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Time resolved EPR on photosystem II particles after irreversible and reversible inhibition of water cleavage with high concentrations of acetate

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Electron transfer in irreversibly acetate-inhibited PS II preparations from *Synechococcus* is investigated with EPR. A 1.4 mT wide EPR line with a decay rate consistent with the $P_{680}^+ Q_A^-$ back reaction is tentatively assigned to be a broadened P_{680}^+ spectrum. With reversible inhibition of water cleavage by the ADRY reagent FCCP added with acetate electron transfer is studied as well. Under these conditions the spectrum of the immediate donor to P_{680}^+ is the same as the spectrum of Z^+ in systems with irreversibly inhibited water cleavage (signal II fast) and the same as that of D^+ (signal II slow). The consequences are discussed.

Photosystem II; Acetate; ADRY; EPR; Signal II; P-680; *(Synechococcus)*

1. INTRODUCTION

Most of the components involved in the light induced reactions of PS II produce short-lived paramagnetic intermediates. Direct observation of EPR signals of some of these states is difficult due to their limited lifetime. One approach to this problem involves the systematic retardation of the processes of interest by inhibiting specific reactions in the OEC, for example by Tris-treatment. However, most of these inhibitions lead to an irreversible destruction of the water cleavage capability, therefore it remains unclear whether the observed EPR signals originate from molecular species relevant for the normal electron route or from a side path not related to the physiological process.

Saygin et al. [1] showed that PS II inhibition by high amounts of acetate is exceptional in two regards:

(i) Under certain conditions the inhibition of oxygen evolution is completely reversible by washing out the acetate.
(ii) The electron flow from the donor side to re-reduce P_{680}^+ is retarded 5000-times.

ADRY reagents acting as additional electron donors are known to destabilize higher oxidation states of the Kok cycle [2–4]. Important here is the fact that in acetate + ADRY-treated samples oxygen evolution can fully be reestablished by removal of the additions even if this is done after prolonged illumination [5].

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Abbreviations: ADRY, reagents accelerating the deactivation reactions of water splitting enzyme Y; D, donor side species, oxidized form (D^+) gives rise to signal II slow (recently identified as tyrosine); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FeCy, potassium ferricyanide; OEC, oxygen evolving complex; P_{680}, primary electron donor in PS II; Pheo, intermediate electron acceptor pheophytin; PPBQ, phenyl-*p*-benzoquinone; PS, photosystem; Q_A, first stable electron acceptor in PS II; Z, electron donor to P_{680}^+ in inhibited systems, oxidized form (Z^+) gives rise to signal II fast

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Due to this total reversibility it is assumed that EPR signals obtained from such reversibly inhibited samples originate from physiological components rather than from naturally not related side paths and reflect the conditions of normal O_2 evolution except for the desired retardation of the electron transfer kinetics.

In this work time resolved EPR experiments on acetate and acetate + ADRY-treated samples have been performed in the $g = 2$ region. Signals of P_{680}^+ and the donor to P_{680}^+ have been observed. In samples with acetate only the re-reduction of P_{680}^+ is mainly due to a cyclic electron back reaction from Q_A^- to P_{680}^+ while in samples treated with acetate + ADRY re-reduction of P_{680}^+ is found to result from the retarded electron flow from the donor side. In the latter case the spectrum observed for the immediate donor to P_{680}^+ turns out to be the same as that observed as signal II fast (Z^+) under irreversible conditions (e.g. in Tris-treated PS II [6]) as well as that of signal II slow (D^+).

The well-known D^+ (signal II slow) formerly assigned as a plastohydroquinone radical [6] has recently been identified as a tyrosine radical [7]. In addition it was shown that the Tyr-160 residue in the D2 protein of PS II is involved in the formation of this tyrosine radical and signal II slow [8]. It has also been suggested that Z^+ signal II fast is associated with the corresponding Tyr-161 residue in the homologous D1 protein. Because under our reversible inhibition of water cleavage the spectrum of the immediate donor to P_{680}^+ is identical to that of D^+ it is very likely that the immediate donor in a physiological system should be assigned to a tyrosine radical as well.

