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LIGHT-INDUCED CHANGES OF ABSORBANCE AND ELECTRON SPIN RESONANCE IN SMALL PHOTOSYSTEM II PARTICLES

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SUMMARY

Photosystem II reaction center components have been studied in small system II particles prepared with digitonin. Upon illumination the reduction of the primary acceptor was indicated by absorbance changes due to the reduction of a plastoquinone to the semiquinone anion and by a small blue shift of absorption bands near 545 nm (C550) and 685 nm. The semiquinone to chlorophyll ratio was between 1/20 and 1/70 in various preparations. The terminal electron donor in this reaction did not cause large absorbance changes but its oxidized form was revealed by a hitherto unknown electron spin resonance (ESR) signal, which had some properties of the well-known signal II but a linewidth and *g*-value much nearer to those of signal I. Upon darkening absorbance and ESR changes decayed together in a cyclic or back reaction which was stimulated by 3-(3,4 dichlorophenyl)-1,1-dimethylurea. The donor could be oxidized by ferricyanide in the dark.

Illumination in the presence of ferricyanide induced absorbance and ESR changes, rapidly reversed upon darkening, which may be ascribed to the oxidation of a chlorophyll *a* dimer, possibly the primary electron donor of photosystem II. In addition an ESR signal with 15 to 20 gauss linewidth and a slower dark decay was observed, which may have been caused by a secondary donor.

INTRODUCTION

Small particles enriched in the reaction center of photosystem II and devoid of photosystem I have been prepared by treatment of chloroplasts with Triton X-100 [1, 2] and with digitonin [3]. Both types of particles apparently have a similar composition and might very well be a structural unit existing as such in intact chloroplasts. The particles have a high system II activity with artificial electron donors and acceptors and potentially they provide a very valuable tool in the study of primary reactions in photosystem II.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Some light-induced reactions of endogenous components have been reported. The Triton particles efficiently reduced cytochrome b_{559} at room temperature [4] and their 3-(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU)-sensitive fluorescence rise in the presence of an artificial electron donor [5] probably indicates some plastoquinone reduction [6]. C550, however, which seems to be a reliable indicator for the redox state of the primary acceptor, was reported to be largely in the reduced state. After addition of ferricyanide its photoreduction was measured at liquid nitrogen temperature [5]. This was accompanied by absorbance changes near 690 nm. Similar changes have been observed in chloroplasts at low temperature [7, 8] and at room temperature after deoxycholate treatment [9] and appear to be the normal complement of C550, indicating the redox state of the primary acceptor rather than the donor. In addition, illumination at low temperature in the presence of ferricyanide induced an irreversible ESR signal [5] comparable to the one observed in intact chloroplasts [10] which is probably due to a secondary electron donor [11]. The digitonin particles exhibit a relatively small C550 photoreduction at low temperature, which is accompanied by an oxidation of cytochrome b_{559} [3]; in these particles no significant changes at room temperature have been reported yet. This paper reports on primary reactions of the digitonin particles at room temperature, studied by optical and ESR spectroscopy.

MATERIALS AND METHODS

The preparation of system II particles free from System I activity is described in detail in ref. 3. Briefly, a grana preparation obtained by digitonin treatment of spinach chloroplasts was fractionated by a further digitonin treatment and small particles containing photosystem I, photosystem II and the accessory pigment protein complex of system II were separated by density gradient centrifugation and ion exchange chromatography. Absorbance changes were measured in a split beam apparatus as described earlier [6] except where indicated otherwise in the legends.

Electron spin resonance (ESR) was measured with a Varian E9 spectrometer with 100 kHz modulation. The microwave frequency was between 9.54 and 9.55 GHz. Precise g-values can as yet not be given, but may be estimated relative to signal I and signal II, which were centered at about 3396 and 3392 gauss, respectively, measured in chloroplasts using the same cuvette and instrument settings. The sample was contained in a flat cell (0.2 mm), which could be illuminated by continuous light or xenon flashes through the slotted front side of the cavity. To allow direct comparison with absorbance changes (and to save material) the same sample was used, (chlorophyll concentration 100 μg/ml) and the signal to noise ratio was enhanced by averaging when necessary. All experiments were done at room temperature.

