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A HIGH POTENTIAL ACCEPTOR FOR PHOTOSYSTEM II

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Summary

The effects of ferricyanide on Photosystem II reactions have been investigated by measurements of microsecond and millisecond prompt fluorescence and microsecond-delayed fluorescence in dark-adapted chloroplasts:

(1) Titrations using ferri-ferrocyanide mixtures on: (a) the fast phase of the increase in fluorescence yield observed during a xenon flash, and (b) the normalised area above the millisecond fluorescence induction curve for chloroplasts inhibited by DCMU, showed a pH dependent mid point potential of 400 mV at pH 7.0 which varied by approx. —60 mV/pH unit between pH 6 and 8.5.

(2) A saturating laser flash induced a fluorescence increase (as monitored by a weak measuring beam) of only 50% of that reached following a second flash in chloroplasts preincubated with ferricyanide and inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) prior to illumination. In the absence of ferricyanide, the fluorescence level reached after a single flash was initially close to that measured after a second flash (although the level subsequently declined).

(3) The initial amplitude of the microsecond-delayed fluorescence excited by a single laser flash was diminished in chloroplasts dark-adapted with ferricyanide. In the presence of DCMU and ferricyanide, the amplitude was also diminished for the first flash of a series, but subsequently enhanced above the level obtained in chloroplasts in the presence of DCMU alone.

(4) The above effects were not seen if DCMU was added to the chloroplasts

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mops, 3-(*N*-morpholino)propane sulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

before ferricyanide, or if the period of incubation with ferricyanide was much less than 4 min.

(5) These results suggested the presence of a second acceptor Q2, with E_{m7} = 400 mV and n = 1, before the DCMU block in Photosystem II. There is 0.35—1 equivalent of the acceptor per reaction centre, and its reduction occurs within <5 μs. The role of the acceptor in double turnovers of the photochemistry during a single flash and its likely operating redox potential are discussed.

Introduction

It has been suggested recently that during a period of dark adaptation, the PS II reaction centres relax to a state in which a short flash (<5 μs) induces two turnovers of *P*-680 (primary donor to the photosystem) [1—7], leading to double hits in the reaction centre. Such an effect could only occur if the relaxation time of the closed reaction centre (P^+Q^-) to the open state (PQ) was within the time of the flash, which in turn implies and requires a rapid rate of electron transfer between the reaction centre and any secondary donors and acceptors. The rate of re-reduction of P^+ is very rapid [1,8—11]; van Best and Mathis [12] recently measured a half-time for the reaction of about 30 ns. However, the rate of reoxidation of Q^- is about 500 μs, which is well in excess of the flash duration [13,14]. Glaser et al. [1] therefore, suggested that a second electron acceptor must exist, operating either in parallel or in series with Q, and with a reaction time of less than 1 μs.

Ikegami and Katoh [15] observed earlier that in the presence of ferricyanide, the area above the fluorescence induction curve measured in continuous light after the addition of DCMU, which was presumed to be proportional to the acceptor pool limited by DCMU, was twice that measured in the absence of ferricyanide. They suggested that this might be indicative of the presence of a relatively high potential second acceptor, and titrated a component with a redox potential of approx. 360 mV at pH 7.8. The addition of DCMU before ferricyanide prevented the effect [15].

Diner [4] and Joliot and Joliot [5] have shown other evidence for a second acceptor, but the component which they measured apparently had a much lower redox potential than that of Ikegami and Katoh [15].

We have reinvestigated the Ikegami-Katoh effect and extended the previous work by studying the effect of ferricyanide on other Photosystem II phenomena, in an attempt to relate this to the suggested double hit reaction of Glaser et al. [1], Diner [4] and Joliot and Joliot [5]. Our observations show the presence of a second equivalent of acceptor before the DCMU block which has a pH dependent midpoint redox potential, and which reacts within 5 μs of flash activation. Kok and Velthuys [6,7] have independently measured the effect of ferricyanide on the yield of oxygen as a function of flash number, and also interpreted their results as reflecting the presence of a second acceptor before the DCMU block.

Materials and Methods

Preparation of chloroplasts

Chloroplasts were prepared from market or greenhouse spinach by homogenisation of chopped deveined leaves for 15 s in a medium containing 0.4 M sucrose, 50 mM potassium phosphate buffer (pH 7.8) and 10 mM sodium chloride. The homogenate was filtered and chloroplasts were separated by conventional centrifugation procedures [16]. Market lettuce, peas and perpetual spinach were used in some experiments and for some experiments an extraction medium containing 50 mM Tricine (pH 7.8) and 250 mM NaCl was used. These variations in material had no marked effect on the behaviour of chloroplasts in the experiments described.

Tris-washed chloroplasts were prepared from chloroplasts prepared as above, using essentially the method of Yamashita and Butler [17].

Chloroplasts were stored in the dark on ice until use. The total chlorophyll was assayed according to the procedure of Arnon [18]. The chloroplasts were diluted before use as described in the figure legends to give a final concentration of chlorophyll between 5 and 10 μg/ml.

