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# Complete tracking of transient proton flow through active chloroplast ATP synthase

**(photosynthesis/ATPase/phosphorylation/chemiosmotic mechanism)**

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ABSTRACT **Proton pumping in thylakoid membranes and backflow of protons through the active ATP synthase CF0–CF1 (where CF0 is the proton channel and CF1 is the catalytic portion) were investigated by flash spectrophotometry. A steady pH difference across the membrane was generated by continuous measuring light, supplemented by voltage transients that were generated by flashing light. In the presence of $P_i$ and ADP, the electric potential transients elicited transients of proton flow via CF0–CF1, typically 1.3 $H^+$ per CF1 and flash group. Proton flow was blocked by CF0–CF1 inhibitors: *N,N'*-dicyclohexylcarbodiimide, acting on the channel component CF0, and tentoxin, acting on the catalytic component CF1. The half-rise time was 40 ms in $^1H_2O$ and 78 ms in $^2H_2O$. ATP synthesis under conditions of flashing light and transient proton flow was characterized by a $K_m(P_i)$ of only 14 $\mu$M, contrasting with a $K_m$ of several hundred micromolar for continuous ATP synthesis at high rate. This might reflect a resistance to $P_i$ diffusion. The degree of proton delocalization in the chemiosmotic coupling between redox reactions and ATP synthesis is under debate. In thylakoids, it has been proposed that intramembrane proton buffering domains act as ducts for protons between pumps and ATP synthases. In this work, transient proton flow by way of CF0–CF1 was completely tracked from the lumen, across the membrane, and into the suspending medium. Proton uptake from the lumen and charge flow across the membrane occurred synchronously and in stoichiometric proportion. The uptake of protons from the lumen by CF0–CF1, half completed in 40 ms, was preceded by release of protons from water oxidation into the lumen, half completed in <1 ms. Hence, pumps and ATP synthases were coupled through the lumen without involvement of intramembrane domains.**

The chemiosmotic theory of oxidative and of photophosphorylation places proton pumps and proton translocating ATP synthases in a membrane and couples them by lateral proton flow through two aqueous bulk phases at different electrochemical potentials of the proton (1, 2). Though supported by a vast body of evidence, the delocalized coupling concept has been challenged from its origin (3). In one class of alternative models, proton pumps and ATP synthases are thought to be coupled in pairs rather than sharing common bulk phases (localized coupling or microchemiosmotic concepts, e.g., ref. 4). In another class, proton flow is thought to be contained in the membrane (e.g., ref. 5) or confined to the membrane/water interface (e.g., refs. 6 and 7), and the protons in these localized domains are supposed not to be equilibrated with adjacent aqueous phases. The bulk of the information interpreted to favor localized coupling concepts was obtained using mitochondria. It resulted from an evaluation of the energetics of phosphorylation or from a comparison of the rates of stationary electron flow and of ATP synthesis in the presence of inhibitors to the former and the latter (reviewed in refs. 4 and 8). Unambiguous conclusions were not reached, as clearly stated in articles from laboratories that had supported localized concepts (9, 10). In none of these studies was proton flow by way of the ATP synthase kinetically and spatially resolved. In this investigation, carried out on thylakoids, proton flow was completely tracked with the aim of finding out whether or not ATP synthesis required protons to pass through localized domains, avoiding the bulk phases. Thylakoids are of particular interest in this context, as the existence of proton-buffering domains in the membrane seems established (5, 11, 12). It has been proposed that these domains can act as proton ducts between pumps and ATP synthases (5).

When thylakoids are exposed to repetitive flashes plus continuous background light, they can synthesize ATP as a consequence of proton translocation. This occurs, if the flash-induced transmembrane voltage exceeds the threshold level for activation of the ATP synthase (13–15). Charge flow linked to ATP synthesis has been detected spectrophotometrically through electrochromic absorption changes of intrinsic chloroplast pigments (13). In this study, in addition to charge flow, pH transients were recorded in the lumen of thylakoids and in the suspending medium. In this way, transient proton flow through the active ATP synthase was completely tracked as proton uptake from the lumen, as charge flow across the membrane, and as proton release into the medium.

