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INVESTIGATION OF THE APPARENT INEFFICIENCY OF THE COUPLING BETWEEN PHOTOSYSTEM II ELECTRON TRANSFER AND ATP FORMATION

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The maximum phosphorylation efficiency achieved with synchronous turnovers of Photosystem II (PS II) in spinach chloroplast lamellae is 0.3 molecules of ATP per pair of electrons transferred. This is the same as the efficiency observed for PS II operating alone in continuous light and would seem to indicate less than 50% coupling efficiency. Flash-induced ATP synthesis associated with both photosystems acting in unison closely approaches twice the flash-induced ATP synthesis associated with the Photosystem-I-dependent oxidation of duroquinol (itself 0.6) and comes close to equalling the highest efficiency observed in steady-state PS I + PS II electron transport. The anomalously low coupling efficiency seen when PS II is operating alone can be overcome by a ΔpH of two units imposed before flash illumination, or by a prior flash series involving the entire electron transfer chain. In contrast, prior electron transport through PS II alone is only slightly effective in enhancing the coupling efficiency of subsequent PS II turnovers. (It should be noted that in all cases where supplementary energy was provided, either by a proton gradient or by prior illumination, this supplementary energy was always below the energetic threshold for phosphorylation. Furthermore, the enhancement of PS II coupling efficiency by supplementary energy persisted even after a large number of subsequent PS II-inducing flashes). The efficiency of flash-induced ATP synthesis associated with whole-chain electron transfer or with PS-I-dependent duroquinol oxidation is also enhanced by the supplementary energy, but only during the first few inefficient flashes, suggesting that in this case the supplementary energy may simply be contributing to the initial build-up of an energetic threshold for ATP synthesis. This cannot be the case when the same supplementary energy contributes to the efficiency of the PS II reaction, since the enhancement then persists for a long time and contributes to an essentially constant flash yield of ATP. Our results imply that during electron transfer involving both photosystems, PS II participates in generating about half of the total ATP, whereas it operates inefficiently only when operating alone. Since hydrogen ions produced by PS I are able to raise the efficiency of subsequent PS-II-dependent phosphorylation, at least some cooperation between the two photosystems takes place and this suggests some donation of protons from PS I to PS II. However, the inability of PS II alone to achieve high efficiency, even with prolonged pre-illumination, would seem to indicate some functional distinction of protons from the two photosystems.

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Abbreviations: PS I and II, Photosystem I and II; ATP/e_2, phosphorylation efficiency, number of ATP molecules synthesized per pair of electrons transferred; Chl, chlorophyll; Δp, protonmotive force; ΔpH, transmembrane proton activity difference; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DMQ, 2,5 or 2,6-dimethylbenzoquinone; DAD_{ox}, diiminodurene; $TMPD_{ox}$, oxidized *N',N',N,N*-tetramethyl-*p*-phenylenediamine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Mes, 4-morpholineethanesulfonic acid; Mopso, 3(*N*-morpholino)-2-hydroxypropanesulfonic acid; Heppso, *N*-hydroxyethylpiperazine-*N'*-2-hydroxypropanesulfonic acid.

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Introduction

It is generally thought that in the linear electron transport sequence from water to terminal electron acceptors such as NADP, methyl viologen or ferricyanide, there are two hydrogen ion-producing reactions that are coupled to ATP formation: the oxidation of water associated with Photosystem II (PS II), and the oxidation of plastoquinol associated with Photosystem I (PS I). However, these two reactions are apparently not equivalent in their capacity to provide energy for ATP formation. One measure of phosphorylation efficiency is the ratio of the amount of ATP made per pair of electrons transferred through a given pathway (ATP/e_2). When assayed in continuous light, the measured ATP/e_2 values for reactions involving the entire electron transport chain approach 1.3 [1]. This is very close to the theoretical maximum of 1.33, a value based on a proton-to-electron stoichiometry of 1 per hydrogen ion-producing reaction and the requirement for three protons in the synthesis of one molecule of ATP. Phosphorylation associated with the oxidation of plastoquinol can have ATP/e_2 values of 0.5–0.6, or nearly one-half of the yield of the complete chain. This is true when ferrocyanide is used as an electron donor to PS II in chloroplasts incapable of water oxidation [2] and for the PS I-supported oxidation of plastoquinol analogs [3]. However, when PS II is assayed separately, as when electron transport is from water to a lipophilic acceptor in the presence of an intersystem inhibitor such as DBMIB, much lower ATP/e_2 values are observed, typically about 0.3 [4,5]. Although this discrepancy has been noted for several years, no satisfactory explanation has emerged. One aim of this study was to clarify the contribution of PS II to the overall yield of ATP and, if possible, to identify the causes of the observed poor coupling efficiencies noted when PS II operates alone.

An additional motivation for further characterizing phosphorylation associated with partial reactions of the electron transport chain comes from current discussion of local phenomena in energy coupling. According to the most orthodox versions of the chemiosmotic model, coupling is mediated through a homogeneous protonmotive force whereby there should be no distinction between protons originating from different redox reactions. But in addition to the unexplained difference in coupling efficiency between PS I and PS II driven reactions, a system-specific difference in pH sensitivity of the ATP/e_2 ratio [6] as well as a peculiar insensitivity of PS II phosphorylation to inhibition by $HgCl_2$ [7] has been reported. Thus, if electron transport is more directly coupled to ATP synthesis than just through the bulk phase protonmotive force, local interactions may differ for the coupling events of each photosystem.

In the work described here, we have re-examined the question of the efficiency of PS II-dependent phosphorylation using pre-steady-state conditions of illumination. The use of single-turnover flashes, when combined with a sensitive method for measuring ATP synthesis, makes it possible to address these questions in new ways. If localized coupling phenomena do occur, it is likely that they will be more evident under conditions of limited energization where the rate of energy input cannot increase in response to utilization or loss. In addition, the onset of phosphorylation cannot occur until a sufficiently energetic state has been achieved and, thus, serves as a useful parameter to assess the role of various reactions in the formation of the high energy state.

We found that in flashing light, the ATP/e_2 values for PS II approached a maximum of about 0.3, as in continuous light. This efficiency was constant from about 50 to 600 flashes when an acceptor system consisting only of 1.0 μM DBMIB and 0.1 mM ferricyanide was used. However, the efficiency of PS II phosphorylation was raised to ATP/e_2 values in excess of 0.5 by imposing a ΔpH of 2 units (itself incapable of phosphorylation) prior to flash illumination, and was also raised by providing a prephosphorylating flash series involving both photosystems. In contrast to prephosphorylation electron transport through both photosystems, electron transport through PS II alone was only slightly effective in raising the efficiency of subsequent PS II turnovers in supporting ATP formation. These results suggest that during electron transport involving both photosystems, PS II participates in generating half the total yield of ATP and operates inefficiently only when operating alone. In addition, since protons generated by PS I can raise the efficiency of later PS

II-dependent ATP synthesis, some pooling of protons contributed by the two systems is possible. However, the inability of PS II alone to achieve high efficiency suggests some difference between the function of protons from PS I and the function of protons from PS II.

