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LASER-FLASH-ACTIVATED ELECTRON PARAMAGNETIC RESONANCE STUDIES OF PRIMARY PHOTOCHEMICAL REACTIONS IN CHLOROPLASTS

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SUMMARY

Electron paramagnetic resonance studies of the primary reactants of Photosystems I and II have been conducted at cryogenic temperatures after laser-flash activation with monochromatic light.

P-700 photooxidation occurs irreversibly in chloroplasts and in Photosystem I fragments after activation with a 730 nm laser flash at a temperature of 35 °K. Flash activation of chloroplasts or Photosystem II chloroplast fragments with 660 nm light results in the production of a free-radical signal ($g = 2.002$, linewidth ~ 8 gauss) which decays with a half-time of 5.0 ms at 35 °K. The half-time of decay is independent of temperature in the range of 10–77 °K. This reversible signal can be eliminated by preillumination of the sample at 35 °K with 660 nm light (but not by 730 nm light), by preillumination with 660 nm light at room temperature in the presence of 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea (DCMU) plus hydroxylamine, or by adjustment of the oxidation-reduction potential of the chloroplasts to −150 mV prior to freezing. In the presence of ferricyanide (20–50 mM), two free-radical signals are photoinduced during a 660 nm flash at 35 °K. One signal decays with a half-time of 5 ms, whereas the second signal is formed irreversibly. These results are discussed in terms of a current model for the Photosystem II primary reaction at low temperature which postulates a back-reaction between $P\text{-}680^+$ and the primary electron acceptor.

INTRODUCTION

Significant advances in the understanding of low-temperature reactions of chloroplast Photosystem II have been made in recent years [1]. Three components have been associated with the reaction-center complex of the photosystem: *P*-680, the reaction-center chlorophyll [2, 3]; *C*-550, a component that monitors the oxidation-reduction state of the primary electron acceptor, A [4–6]; and cytochrome b_{559}, an electron carrier capable of undergoing low-temperature photooxidation via Photosystem II [3, 5, 7, 8–10].

Most studies of the reaction-center components of Photosystem II have involved measurement of changes of either absorbance or fluorescence yield at temperatures of 77 °K or lower after continuous illumination of chloroplasts, although electron paramagnetic resonance (EPR) techniques have recently been applied to this system. A light-induced free-radical EPR signal has been detected at low temperatures in a Photosystem II reaction [11]. The production of this free radical during continuous illumination required a high oxidation-reduction potential [11, 12] and led to a model in which the free-radical signal originated from the oxidized form of *P*-680 (*P*-680$^+$) [12]. According to this model, *P*-680$^+$ was formed irreversibly after continuous illumination at low temperatures in an oxidation-reduction potential range where possible secondary electron donors were chemically oxidized.

Recently, there have been a number of studies of the flash-induced behavior of the Photosystem II reaction-center components in attempts to probe the details of the primary photochemical event [3, 10, 13–17]. These studies have led to the postulation that *P*-680$^+$ is not stable at low temperatures and either reacts with a secondary electron donor or participates in a back reaction with the primary electron acceptor [1, 14–18]. To test the proposed role of a Photosystem II free radical whose signal we previously detected, we have initiated a series of studies of laser-flash-induced EPR changes in chloroplasts and Photosystem I and Photosystem II chloroplast fragments at low temperatures. Our results show different kinetic responses for the two chloroplast photosystems at low temperatures and, in addition, provide direct evidence for a back reaction in the primary electron couple of Photosystem II.

METHODS

Whole chloroplasts and washed, broken chloroplasts were prepared from greenhouse-grown spinach as previously described [19, 20]. Digitonin-treated chloroplast fragments enriched in Photosystem I or Photosystem II were prepared by the procedure of Anderson and Boardman [21]. Triton treatment of the Photosystem II fragments to produce a further enriched Photosystem II preparation was by the procedure of Boardman [22] as modified by Kitajima and Butler [23]. This latter preparation was found to be devoid of *P*-700, in agreement with previous findings [22, 23]. Chloroplast samples (0.3 ml) were frozen to 77 °K in quartz EPR tubes prior to examination in the EPR instrument at 35 °K.