2. MATERIALS AND METHODS

PS II particles were prepared from *Synechococcus* according to Schatz and Witt [9]. For EPR purposes the samples were pelleted by centrifugation in a Beckman 60 Ti rotor for 16 h at 50000 rpm. This centrifugation removed substantial amounts of the phycocyanin pigment. The pelleted PS II particles were resuspended in 500 mM mannitol, 20 mM Mes-NaOH (pH 6.5), 20 mM $CaCl_2$ and 10 mM $MgCl_2$ and adjusted to 1 mg Chl/ml. As determined by optical absorption and O_2 yield experiments [9] an average sample contained one active OEC per 100 Chl molecules with a PS II/PS I ratio of >30. The material was stored in the dark at 77 K.

For the experiments, the thawed sample was incubated in darkness for 15 min with acetic acid/sodium acetate at a final concentration of 600 mM (total sample, pH 5.5). An artificial acceptor system of 10 mM FeCy and 1 mM PPBQ was included. When indicated, 100 μM of the ADRY reagent FCCP dissolved in methanol were added immediately before the experiment giving a 10-fold surplus for each RC. The overall methanol concentration did not exceed 5%.

EPR experiments were carried out with a Varian E-line X-band spectrometer. To improve the spectrometer's time resolution the 100 kHz input filter of the lock-in amplifier as well as the low pass filter immediately following the phase detector were removed [10] yielding a total time response of $t_{1/2} = 20\ \mu s$. The E-231 (TE102) cavity's front-end piece was substituted by a specially designed grid window allowing a light transmission rate of 70%. Continuous illumination was provided by a 600 W tungsten halogen lamp followed by an 80 mm water filter, and by Schott OG530 and KG2 glass filters. For observation of the kinetics the beam of a frequency-doubled Nd-YAG laser (Quanta-Ray DCR-2, λ = 532 nm, pulse energy 20 mJ, pulse width 6 ns) was spread by a cylindrical concave lens and diffused by an opalescent glass immediately before entering the cavity to achieve a homogeneous illumination of a sample area of 3 cm^2. A total of 1 ml sample was pumped through a flat cell inside the resonator back to a reservoir cooled to 0°C. The 0.3 mm flat cell contained 10% of the total sample volume. The sample was changed after 18000 flash excitations when inhibited with acetate and after 2000 flashes when treated with acetate and FCCP. The latter more frequent change was necessary due to poisoning effects of FCCP and the long duration of the experiments because of the low repetition rate.

Signal averaging was carried out with a Gould 4500 transient digitizer. To gain full spectral information transients were taken at a sufficient number of equidistant magnetic field points and stored in the computer. This data set was analysed in two different ways:

(i) All stored transients were digitally scanned with an appropriate time window ('boxcar-window'). Plotting the numerical value obtained in these windows against the magnetic field yields the spectrum of the transient. Baseline effects were eliminated using the post transient level as a reference (applied in fig.2).

(ii) All stored transients were fitted to a multiexponential decay by a least square algorithm. Plotting the coefficients for each of the chosen exponentials against the magnetic field gives the spectrum of the associated kinetics (applied in fig.3).

Flash artifacts were eliminated by subtracting an average of off-resonance transients. Data acquisition as well as field sweep, sample flow and laser excitation were under computer control.

3. RESULTS

3.1. *PS II particles with acetate only*

Fig.1 shows the EPR signal II spectra in acetate-treated PS II preparation, recorded under continuous illumination and 5 min after illumination. In the latter case the signal is reduced by a factor of about two. We conclude that under illumination

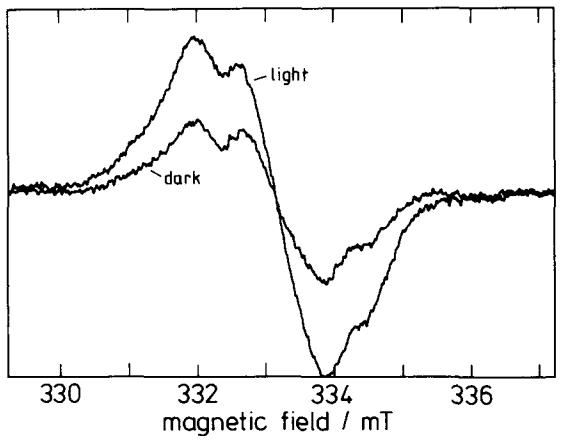

Fig.1. Signal II of acetate only treated PS II particles under continuous illumination and 5 min after illumination, time constant 0.25 s, scan speed 2 mT/min. Microwave power 20 mW, modulation amplitude 0.4 mT, microwave frequency 9.345 GHz.

the oxidized form of electron donor Z is accumulated, and that signal II slow of D^+ is responsible for the signal in the dark. The decay kinetics for the light-induced contribution shows several time constants between 100 ms and $\gg 16$ s. Similar results were obtained by Damoder and co-workers [11] after chloride depletion.

