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A third site of proton translocation in green plant photosynthetic electron transport

(chemiosmotic hypothesis/electrogenic reaction/plastohydroquinone oxidation/photophosphorylation/tetraphenylboron)

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ABSTRACT **Prereduction of the intersystem pool in isolated spinach chloroplasts leads to the appearance of a slow phase in the field-indicating 515-nm change induced by a flash. Measurements with the pH-indicator dye cresol red show that a proton uptake is associated with this slow 515-nm change. When water is the electron donor to photosystem II, electron transfer to ferricyanide is associated with the uptake of more than one proton per electron. Tetraphenylboron upon oxidation by system II releases a proton directly into the medium; yet, flash-induced electron transport from tetraphenylboron to ferricyanide is accompanied by a net uptake of protons from the medium. The above four results demonstrate the existence of two proton translocation sites in the chain between the two photoacts; the first two observations locate the new site at the oxidizing side of the plastoquinone pool.**

Photosynthetic electron transport from water to $NADP^+$ is coupled to a release of protons on the inside and an uptake of protons on the outside of the thylakoids in the chloroplasts. As a consequence of this vectorial ion transport, the process is electrogenic—i.e., it generates an electrical field across the thylakoid membrane. The field induces absorption changes of pigments in the membrane, the largest of which peaks at about 515 nm (for reviews see refs. 1 and 2).

The proton translocation is commonly believed to occur as follows. The primary reactions of photosystems II and I each result in the transfer of an electron across the membrane. Electron donation by water to system II and by plastohydroquinone to system I occur on the inside and each release one proton. Uptake on the outside occurs by the binding of protons in the reduction of plastoquinone by system II and in the reduction of the terminal acceptor by system I. The "H^+/e" of 2 implied by this scheme was supported by pH measurements and was also consistent with observations of the 515-nm absorption change; excitation of systems I and II induced instantaneous absorption changes of equal size (1, 2).

Recently, however, Fowler and Kok (3) in this laboratory, reported H^+/e ratios higher than 2. In addition, under some conditions whole algae, illuminated by short flashes, revealed a slow phase in the 515-nm absorption increase (4, 5), which implied that the two photoacts are not the only electrogenic electron transfer reactions.

In this report I show that a similar slow phase in the generation of the electrical field can be observed with isolated chloroplasts and that it appears associated with electron flow through the chain that connects the photoacts of system II and system I. The evidence is supplemented by three types of pH measurements that similarly indicate the presence of an additional site of proton translocation, located at the oxidizing side of the plastoquinone pool.

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MATERIALS AND METHODS

Spinach chloroplasts were isolated according to ref. 6. The isolation medium contained 400 mM sucrose, 10 mM NaCl, and 50 mM Tris·HCl (pH 7.5). For the pH measurements the chloroplasts were washed with a lightly buffered medium that contained 400 mM sucrose, 2 mM KCl, 0.5 mM $MgCl_2$, and 1 mg of bovine serum albumin per ml (pH 7.6). The chloroplasts were stored concentrated (5 mg of chlorophyll per ml) at 0°C in the dark before the experiments were started.

Absorption changes were measured with a split-beam spectrophotometer. The measuring beam was turned on a short time (≥2 ms) before the actinic illumination with brief saturating flash(es). I used an EG&G FX101 Xe flash tube (1000 V, 4 μF) and red cutoff filters to provide the flashes. Flash-induced intensity changes of the light transmitted by the reaction cuvette (2-mm pathlength) were recorded with a Biomation 802 transient recorder. For pH measurements I used the indicator dye cresol red (200 μM), monitored at 573 nm, at a pH of 7.5–7.6. For each measurement, 3–5 μl of the concentrated chloroplast suspension was diluted in the dark with 0.25 ml of reaction mixture to a final chlorophyll concentration of 100 μg/ml (unless indicated otherwise). The standard reaction mixture contained 200 mM sucrose and either 50 μM ferricyanide or 100 μM methylviologen. If the chloroplasts were to be preilluminated, the concentrated suspension (which was contained in a transparent syringe before being mixed with the reaction medium) was held close to a 15-W light bulb for a few seconds. Reduction of the pool was checked by performing a fluorescence-induction measurement (7). I observed that 1 min after preillumination the pool was still 60% reduced (cf. refs. 7 and 8).