EPR spectra were obtained at 35 °K unless indicated otherwise. This temperature was chosen as nearly optimum for signal-to-noise ratio and freedom from microwave saturation at the powers used, as previously described [11, 12]. Kinetic EPR response was measured using the 100 kHz magnetic field modulation system with its associated amplifier and phase-sensitive detector. In this mode of operation, an instrumental time constant of 1 ms was measured. The output of the phase-sensitive detector was coupled to an analog-to-digital converter (Digital Equipment Corp. Model AX08) and the memory of a small computer (Digital Equipment Corp. Model PDP-8L) was programmed to serve as a digital storage device with a maximum clock rate of one point per 100 μs. Microwave parameters for the kinetic traces are 2 mW of microwave power incident to the TE_{012} cylindrical cavity ($Q_{\text{loaded}} = 5000$) and 10 gauss of 100 kHz modulation. In most cases, kinetic traces were taken at the positive peak of the first-derivative EPR spectrum.

The single-flash kinetic EPR experiments reported here measure the decay following the production of a free-radical state by a single intense flash. Although a discussion of kinetic EPR measurements has recently been published [24], it is important to recognize the exact conditions imposed by the EPR method and the effect of these conditions on the interpretation of the experimental results contained in this paper. The laser flash produces an accumulation of the free-radical state with an equal number of spins in the $S_z = +1/2$ and $-1/2$ states (spin "up" and spin "down" states). The observation of a net EPR absorption depends, however, on a difference in population between these S_z states, a condition depending on the thermal interaction between the spin system and the surroundings (in the simplest case, the spin-lattice relaxation time, T_1) and the magnitude of the microwave radiation field in the sample. As discussed in detail for the response of a bacteriochlorophyll (*P*-865) to a step-function increase in light (producing *P*-865$^+$), McElroy et al. [24] have shown that the observed EPR rise-time is dependent solely on the initial photochemical reaction rate (k_i) when $T_1 \ll 1/k_i$. In our experiments, the initial rate, k_i, is large enough for a considerable free-radical concentration to be produced during the 0.2 μs duration of the laser flash. Then, because of noise components in the microwave-detection system (principally in the microwave detector), we limit the bandwidth of the system so that the first observation of the EPR signal is made an ms, after the laser flash. In all cases, the experimental rise-time, T_r, equals 1 ms, demonstrating that T_1 for these kinetic signals is less than or equal to 1 ms. Estimation of T_1 based on saturation data and spectrometer constants shows T_1 about 5 μs at 35 °K, the temperature at which the kinetic data were obtained. A corrollary of this is that the observed 5 ms decay is a true indication of a chemical reaction and is not a function of T_1 because $T_1 \ll 5$ ms at 35 °K.

The samples were flash activated in the EPR cavity through a 100 cm lightpipe by a coaxial flash lamp-pumped pulsed dye laser. Use of this system permits variation of the flash excitation wavelength by choice of dye combination. Approx. 90 joules of electrical energy were discharged into a coaxial flash lamp dye cell (Model DL-10, Phase-R Corp., New Durham, New Hampshire) placed in an untuned laser cell with 99 % and 40 % reflecting mirrors 43 cm apart. The laser-pulse widths were about 0.2 μs (full width at half-maximum); these were optically matched to the entrance optical window of the fiber optics by a small f: 1 lens system.

