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OXIDATION–REDUCTION POTENTIAL DEPENDENCE OF LOW-TEMPERATURE PHOTOREACTIONS OF CHLOROPLAST PHOTOSYSTEM II

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SUMMARY

The light-induced free-radical signal of Photosystem II (observed after illumination at 77 °K) has been studied in chloroplasts as a function of the oxidation–reduction potential established prior to freezing. The intensity of the light-induced signal is unchanged in the potential region of +590 mV to +760 mV. At higher potential (+850 mV), there is a 30% decrease in signal intensity. The light-induced signal decreases to zero in the low-potential region, with a midpoint potential of +475 mV. These results are considered in terms of a Photosystem II reaction-center complex in which the light-induced free-radical signal arises from the oxidized form of the reaction-center chlorophyll, and this chlorophyll molecule is capable of being reduced at liquid-nitrogen temperature by a secondary electron donor which has a midpoint oxidation–reduction potential of +475 mV.

INTRODUCTION

We recently reported[1] the detection of a new free-radical electron paramagnetic resonance (EPR) signal in spinach chloroplasts and our results indicated that this signal arises from a carrier involved in the primary reaction of chloroplast Photosystem II. The component responsible for the EPR signal was not identified in the previous study, although it was suggested that the free radical was associated with the oxidized form of the reaction-center chlorophyll of Photosystem II, previously referred to as P680 (refs 2–4).

In this paper, we report an examination of the effect of oxidation–reduction potential on the photo-induced EPR change of Photosystem II at 77 °K. Evidence will be presented that the EPR signal is due to the oxidized reaction-center chlorophyll of Photosystem II and that this component is capable of undergoing further reactions at 77 °K after the primary photochemical event.

MATERIALS AND METHODS

Whole chloroplasts were prepared from greenhouse spinach as described[5]. Digitonin chloroplast fragments enriched in Photosystem II (D-10) were prepared by the procedure of Hauska *et al.*[6] and were resuspended in 50 mM potassium phosphate

buffer, pH 7.6, *plus* 200 mM NaCl. Chlorophyll concentration and the chlorophyll *a*: chlorophyll *b* ratio were measured by the method of Arnon[7].

Oxidation–reduction potentials were measured at 5 °C with a Radiometer PK-149 combined platinum–calomel electrode in a cell similar to that used by Dutton[8]. The electrode was standardized against a saturated quinhydrone solution at pH 7.0 ($E_m = +296$ mV at pH 7.0, ref. 9). All oxidation–reduction potentials are reported relative to the standard hydrogen electrode.

All titrations were performed aerobically. In titrations in the +400 mV to +600 mV region, $K_3Fe(CN)_6$ was added to raise the potential to the starting level and titrations were done reductively with either sodium ascorbate or $K_4Fe(CN)_6$. When a desired potential was attained, the reaction mixture was allowed to equilibrate for approximately 1 min; a sample was then removed, placed in a standard X-band quartz EPR tube (3 mm internal diameter), and frozen in liquid nitrogen. In experiments at oxidation–reduction potentials greater than 600 mV, the oxidant K_2IrCl_6 was used. In these titrations, it was found that stable potentials could be achieved by adding solid K_2IrCl_6 to the reaction mixture to the desired final concentration and then allowing time for equilibration with the system (approx. 5 min).

EPR spectra were obtained at 77 °K (refs 1,10) as the Photosystem II free-radical signal is more easily saturated at lower temperatures. Samples were illuminated directly in the EPR cavity for 30 s with monochromatic light (645 or 715 nm) obtained with Baird-Atomic interference filters of 10-nm half-band width. The incident light intensity on the sample was $5 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}. In some light-induced studies, wide-band red light from a Corning 2-58 filter was used for illumination.

Digitonin was purchased from the Sigma Chemical Co.; K_2IrCl_6, from Alfa Inorganics (Ventron Corp.).

RESULTS

When chloroplasts are poised at an oxidation–reduction potential of +250 mV (by the addition of 1 mM hydroquinone) and a sample is examined by EPR spectroscopy at 77 °K, a broad free-radical signal, identical to Signal II (refs 11, 12), is present in the dark (Fig. 1A). Illumination of this sample at 77 °K with far-red light (715 nm), which activates primarily Photosystem I, results in the appearance of a second free-radical signal centered at $g = 2.0026$ and having a linewidth of 8 G (Fig. 1B). This signal, referred to as Signal I (refs 11, 12), has been shown to originate from the oxidized form of a specialized form of chlorophyll, P700, which is the reaction-center chlorophyll of chloroplast Photosystem I. If the same sample is next illuminated with ced light (645 nm), which activates Photosystem II as well as Photosystem I, no additional free-radical signals are photo-induced (Fig. 1C).