Materials and Methods

Chloroplast isolation. Chloroplast thylakoid membranes were isolated from commercial spinach (*Spinacea oleracea* L.) as described elsewhere [8], except that the final wash was with a medium of the same composition as the reaction or incubation medium (with the omission of isotope, inhibitors and electron transport mediators).

Measurement of electron transport and ATP formation during continuous illumination. Steady-state measurements of electron transport and phosphorylation were carried out in a water-jacketed chamber at $20 \pm 0.2°C$. Illumination was provided by a 750 W tungsten lamp, and filtered through both a red filter (Corning 2-62) and a round-bottomed flask containing a solution of 0.15 M ferrous ammonium sulfate/0.25 M H_2SO_4. The latter served both to focus the light beam and to absorb heat. Electron transport, measured as oxygen evolution or consumption, was monitored with a Clark-type oxygen electrode calibrated from the light-induced reduction of a known amount of ferricyanide by a chloroplast suspension. Each 2 ml reaction contained 22–33 nmol chlorophyll and was continuously stirred during the 1 min illumination period. Other reaction conditions are given in the figure and table legends. Steady-state phosphorylation was measured as $^{32}P_i$ incorporation into ATP following organic extraction of the unreacted phosphate as phosphomolybdate as described previously [9].

Measurement of flash-induced ATP formation. Measurements in flashing light were carried out in a water-jacketed reaction chamber at $4 \pm 0.2°C$ unless otherwise stated. Saturating single-turnover flashes with a half-width of 6 μs were provided by either a single xenon lamp (Model FX-193 flashtube, E.G.&G., Salem, MA) or by two xenon lamps (Model FX-200) which were discharged simultaneously on opposite sides of the sample. Unless otherwise stated, the light was filtered through a yellow filter (Corning glass 3-71) and the flash frequency was 5 Hz.

In all experiments in which valinomycin was used to accelerate the decay of the membrane potential, the concentration of valinomycin was 0.4 μM and the concentration of K^+ was at least 20 mM. It was previously shown [10] that higher concentrations of valinomycin and/or K^+ than these did not further increase the amount of illumination required before ATP formation is observed. Also, under these conditions there was no significant absorption change at 518 nm attributable to the electrochromic shift.

The ATP synthesis resulting from a series of single turnover flashes was measured as the incorporation of $^{32}P_i$ (ICN) into ATP according to the method of Smith et al. [11] as described previously [10]. The specific activity of the $^{32}P_i$ in the reaction media was generally 10–15 Ci/mol. For calculation of ATP/e_2 values, the levels of ATP found in nonilluminated samples (which ranged from less than 0.05 to 0.20 mmol ATP/mol Chl) were first subtracted from the flash-generated ATP levels.

Measurement of flash-induced electron transfer. The amount of electron transport occurring per flash was determined from the flash-induced pH change due to the release of protons from water oxidation in the presence of an uncoupler [10]. When ferricyanide is used as the final electron acceptor, protons from water oxidation are responsible for the only irreversible pH change associated with linear electron transport, and proton production is stoichiometric with electron transport. The pH change from a series of 50 flashes was measured with a glass electrode in the same apparatus used for phosphorylation reactions. The electrode was connected to a Keithley 610C electrometer and pH changes were monitored on a strip chart recorder with a full scale expansion of 0.06 pH units. pH changes were calibrated for each sample by addition of a standard HCl solution containing 10 or 20 nmol H^+. The 4 ml stirred reaction mixture contained 100 mM sorbitol, 25 mM KCl, 2 mM $MgCl_2$, 0.5 mM Tricine-KOH (pH 8.1), 0.2 mM ferricyanide, 0.2 mM 2,5-dimethylquinone, chloroplasts containing 175 nmol chlorophyll, and 1.5 μM nigericin to allow rapid equilibration of proton across the thylakoid membrane. It was not feasible to measure electron

transport from each chloroplast preparation. However, consistent values for electrons transferred per flash were obtained from determinations made at various times of the year over a 2-year period. The average value of 1.60 mmol electrons transported/mol Chl per flash was used for reactions involving both photosystems. For PS I operating alone, a value of 1.67 mmol electrons/mol Chl per flash was estimated from pH changes associated with electron transport from TMPD to methyl viologen [12]. The amount of electron transport associated with PS II reactions conducted in the presence of 1 μM DBMIB was found to be somewhat dependent on the flash frequency. The averaged values of 1.43, 1.23, and 1.00 mmol electron/mol Chl per flash were used for measurements at flash frequencies of 5, 10, and 20 Hz, respectively. In all cases, the standard deviations of these measurements were less than 10% of the mean values. Flash-induced electron transfer was measured at 4 and 20 °C, and no significant difference was found. Also, there was no significant effect of valinomycin (0.4 μM) or gramicidin (1 μg/ml) on the flash yield.

Measurement of PS II-dependent proton accumulation. Proton uptake associated with PS II was measured at 4 °C using the same system described for phosphorylation and electron transport measurements. Chloroplast thylakoid membranes were isolated as usual and then rinsed in resuspension medium containing 1.0 mM rather than 5.0 mM buffer. In addition to the thylakoid membranes, the 4 ml stirred reaction mixture contained 100 mM sorbitol/25 mM KCl/0.4 mM Heppso-KOH (adjusted to pH 7.9 prior to each measurement)/2 mM $MgCl_2$/0.2 mM 2,6-dimethylquinone/1.0 μM DBMIB. Because the decay of the pH change was slow, except after very large flash numbers, the number of protons pumped could be estimated from a linear extrapolation of the decay. For these data, this method was as accurate and reproducible as when the data were computer fit to a single exponential decay, using an iterative nonlinear least squares program [13].

