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Light-induced charge separation in Photosystem I at low temperature is not influenced by vitamin K-1

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The photoreduction of iron-sulfur centers was studied at low temperature in Photosystem I particles from spinach and the cyanobacterium *Synechocystis* 6803, which contain various amounts of vitamin K-1 (recently tentatively identified as the acceptor A_1). The irreversible charge separation that was progressively induced at low temperature between P-700 and F_A (or F_B) by successive laser flashes was studied at 15 K. Its maximum amount after a large number of flashes was shown to be fairly independent of the number (0, 1 or 2) of vitamins K-1 per reaction center. Moreover, the first flash yield of this charge separation was diminished by only about 50% when vitamin K-1 was completely absent from the particles by comparison with particles containing one or two vitamin K-1 per reaction center. When F_A and F_B were prereduced, the iron-sulfur center F_X was also reversibly photoreduced at 9 K in the absence of vitamin K-1. The implications of these results for the electron pathways of Photosystem I are discussed and it is proposed that a direct electron transfer from A_0^- to the iron-sulfur centers is highly efficient at low temperature.

Introduction

In addition to the primary donor P-700, the electron transfer components of Photosystem I (PS I) are thought to consist of five different electron acceptors (see, for example, Refs. 1 and 2). The primary electron acceptor, called A_0, is most probably a chlorophyll molecule [3]. It is generally considered that, after the primary charge separation ($P\text{-}700^* \ldots A_0 \rightarrow P\text{-}700^+ \ldots A_0^-$), an electron from A_0^- is passed onto a secondary acceptor A_1, and that the following steps of electron transfer involve three different iron-sulfur centers, called F_X, F_B and F_A [1,2].

An EPR signal with a g value around 2.005 has been tentatively ascribed to the reduced form of a secondary acceptor, called A_1 [4,5], and the identification of A_1 as a quinone molecule has recently proceeded from both EPR [6,7] and absorption data [8]. Moreover, it has been shown recently that the transient absorption signals with a decay half-time of about 120 μs and corresponding to the recombination reaction at low temperature $P\text{-}700^+ \ldots A_1^- \rightarrow P\text{-}700 \ldots A_1$ [9], are fairly consistent with A_1's being vitamin K-1 [10]. This identification was based on the fact that vitamin K-1 is the only quinone present in PS I reaction centers [11–13] and the flash-induced difference spectrum in the ultraviolet closely resembles that of reduced vitamin K-1.

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Abbreviations: PS I, Photosystem I; ΔA, absorption change; DCIP, dichlorophenolindophenol.

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The electron transfer steps involving the PS I electron acceptors have been studied extensively at low temperature. In 1971, it was established that an irreversible light-induced charge separation in PS I occurs at low temperature: P-700 ... $F_A \xrightarrow{h\nu}$ P-700$^+$ ... F_A^- and this observation led to the discovery of the iron-sulfur center F_A by EPR [14]. It was then shown that the center F_B can also be irreversibly photoreduced at low temperature, especially when F_A is prereduced [15]. The reversible light-induced reduction of iron-sulfur center F_X when F_A and F_B are prereduced also became a routine observation in PS I reaction centers following the first detection of F_X^- at low temperature [16]. Subsequently, it was shown that the yield of reduction of F_A following a saturating laser flash at low temperature (at most 25 K) was between 0.2 and 0.4 for the first flash [9,17] and was even smaller for the next flashes [9]. A competition process between forward electron transfer from A_1^- to F_A (or F_B) and a recombination reaction between P-700$^+$ and A_1^- was proposed to explain these yield values [9]. If A_1 is vitamin K-1 and if it participates in forward electron transfer at low temperature, extraction of vitamin K-1 should essentially inhibit the light-induced reduction of F_A and F_B at low temperature and it should also greatly affect the reduction of F_X when F_A^- and F_B^- are prereduced.

Therefore, we studied the light-induced reduction of PS I iron-sulfur centers in different kinds of PS I reaction center containing various amounts of vitamin K-1. We report that although the extraction of vitamin K-1 somewhat decreases the yield of the irreversible photoreduction of F_A (or F_B), it does not dramatically change the light-induced reduction of the iron-sulfur centers F_A, F_B and F_X at low temperature.

Material and Methods

Biological material

The preparation of PS I particles from spinach, including the diethyl ether treatment, has been described previously [18,19]. In brief, the PS I particles, obtained after solubilization of the thylakoid membranes with digitonin, were lyophilized and then extracted with dry or water-ticles were solubilized with 20 mM phosphate buffer (pH 8) containing 0.1–0.2% Triton X-100 by incubation for 15 min at 0–4 °C. Insoluble greyish-white materials were removed by centrifugation. The blue-green supernatant was diluted about 2–4-times with 20 mM phosphate buffer (pH 8) or 0.1 M glycine-NaOH buffer (pH 10) according to the purpose of experiments. The temperature was maintained between 0 and 5 °C during all treatments to prevent damage of the samples.

