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HETEROGENEITY OF SYSTEM II SECONDARY ELECTRON ACCEPTORS IN TRIS-WASHED CHLOROPLASTS

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Tris-washed chloroplasts were submitted to saturating short flashes, and then rapidly mixed with dichlorophenyldimethylurea (DCMU). The amount of singly reduced secondary acceptor was estimated from the DCMU-induced increase in fluorescence, caused by the reverse electron flow from secondary to primary acceptor. The back-transfer from the singly reduced secondary acceptor to the primary acceptor Q induced by DCMU addition affects only a part (60%) of the variable fluorescence (ΔF_{max}). As previously shown, the quenchers involved in this phenomenon, 'B-type' quenchers, are different from those controlling the complementary part of the fluorescence, the non-B-type. In this report, we show that at pH 8.5 in the B-type systems, there exist two kinds of secondary electron acceptors: B, a two-electron acceptor, the corresponding Q accounting for 40% of the variable fluorescence; B′, a one-electron acceptor, the corresponding Q accounting for 20% of the variable fluorescence. The lifetimes of B^- and B'^- in the absence of DCMU are 40 and 1 s, respectively. The primary acceptors of the B and B′ systems can be considered as corresponding to the Q_1s defined previously (Joliot, P. and Joliot, A. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoynoglou, G., ed.), pp. 885–899, Balaban International Science Services, Philadelphia). The B′ centers seems to be equivalent to the Q_β centers as defined by other workers (Van Gorkom, H.J., Thielen, A.P.G.M. and Gorren, A.C.F. (1982) in The Function of Quinones in Energy Conserving Systems (Trumpower, B.L., ed.), Academic Press, New York, in the press).

Introduction

Some years ago, Velthuys [1] and Bouges-Bocquet [2] showed independently that electron transfer between the two photosystems occurred through an intermediate B which can accept electrons one by one from the primary acceptor Q and transfers them in two's to the plastoquinone pool.

More recently, Velthuys [3] suggested that the secondary acceptor B was a plastoquinone of the pool, tightly bound to Q in its plastosemiquinone form only and that the mode of action of DCMU is a competition with the plastoquinone for the binding to the secondary acceptor binding site. A similar mechanism was demonstrated by Lavergne [4] using an exogenous quinone.

The QB^- state is therefore unfit for DCMU binding. In the odd state of the centers, there exists a fast equilibrium between Q^-B and QB^- and DCMU binds to the fraction of centers in the Q^-B state. Lavergne (unpublished results) showed that the rate and equilibrium constant of DCMU binding to centers in the odd state are indeed

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PS, photosystem.

lowered by a factor close to the equilibrium constant $[QB^-]/[Q^-][B]$.

This mechanism offers a simple explanation for the back-transfer of an electron from B^- to Q occurring in the dark upon DCMU addition. [1].

This back-transfer from B^- to Q affects only a part (approx. 60%) of ΔF_{max}. We studied this phenomenon in detail [5,6] and showed that it was not due to an incomplete reverse transfer but rather to a heterogeneity of PS II acceptors, part of them being connected to B (B-type acceptors) and part of them not (non-B-type acceptors). In recent papers, Lavergne [7,8] described the main characteristics of the two types of acceptors and we will give no further information on that type of heterogeneity.

Other heterogeneous models have been proposed by different authors [9–18]. Joliot and Joliot [24] proposed a complex PS II center with two parallel primary acceptors, Q_1 and Q_2. In the presence of hydroxylamine, Q_1 is reduced with a high efficiency by a first photoact whereas Q_2 is reduced with less efficiency by further flashes. Q_2 is not a plastoquinone; it does not give rise to 550, 515 and 320 nm absorption changes.

Melis and Homan [11] have defined two types of centers, Q_α and Q_β, differing by their antenna size and composition. No energy transfer occurs between PS II_β centers. They were further characterized by Van Gorkom et al. [16] and Thielen and van Gorkom [17] who showed that in β centers the electron transport does not proceed via the two-electron accumulating device.