Results

As reported previously (e.g., Refs. 4 and 5), the phosphorylation efficiency (ATP/e_2) of PS II in continuous light is about half the assumed theoretical maximum of 0.66. Electron transport in continuous light from water to oxidized diaminotoluene or to dimethylquinone supports ATP synthesis with an ATP/e_2 of about 0.3, although when the acceptor is oxidized tetramethylphenylenediamine ($TMPD_{ox}$), somewhat higher values were observed (Table I). In contrast, electron transport from water to methyl viologen is seem-

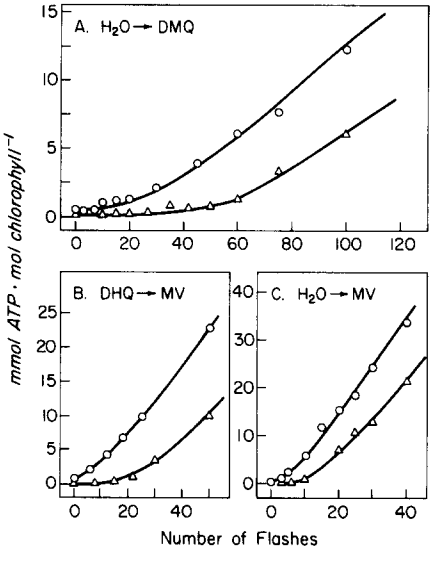

Fig. 1. Dependence of ATP formation associated with different segments of the electron transport chain on the number of single turnover flashes. (A) Flash-induced ATP synthesis associated with the PS II-dependent reduction of dimethylquinone. The 2 ml reaction mixture contained chloroplast thylakoid membranes to a concentration of 33 μM Chl, 100 mM sorbitol, 15 mM Heppso-KOH (pH 7.9), 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM $Na_2H^{32}PO_4$, 0.1 mM ADP, 0.2 mM 2,6-dimethylquinone, and 1.0 μM DBMIB. The valinomycin concentration, when present (lower trace, △———△), was 400 nm. The reaction was continuously stirred and thermostatted at 4 °C. The flash frequency was 5 Hz. (B) Flash-induced ATP synthesis associated with the PS I-dependent oxidation of duroquinol. The reaction medium contained 100 mM sorbitol, 15 mM KCl, 4 mM Tricine-KOH (pH (8.1), 0.5 mM $MgCl_2$, 0.5 mM $Na_2H^{32}PO_4$, 0.5 mM ADP, 100 μM methyl viologen, 0.5 mM duroquinol, 10 μM DCMU, and chloroplast thylakoid membranes to a concentration of 17 μM Chl. The flash frequency was 10 Hz, and the other conditions as in (A). Points shown are the averages of two samples. (C) Flash-induced ATP synthesis associated with whole chain electron transfer. The reaction conditions were as given for (B) except that duroquinol and DCMU were omitted from the reaction mixture and the chlorophyll concentration was 20 μM.

TABLE I

STEADY-STATE ATP SYNTHESIS ASSOCIATED WITH VARIOUS PS II ELECTRON ACCEPTORS

The reaction media contained 80 mM sorbitol, 20 mM Tricine-KOH (pH 8.1) [a] or (pH 7.8) [b,c], 15 mM KCl, 3 mM $MgCl_2$, 1 mM ADP, 5 mM $Na_2H^{32}PO_4$ (52 mCi/mol) plus 0.1 mM methyl viologen [a] or 1.5 mM potassium ferricyanide, 0.5 mM 2,5-diiminotoluene or TMPD and 1.0 μM DBMIB [b] or 0.5 mM potassium ferricyanide, 0.5 mM 2,6-dimethylbenzoquinone and 1.0 μM DBMIB [c].

Electron transport reaction	Rate of phosphorylation (mmol ATP/mol Chl per s)	ATP/e_2
$H_2O \rightarrow$ methylviologen [a]	390	1.19
$H_2O \rightarrow$ 2,5-diiminotoluene [b]	20	0.27
$H_2O \rightarrow$ 2,6-dimethylquinone [c]	60	0.34
$H_2O \rightarrow$ oxidized *N,N,N′,N′*-tetramethyl -p-phenylenediamine [b]	100	0.45

ingly tightly coupled, since the efficiency is about 90% of the suggested theoretical maximum of 1.33.

The yield of ATP resulting from a series of 6-μs flashes is shown in Fig. 1A for the PS II-mediated reduction of dimethylquinone. The concave shape of the upper trace indicates that the chloroplast preparation acquires the capacity for phosphorylation gradually; an almost linear slope was achieved only after 40 flashes. When the membrane potential component of the driving force was eliminated by valinomycin-facilitated movement of K^+ (lower trace), at least 25 flashes were necessary for ATP synthesis to occur at all, and a constant yield per flash was observed only after about 60 flashes. For comparison, similar experiments for a PS I reaction (Fig. 1B), flash-induced electron transfer from duroquinol to methyl viologen, and for a reaction involving both photosystems (Fig. 1C), flash-induced electron transfer from water to methylviologen, are shown. In the absence of valinomycin, there was almost no perceptible delay in the onset of efficient phosphorylation for PS I acting alone (Fig. 1B) or in combination with PS II (Fig. 1C) and full efficiency was achieved after relatively few flashes. In the presence of valinomycin, many fewer flashes were required to initiate ATP synthesis when PS I was involved (Fig. 1B and C) than when PS II was operating alone (Fig. 1A).

Representative phosphorylation efficiencies for a number of flash-induced electron transport reactions are assembled in Table II. The ATP/e_2 ratios for whole chain electron transport from water to ferricyanide or methyl viologen were

TABLE II

COMPARISON OF THE PHOSPHORYLATION EFFICIENCY OF DIFFERENT ELECTRON TRANSPORT REACTIONS IN REPETITIVE SINGLE TURNOVER FLASHES OF LIGHT

Data were compiled from several experiments. The basic reaction medium contained 100 mM sorbitol, 25 mM KCl, 4 mM Tricine-KOH (pH 7.8), 3 mM $MgCl_2$, 0.5 mM $Na_2H^{32}PO_4$, 0.1 mM ADP, plus cofactors as indicated at the following concentrations. PS I and II: 0.1 mM methyl viologen of 0.2 mM ferricyanide. PS I: 0.5 mM duroquinol (DHQ). PS II: 0.2 mM diiminodurene (DAD_{ox}), 0.2 mM 2,5-diiminotoluene (2,5-DAT_{ox}), 0.2 mM oxidized TMPD ($TMPD_{ox}$), 0.2 mM 2,5-dimethylbenzoquinone (2,5-DMQ), all plus 0.2 mM excess ferricyanide. For PS I assays 5 μM DCMU was used to inhibit PS II, and for PS II assays, 1 μM DBMIB was included to inhibit electron transport to PS I.

