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QUANTUM YIELD AND RATE OF FORMATION OF THE CAROTENOID TRIPLET STATE IN PHOTOSYNTHETIC STRUCTURES

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Summary

The formation of the triplet state of carotenoids (detected by an absorption peak at 515 nm) and the photo-oxidation of the primary donor of Photosystem II, *P*-680 (detected by an absorption increase at 820 nm) have been measured by flash absorption spectroscopy in chloroplasts in which the oxygen evolution was inhibited by treatment with Tris. The amount of each transient form has been followed versus excitation flash intensity (at 590 or 694 nm). At low excitation energy the quantum yield of triplet formation (with the Photosystem II reaction center in the state Q^-) is about 30% that of *P*-680 photo-oxidation. The yield of carotenoid triplet formation is higher in the state Q^- than in the state Q, in nearly the same proportion as chlorophyll *a* fluorescence. It is concluded that, for excited chlorophyll *a*, the relative rates of intersystem crossing to the triplet state and of fluorescence emission are the same in vivo as in organic solvent. At high flash intensity the signal of $P\text{-}680^+$ completely saturates, whereas that of carotenoid triplet continues to increase.

The rate of triplet-triplet energy transfer from chlorophyll *a* to carotenoids has been derived from the rise time of the absorption change at 515 nm, in chloroplasts and in several light-harvesting pigment-protein complexes. In all cases the rate is very high, around $8 \cdot 10^7\ s^{-1}$ at 294 K. It is about 2—3 times slower at 5 K. The transitory formation of chlorophyll triplet has been verified in two pigment-protein complexes, at 5 K.

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Abbreviations: Chl, chlorophyll; ^{3}Car, carotenoid triplet state; PS, photosystem; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, dichlorophenolindophenol.

Introduction

Several types of experiment have permitted to conclude that in photosynthetic bacteria in which the electron flow is blocked the primary charge separation is followed by a recombination which populates a specific triplet state (P^R) of the primary donor P. When a carotenoid molecule is present in the reaction center a rapid triplet-triplet energy transfer populates its triplet state (^{3}Car) at the expense of P^R. These properties are of great importance since they provide some insight into the mechanism of charge separation and stabilization [1,2]. In higher plants to electron acceptors of Photosystem II exhibit many similarities to the bacterial ones, and the recent observation of a photoreduced pheophytin in subchloroplast particles enriched in Photosystem II further strengthens the comparison [3]. It has been proposed that a specialized molecule of pheophytin *a* (I) serves as a very primary electron acceptor to *P*-680 and as a donor to the primary plastoquinone molecule PQ_1 (state Q). When PQ_1 is reduced (state Q^-) the fluorescence yield of chloroplasts increases and the additional fluorescence has been proposed to originate from a back-reaction between $P\text{-}680^+$ and I^- [4]. If the radical-pair mechanism holds for Photosystem II it is then possible to expect a specific triplet state to be populated by the back-reaction.

The triplet state of carotenoids (^{3}Car) has been observed for some time in chloroplasts [5—8]. It is characterized by an intense absorption peak around 515 nm and by a fast decay, with a half-time of about 2 μs in aerated samples. ^{3}Car has also been detected by its ability to quench the fluorescence of chlorophyll *a* [9—14]. This triplet state is most probably populated by energy transfer from triplet chlorophyll *a*. The sites of energy transfer, however, are not well established. Some results tend to indicate that ^{3}Car is populated from chlorophyll triplets formed in a non specific manner [14], whereas others tend to favor a more specific mechanism occurring when the Photosystem II centers are blocked [9—11,15]. The quantum yield of formation is also largely uncertain, ranging from 3—4% to nearly 100% [12,15,16].

In this work we have attempted to measure in chloroplasts the quantum yield of ^{3}Car formation relative to that of *P*-680 photo-oxidation, and to compare it to the fluorescence of chlorophyll *a*. For chlorophyll *a* dissolved in a polar solvent the quantum yields for fluorescence and for triplet state formation are 32 and 64%, respectively (the direct internal conversion has a very low probability), implying that the rate of triplet state formation is twice that for fluorescence [17]. The occurrence of a specific mechanism for triplet formation at the reaction center is expected to alter significantly the relative importance of the two de-excitation routes under consideration. We also measured the rate of formation of ^{3}Car in several materials (chloroplasts and subchloroplast particles) under the assumption that this rate would be different and that some information might be gained from the comparative study.

