ELECTRON AND PROTON TRANSFER EVENTS IN CHLOROPLASTS DURING A SHORT SERIES OF FLASHES

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1. Introduction

The popular 'vectorial Z' scheme of green plant photosynthesis is unable to account for a number of observations concerning the translocation of protons and the role of plastoquinone and cytochromes in the rate limiting electron flow between the photosystems [1]. A recently-proposed recplacement scheme modifies it to incorporate a site of proton translocation located between plastoquinone and plastocyanin [1,2]. Evidence for this new scheme was obtained by measuring the flash-generated transmembrane electrical field, the proton uptake, and the redox reactions of cytochrome *f* and cytochrome b_6, and by noting how these phenomena are affected by prereduction of the plastoquinone pool [2,3].

This paper examines more closely the electron and proton transfer events in dark-adapted chloroplasts exposed to a series of short saturating flashes. The data illustrate the field-indicating ΔA_{515}, the cytochrome *f* redox change, and the proton release inside the thylakoid measured by the neutral-red technique developed in [4,5]. The results confirm the involvement of the contended 'extra' site of proton translocation in linear electron flow and thereby support the earlier proposed scheme.

2. Methods

Broken chloroplasts were prepared by standard procedures [3], suspended at 5 mg chl/ml in medium containing 400 mM sucrose, 2 mM KCl, 0.5 mM $MgCl_2$ and 1 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (Hepes)–NaOH (pH 7.45), and stored dark and cold (4°C). About 30 s before each measurement, a 5 μl sample was diluted 50-fold with reaction mixture of room temperature. The standard reaction mixture contained 400 mM sucrose (omitted in fig.1), 100 μM methylviologen, and 1 mM Hepes–NaOH (pH 7.45). Absorbance changes were induced by 4 consecutive flashes (at 1.2 s intervals) and measured as in [2,3]. (By applying the measuring beam only during measuring periods, a relatively high intensity could be used while still keeping its total actinic effect insignificant.) A small home-built computer (based on Heathkit ET 3401, with 4 kilobytes of RAM, and audio cassette mass storage), interfaced with the transient recorder (Biomation 802), was used for signal processing (storage, additions, subtractions).

Proton release inside the chloroplast thylakoids was measured at 553 nm as the difference between alternate measurements with and without 20 μM neutral red added to the standard reaction mixture. This method is based upon that in [5], i.e., added buffer of pH ~7.4 easily overrides buffering (and color change) by neutral red in the outside medium, but not inside the thylakoid where neutral red is bound and thereby accumulated.

Cytochrome *f* behavior was measured by its spectral changes [6] as the difference between alternate measurements with 553 nm and with 540 nm measuring light. Changes of the transmembrane electrical field were measured by its electrochromic effect [7] as the difference between alternate measurements with 515 nm and with 500 nm measuring light.

3. Results and discussion

3.1. *Proton release by PS 2*

Two processes contribute to the proton release

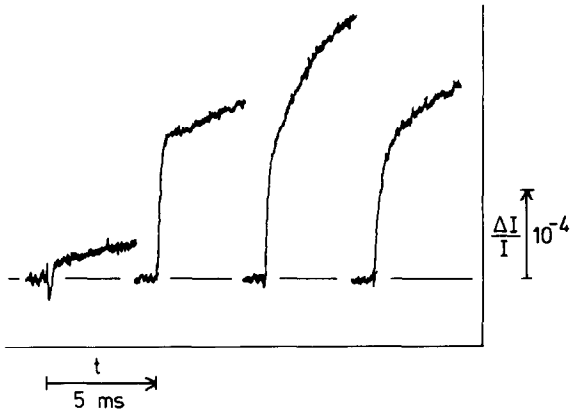

Fig.1. Proton release inside chloroplast thylakoids, recorded as absorbance increase of trapped neutral red, during the first 4 ms after each of 4 flashes spaced by 1.2 s intervals. Average difference of 64 pairs of measurements with and without 20 μM neutral red. Dark time before the measurements, 2–6 h. Electronic time response (10–90%), 100 μs. Measuring beam wavelength, 553 nm (10 nm half band width). Optical pathlength, 2 mm. Chlorophyll (chl) 100 μg/ml.

