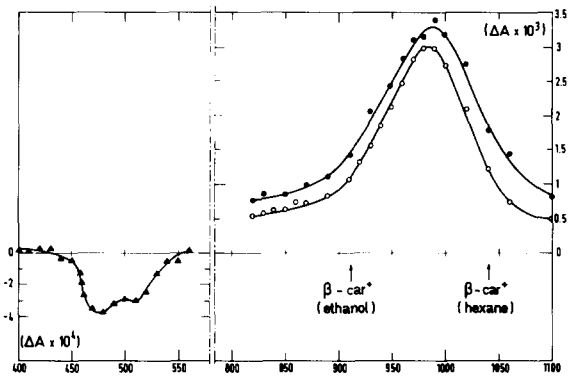

Fig. 8. Flash-induced absorption spectra measured with chloroplasts and PS II particles at 21°C. (○———○) Chloroplasts in 50 mM Tricine, pH 7, 10 mM NaCl, 0.5 mM $K_3Fe(CN)_6$, 2 μM ANT2p. $A_{678} = 1.2$. Each point represents the averaged effect of two 30-ns flashes, measured at 150 μs after the flash. (●———●) PS II particles in 20 mM Mes, pH 5.9, 0.5 mM $K_3Fe(CN)_6$, 4 μM ANT2p. $A_{674} = 0.41$. Other conditions as for chloroplasts. (▲———▲) PS II particles in the same medium. $A_{674} = 0.50$. Excitation by a 1 μs xenon flash. ΔA was measured between 0.5 and 3.5 ms after the actinic flash. Spectra normalized at 490 nm (see text). The right-hand and the left-hand scales apply to the measurements in the infrared and in the visible, respectively. car, carotene.

lipophilic anions ANT2p and FCCP. The stimulation of ΔA at 980 nm by TPB, ANT2p and FCCP was also observed with Tris-treated chloroplasts and with PS II particles (Figs. 12 and 13) and F II particles. In chloroplasts, the stimulation was neither effected by uncoupling agents like gramicidin D or nigericin nor by the lipophilic monovalent cations triphenylmethylphosphonium and triphenyltin. In the presence of 9 μM TPB and 10 μM DCMU, when a saturating preilluminating flash was given 50 ms before the actinic flash, the ΔA at 980 nm was largely suppressed.

Fig. 8 shows the spectrum of flash-induced ΔA measured in chloroplasts and PS II particles in the presence of 2 μM ANT2p. Points in the infrared region were measured when the ΔA reached its peak value at approx. 150 μs after the flash. In chloroplasts (open circles) and in PS II particles (filled circles) there is a well formed peak near 985 nm. This near-infrared spectrum is essentially identical with the spectrum measured in both chloroplasts and PS II particles at low temperature in the absence of of lipophilic anions (Figs. 2 and 3). The spectrum of flash-induced ΔA of PS II particles in the visible spectral region was measured with a different apparatus. This spectrum (Fig. 8, triangles) was measured as the difference between the points 0.5 and 3.5 ms after the flash (raw data, Fig. 12). The flash used for the measurements in the visible region was not saturating. In order to permit a direct comparison of ΔA, one point was measured at 490 nm during the infrared measurements with a measuring light of low intensity, and the results were used to normalize the visible spectrum with the infrared spectrum. The similarity between the spectra of Fig. 8 and those of Figs. 2 and 3 suggests that the species that is stimulated by lipophilic anions is also a carotenoid cation.

An infrared spectrum similar to those of Fig. 8 was also obtained by flash excitation of F II particles at 21°C, without lipophilic anions. The maximum ΔA at 980 nm was about 10-times smaller than that with 5 μM ANT2p. Assuming an extinction coefficient of 175000 $M^{-1} \cdot cm^{-1}$ for C^+ and 50 chlorophylls per reaction center, we found that only one C^+ was formed per 55 reaction centers. ΔA at 980 nm is the same after the first or the second flash, under conditions where the donor D_1 was oxidized by the first flash and was not rereduced at the time of the second flash [18]. We thus conclude that C cannot be equated with D_1. The same conclusion can be drawn from

similar experiments made with Tris-treated chloroplasts (data not shown).

