Biochimica et Biophysica Acta, 440 (1976) 346–355

BBA 47135

FLASH-INDUCED ABSORPTION CHANGES OF THE PRIMARY DONOR OF PHOTOSYSTEM II AT 820 nm IN CHLOROPLASTS INHIBITED BY LOW pH OR TRIS-TREATMENT

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(Received January 22nd, 1976)

SUMMARY

A comparative study is made, at 15 °C, of flash-induced absorption changes around 820 nm (attributed to the primary donors of Photosystems I and II) and 705 nm (Photosystem I only), in normal chloroplasts and in chloroplasts where O_2 evolution was inhibited by low pH or by Tris-treatment.

At pH 7.5, with untreated chloroplasts, the absorption changes around 820 nm are shown to be due to *P*-700 alone. Any contribution of the primary donor of Photosystem II should be in times shorter than 60 μs.

When chloroplasts are inhibited at the donor side of Photosystem II by low pH, an additional absorption change at 820 nm appears with an amplitude which, at pH 4.0, is slightly higher than the signal due to oxidized *P*-700. This additional signal is attributed to the primary donor of Photosystem II. It decays ($t_{\frac{1}{2}}$ about 180 μs) mainly by back reaction with the primary acceptor and partly by reduction by another electron donor. Acid-washed chloroplasts resuspended at pH 7.5 still present the signal due to Photosystem II ($t_{\frac{1}{2}}$ about 120 μs). This shows that the acid inhibition of the first secondary donor of Photosystem II is irreversible.

In Tris-treated chloroplasts, absorption changes at 820 nm due to the primary donor of Photosystem II are also observed, but to a lesser extent and only after some charge accumulation at the donor side. They decay with a half-time of 120 μs.

INTRODUCTION

In spite of earlier information obtained from careful studies of the fluorescence of chlorophyll in vivo [1], the primary reactions in Photosystem II of green plants remain largely controversial. The primary electron acceptor has been rather well characterized (see e.g. refs. 1–6), but the properties of the primary donor appeared to

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Tris, tris(hydroxymethyl)-aminomethane; Tricine, *N*-tris(hydroxymethyl)methylglycine; MES, 2-(*N*-morpholino)ethanesulphonic acid.

be more difficult to analyze. Döring et al. [7, 8] have reported absorption changes in the bands of chlorophyll *a* with a lifetime of 200 μs. Later work from the same laboratory revealed an additional transient, with a lifetime of 35 μs [9]. Döring [10] attributes these absorption changes to the oxidation of the primary donor of Photosystem II, named Chlorophyll a_{II}. With subchloroplasts particles from spinach, Van Gorkom et al. [11, 12] also obtained evidence for the photobleaching of the primary donor of Photosystem II (named *P*-680 by analogy with *P*-700, the primary donor of Photosystem I). Their parallel use of absorption and of EPR spectroscopy allowed them to conclude that a radical cation is formed from a chlorophyll dimer.

In a study of rapid flash-induced fluorescence transients Duysens et al. [13] and Den Haan et al. [14] came to the conclusion that, in physiological conditions, the primary donor of Photosystem II oxidizes the first secondary donor in less than 1 μs. This proposal conflicts with the measurements of absorption changes, unless these concern disturbed Photosystem II reaction centers as in the case of subchloroplast fragments. In this study we will make use of the typical absorption band of the oxidized primary donor of Photosystem II, around 820 nm (compare Refs. 12, 15–17) which has been observed at low temperature [17] and shown to be distinct from the Photosystem I contribution. At low temperature, this species has also been observed by EPR [18, 29], which strongly suggests its radical ion character.

In flash experiments under physiological conditions at 15 °C, we could not detect any Photosystem II contribution at 820 nm in times beyond 60 μs after the flash. However in chloroplasts in which O_2 evolution is blocked either by acid-treatment [19] or Tris-treatment [20] we observe a fairly large absorption increase which can be attributed to the oxidation of the primary donor of Photosystem II. This species disappears largely by back reaction with the primary acceptor, as at low temperature [6, 17]. These results fit nicely with recent results on prompt and delayed fluorescence in chloroplasts inhibited by low pH [19].