## MATERIALS AND METHODS

**Chloroplasts.** Broken pea chloroplasts were prepared according to the procedure for "stacked thylakoids" in ref. 16, except that buffers were omitted in the final suspending medium. All experiments were carried out at room temperature. Under continuous illumination, and with pyocyanin as cofactor, these chloroplasts synthesized ATP at rates of $\approx$1000 $\mu$mol of ATP per mg of chlorophyll per hr.

**Flash Photometric Experiments.** In the flash photometric experiments a thylakoid suspension was excited with groups of three flashes from three xenon tubes placed around the optical cell. The measuring light intensity was usually 120 $\mu W/cm^2$, and it could be varied between 100 and 200 $\mu W/cm^2$ without changing the results. The excitation wavelength was >660 nm, and the flash energy was saturating. Transient absorption changes were measured by a standard procedure (e.g., ref. 16) refined as follows: (*i*) automatic sampling and averaging the transmitted intensity before the flash (accuracy >0.1%) (17) and (*ii*) control of rarely occurring flash failure. This was indispensable when aiming at high resolution of small transient absorption changes (see below). A sample was exposed to the measuring light for at least

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Abbreviations: CF1 and CF0, catalytic portion and proton channel of the chloroplast ATP synthase; DCCD, *N,N'*-dicyclohexylcarbodiimide.

90 s and to 10 flash groups before averaging over the response to typically 20 flash groups at 0.2 Hz.

**Thylakoid Suspension.** The thylakoid suspension was contained in an optical cell with a 2-cm path length. In addition to 10 $\mu$M chlorophyll, the medium contained 3 mM $MgCl_2$ and 10 $\mu$M methyl viologen. The pH was adjusted to 8.3 by NaOH or HCl, and it was stable to within 0.1 unit during sampling. In some experiments (see Fig. 1), superoxide dismutase at 6 units/ml (Sigma) was added to accelerate proton uptake from the medium by photosystem I. For concentrations of added ADP and $P_i$, see figures. In some experiments 7.5 $\mu$M *N,N'*-dicyclohexylcarbodiimide (DCCD) was added to the suspension, and sampling was started after a 10-min incubation; in others 50 $\mu$M tentoxin was added. These agents block the proton channel (CF0) (e.g., ref. 18) and the catalytic component (CF1) (19) of the ATP synthase, respectively.

**pH Transients.** pH transients in the lumen and in the suspending medium were obtained as differences between transient absorption changes measured with and without an appropriate pH-indicating dye. The indicator of flash-induced pH transients in the lumen (wavelength, 548 nm) was 13 $\mu$M neutral red with bovine serum albumin at 2.6 mg/ml added to buffer the suspending medium. Its exclusive and quantitative response to transients of the lumenal surface pH has been established elsewhere (20–22). Although the pH was 8.3 in the medium, neutral red with its apparent pK ranging between 6.7 and 7.8 [depending on the lumenal surface potential (21)] was responsive. This was attributable to a more acid pH in the lumen, maintained by the measuring light. pH transients in the medium were measured by using 13 $\mu$M *m*-cresol purple (pK 8.4; wavelength, 575 nm). Transients of the transmembrane voltage were measured by way of intrinsic electrochromic pigments (wavelength, 524 nm) (23–25).

**ATP Synthesis.** ATP synthesis caused by excitation with repetitive flashes (5 Hz; wavelength, >630 nm) was measured by luminescence of luciferin. The optical cell contained 10 ml of a thylakoid suspension with 10 $\mu$M chlorophyll/3 mM $MgCl_2$/10 $\mu$M methyl viologen/10 mM tricine·KOH, pH 8/100 $\mu$M ADP/100 $\mu$M $P_i$ as indicated in Fig. 4. ADP was purified by ion-exchange chromatography as in ref. 26. The content of one vial of ATP monitoring reagent (LKB) was dissolved in 1.5 ml of distilled water, and 150 $\mu$l was added to the thylakoid suspension. Luminescence was calibrated by adding aliquots of ATP standard (LKB).