In this report, we present a further characterization of the B-type centers by studying the concentrations and lifetimes of their secondary electron acceptors.

To do so we measured, under various conditions, the fluorescence yield after DCMU addition which reflects the amount of reduced secondary acceptors present before the addition. We used Tris-washed chloroplasts to eliminate the influence of the S states.

At pH 8.5, we will show that there are two sorts of secondary electron acceptors: a two-electron acceptor, B, stable in its singly reduced form; and an acceptor, B′, storing one electron only and rapidly reoxidized by a forward reaction after every flash of a series. We will compare our results with other heterogeneous models.

Materials and Methods

Tris-washed chloroplasts were prepared from greenhouse peas in the following way: The leaves were rinsed in ice water and then homogenized for 15 s in a blender in a medium (0.3 M NaCl, 5 mM $MgCl_2$, 50 mM Tricine, pH 7.5) containing ascorbate (0.5 mM) and bovine serum albumin (approx. 1 mg/ml). The homogenate was strained through a nylon cloth and the filtered liquid was centrifuged at 2900 × *g* for 10 min. The chloroplasts were resuspended in 0.8 M Tris-HCl pH 8.5. They were stirred at 4°C during 20 min under daylight. These chloroplasts were again pelleted at 2900 × *g* for 10 min, washed twice in 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$ and resuspended to a chlorophyll concentration of approx. 6 mg/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 further diluted in the same medium to a final chlorophyll concentration of 20 μg/ml. The pH was adjusted with Mes buffer (pH 6) or Tris buffer (pH 8.5), both at 50 mM final concentration.

The experimental setup was as described in Ref. 5: A sample of chloroplast suspension flows to a preillumination chamber where it is excited by a variable number of short saturating flashes (General Radio, Strobotac, 2 μs duration at half peak, energy 0.1 J) spaced at a given time (from 0.1 to several seconds). After a variable dark time (from 0.1 to several seconds), another flow step (0.3 s duration) allows mixing with an equal volume of DCMU ($2 \cdot 10^{-4}$ M) and the transfer to the observation chamber. Fluorescence kinetics can then be recorded. A second flash from a flash pumped dye laser (duration 1 μs) can provide saturating illumination in the observation chamber. A gating system was used to protect the photomultiplier during the flash.

The fluorescence is monitored by synchronous detection of the fluorescence excited by a train of detecting flashes (from a modulated laser) synchronized with the address advance of a multichannel analyzer. The intensity of the flashes was adjusted to give a negligible actinic effect. Five to ten accumulations were necessary to obtain a satisfactory signal-to-noise radio. In order to eliminate luminescence artefacts, the recording obtained in the absence of the detecting flashes was substracted from the overall signal.

The initial fluorescence level (F_0) is detected when no actinic flashes are fired; the addition of DCMU does not modify this level, showing that the secondary acceptors are completely oxidized in dark-adapted Tris-washed chloroplasts.

The maximum fluorescence level (F_{max}) is obtained after several actinic flashes with Tris-washed chloroplasts in the presence of NH_2OH and DCMU at pH 8.5.

The maximum variable fluorescence (ΔF_{max}) is equal to ($F_{max} - F_0$).

Experimental Results

Fluorescence decay after a saturating flash with DCMU present

This section gives information on the rate of recombination kinetics in Tris-washed chloroplasts between Q^- and the positive charge stored on the donor side. At pH 6.0, the fluorescence decay is very fast (20 ± 5 ms) (data not shown).

At pH 8.5, the decay depends strongly on the time allowed for dark adaptation at 0°C: If the dark time is longer than 2 h, 70% of ΔF_{max} decays very slowly with a half-time of 10 min, and (20 ± 5)% of ΔF_{max} decays with a half-time of 0.25 s. If preillumination occurs shortly before DCMU addition, the fluorescence decay is multiphasic: 30% of ΔF_{max} decays with a half-time of less than 25 ms, 20% of ΔF_{max} decays with a half-time of 0.25 s and the rest decays very slowly with a half-time of 10 min.