Material and Methods

Biological material

For the preparation of chloroplasts, around 40 g spinach leaves were ground

for 10 s with 200 ml buffer (0.35 M sucrose/10 mM NaCl/50 mM Tris, pH 7.8) in a mixer. The brei was filtered through a nylon mesh (10 μm openings) and the filtrate was centrifuged for 3 min at 5000 $\times g$. The pellet was homogenized with 50 ml of Tris (0.2 M, pH 9.0) and incubated for 10 min under ambient light. The suspension was centrifuged (5 min at 10000 $\times g$). The pellets were homogenized with 2 ml of the buffer and kept in ice. Before each series of measurements a known amount of the suspension was diluted (30—50 times) with the buffer in a regular 10 $\times$ 10 mm cuvette, with four clear windows, and appropriate chemicals were added (DCMU, potassium ferricyanide or sodium ascorbate, see text). Final chlorophyll concentrations ranged from $1.7 \cdot 10^{-5}$ to $3.0 \cdot 10^{-5}$ M. All preparative operations were done at 4°C. Subchloroplast particles were kindly prepared by Drs. P. Delepelaire (CP-III and CP—IV complexes, according to Ref. 18), S. Reinman (CP-II complex, according to Ref. 19) and K. Satoh (F-III complex, according to Ref. 20). Broken thylakoid membranes were prepared by a mild sonication of chloroplasts suspended in 50 mM Tris-HCl buffer, pH 8.0.

Spectroscopic measurements

For the measurement of flash-induced absorption changes (ΔA) due to ^{3}Car and to P-680$^+$, the cuvette was excited with the light beam from either a ruby laser (λ = 694 nm: duration, 8 ns) or a dye laser pumped by the frequency doubled pulse from a YAG laser (broadband light around 590 nm; duration, 15 ns). Both types of pulse were attenuated in a controlled manner with calibrated neutral filters (Schott, type NG). The beam was directed onto a piece of ground glass; exciting light was collected by a lucite pipe, the exit of which was cut to the shape of the cuvette. The relative energy of each laser pulse was measured and only those pulses having energies within 15% of the average were used for experiments. For the measurement of ΔA we used the same arrangement as in Refs. 14 and 21. In brief, the measuring light was provided by a pulsed tungsten lamp and filtered with appropriate interference filters (515 or 820 nm). The light went then through the cuvette and through another interference filter of the same wavelength and was measured with a photodiode. After proper AC amplification, the photodiode current was measured through 50 Ω by a transient digitizer equipped with a 1 MHz amplifier (for ΔA at 820 nm) or a 5 MHz amplifier (for ΔA at 515 nm).

On its fourth face the cuvette could be excited with short xenon flashes (about 5 μs) of intensity sufficient to saturate the Photosystem II photochemistry. In order to measure the variations of relative chlorophyll *a* fluorescence yield in a given cuvette, we made use of another xenon flash (1 μs duration, low energy) the light of which was filtered with blue filters (Corning CS-4-96 and Schott BG 18/3) and directed on to the cuvette by a piece of glass inserted in the measuring beam for ΔA (between the first filter and the cuvette). Light emitted by the chloroplasts was measured with the same photodiode as for ΔA measurements, but the second filter was replaced by red filters (Corning 2-58 and 4-77). All these measurements were done at 21°C.

Measurement of absorption changes in the nanosecond range

The material was contained in a 10 $\times$ 10 mm cuvette with four clear

windows (measurements at 294 K) or in a 2-mm path cuvette inclined at 45° to the mutually perpendicular measuring and exciting beams (see Ref. 14) (for measurements at low temperature 60% glycerol was added). The exciting pulse was provided by the YAG-pumped dye laser. The measuring light was provided by a xenon flash of about 100 μs duration (see ref. 14 or 16). Matched interference filters ($\Delta\lambda$ = 2—3 nm) and blue-green glass filters (Corning CS-4-96 and Schott BG 38) were placed on the measuring beam, before the cuvette and in front of the photomultiplier. This one (type XP 1141, Radiotechnique) was especially wired for linearly measuring high light intensities (see Refs. 16 and 21). The photomultiplier output (I_{max} was included between 1 and 3 mA) was loaded with 50 Ω and fed into a 7912 Tektronix digitizer equipped with a 7A16A amplifier.