Measurement of fluorescence yield in the microsecond range

The rapid change in the yield of fluorescence within 50 μs of flash excitation was measured by using a fluorimeter controlled by a digital minicomputer, in a method similar to that of Duysens [9]. Excitation of photosynthesis was provided by a xenon flash lamp (20 μs half-bandwidth, Osram X1E100) screened by a glass filter (Corning 9782); fluorescence was detected at right angles to the actinic light by an EMI 9558QB photomultiplier with a red cut-off filter (Wratten 70) and an interference filter with emission peak at 695 nm (Balzer B-40). The apparatus was modified to facilitate measurements under conditions of controlled redox potential by incorporating a flow cell, pump, and thermostated, stirred reaction vessel. Flash groups were synchronised with the pump and sufficient time elapsed between flash groups to ensure a complete change of contents of the flow cuvette. The complete flow system was shielded from the dim ambient light by black cloth.

The fluorescence kinetics were monitored via a DL905 transient recorder (Datalab Ltd., Mitcham, Surrey, U.K.) by a minicomputer (Digital Equipment Co., PDP 11/10), and processed to give a direct visual display of the fluorescence yield change over the 50 μs duration of the flash. The computer was also programmed to print out values for the relative yields at different times from the start of the flash as a function of flash number in the group.

Measurement of fluorescence yield changes over longer time ranges

(a) Changes induced by continuous illumination. A conventional fluorimeter with the photomultiplier (EMI 9558QB) mounted at right angles to the actinic source (Quartz-iodide bulb; Philips 55 W, shielded by a Corning 9782 filter unless otherwise stated) was used. For some experiments a xenon flash (as above) or Q-switched laser (Laser Associates, Slough, U.K., Model 252 YAG laser, frequency doubled to emit at 530 nm; pulse width 20 ns) was connected to the apparatus by a light guide, also at 90° to the photomultiplier. The out-

put of the photomultiplier was fed via the transient recorder to the computer (as above) and could be monitored on a storage oscilloscope connected in parallel. The computer was programmed to display the fluorescence trace and to print out values for the initial fluorescence (F_0), maximal level of fluorescence (F_{max}), and the area above the induction curve (S), normalised by division by the variable yield ($F_{max} - F_0$). This value is referred to as S/F_v as suggested by Ikegami and Katoh [15]. The accumulated traces could be output to an X-Y recorder (Bryans, Mitcham, Surrey, U.K., Model 24000, A4) either directly or after normalisation to equivalent levels of F_{max}.

(b) Changes in fluorescence yield induced by a single saturating flash. The apparatus in (a) was used, but with the actinic light at a much reduced intensity so as to monitor the fluorescence yield. The actinic flash was provided by the Q-switched laser (details as above), fired 40 ms after the shutter of the measuring beam was opened. The intensity of the measuring beam was such that no change in fluorescence level (F_0) was apparent over 500 ms illumination in the presence of DCMU.

Measurement of delayed fluorescence in the microsecond time range

Delayed fluorescence was measured from approx. 7 to 200 μs after a series of saturating laser flashes. The laser light was filtered through Corning 9782 and Wratten 74 filters and the intensity adjusted to be just saturating. The delayed fluorescence was detected by a photomultiplier (EMI 9558QB) using the same filter combination as described for measurement of prompt fluorescence. The photomultiplier was gated on by applying a negative voltage (200 V) between the cathode and the first dynode. In the quiescent state the dynode was held at a voltage positive with respect to the cathode. The circuit enabled the photomultiplier to be turned on approx. 7 μs after the laser flash. Subtraction of small optical (prompt fluorescence) and electrical artifacts, and signal averaging, was performed using the computer. The traces were stored on magnetic tape and the decays were deconvoluted using a simple curve fitting program written in BASIC.

Measurement of redox potential

Redox potentials of the reaction media were adjusted with $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ mixtures and monitored by a conventional platinum/calomel electrode pair using a pH meter (Pye model 79, Cambridge, U.K.). The electrodes were routinely checked using an equimolar mixture of ferri- and ferrocyanide at 10 mM concentration in 100 mM KCl. The potential for this couple was taken as 436 mV. For most experiments, the redox potential was adjusted by varying the amount of ferrocyanide, to avoid changes in the actinic or measuring light due to changes in absorption due to changing concentrations of ferricyanide. However, when corrected for this effect, essentially identical results were obtained when sodium ascorbate or sodium isoascorbate was used as reductant instead of or in addition to ferrocyanide. Ascorbate was used in some experiments for potentials at the lower end of the buffering range of the ferri-ferrocyanide couple.

Results

Effect of ferricyanide on 0—50 μs fluorescence yield

Fluorescence yield changes accompanying a flash over 50 μs were measured for each of a group of 8 flashes. The changes were similar to those previously reported [9,11] and showed a damped oscillation with a periodicity of 4 and maxima on the first and fifth flashes of the series following dark adaptation. Preincubation with ferricyanide produced changes which for all flashes except the first were similar to the control (but seen Discussion). The amplitude of the first flash was markedly diminished (Fig. 1). Zankel [19] originally reported this effect but did not offer any explanation or characterise the phenomenon in detail.

Over the time scale measured, most of the diminution in amplitude of the first flash appeared to be due to a smaller amplitude for the fast phase of the fluorescence rise. Under the conditions of our experiments the flash intensity was not sufficient to saturate the rate of the fast fluorescence rise. Although this had the advantage of minimising the triplet artifact [9], we are unable to relate the effect of ferricyanide to any kinetic phenomenon except insofar as the effect appeared to be occuring with the rise time (approx. 5 μs) of the fast phase. We were also able to observe a diminution of the amplitude of the first flash of the fluorescence yield rise in the presence of ferricyanide in Tris-washed chloroplasts (the fluorescence was quenched on all subsequent flashes) (not shown).