MATERIALS AND METHODS

Freshly prepared spinach chloroplasts in Tricine buffer (50 mM Tricine, 10 mM KCl, 2 mM $MgCl_2$, 0.4 M sucrose, pH 7.5) were diluted just before measurements 10–20 times in buffer without sucrose. Depending on the pH required, the Tricine was replaced by MES or succinate. For Tris-treatment, the chloroplasts were exposed for 5 min to a 0.2 M Tris · HCl buffer (pH 9.0), centrifuged and resuspended in Tricine buffer, pH 7.5.

Absorption changes were measured with an apparatus similar to the one described in Ref. 17. We used a perspex cuvette with an optical path for the measuring beam of 10 mm. Flash excitation was performed by means of two electronic flashes (General Radio, Stroboslave), filtered by means of Corning CS 4-96 optical filters, on either side of the cuvette, perpendicular to the measuring beam; these flashes could be separated by an adjustable time interval (see e.g. Fig. 1). The optical path for the flashes was 5 mm; each flash was saturating. Each time before flashing, the sample in the cuvette was changed for a dark-adapted one, using a flow system with a magnetic valve. All experiments were done at 15 °C. The measuring beam, after having passed 10 cm of water as a heat filter, was usually filtered by a Schott RG 10 (5 mm) filter and a Wratten 48 A filter. For some experiments, the Wratten filter was replaced by an

appropriate interference filter or a Schott RG N9 5 mm filter (providing a far-red background illumination). After having passed the sample, the measuring beam passed a monochromator (Huet M 25, 10 nm bandwidth) and a Schott RG10 (1 mm) filter before reaching the photomultiplier. In order to obtain a sufficient signal to noise ratio, several experiments were accumulated by means of a multichannel analyzer which permitted a time resolution of 30 μs.

RESULTS

Chloroplasts at neutral pH

Flash excitation of a chloroplast suspension at pH 7.5 induces an absorption increase at 820 nm. In order to separate the Photosystem I and Photosystem II contributions, we compared the absorption changes at 820 and 705 nm. The comparison provides easily interpretable information only if it is performed under the same light conditions. In the experiment shown in Fig. 1, a background of far-red light is given by means of a RG N9 filter in the measuring beam; using this filter the absorption changes at both wavelengths could be followed under exactly the same light conditions. The decay kinetics at 820 and 705 nm appeared to be very similar. Due to the far-red background the reduction in the dark of the oxidized *P*-700 was rather slow (compare Ref. 21). The intensity of the background light was adjusted in order to obtain the maximum amplitude of the absorption changes at both wavelengths; in too strong far-red background light, the *P*-700 stayed in its oxidized state and no change could be seen either at 705 nm or at 820 nm. When 1 mM ferricyanide was added to the chloroplasts the decay of the signals at both wavelengths became very slow, but the amplitudes of the signals after the first flash remained as high as without ferricyanide. From these results, we conclude that with approx. 60 μs lost after the flashes and a time resolution of 30 μs, the absorption changes at 820 nm at pH 7.5 are due to *P*-700 only. Virtually nothing is seen which could be attributed to Photosystem II.

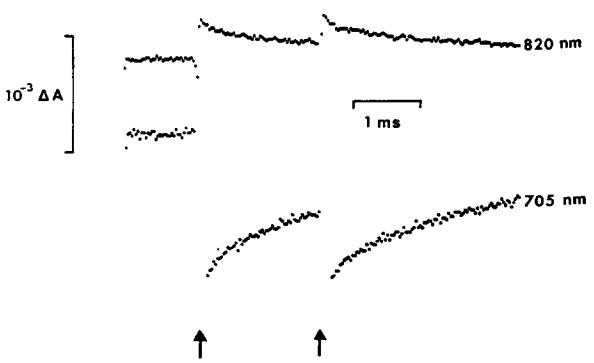


Fig. 1. Flash-induced absorption changes at 820 and 705 nm in chloroplasts, 20 μg chlorophyll/ml, suspended in Tricine buffer pH 7.5. As the bandwidth of the monochromator was too large (10 nm) the change at 705 nm cannot be taken as quantitative. Moreover, at 705 nm some flash-induced fluorescence reached the photomultiplier, which was disturbed for about 150 μs. The signals shown are an average of 100 experiments. The upward arrows indicate when the flashes were given.