Fig. 9 shows spectra of flash-induced ΔA measured in the ultraviolet spectral region with PS II particles. Experimental points corresponding to no additions or to the addition of 5 μM ANT2p are represented by filled or open symbols, respectively. ΔA was measured at 200 μs (circles) and 1 ms (squares) after the actinic flash. The spectra have the same shape, with a trough near 265 nm and a positive peak near 320 nm. These features have been attributed to the reduction of the primary plastoquinone electron acceptor [30]. The similarity of the spectra in the absence and presence of ANT2p suggests that the carotenoid oxidation does not give rise to a significant ΔA around 320 nm and confirms that Fig. 4 does indeed demonstrate the parallel formation of C^+ and reduced plastoquinone. The slight overall decrease in signal amplitude in the presence of ANT2p can be attributed to an aging process that takes place at higher ANT2p concentration (see Fig. 10).

Fig. 10 shows the effect of concentration of three different lipophilic anions on the flash-induced ΔA at 980 nm in chloroplasts. In panel c it is shown that ANT2p causes a dramatic stimulation of ΔA. The stimulation is half-maximal at 2 μM and 15% maximal at 80 nM ANT2p. At concentrations higher than 20 μM, the signal decreases. Panel b shows that FCCP also stimulates ΔA, but the effect is rather weak. Panel a shows that with TPB, ΔA is greatly stimulated and reaches higher levels than with ANT2p. ΔA appears to be somewhat smaller after the first flash (circles, panel a) than after the second or third flashes (triangles and squares, panel a) in a sequence. The presence of 1 mM $K_3Fe(CN)_6$ allows a 20% greater flash-induced ΔA at 980 nm immediately after the addition of oxidant and TPB (data not shown), but then there is a strong decrease in the light-induced signals that takes place in several minutes. If one uses the previous estimate for the extinction coefficient of C^+ at 980 nm, then at the highest TPB concentration used

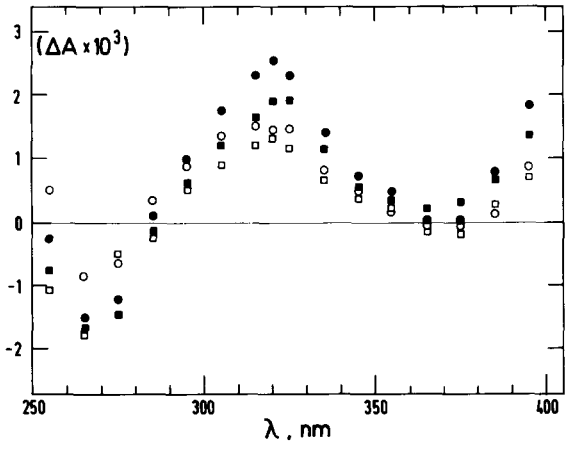

Fig. 9. Difference spectrum of flash-induced ΔA in the ultraviolet region measured at 200 μs (○,●) and 1 ms (□,■) after a saturating 1 μs xenon flash in PS II particles at 20°C. $A_{674} = 0.5$ (50 mM Mes, pH 5.9, 0.1 mM $K_3Fe(CN)_6$). (●,■) No ANT2p, (○,□) 5 μM ANT2p.