The spectrum of D^+ in fig.1 is identical to that obtained in other PS II preparations [6]. Under fast repetitive excitation conditions (10 Hz laser pulse repetition) the major part of Z remains oxidized. For this reason P^+_{680} is left without its normal electron source for re-reduction to P_{680}. Fig.2a shows a typical transient for the acetate-inhibited PS II particles. Two decay times can be identified and are used to analyze the transients at 161 magnetic field points. The main transient component has an exponential decay of $t_{1/2} = 530\ \mu s$. Its spectrum is 1.4 mT wide around $g = 2.0031$ and has a slightly asymmetric shape (fig.2b, broken line). A faster component with $t_{1/2} = 170\ \mu s$, $\Delta B_{pp} = 0.8$ mT, centered at $g = 2.0027$ (fig.2b, solid line), has only $\simeq 10\%$ intensity.

The broad line shape of the dominant signal is rather unexpected. The inset in fig.2b shows this signal together with the typical signal II. Note that the broad signal (broken line) exhibits neither the typical structure of the signal II nor the increased g factor.

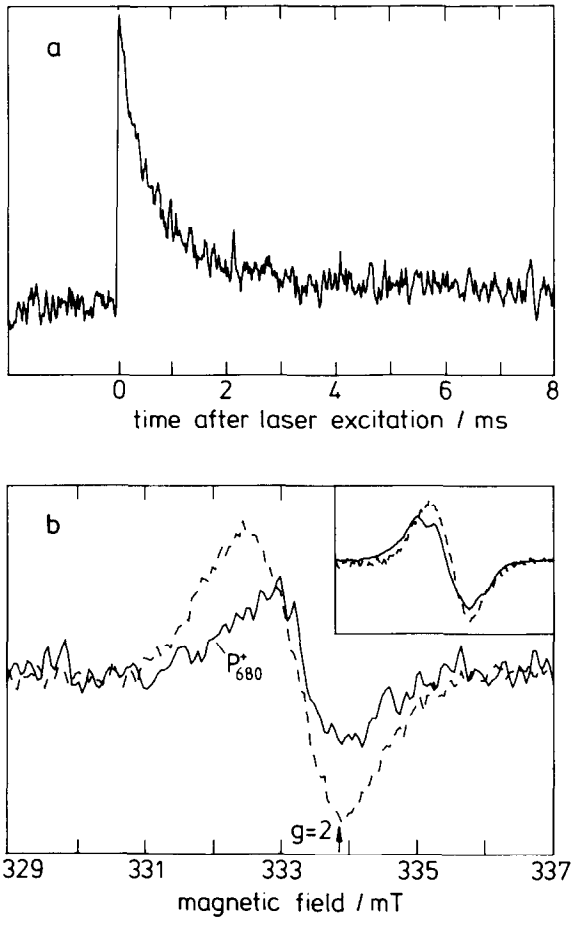

Fig.2. Acetate only treated PS II particles. (a) Flash-induced transient at 332.5 mT (average of 3840 events). The decay curve can be fitted with a double-exponential, the time constants are 170 and 530 μs. (b) Spectra of the 170 μs transient (solid line) and the 530 μs transient (broken line) as obtained by appropriate time windows. 161 field points spaced 0.05 mT were scanned 12 times. Per field point and scan 64 events were averaged, so each of the 161 transients is the total average of 768 events. Microwave power 40 mW, modulation amplitude 0.4 mT, laser repetition rate 10 Hz. (Inset) The 530 μs spectrum in relation to signal II as obtained just before the flash excitation, identical to signal II (dark) in fig.1. All transients are on top of a signal II spectrum.