The PS I particles from *Synechocystis* 6803 were prepared and extracted with organic solvents. 10 g wet weight frozen cells were thawed in 0.4 M sucrose/50 mM Tricine (pH 7.6)/10 mM KCl and broken by three passes through a French press at 138 MPa. The washed membrane fraction was solubilized using 1% octylglucoside/0.5% sodium cholate at a chlorophyll *a* concentration of 1.5–2 mg/ml. PS I particles were sedimented at 200 000 × *g* for 1 h following two preliminary centrifugations at 10 000 × *g* for 10 min. The PS I particles were washed twice with deionized water and lyophilized. The lyophilized preparation was evacuated and stored at −80 °C. Typically, 20–40 mg of PS I powder were extracted with 3 × 25 ml dry hexane at room temperature. This treatment resulted in a PS I preparation containing one vitamin K-1 per P-700 and no change in antenna chlorophyll *a* (see table below). Extraction with hexane containing 0.3% methanol resulted in a preparation completely devoid of vitamin K-1 and approx. 50% of the antenna chlorophyll was removed. After extraction, both types of PS I particle were evacuated to remove residual solvent and then rehydrated using 50 mM Tricine (pH 7.6)/0.2% Triton X-100 at a ratio of 1 ml/10 mg starting material. After 1 h at 4 °C, the resuspended preparation was centrifuged at 10 000 × *g* for 10 min to remove insoluble material and then concentrated for EPR studies using Centricon 30 microconcentrators (Amicon Co., Danvers, MA, U.S.A.) at 5000 × *g* for 10–15 min.

Assay of reaction center concentration

From the chemical assay of PS I particles, using an extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ for the red maximum of P-700 [20], the following chlorophyll to P-700 ratios were found: 150 for

control spinach particles before extraction, 60 when the extraction was conducted with dry diethyl ether and 13 when the diethyl ether was saturated with water; 120 for control *Synechocystis* particles (± Triton) and for particles extracted with hexane alone and 70 for particles extracted with hexane/methanol. Chlorophyll concentration was determined optically on 80% acetone extracts. The EPR signals of (F_A^-, F_B^-) were also measured under highly reducing conditions and allowed a comparison of the reaction center concentrations of the different PS I particles (except for the highly enriched spinach particles, which exhibit a somewhat distorted EPR signal [21]). The relative concentrations of reaction centers thus obtained were in close agreement with the optical measurements.

Assay of PS I particles for vitamin K-1 content

The vitamin K-1 content of spinach particles was determined as described in Ref. [25]. For *Synechocystis* particles, the vitamin K-1 content was determined by extraction of the particles with chloroform/methanol (2 : 1). The extracts were reduced in volume and analyzed by HPLC using a 30-cm reversed-phase analytical column (μBondapak C18, Waters, Millipore) and ethanol as eluent. The retention time of authentic vitamin K-1 standards (Sigma) was about 7 min.

EPR measurements

EPR spectroscopy was carried out with an X-band Bruker ER200D spectrometer equipped with an Oxford ESR 900 helium cryostat. Experiments involving a sequence of laser flashes were conducted with an optical transmission cavity. The samples were maintained at 15 K during acquisition of the EPR spectra and were excited inside the cavity at the same temperature with light pulses from a YAG-laser which was frequency-doubled ($\lambda = 532$ nm, 11 ns). Experiments with a 50% attenuation of the laser intensity were performed by placing a neutral density filter immediately in front of the cavity. The light-induced detection of F_X^- was conducted using a standard cavity (TE 102 mode) and continuous illumination was provided by a 800 W tungsten-iodine lamp whose beam was filtered to remove infrared light (water cuvette + Calflex filters) and concentrated onto the cavity window using a plexiglass light pipe. Samples prepared under highly reducing conditions (20 mM sodium dithionite at pH 10; freezing under illumination; F_A, F_B and F_X completely reduced) were also studied with both cavities. The EPR sample tubes were calibrated and, in the case of successive flashes experiments, contained PS I particles with a fairly low chlorophyll concentration (less than 500 μg/ml). Comparison of the EPR radical signals were performed for all samples under subsaturating conditions of microwave power (0.2–2 μW at 15 K) by a double integration procedure. This procedure was necessary due to the fact that the radical signals exhibited different lineshapes from one kind of PS I particle to another (7.5–8.5 G for small number of flashes, see Results).

Absorption measurements

Nanosecond absorption changes at 820 nm were measured as described in Ref. 22, with the modifications described in Ref. 23. In brief, excitation laser pulses at 532 nm were of 30 ps duration (full width) and the measuring light was detected by a rapid response silicon photodiode (Lasermetrics 3117). The signal from the diode was first amplified (10-20-1C amplifier from Nuclétudes: bandwidth 500 Hz–500 MHz) and then recorded by a Tektronix 7912 digitizer interfaced with a signal averager (Didac, Intertechnique). The PS I particles were contained in a plexiglas cuvette which was inserted in a cryostat cooled with helium gas [24]. The pathlengths for excitation and detection were 5 and 10 mm, respectively.