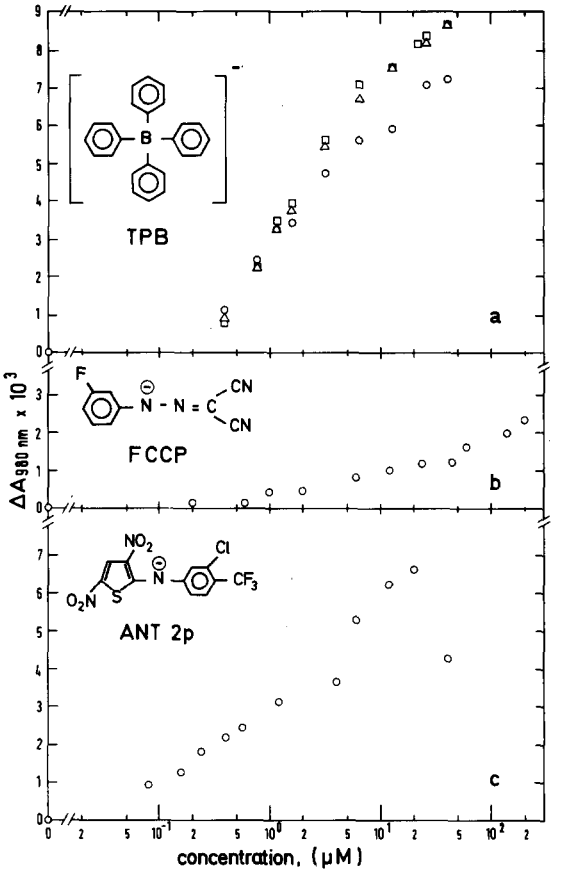

Fig. 10. Effect of lipophilic anion concentration on ΔA at 980 nm induced by one 30 ns saturating flash in chloroplasts (50 mM Tricine, pH 7.0, 10 mM NaCl). $A_{678} = 1.0$. (a) TPB, no $K_3Fe(CN)_6$; (○,△,□) first, second and third flash (separated by 15 s). (b) FCCP, 0.5 mM $K_3Fe(CN)_6$. (c) ANT2p, 0.5 mM $K_3Fe(CN)_6$.

there is approximately one C^+ formed per 660 chlorophylls (i.e. approx. 80% of the reaction centers).

In chloroplasts in the presence of ANT2p, the flash induced ΔA at 980 nm decays with $t_{1/2} \simeq 1.9$ ms. The decay is accelerated in the presence of benzidine (Fig. 11, inset), a lipophilic electron donor of high mid-point potential ($E_{m,7} = +0.55$ mV). In this experiment the redox potential was maintained near + 370 mV by a mixture of ferro- and ferricyanide, so that the benzidine was more than 99% reduced. The pseudo-first-order rate constant for the decay was estimated from the reciprocal of the time required for the signal to decay to $1/e$ of its maximum value. This rate constant, k, plotted versus benzidine concentration, gives a straight line (Fig. 11, filled circles) which indicates that benzidine quenches the 980 nm absorbing species by a second-order mechanism. This result reinforces our identification of the species as a cationic form. Similar data were obtained with PS II particles (Fig. 11). The second-order rate constant for quenching is $7.7 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ in chloroplasts and about twice as large with PS II particles ($1.6 \cdot 10^7$ $M^{-1} \cdot s^{-1}$). The lifetime of C^+ is also decreased by reduced dichlorophenolindophenol. We found no acceleration by TPB.

