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A NEW EPR SIGNAL ATTRIBUTED TO THE PRIMARY PLASTOSEMIQUINONE ACCEPTOR IN PHOTOSYSTEM II

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A study of signals, light-induced at 77 K in O_2-evolving Photosystem II (PS II) membranes showed that the EPR signal that has been attributed to the semiquinone-iron form of the primary quinone acceptor, Q_A^-Fe, at $g = 1.82$ was usually accompanied by a broad signal at $g = 1.90$. In some preparations, the usual $g = 1.82$ signal was almost completely absent, while the intensity of the $g = 1.90$ signal was significantly increased. The $g = 1.90$ signal is attributed to a second EPR form of the primary semiquinone-iron acceptor of PS II on the basis of the following evidence. (1) The signal is chemically and photochemically induced under the same conditions as the usual $g = 1.82$ signal. (2) The extent of the signal induced by the addition of chemical reducing agents is the same as that photochemically induced by illumination at 77 K. (3) When the $g = 1.82$ signal is absent and instead the $g = 1.90$ signal is present, illumination at 200 K of a sample containing a reducing agent results in formation of the characteristic split pheophytin$^-$ signal, which is thought to arise from an interaction between the photoreduced pheophytin acceptor and the semiquinone-iron complex. (4) Both the $g = 1.82$ and $g = 1.90$ signals disappear when illumination is given at room temperature in the presence of a reducing agent. This is thought to be due to a reduction of the semiquinone to the nonparamagnetic quinol form. (5) Both the $g = 1.90$ and $g = 1.82$ signals are affected by herbicides which block electron transfer between the primary and secondary quinone acceptors. It was found that increasing the pH results in an increase of the $g = 1.90$ form, while lowering the pH favours the $g = 1.82$ form. The change from the $g = 1.82$ form to the $g = 1.90$ form is accompanied by a splitting change in the split pheophytin$^-$ signal from approx. 42 to approx. 50 G. Results using chloroplasts suggest that the $g = 1.90$ signal could represent the form present in vivo.

Introduction

Recent advances in the understanding of Photosystem II (PS II) primary photochemistry have come from EPR work (reviewed in Ref. 1) based on the analogy between this photosystem and the better characterized reaction centre of purple photosynthetic bacteria (reviewed in Refs. 2 and 3).

In the bacterial reaction centre, the primary quinone acceptor exhibits an unusual EPR signal when in the semiquinone state [4,5]. This signal which consists of features at $g = 1.82$ and $g \approx 1.65$ has been well characterized biochemically and spectroscopically and it is thought that it arises from an interaction between the semiquinone and a ferrous iron atom (Q_A^-Fe) [2,3]. When the more

Abbreviations: Mes, 4-morpholineethanesulphonic acid; Mops, 4-morpholinepropanesulfphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS II, Photosystem II; BPh, bacteriopheophytin; Chl, chlorophyll; Cyt, cytochrome; Ph, pheophytin.

primary acceptor, bacteriopheophytin (BPh), is trapped in its reduced form in the presence of this semiquinone-iron complex, the BPh^- signal is split about the $g = 2$ region because of an interaction between the BPh^- and the Q_A^-Fe [6,7].

In 1980, Klimov et al. [8] demonstrated that a signal almost identical to the split BPh^- signal of bacteria could be generated in PS II under conditions identical to those originally used by Tiede et al. [6] to trap BPh^-. This work indicated that a pheophytin intermediate acceptor and a semiquinone-iron complex existed in PS II. Shortly afterwards, the discovery and characterization of a spin-polarized triplet state of P-680 [9,10], analogous to that reported in bacteria [11] strengthened the analogy between PS II and the purple bacteria. Klimov et al. [12] went on to show that the presence of iron and plastoquinone was required to form the split Ph^- signal, but the characteristic signal expected from the semiquinone-iron complex itself could not be observed. Later, a rather poorly resolved signal at $g = 1.82$ was photoinduced in purified PS II particles isolated from a PS I-less mutant of a green alga [13]. This was attributed to the long sought after semiquinone-iron complex in PS II [13].

With the advent of preparations of pure PS II membranes from higher plants, much clearer spectra of the semiquinone-iron signal were obtained and the $g \approx 1.65$ feature was resolved [14,15], just as seen previously in bacteria.