Chloroplasts at low pH

At a pH below 5, the amplitude of the absorption change at 820 nm increases; at pH 4.0 it is more than twice that at pH 7.5 (Figs. 2 and 5). At this low pH, a biphasic decay is recognized (Fig. 3); the rapid phase has a half-time of 180 ± 30 μs. A second flash given shortly after the first gave nearly the same effect (Fig. 3). The small decrease in amplitude between the first and second flashes varies with the time interval between the flashes; the maximum decrease is about 15 % (Fig. 4). DCMU (3-(3',4'-dichlorophenyl)-1,1-dimethylurea) added to a final concentration of 10 μM did not change the signals significantly. At 705 nm (with a measuring beam of low intensity) the 180 μs phase was absent and a second flash brought the absorption decrease to the same level as the first.

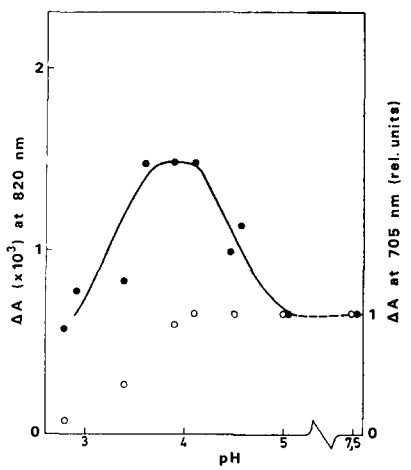


Fig. 2. The pH dependence of the amplitude of the absorption changes at 820 nm (●–●) and at 705 nm (○–○). The chloroplasts at 50 μg chlorophyll/ml were suspended in Tricine, MES or succinate buffer depending on the pH. The data were taken from the average of 20 experiments.

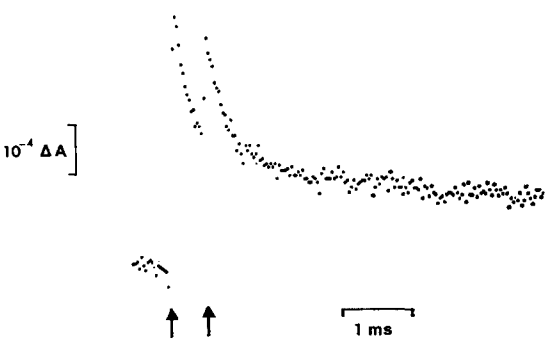


Fig. 3. Flash-induced absorption changes at 820 nm in chloroplasts suspended in succinate buffer pH 4.0, at 20 μg/ml chlorophyll. The signal shown is the average of 40 experiments.

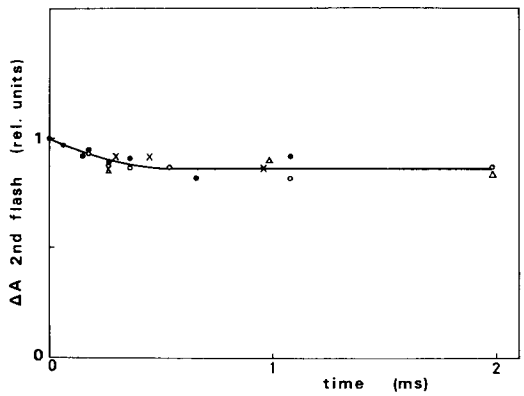


Fig. 4. The effect of a second flash relative to the first as a function of the time between flashes, for the absorption changes at 820 nm, in chloroplasts at pH 4.0 (compare Fig. 3). The figure shows data from 4 independent series of experiments.

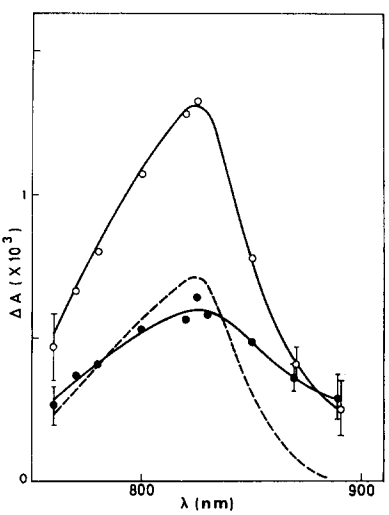


Fig. 5. Spectra of the flash-induced absorption changes in chloroplasts, 50 μg chlorophyll/ml, at pH 7.5 (●–●) and at pH 4.0 (○–○). The dashed curve represents the difference between the two former curves. The data were taken from the average of 40 experiments. At wavelengths where we had a poor signal to noise ratio, error bars are inserted.