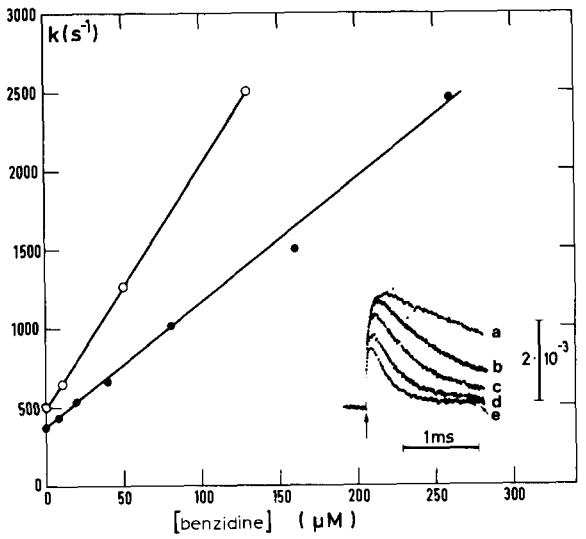

Fig. 11. Plot of the rate constant k of decay for ΔA at 980 nm induced by a 30 ns flash in chloroplasts and a 1 μs flash in PS II particles as a function of benzidine concentration, at 21°C. (●———●) Chloroplasts: $A_{678} = 1.83$ (50 mM Tricine, pH 7, 10 mM NaCl, 2 μM ANT2p, 80 μM $K_3Fe(CN)_6$, 800 μM $K_2Fe(CN)_6$) (○———○) PS II particles: $A_{674} = 0.5$ (same medium, except Mes, pH 5.9, and no NaCl). Inset: raw kinetic data obtained with chloroplasts; experiments a–e correspond to 0, 10, 20, 40 and 65 μM benzidine, respectively.

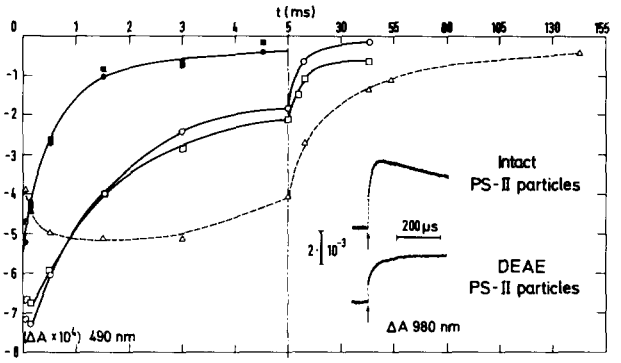

Fig. 12. Kinetics of flash-induced ΔA at 490 and 980 nm (inset) measured in PS II particles at 21°C. For experiments at 490 nm, $A_{674} = 0.5$ (50 mM Mes, pH 5.9, 5 μM ANT2p). (○,□) intact particles. (●,■) Intact particles plus 50 μM benzidine. (△) DEAE-Sephadex particles (no benzidine). (○,●) Addition of 100 μM $K_3Fe(CN)_6$ (□,■) Addition of 100 μM $K_3Fe(CN)_6$ and 1 mM $K_2Fe(CN)_6$. For experiments at 980 nm, $A_{674} = 1.2$ for intact particles and 0.9 for DEAE-Sephadex particles (20 mM Mes, pH 5.9, 5 μM ANT2p, 300 μM $K_3Fe(CN)_6$). Actinic flash: 1 μs xenon flash for experiments at 490 nm and 30 ns flash for experiments at 980 nm.

Fig. 12 shows the kinetics of decay of flash-induced ΔA at 490 and 980 nm (inset) with two different kinds of PS II particles in the presence of ANT2p. In intact particles (open circles and inset, upper trace) ΔA decays with $t_{1/2} \simeq 1.7$ ms. The decay was unchanged when the redox potential was lowered from a high value near +500 mV (ferricyanide alone) to +370 mV (ferri- and ferrocyanide, open squares). DEAE-Sephadex PS-II particles that had been passed over a DEAE-Sephadex column and were missing a tertiary electron donor [39] decayed much more slowly (Fig. 12, triangles and inset, lower trace). This suggests that the missing donor could be involved in the reduction of C^+. The figure shows that benzidine also accelerates the recovery of the bleaching at 490 nm.

With PS II particles in the presence of ANT2p, we attempted to correlate the kinetics of ΔA at

