Biochimica et Biophysica Acta 893 (1987) 49–59
Elsevier

BBA 42564

Primary radical pair in the Photosystem II reaction centre

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(Received 17 February 1987)

Key words: Photosynthesis; Electron transfer; Triplet state; Pheophytin; Carotenoid

Primary Photosystem II (PS II) reactions have been studied by flash absorption spectroscopy with nanosecond resolution. In isolated reaction centers which are devoid of the 47- and 43-kDa chlorophyll-binding polypeptides, an absorption change (ΔA) is induced immediately by a flash. The difference spectrum (450–560 nm, 820 nm) indicates the formation of a radical pair (P-680$^+$, Pheo$^-$) decaying with $t_{1/2} = 40$ ns at 120 K. At 140 ns after the flash the ΔA can be attributed to the triplet state of P-680 (^{3}P-680) and of a carotenoid (^{3}Car, $\lambda_{max} = 545$ nm). ^{3}Car is formed with a yield of $\approx 3\%$ and it rises with $t_{1/2} \approx 12$ ns. At 276 K, the radical pair decays, with $t_{1/2} \approx 32$ ns. One radical pair is formed per 20 chlorophylls. The data are best interpreted if ^{3}P-680 is formed as a product of radical pair recombination with a yield of 23% at 276 K (80% at 10 K) and if ^{3}Car is formed only in a minority of pigment complexes. The ΔA at 820 nm disappear under conditions (addition of dithionite and methyl viologen, plus continuous illumination) designed to reduce pheophytin. The signals reappear after turning off the continuous light. More intact PS II particles (core complex) were also studied. At 820 nm, the data show that P-680$^+$ is formed under oxidizing conditions and is re-reduced in the microsecond time range. Under reducing conditions the primary biradical decays with $t_{1/2} = 25$ ns. ^{3}P-680 is formed, with properties analogous to those in isolated reaction centers. ^{3}Car is also formed ($\lambda_{max} = 535$ nm). The results show that the PS II reaction center has strong functional analogies with the reaction center of purple bacteria. The behavior of the carotenoid is a remarkable exception. The primary biradical decays much more slowly than expected on the basis of fluorescence measurements.

Introduction

In photosynthetic organisms, several classes of reaction centers share common properties in their conversion of light energy into chemical energy. In particular, the photosystem II (PS II) reaction center of plants and the reaction center of purple bacteria have many similar structural and functional properties, the detailed study of which has often been a source of experimental and conceptual progress [1–6]. The reaction center of purple bacteria has long been isolated in a pure form, as required for many spectroscopic and biochemical investigations [7]. Similar perspectives are offered by the recent preparation from PS II of a rather simple pigment-protein complex which resembles an isolated reaction center [8]. It comprises two

Abbreviations: ^{3}Car, carotenoid triplet state; P-680, primary donor of Photosystem II; ^{3}P-680, triplet state of P-680; Pheo, pheophytin; P, primary electron donor; I, primary electron acceptor; PS II, Photosystem II.

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polypeptides of 32–34 kDa apparent molecular mass, also known as D_1 and D_2 polypeptides, cytochrome *b*-559, five chlorophyll *a*, two pheophytin *a* and one β-carotene molecules. Photoreduction of one pheophytin by continuous light under reducing conditions showed that this preparation is photochemically active, and the observation of a spin-polarized triplet state indicates that the primary photochemistry functions normally [9]. In this work we applied flash excitation techniques to understand the functional properties of this PS II preparation, by direct observation of the primary radical pair and of the products of its evolution. The results are in good agreement with the preparation being the PS II reaction center, as will be described below.

In bacterial reaction centers the primary photochemical act produces the biradical P^+I^- (P, primary electron donor; I, primary acceptor, bacteriopheophytin). When electron transfer away from I is impossible, the biradical state lasts for a few nanoseconds and eventually recombines, leading to four neutral states: the excited singlet state and the ground state of P, the triplet state of P (^3P), and the triplet state of a carotenoid (^3Car). Formation and decay of these states can be followed by measuring flash absorption transients due to P^+I^-, ^3P or ^3Car [10,11], EPR signals of the triplet states [12,13] or delayed light emission [14,15]. These studies led to a reasonable understanding of the reaction routes and mechanisms, as well as of the role of the several molecules involved. Similar detailed studies have not been performed on PS II, essentially because of the inavailability of isolated reaction centers: in larger membrane fragments, the signals were of small amplitude and the properties under investigation were made more complex by the interaction of the reaction center with its associated light-harvesting antenna. A spin-polarized triplet state has been evidenced by EPR and attributed to the primary donor, P-680 [16]. Absorption data gave information on its kinetics of decay, absorption spectrum and yield of formation [17,18]. Nanosecond absorption data are particularly scarce. A duration of a few nanoseconds for the primary biradical has been reported, together with a rapid formation of ^3Car, but the experiments lacked signal-to-noise ratio [19,20]. There are now many experimental arguments in favor of pheophytin *a* being the primary acceptor I [21], but the fate of the biradical P^+I^-, when forward electron transfer is blocked because the quinone Q_A is either reduced or absent, is poorly understood. This block in electron transfer leads to an increased chlorophyll fluorescence yield, the origin of which is the object of two widely different theories: the increased fluorescence may be a delayed light resulting from the P^+I^- charge recombination, or it might reflect the reduced ability to realize the $P^*I \rightarrow P^+I^-$ charge separation when Q_A is reduced [21–27]. The present work produces some data in partial answer to the above-mentioned questions. The biradical P^+I^- is studied in PS II reaction centers (absorption spectrum, yield of formation, routes of evolution), and similar properties are studied in the PS II core complex, where the reaction center is associated with an antenna of about 60 chlorophyll molecules.