The fluorescence yield rise in the presence of DCMU

The effect of preincubation with ferricyanide on the fluorescence yield

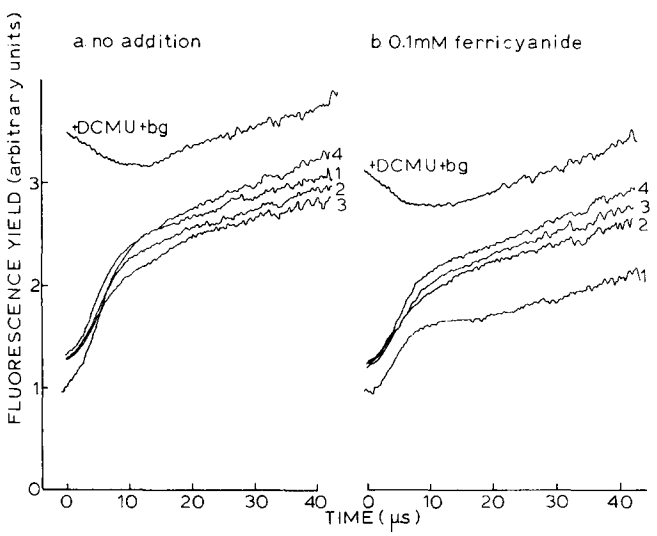

Fig. 1. Fluorescence yield measurements during first 4 flashes (time between flashes was 250 ms) following 10 min dark adaptation of chloroplasts in the absence (a) or presence (b) of ferricyanide (0.1 mM). Chloroplasts were suspended in a buffer containing 0.4 M sucrose, 50 mM Mops, pH 7 and 10 mM $MgCl_2$ (chlorophyll concentration 2 μg/ml). Each trace was an average of 4. Upper lines show fluorescence yield of chloroplasts in the presence of 10 μM DCMU continuous blue background illumination (1 mW · cm^{-2}).

change on the first flash of a series given to chloroplasts preincubated in the dark for 10 min was unaffected by the addition of DCMU after the period of dark adaptation and prior to the measurement. In the presence of DCMU, the level of fluorescence elicited by a second and subsequent flashes was close to maximal.

If DCMU was added before the ferricyanide, then we did not observe any effect of preincubation with ferricyanide on the first flash. A similar effect of order of addition was previously observed by Ikegami and Katoh, measuring the area above the fluorescence rise curve [15].

Apparent mid-point potential of the ferricyanide effect

Titration of the effect of ferricyanide on the fast phase (approx. 10 μs) of the fluorescence yield rise following the first flash by varying ferri-ferrocyanide mixtures showed that the effect was half complete with $E_{m7} \approx 400$ mv.

When the experiments were repeated at different values of pH, the apparent mid-point of the effect was found to vary (Fig. 2). Our experimental precision was not sufficient to show unambiguously either the slope of the pH dependence or the n value of the titration (Fig. 3). In particular, below pH 7, the diminishing amplitude of the fluorescence yield change over this time scale [8] made measurements increasingly imprecise. However, similar titrations of an apparently related phenomenon (Fig. 4 and see below) suggested a slope of —60 mV/pH unit for the pH-dependence and an n-value of 1 for the effect.

Effect of ferricyanide on the fluorescence induction in the presence of DCMU: Ikegami-Katoh effect [15]

The mid-point potential and dependence on order of addition of DCMU and ferricyanide observed in the experiments on the rise of fluorescence yield

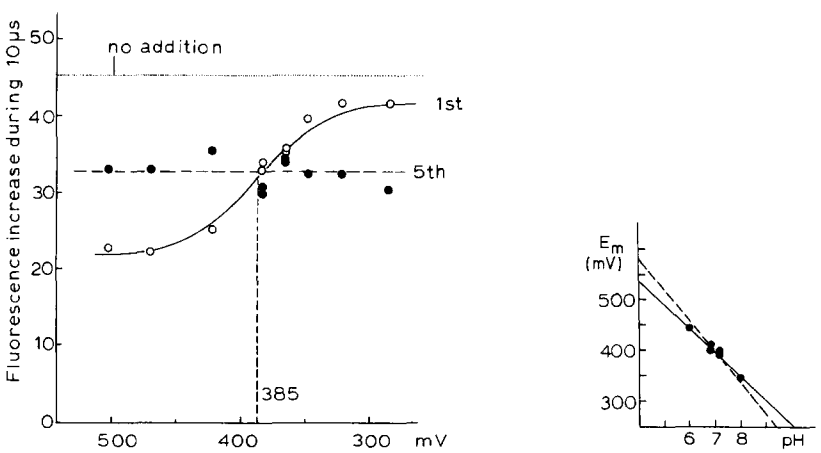

Fig. 2. Effect of redox potential on extent of fluorescence yield increase during first 10 μs of flash, for 1st and 5th flash. Same conditions as in Fig. 1. ———, theoretical curve for titration of a one electron reaction with midpoint potenital 385 mV. - - - - - -, fluorescence increase on first flash with no additions. Vertical scale percentage of maximal variable portion of fluorescence increase.

Fig. 3. Effect of pH on redox potential of titrations in Fig. 2. Solid and broken lines show —30 and —60 mV/pH unit Nernst equation curves for a one electron transition (n = 1).