In presence of a far-red background illumination at pH 4.0, *P*-700 stayed in its oxidized state as at pH 7.5: no absorption change at 705 nm was observed. At 820 nm the slow phase was absent in that case, but the fast phase was still observed, decaying with $t_{\frac{1}{2}}$ approx. 180 μs; a second flash was still less effective than the first. The absorption changes at 820 nm were completely inhibited by 10 μM DCMU if a strong far-red background illumination was used.

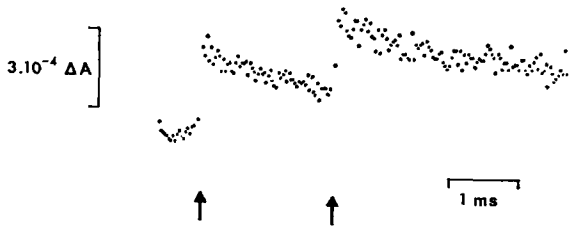


Fig. 6. Flash-induced absorption changes at 820 nm in chloroplasts, 30 μg chlorophyll/ml, at pH 3.9, in the presence of 1 mM ferricyanide. Average of 20 experiments.

Due to the fact that at low pH fast stabilisation reactions at the donor side of Photosystem II are inhibited or retarded [19], we are apparently able to see absorption changes which we attribute to the primary donor of this photosystem. Analogous results were obtained by Mathis and Vermeglio [17] at liquid nitrogen temperature.

The difference spectra of the flash-induced absorption changes at pH 7.5 and at pH 4.0 are shown in Fig. 5. As we attribute the absorption changes at pH 7.5 to *P*-700 only and the additional changes at pH 4.0 to the primary donor of Photosystem II, the difference between the two curves represents the spectrum of this last species. It appears that the spectra of both oxidized primary donors are rather similar in this spectral range. As at low temperature [17] however, the spectrum of $P\text{-}700^+$ is significantly broader: at 890 nm absorption changes are due only to Photosystem I. The shape of the spectrum of the oxidized primary donor of Photosystem II is confirmed by an experiment at low pH under far-red background illumination, in which case only Photosystem II contributes as *P*-700 stays in the oxidized state (results not shown).

Addition of 1 mM ferricyanide to the chloroplasts at low pH oxidized the *P*-700. At pH 4.1, this addition did not appreciably influence the 180-μs phase in the decay, but at pH 3.7 this phase was no longer visible. A comparably abrupt effect has been observed in luminescence experiments [19]. The absorption changes which remain at pH below 4 (Fig. 6) have a slow decay, and the level attained after the second flash is above the first. The corresponding difference spectrum presents a very broad peak around 820 nm. It is not clear whether under these conditions we still observe the primary donor of Photosystem II. Perhaps we observe the radical cation of a chlorophyll that is not the primary donor of one of the two photosystems. At a pH under 4.0, in the presence of ferricyanide, the chloroplasts were subject to a strong effect of aging. Moreover, an important clumping of the chloroplasts severely complicated the measurements and prevented a more detailed investigation. Without ferricyanide, at pH values below 3.0, a signal remains which has a relatively slow decay (half-time of a few ms) and which again cannot be ascribed to oxidized *P*-700 and perhaps not to the primary donor of Photosystem II either.