Results and Discussion

Measurement of P-680$^+$ and ^{3}Car at different laser energies

For each series of measurements the chloroplast concentration was kept identical, but the state of the Photosystem II reaction center was not the same. For the measurement of *P*-680$^+$, 5 mM of ferricyanide was added to the suspension in order to chemically oxidize *P*-700, and the chloroplasts were excited by two xenon flashes 100 ms and 50 ms before the laser flash. When the laser was fired the Photosystem II centers were open (state Q, PQ_1 oxidized), but the donor (D) to *P*-680 was oxidized (see Ref. 21). The laser flash induced a charge separation (*P*-680$^+$, PQ_1^-) which lasted for about 200 μs and disappeared largely by back-reaction. It is very difficult to measure ^{3}Car under these conditions because the measuring light at 515 nm has an actinic effect (this effect can be easily controlled by fluorescence measurements, see below) and tends to place the centers in the state Q^-. Thus we decided to place all the centers in the state Q^- (PQ_1 reduced) by adding $5 \cdot 10^{-6}$ M DCMU/1 mM ascorbate and firing two xenon flashes 100 ms and 50 ms before the laser flash.

The kinetic behaviour at the two wavelengths is shown in Fig. 1. At 820 nm the flash-induced ΔA decays in a biphasic manner, with a major phase ($t_{1/2} \approx$ 100 μs) and a much slower one. The fast phase corresponds to a back-reaction

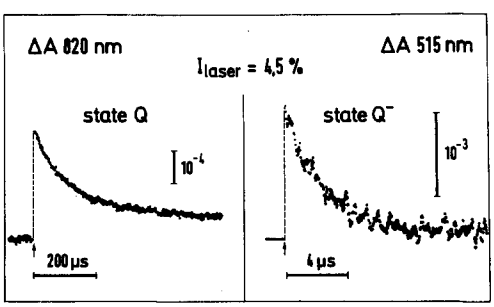

Fig. 1. Kinetics of flash-induced ΔA at 820 and 515 nm, in Tris-treated chloroplasts (chlorophyll concentration, 30 μM). Excitation by the ruby laser, attenuated to 4.5% of its maximum energy. Pre-illumination by two flashes. ΔA at 820 nm: addition of 5 mM potassium ferricyanide. ΔA at 515 nm: addition of 5 μM DCMU and 1 mM ascorbate.

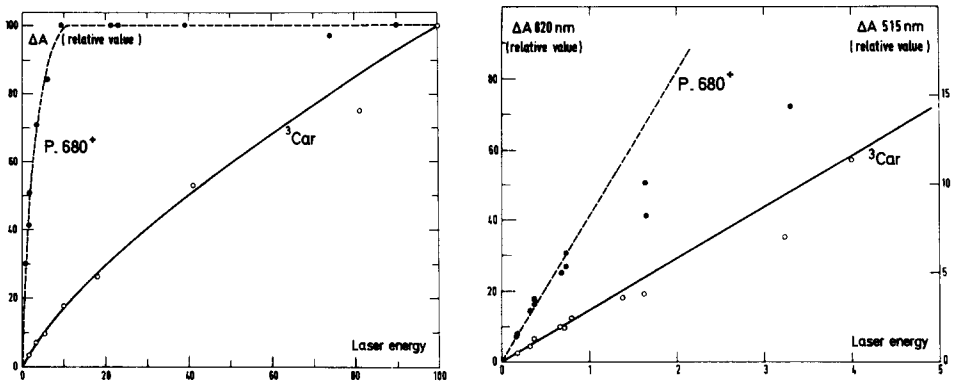

Fig. 2. Effect of ruby laser energy on the magnitude of absorption change (measured immediately after the flash) at 820 nm (dots, broken lines) and 515 nm (open circles, continuous lines). Conditions similar to those of Fig. 1. Chlorophyll concentration:28 μM. $\Delta A = 100$ corresponds to $3.5 \cdot 10^{-4}$ at 820 nm and to $16.3 \cdot 10^{-3}$ at 515 nm. The right-hand side of the figure is an enlargement showing the behaviour at low laser energy. The laser energy is expressed in relative units. $I = 100$ corresponds to approx. 10 mJ light falling on the cuvette, but the geometrical arrangement precludes precise measurement of the energy.