Materials and Methods

Biological materials

The PS II reaction center was purified from spinach thylakoids as described previously [8]. Polypeptide analysis by gel electrophoresis and by immunological detection revealed only the D_1 and D_2 polypeptides and cytochrome *b*-559 [28]. The material was kept frozen at −70 °C in 50 mM Tris-HCl (pH 7.2)/30 mM NaCl/10% (w/v) glycerol/0.05% Triton X-100, at a chlorophyll concentration of $(1–2) \cdot 10^{-4}$ M. The PS II core complex was purified as described previously from digitonin extracts of spinach thylakoid membranes by two-step chromatography using DEAE-Toyopearl 650S (Toyo Soda, Yokyo, Japan) [29]. This material was kept frozen at −70 °C in 50 mM Tris-HCl (pH 7.2)/50 mM NaCl/10% glycerol/0.2% digitonin at a chlorophyll concentration of about 10^{-3} M. This PS II core complex was previously named 'reaction center'; a new name is now chosen because of the recent isolation of the reaction center, as mentioned above, and in agreement with a proposal on nomenclature [30]. The PS II particles were thawed before use, kept in darkness on ice, and diluted as described in Results.

Spectroscopic measurements

Absorption changes at 820 nm in the nanosecond range were measured with an apparatus essentially as described in Ref. 31, with modifications of individual components [32]. The excitation pulse was from a frequency-doubled mode-locked neodymium-YAG laser (532 nm; duration about 20 ps; 0.2–5 Hz repetition rate; energy of 7 mJ before attenuation). The measuring light from a laser diode (Telefunken, type TXSK, 820 nm) was focused through the cuvette on a silicon photodiode (Lasermetrics, model 3117), the output of which was amplified 100 × in two stages (Nuclétude, 1 GHz). The cuvette (height = 8 mm; optical paths as given in figure legends) was cooled at +3° C. The response time of the apparatus was $(0.5–1.0) \cdot 10^{-9}$ s.

Measurements in the microsecond time domain were performed as previously [33], with a 10 × 10 mm cuvette at 21° C, or with a 1.1 mm wide plexiglass cuvette for measurements at low temperature, in a cryostat cooled by a flow of helium gas.

For measurements in the 450–560 nm region, with nanosecond time resolution, a new set-up was built, using a frequency-doubled nanosecond YAG laser as source of excitation light. This pulse (of 9 ns f.w.h.m.) was used after pumping a dye laser with rhodamine 6G (broadband around 595 nm). The measuring light was provided by a xenon flash tube (USSI, type 1CP3) operated at 700 V, with a capacitor of 24 μF and with insertion of an inductance for lengthening its duration (around 20 μs f.w.h.m.). After passing the cuvette, the measuring light went through a monochromator (Jobin-Yvon, model H25) and was measured with a silicon photodiode (EGG, model FND100). After a 10 × amplification the signal went through a high-pass filter ($t_{1/2} = 160$ ns) and was then recorded in numerical form by a transient digitizer (Tektronix, type R7912). The time response of the apparatus was about 14 ns f.w.h.m. for a pulse and 20 ns (a rise from 10 to 90%) for a step function.

Results

Nanosecond absorption changes with PS II reaction centers

Excitation of PS II reaction centers with a picosecond laser pulse induces an absorption increase at 820 nm, which decays essentially as a single exponential with $t_{1/2} = 32$ ns (Fig. 1). The absorption rises within 1 ns and the decay includes no significant component of 1–2 ns (Fig. 1a). Varying the laser intensity, it appears that the initial ΔA shows a tendency to saturate, but a true saturation was clearly not achieved (Fig. 1b). If we suppose that the ΔA is due to the formation of a radical pair with P-680$^+$ and reduced pheophytin, with respective extinction coefficients of 7000 and 5400 $M^{-1} \cdot cm^{-1}$ at 820 nm [34,35], it turns out that one biradical is formed per 20 chlorophylls, at our maximum laser intensity. As shown in Fig. 1c, the absorption decay in fact includes a slow component which is due to the triplet state of P-680 (see below).