The large and easily observable semiquinone-iron signals obtained in this kind of PS II preparation [16] lead us to ask the question: why did we (and other groups) have so much difficulty seeing this signal earlier? This is answered in part by the fact that the iron interaction is more labile in PS II than it is in bacteria and, in the majority of purified PS II particles, very little of the iron interaction survives the first encounter with detergent [1]. In this paper, we provide another answer to this question: the Q_A^-Fe species gives rise to two kinds of EPR signal, the 'classical' $g = 1.82$ signal at low pH and a new $g = 1.90$ signal at higher (more physiological?) pH values. Some of these results have been reported in a preliminary form earlier [17].

Materials and Methods

Oxygen-evolving PS II membranes were prepared according to the method of Berthold et al. [16] with the modifications used earlier [15] or those introduced by Ford and Evans [18]. The membranes were usually stored at high concentration (Approx. 10 mg/ml) at −80°C in darkness in the following buffer: 0.4 M sucrose/15 mM NaCl/5 mM $MgCl_2$/20 mM Mes (pH 6.0).

The pH treatments were carried out as follows. Stored membranes were thawed in darkness and diluted (approx. 0.3 mg/ml) into an appropriate resuspension buffer: 5 mM $MgCl_2$, 15 mM NaCl and 50 mM of either Mes (pH 6.0), Mes (pH 6.5), Mops (pH 7.0), Hepes (pH 7.5), Hepes (pH 8.0) or Hepes (pH 8.5). The membranes were incubated in these buffers for 1 h at 4°C in darkness before being pelleted by centrifugation at $35000 \times g$ for 30 min. The pellets were resuspended in buffers at the same pH as used for the incubation but in which 4 M sucrose, 15 mM NaCl and 5 mM $MgCl_2$ were present. In some cases, samples were washed with 0.8 M Tris (pH 8.2), or glycine (pH 9.0) was added directly to EPR samples (see legends).

EPR samples were made up as described previously [14] and measurements were made using a Bruker ER-200t-X-band spectrometer fitted with an Oxford Instruments liquid helium temperature cryostat and control system. Illuminations at 77 K were done in an unsilvered dewar containing liquid nitrogen using a 800 W projector. Illumination at 20°C of reduced samples was done using the same projector with an attenuated voltage. The sample was maintained at 20°C in a water-bath and anaerobicity was maintained by blowing O_2-free argon over the sample.

For the experiments with chloroplasts, the chloroplasts were prepared in the course of a PS II membrane preparation as described above, but before Triton treatment, the chloroplasts were instead suspended at approx. 0.5 mg Chl/ml in either 50 mM Hepes (pH 8.0)/5 mM $MgCl_2$/15 mM NaCl or 50 mM Mes (pH 6.0)/5 mM $MgCl_2$/15 mM NaCl. The chloroplasts were left to incubate in these buffers for 1 h in darkness at 4°C before being pelleted by centrifugation. The pellets were resuspended at high concentration in

buffers containing 0.4 M sucrose, 15 mM NaCl and 5 mM $MgCl_2$ and either 50 mM Mes (pH 6.0) or 50 mM Hepes (pH 8.0). The chloroplast suspension was put into EPR tubes in the presence of the appropriate additions (see legends) and centrifuged to concentrate the chloroplasts in the EPR tubes.

Results and discussion

Evidence that a signal at $g = 1.90$ arises from $Q_A^- Fe$

Illumination at 77 K of PS II membranes results in stable photoreduction of Q_A largely at the expense of cytochrome *b*-559.

$$\text{Cyt } b\text{-559P-680PhQ}_A\text{Fe} \xrightarrow{77\text{ K } h\nu} \text{Cyt } b\text{-559}^+ \text{ P-680PhQ}_A^- \text{Fe}$$

The two radicals formed under these conditions can be observed by EPR. The oxidized cytochrome *b*-559 gives rise to a signal at $g \approx 3.0$, first reported by Malkin and Vänngård [19] (not shown but see Ref. 15), while the semiquinone-iron signal is manifest as a number of resonances at high field (Fig. 1A). As well as the usual $g = 1.82$ signal and associated low-field feature at $g \approx 1.67$, a new signal at $g = 1.90$ is also photoinduced. The relative proportions of the $g = 1.82$ and $g = 1.90$ signals photoinduced varied from preparation to preparation. In particular, it was found that in samples washed and resuspended in Tris (pH 8.2), no $g = 1.82$ could be detected and instead a large $g = 1.90$ signal was present. A spectrum from such a sample is shown in Fig. 1B. The photoinduced signal consisted of a broad resonance centred at $g = 1.90$ with an associated low-field feature at $g \approx 1.64$.