## RESULTS

**Complete Tracking of CF0–CF1-Mediated Proton Flow.** Fig. 1 shows the time course of flash-induced absorption changes of *m*-cresol purple (*A*), of intrinsic electrochromic pigments (*B*), and of neutral red (*C*), in the presence of $P_i$ and of ADP. As illustrated in the schematic drawings (Fig. 1 *Right*), the traces indicate pH transients in the medium (*A*), transients of the transmembrane voltage (*B*), and pH transients in the lumen (*C*). Fig. 1*C* shows a rapid acidification of the lumen during the first group of three flashes (for higher time resolution, see Fig. 2). This was attributable to the rapid deposition of three protons per photosystem II by water oxidation. It was followed by a slower acidification of smaller extent, visible only in the sample preincubated with DCCD. This was attributable to the oxidation of plastoquinol, caused by about one turnover of photosystem I (25). Similar behavior was observed during the second group of flashes. The slow relaxation of the pH transient (half-decay, 5–10 s, out of recorded time range) reflected the low proton conductance of the thylakoid membrane. In the absence of DCCD (i.e., with the proton channel unmodified) the acidification was diminished. This might indicate either less proton deposition or, alternatively, partial compensation of proton deposition by proton efflux. The latter view was supported by traces in Fig. 1 *A* and *B*. Traces in Fig. 1*B* show the transmembrane voltage. The decay was more rapid in the absence of the DCCD block to CF0. It is noteworthy that traces obtained in the absence of $P_i$ and of ADP (without incubation with DCCD) were similar to those shown in Fig. 1 but with the DCCD block to CF0. For the pH transients in the lumen, this is shown in Fig. 2 (compare traces in *C*, *D*, and *A*). For electrochromism, refer to figure 1 in ref. 13. Traces in Fig. 1 *B* and *C* suggested that ADP and $P_i$ activated transient efflux of protons from the lumen and across the membrane. This flow was also apparent as diminished alkalinization of the medium (Fig. 1*A*). The accelerated decay of the transmembrane voltage in the presence of ADP and $P_i$ was in agreement with earlier observations of extra charge flow stoichiometrically coupled to ATP synthesis (3 equivalents per mol of ATP) and driven by flash-induced voltage transients (13, 14). Traces in Fig. 1*B* reproduced the observation (13) that the extra proton conductivity of the membrane ceased, once the voltage had dropped below a threshold level.

Because of the buffering capacity of ADP and $P_i$, measurements of pH transients in the suspending medium were only