Acid-pretreated chloroplasts resuspended at neutral pH

Chloroplasts treated for a few minutes at pH 3.9–4.1 and resuspended in Tricine buffer (pH 7.5) still showed the absorption changes at 820 nm attributable to Photosystem II, but the decay was somewhat faster: $t_{\frac{1}{2}}$ approx. 120 μs. The acid inhibition of the secondary reactions is apparently irreversible. As at low pH, without background

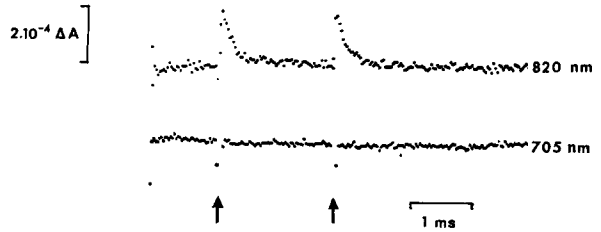


Fig. 7. Flash-induced absorption changes at 820 and 705 nm in chloroplasts washed with succinate buffer pH 3.9 and resuspended in Tricine buffer pH 7.5, at 15 μg chlorophyll/ml, in the presence of a strong far-red background light. Average of 20 experiments.

illumination DCMU had little or no effect on the absorption changes. In the presence of strong far-red background illumination, the *P*-700 is completely oxidized (Fig. 7) and the absorption changes at 820 nm can be attributed to the primary donor of Photosystem II. The effect of the second flash is the same as the first (as without background light), also with a shorter time between flashes than shown in Fig. 7. With background light, in the presence of 10 μM DCMU, the Photosystem-II absorption changes are completely inhibited.

Tris-washed chloroplasts

With Tris-washed chloroplasts and in the absence of far-red background light, the absorption changes at 820 nm are very similar to the *P*-700 changes measured at 705 nm; perhaps small changes immediately after the flash were due to Photosystem II, but even after the second flash (2 ms after the first) no clear Photosystem-II contribution could be detected. In the presence of a strong far-red background light, which oxidized *P*-700, as in the acid-inhibited chloroplasts, absorption changes at 820 nm were obtained which could be attributed to Photosystem II. The amplitude of the change was about 30–40 % of the maximum amplitude obtained with acid-washed chloroplasts; the decay time of the signal was: $t_{\frac{1}{2}}$ approx. 120 μs. The changes were completely inhibited by DCMU, as in the case of acid-inhibited chloroplasts in the presence of background light. By analogy with the experiments on acid-inhibited chloroplasts, we propose that these fast decaying absorption changes can be attributed to the primary donor of Photosystem II.

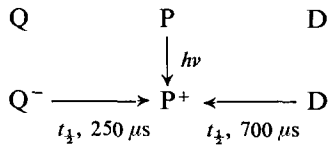
DISCUSSION

At normal pH, we do not see any contribution of Photosystem II to the signal at 820 nm (Fig. 1). Our results thus suggest that the 120–200 μs phase in the absorption changes at 680 nm reported by Döring [10] represents only a small contribution to the dark reduction of the oxidized primary donor. In agreement with the experiments of Duysens et al. [13] and Den Haan et al. [14] we suppose that the electron donation to the oxidized primary donor of Photosystem II is too fast to permit its observation under our conditions.

At low pH, a flash-induced absorption increase is visible which can be attributed to Photosystem II, apparently due to an irreversible inhibition of secondary reactions. As at liquid nitrogen temperature [17] we are led to conclude that the decay

of the signal at 820 nm which we attribute to the oxidized primary donor of system II is mainly due to the reverse reaction with the primary acceptor: $P^+Q^- \rightarrow PQ+h\nu$ (Q is the primary acceptor, [1]). This conclusion is supported by the following observations: (1) The effect of the second flash, in dark-adapted chloroplasts, even in the presence of DCMU: apparently the second flash finds the reaction center largely in the reopened state. (2) The strong luminescence which is seen under the same conditions and which has a decay time very close to the decay time of the fast absorption change seen at 820 nm [19, 22].

As addition of DCMU had no significant influence on the absorption changes in the absence of background light, we suppose that the reoxidation of Q^-, even in the absence of DCMU, in a way other than via the back reaction, is slow compared to the phenomena described here. The fact that the second flash has a slightly smaller effect than the first (Figs. 3 and 4) may be explained by a relatively slow electron donation to the reaction center competing with the back reaction. From the decrease of about 15 % relatively to the first flash (Fig. 4), i.e. about 30 % of the system II contribution, we assume an electron donation of $t_{\frac{1}{2}}$ approx. 700 μs under these conditions. A second flash had the same effect as the first at 705 nm. The main reactions which we suppose to occur at pH 4.0 are summarized in the following scheme:



At pH 4.5 the amplitude of the absorption change attributed to System II is about half that at pH 4, but the decay is as fast ($t_{\frac{1}{2}}$ approx. 180 μs). We conclude that at pH 4.5 two types of reaction center are present, some with a fast secondary donor and others with an inhibited one. This means that the inhibition by low pH is abrupt, which agrees with the conclusion reached by Van Gorkom et al. [19] based on fluorescence experiments.