Fig. 2 shows that addition of sodium dithionite to both kinds of sample results in the presence in the dark of the $g = 1.90$, $g = 1.82$ and $g \approx 1.65$ signals to an extent similar to that photoinduced (compare with Fig. 1).

Illumination of the chemically reduced samples at 200 K resulted in the formation of the split Ph^- signal in both samples (Fig. 3).

$$\text{DP-680PhQ}_A^- \text{Fe} \xrightarrow{200\text{ K } h\nu} \text{D}^+ \text{ P-680Ph}^- \text{Q}_A^- \text{Fe}$$

In this reaction, D, the ultimate source of the electron, is probably dithionite.

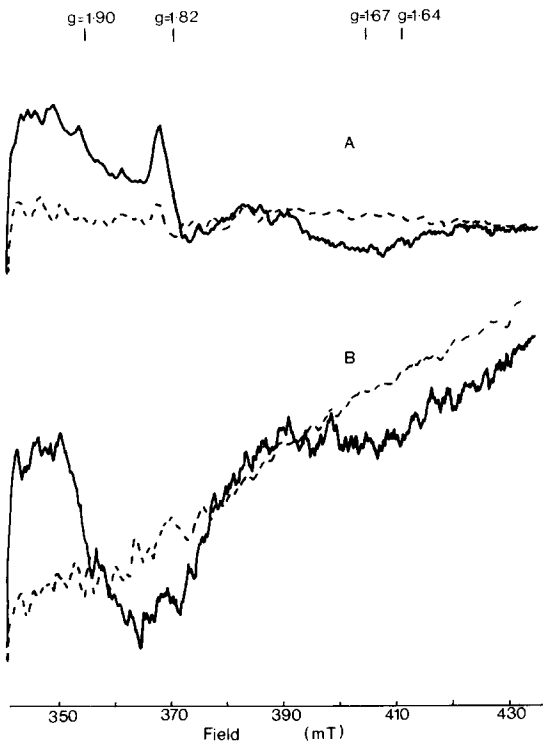

Fig. 1. EPR signals photoinduced in PS II membranes by 10 min illumination at 77 K (solid lines). The broken lines show spectra recorded in the dark prior to illumination. A was in Mes buffer (pH 6.0), B was washed and resuspended in Tris (pH 8.2). Instrument conditions were as follows: microwave power, 8 dB down from 200 mW (approx. 35 mW); temperature, 4–5 K; modulation amplitude, 20 G; microwave frequency, 9.45 GHz; instrument gain, $A = 2.5 \cdot 10^5$, $B = 5 \cdot 10^5$. Chlorophyll concentration in B (approx. 5 mg Chl/ml) was approximately half of that in A.

The fact that the split signal is formed to a similar extent in samples where the $g = 1.82$ signal is absent and the $g = 1.90$ signal is present is a strong indication that the $g = 1.90$ signal also arises from the semiquinone-iron complex. In neither sample were changes in the semiquinone-iron signals observed to result from 200 K illumination.

It is of note that the splitting of the split Ph^- signal changes slightly with pH in that, in the presence of the $g = 1.90$ signal alone, the splitting is approx. 50 G, while in the presence of a mixture of the $g = 1.90$ and $g = 1.82$ forms, the splitting is approx. 42 G. In a sample where only the $g = 1.82$ signal was present (i.e., in the presence of *i*-dinoseb), the splitting of the split Ph^- signal was