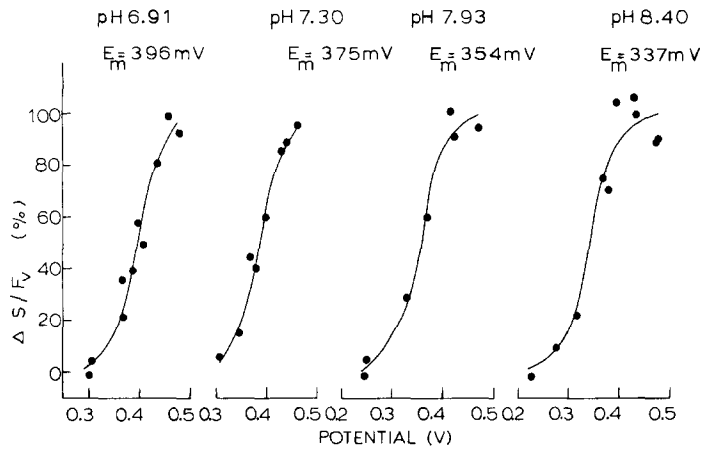

Fig. 4. Effect of ferricyanide on fluorescence induction in the presence of DCMU at various values of pH. Solid lines show theoretical Nernst equation curves for $n = 1$. The normalised areas above the fluorescence induction curves (S/F_V see Methods) are expressed as percentage of maximum change at highest redox potential (S/F_V (%)). Chloroplasts were diluted in buffers containing 0.25 M NaCl plus 50 mM each of Mes, Mops, or Tricine, adjusted to the required pH, and at chlorophyll concentrations of 2--5 μg/ml.

(above) are strikingly similar to those reported by Ikegami and Katoh [15] (see Introduction). We therefore reinvestigated the latter effect in order to characterise the redox potential dependence over a wider range of values of pH.

The effect of varying the redox potential during the preincubation period on the area above the induction curve at several values of pH is shown in Fig. 4. Fig. 5 shows the values for E_m from a series of titrations plotted as a function of pH. Although the points showed considerable scatter between groups of

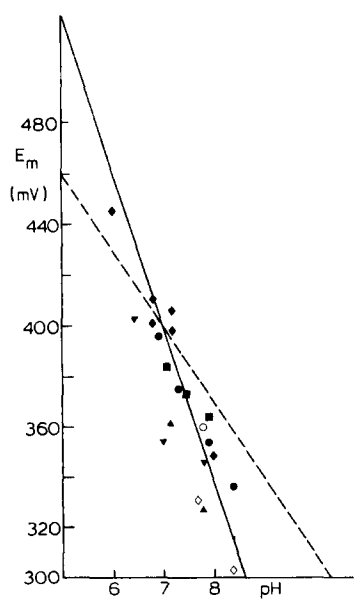

Fig. 5. Values of redox potential from experiments on several preparations (one symbol per preparation) plotted as a function of pH. The value reported by Ikegami and Katoh [4] (○) and points from Fig. 3 (♦) are shown.

titrations, the mid-points at different values of pH within a group showed a more consistent trend, with a slope −60 mV/pH unit characteristic of an H-carrying redox couple. Included in Fig. 5 are values from the titrations shown in Fig. 3 for the effect of ferricyanide on the rapid fluorescence rise. It can be seen that these values are within the range of scatter for the results from our measurements on the area above the induction curve. We have also included the value reported by Ikegami and Katoh [15] which falls close to the same curve. Titrations performed on chloroplasts preincubated with 10 μM FCCP yielded similar redox potentials for equivalent values of pH (not shown).

From the close matching of the preincubation requirements and of the redox potentials it seems likely that the Zankel effect and the Ikegami-Katoh effect reflect the same component. However, the effect of ferricyanide on the fluorescen yield rise could be seen as well in the absence as in the presence of DCMU. This suggests that the Ikegami-Katoh effect cannot be attributed simply to a loss of B^- caused by oxidation by ferricyanide, and the consequent lower level of reduction of Q on addition of DCMU [20,21], as suggested by Joliot and Joliot [5]. In any case, if such a mechanism was operating, we would expect the effect to be reflected in a change in the level of F_0 as well as in the area above the curve [20,21]. Similarly, our experiments preclude explanations based on electron transfer to known secondary acceptors, since the reduced extent of the change in fluorescence yield was apparent in the Zankel effect within about 5 μs, long before any substantial reoxidation of Q^- by B or B^- would have occurred.

The size of the pool associated with the quenching state

Ikegami and Katoh [15] estimated the size of the pool of extra oxidising equivalents after incubation with ferricyanide from the area above the fluoresence induction curve in the presence of DCMU after normalising by division by the variable fluorescence, and we have adopted this procedure in the treatment of our results above. Joliot (personal communication) has pointed out that this treatment may over estimate the extra pool size, especially if for any reason the rate of excitation of the reaction centre is less in the presence of ferricyanide. In order to obtain an estimate of the range of possible values for the extra pool size, we have estimated the area above the induction curve under different conditions of preincubation (Fig. 6i and ii), and show in Table I results either unadjusted or divided by F_0 or F_v, together with values for the half-time of the induction. The half-time might be expected to vary with the rate of filling of the pool, or, at a constant rate of electron donation, to reflect the pool size. These results show a variability for extra pool size of between 35 and 77%. Results from similar experiments have shown values as high as 120% for the value for S/F_v. The rather wide variability in these values is associated with several effects of ferricyanide on the fluorescence induction for which we do not at present have a ready explanation. Some of these are apparent in Fig. 6. Preincubation with ferricyanide usually resulted in a lower value of F_0 and of F_{max}, with a greater effect on F_{max}, so that F_v was also lower. These effects were variable from preparation to preparation. Such preincubation also gave rise to an extensive 'tail' in the fluorescence induction, and it is the 'tail' which contributes much of the additional area. In order to get more informa-

TABLE I

AREA ABOVE INDUCTION CURVE MEASURED FOR 1 s IN THE PRESENCE OF DCMU FOR CHLOROPLASTS PREINCUBATED IN THE DARK WITH OR WITHOUT FERRICYANIDE

Conditions as for Fig 6 except that in C, the chloroplasts were dark adapted for 10 min before addition of ferricyanide in the dark. Areas measured by weight, were normalised to F_0 and F_v in the dark sample for particular experiment, A, B, and C. Values obtained for dark-adapted chloroplasts in the presence of DCMU were arbitrarily taken to equal 100, other normalised values expressed relative to 100. $t_{1/2}$ = time taken to half F_v.