Results

Photochemistry at low temperature under moderately reducing conditions — EPR study

Different kinds of PS I particle were poised with sodium ascorbate and frozen in the dark, so that before flash illumination at low temperature, P-700 was reduced and the iron-sulfur centers F_A, F_B and F_X were oxidized. The extent of stable charge separation between P-700 and F_A (or F_B) was then studied by measuring the EPR signals of the reduced iron-sulfur centers and of oxidized P-700 after photoactivation using saturating laser flashes at 15 K.

Fig. 1 shows the light-induced EPR signals obtained after 1, 30 and 100 flashes with *Synechocystis* particles before extraction (two vitamin K-1 per P700; chlorophyll to P-700 ratio $R \approx 120$; [P-700] = 4.5 μM) and after extraction with hexane/methanol (no detectable vitamin K-1; $R \approx 70$; [P-700] = 4.4 μM). Several distinct features emerge from the observation of the iron-sulfur EPR signals (Fig. 1, upper part). Considering the unextracted PS I reaction centers, the first flash signal can be ascribed solely to F_A^- (g values of 1.86, 1.94 and 2.05) whereas a peak at $g = 1.89$, which is most probably due to F_B^-, is developed by a larger number of flashes. Moreover, a detailed examination of the EPR spectra obtained after activation by a number of flashes comprised between 1 and 30 (not shown) reveals that the F_B^-/F_A^- ratio of photoinduced signals increases continuously with the flash number. The EPR lineshape was clearly different for the extracted PS I particles, where the prominent peak at $g = 1.89$ indicates that the extent of photoreduction is as large for F_B as for F_A. However, in that case, the EPR lineshape appears identical all along the flash sequence.

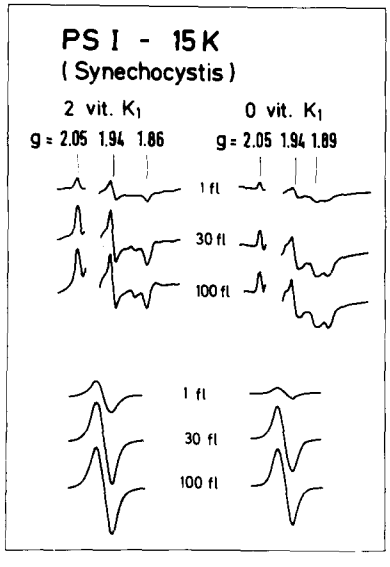

Fig. 1. Irreversible charge separation induced at 15 K by 1, 30 and 100 laser flashes ($\lambda = 532$ nm; 11 ns) in *Synechocystis* PS I particles poised at pH 8 with sodium ascorbate (4 mM) and DCIP (0.4 mM) and frozen in the dark. EPR spectra measured with an optical transmission cavity. Left part, original particles containing two vitamins K-1 per reaction center. Chlorophyll concentration, 490 μg/ml; $R \approx 120$; [P-700] $\approx$ 4.5 μM. Right part, PS I particles extracted with hexane/methanol and containing no detectable vitamin K-1. Chlorophyll concentration: 280 μg/ml; $R \approx 70$; [P-700] $\approx$ 4.4 μM. Upper part, EPR difference spectra (after subtraction of the dark baseline) of the iron-sulfur centers F_A and F_B. Instruments settings: temperature, 15 K; microwave power, 20 mW; modulation amplitude, 10 G. Lower part: EPR difference spectra of the radical region around $g \approx 2.0$ ($P\text{-}700^+$). Instrument settings; temperature, 15 K; microwave power, 20 μW; modulation amplitude, 2 G. Linewidths of the radicals: left part: 8.5, 9.2 and 9.2; right part: 8.0, 8.4, 8.5 for 1, 30 and 100 flashes, respectively. The right and left parts of the figure can be directly compared (same gain of the spectrometer and the same scale of the drawings).

The lower part of Fig. 1 shows the light-induced radicals measured at about $g = 2.00$ on the same samples and after the same number of flashes. The linewidths of these gaussian signals are comprised between 8 and 8.5 G, which is the characteristic linewidth of $P\text{-}700^+$ at low temperature, for a small number of flashes (less than 15), but they increase (up to 9–9.5 G) for a larger number of flashes. This is probably due to the fact that some radicals other than $P\text{-}700^+$ and not related to PS I photochemistry are formed by the laser light. A low quantum yield of formation of these radicals would preclude their observation for a small number of flashes but their relative extent becomes more important for a larger number of flashes. As a matter of fact, the EPR signals of the iron-sulfur centers appear to be practically fully developed after 100 flashes, whereas the radical signals become significantly broader and larger following 300 more flashes.