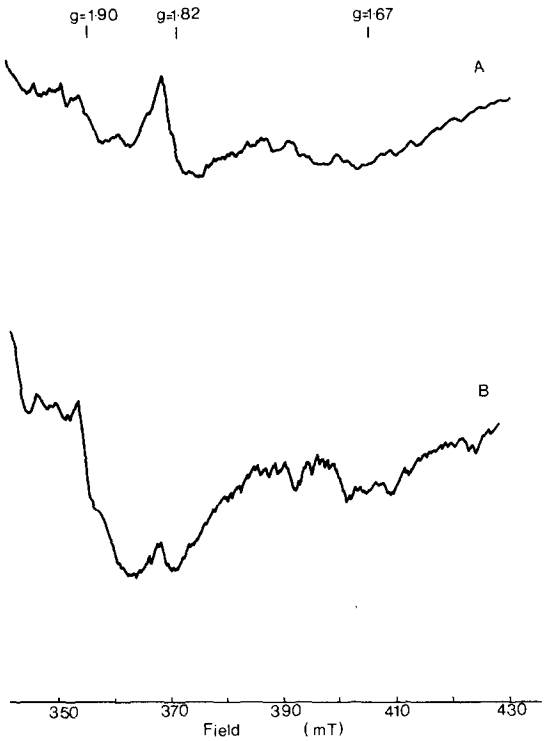

Fig. 2. EPR signals chemically reduced in PS II membranes by anaerobic incubation for 10 min in the presence of sodium dithionite (50 mM). A is at pH 6.0, B is washed and resuspended in Tris (pH 8.2). EPR conditions and chlorophyll concentrations were exactly as in the legend to Fig. 1.

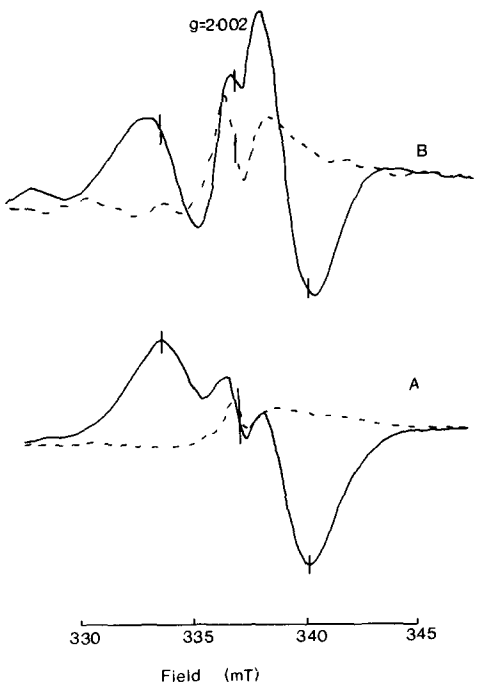

Fig. 3. The split pheophytin$^-$ signal photoinduced in samples having largely the $g = 1.90$ (upper trace, B) and largely the $g = 1.82$ (lower trace, A) form of the semiquinone-iron. The broken lines were recorded in the dark in samples previously incubated with sodium dithionite (50 mM) for 10 min at 20°C. The solid lines show the signals recorded after 4 min of illumination at 200 K. The lower trace (A) was recorded using membranes at pH 6.0. The upper trace (B) was recorded using membranes washed and resuspended in Hepes (pH 8.5). A spectrum recorded on a sample washed and resuspended in Tris (pH 8.2) gave an almost identical spectrum to that shown in B. Instrument setting, were as in Fig. 1, except that the modulation amplitude was 10 and the gain was $5 \cdot 10^4$ in both cases. Chlorophyll concentration was approx. 10 mg Chl/ml in both samples. The vertical lines at $g = 2.002$ indicate a g-marker at this value. The vertical lines at higher and lower field positions mark equivalent field positions in the upper and lower spectra.

only 33 G [15]. The split Ph$^-$ signal induced in a sample in which the $g = 1.90$ semiquinone-iron signal is largely present (upper trace Fig. 3B) exhibits a different shape as well as a different splitting (see also Fig. 2 of Ref. 15).