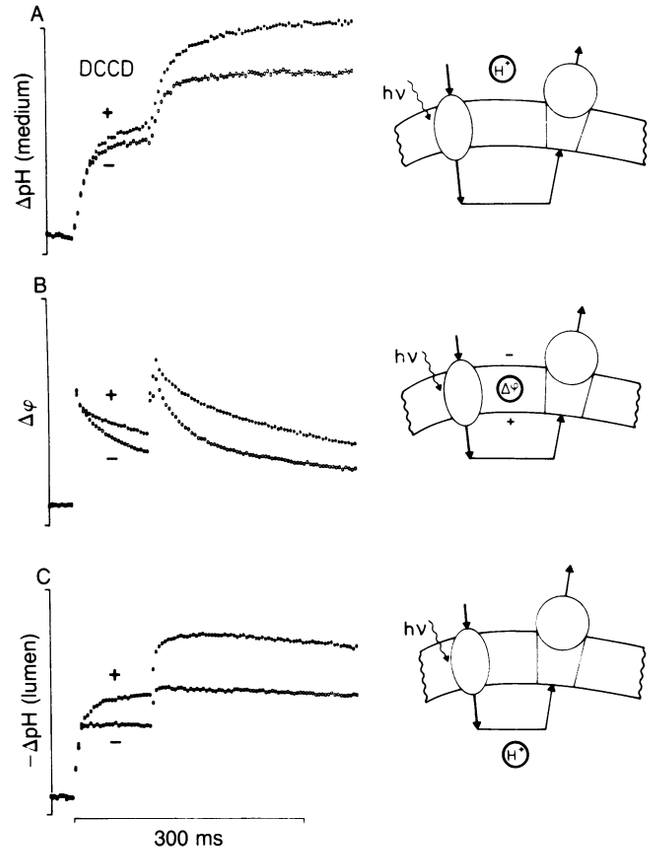


FIG. 1. (*Right*) Illustration of the thylakoid membrane with light-driven proton pump, ATP synthase, and proton flow (arrows). Encircled is the measured pH or voltage described by the traces on the right: namely, pH transients in the medium (*A*), voltage transients across the membrane (*B*), and pH transients in the lumen (*C*). (*Left*) Transient changes of these values under excitation with two groups of three flashes in the presence of 20 $\mu$M ADP and 60 $\mu$M $P_i$. Larger transient changes were observed after a 10-min incubation with 7.5 $\mu$M DCCD, modifier of F0-type proton channels (18). The thylakoid suspension was excited with two groups of three flashes each, with 3 ms between consecutive flashes in a group, 100 ms between two successive groups, and a 5-sec repetition period. In these samples one group of three flashes turned over photosystem II thrice, whereas photosystem I, due to its slower relaxation, was turned over approximately once. This was checked via the absorption changes of chlorophyll P700. Full-scale in *A* is $1.6 \times 10^{-3}$, in *B* is $7 \times 10^{-3}$, and in *C* is $1.5 \times 10^{-3}$, the relative change of transmitted intensity.

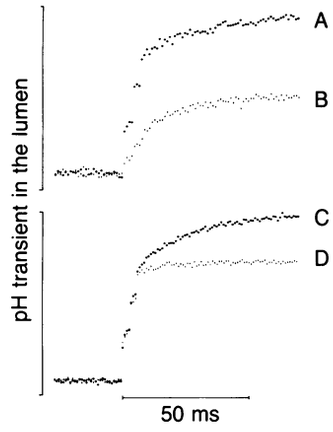


FIG. 2. pH transient in the lumen. (*A*) With 45 $\mu$M ADP and 15 $\mu$M $P_i$ but after preincubation with 7.5 $\mu$M DCCD. (*B*) With 0.3 nM gramicidin. (*C*) Without ADP and $P_i$. (*D*) With 15 $\mu$M $P_i$ and 45 $\mu$M ADP. Full-scale for the pH transients is $7.5 \times 10^{-4}$.

feasible because of (*i*) the unexpectedly low $K_m$ for $P_i$, which allowed us to use low concentrations, and (*ii*) by choice of pH 8.3 for the medium, more than one unit above the pK of $P_i$. The total buffering capacity of the thylakoid suspension, with 10 $\mu$M chlorophyll/20 $\mu$M ADP/60 $\mu$M $P_i$, was <30 $\mu$M protons per pH unit. Traces representing transients of the medium pH were more often distorted by drift than those of voltage and lumenal pH.

**Transient Proton Flow Required ADP and $P_i$ and Could Be Blocked Either at CF0 or at CF1.** Fig. 2 shows pH transients in the lumen, similar to traces in Fig. 1*C*, but at higher time resolution. After excitation with three flashes, three steps of rapid proton deposition by water oxidation were discernible. In the absence of $P_i$ and ADP (traces in Fig. 1*C*) or with these agents but with CF0 blocked by DCCD (traces in Fig. 1*A*), a slower phase of proton deposition was obvious, which was attributable to plastoquinol oxidation. In the presence of $P_i$ and ADP, this was virtually eliminated since it was compensated by proton uptake from the lumen. The $IC_{50}$ of the DCCD block to this extra proton uptake was about 4.5 $\mu$M. The extent of the flash-induced electron transport and of proton pumping was not influenced by DCCD, unless the concentration was raised to 20 $\mu$M [at 10 $\mu$M chlorophyll]. It was safe to assume that preincubation with low concentration of DCCD, say at 7.5 $\mu$M, acted specifically on CF0 (see ref. 18 for review).

It was logical to expect that transient proton flow would also be blocked by an antagonist of CF1, tentoxin. This fungal tetrapeptide inhibits the ATPase activity of soluble CF1 as well as photophosphorylation with $IC_{50}$ ranging from nM to $\mu$M, depending on the plant species. For CF1 from pea we determined that $IC_{50}$ was equal to 250 nM (unpublished result). Fig. 3 shows transients of the lumenal pH (*B*) and of the transmembrane voltage (*A*). They are similar to those in Fig. 1, but the ADP-, $P_i$-induced extra flow of protons was blocked by tentoxin. It is obvious that transient proton flow through CF0–CF1 could also be blocked at the catalytic component of the enzyme CF1.

