Biochimica et Biophysica Acta, 590 (1980) 353—359

BBA 47832

THE EFFECT OF pH ON THE REDUCTION KINETICS OF *P*-680 IN TRIS-TREATED CHLOROPLASTS

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(Received July 2nd, 1979)

Key words: Oxygen evolution; pH effect; Electron transfer; Photosystem II; Flash absorption spectroscopy; (Chloroplast)

Summary

The primary donor of Photosystem II (PS II), *P*-680, was photo-oxidized by a short flash and its rate of reduction was measured at different pH values by following the recovery of the absorption change at 820 nm in chloroplasts pretreated with a high concentration of Tris. The re-reduction is biphasic with a fast phase (dominant after the first flash) attributed to the donation by a donor, D_1, and a slow phase (usually dominant after the second flash) attributed to a back-reaction with the primary acceptor.

It is found that pH has a strong influence on the donation from D_1 ($\tau = 2$ μs at pH 9, 44 μs at pH 4), but no influence on the back reaction ($\tau \approx 200$ μs). pH also influences the stability of the charge separation since the contribution of donation from D_1 at the second flash increases at lower pH, getting close to 100% at pH 4.

In oxygen-evolving photosynthetic organelles, excitons channelled to the PS II reaction centers lead to the photo-oxidation of the primary donor *P*-680. In previous work, we monitored the kinetics of re-reduction of *P*-680$^+$ by the absorption changes at 820 nm. We have found that *P*-680$^+$ is reduced under physiological conditions with $t_{1/2} \simeq 30$ ns [1] and that this half-time is very much increased by treatments which inhibit oxygen evolution: 120 μs after treatment at pH 4.0 [2], 20—40 μs or 5 and 22 μs after incubation with a high concentration of hydroxylamine [1,3—5], 6 μs (first flash) or 130 μs (sub-

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Abbreviations used: Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; Mes, 4-morpholine-ethanesulphonic acid.

sequent flashes) after treatment with Tris [5]. It was shown that the slow reduction (100—200 μs) is due to a back-reaction with the primary acceptor and that the fast phase (5—40 μs) is due to the reduction of P-680$^+$ by a donor, D_1, which is either an accessory donor or a modified state of the physiological first donor to P-680. These results are in general agreement with data found by other authors using different techniques (reviewed in Refs. 1 and 5).

In this work we investigated the effect of pH on the kinetics of reduction of P-680$^+$ following a short ruby laser flash in Tris-treated chloroplasts. We found that pH has a large effect on the kinetics of reduction after the first flash (donation by D_1). It has no effect on the kinetics of the back-reaction. The kinetics of reduction after the second flash are affected, however, probably by changing the amount of remaining fast donation. Magnesium ions have a small effect on the kinetics. The effect of pH may help in understanding a few unexplained properties of the behaviour of P-680$^+$ and reveal important properties of the donor side of PS II.

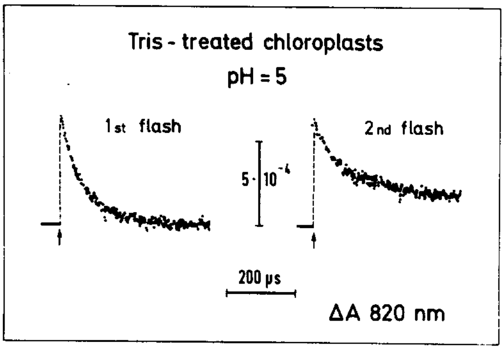
Materials and Methods

Spinach chloroplasts were prepared and treated with Tris (0.2 M, pH 9.0) as previously described [5]. Chloroplast pellets were homogenized in a small amount of distilled water, kept on ice and diluted just before each experiment in a 10 × 10 mm cuvette. The medium contained 10 mM KCl, 2 mM $MgCl_2$, 5 mM potassium ferricyanide and 50 mM of buffer (succinate (pH 4.0), Mes (pH 5.0 and 6.0), Tricine (pH 7.0 and 8.0) or Tris (pH 9.0)).