A characteristic of the semiquinone-iron complex in bacteria is that under some circumstances it can reluctantly undergo a second reduction step. This reaction can take place over a long incubation period at very low potential and high pH (less than -500 mV, pH 10) [20] or if illumination is given at ambient temperature in the presence of sodium dithionite [21]. When PS II membranes are submitted to the latter treatment, the EPR signal attributed to Q_A^-Fe at $g = 1.82$ and $g \approx 1.65$ disappears [17]. In the same sample, the $g = 1.90$ signal also disappears (Fig. 4A, B). Similarly, in the sample where only the $g = 1.90$ signal was present, illumination at 0°C resulted in disappearance of this signal (Fig. 4C, D). At the same time, a large $g \approx 2.00$ radical was induced (not shown). This signal is presumably due to Ph$^-$, but in the absence of the semiquinone. When the sample was thawed under argon and re-frozen after a few minutes ($t_{1/2} \approx 5$ min) in darkness, the Ph$^-$ radical had largely disappeared. Further illumination of the samples at 200 K did not result in the formation of the split Ph$^-$ signal, instead the

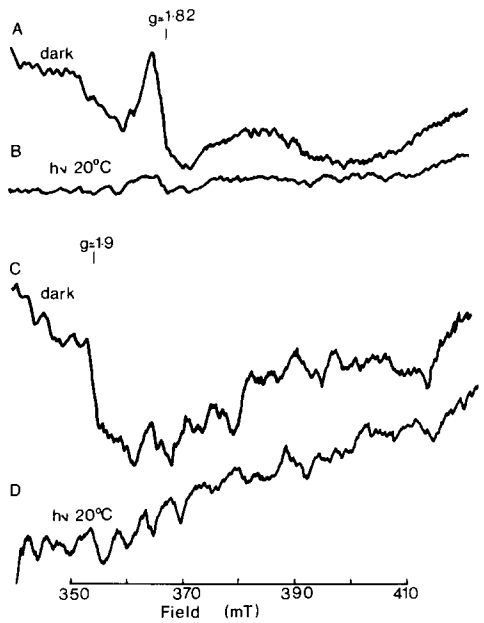

Fig. 4. The effect of illumination of PS II membranes at room temperature in the presence of sodium dithionite (50 mM). A and B were at pH 6.0 with a chlorophyll concentration of approx. 10 mg/ml. C and D were washed and suspended in Tris (pH 8.2), with a chlorophyll concentration of approx. 5 mg/ml. Illumination was carried out in a 20°C water-bath and samples were frozen in darkness approx. 2 s after the end of the illumination period. Instrument conditions were as in Fig. 1. The gain for C and D was twice that in A and B.

unsplit Ph^- radical with ΔH of approx. 13 G was regenerated (not shown).

$$DP\text{-}680PhQ_A^- Fe \xrightarrow{h\nu\ 20°C} D^+ P\text{-}680Ph^- Q_A^{2-} Fe$$

$$\xrightarrow{\text{dark } 25°C} DP\text{-}680PhQ_A^{2-} Fe \xrightarrow{200\ K\ h\nu} D^+ P\text{-}680Ph^- Q_A^{2-} Fe$$

In these reactions, the ultimate source of the electrons is again probably dithionite, D. These reactions are analogous to those thought to take place in bacterial reaction centres [21]. The same analogy was used earlier to explain the absence of the split signal when illumination was given to PS II particles at room temperature [8].

Thawing of the sample resulted in rapid disappearance of the Ph^- radical but the Q_A^-Fe signal could only be regenerated by oxidation of the sample followed by re-reduction or photoreduction of the semiquinone (see Ref. 17). Similar behaviour was observed in bacterial systems [20] and it is imagined that, having undergone the second reduction step very unwillingly, the quinone environment has changed, or perhaps it even has left the reaction centre. Thus, reoxidation probably occurs as a two-electron step at higher potential before the quinone returns to its normal conformation or site.

The data presented above is taken as good evidence that the $g = 1.90$ signal arises from Q_A^-Fe. The existence of two EPR forms (i.e., $g = 1.82$ and $g \approx 1.67$ vs. $g = 1.90$ and $g \approx 1.64$) presumably reflects a difference in the interaction between the semiquinone and the iron induced by an environmental change.

Conditions controlling the existence of the two resonance forms of Q_A^-Fe

pH. The first observations made of samples exhibiting only the $g = 1.90$ form of Q_A^-Fe were in experiments where PS II membranes were washed and resuspended in Tris at pH 8.2. In subsequent experiments, the membranes were washed and resuspended in buffer with sucrose, NaCl, $MgCl_2$ and Mes (pH 6.0) after Tris treatment and the normal distribution of $g = 1.82$ and $g = 1.90$ signals was observed. This indicated that pH rather than Tris-washing was responsible for the effect.