RESULTS

A Slow Phase in the Flash-Induced 515-nm Change in Isolated Chloroplasts. The slow 515-nm absorption increase in intact algae has been shown to be associated with electron donation to photosystem I. It occurs only under conditions in which a rapid (within 10 ms) rereduction of the system I donors takes place after the flash—i.e., after the first few flashes of a sequence given after a dark period. It is not observed after steady-state flashes, in which case the electron donors remain mostly oxidized (5).

This suggests that the secondary 515-nm absorption increase (in the 1- to 50-ms time range) occurs only when an appreciable fraction of the plastoquinone pool between the photosystems is reduced at the time of the flash. Therefore, I searched for a slow phase in the flash-induced 515-nm absorption change of isolated chloroplasts under conditions in which the pool was reduced. To obtain this reduction, I preilluminated the chloroplasts in the absence of an electron acceptor.

Abbreviation: Ph_4B, tetraphenylboron.

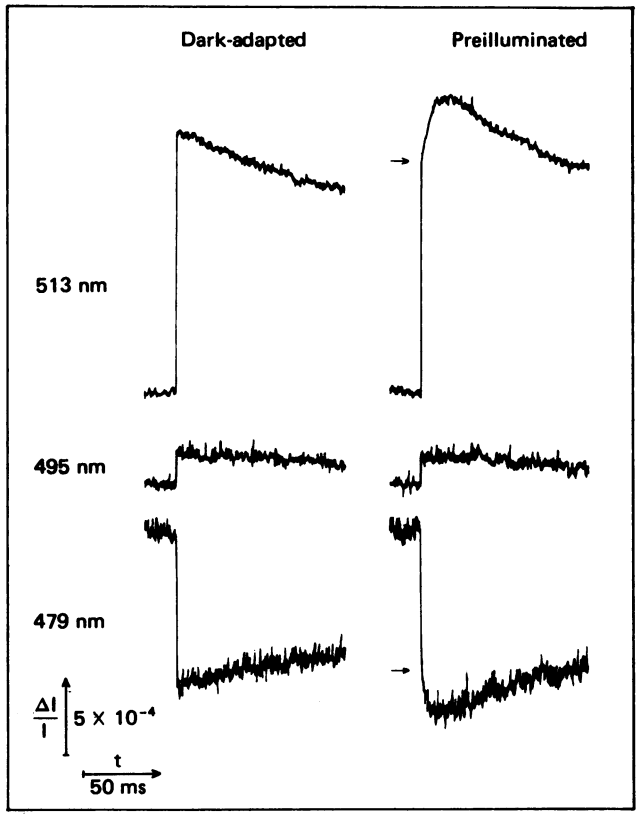

FIG. 1. Time course of the flash-induced absorption changes at 513 nm, 495 nm, and 479 nm, observed with isolated chloroplasts that were either dark-adapted (left-hand side) or preilluminated for 5 s with white light 30 s before the measurement (right-hand side). Photosystem I electron acceptor (100 μM methylviologen) was added 15 s before the flash. Chlorophyll concentration, 80 μg/ml.

Fig. 1 shows observations of the flash-induced absorption increase at 513 nm and the decrease at 479 nm, which are both largely due to generation of field. At 495 nm the electrochromic effects are minimal. The left-hand side of Fig. 1 shows the "control" experiment in which fully dark-adapted chloroplasts were used. The right-hand side shows the results of an experiment that was carried out with chloroplasts that were preilluminated. Clearly, after rapid changes similar to those seen in the control, the changes at 513 and 479 nm in this case each contained a slow phase. These slow phases could be seen after several successive flashes.

