Spectral and kinetic pH-dependence of fast and slow signal II in tris-washed chloroplasts

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We focus this report on the relationship between signal II fast and slow during a flash sequence for Tris-washed chloroplasts at different pH-values. The pH influences both the redox state and spectral form of signal II slow in dark-adapted chloroplasts. At pH 6.0, signal II slow is oxidized and does not influence the kinetics of signal II fast equally formed on each flash. At pH 8.5, signal II slow is mainly reduced in the dark and the first flash produces signal II slow and no signal II fast. Signal II fast appears on the following flashes only. Signals II fast and slow are connected to the same center and signal II fast is observed only if signal II slow is oxidized.

Chloroplast, Tris-washed *Photosystem II* *pH effect* *Signal II*

1. INTRODUCTION

System II centers appear as intricate molecular assemblies in which several electron acceptors and donors are involved. Different heterogeneous models have been proposed and are all focused on the properties of photosystem II acceptors [1–8]. It is difficult to study the donor side of oxygen-evolving chloroplasts because of the complexity of the oxygen-evolving enzyme and the lack of well-resolved absorbance changes. To characterize the donor side, we used Tris-washed chloroplasts as a simplified material and studied the kinetics of paramagnetic signals corresponding to the oxidized form of donors. A fast signal II arises from the oxidized from of Z^+ (denoted D_1 in [22]) a donor located on the main pathway to oxygen in untreated chloroplasts. A slow signal II is also observed and attributed to a donor D located on a sidechain. Both signals have similar spectra already described in [9–13]. In [14] we already mentioned that fast signal II decay was biphasic at high pH and that it was pH dependent.

Symbols: Z and D, species from which, respectively, fast signal II and slow signal II originate in ESR spectroscopy.

We will focus this report on the relationship between fast and slow signal II during a flash sequence for dark-adapted chloroplasts at different pH-values. We will also mention spectral characteristics at pH 6 and 8.5. The kinetics observed for the donor side will be compared to what is known of the acceptor side.

2. MATERIAL AND METHODS

Tris-washed chloroplasts were prepared from greenhouse peas as follows: The leaves were rinsed in ice water and then homogenized for 15 s in a blender in a (0.3 M NaCl/5 mM $MgCl_2$) 50 mM Tricine (pH 7.5) medium containing ascorbate and bovine serum albumin. The homogenate was strained through a nylon cloth, the filtered liquid was centrifuged at $2900 \times g$ for 10 min. The chloroplasts were resuspended in 0.8 M Tris (pH 8.5). They were stirred at $\sim 4°C$ for 20 min under daylight. These chloroplasts were again pelleted at $2900 \times g$, by 10 min centrifugation, washed twice in 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$ and resuspended to ~ 6 mg chl/ml in a medium containing 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$. They were stored in an ice bucket in the dark until use. For the experiments, the chloroplasts were

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further diluted in the same medium to 3–4 mg chl/ml final conc. The pH was adjusted with MES buffer (pH 6) of Tris buffer (pH 8.5) both 50 mM final conc. Tris-washed chloroplasts are first adjusted to the desired pH, then kept in the dark for several hours before the measurements. In pH jump experiments, the first pH is given by a diluted buffer (10 mM), the second pH is imposed by a concentrated buffer (100 mM). The EPR experiments were performed with a Bruker B ER 420 spectrometer. Microwave power of 25 mW and modulation amplitude of 6.3 G were used. Data were collected and stored on a Nicolet signal averager model 535. For the spectra the scan rate was 100 G/2 min. All experiments were performed at room temperature (~20°C). The light sources were a 900 W Xenon lamp for continuous white illumination and a model DL 2100 C pulsed dye laser (phase R corporation) with a pulse duration of 1 μs for illumination by flashes. The flash intensity was saturating and flash frequencies of 0.05 or 0.1 Hz were used.

During continuous illumination, the main signal observed is signal I (corresponding to $P700^+$) which overlaps largely signal II. However, the band width of signal I is narrower than that of signal II and a field value corresponding to a region where signal I is negligible can be chosen to measure pure signal II.

3. RESULTS

3.1. *Comparison of spectral characteristics and of radical concentrations at pH 6 and 8.5*

In untreated chloroplasts, D is normally oxidized. If it is reduced chemically, it can be oxidized by S_2 and S_3 states with half-times of several seconds [21]. We studied the oxido-reduction state of D in Tris-washed chloroplasts and its connection with photosystem II centers. We also compared the maximal concentration of D^+ and Z^+ to check whether it is likely that each photosystem II possesses both donors.

3.1.1. Radical concentration

At pH 6.0 a large signal II is observed for dark-adapted chloroplasts, a laser flash induces only a slight increase in the amplitude of the signal stable in the dark and the amplitude of signal II is approximately doubled during continuous illumination. At pH 8.5, a smaller signal II is detected prior illumination for dark-adapted chloroplasts. After a flash, the amplitude of the stable signal is much larger and during continuous illumination the amplitude is approximately double that observed for the stable signal after a flash.