More systematic pH experiments are shown in Fig. 5. Samples were washed and incubated for 1 h in a buffer at a given pH and then concentrated for EPR experiments. It can be seen that a gradual diminution of the $g = 1.82$ signal occurs as the pH is increased from 6.0 to 8.5. At the same time, the $g = 1.90$ signal gradually increases. At pH 9.0, the $g = 1.82$ signal is almost completely absent. This effect is reversible by 50–60% when a sample at pH 8.5 is washed and resuspended at pH 6.0. The long incubation periods and the fact that some clumping of membranes occurs, making penetration of buffers difficult, probably accounts for some of the irreversibility.

Herbicides In a recent publication, we reported that some herbicides induce changes in the size and shape of the Q_A^-Fe signals in PS II [15]. Of particular note was that *i*-dinoseb apparently converted the $g = 1.90$ form of the signal into the $g = 1.82$ form [15]. In a sample where the $g = 1.90$

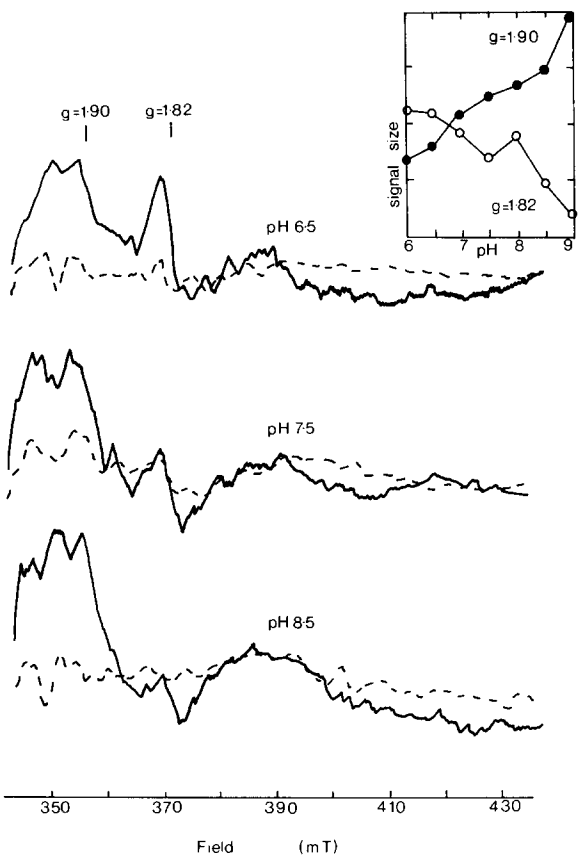

Fig. 5. The effect of pH on the relative sizes of the $g = 1.90$ and $g = 1.82$ signals in PS II membranes. Treatments in buffers at various pH values were carried out as described in Materials and Methods. Broken lines are spectra that were recorded in darkness, while solid lines were recorded after 10 min of illumination at 77 K. Instrument conditions were as described in Fig. 1A. The chlorophyll concentration was the same in all samples (approx. 10 mg Chl/ml). The inset is a plot of the size of the light-induced $g = 1.90$ (●) and $g = 1.82$ (○) signals recorded at a series of pH values.