**The Extent of Transient Proton Flow.** Fig. 3*C* shows the difference between each pair of traces on the left, superimposed on each other. Before superposition, the ordinate scales were normalized for stoichiometric correspondence between protons leaving the lumen (Fig. 3*B*) and charges passing through the membrane (Fig. 3*A*). The rationale for normalization was the following. Under conditions of only linear electron flow and under excitation with one single-turnover flash of light, each photosystem translocates one electron across the thylakoid membrane: i.e., two electrons are translocated per electron transport chain cycle. Under the same conditions, two protons per chain cycle are deposited in the lumen, one rapidly, attributable to water oxidation, and the other one more slowly, resulting from plastoquinol oxidation (reviewed in ref. 25). When the suspension was excited with a group of three flashes spaced 3 ms apart, photosystem II was turned over thrice, whereas photosystem I was turned over only a little more than once, predominantly after the first flash. The latter was confirmed by way of the absorption changes of chlorophyll P700 (data not shown). Accordingly the jump of the electrochromic absorption changes on the first flash was twice as big as on the second and third flashes (see Fig. 3*A*). Likewise, there were three rapid steps of proton deposition by water oxidation and only one slower step by plastoquinol oxidation (Fig. 3*B*, +tentoxin). Hence for stoichiometric adjustment of the ordinate scales, the rapid jump of the voltage upon the first flash (Fig. 3*A*) was set twice as big as the rapid pH jump in the lumen (Fig. 3*B*). This implied relative downscaling of the voltage transient in Fig. 3*A* by a factor of 1.5. According to this argument the ordinate scale in Fig. 3*C* represented the translocation of 1 $H^+$ per electron transport chain cycle. One of the two superimposed traces in Fig. 3*C* represented the number of protons that had left the lumen. The other represented the number of charges that had crossed the membrane. They were identical within noise limits: i.e., any proton leaving the lumen was immediately apparent as a charge crossing the membrane.

The extent of CF0–CF1-mediated proton translocation in Fig. 3*C* was 0.8 $H^+$ per linear electron transport chain cycle.

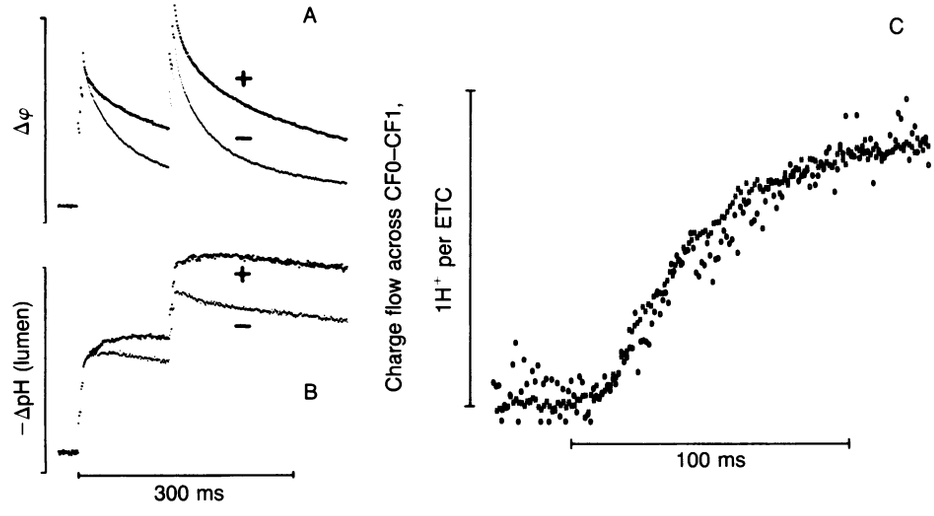


FIG. 3. Transients of the transmembrane voltage $\Delta\varphi$ (*A*) and of the lumenal pH (*B*) in the presence of 20 $\mu$M ADP and 60 $\mu$M $P_i$ and with (+) and without (−) tentoxin. Full-scale in *A* for $\Delta\varphi$ was $5 \times 10^{-3}$ and in *B* for $-\Delta$pH was $7.3 \times 10^{-3}$. The difference between each pair of traces in *A* and *B* was plotted in *C* after renormalization (see text). Traces in *C* give the number of protons that left the lumen as measured with neutral red (circles) and that crossed the membrane as measured with electrochromism (stars). Full-scale in *C* was 1 $H^+$ per electron transport chain cycle (ETC).