Description of the observed fluorescence transients when DCMU is added after a flash

As indicated in the Introduction, when DCMU is added to preilluminated chloroplasts, it causes a reverse electron transfer from the singly reduced acceptor B^- to Q in the B-type centers. The result of this back-transfer is a transitory increase in fluorescence corresponding to the formation of

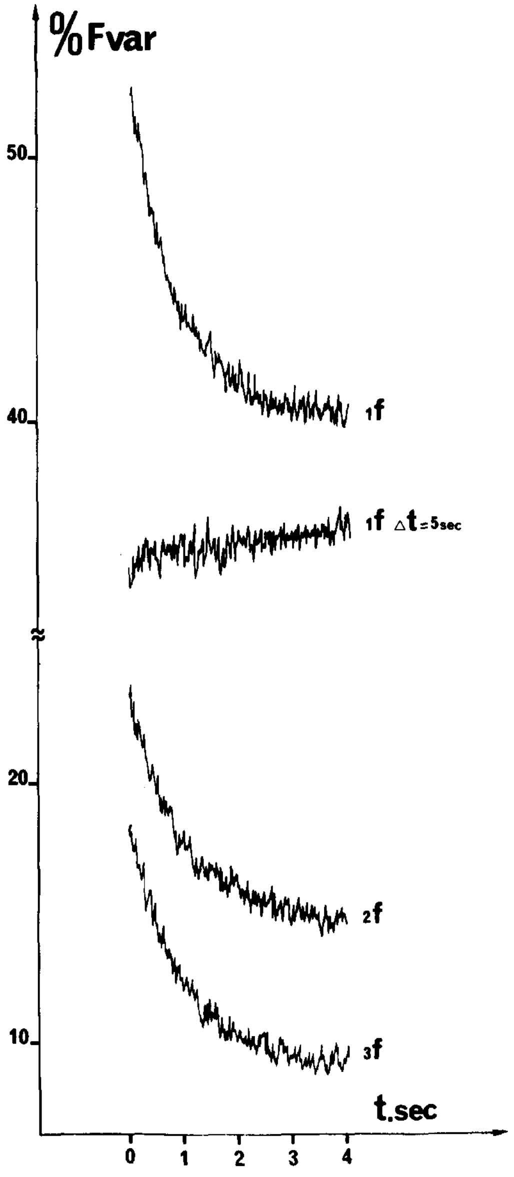

Fig. 1. Recordings of the fluorescence transient observed after preilluminating flashes and DCMU addition at pH 8.5. (Upper trace) 1f: The recording starts 0.4 s after the flash, 0.3 s after DCMU addition. (Middle trace) 1f, $\Delta t = 5$ s: A flash is given, after 5 s darkness, DCMU is added, the recording starts 0.3 s after the mixing (Lower trace) 2f, 3f: Two or three flashes spaced at 0.5 s are given, then DCMU is added. 1 s after the last flash, the recording starts 0.3 s after DCMU addition. The fluorescence levels are represented as % of the maximum variable fluorescence observed with DCMU and NH_2OH present.

Q^- if its reoxidation by positive charges of the donor side is slower than its reduction. In that case the amount of B^- can be deduced from the peak of the fluorescence transient upon DCMU mixing.

At pH 8.5, with the high concentration used for DCMU the fluorescence increase is achieved in the time required, in our flow method, to bring the sample to the observation chamber (300 ms). Therefore, we cannot follow the increase in fluorescence and only detect the kinetics after the initial Q^- formation.

When DCMU is added shortly after the flash the fluorescence transient recording starts from a high fluorescence and decays accordingly with the rate of Q^- reoxidation as shown in Fig. 1 (curves 1f, 2f, 3f,). If DCMU is added some seconds after the flash, the fast fluorescence decay is no longer observed; the fluorescence rises only to the level to which the fluorescence had rapidly decayed in the preceding experiment (Fig. 1, curve 1f; $\Delta t = 5$ s). We therefore conclude that a part of the singly reduced secondary acceptor which we call B'^- rapidly disappears in the dark in the absence of DCMU and that the corresponding Q^- (when formed) undergoes a fast reoxidation.