RESULTS

The spectrum of light-induced absorbance changes, which was measured in the presence of 5 μM ferricyanide in order to improve reversibility, appeared to contain all bands previously shown to be associated with the reduction of the primary electron acceptor of photosystem II (Fig. 1). In the ultraviolet the formation of a plastosemiquinone anion is seen [9], providing a quantitative measure for the reaction

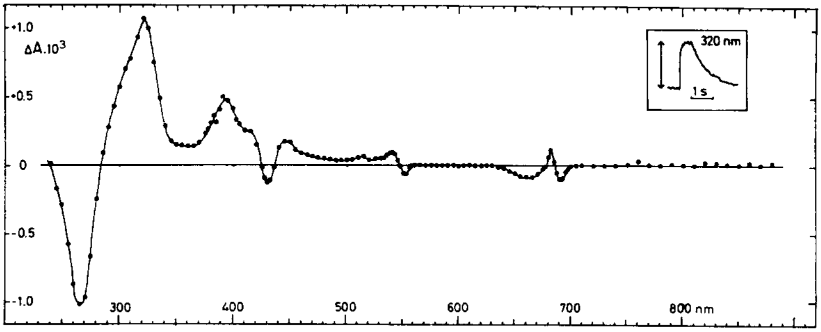

Fig. 1. Light minus dark difference spectrum of absorbance changes measured in the presence of 5 μM ferricyanide. Chlorophyll concentration 100 μg/ml. Optical pathlength 1.1 mm, bandwidth adjusted to the wavelength interval between measurements. Saturating illumination (about 50 nE $cm^{-2} \cdot s^{-1}$ absorbed) with a broad band red or blue light.

center concentration, which varied from 1 per 20 to 1 per 70 chlorophylls in different batches (assuming a differential extinction coefficient of 13 $mM^{-1} \cdot cm^{-1}$ at 320 nm [12]. The band shifts centered at 545 nm (C550) and at 685 nm ("C690") were present. In addition to these absorbance changes a slight chlorophyll bleaching with similar kinetics was indicated by the decrease at 430 nm and at 660 nm, which was not observed in similar measurements on chloroplasts treated with deoxycholate [9]. If it is due to chlorophyll oxidation the extent would account for about one chlorophyll per 20 reaction centers. It was observed in the absence of ferricyanide as well.

The absorbance changes described above were accompanied by an ESR signal of unknown nature. As illustrated in Fig. 2A the ESR spectrum in the $g = 2$ region appeared to contain the well known signal II in the dark. Illumination induced an asymmetrical signal with a g-value nearer to that of signal I than that of signal II. The shape of the light-induced spectrum was irreproducible and might change even between successive scans of the same sample, as illustrated in Fig. 2B. The linewidth ($\Delta H_{\text{p-p}}$) varied from 8 to 10 gauss. Some hyperfine structure, although hardly resolved at all, the asymmetry and the variation of the spectrum, and its saturation at low microwave power (Fig. 3), are properties which resemble those of signal II but the linewidth and g-value differ from signal II [13]. A very similar ESR signal could be induced chemically by addition of 1 mM ferricyanide in the dark (Fig. 2C). The concentration of the ESR component varied in the same way as that of the semiquinone in different batches of particles and was estimated to be at least in the same order of magnitude. In Fig. 4 the kinetics of light-induced absorbance and ESR changes are compared. The rise of both changes was fast (less than 500 μs for the ESR signal, flash experiments not shown) and their dark decay had a half time of 3 to 5 s. Both changes were induced efficiently by a single xenon flash. The dark decay of both changes was accelerated about five-fold by 15 μM DCMU. Ferricyanide, however, affected the two phenomena differently: at 5 μM it accelerated the dark decay of the 320 nm absorbance change but it increased the half-life of the ESR signal to about 20 s.