Electron transport reaction	Number of flashes	ATP/e_2 (averaged over flash period)
Photosystems I and II		
$H_2O \rightarrow$ ferricyanide	0–50	0.89
$H_2O \rightarrow$ methylviologen	0–75	1.00
Photosystem I		
$DHQ \rightarrow$ methylviologen	0–50	0.49
	50–100	0.68
Photosystem II		
$H_2O \rightarrow DAD_{ox}$	0–100	0.20
$H_2O \rightarrow$ 2,5-DAT_{ox}	0–100	0.22
$H_2O \rightarrow TMPD_{ox}$	0–75	0.29
	0–200	0.35
	0–500	0.36
$H_2O \rightarrow$ 2,5-DMQ	0–75	0.20
$H_2O \rightarrow$ DBMIB	0–200	0.26
	0–600	0.29

comparable and generally greater than 0.8 when averaged over zero to 50 or so flashes. Variability in the calculated efficiency was due to varying degrees of coupling at early flash numbers. When the ultimate slope of ATP yield vs. flash number is considered, then a consistent efficiency of 1.0–1.2 was achieved for whole chain phosphorylation.

The flash-induced oxidation of duroquinol, a DBMIB-sensitive reaction that is presumed to utilize the native coupling site [13], was seen to be associated with very high ATP/e_2 ratios (Table II), actually achieving the predicted maximum of 0.66, a value nearly identical to that observed in continuous light [3]. Similarly high values (i.e., greater than 0.6) were obtained with two other plastoquinol analogs, thymolquinol and trimethylquinol. Like duroquinol, the PS I-dependent oxidation of these two quinols is also inhibited by DBMIB.

The flash-induced PS II electron transport associated with the highest efficiencies was the reduction of $TMPD_{ox}$. Averaged over a group of 200 flashes, an ATP/e_2 value of 0.35 was obtained (Table II) and did not increase further with flash number. Of the quinonediimine acceptors that were studied, diiminodurene and diiminotoluene, were similar to each other in their effects. Both supported ATP synthesis with efficiencies about 30% lower than that supported by the $TMPD_{ox}$ system. The PS II-dependent reduction of dimethylquinone supported an ATP/e_2 ratio of only 0.20 when averaged over the initial 75 flashes.

The higher efficiencies seen when $TMPD_{ox}$ was the electron acceptor may have resulted from an added contribution from unanticipated oxidation of plastoquinol. It has previously been reported [14] that under certain conditions, TMPD may allow a bypass of electron flow around the DBMIB block. While the presence of ferricyanide in these PS II assays is believed to prevent the shuttling of electrons to PS I, $TMPD_{ox}$ might nevertheless accept some electrons from plastoquinol at the inner side of the membrane where little ferricyanide penetrates. Since plastoquinol would liberate two protons upon oxidation and these would not be balanced by any proton uptake during the reduction of the pure electron acceptor $TMPD_{ox}$, the H^+/e^- might be somewhat elevated by this unanticipated production of protons.

In our spinach thylakoid preparations, ferricyanide cannot be reduced directly by PS II in continuous light unless lipophilic mediators are present. However, in flashing light, ferricyanide may oxidize plastoquinol rapidly enough during the dark intervals to sustain electron transport or, a more likely possibility, the 1.0 μM DBMIB present as an inhibitor may be sufficient to act as a lipophilic-mediating electron carrier. In any case, an acceptor system consisting of 1.0 μM DBMIB and 0.1 mM ferricyanide is adequate to support flash-induced PS II phosphorylation at 5 Hz (Table II). This is seen more clearly in Fig. 2, where it is evident that a constant yield per flash was reached after about 50 flashes. Although the data are not included in this figure, the slope remained linear from 50 to 600 flashes with an efficiency of about 0.3, typical of these artificial PS II reactions.

Since the measured ATP/e_2 ratios for PS II are consistently much less than the commonly predicted 0.66, the possibility that the stoichiometry of proton accumulation is less than one proton per electron was investigated. Proton uptake associated with the reduction of dimethylquinone in the

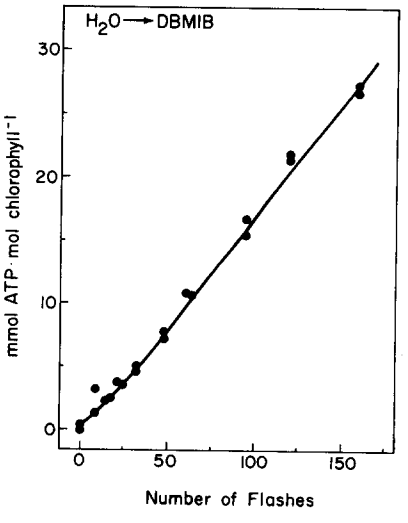

Fig. 2. Dependence of ATP formation on the number of Photosystem II turnovers with only DBMIB as a lipophilic exogenous oxidant. The reaction medium was composed of 100 mM sorbitol, 25 mM KCl, 10 mM Heppso-KOH (pH 7.8), 3 mM $MgCl_2$, 0.5 mM $Na_2H^{32}PO_4$, 0.1 mM potassium ferricyanide, 1.0 μM DBMIB, and chloroplast thylakoids to a concentration of 33 μM Chl. Other conditions of the experiment are as given for Fig. 1A.

presence of 1.0 μM DBMIB was measured with a glass electrode in flashing light. Since dimethylquinone takes up two protons per electron pair when reduced at the pH values of concern here, the measured proton uptake in the absence of an uncoupler should equal the number of protons released inside the vesicle upon the oxidation of water. Flash-induced electron transfer was determined from measurements of the H^+ release associated with water oxidation in the presence of ferricyanide and an uncoupler as described in Materials and Methods. If a fraction of protons from water oxidation were released to the outer aqueous phase, this would cancel a part of the alkalization due to protonation of reduced dimethylquinone, and the measured H^+/e^- would then be less than 1. In Fig. 3, the proton uptake observed after a series of flashes given at 5 or 20 Hz is shown as a function of flash number. At both frequencies, there was a rapid initial phase of uptake, but after about four flashes, the increase in proton uptake with flash number appeared linear. The intercepts, extrapolated from the linear phase by regression analysis, were approximately the same: 7.8 mmol H^+/mol Chl at 20 Hz and 8.6 mmol H^+/mol Chl at 5 Hz. This intercept may represent H^+ binding and is most likely not directly related to protonation of the electron acceptor. Surprisingly, there was a clear difference in slope at the two flash frequencies. Even when adjusted for the reduced electron transfer that occurs at 20 Hz when DBMIB is present, the apparent H^+/e^- was only 0.53 at 20 Hz as compared to 0.91 at 5 Hz.