Similar results were obtained when either methylviologen or 50 μM ferricyanide was used as the electron acceptor of system I. The addition of the system II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron) between the preillumination and flash did not inhibit the slow 515-nm absorption increase.

Fig. 2 shows the spectrum of the slow phase observed with preilluminated chloroplasts. Plotted as a function of wavelength is the change of transmission between 2 ms and 30 ms after the flash. For each point I subtracted the decay that occurred during the same period in a parallel experiment with dark-adapted chloroplasts. The spectrum closely matches that of previously measured "immediate" electrochromic effects in chloroplasts (the rapid rise in Fig. 1; see ref. 9). In addition, there is an increase in absorption around 554 nm that reflects the reduction of cytochrome *f*. I found (not shown) that only part of the cytochrome *f* was oxidized by the flash; most of this was rereduced within 10 ms, as has been observed in (dark-adapted) whole algae (5). The behavior of other cytochromes under these conditions is unclear. The flash after preillumination causes

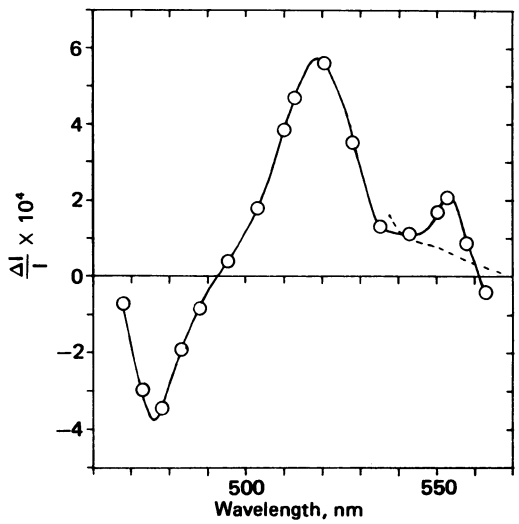

FIG. 2. Spectrum of the "extra" absorption change between 2 ms and 30 ms after the flash. Preilluminated chloroplasts under conditions as used for Fig. 1. The absorption change between 2 ms and 30 ms observed with dark-adapted chloroplasts has been subtracted. Measuring light half-bandwidth, 2.5 nm. Around 550 nm the shape of a pure (gramicidin-sensitive) electrochromic spectrum is indicated by a broken line. Chlorophyll concentration, 80 μg/ml.

partial reduction of cytochrome *b*-563; its reoxidation, however, seems slower than the secondary 515-nm absorption increase.

In whole algae the extent of the slow 515-nm change can equal the size of the instantaneous field increase due to photosystem I (4). Quantitative evaluation of the results of Fig. 1 is complicated by the uncertain time-course of decay in the preillumination experiment and by the unknown percentage of double hits in system I [which can be considerable (10), so that appreciably more than half of the fast phase might be due to system I].

Estimating that the amplitude of the slow phase at 513 nm in Fig. 1 amounts to 1/3 to 1/2 of the gramicidin-sensitive (9) fraction (85–90%) of the rapid phase, I compute that at least half, and possibly all, of the electrons passing through system I contribute to the slow change. The highest estimate assumes negligible double hitting of system I in my experiments and seems therefore rather unlikely.