In the presence of 1 mM ferricyanide light-induced changes were quite different. Absorbance changes had a rapid dark decay (in the order of 50 ms) and steady

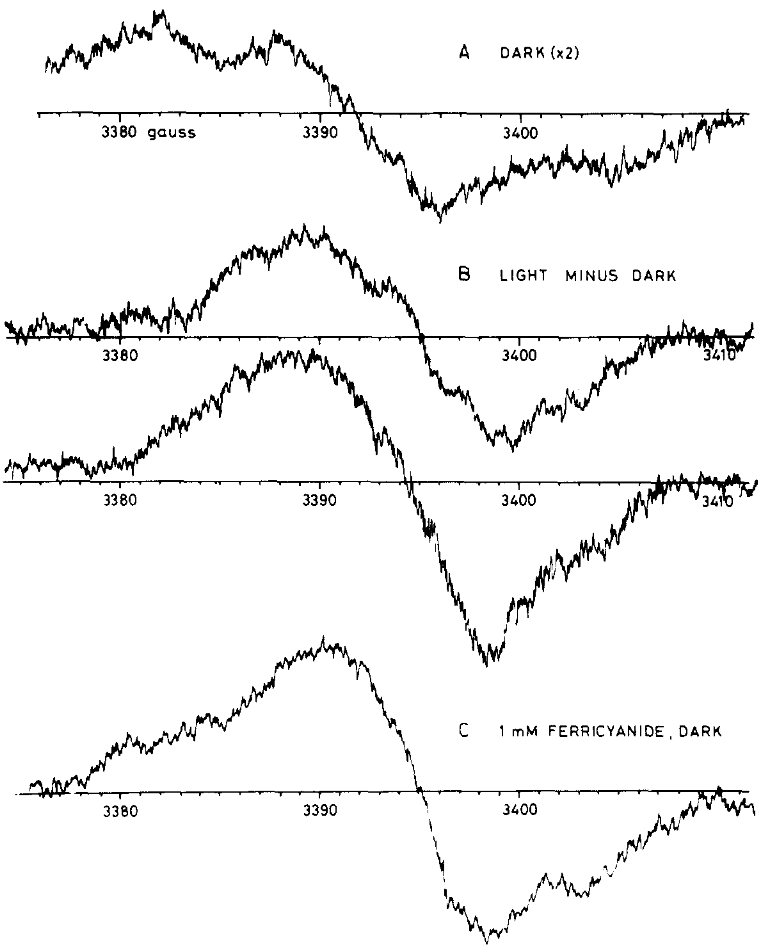

Fig. 2. First derivative ESR spectra. A, dark spectrum in the presence of 5 μM ferricyanide, 4 gauss modulation, microwave power 20 mW, vertical scale 2 times enlarged relative to the other spectra; B, light-induced difference spectra (2 different scans of the same sample are shown), no additions; 1.25 gauss modulation; microwave power 5 mV. These are single scans on 5 times more concentrated particles; C, dark spectrum in the presence of 1 mM ferricyanide, 4 gauss modulation, microwave power 20 mW.

state measurements required a high illumination intensity. The light minus dark difference spectrum (Fig. 5) can be interpreted as the oxidation of a chlorophyll *a* dimer [6], which might be the primary electron donor in photosystem II, P680. The absorbance changes were specific for the system II particles: in the fraction containing system I (F1), and the accessory complex of system II (F3), respectively, no such changes could be measured (less than 1 % on a chlorophyll basis). In different batches the magnitude of these absorbance changes was quantitatively correlated with the semiquinone concentration (measured as in Fig. 1). The P680 measurements thus provide a very convenient method of determining the system II reaction center concentration during fractionation and purification of the particles. Optimal conditions are given in Fig. 6. The particles retained P680 activity for months and no

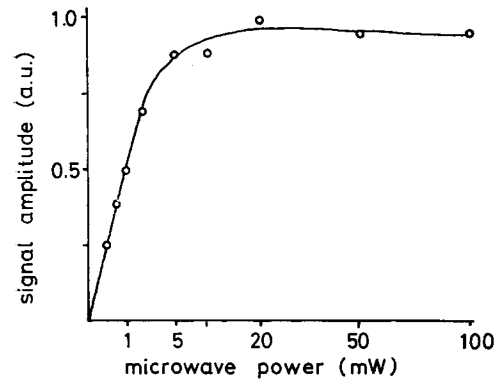

Fig. 3. Microwave power dependence (square root scale) of the light-induced ESR signal shown in Fig. 2B, measured at 3390 gauss, 5 gauss modulation.

decrease in activity of frozen particles has yet been detected. The illumination itself during the measurement is destructive, however: strong illumination in the presence of ferricyanide inactivated the particles within minutes and caused a slow bleaching of bulk pigments, of which only an insignificant part was reversible in the dark. The P680 absorbance changes were insensitive to DCMU but were decreased by ferrocyanide (50 % at 100 μM) which may act as a system II electron donor [6].