Similar experiments were conducted with spinach PS I particles, either before (two vitamins K-1 per P700; chlorophyll to P-700 ratio $R \approx 150$; [P-700] = 3.7 μM; Fig. 2, left) or after (no detectable vitamin K-1 per P700 [25]; chlorophyll to P-700 ratio $R \approx 13$; [P-700] = 7.8 μM; Fig. 2, right) an extraction step with diethyl ether which was saturated with water. The behavior of the unextracted spinach particles is very similar to the behavior of unextracted *Synechocystis* PS I particles. A varying ratio of F_A and F_B reduction is also observed along with the flash number. Such a change has not been observed previously in similar spinach PS I particles [9] and may be a consequence of lyophilization, as this supplementary

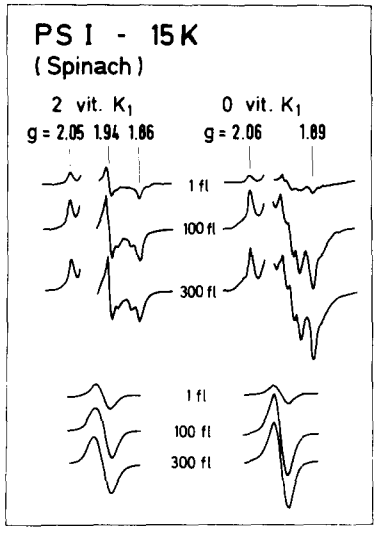

Fig. 2. Irreversible charge separation induced at 15 K by 1, 30 and 100 laser flashes ($\lambda = 532$ nm; 11 ns) in spinach PS I particles. EPR spectra measured with an optical transmission cavity. Left part, original particles containing two vitamins K-1 per reaction center, poised at pH 8 with sodium ascorbate (4 mM) and DCIP (0.4 mM) and frozen in the dark. Chlorophyll concentration, 460 μg/ml; $R \approx 150$; [P-700] ≈ 3.4 μM. Right part, particles extracted with water-saturated diethyl ether containing no detectable vitamin K-1, poised at pH 10 with 6 mM sodium dithionite and frozen immediately in the dark. Chlorophyll concentration, 90 μg/ml; $R \approx 13$; [P-700] ≈ 7.8. Upper part, EPR difference spectra (after subtraction of the dark baseline) of the iron-sulfur centers F_A and F_B. Instrument settings, temperature, 15 K; microwave power, 20 mW; modulation amplitude, 10 G. Lower part: EPR difference spectra of the radical region around $g \approx 2.0$ ($P\text{-}700^+$). Instrument settings: temperature, 15 K; microwave power: 2 μW; modulation amplitude, 2 G. Linewidths of the radicals: left part: 7.4, 9.2 and 10.1; right part: 7.8, 7.8, 7.5 for 1, 30 and 100 flashes, respectively. The right and left parts of the figure can be directly compared (same gain of the spectrometer and same scale of the drawings).

step appears to be the most important difference between the two preparations that were studied here and in Ref. 9.

In the case of extracted spinach particles, the experiments shown in Fig. 2 were performed under a somewhat different initial redox state by adding sodium dithionite in excess at pH 8 in the dark before freezing (however, it should be mentioned that spinach particles in the presence of ascorbate give essentially the same results as described above with extracted PS I particles from *Synechocystis*). Under these conditions, the dark EPR spectrum (not shown) indicates some reduction of F_A and F_B (less than 20%). The lineshape of the light-induced EPR signals was observed to be more complicated, due to the fact that illumination at low temperature then gave rise to EPR signals corresponding to reaction centers where F_A^- and F_B^- are both reduced (features distinct from the isolated F_A^- or F_B^- spectra at $g = 1.92$ and 1.96 [21]), in addition to EPR signals of isolated F_A^- or F_B^- corresponding to reaction centers where the iron-sulfur centers were initially oxidized. Therefore, due to the interaction between F_A^- and F_B^- [1,2], the light-induced EPR signals of iron-sulfur signals (Fig. 2, upper right) do not represent true EPR signals. However, as their lineshape is conserved throughout the flash sequence, these signals can be used to estimate the advancement of irreversible charge separation.