between $P\text{-}680^+$ and PQ_1^- and the slower one (10—20% of the total) may correspond to $P\text{-}680^+$ in those centers where the electron transfer from PQ_1^- to PQ_2 ($t_{1/2} \approx 600$ μs; Conjeaud, H. and Mathis, P., unpublished results) has occurred before the back-reaction. At 515 nm the flash-induced ΔA decays exponentially with $t_{1/2} = 1.9$ μs, in agreement with previous measurements for ^{3}Car. At 515 nm, a large absorption change is usually due to an electrochromic shift of the carotenoid absorption. This effect is small under our exprimental conditions because a large fraction of the reaction centers is brought in an active state before the laser flash. The electrochromic shifts decays slowly (in milliseconds) and is thus kinetically easily distinguishable from ^{3}Car. Varying the laser energy did not significantly alter the kinetics at both wavelengths. The magnitude of ΔA measured immediately after the flash has been plotted versus laser energy in Fig. 2, for a particular experiment with the ruby laser. At 820 nm, a clear saturation behaviour is observed, whereas at 515 nm the signal continues to increase at our maximum laser intensity, in agreement with a previously reported behaviour for ^{3}Car [7,8,14]. Around 820 nm, the absorption spectrum of $P\text{-}680^+$ is nearly the same as that of the chlorophyll *a* cation radical [23], the extinction coefficient of which is 7000 $M^{-1} \cdot cm^{-1}$ [24]. Accepting the same value for $P\text{-}680^+$, we find that one $P\text{-}680^+$ is formed for 450 chlorophyll molecules. For ^{3}Car a value of 100 000 $M^{-1} \cdot cm^{-1}$ can be accepted for its extinction coefficient at 515 nm [16]. At our maximum laser energy we then find one ^{3}Car per 160 chlorophyll molecules.

In Fig. 2, it is apparent that, at low laser energy, the amounts of $P\text{-}680^+$ and of ^{3}Car formed by the flash are proportional to the flash energy. With the previous assumptions for the extinction coefficients, it is possible to derive a relative quantum efficiency for the formation of ^{3}Car (QE^T) and for the formation of P-680$^+$ (QE^{+-}). Accepting for the moment that $QE^{+-} = 100$, it appears that $QE^T = 24.9$. With the dye laser the results were very similar to those of Figs. 1 and 2 and we obtain a value $QE^T = 31$.

Formation of ^{3}Car and chlorophyll a fluorescence yield

Under our experimental conditions we have measured the increase of chlorophyll *a* fluorescence yield in going from state Q to state Q^- by monitoring the fluorescence excited by a very weak xenon flash. We have found an increase by a factor 3.5. We attempted to measure whether the formation of ^{3}Car increases in the same proportion. This measurement is difficult to make, for two reasons. First, the measuring light at 515 nm has some actinic effect and tends to bring some of the Photosystem II centers into the state Q^-. The measuring light intensity cannot be kept too low, however, since we need a good time resolution and a reasonable signal-to-noise ratio. Second, the measurement has to be done with a weak laser flash since, as shown in Fig. 2, the quantum yield of ^{3}Car formation decreases very much when the laser energy is increased.

The experimental conditions chosen were a compromise between the different requirements. The measurements were performed with Tris-treated chloroplasts ($1.6 \cdot 10^{-6}$ M of chlorophyll). We used the dye laser and its energy was chosen so as to effect about 50% of the *P*-680 oxidation, i.e., at a laser energy where the quantum yield of ^{3}Car formation was already slightly decreased (see Fig. 2). With dark-adapted chloroplasts, without addition, the flash excitation led to the formation of one ^{3}Car per 4850 chlorophylls, in average of 24 experiments. With chloroplasts brought in the Q^- state (addition of 1 mM ascorbate/10 μM DCIP and 10 μM DCMU; one saturating xenon flash was given before the laser flash), we obtained the formation of one ^{3}Car per 2560 chlorophylls. The 515 nm light had an actinic effect, which could be evaluated by measuring the fluorescence induced by the weak xenon flash with chloroplasts illuminated with the measuring light at 515 nm (relative fluorescence yield = 100) and with chloroplasts brought into pure Q or Q^- states (relative fluorescence yields = 71.5 and 250, respectively). Thus the fluorescence yield increases 2.5-times and the triplet yield 1.9-times in going from the mixed state to the Q^- state. The figure for the triplet yield may be somewhat underestimated since, at our flash intensity, the quantum yield of ^{3}Car formation is slightly reduced. Our result, although not being fully conclusive, nevertheless indicates that ^{3}Car formation and fluorescence yield increase in nearly the same proportion upon formation of the state Q^-.