As shown in Fig. 2, a different pattern of light-induced reactions is observed when chloroplasts are poised at an oxidation–reduction potential of +560 mV (by the addition of 20 mM $K_3Fe(CN)_6$) and examined at 77 °K. At this oxidation–reduction potential, P700 is oxidized and the dark signal is now the combination of Signal II and a signal from oxidized P700 (Fig. 2A). Illumination of this sample with far-red light no longer produces any light-induced change because P700 has been chemically oxidized (Fig. 2B), but a subsequent illumination with red light (Fig. 2C) results in a large photo-induced signal produced by the Photosystem II free radical.

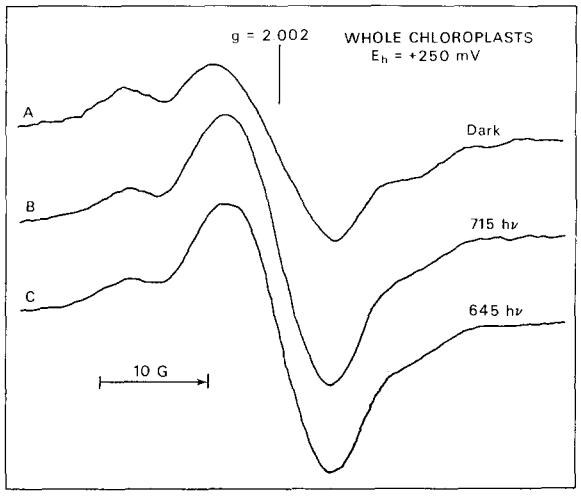

Fig. 1. Low-temperature light-induced free-radical signals in chloroplasts poised at $E_h = +250$ mV. The reaction mixture contained 0.3 M sucrose, 50 mM Tris–HCl buffer (pH 7.8), 10 mM NaCl, and whole chloroplasts (0.5 mg/ml). The oxidation–reduction potential of the system was adjusted to +250 mV by the addition of hydroquinone. A sample was then removed for EPR examination at 77 °K. (A) Dark, (B) Illuminated at 77 °K with 715-nm light, (C) Illuminated at 77 °K with 645-nm light. First-derivative EPR spectra were recorded at 77 °K with the following instrument setting: frequency, 9.22 GHz; power, 0.5 mW; modulation amplitude, 2 G; scan rate, 4 G/5.

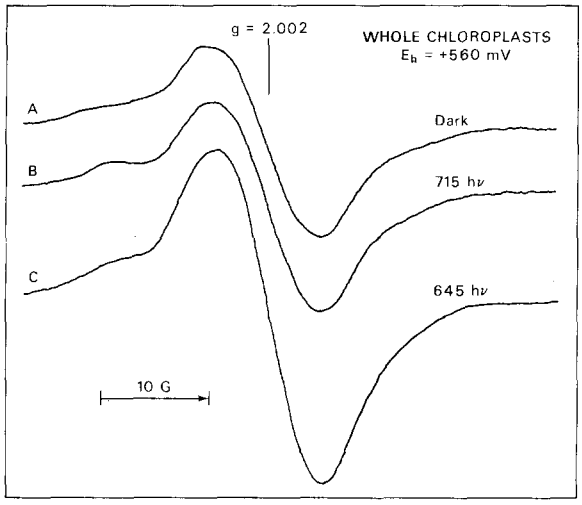

Fig. 2. Low-temperature light-induced free-radical signals in chloroplasts poised at $E_h = +560$ mV. Reaction mixture as in Fig. 1. $K_3Fe(CN)_6$ was added to adjust the oxidation–reduction potential to +560 mV. EPR spectra were recorded at 77 °K as described in Fig. 1. (A) Dark, (B) Illuminated at 77 °K with 715-nm light, (C) illuminated at 77 °K with 645-nm light.

These results indicate that at 77 °K and at an oxidation–reduction potential of +560 mV a Photosystem II light-induced free-radical signal can be observed but that

at a lower oxidation–reduction potential ($E_h = +250$ mV) this signal is not detected after continuous illumination.

To study the oxidation–reduction potential dependence of the Photosystem II change, the effect of higher potential ($E_h > +400$ mV) was examined in greater detail. In these experiments we have used Photosystem II chloroplast fragments (D-10) because of the enrichment of the Photosystem II free-radical signal in this preparation (see ref. 1). Since the D-10 preparation has approx. 5–10 times more photo-induced Photosystem II signal than signal from oxidized P700, no correction is necessary for the contribution of the P700 free radical to the light-induced changes in the $g = 2.00$ region.

