Biochimica et Biophysica Acta 890 (1987) 106–115
Elsevier

BBA 42068

Stoichiometries and energetics of proton translocation coupled to electron transport in chloroplasts

Roger P. Hangarter *, Robert W. Jones **, Donald R. Ort and John Whitmarsh

Department of Plant Biology, USDA / ARS, University of Illinois, Urbana, IL 61801 (U.S.A.)

(Received 21 July 1986)

Key words: Proton translocation; Electron transport; Cytochrome *b/f* complex; Photosystem II; Thylakoid vesicle

We have investigated the stoichiometry of electron transport-driven proton uptake from the external medium and proton release into thylakoid vesicles of chloroplasts during single-turnover flash illumination. Proton uptake from the external medium was measured using a glass pH-electrode. For each electron originating from the oxidation of water by Photosystem II and ultimately forming H_2O_2 via the photoreduction of methyl viologen, approx. 2.7 protons were taken up from the external medium. For each electron originating from the oxidation of duroquinol by the cytochrome *b/f* complex and ultimately forming H_2O_2 via methyl viologen under conditions in which water oxidation was inhibited by diuron, approx. 1.7 protons were taken up from the external medium. It is presumed that for each electron involved in the formation of H_2O_2 that one proton is taken up. Therefore, these results indicate that the electron-transfer reaction from H_2O to Photosystem I involves the uptake of approx. 1.7 protons per electron, and that in the reaction from duroquinol to Photosystem I approx. 0.7 protons per electron are taken up. Proton release within the thylakoid vesicles was measured spectrophotometrically using the pH-sensitive dye neutral red. Flash-induced absorbance changes due to neutral red indicated that for each proton released within the thylakoid vesicle due to water oxidation approx. 1.9 protons appear inside the vesicle as a result of quinol oxidation. The flash-induced formation of ATP was determined by the incorporation of $^{32}P_i$ into ATP. The initiation of flash-induced ATP formation by Photosystem II operating alone required nearly twice as many flashes as were necessary when the Photosystem I-dependent turnover of the cytochrome *b/f* complex was responsible for proton accumulation, and nearly three times more than when Photosystem II and the cytochrome *b/f* complex operated together. This is taken to indicate that both of the reactions that result in proton accumulation within the vesicle contribute energetically to ATP synthesis and contribute in proportion to their measured H^+/e^- ratio. All of these flash-induced measurements, proton uptake from the external medium, proton release inside the vesicle, and the filling of the energetic threshold pool required for the

* Current address: Botany Department, The Ohio State University, 1725 Neil Avenue, Columbus, OH 43210, U.S.A.

** Current address: Center for Arid Studies, University College of North Wales, Bangor, Gwynedd LL57 2UW, Wales, U.K.

Abbreviations: Chl, chlorophyll; Cyt, cytochrome; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DMQ, 2,5-dimethylquinone; DQH_2, duroquinol; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; FeCN, ferricyanide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; P_i, inorganic phosphate; P-700, the primary donor of Photosystem I; PS I, Photosystem I; PS II, Photosystem II.

Correspondence: Dr. J. Whitmarsh, Department of Plant Biology, USDA/ARS, 289 Morrill Hall, University of Illinois, 505 South Goodwin Avenue, Urbana, IL 61801, U.S.A.

onset of ATP synthesis, demonstrate that for each electron transferred from water through the cytochrome *b/f* complex to Photosystem I nearly three protons are accumulated within the thylakoid membrane. Thus, these data indicate that oxidation of quinol by the cytochrome *b/f* complex is able to support the translocation of two protons per electron during flash energization.