The absorption transient is not influenced by the addition of ferricyanide (0.5 mM) or dithionite (1 mM). With the addition of dithionite and 10 μM methylviologen, under continuous illumination by a 600 W halogen lamp, the size of the

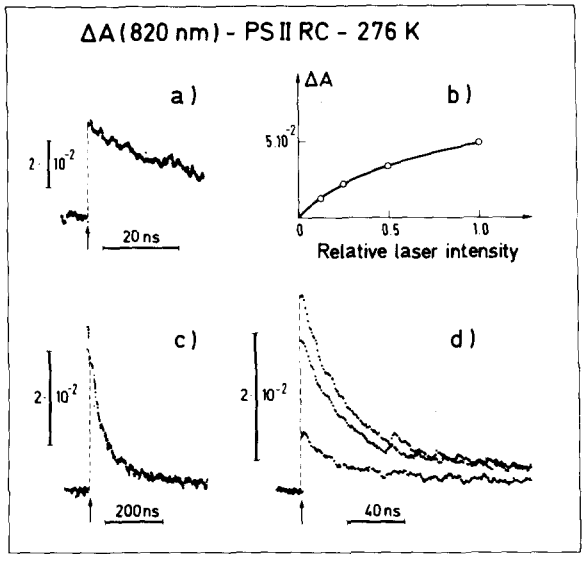

Fig. 1. Absorption changes at 820 nm induced in PS II reaction centers ($2.0 \cdot 10^{-5}$ M chlorophyll) at 276 K by a picosecond laser flash. Optical paths in the cuvette: 30 mm (measure); 3 mm (excitation). a, b, c, No addition; d, addition of 1 mM dithionite and 10 μM methyl viologen; upper, middle and lower traces, before, after and during continuous illumination. b, Initial ΔA in condition a, at different laser intensity. a, c, d, maximum laser intensity. Average effect of two (a) or eight flashes (b, c, d).

signal is greatly reduced, but it comes back close to its initial level when light is turned off (Fig. 1d). These conditions, with continuous light, have been shown to induce the reduction of pheophytin in the reaction centers [8], and it is understandable that the transient absorption disappears. With our time resolution we see no faster kinetics which would indicate an electron acceptor more primary than pheophytin or a long-lived excited state. As shown in Fig. 1d, a decrease in the amplitude of the fast phase of decay is accompanied by an equivalent decrease in the slow phase, showing a correlation between both properties.

In the blue-green part of the spectrum (450–560 nm), similar measurements were performed using another set-up, as described in Materials and Methods, with a nanosecond laser for excitation and a xenon flash for analysis. The measurements were done at a lower temperature, 120 K, to avoid potential aging and damaging of the sample, due to the long duration of the experiments and to the actinic affect of the measuring light. Absorption kinetics were dependent on wavelength (Fig. 2). In the 450–500 nm region an initial increase is followed by a decay, with an overall $t_{1/2}$ of 47 ns. In fact, the decay is biphasic, with a fast phase ($t_{1/2} \approx 40$ ns) followed by a slower phase (with the AC-coupling used for the experiments of Fig. 2, a non-decaying ΔA would apparently decay with $t_{1/2} = 160$ ns). Around 540 nm, the ΔA rises with some delay and then decays only little in our time scale (Fig. 2, 545 nm).

From traces obtained at different wavelengths we have measured the signal at 13, 23 and 140 ns after a time defined as time zero (this is somewhat arbitrary since the actinic flash has a duration not negligible compared to the kinetics observed; time zero is the time where ΔA starts to rise at 465 nm; see Fig. 2). The difference spectrum largely varies with time (Fig. 3). At 13 ns, presumably only the initial species is present. The spectrum includes a large peak at 460 nm and two well-defined troughs at 515 and 545 nm. At 23 ns, the ΔA has increased somewhat at 460 nm (this increase presumably reflects the apparatus response time) and more strongly in the green region. At later times, the ΔA continues to increase around 540 nm and decreases in the blue. At 140 ns the features observed at 13 ns have disappeared; two peaks are present at 470 and 540 nm. As shown below from measurements on a slower time scale, these peaks can be attributed respectively to the triplet state of P-680 and β-carotene. The decay of absorption at 465 nm mainly reflects the decay of the biradical. Its apparent $t_{1/2}$ increases by a factor of 1.4 when

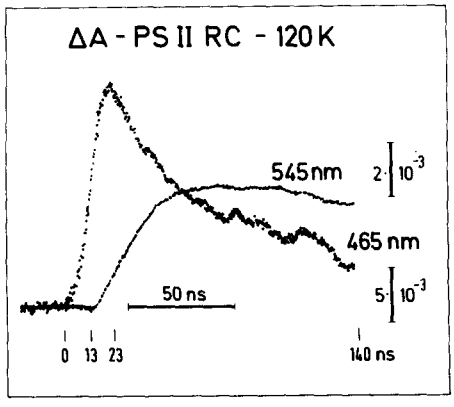

Fig. 2. Absorption changes at 465 and 545 nm induced in PS II reaction centers with 65% glycerol ($2.4 \cdot 10^{-5}$ M chlorophyll) at 120 K by a nanosecond laser flash (595 nm). Optical paths in the cuvette, 10 mm (measure); 6 mm (excitation). Average effect of 20 (465 nm) or 10 (545 nm) flashes. Vertical bars indicate the times where measurements were made for the spectrum of Fig. 3.