Two dye combinations were used. For emission at 660 nm, a solution of 80 μM cresyl violet perchlorate (Eastman Organic Chemicals No. 11884) and 50 μM Rhodamine 6G (Eastman Organic Chemicals No. 10724) in methanol produced 250 mJ of light in a band around 660 nm. For emission at 730 nm, a solution of 80 μM oxazine-1-perchlorate (Pilot 740, Pilot Chemicals, Div. New England Nuclear Corp., Watertown, MA) and 100 μM Rhodamine 6G in methanol produced 200 mJ of light in a band at 730 nm. Spectra of the laser flashes were obtained by illuminating the entrance slit of a Jarrell-Ash one-meter spectrograph with the fiber optics and recording the spectrum photographically. Known Hg I lines (mostly in second order) were used to calibrate the spectrograph. Laser output energies were monitored by the use of a ballistic thermopile (Korad Model 99, Aanta Monica CA) and a DC amplifier meter (Korad Model 102, Santa Monica, CA). Power measurements at the end of the fiber optics gave about 200mJ of light at 660 nm and 125 mJ of light at 730 nm entering the EPR cavity. The storage of data was

initiated by the laser flash; extreme care had to be taken to eliminate "flash artifacts" caused by the laser excitation. The success of this elimination was judged by taking kinetic data at the "zero-crossing" of the first-derivate EPR spectrum and observing no net effect of the laser flash.

The samples were continuously irradiated in the EPR cavity with monochromatic light (660 or 730 nm) obtained with Baird-Atomic interference filters of 10 nm half-band width. The incident light intensity on the samples was 5×10^4 ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}.

RESULTS

Flash-induced changes with far-red light

To study the flash-induced behavior of Photosystem I at 35 °K, untreated chloroplasts and Photosystem I chloroplast fragments were illuminated with 730-nm laser flashes. In preliminary studies it was found that this wavelength of light did not elicit any Photosystem II primary photoreactions under our experimental conditions (see also ref. 17). As shown in Fig. 1, in both untreated chloroplasts and Photosystem I fragments, after flash-induced activation with 730 nm light a rapid photoreaction occurs, as monitored by EPR absorption in the $g = 2.002$ region. This photoreaction results in the production of the free-radical EPR signal of P-700$^+$. (The kinetic traces are monitored at the low-field maximum of the P-700$^+$ signal as determined in a separate experiment.) The rise-time of the EPR signal of P-700$^+$ is 1 ms (instrument-limited). There is little decay observed in the EPR signal over the 40 ms period of the kinetic trace, indicating that P-700$^+$ is formed irreversibly at this temperature.

The EPR spectrum of the 730 nm flash-activated signal in untreated chloroplasts and in Photosystem I fragments was identical to that of the P-700$^+$ signal produced either by a continuous irradiation at 35 °K or by chemical oxidation.

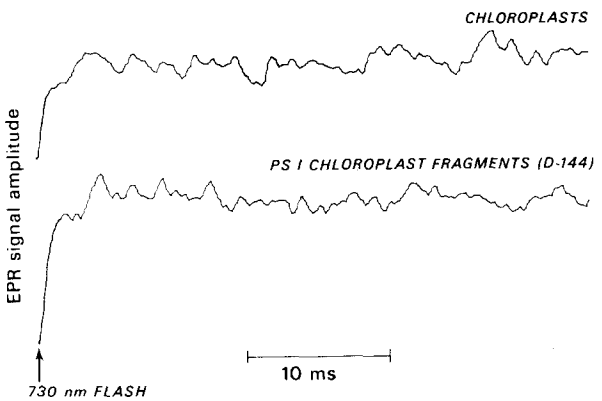

Fig. 1. Kinetics of P-700 photooxidation at 35 °K after a 730 nm laser flash. Chloroplasts or Photosystem I (PS I) chloroplast fragments at a chlorophyll concentration of 0.25 mM in 50 mM potassium phosphate buffer (pH 7.2) plus 20 mM NaCl were incubated with 5 mM ascorbate at 4 °C in darkness prior to EPR examination at 35 °K. EPR settings: frequency, 9.22 GHz; microwave power, 2 mW; modulation amplitude, 10 G.