Flash-induced absorption changes (ΔA) were measured at 820 nm, at room temperature, as previously described [1,5]. Except where mentioned, the excitation ruby laser pulse was of saturating intensity. The chloroplasts were either dark-adapted or pre-illuminated with a short xenon flash. With our experimental set-up the signals (kinetics of flash-induced absorption change) are obtained in a numerical form by a signal averager. They were transferred to floppy disks and analyzed in a computer in terms of exponential components. We found that the decays could be correctly approximated by two exponential components over the entire pH range covered. In a previous work [5] a third component had been introduced, but we found here that its contribution is too weak to be safely considered.

Results

Chloroplast suspensions were supplemented with 5 mM ferricyanide in order to largely chemically oxidize P-700. The flash-induced absorption increase is attributed mostly to P-680$^+$ and its decay to the reduction of P-680$^+$. With dark-adapted chloroplasts, some results are shown in Figs. 1 and 2 (left traces) for pH 5 and 8. The recovery presents a fast phase (τ = 20 μs at pH 5 and 3.5 μs at pH 8) and a minor slow phase. At pH 8 the fast phase is not well resolved in Fig. 2 because of the lost time (approx. 2 μs) resulting from a perturbation of the detecting device (1 MHz electric band-width) by a laser artefact. This fast phase is better seen in Fig. 3 in which the perturbation is reduced by using a 5 MHz amplifier and by decreasing the laser energy so as to excite



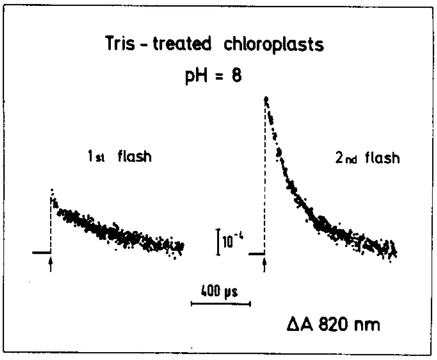
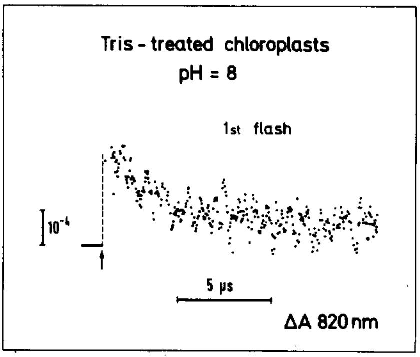


Fig. 1. Absorption changes at 820 nm induced by a ruby laser flash in Tris-treated chloroplasts (pH 5.0). Left trace: first flash to dark-adapted chloroplasts. Right trace: a xenon flash preceded the laser by 100 ms. Chlorophyll, 4.5 · 10^{-5} M. Average of four experiments. Amplifier band-width, 1 MHz.

Fig. 2. Same as Fig. 1, but at pH 8.0.

approx. 35% of the PS II centers. The initial ΔA, measured just after the laser flash, does not vary with pH. Considering the entire pH range (Fig. 4) it appears that the magnitude of the fast phase (which represents 75—90% of the total ΔA) varies very little with pH, whereas its lifetime varies considerably, from 44 μs at pH 4.0 to 3.5 μs at pH 8.0. The lifetime is even shorter at pH 9.0 (approx. 2 μs, at the limit of our possibility of detection). A slow phase with an average lifetime of 200 μs (little or no pH-dependence) must be taken into account to fit the total decay of ΔA. It is probably largely due to P-680$^+$ but we cannot exclude other contributions (such as a small contribution of P-700$^+$).

Following laser-flash excitation of chloroplasts which have been pre-illuminated by one xenon flash, the reduction of P-680$^+$ is slower than after



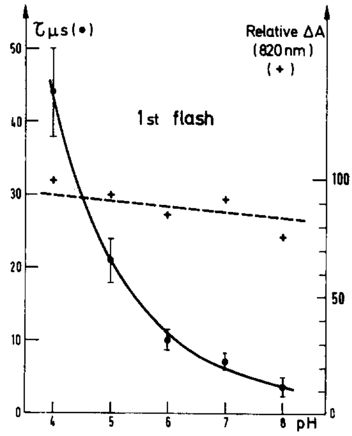


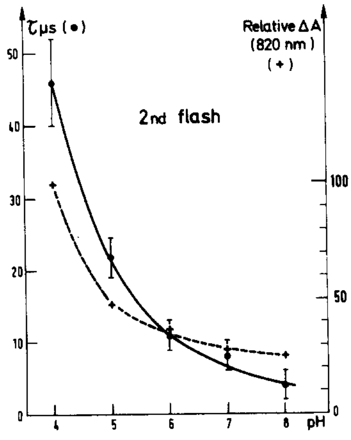
Fig. 3. Chloroplasts at pH 8.0 were dark-adapted and excited by a non-saturating ruby laser flash. The flash was kept non-saturating (excitation of approx. 35% of the Photosystem II centers) to decrease the perturbation by the flash artefact. Measurement with a 5-MHz band-width amplifier. Chlorophyll concentration, 5 · 10^{-5} M. Average of 12 experiments.