	Area	Area/F_0	Area/F_v	$t_{1/2}$ (ms)
(A) 10 μM DCMU (F_0 = 29.25, F_v = 88.75)				
Dark-adapted for 10 min	100.0 *	100.0	100.0	35.0
Dark-adapted for 10 min, 1 laser flash, 5 min, dark	79.8	77.8	76.0	32.0
Dark-adapted for 10 min, 1 laser flash	31.0	30.6	33.9	18.0
(B) 0.2 mM FeCN added in dim light, 10 μM DCMU (F_0 = 24.0, F_v = 77.0)				
Dark-adapted for 10 min	136.0	164.7	152.4	53.0
Dark-adapted for 10 min, 1 laser flash, 5 min, dark	80.2	97.7	93.1	35.0
Dark-adapted further 2 min	31.9	39.1	33.9	21.0
Dark-adapted 10 min, 1 laser flash	65.6	80.9	76.2	24.0
(C) 0.2 mM FeCN added after 10 min dark, 10 μM DCMU (F_0 = 26.0, F_v = 77.5)				
Dark-adapted for 10 min	149.0	167.5	177.8	57.0
Dark-adapted for 10 min, 1 laser flash, 5 min, dark	93.0	106.1	110.1	43.0
Dark-adapted further 2 min, 1 laser flash	29.0	33.5	33.9	25.0
Dark-adapted for 10 min, 1 laser flash	69.0	78.1	76.2	25.0

* Area, and area normalized to F_0 or F_v for dark-adapted chloroplasts with DCMU arbitrarily taken to equal 100. All values of F_0 and F_v in the left-hand column are in comparable, but arbitrary units.

tion about the pool size, we have investigated the effect of a single laser flash immediately before the continuous light used to measured the induction curve.

Effect of preilluminating flashes on the fluorescence induction in the presence and absence of ferricyanide

The effect of a preilluminating flash on the induction curve and on the level of fluorescence measured by a weak measuring beam are shown in Figs. 6 and 7 and in Table I. Effects qualitatively similar to those shown here for a laser flash were observed using a xenon flash of 5—10 μs duration. A laser flash given to dark adapted, DCMU inhibited chloroplasts ~75 ms before the continuous light gave rise to the initial fluorescence change above F_0 of 75% the maximal level. In a similar experiment with ferricyanide treated chloroplasts the initial fluorescence change was 57% of the maximal. In other preparations these values varied between 70 and 80% for normal chloroplasts and 40 and 60% for ferricyanide-treated chloroplasts. With both types of chloroplasts, when the same experiment was repeated on the preilluminated sample, the initial fluorescence level was 90% of the maximal. A similar effect was seen when the change in fluorescence was monitored with a weak measuring beam (Fig. 7). In this case it could be shown that a single laser flash given to ferricyanide treated chloroplasts abolished the quenching state, so that a second or subsequent flashes showed the increased initial fluorescence level.

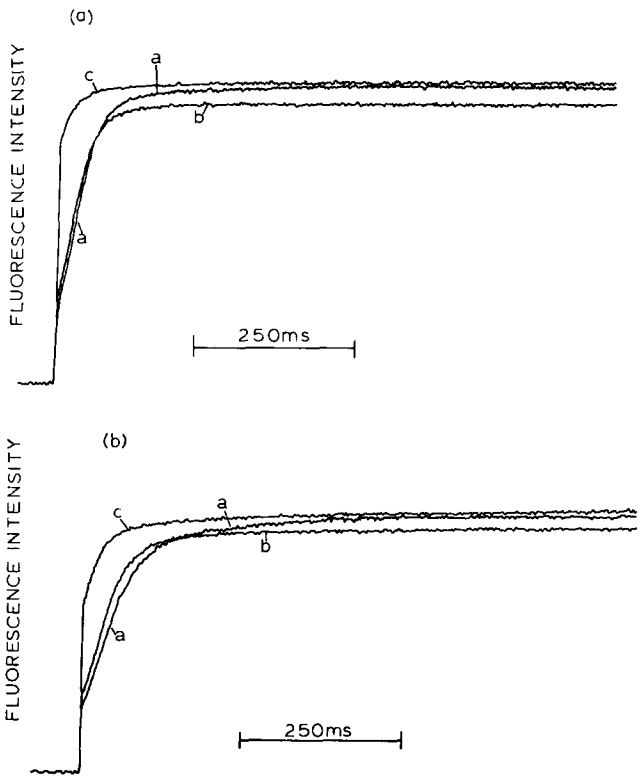

Fig. 6. Fluorescence induction of normal or ferricyanide treated chloroplasts inhibited by DCMU. Chloroplasts were suspended in buffer containing 0.4 M sucrose, 0.01 M NaCl, 0.05 M phosphate (pH 7.8) at 10 μg/ml chlorophyll concentration. Actinic light was shielded by Corning 9782 and Schott GG495 filters. (No absorption of actinic light by ferricyanide with this filter combination). (a) Chloroplasts preincubated in the dark for 10 min prior to addition of 10 μM DCMU. a. fluorescence induction in dark sample. b. fluorescence induction in dark sample with one laser flash 5 min before measurement. c. fluorescence induction in dark sample with one laser flash ~75 ms before measurement. (b) Chloroplasts preincubated in the dark for 10 min with 0.2 mM ferricyanide. Ferricyanide was added to the suspension under the dim light of the laboratory.