Measurements of the rate of formation of ^{3}Car

The rise time of the absorption change at 515 nm has been measured with broken thylakoid membranes (hereafter named chloroplasts), as described in Material and Methods. In order to have a reasonable signal-to-noise ratio (the fluctuations of the xenon flash profile are the major source of the noise) the measurements had to be done under conditions of rather strong excitation, giving a ΔA of $(1—3) \cdot 10^{-2}$ (about one ^{3}Car per 200 chlorophyll molecules). Under those conditions the ΔA values at 515 nm due to the electron carriers are negligible and they are essentially due to ^{3}Car. Moreover these measurements are effected with a strong measuring light which probably saturates the reaction center photochemistry. ΔA values at 515 nm were measured under identical conditions with chloroplasts and with degassed chlorophyll *a* dissolved in ethanol. A typical result (Fig. 3) shows that the curve for chloroplasts rises slightly slower than the curve for chlorophyll *a*.

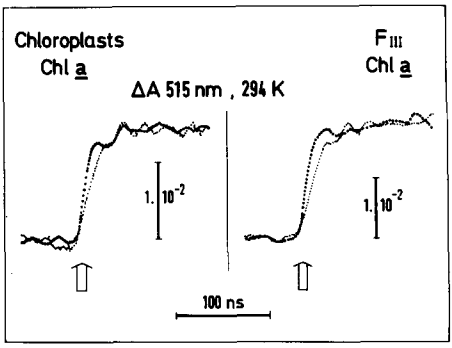

Fig. 3. Submicrosecond kinetics of ΔA induced in broken thylakoid membranes (chloroplasts, left, fainter line), in F_{III} particles (right, fainter line) and in two samples of Chl *a* in ethanol (intense lines). Average of 20 experiments for each trace. The signals for Chl *a* have been adjusted to the same size as the signal with chloroplasts or F_{III}. Experiments at 294 K. Chlorophyll concentration: chloroplasts, $4 \cdot 10^{-5}$ M; F_{III}, $1.8 \cdot 10^{-5}$ M.

The curves can be analyzed as follows. With chlorophyll *a* we assume that the following reactions take place:

$$\text{Chl } a \xrightarrow{h\nu} \text{Chl}^*a(\text{singlet}) \rightarrow {}^3\text{Chl } a(\text{triplet})$$

The time course for chlorophyll *a* represents the process of excitation (by the flash of a finite duration), of intersystem crossing, and also the response time of our apparatus. Let us call this time course $X(t)$ and $Z(t)$ the equivalent curve obtained with chloroplasts. The latter now also includes the transfer time from ^{3}Chl *a* to carotenoids ($^3\text{Chl } a \rightarrow {}^3\text{Car}$) which we assume is a first-order process with a rate k_T. It can be shown (Paillotin, G, personal communication, and Ref. 16) that:

$$X(t) - Z(t) = \frac{1}{k_T} \cdot \frac{dZ}{dt} .$$

This relation permits a direct determination of k_T from two curves in Fig. 3. On the average we find, for chloroplasts at 294 K, $k_T = 8.3 \cdot 10^7\ s^{-1}$ and thus the rise-time τ is 12 ns. This rise-time is shorter than that previously reported (29 ns in Refs. 16 and 25), probably because of a more rapid apparatus response time. We assume that τ is the lifetime of ^{3}Chl *a* in the chloroplasts, or equivalently the lifetime of formation of ^{3}Car. Upon lowering the temperature the transfer becomes somewhat slower (Table I).