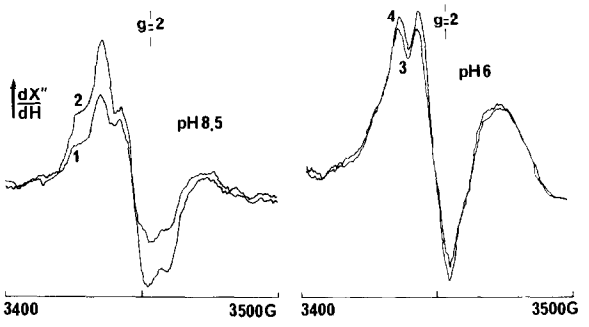


Fig.1. Signal II spectra: (3,4) pH 6; (1,2) pH 8.5; (1,3) dark-adapted samples; (2,4) recorded in the dark after a saturating laser flash. Instrument settings: 4 scans averaged; signal gain, 8×10^5; modulation amplitude, 6.3 G; time constant, 0.2 s; room temperature; total conc. chl. 4 mg/ml; scan rate, 2 min for 100 G.

Therefore, we conclude that for dark-adapted chloroplasts D is mainly oxidized at pH 6, partly reduced at pH 8.5 (fig.1).

During the illumination the signal corresponds to the total amounts of D^+ and Z^+, the stable signal in the dark following the illumination corresponds to D^+ only. The amplitude of signal II during illumination being twice that of the dark signal, the maximal concentrations of D^+ and Z^+ are roughly estimated as equal.

3.1.2. Spectral forms of signal II slow at pH 6 and pH 8.5

The spectrum of signal II has a hyperfine structure with 6 bands (named A to F from low-to-high field values). The relative amplitudes of the bands are very different at pH 6 and 8.5 (fig.1): at pH 6, 3 main peaks are observed: B, C, E, A and F appear as shoulders. At pH 8.5, B and E bands are larger than the others but A, C and F appear clearly as peaks.

The two spectral forms of signal II have been described under various conditions. The shape of the spectrum was dependent upon the salt content of the medium used [15]. A macroscopic orienta-

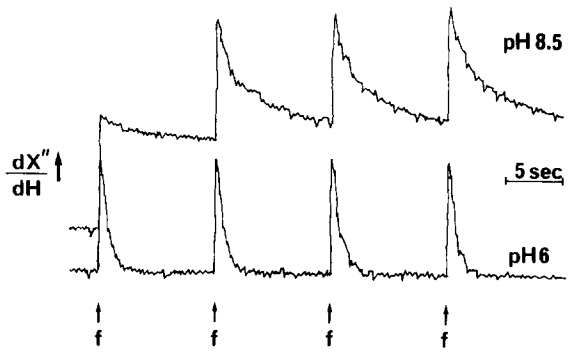


Fig.2. Signal II kinetics during a sequence of flashes for dark-adapted samples at pH 6 and 8.5. The time between flashes is 10 s. Instrument settings 4 scans averaged, time constant 50 ms.

tion of the chloroplasts (by a flow system, a large magnetic field or a drying technique on quartz slides) results in a spectral form analogous to what we observe at pH 6.0. When the chloroplasts are randomly oriented, the spectrum is more like that observed at pH 8.5 [16–18].

In [19] signal II was thought to arise from several paramagnetic centres with different characteristics.

Here, we checked that the salt concentration, and the magnetic field for EPR measurements (~3500 G) have no effect on the spectra at both pH values.

The most likely explanation seems to be a pH-induced change in the membrane structure which leads to different environments for the radicals giving rise to signal II. In [20] a 20% decrease in the thylakoid membrane thickness upon acidification [20]. The different protein environment may also influence the redox state of D: pH 6 favours its oxidation; pH 8.5 favours its reduction. A pH jump experiment was done in both ways: 6–8.5, 8.5–6 to see if the changes in spectrum and oxido-reduction state were rapid and concomitant.

If chloroplasts at pH 6 are transferred to pH 8.5, the change in spectral form and the reduction of D are achieved within the time needed to record a spectrum (some minutes). If chloroplasts at pH 8.5 are transferred to pH 6.0, the change in spectral form is readily observed but no appreciable oxidation of D is observed in the first minutes (time needed to record the spectrum).

3.2. *Kinetics of signal II fast during flash sequence*

In Tris-washed chloroplasts, the reduction of P^+ occurs in the microsecond time range [22]. The fast signal II rise time is therefore shorter than the time resolution of our spectrometer and we only follow its reduction kinetics.

Under repetitive flashes D^+ is formed and, as observed in [14], the fast signal II formed by a flash decays in a biphasic way at high pH-values. In this work with Tris-washed pea chloroplasts we found at pH 8.5 a half-time of 0.5 s for 35% of the amplitude and 3 s for 65% of the amplitude. When lowering the pH, the fast phase (0.5 s) becomes predominant. These values are slightly different from the preliminary results, we had obtained with spinach chloroplasts [14].