Flash-induced changes with red light

When chloroplasts (plus 5 mM ascorbate) are flash-activated at 35 °K with 660 nm light, which activates Photosystem II as well as Photosystem I, a component is formed rapidly and decays with a half-time of 5.0±0.5 ms (Fig. 2). In these experiments, the chloroplasts had been preilluminated with 730 nm light for 30 s at 35 °K to eliminate any changes arising from Photosystem I. Under these conditions, the flash-induced change was almost completely reversible. If samples had not been preilluminated with 730 nm light, an irreversible component, $P\text{-}700^+$, was also formed during the 660 nm flash. The half-time of decay (5.0±0.5 msec) of the reversible component in untreated chloroplasts was found to be independent of temperature over the range from 11 to 77 °K.

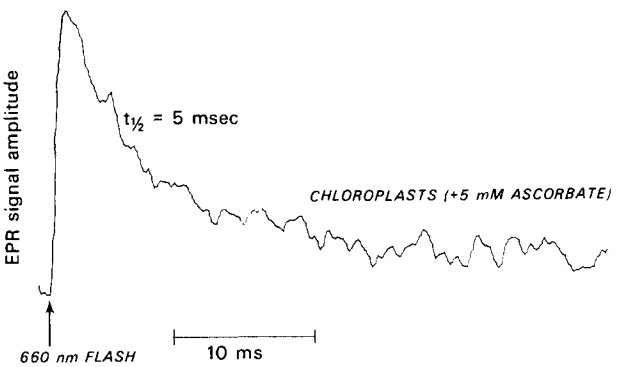

Fig. 2. Kinetics at 35 °K in the $g = 2.00$ region in untreated chloroplasts after a 660 nm laser flash. The reaction mixture and EPR conditions were as in Fig. 1. The sample was illuminated for 30 s with 730 nm light prior to flash-activation.

A similar, rapidly decaying component was observed when chloroplast fragments enriched in Photosystem II (prepared by digitonin and digitonin plus Triton treatment) were flash-activated with 660 nm light at 35 °K. Because the reversible component is flash-activated only by Photosystem II light (660 nm) and is enhanced in preparations enriched in Photosystem II activity, it appears that the reversible component is associated with Photosystem II. Support for this conclusion comes from studies with Photosystem I fragments, which have been shown to be devoid of significant amounts of the Photosystem II reaction center [4]. When these fragments are flash-activated at 35 °K with a 660-nm laser flash, no reversible component is detected and the kinetic traces are identical to those observed after a 730 nm flash (see Fig. 1).

The spectrum of the reversible component in untreated chloroplasts obtained by flash excitation with 660 nm light at varying field positions indicates that the component is a free radical with a g-value of 2.002 and a linewidth of approximately 8 gauss. This spectrum is similar to those obtained from positive cation chlorophyll free radicals both in vivo and in vitro [25–27]. No reversible flash-induced changes were detected at any field position other than those associated with the $g = 2.002$ free radical.

When chloroplasts were subjected to repeated 660 nm flashes at 35 °K there was a gradual decrease in the intensity of the rapidly decaying component. Thus,