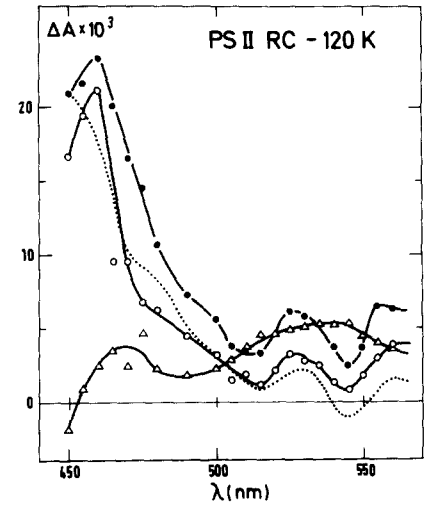

Fig. 3. Difference spectra measured under the conditions of Fig. 2. Measurements were made at 13 (○), 23 (●) and 140 (△) ns, as defined in Fig. 2. A drawn curve (dashes) was calculated for the radical pair ($P\text{-}680^+$, $Pheo^-$) from data of Refs. 8, 36 and 37. This curve was normalized at the maximum ΔA with the data at 13 ns.

temperature decreases from 135 K (≈ 40 ns) to 5 K (≈ 55 ns). The absorption increase at 545 nm mainly reflects formation of the carotene triplet state (^{3}Car); it has a rise in $t_{1/2}$ of about 12 ns, which is somewhat limited by the apparatus time-response. This rise appears to be faster than the biradical decay. A better signal-to-noise ratio and a faster time response would be required to establish that point.

It is worth noticing that the spectrum at 13 ns corresponds reasonably well to that due to the formation of P-680$^+$ and reduced pheophytin as shown in Fig. 3 by comparison with published spectra [8,36,37]. The troughs at 515 and 545 nm are clearly attributable to pheophytin reduction [8]. The more positive absorption in the 540 nm region could well be due to a contribution of ^{3}Car already at 13 ns.

Measurements in the microsecond range with PS II reaction centers

Excitation of PS II reaction centers with a nanosecond laser pulse induces absorption transients in the microsecond time domain which were measured at various temperatures. Some data obtained at 50 K are shown in Figs. 4 and 5. At 540 nm the ΔA decays biphasically with $t_{1/2}$ of 9 ± 1 μs and 0.9 ± 0.1 ms. A decomposition of the kinetics gives a band centered at 540 nm for the fast component, which is very probably due to ^{3}Car. The slow phase gives a flat spectrum between 490 and 560 nm, and an increase toward 460 nm. In

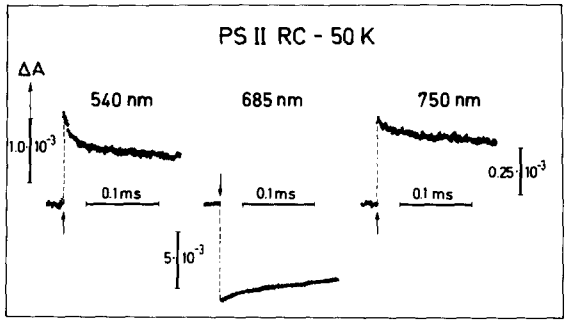

Fig. 4. Absorption changes at 540, 685 and 750 nm induced in PS II reaction centers ($2.2 \cdot 10^{-5}$ M chlorophyll) at 50 K by a nanosecond laser flash (595 nm). Addition of 65% glycerol. Cuvette thickness, 1.1 mm. Averaged effect of eight (540 and 750 nm) or four (685 nm) flashes.

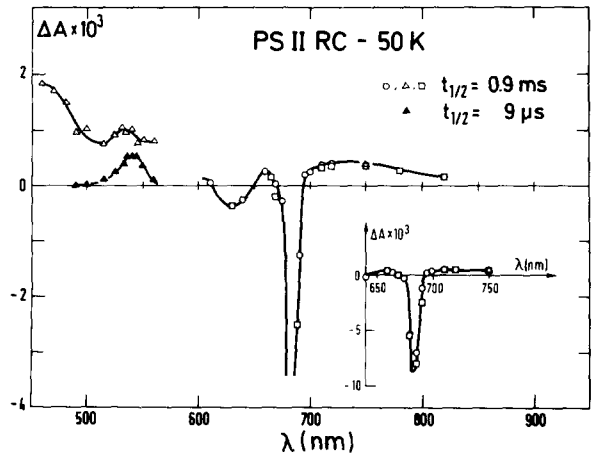

Fig. 5. Difference spectrum of flash-induced ΔA measured under the conditions of Fig. 4. Open symbols, signal component decaying with $t_{1/2}$ = 0.9 ms (three different cuvettes were used; the results are noted with different symbols). Filled triangles, signal component decaying with $t_{1/2}$ = 9 μs. Averaged effect of 4–20 flashes.

the red and infra-red, its spectrum includes a strong and sharp bleaching at 680–685 nm and a broad band at 730–750 nm, both features being typical of the formation of a chlorophyll *a* triplet state [34,38]. The kinetic and spectral properties fit those reported for the triplet state of P-680, in larger PS II particles with reduced Q_A [17,18].