3.2. *PS II particles with acetate plus ADRY*

Addition of the ADRY reagent FCCP to the acetate-incubated sample prior to first illumination permits repetitive excitation experiments on a reversibly inhibited sample [5].

Two transient signals could be observed on top of a signal II slow spectrum (fig.3a). The first one

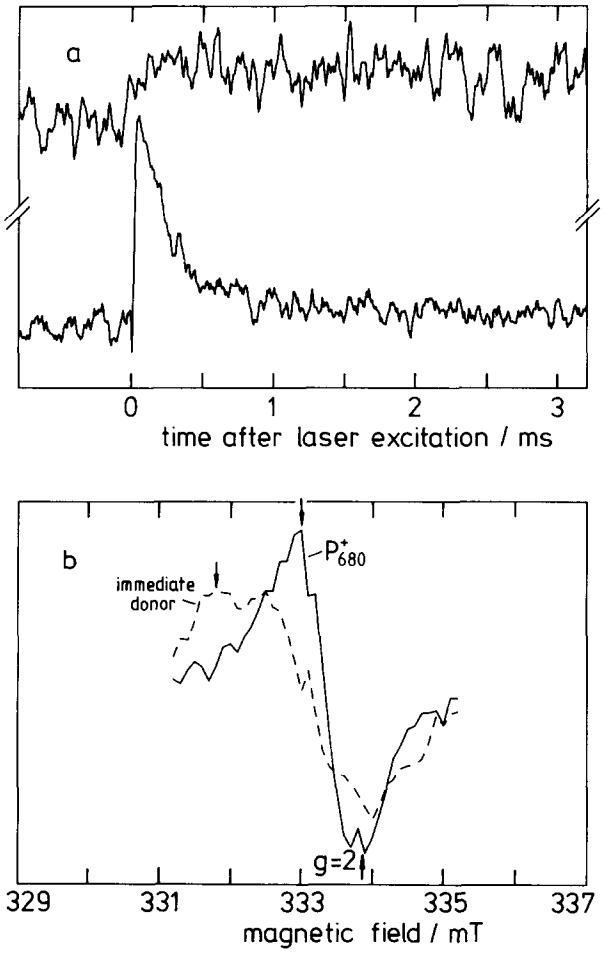

Fig.3. Acetate + ADRY-treated sample. (a) Top, flash-induced transient at 331.8 mT; bottom, transient at 333 mT, decay time 170 μs. The respective field positions are marked by arrows in b. (b) Spectra of the coefficients of $f = \alpha \cdot \exp(-\ln 2 \cdot t/170\,\mu s) + \beta(1 - \exp(-\ln 2 \cdot t/170\,\mu s))$. 41 field points spaced 0.1 mT were scanned 8 times. Each transient represents 512 events. Microwave power 10 mW, modulation amplitude 0.5 mT, laser repetition rate 1 Hz. Solid line (coefficients α): spectrum associated with the transient in a (top); broken line (coefficients β): spectrum associated with the transient in a (bottom), magnified four times.

(fig.3b, solid line) (apparatus-limited rise-time, decay $t_{1/2}$ = 170 μs, g = 2.0027, ΔB_{pp} = 0.8 mT) clearly represents P^+_{680} [12–16] while the other one, whose rise time corresponds to the decay of the P^+_{680} transient, displays a signal II line shape with zero crossing at g = 2.0044 (fig.3b, broken line). It decays within several milliseconds (not shown) in agreement with data published for similar ADRY reagents [2]. This spectrum of the immediate donor as well as D^+ in fig.1 is for all practical purposes identical to the spectra of D^+ and Z^+ of Tris-washed samples [6].

4. DISCUSSION

The most important effect of acetate on PS II is the retardation of both the forward reaction to P^+_{680} (5000-times slower) and the back reaction from Q_A^- (about 5-times slower). It has been shown that acetate inhibits the OEC by competing with bound water or cofactors of water cleavage [11,17–20]. With our high overall concentrations we assume this still to be the case. On the acceptor side acetate may deplete bicarbonate and thus retard the electron flow from Q_A to Q_B and to the plastoquinone pool [21,22]. Thus the electron flow is disturbed on either side of the reaction center forcing electrons into a cyclic path involving P^+_{680} Q_A^- recombination after light excitation, while in the presence of an external lipophilic donor a slow forward reaction can be observed.