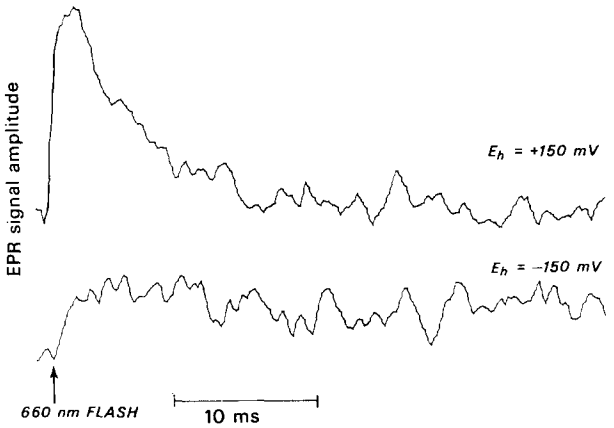

Fig. 3. Effect of oxidation-reduction potential on the kinetics at 35 °K in the $g = 2.00$ region in chloroplasts. The reaction mixture was as in Fig. 2 except that the following oxidation-reduction potential mediators were present: 1,2-naphthoquinone (40 μM), 5-hydroxy-1,4-naphthoquinone (25 μM), indigodisulfonate (50 μM), and anthraquinonedisulfonate (25 μM). The reaction mixture was in an anaerobic cell similar to that used by Dutton [32] and the oxidation-reduction potential was adjusted to the desired level by the addition of small aliquots of a sodium dithionite solution. Samples were removed anaerobically into anaerobic EPR tubes prior to freezing.

after 10 laser flashes, only 30–50 % of the intensity was observed, as compared with the first flash.

The association of the reversible component with Photosystem II has been strengthened by a study of the dependence of the reversible signal on the oxidation-reduction potential of the sample established prior to freezing. As the primary electron acceptor of Photosystem II has a midpoint oxidation-reduction potential of about 0 mV (pH 7) [5], no Photosystem II changes would be expected to occur when the acceptor is reduced prior to illumination at low temperature. As shown in Fig. 3, when the potential of a chloroplast suspension was adjusted to +150 mV prior to freezing, activation by a 660 nm flash indicated the presence of the rapidly decaying component. However, after the potential of the suspension had been adjusted to a potential at which the Photosystem II primary electron acceptor is reduced (−150 mV), the reversible component was no longer present and the only component detected was formed irreversibly. This irreversible component could be eliminated by pre-illumination with 730 nm light, indicating its association with $P\text{-}700^+$.

It was found that the rapidly decaying component could be eliminated by Photosystem II preillumination of chloroplast fragments at 35 °K prior to flash activation. As shown in Fig. 4, preillumination for 30 s with 660 nm light resulted in the absence of any flash-activated components. A similar experiment with 730 nm preillumination indicated that this Photosystem I light had no effect on the rapidly decaying component observed in the subsequent 660 nm flash.

The reversible free-radical signal could also be eliminated by preillumination of the sample at room temperature in the presence of DCMU (10 μM) and hydroxylamine (500 μM). Under these conditions, the electron acceptor of Photosystem II accumulates and any subsequent photochemistry would be expected to be inhibited [5, 28]. Subsequent flash activation of this treated sample at 35 °K elicited only a

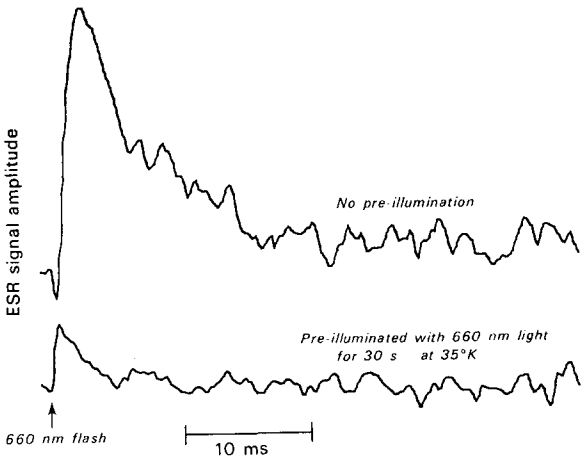

Fig. 4. Effect of preillumination with 660 nm light on Photosystem II kinetics at 35 °K after a 660 nm laser flash. Conditions were as in Fig. 2 except that the sample was preilluminated for 30 s with 660 nm light prior to flash-activation.

small, light-induced EPR change.