Temperature influences both the size and the kinetics of ΔA in most spectral regions. The effect is rather weak, however, for ^{3}Car measured at 540 nm: the $t_{1/2}$ of decay varies from 5 μs at 240 K to 9 μs at 5 K, in agreement with previous reports [39], and the size of the signal also varies little (Fig. 6). The properties of ^{3}P-680 have been studied at 540 and 750 nm, for broad absorption bands presumably little affected by temperature, rather than at 680–685 nm where low temperature induces a narrowing of the band and a concomitant increase in the maximum intensity. The decay of ^{3}P-680 varies from 30 μs at 276 K to 0.9 ms at 5 K, being nearly temperature-independent between 120 and 5 K. The relative yield of ^{3}P-680 increases upon lowering the temperature, as shown in Fig. 6. This increase is qualitatively similar to that reported for the triplet of the primary donor in reaction centers from carotenoid-less photosynthetic bacteria [10,40]. The behaviour of ^{3}Car,

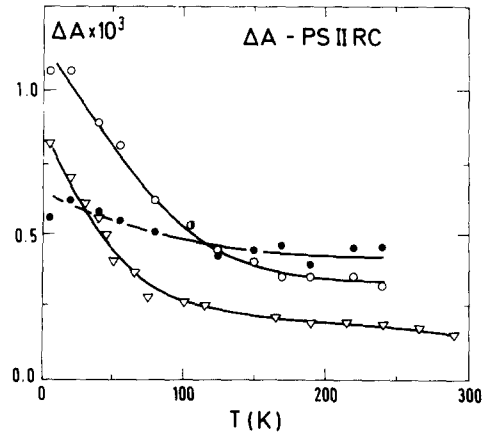

Fig. 6. Influence of temperature on the size of ΔA induced in PS II reaction centers, under the conditions of Fig. 4. Averaged effect of eight flashes for each point. Measurements at 540 and 750 nm correspond to two separate experiments. 750 nm (▽), total ΔA; 540 nm, fast (●) and slow (○) phases of decay.

however, is totally different from that reported in carotenoid-containing bacterial reaction centers, in which the formation of ^{3}Car disappears steeply below 60 K [11,40].

Experiments with PS II core complexes

The PS-II core complexes used in this work were prepared by treatment with digitonin. They comprise the reaction center and the PS II core antenna of about 60 chlorophyll *a* [41]. Flash excitation of the PS II core under oxidizing conditions with a picosecond laser pulse produces an absorption increase at 820 nm (Fig. 7a) that we attribute to the oxidation of P-680 (see below). The signal practically does not decay within 150 ns. Assuming an extinction coefficient of 7000 $M^{-1} \cdot cm^{-1}$ for P-680$^+$, the ΔA indicates one P-680$^+$ per 78 chlorophyll *a*. Addition of dithionite to the core particles results in a 10% larger ΔA which decays nearly as in reaction centers (Fig. 7b,c). There is, however, a small (15%) fast phase ($t_{1/2} = 4$ ns), while the major part of the signal decays with $t_{1/2} = 25$ ns. The ΔA saturates clearly with laser intensity (Fig. 7d). With the hypothesis that, with dithionite, the ΔA is due to the primary biradical (P-680$^+$, Pheo$^-$), we obtain one biradical per 125 chlorophyll *a* at saturating excitation.

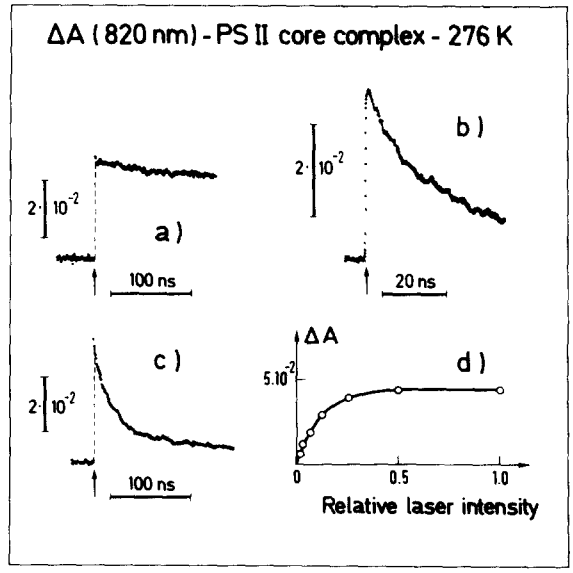

Fig. 7. Absorption changes at 820 nm in PS II core particles ($4.5 \cdot 10^{-4}$ M chlorophyll) at 276 K by a picosecond laser flash. Optical paths in the cuvette, 10 mm (measure); 1 mm (excitation). Addition of 80 μM potassium ferricyanide. a, No further addition; b,c,d, further addition of 12 mM dithionite. The number of flashes averaged was five (a,c,d) or ten (b). Laser intensity, 50% of the maximum for a,b,c.

Measurements in the microsecond range at 820 nm show that, with addition of ferricyanide, P-680 is oxidized and then re-reduced by the donor Z in a pH-dependent manner, as expected ($t_{1/2} = 40$ μs

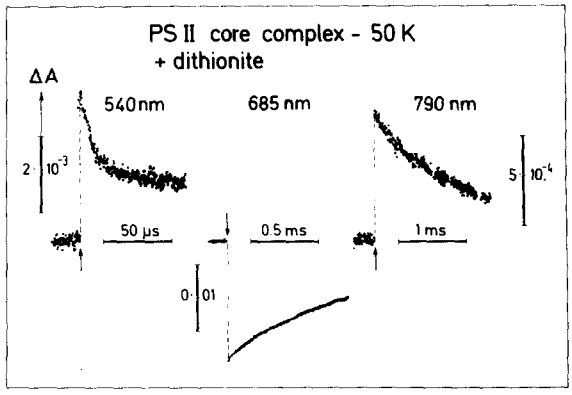

Fig. 8. Absorption changes at 540, 685 and 790 nm induced in PS II core particles ($2.2 \cdot 10^{-4}$ M chlorophyll) at 50 K by a nanosecond laser flash (595 nm). Addition of 65% glycerol and 10 mM dithionite. Cuvette thickness, 1.1 mm. Average effect of four flashes.