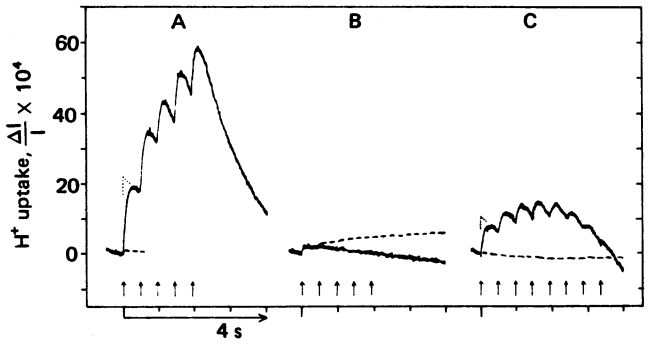

FIG. 3. Flash-induced proton uptake, measured with cresol red at 573 nm, by dark-adapted chloroplasts in the presence of (*A*) 50 μM ferricyanide or (*B*) ferricyanide plus 20 μM diuron and (*C*) by chloroplasts preilluminated as in Fig. 1 before addition of ferricyanide and diuron. Each upward arrow indicates the firing of a flash. The broken lines represent "background" (non-pH) absorption changes that were measured by performing the experiments in the presence of 5 mM Hepes, pH 7.6.

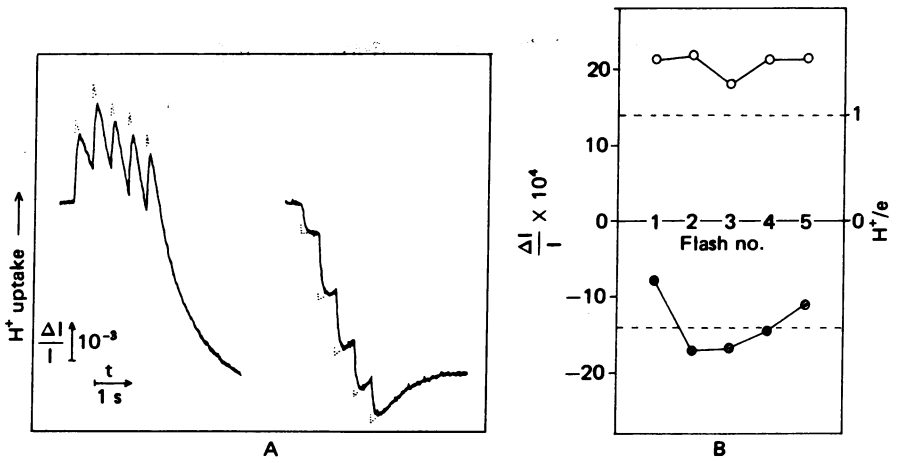

FIG. 4. (*A*) Proton uptake (left-hand trace). Net evolution in the presence of 4 μM gramicidin D (right-hand trace). Dark-adapted chloroplasts illuminated by a series of five flashes in the presence of 50 μM ferricyanide. The dotted lines indicate the manner in which the flash yields were extrapolated. (*B*) Plot of the extrapolated uptake yields (○) and net evolution yields (●) obtained from Fig. 4*A*. For evolution, the average yield of the first four flashes is indicated by a broken line. This amplitude should equal 1 H^+ per system II reaction center and is used to calibrate the uptake yield (right-hand ordinate).

Proton Uptake Associated with Oxidation of the Quinone Pool by System I. The slow 515-nm phase observed in the previous section suggests that the electron transport chain between the two photosystems contains a site of proton translocation at the oxidizing side of the plastoquinone pool. To test this hypothesis, I measured proton uptake by chloroplasts that were suspended in a medium buffered by the (nonpenetrating) dye cresol red (11, 12). Ferricyanide was used as the electron acceptor for system I. Fig. 3*A* shows the proton uptake induced by a series of five flashes in a control (dark-adapted) sample. Because ferricyanide is not protonated upon reduction, only the electron flow through the intersystem chain is associated with proton uptake (3, 11, 12). The experiment of Fig. 3*B* was performed in the presence of diuron. Reduction of the pool is completely inhibited and so is proton uptake. The very slow proton release is probably due to the residual evolution by system II of a few protons on the inside (less than one per center). In the experiment of Fig. 3*C*, the chloroplast sample was preilluminated prior to dilution with the diuron-containing reaction medium. Due to the preillumination, the flashes again induced proton uptake. This uptake cannot be associated with the reduction of plastoquinone by system II. Rather, it must be associated with oxidation of the quinone pool by system I.