The rest of the singly reduced acceptor decays more slowly in the dark (it is still present after 5 s darkness: Fig. 1, curve 1f; $\Delta t = 5$ s). The corresponding Q^- undergoes no fast reoxidation (as shown by the absence of decay in Fig. 1, curve 1f;

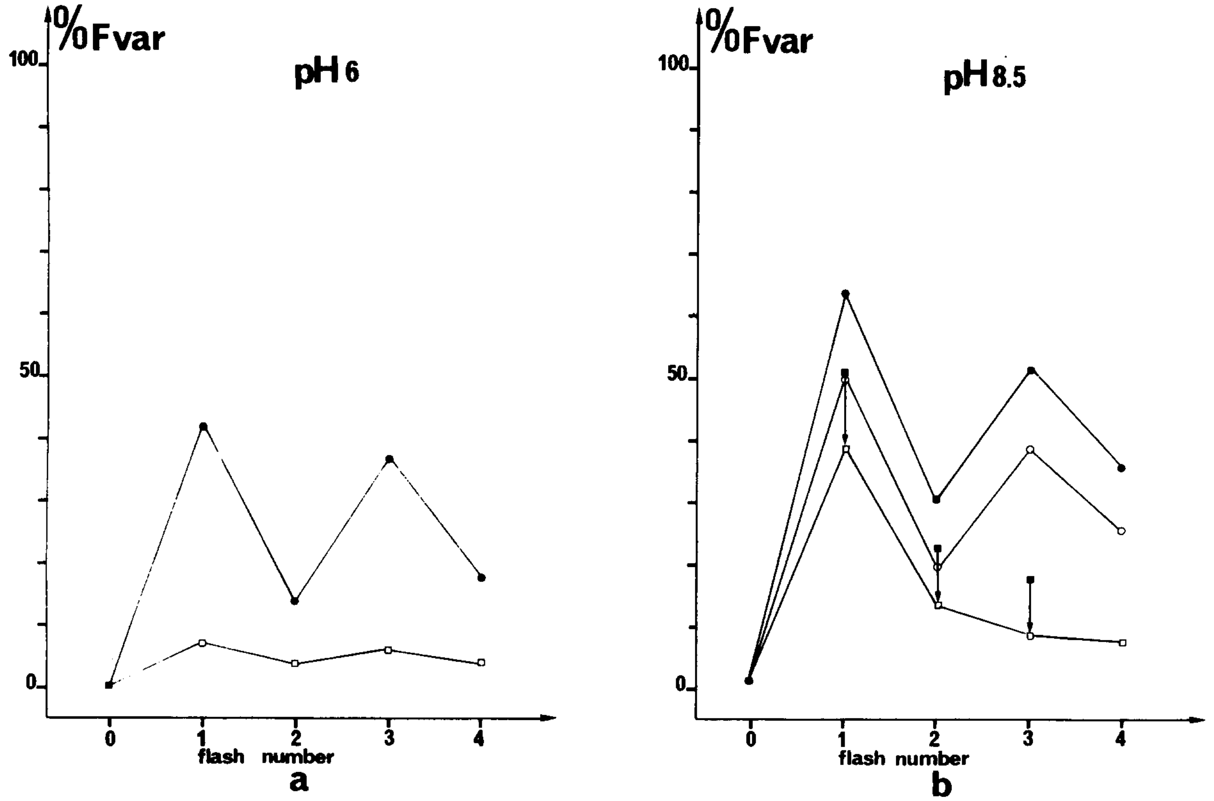

Fig. 2. Amplitude of the DCMU-induced fluorescence increase as a function of the preilluminating flashes number. The spacing between the flash is 0.5 s. DCMU is added 0.1 s after the last flash (unless otherwise stated). Detection starts 0.3 s after DCMU addition. (a) Curve 1 (●): Tris-washed chloroplasts at pH 6.0 are preincubated in the dark with 10 mM NH_2OH. Curve 2 (□) No donor is present. (b) Curve 1 (●): Tris-washed chloroplasts at pH 8.5 are preincubated with 10 mM Nh_2OH. Curve 2 (○): Same as curve 1 except that DCMU is added 5 s after the last flash. Curve 3 (■ and □): No donor is present. The full and open squares represent the maximum and asymptotic fluorescence levels, respectively, of the fluorescence transient recording (shown in Fig. 1 in curves 1f–3f).