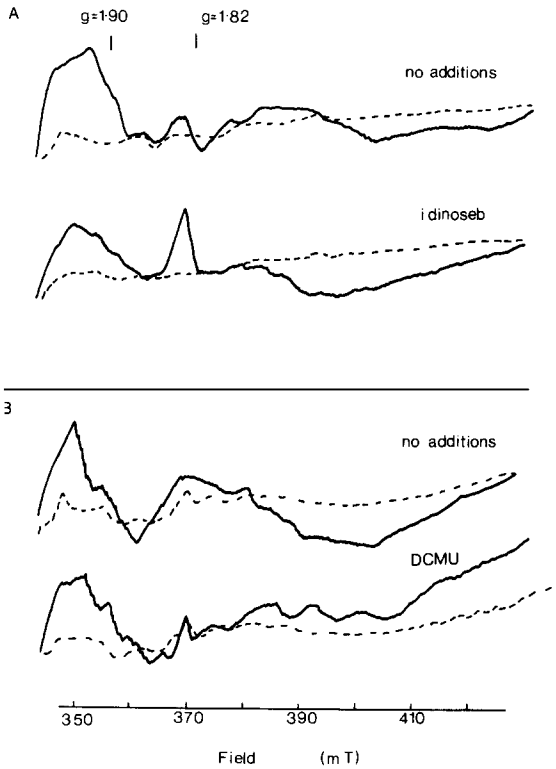

Fig. 6. The effect of herbicides on the relative contributions of the two resonance forms of the primary semiquinone-iron complex. Broken lines were recorded in darkness, while solid lines were recorded after 10 min of illumination at 77 K. A shows spectra recorded in PS II membranes at pH 8.5 in the presence (lower) and absence (upper) of *i*-dinoseb (approx. 900 μM). The samples in A were washed and resuspended in Hepes (pH 8.5). B shows samples at pH 9.0 in the presence (lower) and absence (upper) of 400 μM DCMU. The samples in B were washed and resuspended in Hepes (pH 8.5) glycine (pH 9.0) had been added to a final concentration of 200 mM. EPR conditions were as in Fig. 1, except the gain was $2.5 \cdot 10^5$ and the chlorophyll concentration was the same in all samples (approx. 10 mg Chl/ml).

form was dominant (pH 8.5) *i*-dinoseb results in a significant increase of the $g = 1.82$ form apparently at the expense of the $g = 1.90$ form (Fig. 6A).

It has also been reported that DCMU addition increases the intensity of the $g = 1.82$ signal [15,17,27]. At pH 6.0, it was shown that the effect of DCMU on the $g = 1.82$ signal was much more marked than on the $g = 1.90$ signal [15]. In agreement with this observation, Fig. 6B shows that at pH 9.0, where the $g = 1.90$ form dominates, DCMU has little effect. The slight decrease in the $g = 1.90$ signal and the $g \approx 1.65$ signal in the presence of DCMU may be significant, since DCMU-induced decreases in these signals have been clearly observed in oriented PS II membranes (Rutherford, A.W., unpublished data).

The Q_A^-Fe signal in chloroplasts

The pH dependence of the two resonance forms of Q_A^-Fe described above indicates that at pH values normally used for studies of chloroplasts

and PS II particles (i.e., pH 7.5–8.0), the classical $g = 1.82$ form of the signal should not be favoured.

This could explain the inability of workers in the past to observe an EPR signal attributable to Q_A^-Fe. To test this possibility, experiments were carired out with unfractionated chloroplasts washed and suspended in buffers at pH 6.0 or 8.0.

Samples were frozen in darkness in the presence of DCMU and spectra of the dark-adapted samples were obtained by EPR. Illumination was given at 77 K to form Q_A^-Fe at the expense of cytochrome *b*-559. Spectra taken under these conditions show large contributions from the formation

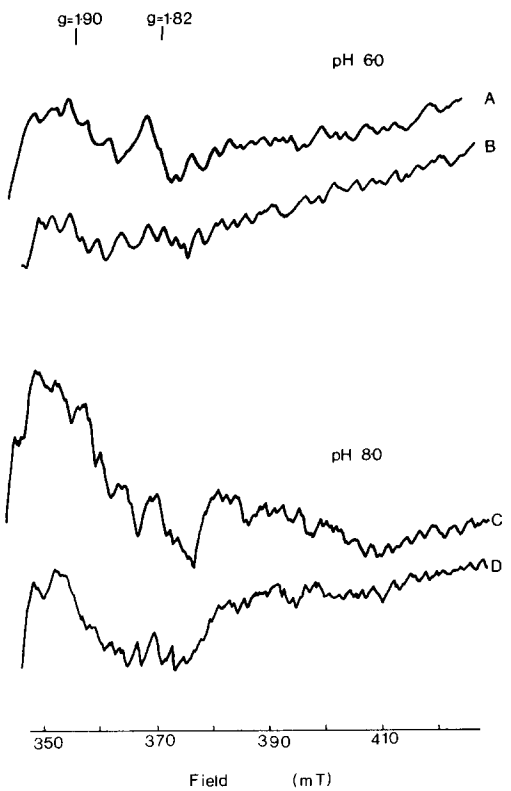

Fig. 7. EPR signals of the primary semiquinone-iron complex photoinduced in chloroplasts. The two upper traces (A and B) were recorded in a sample washed and resuspended in Mes (pH 6.0). The lower traces (C and D) were recorded in a sample washed and resuspended in Hepes (pH 8.0). A and C were recorded after 30 min of illumination at 77 K followed by incubation in darkness at 200 K for 5 min. B and D were recorded in darkness prior to illumination. EPR conditions were as in Fig. 1. Chlorophyll concentrations in the samples are not known (more than 4 mg Chl/ml), since samples were centrifuged into the EPR tubes. Both samples contained 400 μM DCMU.