In the presence of 1 mM ferricyanide the ESR signal described above was observed in the dark (Fig. 2C). Strong illumination induced two additional components (Fig. 7). The 15 to 20 gauss wide ($\Delta H_{\text{p-p}}$) signal had a dark decay of several

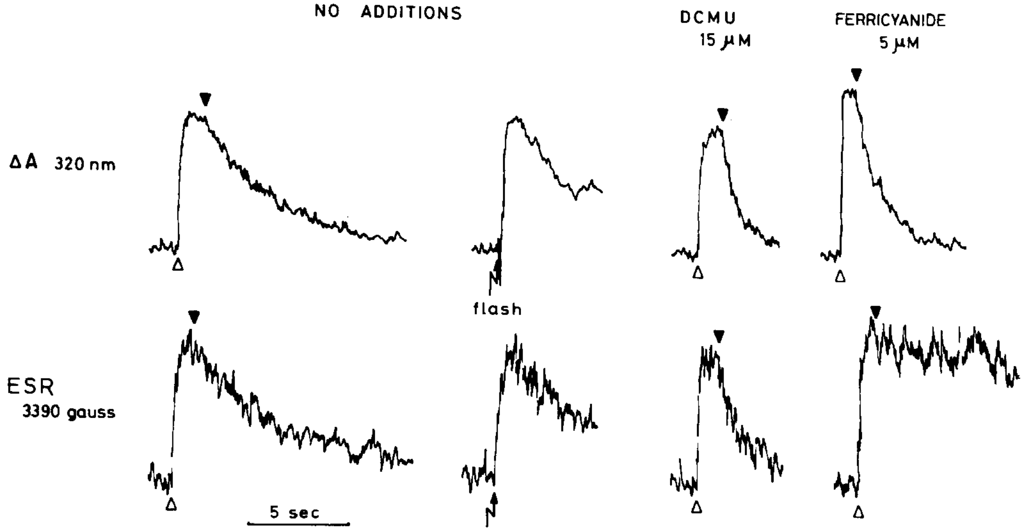

Fig. 4. Kinetics of light-induced absorbance changes at 320 nm, 5 nm bandwidth and ESR changes at 3390 gauss, 8 gauss modulation, measured in the same sample. Open and solid pointers indicate beginning and end of illumination. The flash was an 8 μs xenon flash.

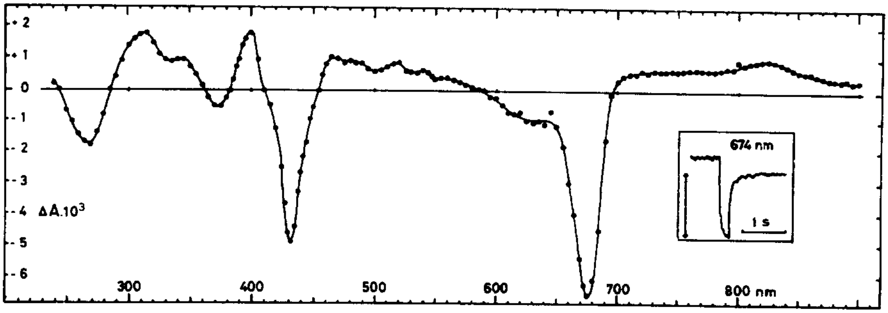

Fig. 5. Light minus dark difference spectrum of absorbance changes measured in the presence of 1 mM ferricyanide. The extent of the rapid dark decay (see kinetic curve, insert) is plotted. Chlorophyll concentration 75 μg/ml, pH 4. Optical pathlength 1.2 mm, bandwidth adjusted to the wavelength interval between measurements. Absorbed intensity about 300 nE $cm^{-2} \cdot s^{-1}$ red or blue. Red and blue regions of the spectrum were calibrated to each other in separate measurements with green illumination. Gradual inactivation of the sample was corrected for by checking it regularly, at a fixed wavelength. The measurement of a complete difference spectrum took about four samples.