The maximal amount of light-induced charge separation can be compared between different kinds of PS I particles from such experiments by measuring the EPR signals of $P\text{-}700^+$ or F_A^- (F_B^-) induced by a large number of flashes after normalization for the concentration of reaction centers. The EPR radical signals were preferred for this purpose, because a quantitative comparison between EPR signals of iron-sulfur centers in different PS I particles is more difficult, due to the varying proportion of reduction of F_A and F_B from one kind of reaction center to another. Nevertheless, an approximate quantitation of iron-sulfur EPR signals was performed and gave essentially the same results. Due to the fact that, as mentioned above, some radicals different from $P\text{-}700^+$ probably appear for large number of flashes, the maximum extent of $P\text{-}700^+$ light accumulation was obtained, therefore, by measuring the radical signal, by a double integration procedure and under non-saturating conditions of microwave power, after 30 flashes and by multiplying this signal by a correction factor. This correction factor should correspond to the ratio between the extent of irreversible charge separation for a very large number of flashes (300–500 in these experiments) and for 30 flashes and was measured from the EPR signals of iron-sulfur centers. The maximal extents of charge separation are shown in column 3 of Table I, after normalization to the

TABLE I

n.d., non detectable.

PS I particles	Chlorophyll per P-700	Vitamin K-1 per P-700	Maximum extent of light-induced irreversible charge separation (A.U.)	First flash yield $S_{1st\ fl.}/S_{h\nu\ max}$
Spinach				
Control particles	150	2.2 [25]	1.01	0.46
Extracted with dry diethyl ether	60	n.d. [25]	0.78	0.22
Extracted with water-saturated diethyl ether	13	n.d. [25]	0.91	0.21
Synechocystis 6803				
Control particles				
−Triton	120	2.1 ± 0.2	1.00	0.38
+Triton	120	2.1 ± 0.2	1.13	0.38
Extracted with hexane	120	1.1 ± 0.25	1.05	0.39
Extracted with hexane/methanol	70	n.d.	0.93	0.16

concentration of reaction centers. These values were normalized by reference to a value which was arbitrarily set to 1 for the unextracted PS I particles of *Synechocystis* which were not exposed to Triton, so that it allows a simple estimation of the effect of vitamin K-1 extraction. These results are shown for different kinds of PS-I particle that we have also studied besides the four different reaction centers represented in Figs. 1 and 2: for original unextracted particles of both organisms, the extent of irreversible charge separation appears very similar, 1.01 for spinach versus 1.00 for *Synechocystis* and 1.13 for *Synechocystis* particles in the presence of Triton. This extent appears identical (1.05) for *Synechocystis* reaction centers which have been extracted with hexane alone and which contain one vitamin K-1 per reaction center. Moreover, it appears to be only slightly smaller in reaction centers where no vitamin K-1 was detected (0.78 and 0.91 for spinach particles with $R \approx 60$ and 13, respectively, 0.93 for *Synechocystis* particles with $R \approx 70$). Considering the uncertainties in the estimation of chlorophyll to P-700 ratios as well as in the quantitation of EPR signals, these differences appear to be hardly significant.

The development of irreversible charge separation throughout the flash sequence is shown in greater detail in Figs. 3 and 4 for *Synechocystis* and spinach PS I particles respectively. In these

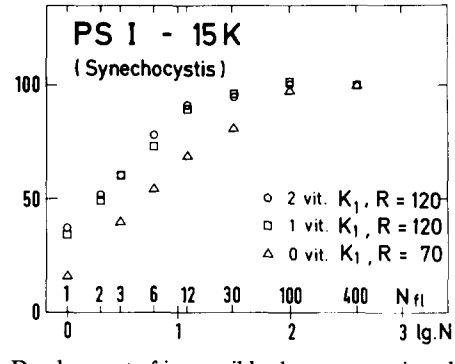

Fig. 3. Development of irreversible charge separation along the flash sequence for different PS I particles from *Synechocystis*. ○, original particles containing two vitamins K-1 per reaction center (same sample as in Fig. 1). □, particles extracted with hexane alone containing one vitamin K-1 per reaction center. △, particles extracted with hexane/methanol containing no detectable vitamin K-1 (same sample as in Fig. 1). The amounts of irreversible charge separation were determined from the P-700$^+$ EPR signals for $n_{flashes} \leq 30$, from the iron-sulfur EPR signals for $n_{flashes} \geq 30$ and after normalization of these amounts for $n_{flashes} = 30$ (see text for discussion). These quantities were calculated in arbitrary units considering that their maximum values, which are obtained after a large number of flashes (100 or 400), were normalized to 100.

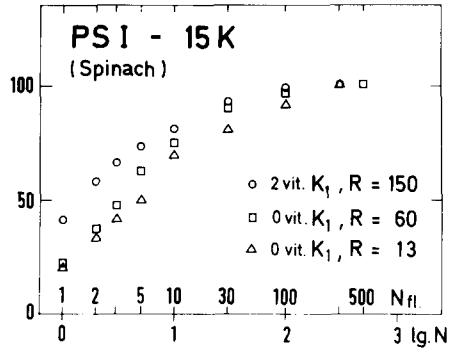

Fig. 4. Advancement of irreversible charge separation along the flash sequence for different PS I particles from spinach. ○, original particles containing two vitamins K-1 per reaction center (same sample as in Fig. 2). □, particles extracted with dry diethyl ether containing no detectable vitamin K-1. △, particles extracted with water-saturated diethyl ether containing no detectable vitamin K-1 (same sample as in Fig. 2). The normalization procedure is the same as in Fig. 3.