Since the apparent H^+/e^- changed with flash frequency, the effect of flash frequency on PS II phosphorylation was investigated. In Fig. 4, the ATP/e_2 ratio for PS II with dimethylquinone as acceptor is shown as a function of frequency for the initial 30 flashes (lower trace), and averaged from 30 to 75 flashes (upper trace). During the first 30 flashes, when a membrane potential is a critical component of the driving force, the efficiency increased steadily with frequency. However, at the larger flash numbers, where the more slowly decaying ΔpH was becoming an increasingly important component of the driving force for ATP synthesis, there seemed to be an optimum yield per flash at 5 Hz, a finding consistent with

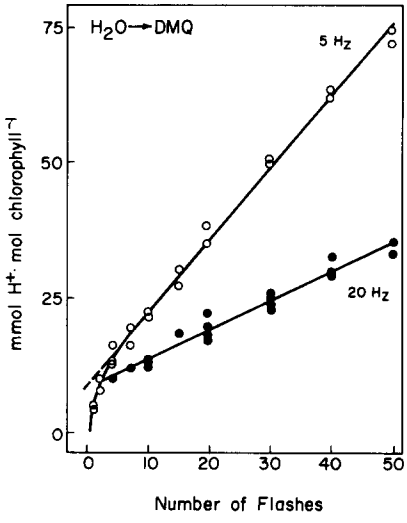

Fig. 3. Flash-induced proton uptake associated with reduction of dimethylquinone by PS II. The reaction medium contained chloroplast thylakoid membranes to a concentration of 44 μM Chl, 100 mM sorbitol, 25 mM KCl, 2 mM $MgCl_2$, and 0.3 mM Heppso-KOH (pH 7.9), 0.2 mM 2,5-dimethylquinone, and 1.0 μM DBMIB. The flash frequency wsa 5 Hz (○) or 20 Hz (●). The slope calculated from 4–50 flashes gives a value for the H^+/e^- of 0.53 at 20 Hz and 0.91 at 5 Hz.

the greater proton uptake seen in Fig. 5. Although it appears that a reduced H^+/e^- may partially explain the low ATP/e_2 for PS II, particularly at high flash frequencies, it is evident that rapid release of protons to the outer phase is not the primary explanation. Chloroplasts isolated from the spinach used in the measurement of H^+/e^- at 5 Hz, which were efficient in pumping protons, still displayed the typically low ATP/e_2 value characteristic of PS II when operating alone.

The initial increase in the efficiency of phosphorylation with increasing flash number suggested that tighter coupling was associated with increased energization of the thylakoid system (see Fig. 1). Indeed, it was found that the efficiency of PS II phosphorylation could be improved by imposition of an added protonmotive force which was by itself too small to drive ATP synthesis (Fig. 5). Thylakoid membranes were either incubated at pH 5.9 or at pH 6.9 and then injected into a phosphorylation medium at pH 7.9, 3 s prior to the flash series. This meant that in addition to the light-induced protonmotive force an additional

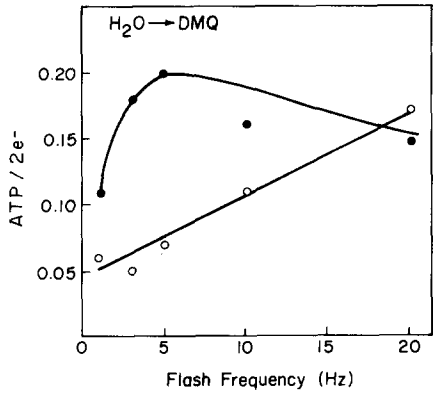

Fig. 4. Phosphorylation efficiency of the Photosystem II reaction, H_2O to dimethylquinone as a function of flash frequency. (○) ATP/e_2 calculated from zero to 30 flashes; (●) ATP/e_2 calculated from 30 to 75 flashes. Chloroplast thylakoid membranes were suspended to a concentration of 22 μM in a reaction medium containing 100 mM sorbitol, 20 mM KCl, 15 mM Tricine-KOH (pH 7.8), 3 mM $MgCl_2$, 0.1 mM ADP, 0.2 mM 2,6-dimethylquinone, and 1.0 μM DBMIB. 15 s after chloroplast addition, $Na_2H^{32}PO_4$ was added to a concentration of 0.5 mM. The time before the first flash was adjusted so that the chloroplasts were exposed to phosphate for a maximum of 90 s prior to termination of the reaction, which occurred 5 s after the final flash. Other conditions of the experiment are the same as given for Fig. 1A. Flash-induced electron transfer was determined by measurement of proton release associated with the reduction of ferricyanide by electrons from water. The reaction mixture for electron transfer determination was similar to that given above except for the addition of 0.2 mM potassium ferricyanide and the addition of 1.5 μM nigericin to allow prompt equilibration of the pH across the membrane.

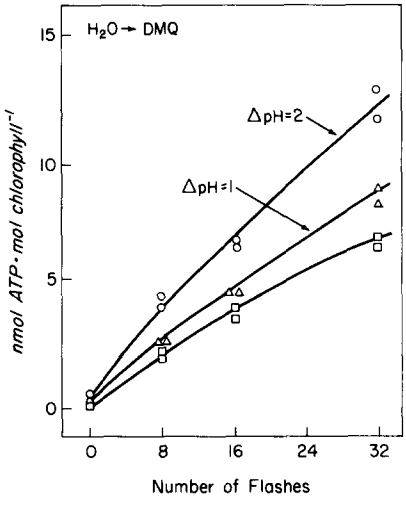

Fig. 5. Enhancement of the flash-induced yield of ATP from Photosystem II by an imposed ΔpH. Thylakoid membranes were resuspended in the following media and incubated for 80 min prior to the assay of phosphorylation: 100 mM sorbitol, 1 mM KCl, 2 mM $MgCl_2$ and either 10 mM Heppso-Bistrispropane (pH 7.9; □———□), 10 mM Mopso-Bistrispropane (pH 6.9; △———△, ΔpH = 1) or 10 mM Mes-Bistrispropane (pH 5.9; ○———○, ΔpH = 2). An aliquot of the suspension was injected into the reaction medium (to 30 μM Chl), which contained 100 mM sorbitol, 10 mM Heppso-Bistripropane (pH 7.9), 1 mM KCl, 0.5 mM $Na_2H^{23}PO_4$, 0.1 mM ADP, 0.2 mM 2,6-dimethylquinone, 0.1 mM potassium ferricyanide, and 1.0 μM DBMIB. The volume of the chloroplast aliquot was small enough so as to have no significant effect on the pH of the phosphorylation reaction medium. The flash series was begun 3 s after addition of chloroplasts, and the reaction was terminated 5 s after the last flash. Conditions were otherwise as described in Fig. 1A.