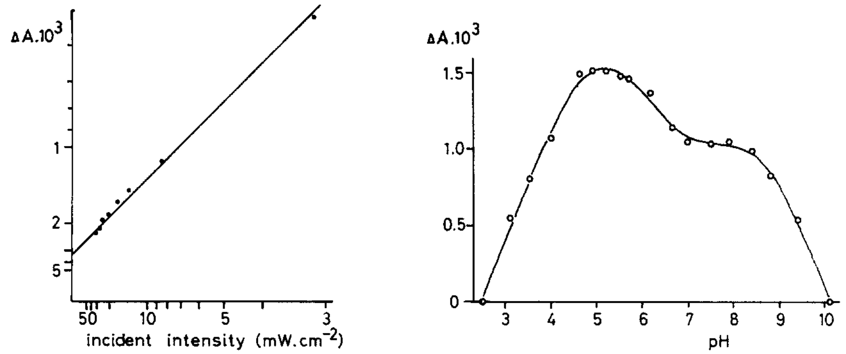

Fig. 6. pH and intensity dependence (double reciprocal plot) of the light-induced 435 minus 460 nm absorbance difference used as a routine assay for P680. Measured in an Aminco DW-2 spectrophotometer, optical pathlength 1 cm; bandwidth 3 nm; actinic light of wavelengths between 630 and 800 nm; the standard reaction mixture contained 7.5 μg chlorophyll/ml, 1mM ferricyanide, and 0.05 M succinate buffer to give a final pH of 5.2. These conditions were optimal except for the 30 mW light intensity which induced only two third of the maximal change, as shown in the figure. For the pH curve the chlorophyll concentration was 5 μg/ml.

seconds and was rather resistant to prolonged illumination. It has not been studied in detail yet and its relation to reaction center activity is uncertain. The 7 gauss wide signal had a dark decay of less than 100 ms and disappeared after prolonged illumination. Its lineshape and *g*-value were not significantly different from those of the well known signal I, just as was found for the ESR signal attributed to oxidized P680 in deoxycholate treated chloroplasts [6]. The shape and the kinetics of the 7 gauss wide signal suggest its tentative assignment to oxidized P680.

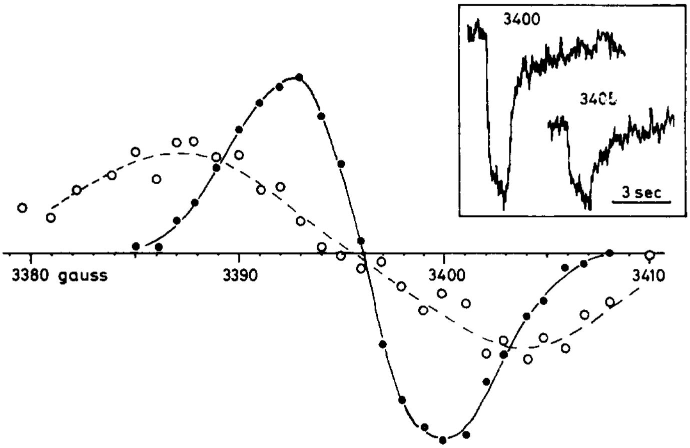

Fig. 7. Light-induced ESR changes in the presence of 1 mM ferricyanide, 4 gauss modulation. Solid circles, component which is inactivated by prolonged illumination; open circles, component resistant to prolonged illumination, average of 4 measurements. The signal amplitude is a minimal estimate of the initial value (first illumination) drawn on the same scale as Fig. 2 and measured on the same sample. Insert: kinetics measured with a time constant of 0.1 s and 2.5 gauss modulation (average of 50).