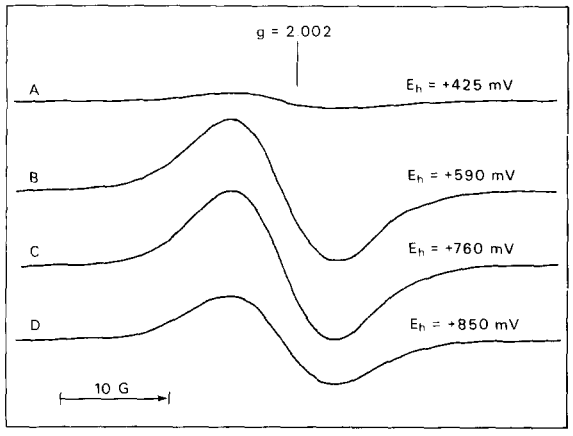

Fig. 3. Light *minus* dark EPR spectra of free-radical signal in Photosystem II chloroplast fragments at defined oxidation–reduction potentials. The reaction mixture contained 50 mM potassium phosphate (pH 7.6), 200 mM NaCl, and D-10 chloroplast fragments (0.25 mg/ml). Oxidation–reduction potentials of +425 mV and +590 mV were obtained by the addition of $K_3Fe(CN)_6$. As described in Materials and Methods, the potential of +760 mV was obtained by the addition of solid K_2IrCl_6 (1 mg/ml reaction mixture); +850 mV, by another addition of solid K_2IrCl_6 (1 mg/ml reaction mixture). Samples were withdrawn at the indicated potentials and illuminated for 30 s at 77 °K with wide-band red light. EPR spectra were recorded as described in Fig. 1.

The light *minus* dark EPR spectra at four oxidation–reduction potentials established prior to freezing at 77 °K are shown in Fig. 3. In Fig. 3A ($E_h = +425$ mV), there is only a small light-induced change after illumination at 77 °K; in Fig. 3B, the potential has been raised to +590 mV and a large light-induced Photosystem II signal is observed. When the potential is raised even further, to +760 mV (Fig. 3C), the light *minus* dark signal is comparable in intensity to the signal observed at +590 mV; at a higher potential (Fig. 3D, $E_h = +850$ mV), a substantial portion of the change persists, although there is a 30% decrease in the signal intensity at this potential. There is considerable damage to the chloroplasts fragments at +850 mV, as evidenced by a large free-radical signal present in the dark, and this damage may be the cause of the diminution of the light-induced change.

The experiment shown in Fig. 3 has been repeated with unfractionated chloroplasts, with similar results. The findings indicate that the Photosystem II free-radical signal can be observed at high oxidation–reduction potentials ($E_h > +800$ mV) and

that the light-induced signal disappears in the potential range between +590 mV and +425 mV.

To determine the midpoint potential (E_m) at which the light-induced change disappears, samples in the +500 mV region have been studied in greater detail. These experiments represent titrations in which the light-induced signal is observed after samples are poised at oxidation–reduction potentials established prior to freezing. A typical titration with D-10 fragments is shown in Fig. 4. The Photosystem II light-induced signal is maximal at potentials greater than +550 mV and decreases in the lower potential range until no signal is detected at potentials lower than +400 mV.

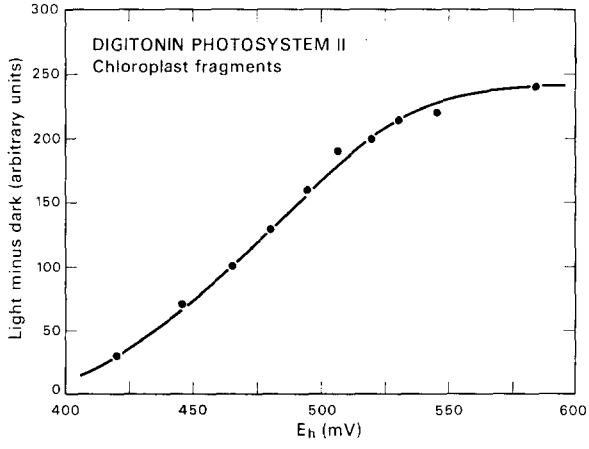

Fig. 4. Titration of light-induced free-radical signal in Photosystem II chloroplast fragments. The reaction mixture contained 50 mM potassium phosphate (pH 7.6), 50 mM $K_3Fe(CN)_6$, and D-10 chloroplast fragments (0.25 mg/ml). The sample was titrated reductively with sodium ascorbate and aliquots were removed at the indicated potentials. Samples were illuminated with wide-band red light at 77 °K for 30 s. EPR spectra were recorded at 77 °K as described in Fig. 1. Titration end-point: at 600 mV, 240 units.