ΔpH of either one or two units was available. In both cases, an enhanced yield of ATP per flash was observed. When a 1-unit pH jump was given prior to flash excitation, the linear slope from 8 to 32 flashes indicated an efficiency of 0.30, a value normally attained only after considerably more flashes. An additional increase in efficiency was seen if a 2-unit pH jump, still below the energetic threshold for ATP synthesis, was added prior to flash-induced phosphorylation. The ATP/e_2 averaged over 16–32 flashes was 0.50, which was considerably higher than the value of 0.25 seen in this experiment in the absence of an imposed potential, or the 0.33 typical of phosphorylation in continuous light. In order to distinguish the effect of a transmembrane pH difference from the effect of a lower internal pH, a comparison of phosphorylation efficiencies was made at external pH value of 6.9 and 7.9 and no difference was found.

In Fig. 6, a similar but less striking enhancement of phosphorylation associated with the PS I-dependent reaction duroquinol to methyl viologen was seen. In this experiment, the control ATP/e_2 averaged over 16–32 flashes was 0.43 and was increased to 0.58 with an added ΔpH of 2 units. However, it is important to make the distinction that this higher efficiency can also be achieved by increasing the number of flashes, which is not true for the enhanced PS II efficiency reported in Fig. 5. For the situation in which both photosystems are turning over in unison (Fig. 7), the imposed subthreshold pH jumps did not enhance the efficiency beyond 20 turnovers. In this

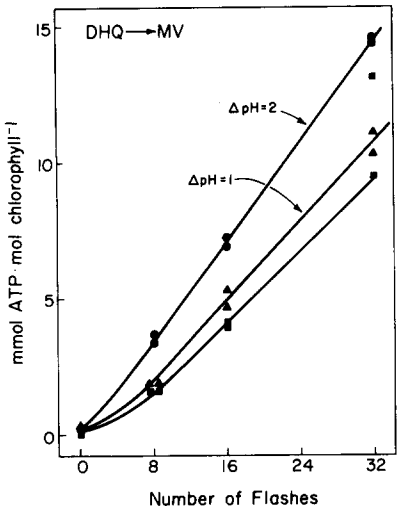

Fig. 6. Effect of an imposed ΔpH on the ATP flash yield associated with the PS I reaction, duroquinol to methyl viologen. Experimental conditions were as in Fig. 5 except the reaction medium contained 100 mM sorbitol, 10 mM Heppso-Bistrispropane (pH 7.9), 1 mM KCl, 2 mM $MgCl_2$, 0.1 mM ADP, 0.5 mM $Na_2H^{32}PO_4$, 0.1 mM methyl viologen, 5.0 μM DCMU, and 0.5 mM duroquinol. (■, control; ▲, ΔpH = 1; ●, ΔpH = 2).

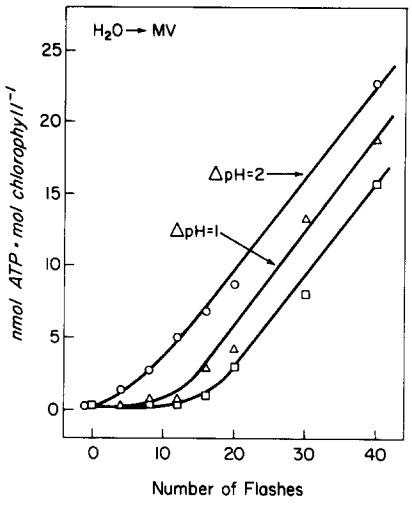

fig. 7. Effect of an imposed ΔpH on the flash yield associated with whole chain electron transfer from water to methyl viologen. Experimental conditions were largely as described in Fig. 6. Nonactin (400 nM) and 20 KCl were present in the phosphorylation reaction media and DCMU and duroquinol were omitted. (□, control; △, ΔpH = 1; ○, ΔpH = 2).

latter case, ATP synthesis in the initial turnovers was prevented by the presence of nonactin and K^+. It is clear that the only effect of the imposed ΔpH was merely to shift the threshold flash requirement for the onset of phosphorylation. In all the experiments where supplemental energy was provided it was less than the threshold energy required for phosphorylation, and this was indicated by the absence of significant recovery of ATP from nonilluminated samples (generally much less than 1.0 mmol ATP/mol Chl).

The enhancement of phosphorylation by an imposed pH difference could be duplicated by prephosphorylating flash series that allowed both photosystems to turnover under nonphosphorylating conditions (Table II). In these experiments, a suspension of chloroplasts was exposed to one or 20 flashes in the presence of ferricyanide and dimethylquinone prior to the addition of $^{32}P_i$, ADP and DBMIB. The latter ingredients were added immediately after the final prephosphorylating flash and after 3 s, a second series of flashes was given. There was very little increase in the dark incorporation of $^{32}P_i$ into ATP immediately following the first flash series, probably due to the rapid relaxation of the membrane potential. However, there was a considerable enhancement of ATP synthesis in the subsequent flash series, presumably due to a more stable ΔpH. After subtracting the small post-illumination phosphorylation component (typically 0.2 mmol ATP/mol Chl), the net ATP/e_2 for 20 flashes was 0.58 for electron transport from H_2O to dimethylquinone. As with the pH jump experiments, this is a much higher value than seen previously in flashing or continuous light. To distinguish between a specific requirement for PS I electron transport and a general increase in stored energy delocalized throughout the inner thylakoid compartment, the experiment was repeated with DBMIB present to inhibit PS I during the pre-phosphorylating flashes as well as during the ATP synthesizing flashes. While it appears that previous PS II electron transport can also enhance the efficiency of subsequent PS II phosphorylation slightly (Table IV), the enhancement was much smaller than that seen when the initial flash series was used to drive electron transport through both photosystems, i.e., in the absence of DBMIB.

TABLE III

EFFECT OF PREILLUMINATION OF CHLOROPLASTS ON THE FLASH-INDUCED ATP YIELDS

At $t = 0$, chloroplasts (22 μM Chl) were added to a reaction medium containing 100 mM sorbitol, 25 mM KCl, 4 mM Tricine-KOH (pH 7.8), 3 mM $MgCl_2$, and 0.2 mM potassium ferricyanide. 15 s later the indicated number of 'prephosphorylating' flashes was given. At $t = 18$ s, the remaining ingredients [a] were added, and at $t = 21$ s, the second flash series was begun. The reaction was stopped 30 s after addition of $^{32}P_i$. The flash frequency was 10 Hz, and the light was filtered through a yellow filter (Corning 3-71).