Chloroplasts pretreated at pH 4.0 and resuspended at pH 7.5 do not behave very differently from chloroplasts at pH 4.0, thus showing that the acid inhibition of secondary reactions is irreversible. However, the decay of the signal at 820 nm due to Photosystem II is somewhat faster. As the second flash has the same effect as the first, even when it is given during the decay of the absorption increase provoked by the first flash, we again attribute the decay to the back reaction between primary donor and acceptor. Apparently secondary electron donation is much slower in these chloroplasts than at pH 4.0. Strong far-red light which is preferentially absorbed by Photosystem I maintains *P*-700 in its oxidized state. In the acid-inhibited chloroplasts, the remaining absorption changes at 820 nm could be attributed to Photosystem II. The fact that DCMU inhibited these "remaining" absorption changes indicates that some secondary electron transfer from the reaction center of System II occurs. Apparently the far-red light also excites System II to some extent, whereas without DCMU, a reoxidation of Q^- occurs which is probably very slow compared to the back reaction.

On the basis of fluorescence experiments, it has been proposed that the oxidized primary donor of system II, P^+, is a fluorescence quencher [14, 23–25] but no direct

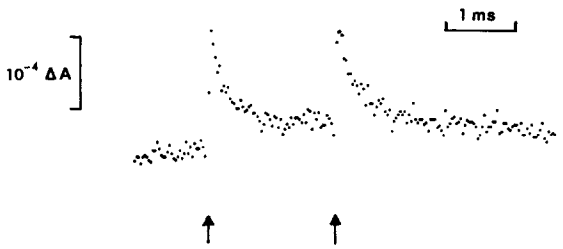


Fig. 8. Flash-induced absorption changes at 820 nm in Tris-washed chloroplasts, 30 μg chlorophyll/ml, in the presence of strong far-red background light. Average of 60 experiments.

evidence for the oxidized state of P^+ was presented. Our experiments strongly support this proposal, as under the conditions where the presence of P^+ is shown, a strong fluorescence quenching also exists (compare Ref. 20).

In Tris-treated chloroplasts absorption changes attributable to Photosystem II are only clearly visible in the presence of a strong far-red background illumination. Apparently a charge accumulation at the donor side of System II is required in these chloroplasts. Probably the inhibition site in Tris-treated chloroplasts is not as close to the primary reactions as it is in acid-treated chloroplasts. Haveman and Lavorel [22] observed a dominant 120 μs phase in the decay of luminescence with Tris-washed chloroplasts. They attributed the high amplitude of this phase in the decay to a charge accumulation at the donor side of System II and explained the 120 μs phase by the "intrinsic" back reaction between primary donor and acceptor. This is essentially confirmed by the experiment shown in Fig. 8, if we assume that the decay of the signal at 820 nm is due to the back reaction of P^+ with Q^-, as with acid-treated chloroplasts.

Velthuys and Amesz [26] and Velthuys [27] proposed that, in Tris-treated chloroplasts, only one positive charge can be stored on the donor side of Photosystem II. Babcock and Sauer [28] concluded that it is stored on the immediate physiological donor to P^+, leading to the EPR signal II_f. We report here that, without background illumination, a first flash, as well as a second one (given 2 ms after the first, i.e. well within the decay time of signal II_f [28]), does not provoke the appearance of absorption changes at 820 nm due to P^+. So our experiments lead us to propose that at least two positive charges can be stored on the donor side of Photosystem II in Tris-treated chloroplasts. It is also possible that the signal II_f is not due to the immediate physiological donor to P^+, as supposed by Babcock and Sauer [28] but must be placed one step further away.

ACKNOWLEDGEMENT

We are much indebted to A. Peronnard for his skilful technical assistance.

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