According to the above rationale for calibration, Fig. 3*B* revealed an initial deposition of 4 $H^+$ per linear electron transport chain cycle. After the voltage had dropped below threshold (see Fig. 3*A*), the remaining 3.2 protons left the lumen only at a very slow rate (via leak conductance, which was insensitive to DCCD, data not shown). How many protons were translocated per CF0–CF1? With the typical figures of 600 chlorophyll molecules per electron transport chain and 1000 chlorophyll molecules per CF0–CF1, 1.3 $H^+$ were transported per CF0–CF1. Assuming a $H^+$/ATP stoichiometry of 3, it implied the synthesis of 0.4 mol of ATP per mol of CF1 per flash group.

**Rate Limiting Factors of Transient Proton Flow via CF0–CF1 and of ATP Synthesis.** The extent of flash-induced transient proton flow via CF0–CF1 depends on competition between this channel and the electric leak conductance of thylakoid membranes (13, 14). Which factors limited the rate of proton flow via CF0–CF1? The extent of transient proton flow as a function of the concentration of ADP and $P_i$ was measured (data not shown). Apparent $K_m$(ADP) of 3 $\mu$M and $K_m(P_i)$ of 14 $\mu$M were determined. In these thylakoid preparations photophosphorylation under continuous light was governed by an apparent $K_m(P_i)$ of 240 $\mu$M (rate = 1000 $\mu$mol of ATP per mg of chlorophyll per hr). Even higher figures have been reported in the literature, $K_m$ of 450 $\mu$M (27).

Measurements of photophosphorylation under a flashing light confirmed the low $K_m(P_i)$. Unfortunately, the measuring light of the spectrophotometric experiments prevented measurements of ATP synthesis via luciferin/luciferase assay under otherwise identical conditions. Therefore, the measuring light was switched off, and the flash frequency was increased. Fig. 4 shows luciferin luminescence (negatively directed) during illumination of thylakoids with a series of flashes with a 200-ms repetition period and during the 40-s interval marked by the time bar. Three traces were plotted, at phosphate concentrations of 20, 80, and 200 $\mu$M. After an induction period, the steady rates of ATP production at 80 and at 200 $\mu$M $P_i$ were approximately equal, while the rate was 57% of maximum at 20 $\mu$M $P_i$. A systematic investigation of the apparent $K_m$ as a function of flash frequency will be presented elsewhere. How much ATP was formed per exciting flash? At saturating concentration of substrate the rate of ATP synthesis was 150 nM in 10 s (see Fig. 4) or 3 nM per flash. With the chlorophyll concentration at 10 $\mu$M and with the molar ratio of CF1/chlorophyll at 1:1000, this was

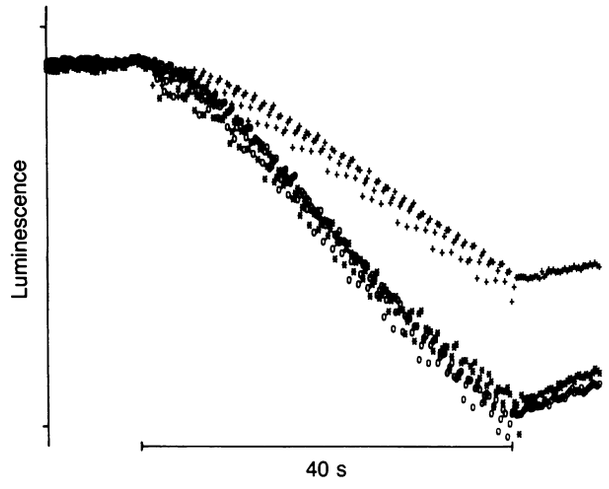


FIG. 4. Luciferin luminescence (negatively directed) under excitation of thylakoids with a series of flashes at a 200-ms repetition period. ADP (100 $\mu$M) was present, and the concentration of $P_i$ was 20 (+), 80 (○), or 200 (*) $\mu$M. Full-scale for luminescence represents 500 nM ATP.