Flash-induced changes in the presence of ferricyanide

Because our previous EPR studies on Photosystem II in chloroplasts [11, 12] revealed the photoproduction of a free-radical signal at low temperatures after preincubation of the samples with concentrations of ferricyanide sufficient to raise the oxidation-reduction potential to about +550 mV, we studied the Photosystem II flash-induced changes in chloroplasts at high oxidation-reduction potentials. Under these conditions, *P*-700 has been chemically oxidized prior to illumination and does not contribute to any EPR changes.

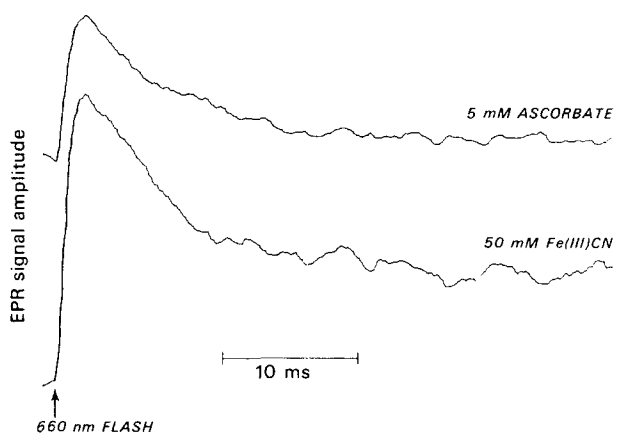

Fig. 5. Effect of ferricyanide on Photosystem II kinetics in the $g = 2.00$ region after a 660 nm laser flash. Conditions as in Fig. 2 except that there was no illumination of the sample prior to flash-activation. Ascorbate or ferricyanide was added to the indicated final concentration prior to freezing.

When chloroplast fragments are preincubated with ferricyanide (10–50 mM) prior to flash activation with 660 nm light at 35 °K, the kinetic pattern differs significantly from those previously obtained (Fig. 5): the magnitude of the flash-induced signal is two to three times greater in the presence of ferricyanide than in the absence of ferricyanide, and only half of the photoinduced signal decays over the 40 msec kinetic period. The oxidation-reduction potential dependence of the irreversible portion of this signal is similar to that observed previously with continuous irradiation at low temperature, suggesting that two different components are flash-activated in the presence of ferricyanide. One component is formed reversibly and the magnitude of this signal is similar to that now observed in the absence of ferricyanide, while the second, of similar or larger magnitude, is formed rapidly but shows little decay at this temperature (35 °K).

A spectrum of the irreversible component was obtained by flash excitation at varying field positions. (Separate samples were required for each point because of the irreversibility of the component at 35 °K). The spectrum of the irreversible component was identical to that of the reversible component within the accuracy of these measurements. Preillumination of preparations at 35 °K with 660 nm light in the presence of ferricyanide followed by activation with a laser flash of the same wavelength showed that both components decreased in parallel fashion. After a 1 min preillumination, no significant flash-induced changes were detected. The kinetic pattern observed with repetitive flashes indicated a gradual decrease in the amplitude of both components with increasing numbers of flashes.

DISCUSSION

Recent studies in several laboratories [1, 14–17] led to the formulation of the following reaction sequence for the Photosystem II reaction center at low temperature.

$$h\nu + \mathrm{D} \cdot P \cdot \mathrm{A} \underset{k_{-1}}{\overset{}{\rightleftharpoons}} \mathrm{D} \cdot P^{+} \cdot \mathrm{A}^{-} \xrightarrow[\text{dark}]{k_2} \mathrm{D}^{+} \cdot P \cdot \mathrm{A}^{-}$$

where P is the reaction-center chlorophyll, P-680, A is the primary electron acceptor, and D is an electron donor.

The evidence on which the proposed Photosystem II back reaction, characterized by rate constant, k_{-1}, is based is compelling, yet indirect. Most of it has come from measurements of the extent of absorbance or fluorescence change following flash-activation [10, 14, 17] and until recently no kinetic evidence for such a back reaction had been obtained through direct observation of any of the components. However, a recent report by Mathis and Vermeglio [16] presented evidence for a reversible C-550 change at 77 °K (half-time of decay, 4.2 ms) in chloroplasts after a laser flash; a result directly supporting this model.