$\Delta t = 5$ s) and amounts to 40% of $\Delta F_{\max}$ (Fig. 1, curve 1f; $\Delta t = 5$ s).

This means that the amount of B'^- can be deduced from the difference between the fluorescence level reached at the end of the DCMU-induced fluorescence increase (this fluorescence increase is not visualized in Fig. 1) and the asymptotic level reached at the end of the fast fluorescence decay following the initial increase (in Fig. 3, insert: this difference is denoted as A_1).

Lifetimes of reduced primary acceptors formed by a reverse reaction

At pH 8.5 after their formation upon DCMU addition, the reduced primary acceptors can undergo a back-reaction with positive charges stored on the donor side.

The lifetimes and amounts of Q^- detected are dependent on the flash number.

(a) After one flash: a biphasic decay is observed.

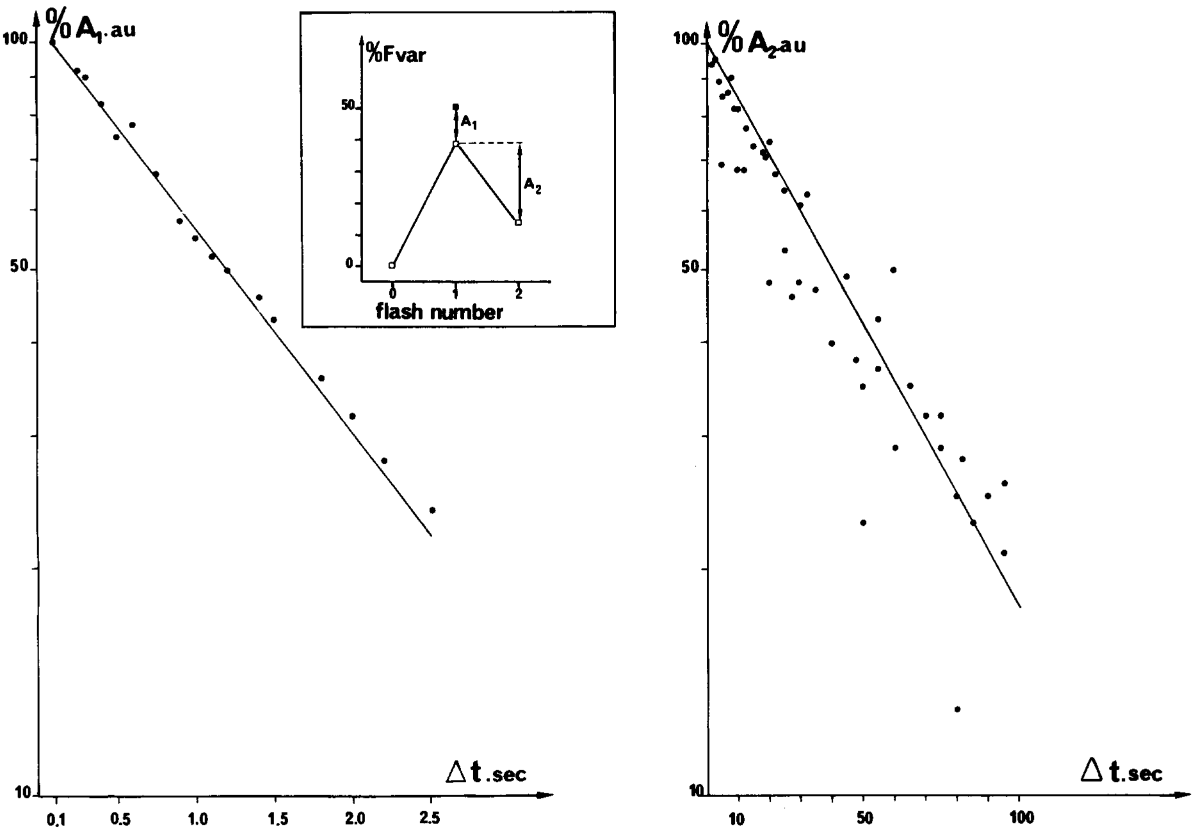

Fig. 3. Amplitude of the DCMU-induced fluorescence transient at pH 8.5 after one flash as a function of the delay Δt (s) between the flash and the DCMU addition. The recording always starts 0.3 s after DCMU addition. In the inset are shown the quantities taken as amplitude A_1 and A_2 for $\Delta t = 0.1$ s: A_1 represents the difference between the maximum fluorescence attained after DCMU mixing and the asymptotic level reached at the end of the fast decay. This difference is normalized to A_1 observed for $\Delta t = 0.1$ s. A_2 represents the asymptotic level as a function of Δt normalized to the difference between the asymptotic levels attained after the fast fluorescence decay (cf. Fig. 1) after one (1F) and two flashes (2F) when $\Delta t = 0.1$ s.