These experiments, together with our own observations and those of Ikegami and Katoh [15] on the area over the induction curve show that the component associated with the quenching state must have a stoichiometry of not more than 1 per Photosystem II centre.

Effects of ferricyanide on delayed fluorescence

The effects of ferricyanide on fluorescence yield could possibly be ascribed to quenching by any of the states P^+Q, PQ, or P^+Q^- since P^+ and Q are both known to quench fluorescence. Components on the donor side of Photosystem II with potentials of about 400 mV have been previously characterised [22,23], and the possibility exists that these may in some way be associated with the effect of ferricyanide. It seemed possible that measurement of delayed fluorescence might distinguish between the different potential quenching states since of the most likely states only P^+Q^- is a substrate for delayed fluorescence and its presence would be expected to give a higher intensity. The initial ampli-

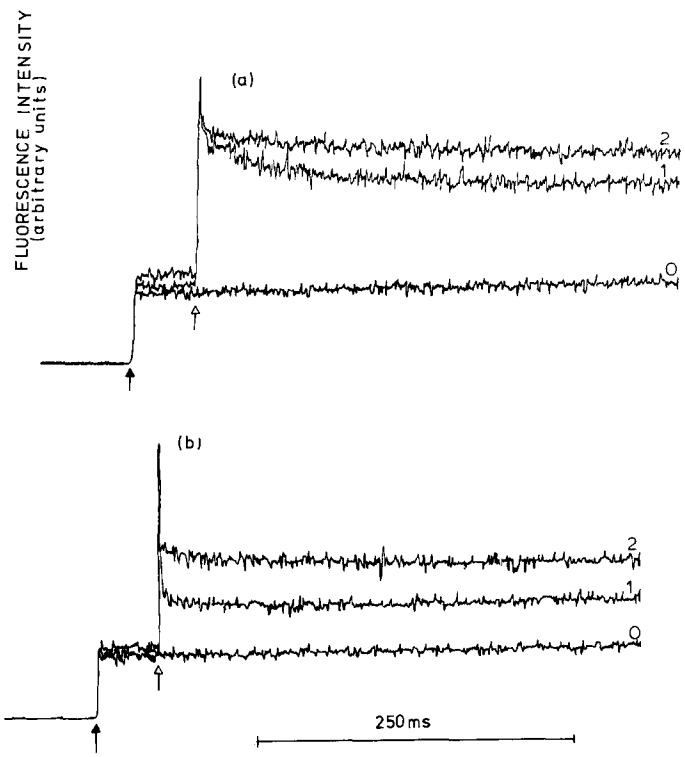

Fig. 7. Effect of saturating laser flash on the fluorescence yield assayed by a weak measuring beam. Chloroplasts (2.7 μg chlorophyll/ml) were suspended in a medium containing 0.4 M sucrose, 50 mM phosphate buffer (pH 7.8) and 10 mM NaCl. The suspension was dark adapted for 4 min either in the absence (a) or in the presence (b) of ferricyanide (0.5 mM). The fluorescence yield was then measured (trace 0), and the chloroplasts dark-adapted for a further 4 min; DCMU was the added to a final concentration of 10 μM, and 15 s later, the fluorescence yield was again assayed by the weak measuring beam. At 50 ms after the opening of the shutter of the measuring beam (⇒), the laser was fired (⇨, trace 1) and the change in yield followed. The chloroplasts were allowed to dark adapt for 2 min, and then the previous cycle of measurement was repeated (trace 2).

tude of the delayed fluorescence measured in dark-adapted chloroplasts over the time range between 7 and 200 μs after a 20 ns laser flash, showed an oscillation of period 4 with minima on the first and fifth flashes, maxima on third and seventh as previously reported [11]. Deconvolution of the decay of the delayed fluorescence, showed that over this time scale, two major components contributed to the decay, with half-times ~10 and ~40 μs. Preincubation with ferricyanide produced a small but consistent decrease in the amplitudes of both components of the decay following the first flash, an effect consistent with more rapid loss of P^+Q^- due to electron transfer from Q^- (Fig. 8a and b). The diminution was observed over the pH range 6—8, and was also present in Tris-washed chloroplasts. In normal chloroplasts, the intensity of delayed fluorescence was enhanced on subsequent flashes after preincubation with ferricyanide, but not in Tris-washed chloroplasts.