Our studies of EPR changes after flash-activation at low temperature indicate the presence of a free-radical component associated with Photosystem II that is formed rapidly and decays with a 5 ms half-time. The half-time for the back reaction between P-680^{+} and A^{-} at low temperatures has been found by several groups to be approx. 4.5 ms, based on measurements of P-680 and C-550 absorbance changes [3, 16] and on measurements of fluorescence yield changes [15]. The finding that the reversible Photosystem II free-radical signal has kinetics similar to those obtained by

other workers for the Photosystem II primary reactants supports our assignment of this signal to a component of the Photosystem II reaction center.

When $P\text{-}680^+$ is formed during a saturating flash, it is apparent that two reaction routes can occur at low temperature: reduction by A^- or reduction by D. In the case in which $P\text{-}680^+$ is reduced by an electron donor (during continuous irradiation), subsequent flash-activation would not produce any additional $P\text{-}680^+$ because A is trapped in the reduced state even though $P\text{-}680^+$ has been reconverted to P-680. Our finding that Photosystem II preillumination at 35 °K eliminates any reversible free-radical signal in subsequent flashes is predicted by this model and indicates that the reversible component is $P\text{-}680^+$. In addition, our failure to detect the free-radical signal during continuous irradiation at low temperature indicates the component cannot be the primary electron acceptor of Photosystem II because this carrier accumulates in the reduced state under these conditions. There is no evidence of any reversible secondary donor reactions associated with Photosystem II, eliminating such donors as the free-radical component. The results obtained with repetitive flashes where the reversible signal is present but with diminished amplitude may be explained in terms of the model if the signal arises from $P\text{-}680^+$ and not from an electron donor (see also ref. 16).

In our previous studies of Photosystem II reactions in chloroplasts during continuous irradiation, we observed a Photosystem II-induced, free-radical signal at low temperatures, but only in the presence of ferricyanide [11, 12]. In contrast to our present findings, this previously observed EPR signal was formed irreversibly at temperatures below 77 °K. On the basis of its EPR parameters and oxidation-reduction properties, we concluded that the irreversibly formed signal might originate from $P\text{-}680^+$. Subsequently, Lozier and Butler [17] concluded that $P\text{-}680^+$ is not stable in the presence of ferricyanide when they could not detect any absorbance changes associated with $P\text{-}680^+$ after low-temperature illumination. Photosystem II flash-activation of chloroplasts in the presence of ferricyanide indicates the formation of two components, one formed reversibly and originating from $P\text{-}680^+$ and a second formed irreversibly. The irreversibly formed component is probably identical to the free-radical component we previously detected during continuous irradiation. Lozier and Butler [17] have suggested that this irreversible signal originates from the oxidized form of an electron donor that is formed when other lower potential donors, such as cytochrome b_{559}, are chemically oxidized. Our finding that the irreversible component is formed in addition to the reversible component during a 660-nm flash suggests that the irreversible component may not be an electron donor to $P\text{-}680^+$. However, the recent finding [29] that this component appears to function in a back reaction with A^- at −50 °C indicates a possible role for it on the oxidizing side of Photosystem II at low temperatures.

To summarize our present findings, we conclude that a back reaction occurs in the primary electron couple of Photosystem II at low temperature. This reaction has a half-time of approx. 5 ms and occurs over the range of potential from about 0 mV to about +600 mV. In addition, electron donors other than cytochrome b_{559} which react with $P\text{-}680^+$ are present in the Photosystem II reaction center. Thus, $P\text{-}680^+$ does not accumulate, even at high oxidation-reduction potentials. The relationship of these results to the findings in other laboratories on irreversible low-temperature Photosystem II absorbance changes [30, 31] is currently under investigation.

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