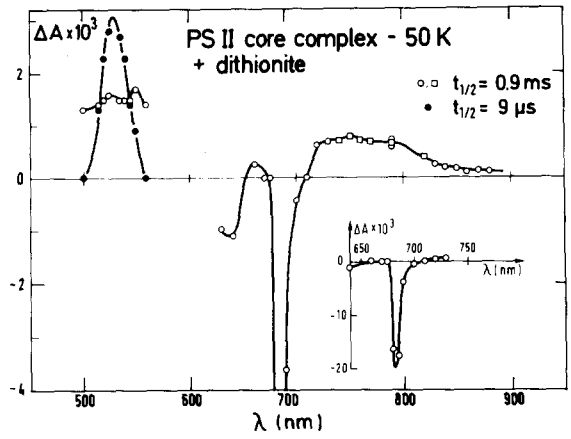

Fig. 9. Difference spectrum of flash-induced ΔA measured under the conditions of Fig. 8. Open symbols, signal component decaying with $t_{1/2} = 0.9$ ms (results from two different cuvettes). Closed circles, signal component decaying with $t_{1/2} = 9$ μs. Averaged effect of 4–10 flashes.

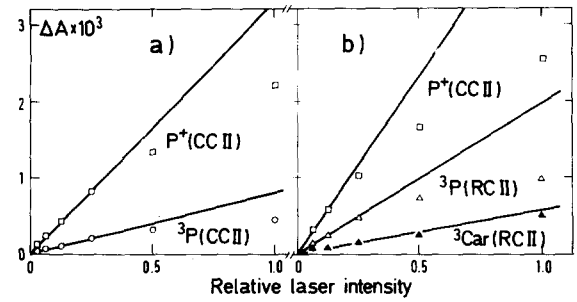

Fig. 10. Effect of nanosecond laser intensity (at 595 nm) on ΔA induced at 50 K in PS II reaction centers (b, open triangles: 0.9 ms decaying component; closed triangles: 9 μs decaying component; $4.4 \cdot 10^{-5}$ M chlorophyll; no addition; measurements at 540 nm) and in PS II core particles (a, squares: addition of 0.2 mM ferricyanide, $1.9 \cdot 10^{-4}$ M chlorophyll, 1.7 ms decaying component; circles: addition of 11 mM dithionite, $1.8 \cdot 10^{-4}$ M chlorophyll, 0.9 ms decaying compoennt; measurements at 820 nm); (b, squares: addition of 0.2 mM ferricyanide, $2.0 \cdot 10^{-4}$ M chlorophyll, 1.7 ms decaying component, measurements at 820 nm). Addition of 65% glycerol. Cuvette thickness, 1.1 mm. Averaged effect of 4–16 flashes.

at pH 5, 20 μs at pH 6.0, 13 μs at pH 6.8, data not shown). We obtain one P-680$^+$ per 60–65 chlorophyll *a*. In the presence of dithionite, the ΔA at 820 nm, at room temperature, is greatly decreased, to 10% of its original value. A study versus wavelength (Figs. 8, 9), at different temperatures permits the demonstration of the formation of two triplet states: the triplet state of P-680 (large bleaching at 680–685 nm, broad band around 750 nm, flat spectrum around 530 nm, temperature-dependent decay: $t_{1/2} = 0.5$ ms at 160 K, 0.9 ms below 120 K) and the triplet state of carotene (well-defined maximum at 530 nm; $t_{1/2} = 7$–10 μs). The state ^{3}Car is relatively more populated than in reaction centers (Figs. 5, 9).

When temperature is decreased, the amount of ^{3}P-680, as measured by the ΔA at 750 nm, increases progressively, nearly as in reaction centers. The formation of ^{3}Car increases slightly (data not shown), as found earlier in thylakoid membranes [39].