Fig. 4. A plot of the lifetime (●) of the fast phase introduced for a best-fit of the decay of ΔA at 820 nm in dark-adapted chloroplasts and of the relative contribution (+) of that phase to the total ΔA.

dark adaptation. Results are shown in Figs. 1 and 2 (right traces). The decay of *P*-680$^+$ can again be described with two exponential phases. At most pH values the major phase is the slow phase, the lifetime of which is approx. 200 μs. This lifetime varies between 150 and 300 μs for unknown reasons, but it does not vary significantly with pH. The fast phase is nearly the same as that obtained in dark-adapted chloroplasts (Fig. 5). Its relative contribution increases at lower pH, being approx. 100% at pH 4.0. The total Δ*A* does not vary significantly with pH.

The experiments on the effect of pH have been performed under various conditions and the following factors were found to have no significant influence: nature of the buffer (other buffers used: phosphate, Hepes and Tris), addition of 0.35 M sucrose, addition of $1 \cdot 10^{-6}$ M gramicidin (to collapse an eventual stable pH difference between the inside and outside of the thylakoid) and removal or inhibition of the coupling factor of phosphorylation (by treatments with EDTA or *N,N'*-dicyclohexylcarbodiimide, respectively (Galmiche, J. and Girault, G., unpublished results). Intermediate pH values were also studied (4.5, 5.5, 6.5 and 7.5) but the data were not analyzed on a computer; hand analysis did not reveal any irregularity.

We found, however, that magnesium ions influence the kinetics of the reactions. Tris-treated chloroplasts were suspended in an Mes buffer at pH 6.0 (titrated with Tris), plus 10 mM NaCl, plus 3 mM ferricyanide, with or without 10 mM $MgCl_2$. After the first flash, the reduction of *P*-680$^+$ is slightly, but significantly, slower without Mg^{2+} ($\tau = 12$ μs) than with Mg^{2+} ($\tau = 9$ μs). After the second flash (Fig. 6), the recovery includes a fast phase ($\tau \approx 10$ μs) and a slow phase ($\tau \approx 200$ μs). It appears that the fast phase is much larger without Mg^{2+} (30% of the total) than with Mg^{2+} (10% of the total) (the time interval between flashes was 50 ms instead of 100 ms for Figs. 1—5). We interpret the fast phase at the second flash as a reduction of *P*-680$^+$ by donor



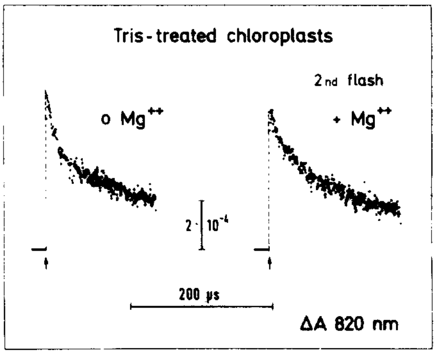


Fig. 5. Same as Fig. 4, but for chloroplasts pre-illuminated by one flash.

Fig. 6. Absorption change at 820 nm induced by a ruby laser flash (given 50 ms after a xenon flash). Tris-treated chloroplasts (chlorophyll concentration, $3.5 \cdot 10^{-5}$ M) in a buffer at pH 6.0 with or without 10 mM $MgCl_2$. Average of four experiments.

D_1 at those reaction centers where D_1^+ has been re-reduced in the time interval between flashes. It thus seems that addition of Mg^{2+} slightly accelerates the reduction of P-680$^+$ by D_1 and substantially stabilizes the charge separation in decreasing the rate of re-reduction of D_1^+.