$$A_2 = \frac{F_{\text{asymptotic}}(1\text{f}, \Delta t) - F_{\text{asymptotic}}(2\text{f}, 0.1\text{ s})}{F_{\text{asymptotic}}(1\text{f}, 0.1\text{ s}) - F_{\text{asymptotic}}(2\text{f}, 0.1\text{ s})}$$

The first phase has a half-time of 0.5 s with an amplitude $\Delta F = 15\%$ of ΔF_{max} (this is the primary acceptor connected to B′).

The second phase is slow ($t_{1/2} \simeq 10$ min) with an amplitude $\Delta F = 40\%$ of ΔF_{max} (this is the primary acceptor connected to B).

(b) After the following flashes only the fast decay is detected; the fluorescence decays back to a low fluorescence level.

Due to the fast decay, the amplitude of the first phase is underestimated in our experiments and from the NH_2OH-DCMU experiment and extrapolation to zero time of mixing we can assume that its true amplitude is closer to 20% of ΔF_{max}.

Lifetimes of reduced secondary acceptors in the absence of DCMU

At pH 6.0, when NH_2OH is present, it prevents any reoxidation of Q^- formed by back-reaction and after DCMU addition, the fluorescence reaches a stable level. The binary oscillations of the DCMU-induced fluorescence rise (Fig. 2a, curve 1) shows the occurrence of the two-electron secondary acceptor B and the stability of the B^- state. With no donor present, no fluorescence increase is observed (Fig. 2a, curve 2). This shows that Q^- is reoxidized as soon as it is formed by reverse electron transfer. We cannot therefore determine the lifetimes of B^- in the absence of NH_2OH at this pH.

At pH 8.5, binary oscillations are also observed for the DCMU-induced transients in the presence of NH_2OH (Fig. 2b, curve 1). With no donor present, a large DCMU fluorescence increase is observed only after the first flash. After the following flashes only a small fluorescence increase is detected.