figures, the data of Figs. 1 and 2 were taken together with the same data obtained for other flash numbers and for other preparations of PS I particles. The extent of light-induced charge separation is plotted for the different PS I particles versus the number of flashes in percentages of the maximum extent that is attained for a large number of flashes (normalization to 1 for all particles for 300–500 flashes). These percentages were obtained taking into account the EPR radical signals for a number of flashes $n \leqslant 30$ (consisting essentially of P-700$^+$), the iron-sulfur EPR signals for $n \geqslant 30$, and normalization of these two sets of data for $n = 30$. Here again, the curves appear very similar for unextracted materials as well as for hexane-extracted *Synechocystis* particles (one vitamin K-1 per center). By contrast, the three different kinds of extracted PS I particles containing no vitamin K-1 that we have studied behave differently, as the photoaccumulation curves are shifted downwards for small number of flashes. The first flash yield (taking a yield of 1 for the full extent of charge separation) can be estimated from such curves and is also shown in column 4 of Table I. The similarities between unextracted materials is again evidenced by this parameter, although a somewhat larger value has been found for spinach (0.46) than for *Synechocystis* (0.38). Moreover, the yield is identical for *Synechocystis* PS I particles containing either one or two vitamins K-1 per reaction center. By contrast, the first flash yield is divided by a factor of about 2 for particles devoid of vitamin K-1 (0.21–0.22 versus 0.46 for spinach PS I and 0.16 versus 0.38–0.39 for *Synechocystis* PS I).

It is also possible to calculate the yield, ρ_n, for irreversible charge separation for any flash, n, from the photoaccumulation data of Figs. 3 and 4 (EPR signals, S):

$$\rho_n = \frac{S_{\text{after } n \text{ flashes}} - S_{\text{after } n-1 \text{ flashes}}}{S_{\text{after 300 flashes}} - S_{\text{after n}-1 \text{ flashes}}}$$

Such calculations show that the flash yields decrease continuously with the flash number for all kinds of PS I reaction centers. They also indicate that, in the absence as well as in the presence of vitamin K-1, fairly high yield values are obtained for the second (no vitamin K-1: 14–16%) and 3rd–6th flashes (no vitamin K-1: 10–15% per flash). Such values exclude the possibility that the yield which was observed for the first flash when no vitamin K-1 was detectable can be ascribed to a minority of reaction centers (that would mean 15–20%) retaining vitamin K-1. Control experiments were conducted with a 50% attenuation of the laser intensity for different samples: spinach PS I (original particles, $R \approx 150$; particles extracted with dry diethyl ether, $R \approx 60$; highly enriched particles, $R \approx 13$) and *Synechocystis* PS I (particles extracted with hexane/methanol, $R \approx 70$). In all cases, the first flash signals were less than 10% different than the signals induced with a 100% laser intensity on a similar sample. Therefore, this indicates that, under the chlorophyll concentrations that were used, the laser flashes were practically saturating in all cases regardless of the antenna size.

Photochemistry of extracted particles under highly reducing conditions — EPR study

An EPR sample containing spinach PS I particles extracted with water-saturated diethyl ether ($R \approx 13$) was also prepared in the initial redox state (P-700...A_0-A_1-(F_X, F_A^-, F_B^-) at pH 10 at room temperature. A reversible EPR signal peaking at $g \approx 1.8$ was observed in this sample at 8–10 K under continuous illumination (Fig. 5). This signal corresponds to the reduced form of the

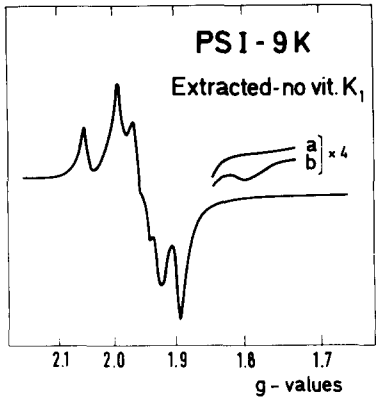

Fig. 5. EPR spectra recorded at 9 K of the iron-sulfur centers of highly enriched PS I spinach particles that were extracted with water-saturated diethyl ether ($R \approx 13$). The particles were poised with 20 mM sodium dithionite at pH 10, illuminated with a flashlight for 1 min and kept in darkness for 2 min before freezing in the dark. The fullscale spectrum was obtained in the dark (same before or after illumination). The signals of the $g \approx 1.8$ region are expanded four times for spectra recorded in the dark (trace a) or under illumination (trace b). Conditions of illumination, 800 W tungsten-iodine lamp whose light was filtered (Calflex + water) to prevent heating of the sample. Chlorophyll concentration, 350 μg/ml. Instrument settings, standard cavity (TE102 mode); modulation, 10 G.