The proton uptake is induced by the first five or six flashes in the sequence, which presumably oxidize the reducing equivalents that were brought into the pool by the preillumination. The extent of the uptake by the first flash is about half of that seen in the control with dark-adapted chloroplasts in the absence of diuron (Fig. 3*A*). Assuming that in the control the flashes translocate 1.5 H^+ per chain (see the next section), the H^+ uptake induced by the first flash in the experiment of Fig. 3*C* amounts to about 0.7 H^+ per chain. This number is in reasonable agreement with the amplitude of the slow 515-nm change estimated in the previous section.

The H^+ to e Ratio in the Presence of Ferricyanide. To estimate the H^+ uptake in terms of protons per electron, I measured the H^+ exchange associated with the electron transport from water to ferricyanide (3, 12). In the absence of gramicidin D each flash induced a rapid uptake which was followed by a slow efflux (leakage) of the protons that were released internally (left-hand pattern in Fig. 4*A*). In the presence of the uncoupler the efflux became rapid so that one only views the net result of the proton uptake on the outside and the proton release on the inside (right-hand pattern). Because protons are released by the electron donor (water) but not bound by the acceptor (ferricyanide), the net result should, on the average, be the release of one proton per electron.

To compute the flash yields of proton uptake and release, I extrapolated the traces to zero time (the time of the flash) as shown by the dotted traces in Fig. 4*A*. The corrected yields are plotted in Fig. 4*B*. Assuming that the average net H^+ yield of the first four flashes was 1 H^+ per electron (dashed line), I compute that the average uptake from the outside was about 1.5 H^+ per electron—i.e., definitely larger than 1 H^+ per electron.

This result indicates that, even when the plastoquinone pool is largely oxidized, electron transfer through the intersystem

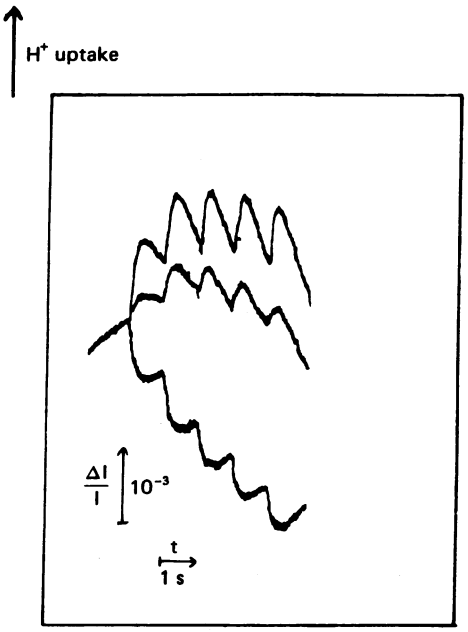

FIG. 5. Proton uptake and evolution by dark-adapted chloroplasts in the presence of sodium Ph_4B. The upper trace represents the uptake in a "control" experiment in the presence of 50 μM ferricyanide. The middle trace was obtained when 40 μM Ph_4B was also present, and the lowest trace, when both Ph_4B and 1 μM gramicidin D were added before the illumination. Chlorophyll concentration, 40 μg/ml.