TABLE I

DECONVOLUTED RISE-TIME OF THE FLASH-INDUCED ΔA AT 515 nm (TO 1—(1/*e*) OF THE MAXIMUM), WITH SEVERAL MATERIALS AND AT SEVERAL TEMPERATURES

T (K)	Deconvoluted rise-time, τ, at 515 nm (ns)				
Material:	Chloroplasts	F_{III}	CP-II	CP-III	CP-IV
294	12	10	13	16	20
77	16	12			22
4	40			35	40

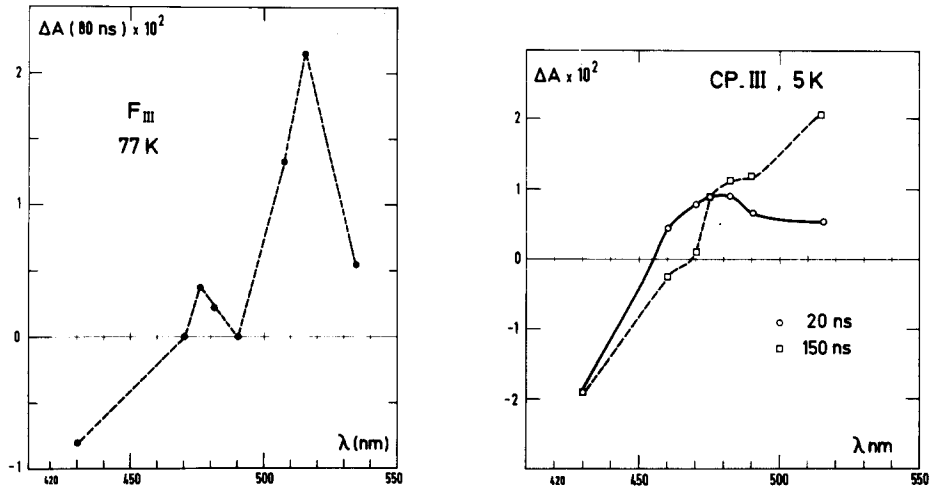

Fig. 4. Difference spectrum of flash-induced ΔA at 80 ns after the flash, with F_{III} particles, at 77 K. 2 mm path cuvette; chlorophyll concentration: $4.5 \cdot 10^{-5}$ M. Number of experiments, 10—30 at each wavelength.

Fig. 5. Difference spectrum of flash-induced ΔA at 20 ns or 150 ns after the flash, with CP-III particles, at 5 K. 2 mm path cuvette; chlorophyll concentration: $3.6 \cdot 10^{-5}$ M. Number of experiments: 20—40.

Similar measurements were conducted with several light-harvesting complexes. In the F_{III} particles the rise-time of the absorption increase at 515 nm is very short too (Fig. 3 and Table I). With these particles we were able to measure a difference spectrum of flash-induced ΔA at 80 ns after the flash. As shown in Fig. 4, this spectrum presents a peak around 515 nm and smaller ΔA values at shorter wavelength. It closely resembles the difference spectrum of ^{3}Car measured about 1 μs after the flash (see Ref. 14). The CP-II particles (light-harvesting complexes prepared with SDS) behave like the F_{III}.

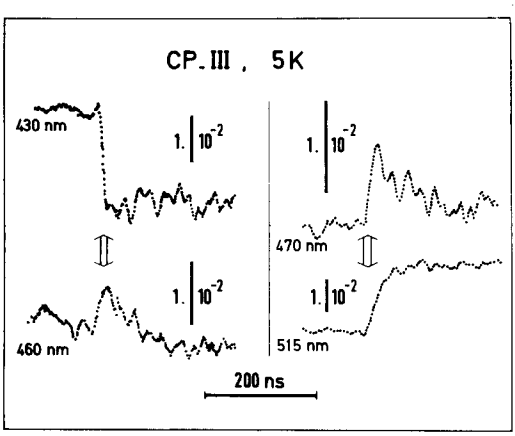

Fig. 6. Submicrosecond kinetics of ΔA induced at 5 K in CP-III particles at 430 nm (average of 40 experiments), 460 nm (40 experiments), 470 nm (40 experiments), 515 nm (20 experiments). 2 mm path cuvette; chlorophyll concentration, $3.6 \cdot 10^{-5}$ M.