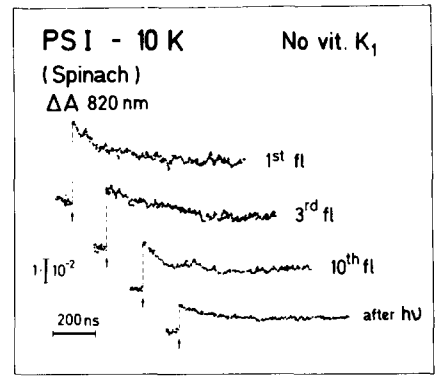

Fig. 6. Absorption transients induced at 820 nm by a laser flash ($\lambda = 532$ nm; duration, 30 ps; energy between 10 and 20 mJ) given at 10 K to highly enriched spinach PS I particles ($R \approx 13$) in a mixture of glycerol and Tris buffer (pH 8). Addition of ascorbate (1 mM) and DCIP (30 μM). The sample was dark-adapted before freezing in complete darkness. $A_{675} = 6.45$ for 1 cm (optical path of the measuring light). From the top to the bottom: first flash signal, third flash signal, average of two signals (10th and 11th flashes) and after illumination: average of 20 signals.

iron-sulfur center F_X, the EPR signal high-field peak of which is shifted from its usual $g = 1.76$–1.78 value [21]. The sample was thawed and frozen again under illumination so as to obtain the total F_X^- signal (not shown; spectrum similar to that in Ref. 21). Comparison between the two spectra shows that about 25% of the iron-sulfur centers F_X^- were reversibly reduced under illumination.

Nanosecond successive flash-absorption experiments at low temperature

Nanosecond absorption experiments at 820 nm were conducted in parallel with the EPR studies with highly enriched spinach PS I particles (no vitamin K-1; $R \approx 13$). As shown in Fig. 6, in all cases, the initial fast rise is followed by a fast decay which can be attributed to a recombination reaction between the primary partners [23,26] and which precedes slower components ($t_{1/2} \gg 1$ μs). Although it is difficult to measure it precisely in single-flash experiments, the halftime of the fast decay can be estimated to be comprised between 70 and 90 ns. The initial signal size decreases along with the flash number, and after a continuous illumination, it finally amounts to about 35% of the first flash initial signal. After continuous illumination, the remaining signal is composed of several decay phases: the fast phase, which is most likely due to a recombination reaction between $P\text{-}700^+$ and A_0^-, has a $t_{1/2} \approx 80$–85 ns and represents 50–55% of the whole signal; and a slower phase with $t_{1/2} \approx 0.8$–1.0 ms, which constitutes the most important part of the remaining signal. The spectrum of this slow phase was measured at 10 K in the red and near infrared regions. It is very similar to the spectrum obtained in CP1-SDS particles that was ascribed to the P-700 triplet state [27]. By comparison with the preceding EPR experiments, the decrease with flash number of the initial signal size can be explained by an irreversible charge separation occurring in some reaction centers after each laser flash. Assuming that there is no subnanosecond decay of the primary radical pair ($P\text{-}700^+ \ldots A_0^-$) at any flash, this irreversible charge separation appears to be achieved in about 65% of the reaction centers after continuous illumination. A more detailed comparison between this absorption experiment and the preceding EPR experiments involving a se-

quence of flashes cannot be made reliably, because the laser pulses used for the absorption experiments were not completely saturating (80% of the signal is left for a 50% attenuation of the laser) and because of the poor signal to noise ratio in single flash nanosecond absorption experiments.

Discussion

The ability of PS I reaction centers to undergo a light-induced irreversible charge separation between P-700 and F_A (or F_B) at low temperature can be considered to be a good indicator of functional electron transfer in PS I. The successive laser flash EPR experiments that are described above (Figs. 1–4 and Table I) indicate that the extent of this irreversible charge separation induced by large number of flashes shows little if any dependence on the content of vitamin K-1 per reaction center. Moreover, the first flash yield was only diminished by a factor of about 2 for samples devoid of vitamin K-1 in comparison to reaction centers containing one or two vitamins K-1 per P-700. These experiments were conducted with both spinach and *Synechocystis* PS I particles. The observed similarities between these two different reaction centers should be stressed, as these particles were not only obtained from different organisms, but also using different detergents (digitonin versus octylglucoside-cholate) and the organic solvents used for extraction were different (diethyl ether or water/diethyl ether versus hexane or hexane/methanol). This suggests that our data most probably represent a general phenomenon.

The question arises if the 50% decrease in first flash yield following the extraction procedure is due to vitamin K-1 depletion by itself or is another consequence of the extraction procedure. Addition of exogenous vitamin K-1 was attempted, but after this addition, the first flash yield was still 50% lower than in the control unextracted particles (unpublished results). Therefore, better conditions for addition of vitamin K-1 should be tested before the reason for this 50% decrease is known, and are now under investigation.