Electron transport reaction	Number of previous flashes	Number of flashes with ADP and $^{32}P_i$	mmol ATP/mol Chl	ATP/e_2
$H_2O \rightarrow$ DMQ	0	0	0.24	–
		20	4.74	0.28
		70	15.50	0.27
	1	0	0.23	–
		20	4.74	0.29
		70	14.20	0.25
	20	0	0.96	–
		20	8.90	0.50
		200	90.50	0.51
$H_2O \rightarrow$ FeCy	0	0	0.18	–
		20	14.30	0.76
		200	189.40	0.99
	1	0	0.23	–
		20	14.60	0.77
		200	194.40	1.02
	20	0	0.49	–
		20	19.40	1.02
		200	203.10	1.07

[a] For the $H_2O \rightarrow$ DMQ sample, 0.1 ml of a solution containing 4 mM ADP, 20 mM $Na_2H^{32}PO_4$, 40 μM DBMIB, and 8 mM 2,5-DMQ, was added. For the $H_2O \rightarrow$ ferricyanide ($H_2O \rightarrow$ FeCy) samples, 0.1 ml of a solution containing 4 mM ADP, 20 mM $Na_2H^{32}PO_4$ was added.

TABLE IV

THE EFFECT OF PREVIOUS PHOTOSYSTEM II ELECTRON TRANSPORT ON ATP YIELDS FROM PS II

The protocol for this experiment was largely as described in Table III. The reaction medium contained chloroplasts lamellae (33 μM Chl) in 100 mM sorbitol, 25 mM KCl, 10 mM Heppso-KOH (pH 7.8), 3 mM $MgCl_2$, 0.1 mM potassium ferricyanide, and 1.0 μM DBMIB. Prior to the phosphorylating flash series, $Na_2H^{32}PO_4$ and ADP were added to final concentrations of 0.5 and 0.1 mM.

Number of previous flashes	Number of flashes with ADP and $^{32}P_i$	mmol ATP/mol Chl	ATP/e_2
0	0	0.74	–
	30	5.19	0.24
	60	9.22	0.23
	150	27.50	0.28
15	0	0.14	–
	15	3.16	0.32
30	0	0.40	–
	30	5.95	0.30

Discussion

The phosphorylation efficiency is an expression of the amount of ATP made for a given amount of electron transfer. The maximum predicted efficiency for PS II, if perfect coupling is achieved, is an ATP/e_2 ratio of 0.66 based on a stoichiometry of one H^+ translocated across the membrane per turnover of the electron transport chain and three H^+ translocated out through the coupling factor per ATP synthesized. It is clear from prior work and the data presented here that the efficiency of PS II operating alone falls short of the predicted maximum and the typical ATP/e_2 of 0.3–0.4 would seem to indicate only about 50% coupling efficiency. This is true for phosphorylation occurring in continuous light (Table I) or in flashing light (Table II). In contrast, very high efficiencies of whole chain phosphorylation can be measured in these chloroplast thylakoid preparations: the frequently observed ATP/e_2 ratio of 1.2 repre-

sents 90% of the maximum predicted coupling efficiency. A number of explanations can be suggested but, as yet, none satisfactorily explains all of the data.

The assumption that every proton produced by water oxidation is released into the inner side of the thylakoid membrane is by no means well established and it has been suggested that perhaps the H^+/e^- for PS II is variable [15,16]. Nevertheless, most measurements of proton release associated with PS II have not detected a sizeable fraction released to the outer aqueous phase. In experiments using either pH-sensitive dyes [17] or rapidly responding glass electrodes [18], proton release was only observed in the external medium on a time scale much longer than the associated electron transport reactions unless an uncoupler was added. An exception to these reports is the study of Gould and Izawa [19], who measured proton uptake associated with electron transport from water to DBMIB and found an H^+/e^- value of 0.5.

In our experiments, it was found that the proton uptake per flash was somewhat variable with reaction conditions and chloroplast preparations and at a flash frequency of 20 Hz was as low as 0.5. Schönfeld and Neumann [20] have shown that the conductance of the thylakoid membrane to protons is dependent on ΔpH and that an abrupt threshold pH difference for significant conductance is observed even in the absence of phosphorylation. It, therefore, seems possible that observed decreases in the apparent stoichiometry of PS II proton accumulation are due to rapid, energization-dependent, efflux through the coupling factor and that the actual H^+/e^- does not vary with flash frequency and is nearly 1. In any case, since H^+/e^- stoichiometries of over 0.9 were observed under conditions giving low ATP/e_2 values, other explanations for the poor efficiency of PS II must be sought.

The increase in the yield of ATP per flash seen with PS II at low flash numbers, and with all reactions in the presence of valinomycin immediately after the onset of phosphorylation, suggests that the coupling efficiency increases as the protonmotive force increases. Gräber and Witt [21] reported that the yield of ATP per flash is a function of both $\Delta\Psi$ and ΔpH, and approaches a limiting ATP/e_2 of 1.33 at high values of the total ΔpH. In explanation, they cited the work of Schröder et al. [22], which indicated a difference in the dependence of phosphorylating and nonphosphorylating proton efflux on the magnitude of the ΔpH. Schröder et al. [22] suggested that the rate of nonphosphorylating proton efflux was proportional to the square of the internal proton concentration. Essentially the same model was proposed by Portis and McCarty [23], however they suggested a third-order relationship between the internal proton concentration and proton efflux associated with ATP synthesis. These models predict that as the internal proton activity and, therefore, the driving force for proton efflux increases, a greater proportion of the proton current will pass through the coupled pathway and the efficiency will increase.

A simple dependence of the ratio of coupled to noncoupled proton efflux as well as the rate of phosphorylation on the magnitude of the ΔpH, fails to accommodate the experimental observation that the phosphorylation efficiency is remarkably constant at vastly different rates of electron transport. Often times, the electron transport rate at a given light intensity is determined by the mediator used and rates of phosphorylation can vary 20-fold without significantly affecting the ATP/e_2. Similarly, electron transfer rates can be varied over an extremely wide range with light intensity with little change in the ATP/e_2 values observed. For example, Saha et al. [24] showed that the rate of ATP synthesis was linearly related to light intensity even at rates of phosphorylation as low as 1 mmol ATP/mol Chl/s. Concavity of the slope was only seen at light intensities less than about 6 kerg $\cdot$ $cm^{-2} \cdot s^{-1}$, implying a constant efficiency above this light level.