DISCUSSION

Most of the difference spectrum in Fig. 1 may be attributed to the reduction of the primary acceptor of photosystem II. The behaviour of absorbance changes associated with this reduction in a variety of materials and circumstances is amply documented in the literature [9, 15, 16]. It must be mentioned that in these particles the primary acceptor was partially in the reduced state and the complete photoreduction could be measured only after its oxidation with 5 μM ferricyanide. Values between 10 and 90 % reduced were observed. The reason for this variation is not clear. A similar situation seems to exist in Triton particles [17, 18, 5]. In subchloroplast fragments prepared with deoxycholate the presence of the reduced acceptor was shown to depend on the donor-side of the reaction center [6].

In the absence of additions the reduction of the primary acceptor must be accompanied by the oxidation of some endogenous electron donor. Any absorbance change caused by the oxidation of this donor should be reflected in the difference spectrum as well. Comparison with similar measurements in deoxycholate-treated chloroplasts [9], where an artificial electron donor was present, does not reveal any large new absorbance change. Although some significant differences may be seen, notably in the region from 350 to 450 nm, their assignment to the endogenous donor requires further study. The assignment of the ESR signal shown in Fig. 2B to the oxidized donor seems reasonable. It is not due to the semiquinone itself because its dark decay was slowed down by a low ferricyanide concentration, which accelerated the dark decay of the semiquinone; moreover it was formed in the dark by a high ferricyanide concentration, which would not be expected of the semiquinone, and an ESR signal of the reduced primary acceptor could not be detected in other material. Since the kinetic behaviour of the semiquinone and the new ESR component were otherwise very similar, since both were induced efficiently by a single flash, and since their concentrations were correlated, they seem to be the final products of one and the same electron transport chain. Apparently their dark decay is due to a cyclic or back reaction which is stimulated by DCMU. The new ESR signal reported here may actually have been observed already by others at low temperature, where it might be rather similar to the signal expected of an oxidized chlorophyll dimer.

The rapidly decaying absorbance and ESR changes measured in the presence of a high ferricyanide concentration ($> 100\ \mu M$) may be interpreted as the oxidation of a chlorophyll *a* dimer, as was discussed in detail in ref. 6. The variability of the difference spectra in deoxycholate-treated chloroplasts was not observed in these particles. The magnitude of the changes seemed to be strictly correlated with the concentration of system II reaction centers. Their assignment to the primary electron donor can only be tentative, however, especially after the recent proposal of a chlorophyll dimer as the secondary donor at low temperature [11]. The broad ESR signal observed in the presence of ferricyanide, which decays in a few seconds in the dark, may be due to a secondary electron donor. Its relative resistance to prolonged illumination does not exclude this possibility. Absorbance changes in the ultra violet region, which may contain a large contribution of the primary acceptor, also appeared to be inactivated more slowly than those in the visible region. A more precise ESR spectrum is clearly needed and the possible relation to a fast transient signal of similar linewidth observed in intact chloroplasts [19] certainly warrants further study.

Taken together the data presented in this paper may be explained by a short linear electron transport chain, consisting of the light reaction and two successive stabilization steps on the donor side of the reaction center, all reactions being reversible:

$$\mathrm{DZPQ} \overset{\text{light}}{\rightleftarrows} \mathrm{DZP^+Q^-} \rightleftarrows \mathrm{DZ^+PQ^-} \rightleftarrows \mathrm{D^+ZPQ^-}$$

The light reaction transfers one electron from a chlorophyll *a* dimer (P) to a plastoquinone molecule (Q), as indicated by the absorbance changes of Figs 5 and 1, respectively. In addition the oxidized chlorophyll dimer is revealed by the ESR signal shown in Fig. 7, solid circles, while the plastosemiquinone anion could not be detected with ESR. The secondary donor Z may upon oxidation cause the ESR signal shown in Fig. 7, open circles. The terminal electron donor in these particles (D) was revealed by the ESR signal of its oxidized form, shown in Fig. 2B. In the absence of additions D is oxidized within 500 μs. Z^+ and P^+ could be measured only during strong illumination in the presence of a ferricyanide concentration which rapidly reoxidized Q^-.

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