The absorption changes at 820 nm induced by successive flashes (Fig. 6) also show that an irreversible charge separation can occur in about two-thirds of the reaction centers in diethyl ether-extracted spinach particles ($R \approx 13$), a value which is comparable with the value obtained in unextracted spinach digitonin PS I particles [9]. These particles contained about two vitamins K-1 per P-700 (Duranton, J. and Moneger, R., unpublished observation). The low-temperature light-induced reduction of center F_X does not appear either to be decreased by the extraction of vitamin K-1 (Fig. 5) when F_A and F_B are prereduced. Therefore, we conclude that all three iron-sulfur centers F_A, F_B and F_X, behave similarly in that their extent of photoreduction at low temperature does not appear to be greatly affected by the content of vitamin K-1. This conclusion can be correlated with a recent optical study performed at room temperature which shows that the photoreduction of PS I iron-sulfur centers is unaffected when vitamin K-1 is photodegradated by ultraviolet irradiation [28].

The completely identical behavior of reaction centers containing one or two vitamins K-1 per P-700 extends a recent observation [29] and confirms that at least one of the two vitamins K-1 which are present in PS I reaction centers does not participate in electron transfer at low temperature. However, the absence of any dramatic effect in the reduction of iron-sulfur centers when all vitamin K-1 is extracted is much more surprising and it completely contradicts some recent models of electron transfer in PS I at low temperature. Previous studies have shown that the formation and the recombination of the radical pair ($P\text{-}700^+ \ldots A_1^-$) is the dominant process in PS I photochemistry at low temperature [9,30], whether F_A and F_B are initially oxidized or reduced. This radical pair decays exponentially with a halftime of about 120 μs and its difference spectrum is consistent with A_1 being vitamin K-1 [10]. We also hypothesized that the reduction of iron-sulfur centers competes with the recombination reaction inside the pair ($P\text{-}700^+ \ldots A_1^-$):

$$P\text{-}700 \ldots A_1 \xrightarrow{h\nu} \ldots \rightarrow P\text{-}700^+ \ldots A_1^- \rightarrow P\text{-}700^+ \ldots FeS^- \qquad \text{(a)}$$

Assuming this competition model, the rate of reduction of iron-sulfur centers was proposed to be highly heterogeneous. It should be noted that

the same heterogeneity was found in the data presented here as the yield of reduction of (F_A, F_B) decreased along with the flash number.

The results presented in this paper can be interpreted basically in two different ways:
(1) The first one is that A_1 is not vitamin K-1, or that a residual vitamin K-1 is so firmly attached to the reaction center that it cannot be extracted and has escaped observation. However, absorption data obtained at room temperature will be presented elsewhere showing that the electron transfer is essentially blocked at the level of A_0 in extracted PS I reaction centers containing no detectable vitamin K-1 ([25], Ikegami and Sétif, unpublished results), which is in contradiction with some recent results obtained after photodegradation of vitamin K-1 by ultraviolet irradiation [28]. Moreover, some reconstitution experiments show that vitamin K-1 can play an essential role in electron transfer reactions of PS I (Ref. 25; see also Ikegami and Sétif, unpublished results and Biggins, J. and Mathis, P., personal communication).
(2) In the second interpretation, the preceding model of electron transfer at low temperature (a) should be reconsidered, as the data suggest a direct electron transfer from the primary acceptor A_0 to the iron-sulfur centers. This transfer could compete with the recombination reactions inside the primary radical pair according to the scheme:

$$\text{P-700-A}_0 \xrightarrow{h\nu} \text{P-700}^+\text{-A}_0^- \rightarrow \text{P-700}^+ \ldots \text{FeS}^- \qquad \text{(b)}$$

The weak differences of behavior between reaction centers with or without vitamin K-1 suggest that reaction (b) holds also in the presence of vitamin K-1 and constitutes the main process for reduction of the iron-sulfur centers at low temperature.

This hypothesis implies that vitamin K-1 is not located on the main electron flow which leads to reduction of iron-sulfur centers, leading to the following simplest model in the presence of A_1:

$$\text{P-700-A}_0 \xrightarrow{h\nu} \text{P-700}^+\text{-A}_0^- \begin{cases} \rightarrow \text{P-700}^+ \ldots \text{FeS}^- \\ \rightarrow \text{P-700}^+ \ldots \text{A}_1^- \end{cases}$$

Finally, it is also pertinent to consider whether such a model is equally valid at room temperature or if it is the result of a modification in the electron pathways at low temperature.

Clearly, many more studies and especially reconstitution experiments are necessary to understand the role of vitamin K-1 in PS I under different conditions of temperature, and are now underway in this laboratory.

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