In the CP-III and CP-IV particles the kinetics appear to be somewhat slower and they are slowed down at low temperature (Table I). At 5 K the transfer time is significantly longer than the apparatus response time and thus the absorption of ^{3}Chl *a* should be detectable in short times after the flash. With CP-III, the difference spectrum is indeed rather different (Fig. 5) when it is measured immediately (20 ns) or some time (150 ns) after the flash. The kinetic properties at different wavelengths are modified accordingly (Fig. 6). A similar behaviour was observed with the CP-III and the CP-IV particles, but we were not able to do these measurements with the other materials. The difference spectrum measured at 20 ns with the CP-III particles at 5 K (Fig. 5) is in good agreement with that of the triplet state of chlorophyll *a* [16,26]. At 150 ns the spectrum resembles that of ^{3}Car, but it is not typical since the difference spectrum of ^{3}Car usually is rather weak around 475—490 nm. Since the CP-III particles contain only chlorophyll *a* and carotenoids as pigments, we make the hypothesis that we are indeed concerned with a carotenoid triplet the absorption band of which is broader than usual.

Conclusions

The first part of our work indicates that the relative quantum yield of ^{3}Car formation (with the Photosystem II reaction center in state Q^-) is about 30% of that of *P*-680 photo-oxidation in open reaction centers. This value was obtained with a dye laser of which the wavelength (about 595 nm) excites in the same proportion the photochemistry of PS I and of PS II [27]. Since electron transfer at both PS I and PS II centers takes place with a quantum yield close to unity [28], we conclude that the quantum yield of ^{3}Car formation in state Q^- is close to 15%. Although this evaluation requires a few hypothesis on the extinction coefficients of ^{3}Car and of *P*-680$^+$ and on the energy distribution in the photosystems, it is the result of a much more direct approach than estimations based on chlorophyll fluorescence quenching. Our value is in good agreement with that obtained by den Haan [12] from a careful study of the fluorescence quenching. Our parallel measurements indicate that the chlorophyll *a* fluorescence and the ^{3}Car yield vary nearly in parallel in going from state Q to state Q^-. If we suppose a strict relation and since we found that the chlorophyll *a* fluorescence yield varies by a factor 3.5, we may propose that the quantum yield of ^{3}Car formation is about 4.5% with the chloroplasts in state Q (open PS II centers).

Since we assume a one-to-one triplet-triplet energy transfer from chlorophyll to carotenoids, we conclude that the quantum yield of ^{3}Chl *a* formation is 4.5% in state Q. It is interesting to compare this last figure with the Chl *a* fluorescence quantum yield of approx. 2% determined in state Q (Ref. 29; see also Ref. 30 for a discussion). The rate of intersystem crossing is twice that of fluorescence for Chl *a* in solution [17]. In the absence of any specific mechanism affecting the relative rates, the same ratio should also hold in chloroplasts. From our measurements we are thus led to a Chl *a* fluorescence quantum yield of 2.2% in state Q, in good agreement with direct fluorescence measurements. It is interesting to note that the minimum losses of quantum of excitation in chloroplasts are thus 6.7% and that some quantum efficiency values of electron

transfer may be slightly overestimated [28].

The relative quantum yields of formation of ^{3}Car and of *P*-680 oxidation are nearly the same with the ruby laser (λ = 694 nm) and with the dye laser ($\lambda \approx$ 595 nm). At 694 nm the PS I center has a much greater probability of being excited than the PS II center. Our results simply show that a negligible formation of ^{3}Car is induced by far-red light specifically absorbed by PS I, in agreement with Joliot et al. [31]. ^{3}Car has been shown to be formed in particles enriched in PS I, but mostly at low temperature (Ref. 14; some data of Ref. 32 may also be interpreted in that manner). The absolute quantum yield of ^{3}Car formation is probably smaller upon excitation at 694 nm than around 595 nm. This factor, in addition to the use of too high flash intensities may explain the low quantum yield of ^{3}Car previously reported (1—2% in Ref. 16).

In conclusion to the transfer time measurements we can say that the transfer from ^{3}Chl *a* to carotenoids is a very fast process which is not much slowed down at cryogenic temperature. The transfer is equally fast in several isolated light-harvesting complexes, in which the respective geometrical arrangement of chlorophylls and carotenoids is thus not greatly perturbed.

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