Introduction

Proton accumulation within chloroplast thylakoid vesicles is the result of both proton release associated with the oxidation of water by Photosystem II and proton translocation associated with the oxidation of plastoquinol by the cytochrome *b/f* complex. The translocation of protons across the thylakoid membrane includes reactions involved in the uptake of protons from the external medium in addition to the reactions involved in the release of protons into the vesicle. It is clear that one of the reactions resulting in the uptake of protons from the external medium is the reduction and subsequent protonation of plastoquinone by the Photosystem II reaction center [1,2]. Patterned after the original proposals of a Q-cycle by Mitchell [3] and a *b*-cycle by Wikström [4] numerous models are being considered for the oxidation of plastoquinol that involve a proposed second reaction for proton uptake from the external medium during intersystem electron transfer (reviewed in Refs. 5 and 6). Thus, in addition to the proton taken up for each electron involved in plastoquinone reduction by Photosystem II and its subsequent release into the vesicle upon plastoquinol oxidation, these models provide for the uptake and release of an additional proton for each electron transferred through the Cyt *b/f* complex to Photosystem I. In either the Q-cycle or *b*-cycle mechanisms quinol oxidation is envisioned to occur near the inner surface of the membrane allowing for the release of one proton for each of the sequential single electron steps of the oxidation. The models have in common that one of the electrons originating from the quinol is transported to Photosystem I via the Rieske FeS center, cytochrome *f* and plastocyanin, whereas the other is directed through the *b*-cytochromes. Although the alternative mechanisms differ in how the reduced *b*-cytochrome leads to quinone reduction and the attendant uptake of protons from the external medium, they share the salient features that two sequential turnovers of a Cyt *b/f* complex result in the net oxidation of one quinol, the transfer of two electrons to Photosystem I, and the translocation of four protons across the membrane.

There are numerous lines of experimental evidence that support the operation, under certain conditions, of some form of proton translocation coupled to an electron cycling through the Cyt *b/f* complex. Among these is the demonstration of a slow electrogenic reaction occurring between the photosystems indicating transmembrane charge transfer associated with the Cyt *b/f* complex [7]. Electrogenic charge transfer by the Cyt *b/f* complex is evident not only from the existence of the slow phase of electrochromic band shift, but also from the slowing effect of a transmembrane electric potential on the turnover of the Cyt *b/f* complex [8,9]. The involvement of Cyt b_6 redox activity in transmembrane electron transfer is indicated by the kinetic correlation between the cytochrome's redox behavior and the formation of at least a significant portion of the slow electrochromic shift [10–12]. One of the most convincing tests for the operation of a proton-coupled electron cycle within the Cyt *b/f* complex, measurements of the number of protons taken up from the external media and the number of protons translocated into the thylakoid vesicle during quinol oxidation, has yielded equivocal results. This is due in part to experimental difficulties in determining proton-to-electron ratios, and in part to the complexity of the plastoquinol-cytochrome *b/f* interaction. While there are several reports of proton uptake from the external medium [9,10,14–16], and proton release into the thylakoid vesicle [17] indicating the operation of modified Q or *b*-cycle, there are other reports that have failed to detect any additional proton translocation associated with plastoquinol oxidation [18,19]. In the work presented here we examine the crucial issue of whether the protons disappearing from the external medium are accumulated within the thylakoid

vesicle and whether they contribute proportionately to membrane energization. We show that flash-induced proton uptake is indeed greater than that predicted for a linear electron-transport scheme and, through two independent methods, we show that these protons are translocated into the thylakoid vesicles. We measured electron-transport-driven proton uptake from the external medium and, using neutral red, we measured pH changes in the internal space of thylakoid vesicles. We found that the protons taken up from the external medium appeared inside the thylakoids. Furthermore, the protons released into the inner aqueous phase were shown to contribute to the generation of the threshold transmembrane pH difference required for the onset of ATP synthesis.

Materials and Methods

Isolation of chloroplast thylakoid membranes

Thylakoid membranes were isolated from commercial spinach (*Spinacia oleracea* L.) as described elsewhere [20] with the exception of the final resuspension medium. For potentiometric and ATP formation measurements the thylakoid membranes were resuspended in a medium containing 100 mM sorbitol/2 mM $MgCl_2$/20 mM KCl/5 mM Mes-KOH (pH 6.5)/5 mM Hepes-KOH (pH 7.0 and 7.5)/5 mM Tricine-KOH (pH 8.0 and 8.5). For spectroscopic measurements the resuspension medium was the same as described above, except that the buffer used was 2 mM Hepes-KOH at pH 7.0, 7.5 and 8.0. The chlorophyll concentration was determined using the specific absorption coefficients for Chl *a* and *b* published by Ziegler and Egle [21].