As mentioned previously, there is good evidence indicating that in the photosynthetic membranes of chloroplasts and bacteria, the membrane conductance to protons is not fixed and depends on the magnitude of the protonmotive force [20,21]. Thus, large decreases in conductance to protons due to small decreases in Δp would allow Δp to be fairly stable at different rates of proton input [21]. At the same time, due to the second (or higher) order dependence of phosphorylating proton efflux on the electrochemical potential, small changes

in Δp give rise to large decreases in the rate of ATP synthesis. Thus, a decrease in electron transport might lead to a large decrease in phosphorylation without substantially reducing the protonmotive force in continuous light, but because of the linear relationship between Δp and the nonphosphorylating proton efflux, there should be little change in the rate of this process. The unavoidable result would be a reduction in efficiency, since for a lower rate of proton input and ATP synthesis, the leak rate would scarcely change. But in fact, large variation in the electron transport rates are usually not accompanied by these predicted changes in coupling efficiency.

Without an adequate model for understanding the relationship between coupling efficiency and the size of the protonmotive force in continuous light, it is even more difficult to explain our observation that subthreshold energy inputs increased the efficiency of subsequent flash-driven PS II-dependent ATP synthesis. The ATP/e_2 for PS II partial reactions could be increased in flashing light if the energetic contribution from electron transport was superimposed upon an artificially induced ΔpH (Fig. 5). The stimulation of ATP synthesis by a preceding series of flashes under nonphosphorylating conditions (Table IV) further supports the idea that subthreshold levels of energization can enhance the efficiency of subsequent phosphorylation. In this case, it appears likely that a pH difference was also involved, since the membrane potential as monitored by the electrochromic change, which formed during the prephosphorylating flash sequence decayed before the phosphorylating flash sequence was given (data not shown). In all the experiments where a subthreshold energy input was provided prior to the phosphorylating reactions, an increased yield of ATP was seen. An imposed ΔpH (Fig. 7) or prior illumination (Table III) led to better coupling of whole chain electron transfer for small numbers of flashes, and an imposed ΔpH raised the efficiency of the duroquinol to methyl viologen reaction for small numbers of flashes. The crucial distinction to note is that in neither of these cases was the efficiency raised above the maximum seen at high flash number or in continuous light. With PS II reactions on the other hand, the enhanced efficiency is nearly twice that seen in continuous light or that seen after large numbers of flashes. The other critical point is that the added energy input was below the threshold for phosphorylation, as indicated by the ATP synthesis measured in nonilluminated control samples. One might expect then to see an increase in the yield of ATP produced by only the initial flashes, since less electron transport would be required to bring the protonmotive force up to the threshold, but thereafter, no enhancement is anticipated. This expectation was fulfilled for reactions involving PS I, either operating alone (Fig. 6) or in combination with PS II (Fig. 7). It was not fulfilled for PS II operating alone (Fig. 5). There, thus, appears to be a functional difference between the imposed Δp present in the dark before the phosphorylating flash series and the Δp produced by PS II during the phosporylating flash series.

One conclusion to be drawn from these observations is that the low ATP/e_2 values typically measured for PS II may not necessarily indicate the contribution of PS II to the phosphorylation efficiency of the electron transport chain when PS I and PS II are working together. Although the nature of the enhancement of phosphorylation by preillumination is not understood, it seems likely that concurrent illumination of PS I would have a similar effect, and that PS II may well contribute nearly half the ATP synthesized during whole chain electron transport. If the PS II ATP/e_2 ratio is close to 0.6, it is then unnecessary to invoke extra proton transport by a Q-cycle to explain the observed whole chain ATP/e_2 values of almost 1.3. Alternatively, if PS II-coupling decreases drastically at low values of the protonmotive force as suggested here, then perhaps under these circumstances a Q-cycle could contribute an extra proton and maintain the overall efficiency. This would explain why ATP/e_2 values greater than 1.3 are not measured, since the extra proton would be translocated only under conditions associated with poor PS II coupling.

Two unexplained problems remain: why is coupling enhanced as the protonmotive force increases and why is PS II alone apparently unable to generate the condition required for maximum coupling?

To address the first question, the relationships between proton fluxes, phosphorylation, and the

magnitude of the protonmotive force need to be reevaluated. One possibility is that there exists a pathway for noncoupled proton efflux which has a higher-order dependence on the size of the protonmotive force (as has been suggested for photosynthetic bacteria by Clark et al. [30]). Such would be the case if noncoupled proton efflux at low Δp values were accounted for by some slippage in the terminal reactions coupling ATP synthesis to the outward movement of protons. An additional possibility is that there is a dependence of the degree of coupling factor activation on the magnitude of the protonmotive force, and PS II-associated phosphorylation is limited primarily by the number of active coupling factors. However, we have found [31] that the pattern of inhibition of phosphorylation by the energy-transfer inhibitor, triphenyltin chloride, is very similar for PS I and PS II-associated reactions, which strongly suggests that the difference in phosphorylation efficiency cannot simply be explained by a difference in the number of available coupling factors.

Regardless of the mechanism by which phosphorylation efficiency increases with the magnitude of the protonmotive force, it is still necessary to understand why PS II alone is apparently unable to achieve a sufficiently energetic Δp and how this deficiency is remedied by the addition of subthreshold amounts of energy. One explanation may lie with the average distance between PS II and the coupling factor. The PS II reaction center and the associated water-oxidizing apparatus are believed to be largely confined to appressed membranes in the grana stacks (e.g., Refs. 32 and 33) and the coupling factor to be present only on stroma exposed, nonappressed membranes [34]. The distribution of the cytochrome b_6-f complexes is probably more homogeneous [33]. In consequence, any lateral resistance to proton movement between the proton-reducing redox reactions and the coupling-factor complex would have its greatest effect on PS II coupling. If there are two parallel routes for lateral proton movement, one confined to the surface of the membrane and the other through the bulk aqueous phases, it has been argued that the route along the surface would be faster [35]. Transfer of protons through hydrogen-bonded chains might be facilitated by the generation of strong proton sinks such as the coupling factor. Without a lateral potential gradient, proton movement would be random, and diffusion of the proton would be limited by the movement of compensating charges. It might be expected that due to the spatial separation, PS II protons would have a greater likelihood of leaking out through the membrane or being buffered by titratable groups in the bulk aqueous phase. If so, the transmembrane protonmotive force at the coupling factor would not be as great as in the grana region and there would be less ATP synthesis resulting in reduced driving force for lateral proton currents. In line with this reasoning, the addition of a small pH difference would allow the initial turnovers to immediately drive phosphorylation, and the current of protons through the interphase region could be established. However, the nature of such a lateral resistance to proton movement is not obvious: proton binding to membrane-buffering groups should be eventually saturated, and losses via noncoupled efflux should be detected in the measurements of H^+/e^-. While initial proton losses due to noncoupled efflux may be masked by compensating proton uptake due to binding to the membrane, as we observed on the first few flashes in Fig. 3, the usefulness of such a model for the mechanism of PS II coupling awaits further testing.

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