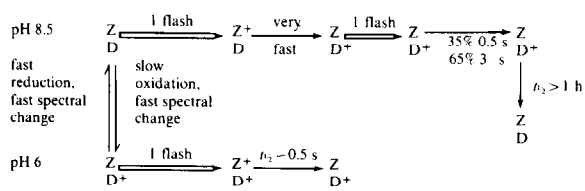
With dark-adapted chloroplasts (dark time ≥2 h) D is in a reduced state at pH 8.5, the first flash produced mostly an irreversible signal (D^+), the reversible fast signal II appears on top of it after the following flashes (fig.2), with the biphasic decay described above.

At pH 6.0, D^+ is present in the dark and the signal II formed by the first flash is completely reversible.

4. DISCUSSION

Our results show that in Tris-washed chloroplasts, D and Z exist in equivalent amounts. If they are both reduced prior to illumination (pH 8.5, long dark adaptation), they are successively oxidized: D^+, then Z^+; and belong therefore to the same system II center. Because of the identity of their spectrum we cannot say if D^+ is formed via Z^+ or directly from P^+.

The pH influences both the redox state of D and the EPR spectrum corresponding to its oxidized form. We believe that this is due to different environments for the radical giving rise to signal II but we have as yet no direct evidence. We can summarize our EPR results in the scheme:



There is no discrepancy between the one-donor capacity found by Conjeaud and Mathis and the two-donor capacity which we detect after a long dark adaptation at pH 8.5. Their dark adaptation time was much shorter (personal communication) and D^+ was most likely in its oxidized state at all pH-values [22].

The biphasic decay seen at pH 8.5 for fast signal II is likely to be due to different system II centers. In a study on Tris-washed chloroplasts [8] we had shown that at pH 8.5 two different types of system II secondary electron acceptors exist: a two-electron acceptor, B and a one-electron acceptor, B'. Various experimental indication, lead us to think that B and B' are the likely substrate for the signal II fast reduction. At pH 8.0, preliminary measurements of absorbance changes at 320 nm also display a biphasic decay (Van Gorkom, personal communication). A direct contribution of fast signal II absorption spectrum can interfere with these measurements and further experiments will be needed for the identification of fast signal II reduction substrates.

REFERENCES

[1] Ikegami, I. and Katoh, S. (1973) Plant Cell Physiol. 14, 829–836.
[2] Melis, A. and Homan, P. (1976) Photochem. Photobiol. 32, 343–350.
[3] Joliot, P. and Joliot, A. (1977) Biochim. Biophys. Acta 462, 559–574.
[4] Eckert, H.J. and Renger, G. (1980) Photochem. Photobiol. 31, 501–511.
[5] Thielen, A.P.G.M. and Van Gorkom, H.J. (1981) FEBS Lett. 129, 57–61.
[6] Lavergne, J. and Etienne, A.L. (1981) in: Proc. 5th Int. Congr. Photosynthesis (Akoyunoglou, G. ed) vol. 3, pp. 939–948, Balaban Int. Sci. Services, Philadelphia PA.
[7] Lavergne, J. (1982) Photobiochem. Photobiophys. 3, 273–285.
[8] Boussac, A. and Etienne, A.L. (1982) submitted.
[9] Babcock, G.T. and Sauer, K. (1973) Biochim. Biophys. Acta 325, 483–503.
[10] Babcock, G.T. and Sauer, K. (1973) Biochim. Biophys. Acta 325, 504–519.
[11] Babcock, G.T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 315–328.
[12] Babcock, G.T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 329–344.
[13] Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
[14] Etienne, A.-L., Boussac, A. and Lavergne, J. (1981) Proc. 5th Int. Congr. Photosynthesis (Akoyunoglou, G. ed) vol. 2, p. 405–413, Balaban Int. Sci. Services, Philadelphia PA.
[15] Gribova, Z.P., Tikhonov, A.N. and Ruuge, E.K. (1977) Biophysics 22, 269–273.
[16] Hales, B.J. and Das Gupta, A. (1981) Biochim. Biophys. Acta 637, 303–311.
[17] Dismukes, G.C., McGuire, A., Blankenship, R. and Sauer, K. (1978) Biophys. J. 239–256.
[18] Garab, G.I., Kiss, J.G., Mustardy, L.A. and Michel-Villaz, M. (1981) Biophys. J. 34, 423–437.
[19] Ruuge, E.K., Tikhonov, A.N. and Blumenfeld, L.A. (1974) Biophysics 19, 1056–1060.
[20] Murakami, S. and Packer, L. (1970) J. Cell Biol. 47, 332–351.
[21] Velthuys, B.R. and Visser, J.W.M. (1975) FEBS Lett. 55, 109–112.
[22] Conjeaud, H., Mathis, P. and Paillotin, G. (1979) Biochim. Biophys. Acta 546, 281–291.