After preincubation in the presence of ferricyanide, and when DCMU was added immediately before the flash sequence, the diminished amplitude of

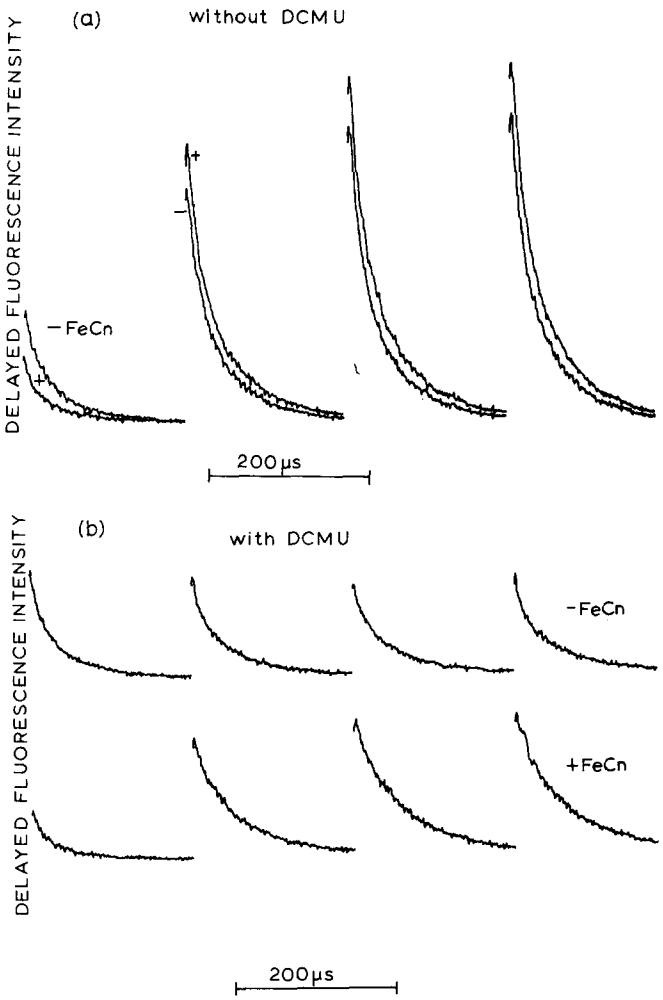

Fig. 8. Effect of ferricyanide on the delayed fluorescence following a series of 4 saturating laser flashes (1/320 ms). Chloroplasts were diluted in 0.4 M sucrose, 50 mM phosphate buffer (pH 7.8) and 10 mM NaCl (chlorophyll concentration 6.6 μg/ml). (A) Chloroplasts dark-adapted in the presence or absence of ferricyanide (0.5 mM) (B) Chloroplasts dark-adapted in the presence or absence of ferricyanide. 5 μM DCMU added prior to flash group.

delayed light after the first flash was also seen (Fig. 8a and b). However, there was an enhancement of the intensity of delayed fluorescence following flashes 2—4. Neither of these effects was seen if DCMU was added after the chloroplasts had been preincubated in the absence of ferricyanide, or if DCMU was added before the preincubation with ferricyanide. The effect of ferricyanide was not mimicked by 20 μM methylviologen and was reversed by including an excess of ferrocyanide in the incubation mixture.

The enhanced delayed fluorescence in the presence of DCMU can be most easily explained as resulting from an increased turnover of the reaction centre as a consequence of the availability of an additional acceptor before the DCMU block. The enhancement was observed up to 7 flashes after the first, suggesting

that this acceptor is regenerated in the dark period between flashes, possibly by a cycle of electron flow around the photosystem.

Discussion

When chloroplasts were incubated with ferricyanide either in the presence or absence of DCMU, a single saturating flash did not remove as much of the quenching state as it does in the absence of ferricyanide (Figs. 6 and 7). The two best characterised fluorescence quenching states are those due to the presence of the oxidized primary donor (P^+) [10] and oxidized primary acceptor (Q) [24] of the Photosystem II reaction centre. Our results could be interpreted in terms of either: (i) a stabilisation of P^+ due to oxidation of a secondary donor by ferricyanide, (ii) a rapid oxidation of the primary acceptor Q, by an additional acceptor, or (iii) donation of electrons from P to an alternative primary acceptor which does not quench fluorescence strongly. Since the initial intensity of delayed fluorescence following the first flash after dark adaptation was lower after preincubation with ferricyanide, it is possible that the delayed fluorescence substrate P^+Q^- was at a lower concentration, indicating that the quenching state is likely to be PQ or P^+Q but not P^+Q^-.

We would therefore suggest that we have identified a component Q2 on the acceptor side of Photosystem II, which is able to compete with Q for electrons from the photochemistry. The properties of Q2 characterised above are as follows: (a) 0.35—1 equivalent of Q2 per Photosystem II reaction centre; (b) reduction of Q2 occurs in <5 μs, and it is not rapidly reoxidized at least in the time scale of our measurements (up to 4 min; Figs. 6 and 7); (c) the midpoint potential at pH 7 (E_{m7}) of Q2 is approx. 390 mV; this value shows a dependence on pH of —60 mV/pH unit over the range 6—8.5 (Figs. 2 and 4) and is not affected by FCCP. Its operating potential is likely to be <300 mV (see below); (d) DCMU does not block the reduction of Q2 but inhibits its oxidation by ferricyanide; (e) on the basis of these properties , Q2 is distinct from both Q and the couple B/B^- [20,21,25] and also from cytochrome b-559 (high potential) [26].

We are not able on the basis of our present results, to reach any firm conclusions as to the relation between Q2 and the photochemical centres.

We may consider a number of possibilities, which are not necessarily exclusive:

(i) Q and Q2 are connected in series, with (a) a rapid (<50 ns), or (b) a slow (<5 μs) electron transfer from Q to Q2.

(ii) Q and Q2 are in parallel, and are alternative photochemical acceptors for electrons from P.