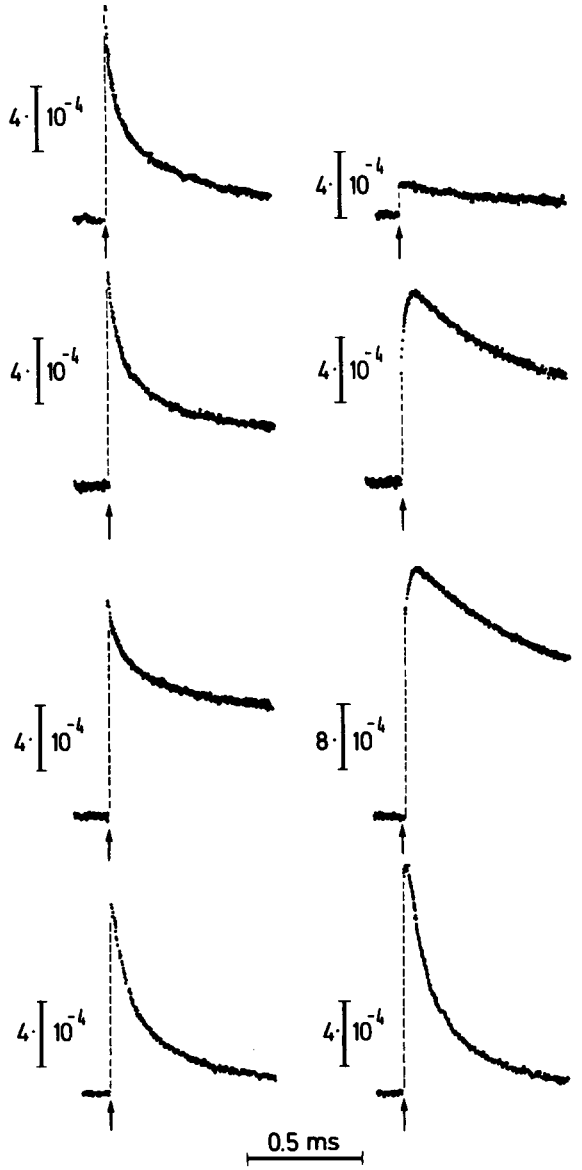

Fig. 13. Kinetics of ΔA at 820 (left-hand traces) and 980 nm (right-hand traces) at 20°C, induced by a 30 ns flash in PS II particles (50 mM Mes, pH 5.9, 100 μM $K_3Fe(CN)_6$). $A_{674} = 2.0$. From top to bottom: no further addition, addition of 0.5 μM ANT2p, addition of 2.5 μM ANT2p, addition of 2.5 μM ANT2p and 200 μM benzidine. One experiment per trace.

820 nm (presumably due mainly to $P\text{-}680^+$) and at 980 nm (due to C^+). Fig. 13 shows the results obtained with intact PS II particles. With no ANT2p, the flash-induced ΔA at 980 nm is quite small. At 820 nm the signal is large and decays biphasically; more than half of the signal decays with $t_{1/2} = 20$ μs and the remainder decays with a $t_{1/2}$ of several hundred microseconds. With 0.5 μM ANT2p, the initial ΔA at 820 nm remains constant but the relative contribution of the slow phase increases; at 980 nm the ΔA is increased 6-fold. When the ANT2p concentration is raised to 2.5 μM, ΔA at 980 nm increases again by more than 2-fold; at 820 nm the initial ΔA is unchanged, but the slow phase is still increased and appears to decay approximately like the signal at 980 nm. The addition of 200 μM benzidine accelerates both the decay time of the 980 nm signal and the slow phase at 820 nm. The results can be most easily explained by postulating that the 980 nm absorbing species contributes to ΔA at 820 nm in the presence of ANT2p. Such a contribution is to be expected from the absorption spectra of carotenoid radical cations in vitro [5,6]. The short-wavelength tail for carotenoid radical cations has an extinction coefficient of the order of 20000 $M^{-1} \cdot cm^{-1}$, greater than that for $P\text{-}680^+$ at 820 nm (approx. 7000 $M^{-1} \cdot cm^{-1}$). This makes it difficult to draw a precise correlation between the formation of C^+ with the decay of $P\text{-}680^+$ in the presence of lipophilic anions at room temperature. In a separate experiment at 77 K, 2 μM ANT2p also stimulated ΔA at 980 nm by 2-fold (data not shown). In the absence of ANT2p at low temperature, the decay at 820 nm does not seem to include a contribution of C^+ after a single flash (Fig. 1), probably because of the lower yield of C^+ formation.