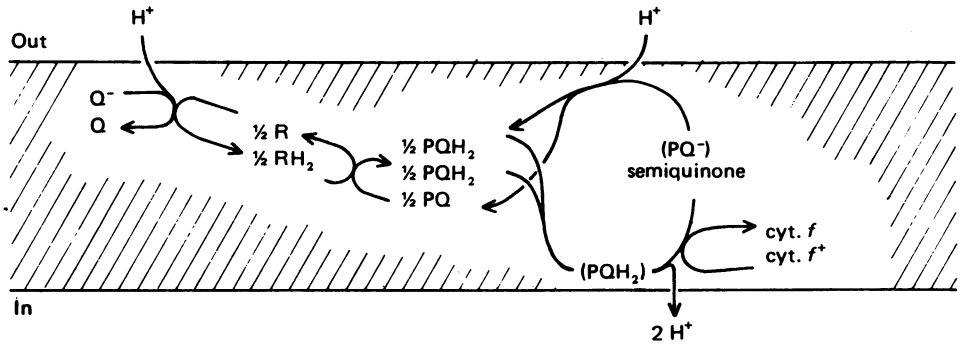

FIG. 6. Simplified scheme to explain the translocation of two protons per electron passing through the intersystem chain. The oxidation of plastohydroquinone (PQH_2) by cytochrome *f* generates the semiquinone anion (as an intermediate product) that crosses the membrane before it can meet with other semiquinones (formed in neighboring chains) and dismutate. Q and R are the primary and secondary acceptor of system II. Cytochrome *f* is a secondary donor of system I. The two proton translocations shown in the scheme are in addition to the release (inside) associated with O_2 evolution and the uptake (outside) associated with substrate reduction.

chain is associated with proton uptake at more than one site, a conclusion for which as yet no supportive evidence from 515-nm measurements exists (see *Discussion*).

H^+ Release in the Photooxidation of Tetraphenylboron Exceeded by H^+ Uptake by the Chain. Tetraphenylboron (Ph_4B) is a very efficient electron donor to system II; it acts in micromolar concentrations, overriding H_2O oxidation completely (13, 14). Fig. 5 shows three measurements of flash-driven proton exchange in dark-adapted chloroplast suspensions containing ferricyanide and cresol red. In one of the experiments, I used a saturating concentration (40 μM) of Ph_4B and added gramicidin D to observe the net H^+ exchange. Interestingly, I found an evolution that was equal to the average release seen in the absence of Ph_4B (see Fig. 4*A*, pattern b). I conclude that, as in water oxidation, one proton is released per electron extracted from Ph_4B (the oxidation product of which is unknown).

In a separate experiment (not shown) it was found that, in the presence of diuron (which inhibits proton uptake, see Fig. 3*B*), the release of a proton into the medium due to the photooxidation of Ph_4B was as rapid in the absence as in the presence of gramicidin D. It thus seems that Ph_4B, in contrast to water, is oxidized on the outside of the thylakoid.

Finally, Fig. 5 shows that, in the absence of diuron and gramicidin D, the oxidation of Ph_4B is still accompanied by an uptake of protons (≈one-third as large as the uptake seen in the control experiment with water as the donor). This uptake occurred despite a concomitant rapid release of 1 H^+/e, which implies that the uptake by the intersystem chain exceeds 1 H^+/e.

DISCUSSION

I have presented four types of experimental evidence for an extra site of proton translocation in the chain connecting the two photosystems, specifically in the oxidation of plastoquinone by system I.

Fig. 6 shows a relatively simple scheme to visualize this extra proton translocation. It involves only compounds that are generally assumed to be engaged in linear electron transport. The oxidation of plastohydroquinone by cytochrome *f* leads to full deprotonation—i.e., formation of the semiquinone anion, with release of both protons on the inside of the thylakoid. The semiquinone anion diffuses towards the outer side of the membrane (the electrogenic step in the process) and reacts with another semiquinone formed by a neighboring center. The dismutation yields a quinone and (half the original amount of) hydroquinone while protons are taken up from the medium. A similar hypothesis has been put forward earlier in relation to ubiquinone reactions in mitochondria (15).

An extensive reduction of the pool was not required for the proton translocation by the proposed site (Figs. 4 and 5) but was apparently essential for the observation of the secondary absorption increase at 515 nm. The scheme of Fig. 6 cannot accommodate this discrepancy. Possibly, under oxidizing conditions the secondary electrogenic reaction is compensated by the coupled transport of a counter ion, or, more likely, is severely retarded so that its effect becomes masked by the simultaneous decay of the field.

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