of P-700$^+$ and centre A$^-$ in PS I (not shown). However the P-700$^+$ centre A$^-$ state back-reacts in the dark at 200 K, while the Cyt *b*-559$^+$ Q_A^-Fe state is largely stable at this temperature. The presence of DCMU prevents any leakage of electrons from Q_A^-Fe to Q_B which occurs at this temperature [22]. Fig. 7 shows traces obtained after illumination at 77 K followed by dark adaptation at 200 K. Virtually no features attributable to PS I components are present in either sample. In the sample at pH 6.0, the peak at $g = 1.82$ is easily discerned, while very little $g = 1.90$ signal seems to be present. In contrast, the chloroplasts at pH 8.0 show only a small $g = 1.82$ signal but a marked signal at $g = 1.90$ is present.

The apparently noisy baseline in these spectra is due to a signal from a component which is usually removed or diminished in the course of preparation of PS II membranes. The signal is composed of very many lines spread over a wide field region and is reminiscent of that reported by other groups and is thought to be some kind of manganese complex [23]. Despite interference from this signal, the main features of the $g = 1.82$ and $g = 1.90$ resonances can be resolved.

It is concluded that the pH dependence of the Q_A^-Fe signals characterized in PS II membranes is also present in chloroplasts and thus the $g = 1.90$ signal is considered to be the normal form at higher, more physiological pH values.

Concluding remarks

In this work, a new EPR signal attributable to Q_A^-Fe has been found and characterized. The existence of this signal at $g = 1.90$ in place of the expected signal at $g = 1.82$ provides an explanation for the difficulties encountered in attempts to observe Q_A^-Fe in chloroplasts and PS II preparations suspended at normal pH values. It is of particular note that an almost identical signal at $g = 1.90$ from Q_A^-Fe has been reported in purple bacteria. The signal was first reported in *Chromatium vinosum* as a degraded form of the Q_A^-Fe which was induced during the course of purification of reaction-centre enriched particles [2]. In contrast, the species *Rhodospirillum rubrum* only exhibits the $g = 1.90$ signal even in whole cells [24]. This indicates that the $g = 1.90$ form can be a

physiological form and does not necessarily indicate any degradation process. The observations in PS II that the two forms of Q_A^-Fe seem to be interconvertable with changing pH, and that the $g = 1.90$ form is that present at normal pH and in chloroplasts, are also taken as evidence that the $g = 1.90$ form is not necessarily a degraded form of the $g = 1.82$ signal.

The pH dependence may reflect a pK on a component in the reaction centre at a pH in the region of pH 7.0–8.0. The results also indicate that the protonation/deprotonation event is not associated with the semiquinone directly. If it were, the protonated ($g = 1.82$) form would not be expected to be photoinduceable at low temperature. Thus, it can be assumed that the components undergoing protonation are present in the dark and could be a protein group close to the quinone-iron complex. Protonation of this group changes the environment of the quinone-iron complex enough to shift one resonance form of the semiquinone-iron ($g = 1.90$) to the other ($g = 1.82$). The protonation/deprotonation event presumably results in a change within the reaction centre which is reflected by a modification of the interaction between the semiquinone and the iron atom. This gives rise to the different g values and signal shapes exhibited by the Q_A^-Fe signal. The existence of a protonatable group associated with the quinone complex in purple bacteria has been invoked from other lines of evidence [25].

Of particular interest is the fact that the herbicide *i*-dinoseb has the same effect as decreasing the pH. It is tempting to speculate that this phenomenon could reflect the herbicide's mode of action – perhaps by perturbing the protonation/deprotonation reactions associated with electron transfer between Q_A^- and Q_B. Evidence for the uptake of a proton as Q_B is reduced to the semiquinone form has already been obtained (Ref. 26 and Rutherford, A.W., Renger, G., Koike, H. and Inoue, Y., unpublished data).

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