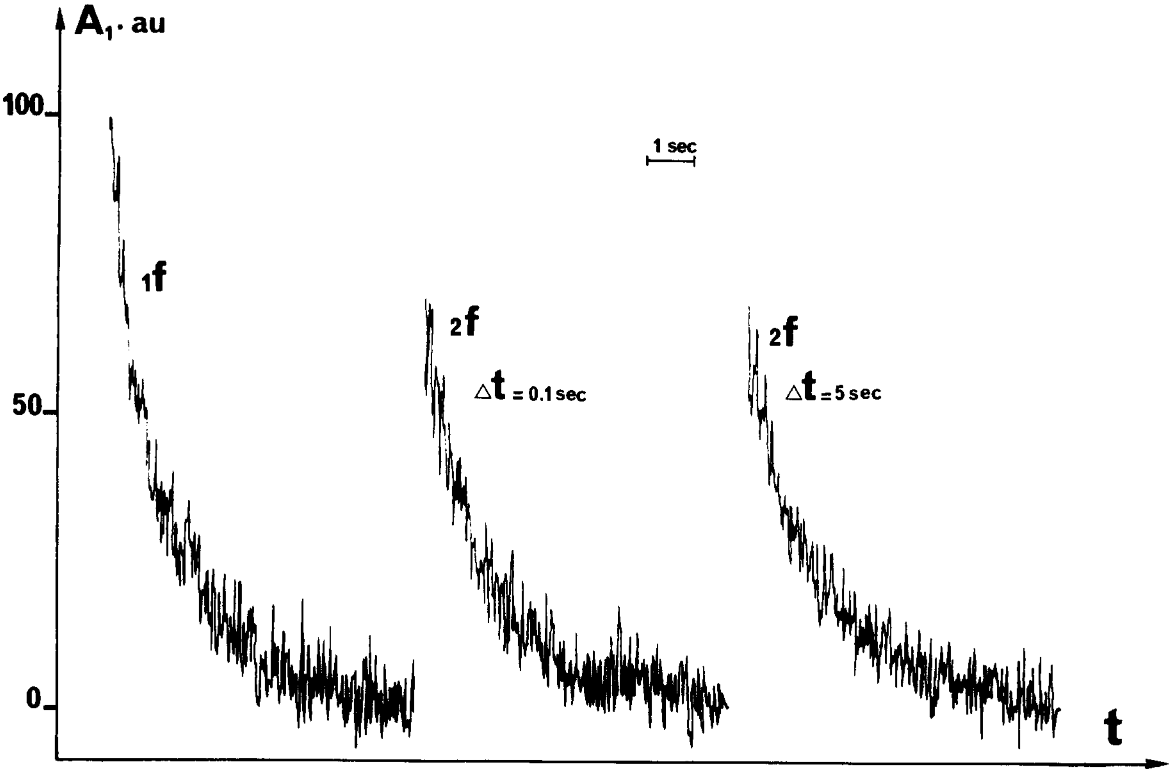

Fig. 4. Fluorescence transients: induced by DCMU addition after preilluminating flashes at pH 8.5. Curve 1f: DCMU is added 0.1 s after one flash. Curve 2f, $\Delta t = 0.1$ s: DCMU is added 0.1 s after two flashes; the spacing between the flashes is 0.1 s. Curve 2f, $\Delta t = 5$ s: the spacing between the flashes is 5 s; DCMU is added 0.1 s after the second flash. The curves were shifted so that all asymptotic levels are arbitrary located at 0, the maximum amplitude of the fast fluorescence transient is chosen to be equal to 100.

The lifetimes of reduced secondary acceptors can be studied by varying the spacing Δt between the last flash and the DCMU addition.

(a) After one flash.

The kinetics are biphasic: B'^- decays rapidly with a half-time of 1 s. B^- decays more slowly with a half-time of 40 s. The amplitude of the corresponding ΔF values are 15 and 40% of ΔF_{max}, respectively.

(b) After the second and following flashes, only B'^- is detected. It decays with a half-time of 1 s. The difference in amplitude of ΔF corresponding to B'^- after the different flashes (it is smaller after the second and following flashes than after the first flash) is due to the nonlinear relationship between the fluorescence level and the amount of Q^- and not to differences in concentration of B'^-.

Are the secondary electron acceptors revealed by DCMU addition both two-electron acceptors?

In Tris-washed chloroplasts there is a donor capacity of at most two. Therefore, B^- cannot be formed on the third flash due to the filling up of the donor side and this explains why no period 2 oscillations are observed in the absence of an exogenous donor.