inside the thylakoid: water oxidation by PS 2, and electron transfer between PS 2 and PS 1 [4,8,9]. Because intersystem electron transfer is slow compared to the donor side reactions of PS 2, the contributions to H^+ release of PS 2 and of the intersystem chain can be distinguished kinetically [4]. The more rapid components of H^+ release are shown in fig.1. The four different curves represent the ms-range H^+ release induced by 4 consecutive flashes. The results show a large dependence on the flash number. As argued in [4,9,10], the probable cause of the flash-number dependency is that with consecutive photoreactions the donor side of PS 2 steps through a cycle of 4 states, called S_0, S_1, S_2, S_3 [11]; in a flash series after dark-adaptation this cycling occurs to a large extent synchronously, with ⩾75% of the centers starting in state S_1 [11]. The first flash induces very little H^+ release; a maximum is obtained with the third flash (and in a longer series, not shown, a second minimum is obtained with the fifth and a second maximum with the seventh flash). Assuming that the individual S-state transitions cause the release of whole numbers of protons, and recognizing that their sum for a full cycle must be 4, we derive from the results that during the transitions of $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_0$ protons are released according to a 1, 0, 1, 2 pattern. This conclusion supports [9,10] versus [4]. Some conclusions in [4], however, are upheld by fig.1. As in [4], ≤100 μs half-time is observed for H^+ release during the S_2–S_3 transition (second flash) and ~1 ms for the H^+ release during the S_3–S_0 transition (third flash, the fastest component in the clearly biphasic release should probably be ascribed to a minority of centers undergoing S_2–S_3).

3.2. *Total proton release inside*

Fig.2A represents an experiment similar to that of fig.1, but this time the measurements were extended over a time-span sufficiently long to observe not only the H^+ release by PS 2 but also most of the H^+ release associated with electron flow between PS 2 and PS 1. Since fig.1 showed that the PS 2 contribution to H^+ release is negligible with the first flash, curve no. 1 of fig.2A should represent exclusively intersystem H^+ release. To help recognize the separate contributions of PS 2 and of the intersystem chain to the H^+ release induced by flashes 2–4, curve 1 was subtracted from curves 2–4, resulting in curves 2′–4′ (fig.2B). These curves are conspicuously biphasic. The fast (few ms) phase should represent the H^+ release by PS 2; and their secondary phase, the intersystem H^+ release occurring in excess of that after the first flash. Fig.2B clearly reveals two features of the measurement:

(i) The intersystem H^+ release oscillates with a periodicity of two, confirming [8,9];

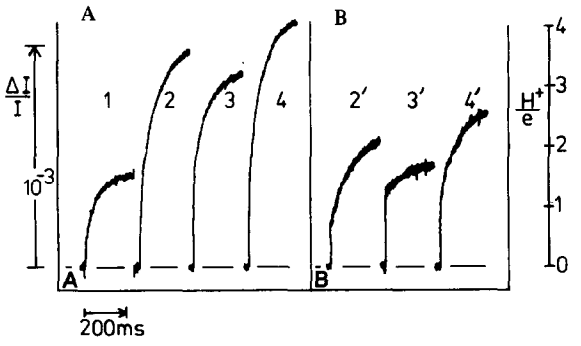

Fig.2. Proton release inside chloroplast thylakoids, recorded as absorbance increase of trapped neutral red, during the first 220 ms after each of 4 flashes spaced by 1.2 s intervals. Average difference of 32 pairs of measurements, with and without 20 μM neutral red. Dark time before the measurements, 1–7 h. Electronic time response, 1 ms. Measuring beam wavelength, 553 nm (3 mm half band width). Curves 2′–4′ represents the difference between curves 2–4 and curve 1. The assumptions underlying the (H^+/e) scale at the right ordinate are discussed in the text.

(ii) The number of protons released with intersystem flow (remember to add back curve 1 to each secondary phase in fig.2B) is appreciably larger than the number of protons released by PS 2.

The oscillation indicates that the extent of intersystem flow is limited by the supply of electrons (to the plastoquinone pool) by the secondary acceptor R of PS 2 [12,13]. Under such conditions the average relative amplitudes of the H^+ release by PS 2 and by intersystem flow should be 1:1 according to the 'classical' Z-scheme (e.g., [4,7]) but 1:2 according to the 'modified' Z-scheme (e.g., [1,3]). The results of fig.2 clearly favor the latter scheme. Based on the 1:2 stoichiometry, the tentative H^+/e scale for the right ordinate of fig.2 assumes a release of 1.5 H^+ by the first flash. The indicated average H^+ release by PS 2 should be ~1, and the given scale is reasonably satisfactory in this respect.