4.1. *Acetate only*

The very slow signal II reduction kinetics ($\geq$100 ms) in the presence of acetate implies that under multiple excitation conditions Z must mainly remain oxidized. The slow reduction also indicates the absence or the inefficiency of external electron donors to Z^+. Consequently, with the laser repetition rate used only a very minor part of Z^+ is able to become reduced. Only this re-reduced portion can donate electrons to P^+_{680} upon the next flash-induced charge separation. In the EPR spectrum the corresponding P^+_{680} signal is observed as the weak narrower contribution with a decay time of 170 μs (see fig.2b, solid line). It has the typical width and g factor of the P^+_{680} signal. The optical data [5] render a correspondingly decaying, weak P^+_{680} contribution confirming this interpretation. However, the major part of oxidized P^+_{680} has to be re-reduced without the involvement of Z. This is possible either by a P^+_{680} Q_A^- back reaction or by another donor.

The optically observed re-reduction of most of P^+_{680} shows [5] a time constant of 530 μs as well. This decay constant is also observed for part (60%) of the re-oxidation of Q_A^-. The remaining part (40%) of Q_A^- decays on a much longer time scale

suggesting an alternate donor of yet unknown identity to contribute to the re-reduction of P_{680}^+ [5].

The excellent agreement in the decay constants of optically observed P_{680}^+ and the 1.4 mT wide EPR line (fig.2b, broken line) suggests the assignment of this line to P_{680}^+.

For kinetic reasons it would also be acceptable to assign this broad EPR line to the 530 μs Q_A^- decay. However, in EPR the Q_A^- is expected as a Q_A^-Fe signal with a large g anisotropy [21,23,24]. A Q_A^- signal in the $g = 2$ region would only be possible if the iron was decoupled from Q_A^- by the acetate. In this case the typical quinone spectrum at $g = 2.0044$ [25] would become observable. Then the broad line of fig.2b could be composed of such an iron decoupled Q_A^- spectrum and a typical P_{680}^+ spectrum. However, no information is available on such a rather unlikely acetate effect.

All other possibilities for a contribution to the 1.4 mT wide line can readily be excluded. If it were assigned to a donor side species it would rise rather than decay with the 530 μs time constant. The intermediate acceptor $Pheo^-$, whose spectrum (1.2 mT, $g = 2.003$) [25,26] in the absence of a paramagnetic Q_AFe complex is well suited for our purpose, is out of the question because the P_{680}^+ $Pheo^-$ back reaction has been found to be only several nanoseconds at room temperature [27], and because no optical absorption change due to this component has been observed [5].

Based on these considerations we rather conclude that the 1.4 mT wide line is a broadened P_{680}^+-spectrum. The nature of the modification remains unclear. The broadening of the EPR signal of P_{680}^+ may be the result of the presence of acetate and the still lasting oxidation of the donor to P_{680}^+.

4.2. *Acetate + ADRY*

Immediately after light excitation a typical P_{680}^+ spectrum rises within experimental time resolution. The immediate donor re-reduces P_{680}^+ within 170 μs, in turn forming a signal II-like spectrum with this rise time (fig.3). This interpretation of the EPR data is in good agreement with optical results [5]. Since the presence of FCCP provides the oxidized immediate donor with electrons, this signal decays within milliseconds. These EPR as well as the optical data prove a retarded forward reaction from the immediate donor to P_{680}^+.

All published spectra of the immediate donor to P_{680}^+ under oxygen evolving conditions were either of low resolution [28] or were indicated as preliminary results [29] and were not conclusive with respect to the comparison with the well documented spectra of Z^+ and D^+.

Our main experimental result shows that the signal of the immediate donor (fig.3b, broken line) is identical to that of D^+ (fig.1) and to that of Z^+ in Tris-washed samples [6]. We assume that because of the total reversibility of oxygen evolution, this forward reaction takes place with unmodified components only retarded 5000 times. Thus the so obtained information should also be relevant for the reaction under oxygen evolving conditions.

Recently, it was shown that D^+ is a tyrosine radical [7,8]. Due to the similarity of the spectra the immediate donor to P_{680}^+ in our reversibly inhibited sample must also be assigned to a tyrosine radical as already suggested by Debus et al. [8].

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