Evaluation of relative yields

The preceding results and additional experiments at low excitation energy (Fig. 10) permit the evaluation of relative yields of formation of the several species observed. With the PS II core complex under oxidizing conditions, the amount of P-680 oxidized by a flash varies linearly with flash intensity, at low intensity (Fig. 10a). Under these conditions, we can write: ΔA (820 nm) $= a \cdot \varepsilon$(P-680$^+$) $\cdot E \cdot Abs \cdot \Phi_0$, where *a* is a constant under defined geometric conditions, *E* is the light energy at the cuvette, *Abs* is the fraction of exciting light which is absorbed (at 595 nm the absorption of the cuvette was always low and the absorption can be considered as homogeneous), and Φ_0 is the quantum efficiency of charge separation. Similar expressions can be written for the formation of ^{3}P-680 (measured at 820 nm, $\varepsilon = 3800$ $M^{-1} \cdot cm^{-1}$ or at 515 nm, $\varepsilon = 15\,000$ $M^{-} \cdot cm^{-1}$; Ref. 34) and of ^{3}Car (measured at 515 nm, $\varepsilon =$ 40 000 $M^{-1} \cdot cm^{-1}$ assuming $\varepsilon = 100\,000$ $M^{-1} \cdot cm^{-1}$ at the absorption peak; Ref. 42). All measurements were done with similar concentrations of PS II core complex, at 50 K, with identical geometric conditions. The comparison of the slopes at low flash intensity (Fig. 10a) gives the following values, after correction for the actual core complex (CC) concentration: the yield of ^{3}P-680 formation is Φ (^{3}P-680, CC) $= 0.5\Phi_0$, and the yield of ^{3}Car formation (data not shown) is Φ (^{3}Car, CC) $= 0.15\Phi_0$. These yields were measured under reducing conditions (Q_A reduced).

Similar measurements were made with PS II reaction centers (RC), at 50 K. In this case, however, we cannot measure directly the primary yield

of charge separation. The measurements were thus done by comparison with P-680 oxidation in core complex (Fig. 10b), after suitable correction for the different absorption of both types of material at 595 nm. The following values were obtained: the yield of ^{3}P-680 formation is Φ (^{3}P-680, RC) = $0.6\Phi_0$, and the yield of ^{3}Car formation is Φ (^{3}Car, RC) = $0.03\Phi_0$ (an extinction coefficient of 100000 $M^{-1} \cdot cm^{-1}$ was assumed for ^{3}Car at 540 nm).

With the temperature behavior depicted in Fig. 6 it is then possible to derive the triplet yields at 276 K, related to the quantum efficiency of charge separation in the PS II core complex: Φ (^{3}P-680) = $0.32\Phi_0$ in the core complex, and 0.23 or $0.32\Phi_0$ in reaction centers based on measurements at 750 or 540 nm, respectively. The ^{3}P-680 yield at 276 K can also be measured in nanosecond experiments, relatively to the biradical yield, from the ΔA remaining 400 ns after the flash, attributed to ^{3}P-680 (with an extinction coefficient of 3800 $M^{-1} \cdot cm^{-1}$ at 820 nm) compared to the initial ΔA due to the biradical (extinction coefficient of 12400 $M^{-1} \cdot cm^{-1}$ at 820 nm). Values of 23 and 33% are obtained for reaction centers and core complex, respectively. With the core complex, the yield of biradical formation is 62% of that of P-680$^+$ formation in the presence of ferricyanide, which brings us to Φ (^{3}P-680, CC) = $0.20 \cdot \Phi_0$, to be compared with $0.32\Phi_0$ obtained from microsecond measurements.

For reaction centers, an important objective is to derive the yield of biradical formation, which should tell us about the nativeness of the structure. If the triplet yield from the biradical is the same as in the core complex, it follows that the yield of biradical formation is $0.6/0.5 = 1.2$ times that in the core complex, at 50 K. The triplet yield from the biradical is effectively nearly the same in both preparations at 276 K, as seen in the preceding paragraph. Assuming this to be the case also at 50 K, we can thus conclude that, within our limits of accuracy, the quantum yield of biradical formation in the PS II reaction is the same as in the core complex (0.5–0.6 Φ_0).

Discussion

On the basis of earlier fluorescence decay measurements, the $t_{1/2}$ of the primary radical pair in PS II was estimated to be about 2 ns [21–24]. Absorption measurements with picosecond or nanosecond set-ups led to similar values, ranging from 1.8 to 6 ns [20,43,44]. Our measurements give widely larger values in reaction centers (32 ns) and core complexes (25 ns). A question to raise immediately is whether we are effectively observing the primary biradical. All our results, however, point in that direction. Firstly, we used simple pigment-protein complexes with only PS II, and thus little chance of contaminating signals. Secondly, our nanosecond data are well analyzed in terms of spectral properties, by a radical pair (P-680$^+$, Pheo$^-$), formed in less than 1 ns, with a high quantum efficiency, and with little temperature influence on its decay. Thirdly, the 25–32 ns transients are not present when they are not expected, i.e., with pheophytin reduced in the reaction centers or with Q_A oxidized in the core complex. A second question then is whether its kinetics of decay are not altered in our preparations. Although this is difficult to answer unambiguously, we have reasons to think that we are observing nearly native properties. In the reaction center the quantum yield is reasonably high, indicating a normal charge separation. In the core complex, several properties indicate that, among the various PS II preparations, it is one of those which are closest to a native state, as evidenced by the fluorescence at low temperature [45] or by the kinetics of electron transfer from Z to P-680$^+$ [41]. It is worth mentioning that there are reports of indirect observations consistent with a slow biradical decay in chloroplasts [46] and in PS II particles [47]. The shorter biradical lifetime in the core complex may be related to the more intact structure of that complex, but also to a coulombic repulsion by the negatively charged quinone Q_A^-. We do not know the origin of the discrepancy between the long biradical lifetimes that we have found here and the short values reported by other groups [20,43,44]. In our work, a 4 ns decay in the core complex was of small amplitude. The discrepancy between absorption and fluorescence data is not too surprising since it has already been reported in bacterial reaction centers and tentatively explained in terms of structural relaxations in the radical pair after its formation [14,15]. Similar phenomena could well take place in PS II.