The short-lived (1 s) acceptor was observed after every flash. If B′ were a two-electron acceptor, a second flash given when it is still mainly in its B'^- state would produce B'^{2-} and no fluorescence increase would be seen. As shown in Fig. 4, the amplitude of the fluorescence increase after two flashes is independent of the spacing between the flashes. Therefore, we conclude that B′ is a one-electron acceptor. When a donor is present, a second argument for B′ being a one-electron acceptor. When a donor is present, a second argument for B′ being a one-electron acceptor is given by the shift downwards observed for the binary oscillation pattern of the DCMU-induced fluorescence increase when DCMU is added 5 s rather than 0.1 s after the flash (Fig. 2b, curves 1 and 2). This shows additionally that in the absence of DCMU, B'^- is reoxidized by a forward reaction.

Discussion

The results reported above confirm the heterogeneity of the acceptor side of PS II. They allow a comparison with the models proposed by other authors.

We confirm that in Tris-washed chloroplasts at both pH 6.0 and 8.5 there exist two types of acceptor systems reacting differently to DCMU addition: the non-B-type acceptors are those in which no reverse electron flow can ever be detected upon DCMU addition, they account for at least 35% of ΔF_{max}. The B-type acceptors are those in which the DCMU can provoke a back-transfer of electrons to Q when it is added to acceptor systems in their semireduced state. They account for at most 65% of ΔF_{max}.

At pH 6.0, in all centers a rapid reoxidation of Q^- occurs after its formation by a flash and when DCMU is present, Q^- formed by a flash is reoxidized by a fast back-reaction ($t_{1/2} \approx 20$ ms). This fact back-reaction prevents the detection of B^- at pH 6.0 by the method described in this paper.

At pH 8.5 in the B-type systems, two different secondary electron acceptors are revealed in our experiments:

(1) B, a two-electron acceptor. In the absence of DCMU its singly reduced form B^- is stable in the dark: $t_{1/2} = 40$ s. When its associated primary acceptor is reduced by DCMU addition after a single flash, it is very slowly reoxidized. The same is true if Q^- is directly formed by a flash in the presence of DCMU. This shows that the positive charge formed by the photoact has been stabilized in the centers on an irreversible donor. Q associated with B accounts for 40% of ΔF_{max}. If two primary acceptors with different quenching efficiencies exist and if centers also differ by their antenna size, the relationship between the fluorescence yield and the complementary area is difficult to translate in amounts of PS II centers.

(2) B′, a one-electron acceptor. Its reduced form is rapidly reoxidized in the dark in the absence of DCMU by a forward reaction insensitive to NH_2OH ($t_{1/2} = 1$ s). When its associated primary acceptor is reduced by a flash in the presence of DCMU it is rapidly reoxidized. The same is true if it is reduced by reverse electron flow. The donor side corresponding to the B′ acceptor seems therefore different from the donor side corresponding to the B acceptor. The primary acceptor connected

to B′ accounts for 15–20% of $\Delta F_{\max}$.

The Tris treatment slows down the reduction of the photooxidized chlorophyll [23]. No appreciable amounts of double photoacts can occur during the flashes. We can therefore conclude that the primary acceptors of the B and B′ systems can be considered as the Q_1s defined by Joliot and Joliot [18].

Thielen and Van Gorkom [17] noticed in similar experiments that the slow component of the complementary area (corresponding to Q_β) was not oscillating with a periodicity of two. They had concluded that Q_β centers were connected to a one-electron secondary acceptor. We can therefore assume that the B′ centers seen under our conditions are similar to the Q_β centers.

Because of the short lifetime of B'^-, DCMU must be added rapidly after the flashes to detect an appreciable amount of Q^- corresponding to B′. The maximum we detected amounts to 20% of $\Delta F_{\max}$ which is comparable to the total amount of Q_β centers. Thielen and Van Gorkom only detected a small amount of Q_β reduced by reverse electron flow but the time elapsed between the flash and DCMU addition cannot in their setup be as fast as in our flow system.

Further experiments are obviously needed for a complete understanding of the heterogeneity of PS II centers. Different mechanisms can take place depending upon pH and illumination conditions.

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