3.3. *Electron flow into cytochrome* f

Measurements of cytochrome *f* are shown in fig.3. The results further document the occurrence of an oscillation of intersystem electron flow under the conditions of dark adaptation and illumination used in this study. Superficially, at least, the correspondence with the H^+ release measurements is quite good. Like H^+ release, cytochrome *f* re-reduction appears to be twice as large after the second and the fourth flash than after the first and the third flash. On the other hand, the amplitude of the observed turnover of cytochrome *f* is small, an av. ~0.15 molecule . 400 chl^{-1} . $flash^{-1}$. The plausible interpretation is that cytochrome *f* rapidly equilibrates with plastocyanin, a component of larger abundance than cytochrome *f* and of similar midpoint potential (e.g., [14]) – even though this interpretation is not supported in detail by actual parallel measurements of plastocyanin and cytochrome *f* [15,16].

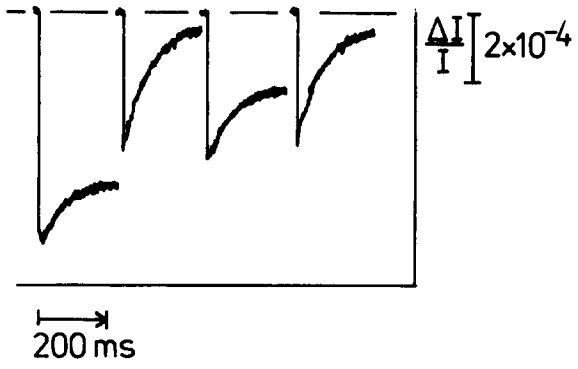

Fig.3. Oxidation (downward) and re-reduction of cytochrome *f* in chloroplasts during the first 220 ms after each of 4 flashes spaced by 1.2 s intervals. Average of 32 pairs of measurement (553 nm minus 540 nm). The transmission increase of 2×10^{-4} represents a reduction of 0.1 molecule cytochrome *f*/400 molecules chl (calculated as in [3]). Dark time before the measurements, 1–5 h. Addition to standard reaction mixture, 1 mM NaCl and 2 μM gramicidin D.

3.4. *Generation of transmembrane field*

Fig.4 shows the increase and the decay of the transmembrane electrical field under the conditions of fig.2. At first sight, it appears that all of the field increase occurs immediately with each flash and thus is completely due to the primary reactions of the photosystems [7]. According to the modified Z scheme, however, intersystem flow generates field also [1–3]. This field generation by secondary electron flow is easily recognized when it occurs rapidly [2,13], but when intersystem electron flow is slow, as under the conditions of fig.2–4, the (supposed) associated field generation is hard to detect because of the appreciable field decay that takes place concurrently.

The oscillation in intersystem flow demonstrated by fig.2,3 helps to make the secondary phase evident. Close examination of fig.4 shows that the decrease of the field after the primary rise is slower and more clearly sigmoidal after the second and the fourth flash than after the first and the third flash. The flash-number dependency is perceived better than

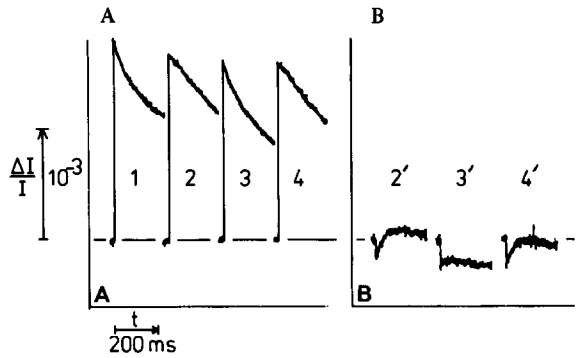

Fig.4. Transmembrane electrical field increase (upward) and decrease, recorded as electrochromic absorbance changes, during the first 220 ms after each of 4 flashes spaced by 1.2 s intervals. Average of 16 pairs of measurements (515 nm minus 500 nm). Dark time before the measurements, 4–5.5 h. Chlorophyll, 60 μg/ml. Curves 2′–4′ represent the difference between curves 2–4 and curve 1.

with the original curves after subtracting curve 1 from curves 2–4, which produces curves 2′–4′. Besides a decrease of the amplitude of the immediate field increase (which suggests an incomplete recovery, between flashes, of the photocenters of PS 2 or of PS 1), a slow phase is evident that is much larger in curves 2′ and 4′ than in curve 3′ of fig.4. This result agrees nicely with the measurements of H^+ release, i.e., the manifestation of a slow phase in curves 2′ and 4′ that is much larger than in curve 3′ of fig.2. Kinetically the correspondence is imperfect, but this is not surprising as the curve manipulation that leads to curves 2′ and 4′ of fig.4 only diminishes, but does not eliminate, the effect of field decay.

Acknowledgements

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