(iii) Q and Q2 are at equal concentrations.

(iv) Q2 is present in only a proportion of centres.

(v) Q2 in its oxidised form is (a) a strong quencher, (b) a weak quencher, or (c) a non quencher.

After a 20 ns laser flash, about 50% of the centres of chloroplasts preincubated with ferricyanide are in a non-quenching state. We can therefore eliminate some combinations of the possibilities listed above (for instance, (i) (b) plus (iii) plus (v) (c)). However, because of the possibility of double turnovers

implicit in (i), (a) or (ii), and the limited accuracy of our measurements of stoichiometry, a large number of plausible models can still be justified. A further complicating factor is the possibility that two types of centres with different quenching properties may normally exist (Q_α and Q_β), as suggested by Melis and Homann [27,28].

Involvement of Q2 in double hits

Q2 may be the second primary acceptor postulated by Glaser et al. [1] to account for the double hits observed in their measurement of *P*-680 re-reduction. The properties of Q2 are remarkably similar to the properties of the component identified by Velthuys and Kok [6,7]. Oxidation of their component was accompanied by the appearance of double hits in the reaction centre which were reflected in double turnovers of the water splitting enzyme. A somewhat similar effect has been reported more recently by Piccioni and Mauzerall [29]. We might therefore have expected to see an advancement in the periodicity of the oscillation of the microsecond fluorescence yield rise in the presence of ferricyanide corresponding to the extent of the double hits, since the flash used for these experiments exceeded the 5 μs reduction time of Q2. Inspection of a number of results revealed a small trend towards this; the ratio of the yields measured for the 5th/4th flashes was consistently lower in the presence of ferricyanide than in its absence. The results of Zankel [19] did not show the same effect presumably because in his experiments a shorter flash (3 μs) was used.

Some important discrepancies exist between our observations, together with those of Velthuys and Kok [6,7], and those of Diner [4], and of Joliot and Joliot [5]:

(a) Diner [4] observed 'double-hits' in the presence of ascorbate and phenylenediamine, conditions under which the redox potential of the system would have been well below that under which our effects were observed.

(b) Diner [4] could see no indication that extra oxidizing equivalents became available to the water splitting reactions, whereas Velthuys and Kok [7] observed a marked enhancement of the oxygen yield on the second flash on preincubation with ferricyanide.

(c) Joliot and Joliot [5] could see no effect of ferricyanide on the fluorescence yield rise at low temperature beyond that attributable to its oxidizing effect on B^-; the double-hits indicated by the 'lag' in the intensity dependence of fluorescence were observed at the normal potential of the system.

Possible effects on the donor side

Our results indicate that reoxidation of Q2 through the DCMU block is very slow, and that even in the absence of DCMU, ferricyanide reacts slowly with Q2. One possibility that we have not considered above, is that in some centres, Q2 may be oxidised rapidly by a DCMU insensitive cycle around the photosystem. Such a cycle would explain some apparent inconsistencies in our results. In particular, it is difficult to understand the stimulation of delayed fluorescence following flashes after the first, seen with chloroplasts inhibited with DCMU after preincubation with ferricyanide, unless pathways exist for regeneration of open traps. If such a pathway did exist, and the rate of electron

transfer from Q to the cycle was rapid compared with the rate of excitation, then we would expect to find a lower F_{max}, and a tail in the induction curve for fluorescence, as are indeed observed.

In view of the similarity between the potentials of Q2, D, the donor to signal 11f [22] and the unidentified donor to Photosystem II observed at low temperatures [23], it is possible that Q2 also serves this function.

Interaction of Q2 with acceptor pool

The reaction of $Q2^-$ with the acceptor pool seems at first to be precluded by its high redox potential. However, calculation of the likely redox potential of the couple B^-/B^{2-}, using the equilibrium constants given by Diner [30], a value of −130 mV for the operating potential of the couple Q/Q^- [31], and a value of 80 mV for the potential of the couple PQ/PQH_2 [32] gives a value of 212 mV. It should be noted that this value depends on assumptions about the possibility of disproportionation and about the equilibration of protons which may be incorrect. The midpoint potential of the couple Q2/Q2H was ~390 mV at pH 7 but decreased by 60 mV/pH unit increase. If we assume that the couple undergoes reduction to the anion, then the appropriate operating redox potential would be that measured above the pK. Our results showed no pK up to pH 8.5, so that the operating potential would be less than 300 mV. If the pK is above pH 10, then the operating potential of the couple could be less than 220 mV, so that we cannot exclude the possibility that Q2 serves as a reductant to the secondary acceptor pool [33].

Some further indication of the equilibrium constant between Q and Q2 may be estimated from the times for the forward and reverse reactions, if it is assumed that Q2 is reduced and oxidised by way of Q, and that in the oxidation by ferricyanide the reaction with Q is rate limiting. Taking the rate constant for the forward reaction as $2 \cdot 10^5\ s^{-1}$ and that for the reverse reaction as $10^{-2}\ s^{-1}$, we find that

$$K = \frac{k_f}{k_r} = \frac{2 \cdot 10^5}{10^{-2}} = 2 \cdot 10^7 \quad (1)$$

$$\Delta E = 2 \cdot 303 \frac{RT}{zF} \log_{10} K = 431\ \text{mV} \quad (2)$$

Assuming an operating mid potential for Q/Q^- as −130 mV, the operating mid-potential for $Q2/Q2^-$ would be ~300 mV, in good agreement with the range of values discussed above.

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