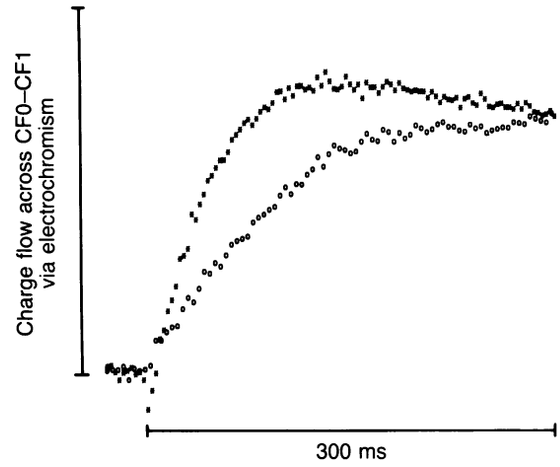


FIG. 5. Transient charge flow across the thylakoid membrane obtained as difference between two voltage transients with and without 30 $\mu$M ADP and 90 $\mu$M $P_i$. One trace was made using $^1H_2O$ (*), and the other trace was made using $^2H_2O$ (○) in the suspending medium, with equal activity of protons and deuterons, i.e., pH-meter reading of 8.3 with $^1H_2O$ and of 7.9 with $^2H_2O$. Full-scale for the charge flow was per 1 electron transport chain cycle.

equivalent to 0.3 mol of ATP per mol of CF1 per flash, close to the amount of ATP calculated from transient proton flow, 0.4 mol of ATP per mol of CF1 (see above).

What limited the rate of ATP synthesis at saturating substrate concentration? Fig. 5 shows the transient charge flow across the membrane at a saturating concentration of ADP and $P_i$, when deuterium was substituted for hydrogen in the suspending medium. The traces are differences between electrochromic absorption changes plus or minus 30 $\mu$M ADP and 90 $\mu$M $P_i$, as exemplified in Fig. 3. The half-rise time of transient charge flow increased from 40 ms in $^1H_2O$ to 78 ms in $^2H_2O$. Presumably, the rate of proton flow across CF0–CF1 was limited by a protolytic reaction step.

**Localized Versus Delocalized Coupling.** It is established in the literature (see the introduction) that thylakoid membranes contain proton-buffering domains that are not normally in equilibrium with the bulk phase unless activated by protonophores. If activated, however, they can transiently trap protons released during water oxidation (11, 12). Trace B in Fig. 2 shows the pH transient in the lumen of thylakoids after the proton trapping domains were activated by addition of 0.3 nM gramicidin. Comparison with traces A, C, and D showed that the protons normally released into the lumen by each of the three successive flashes were no longer detectable by neutral red, indicator of the lumenal surface pH (20–22), but instead they were trapped in the above mentioned domains. It has been argued that these domains serve as ducts for protons, e.g., from water oxidation to CF0–CF1 (8, 28). Fig. 2 clearly shows that without added gramicidin this was not the case. Instead, trace D documents the release of water protons during each of the three flashes (3-ms spacing). It preceded proton uptake by CF0–CF1.

## DISCUSSION

ATP production and transient proton flow via CF0–CF1 were stimulated by flashing light. Transient proton flow across thylakoid membranes was completely tracked by parallel measurement of proton uptake from the lumen, charge translocation across the membrane, and proton release into the medium. Attribution of transient proton flow to CF0–CF1 was unequivocal because of the requirement for ADP and $P_i$ and sensitivity to agents that can block either the channel or the catalytic portion of the ATP synthase. In agreement with earlier work (13, 14), transient proton flow via CF0–CF1

occurred only if the driving voltage exceeded a certain threshold level (see Fig. 1). This is understood in terms of the gating behavior of the chloroplast ATPase/synthase that, without thiol activation, requires ≈2.5 pH units of electrochemical potential difference for activation (see figure 14 in ref. 17). During the short period (100 ms) when the voltage was above threshold, ≈1.3 $H^+$ were translocated per CF0–CF1 (excitation with three flashes spaced 3 ms apart) in comparison with a total of ≈6.3 $H^+$ per CF0–CF1 deposited in the lumen. The remaining 5 $H^+$ leaked out at a much slower rate via leaks that were insensitive to DCCD and tentoxin.