Discussion

The results presented here will be discussed in the same manner as our previous data on Tris-treated chloroplasts [5]. Following the first flash after dark adaptation, P-680$^+$ is reduced by its donor, D_1, in a few microseconds. D_1 stays oxidized for a rather long time and thus, at the second flash, P-680$^+$ is reduced mostly by a back-reaction with the reduced primary acceptor ($t_{1/2}$ for the back-reaction is approx. 130 μs whereas it is at least 600 μs for the electron transfer from the primary to the secondary acceptor). Some fast phase is present, however, due to a partial re-reduction of D_1^+. Our present results thus indicate that pH has no effect on the rate of the back-reaction, but that it strongly influences the rate of electron transfer from D_1 to P-680$^+$.

As shown in Fig. 5, the proportion of donor D_1 which is found in a reduced state by the second flash increases when the pH is lowered, and the pH probably influences the rate of electron return from the acceptors to D_1. At pH 4.0, the second flash is followed by the same kinetics of P-680$^+$ reduction as in the first (Figs. 4, 5). This behaviour is similar to that previously reported for chloroplasts directly brought to pH 4.0 [2], a treatment which inhibits oxygen evolution [6]. In this case P-680$^+$ decays with a half-time of $\simeq$120 μs, mainly by back-reaction, as evidenced by a similar decay of the reduced plastoquinone anion observed at 320 nm [3]. The 120 μs decay ($\tau \simeq 170$ μs) has been confirmed in the course of this work (data no shown) for the first and second flashes. Thus, the difference in the kinetics of reduction of P-680$^+$ when chloroplasts are brought to pH 4.0 either directly ($\tau = 170$ μs) or after Tris treatment ($\tau = 44$ μs) is not due to a slower time response in Ref. 2. After Tris treatment and buffering at pH 4.0, we think that P-680$^+$ is not reduced by a back-reaction but by donation from D_1. We have no proof for that assumption, but rather an indication from the absence of an effect of pH on the back-reaction, and from the increasing contribution of the fast donation at the second flash when pH decreases (Fig. 5). We assume that this is due to a short-circuit electron transfer, made more probable at lower pH values, from one of the plastoquinones to D_1^+. This effect may be related to the slower rate of re-oxidation of the primary acceptor at lower pH. We have no rationale for the apparent complete disconnection of D_1 when chloroplasts are brought directly to pH 4.0 and not when they are first treated with Tris.

On the basis of their luminescence measurements, Kraan et al. [7] already suggested that a 'primary electron donor' of Photosystem II is functioning in different states of protonation. Since the rate of the back-reaction between P-680$^+$ and the primary acceptor is not affected by pH, we propose that, in the electron transfer from D_1 to P-680$^+$, D_1 is the pH-sensitive species. Renger et al. [8] also proposed that the donors to P-680$^+$ are affected by pH in untreated chloroplasts, possibly by direct protonation of a donor, with a pK of approx. 5. As a matter of fact, these authors did not find that the rate of a reaction is affected by pH, but that the magnitude of a 35-μs phase of reduction of P-680$^+$ increases at lower pH values, supposedly at the expense of the sub-microsecond phase of donation (although such complementary behaviour was not established). Our results establish that pH strongly influences the rate of electron donation from D_1 to P-680$^+$. This effect of pH may help in accounting for the scattering of data concerning the slow phases of reduction of P-680$^+$ in chloroplasts (we assume that the main phase of reduction of P-680 under physiological conditions is the 30-ns phase, as described in Ref. 1), since slow phases have been measured at different (or uncontrolled or not measurable) pH values [1,3—5, 9—15]. For most of our own experiments with hydroxylamine [1,3,4], we are of the conviction that the rather slow reduction of P-680$^+$ (20 to 40 μs) was due to unsuitable buffering.

What can the mechanism be by which proton concentration controls the rate of electron donation from D_1 to P-680$^+$? If D_1 is a polypeptide, it is possible that its protonation leads to a conformation less favourable for electron donation. In this hypothesis, our results cannot be accounted for by only two states (protonated and non-protonated) of D_1, giving two different rate constants, since the kinetics at intermediate pH values (6 or 7) cannot be fitted with two exponential components corresponding to the kinetics at extreme pH values. Interpretation of our results would require the existence of at least three protonation states, leading to a progressive change in the rate of donation. The effect of progressive protonation of D_1 might be to increase its positive charge, leading to an electrostatic repulsion (and decreased rate of electron transfer) to P-680$^+$. EPR experiments indicate that signal II_f, which arises probably from D_1 (cf. Ref. 5), is also sensitive to pH [16].