Conclusion

At cryogenic temperatures, electron transfer in PS II gives rise to a long-lived absorption decrease in the blue spectral region and to an absorption increase in the infrared. Both of these absorbance changes are consistent with the formation of a carotenoid radical anion or cation or of a protonated carotenoid. Since the infrared absorbance maxima of the latter species are highly dependent on the polarity of their environment, it is difficult to identify the long-lived species from spectral data alone. Absorbance changes in the blue spectral region similar to those reported here were

recently observed at ambient temperature in the presence of TPB by Velthuys [20] and were attributed to carotenoid bleaching. In the presence of TPB and other lipophilic anions at 21°C, we observe transient light-induced ΔA in the blue and infrared spectral regions which are identical to those observed at low temperature. In addition to the spectral similarities, the 2-fold stimulation by ANT2p of the low-temperature infrared absorbance increase would also suggest that the same species is giving rise to these absorbance changes at low and at ambient temperatures.

For thermodynamic reasons it is unlikely that this species is a carotenoid radical anion. While it is difficult to eliminate a protonated carotenoid, several arguments are in favor of the formation of a radical cation C^+:

(a) At 100 K (Fig. 6), 1 mM $K_3Fe(CN)_6$ induces a 3-fold increase in the flash-induced ΔA at 980 nm. This observation is consistent with the oxidation by ferricyanide of a reductant ($E_m <$ 0.5 V) which competes with the carotenoid for the oxidizing equivalent of $P\text{-}680^+$. This donor could be located either in series or in parallel with the carotenoid. Velthuys [20] observed a similar stimulation by $K_3Fe(CN)_6$ at room temperature in the presence of TPB.

(b) At ambient temperature in the presence of ANT2p, the high-potential reductant benzidine accelerates the relaxation of the carotenoid absorbance change.

(c) DEAE-Sephadex PS-II particles which have lost a tertiary electron donor present in intact particles [39] show a 10-fold slower relaxation of the carotenoid signal compared to intact material.

Since the carotenoid, but not the secondary donor D_1, is photooxidized at low temperature, the carotenoid must be located either between D_1 and P-680 or in parallel with D_1. At room temperature, only a small amount of carotenoid photooxidation is observed even when D_1 has been preoxidized. This observation would tend to eliminate a series model.

It is unclear what, if any, physiological role is fulfilled by the carotenoid. At room temperature, in the absence of lipophilic anions, measurements with 100-ns time resolution failed to reveal any appreciable carotenoid absorbance changes (Fig. 7). Either the carotenoid is not normally functional or it is reduced more rapidly than it is oxidized.

Velthuys [20] has suggested that TPB interacts electrostatically with electron donors which have become positively charged upon photooxidation. Carotenoids undoubtedly occupy regions of low dielectric constant and cation formation could indeed be stabilized by the presence of lipophilic anions. Thus, ANT2p, TPB and FCCP all stimulate carotenoid cation formation, possibly by lowering the E_m value of the carotenoid and permitting $P\text{-}680^+$ to become an effective oxidant. The lipophilic cations triphenyltin and triphenylmethylphosphonium have no effect on carotenoid oxidation. Carotenoids in vitro have been reported to have very positive midpoint potentials for oxidation [33]. Preliminary work of our own has indicated that the single-electron oxidation of some carotenoids occurs near +0.8 V.

Velthuys [20] estimated a maximal carotenoid oxidation of one carotenoid radical cation per 2500 chlorophylls in the presence of TPB following a saturating single-turnover actinic flash. This estimate is based on an extinction coefficient of approx. 250 $mM^{-1} \cdot cm^{-1}$. This estimate assumes total carotenoid bleaching in the blue spectral region and probably underestimates the extent of oxidation. Our estimate is based on extinction coefficients from flash photolysis and pulse radiolysis experiments in vitro and gives one carotenoid radical cation per 660 chlorophylls in chloroplasts in the presence of 40 μM TPB at room temperature.

Acknowledgements

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