With saturating concentrations of ADP and $P_i$, transient proton flow via CF0–CF1 was half completed in 40 ms in $^1H_2O$ and in 78 ms in $^2H_2O$. The isotope effect was not attributable to the influence of $^2H_2O$ on the pH difference before firing of flashes, as could be inferred from the same threshold voltage in both experiments. A similar, very small isotope effect on the pH difference under continuous light at moderate intensity was reported in ref. 29 (see figure 2 therein). The large isotope effect on transient proton flow implied that its rate was limited by a protolytic reaction of CF0–CF1.

A rather low concentration of $P_i$ was required to drive transient proton flow and ATP synthesis under flashing light ($K_m$, 14 μM). This was at variance with the higher $K_m$ under continuous illumination (e.g., 450 μM in ref. 27). This difference was tentatively attributed to the difference between the rates of photophosphorylation under these two excitation conditions. The rate of transient ATP synthesis under flashing light could be calculated from the initial rise of proton flow. As was evident from Fig. 3, in 50 ms, 0.8 $H^+$ was translocated per electron transport chain cycle. With 600 chlorophyll molecules per chain and with a $H^+$/ATP stoichiometry of 3, this was equivalent to an initial rate of 96 μmol of ATP per μmol of chlorophyll per hr, about 10% of maximum rates observed under continuous illumination with pyocyanin as cofactor. Under flashing light, not only the initial rate of ATP synthesis was lower, but during the 5-s time span between flash groups $P_i$ deficiencies could be leveled out. Thus, it is conceivable that the low $K_m$ under flashing light reflects the dissociation equilibrium between $P_i$ and the enzyme, while the high $K_m$ under strong continuous illumination reflects diffusional constraints for $P_i$ (see ref. 27 for similar reasoning for ADP).

The dichotomy between delocalized and localized forms of coupling between redox-driven proton pumps and ATP synthases is more sharply defined for thylakoids than it is for mitochondria. There are two aspects of delocalization relating to the lateral and the normal dimension of the membrane. Along the lateral dimension of thylakoids some proton pumps, namely photosystem II located in the middle of stacked thylakoids, can be as far away as 300 nm from the nearest ATP synthase (30). *Per se*, this implies delocalized coupling. But it also implies lateral losses of the electrochemical potential difference. Since these losses are in the order of a few percent of the transmembrane difference (31), the necessary correction to the original chemiosmotic concept is slight. In the dimension normal to the membrane, the thylakoid membrane contains proton-buffering domains, not generally in equilibrium with the aqueous bulk phases (5, 11) but, when induced, able to trap protons from water oxidation (12). These domains are shared by several photosystems II (32), and it has been argued that they are in the pathway of protons from water oxidation to CF0–CF1 (5).

In this work proton flow across CF0–CF1 was completely tracked. It was demonstrated that protons from water oxidation passed to CF0–CF1 via the lumen. As any proton crossing the membrane via CF0–CF1 was also apparent as a proton leaving the lumen, localized proton trapping domains were obviously not involved. This observation was, of course, restricted to the present experimental situation: room temperature, continuous light plus repetitive flashes, and absence of protonophores that can activate the proton-trapping domains. A different set of experiments, at low temperature, with dark-adapted thylakoids, and after pretreatment with protonophores activating the domains, has revealed that the occupation status of these domains influenced the onset of ATP synthesis as function of the flash number (28, 33). In conclusion and conservatively speaking, photophosphorylation under continuous light seems adequately described by a chemiosmotic mechanism with proton pumps and ATP synthases operating between two laterally extended phases at different electrochemical potentials, the internal lumen, and the partitions between stacked thylakoids. Only in an approximative sense is each of them isopotential (29, 31). They are bulk phases in that they contain many buffering groups to accommodate and to rapidly exchange protons, but due to their narrow width, 5 nm in intact chloroplasts, they are not extended "aqueous bulk" but rather surface regions (21).

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