A slow decay of the primary biradical has also been observed with PS II reaction centers by ps spectroscopy [48].

Quantitative measurements of chlorophyll *a*, pheophytin *a* and cytochrome *b*-559 led to the estimate that there were five chlorophyll *a* per complex in the reaction center preparation [8]. Pheophytin photoreduction and triplet measurements by EPR were consistent with each complex being photochemically active [9], i.e., presumably one P-680 per five chlorophylls *a*. Our flash absorption data give a substantially different value of 20 chlorophylls per P-680. This discrepancy could result from about 75% of inactive complexes. This interpretation is not satisfying, because of the previous measurements of photochemical activity [8,9] and because of our own measurement of a reasonably high quantum efficiency at low flash excitation. It seems highly probable, however, that we were not really able to saturate the formation of the primary radical pair with picosecond pulse. The saturation curve (Fig. 1b) does not indicate a complete saturation. Two factors have to be considered to explain the lack of saturation (in addition to the small antenna size). Indeed, it has been shown, with bacterial reaction centers, that a 'saturating' picosecond pulse induces a photochemical electron transfer only in about half of the reaction centers, presumably because of multi-photon processes [49]. Moreover, we used a linearly polarized (horizontal) laser pulse at 532 nm, a wavelength where the pigment absorption is very weak (see Fig. 4 in Ref. 8) (in these nanosecond experiments at 820 nm, the measuring light was also horizontally polarized). We thus could have a photoselection effect which should induce the saturation curve (Fig. 1b) to present a slow tail at high excitation energy. Thus, we propose that, although we obtain at most one biradical per 20 chlorophylls, this result is not incompatible with an effective ratio chlorophyll/P-680 of 5 in this reaction center preparation. For the core complex, the stoichiometry of chlorophyll *a* per P-680$^+$ is about the same under picosecond (78) or nanosecond (64) excitation; it is about as expected from a previous work [41].

Under our experimental conditions two species can be observed after the biradical has decayed. One of them is presumably the triplet state of P-680, as judged from its difference absorption spectrum, which is nearly the same in reaction centers and in the core complex, and also resembles the ADMR difference spectrum measured in PS II particles similar to our core complex [18]. It is in fact difficult to assign the triplet spectra to P-680 rather than to another chlorophyll *a* which would also absorb around 680 nm. The results obtained with reaction centers are interesting in that respect. The absorption maximum of these complexes is at 673 nm; with only five chlorophyll *a* per complex and one pheophytin absorbing at 682 nm, there is hardly the possibility that a chlorophyll *a* (apart from P-680) absorbs at 680–685 nm. So it is very probable that the narrow bleaching at 680–685 nm is due to the formation of ^{3}P-680 (the spectrum in the green permits the exclusion of a triplet pheophytin). The decay kinetics of ^{3}P-680, as reported here, are as reported before for the spin-polarized EPR signal at low temperature [17]. The yield of ^{3}P-680 formation increases when the temperature is lowered (Fig. 6), as shown before in carotenoid-less bacterial reaction centers [10,40]. The increase of the triplet yield (4-fold between 275 and 10 K) is much greater than the increase of the biradical lifetime (1.4-fold)

The PS II reaction center is known to be highly homologous to the reaction center of purple bacteria. Since it contains one β-carotene per complex, we expected that ^{3}P-680 would be efficiently quenched by that carotenoid, populating ^{3}Car, as happens in wild-type (carotenoid-containing) bacterial reaction centers. This is not the case: ^{3}Car is populated very weakly, with a yield which increases slightly at low temperature, contrasting with the sharp decrease around 60 K in bacteria. In that respect, PS-II appears to be similar to *Rhodopseudomonas viridis* reaction centers where ^{3}Car is not populated, although each reaction center contains a carotenoid [50]. The small amount of ^{3}Car formation in PS II gives the impression of a small heterogeneous pool: it is populated very rapidly ($t_{1/2} \approx 12$ ns), at a rate which is faster than that of biradical decay. It might result from a small contamination by another pigment-protein complex, but immunological controls do not indicate any detectable

contamination, and the absorption maximum of ^{3}Car in the reaction center (540 nm) is significantly different from that in the core complex (525 nm) or in the light-harvesting antenna (518 nm) [39]. It is possible that the β-carotene molecule in the reaction center complex has not kept its native environment, but in the absence of a firm indication for such an alteration we consider these data with isolated reaction centers as supporting our earlier report [41] of a lack of carotenoid triplet formation as a consequence of biradical recombination in PS II. An opposite conclusion has been reached by Klimov et al. [19]; however, their data did not really enable us to distinguish between carotene triplet made in the antenna or in the reaction center.

Acknowledgements

We thank Dr. K. Brettel for his contribution in setting-up the ns apparatus, Mr. O. Nanba for preparation of the PS II reaction center, and Dr. A.W. Rutherford for helpful discussions. Dr. Y. Takahashi's stay in Saclay was supported within the international cooperation launched by the Versailles summit. Ö.H. is the recipient of a postdoctoral fellowship from the Swedish Natural Science Research Council.

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