It is tempting to suggest an alternative mechanism, namely, that D_1 carries both an electron and a proton (or a hydroxyl ion). We previously suggested [5] that D_1 in Tris-treated chloroplasts is the same as that in oxygen-evolving chloroplasts, although in a less efficient state. For example, if D_1 is a proton carrier under physiological conditions, it may operate as follows (OES is the oxygen-evolving system):

$$P^{+\cdot} + D_1H \rightarrow P + D_1^{\cdot} + H^+ \text{ and } D_1^{\cdot} + \text{OES} \cdot H_2O \rightarrow D_1H + (\text{OES} \cdot \text{OH})^{\cdot}$$

Four turn-overs are required for the evolution of one molecule of oxygen. This scheme implies that protons are liberated by the oxygen-evolving system one at a time, i.e., one H^+ at each flash in a sequence of flashes. It offers the advantage that no high charge accumulation is required in the oxygen-evolving system. Experimental data on the H^+ liberation inside the thylakoid during a flash sequence are rather controversial [17—19] and the possibility cannot be excluded that a proton is liberated at each flash by the carrier, D_1, located between the oxygen-evolving system and P-680.

After this article was submitted, Bowes et al. [20] published a work showing that pH influences the fluorescence rise and the luminescence of untreated chloroplasts. This effect is under the control of the S states. It has been interpreted in terms of equilibrium reactions involving two electron carriers between the oxygen-evolving complex and P-680.

Acknowledgements

We thank H. Kramer for his help in some measurements. This work was supported in part by the Solar Energy Program Contract No. 014/76 ESF of the Commission of the European Communities.

References

1 Van Best, J.A. and Mathis, P. (1978) Biochim. Biophys. Acta 503, 178—188
2 Haveman, J. and Mathis, P. (1976) Biochim. Biophys. Acta 440, 346—355
3 Mathis, P., Haveman, J. and Yates, M. (1976) Brookhaven Symp. Biol. 28, 267—277
4 Mathis, P. and van Best, J.A. (1978) in Photosynthesis 77: Proc, 4th Int. Congr. Photosynth. (Hall, D.O., Coombs, J. and Goodwin, T.W., eds.), pp. 387—396, The Biochemical Society, London
5 Conjeaud, H., Mathis, P. and Paillotin, G. (1979) Biochim. Biophys. Acta 546, 280—291
6 Van Gorkom, H.J., Pulles, M.P.J., Haveman, J. and den Haan, G.A. (1976) Biochim. Biophys. Acta 423, 217—226
7 Kraan, G.P.B., Amesz, J., Velthuys, B.R. and Steemers, R.G. (1970) Biochim. Biophys. Acta 223, 129—145
8 Renger, G., Gläser, M. and Buchwald, H.E. (1977) Biochim. Biophys. Acta 461, 392—402
9 Lavorel, J. (1973) Biochim. Biophys. Acta 325, 213—229
10 Den Haan, G.A., Duysens, L.N.M. and Egberts, D.J.N. (1974) Biochim. Biophys. Acta 368, 409—421
11 Gläser, M., Wolff, C., Buchwald, H.E. and Witt, H.T. (1974) FEBS Lett. 42, 81—85
12 Joliot, A. (1977) Biochim. Biophys. Acta 460, 142—151
13 Jursinic, P. and Govindjee (1977) Biochim. Biophys. Acta 461, 253—267
14 Bowes, J.M., Itoh, S. and Crofts, A.R. (1977) 4th, Int. Congr. Photosynth., Book of Abstracts, p. 50
15 Renger, G., Eckert, H.J. and Buchwald, H.E. (1978) FEBS Lett. 90, 10—14
16 Yerkes, C.T. and Babcock, G.T. (1979) 7th Meeting of the American Society for Photobiology, Abstr. No. MPM-C7
17 Fowler, C. (1977) Biochim. Biophys. Acta 459, 351—363
18 Junge, W., Renger, G. and Ausländer, W. (1977) FEBS Lett. 79, 155—159
19 Saphon, S. and Crofts, A.R. (1977) Z. Naturforsch. 32c, 617—626
20 Bowes, J.M., Crofts, A.R. and Itoh, S. (1979) Biochim. Biophys. Acta 547, 336—346