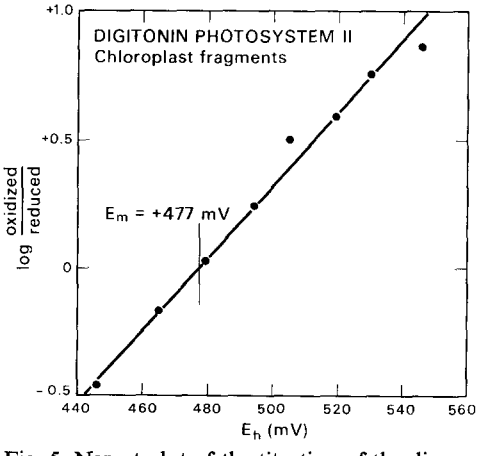

Fig. 5. Nernst plot of the titration of the disappearance of the light-induced free-radical signal in Photosystem II chloroplast fragments. Data from Fig. 4.

As shown in Fig. 5, the titration curve can be fit, within experimental uncertainty, by a Nernst plot for a carrier undergoing a one-electron transition and having a midpoint potential of +477 mV.

A titration of the light-induced Photosystem II signal in unfractionated chloroplasts is shown in Fig. 6. In this experiment, it was necessary to correct for the P700

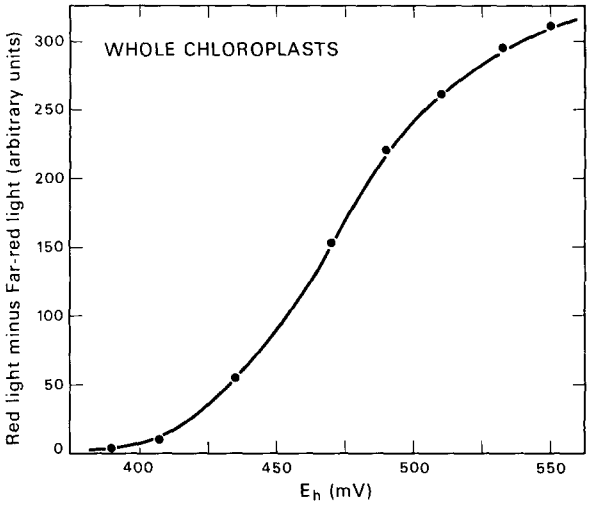

Fig. 6. Titration of light-induced free-radical signal in whole chloroplasts. The reaction mixture contained 0.3 M sucrose, 50 mM Tris–HCl buffer (pH 7.8), 10 mM NaCl, 50 mM $K_3Fe(CN)_6$, and whole chloroplasts (0.5 mg/ml). The sample was titrated reductively with sodium ascorbate and aliquots were removed at the indicated potentials. Samples were illuminated at 77 °K with 715-nm light and then 645-nm light; the difference between the two signals was taken as the Photosystem II free-radical signal. EPR spectra were recorded at 77 °K as described in Fig. 1. Titration endpoint: at 590 mV, 310 units.

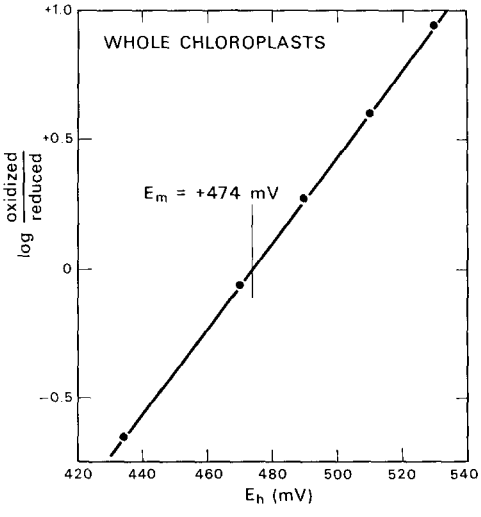

Fig. 7. Nernst plot of the titration of the disappearance of the light-induced free-radical signal in whole chloroplasts. Data from Fig. 6.

light-induced change by illuminating samples with far-red light prior to illumination by red light. The Photosystem II change is then the difference between the signal in red and far-red light. The titration curve is shown in Fig. 6 and the results are similar to those found with the Photosystem II chloroplast fragments. As shown in Fig. 7, the titration can be fit, within experimental uncertainty, to a Nernst plot with a one-electron transition and a midpoint potential of +474 mV.

DISCUSSION

From the similarity between the EPR parameters of the Photosystem II free-radical signal and those of chlorophyll free radicals, we suggested[1] that the Photosystem II signal may originate from the reaction center chlorophyll of Photosystem II. Our measurements of the oxidation–reduction potential dependence of this signal at 77 °K also support this assignment and indicate that additional reactions of Photosystem II, other than the primary reaction, can occur in the photosystem at this temperature.

A relatively high oxidation–reduction potential ($E_h \approx +550$ mV) was necessary to permit observation of the Photosystem II free-radical signal after continuous illumination[1]. We have extended these measurements to an oxidation–reduction potential of +850 mV, and our results indicate that the Photosystem II free radical can still be photoinduced at these extremely high potentials. The extent of the light-induced change at +850 mV was approximately 70% of that detected at a potential of +760 mV. It is not clear if the decrease in the light-induced signal at +850 mV reflects chemical oxidation of the carrier in the dark or is due to a destruction of some component also necessary for the light-induced change, such as the primary electron acceptor. These findings do show that this free-radical signal can be photoinduced at a potential greater than that required for the oxidation of water to oxygen (+815 mV at pH 7.0 and +767 mV at pH 7.8) and that the signal has a midpoint potential greater than +760 mV at pH 7.8. These results are consistent with the assignment of this free-radical signal to a component in the Photosystem II primary reaction.

The finding that the light-induced Photosystem II change occurs at an E_h greater than +760 mV also serves to distinguish this new signal from the high-potential free-radical signal described by Loach *et al.*[13] during room-temperature illumination. In the latter case, the free-radical signal had an E_m of +600 mV ($n=1$). This component would not be expected to show any significant photoinduced changes at an E_h greater than +660 mV.

The reaction center complex of Photosystem II has been considered by Butler[14,15] to consist of more than two components, as shown in the following formula:

$$D \cdot P \cdot A \xrightarrow[77\,^{\circ}K]{h\nu} D \cdot P^{+} \cdot A^{-} \xrightarrow[77\,^{\circ}K]{\text{Dark}} D^{+} \cdot P \cdot A^{-}$$

where P is the reaction center chlorophyll of Photosystem II (the primary electron donor), A is the primary electron acceptor, and D is a secondary donor molecule. A series of investigations has shown that cytochrome b_{559} can function as D in the above reaction and can be photooxidized in a Photosystem II reaction at cryogenic temperatures[3,14–19]. This reaction would be represented by the second half of the

above formula, while the light-induced charge separation is shown in the first part.

Our results indicate that the extent of the Photosystem II light-induced signal in both untreated chloroplasts and Photosystem II chloroplast fragments is unchanged in the potential range +760 mV to +590 mV and is then attenuated (to zero) in a one-electron transition with $E_m = +475$ mV. The disappearance of the light-induced signal at a midpoint potential of +475 mV can be explained by either of two possibilities, with reference to the above-described formulation for Photosystem II reactions at low temperature.

First, the disappearance of the light-induced signal at +475 mV could simply be due to a chemical reduction of A, the electron acceptor, at this potential. However, the midpoint potential of the two components most closely related to A (Q and C550) has been found to be approx. 0 mV (refs 17,20), indicating that the component with an E_m of +475 mV cannot be the primary electron acceptor.

A second possibility, that we believe is compatible with our results, involves the presence of a secondary electron donor, D, which is capable of undergoing oxidation at 77 °K, as shown in the above formulation. Accordingly, the light-induced signal originates from the reaction-center chlorophyll molecule and the midpoint potential of +475 mV is the potential of the secondary donor, D. At a potential lower than +475 mV, D would be chemically reduced and able to donate electrons to the reaction-center chlorophyll at 77 °K. The disappearance of the light-induced signal at +475 mV would then be due to a reduction of the oxidized chlorophyll by D. Therefore, in the steady state, no free-radical signal would be observed.

Butler *et al.*[15] have reported evidence to indicate that at least two secondary electron donors can interact with the reaction center chlorophyll of Photosystem II. One of these donors was cytochrome b_{559} but the other was not characterized. Our findings indicate the presence of an electron donor with a midpoint potential of +475 mV in chloroplasts, a midpoint potential approx. 100 mV more electropositive than that reported for the high-potential form of cytochrome b_{559} (refs 19, 21–24). We have also found that there are no light-induced cytochrome changes at 77 °K in the +500 mV potential region, indicating cytochrome b_{559} cannot be the carrier with an E_m of +475 mV. The identity of this new donor molecule remains unknown since we have been unable to detect any absorbance or EPR changes associated with it. In addition, we cannot as yet attribute any physiological significance to this low-temperature reaction.

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