Measurement of flash-induced electron transfer

The amount of electron transfer occurring per flash was measured using two different techniques. One method employed a glass pH-electrode to measure the irreversible flash-induced pH change resulting from proton release during water oxidation in the presence of nigericin (3 μM). In these experiments the terminal electron acceptor was ferricyanide (0.2 mM). In order to measure electron transfer through Photosystem II alone 2,5-dimethylquinone (0.2 mM) was added to mediate electron transfer from Photosystem II to ferricyanide and DBMIB (0.5 μM) was present to prevent electron flow from plastoquinone through Photosystem I. The alternate method used a Clark-type oxygen electrode to measure, depending on the electron mediator employed, either oxygen uptake or evolution [20]. In order to ensure an oxygen consumption to electron-transfer ratio of 2.0 for the duroquinol to methyl viologen reaction, excess superoxide dismutase was included to eliminate the oxidation of duroquinol by the superoxide radical anion that is produced as an intermediate in the aerobic oxidation of photoreduced methyl viologen. For technical reasons the oxygen flash yield measurements were made at 20°C, whereas proton flash yield measurements were made at 4°C. However, we demonstrated in an earlier study [20], and confirmed here, that flash-induced electron transfer measured at flash frequencies below 20 Hz was constant over the temperature range from 4 to 20°C. Assuming the predicted exponential saturation of the reaction centers as a function of increasing flash intensity, it was determined from light attenuation experiments with calibrated neutral density filters that the unattenuated xenon flashes (6 μs at half peak height) were greater than 98% saturating. Reaction mixtures and further details are given in the figure and table legends.

Measurement of flash-induced proton uptake from the external medium

The amount of proton uptake was determined by measuring the extent of the reversible pH change of the reaction medium induced by a series of single-turnover flashes. The pH changes were measured with a glass pH-electrode and calibrated by addition of a standard HCl solution. We were able to fit the proton efflux occurring after the flash series to a single component exponential decay using an iterative nonlinear least-squares program [23] to calculate the total extent of proton uptake as described previously [9]. The measurements were done at 4°C. Reaction mixtures and further details are given in the figure and table legends.

Measurement of flash-induced proton release into the thylakoid vesicle

The release of protons rapidly equilibrating with

the internal aqueous space of the thylakoid membranes was measured by a modification of the procedure developed by Junge and co-workers [24–26] using the pH-indicating dye neutral red. Light-induced absorbance changes were measured using a laboratory-built spectrophotometer. The pathlength of the measuring beam was 1 cm. The neutral red-dependent response was measured as the absorbance change at 545 nm in the presence of neutral red minus the absorbance change at 545 nm in the absence of neutral red. We determined that the measuring beam resulted in an average reaction center turnover rate of less than once every 30 s. The actinic xenon flashes were filtered by a red (CS 2-58, Corning Glass Co. *, Rochester, NY) blocking filter. The photomultiplier tube was protected from scattered actinic light by a Corning CS 4-96 blocking filter and a Balzers Interference Filter (DT-Green). For technical reasons the absorbance changes due to neutral red reported were measured at 17°C. Measurements done at 4°C gave similar results. Reaction mixtures and further details are given in the figure and table legends.

Measurement of flash-induced ATP formation

The ATP synthesis resulting from a series of single-turnover flashes was measured as the incorporation of $^{32}P_i$ into ATP according to the method of Smith et al. [27] as described previously [20]. The threshold number of flashes required for the onset of ATP synthesis was taken to be the first flash resulting in more than 0.2 mmol ATP per mol Chl [20]. The measurements were done at 4°C. Reaction mixtures and further details are given in the figure and table legends.

Results

Flash-induced proton uptake from the external medium

The ratio of the number of protons taken up from the external medium to the number of electrons transferred from water through the Photosystem I acceptor Methyl viologen to O_2 was 2.7 ± 0.3 for single turnover flash excitation (Table I). Fig. 1 shows that this H^+/e^- value was maintained at turnover rates as high as 60 s^{-1}.

Fig. 2 shows the dependence of proton uptake and electron transfer on the number of saturating single turnover flashes absorbed by the sample at a flash frequency of 10 Hz. The amount of electron transfer per flash was determined from measurements of the irreversible, uncoupler-insensitive, proton release associated with water oxidation and is independent of flash number. However, the flash-yield of proton uptake per flash is decidedly flash number dependent, so the maximum H^+/e^- ratio of 2.6 is observed only during the first 20–30 saturating flashes. For example, if we calculate the H^+/e^- from the slope of the line between 50 and 80 flashes the apparent value drops to 1.6. While this decline in H^+ uptake most likely reflects an actual change in the H^+/e^- ratio during the later flashes, we believe the actual proton uptake is likely to be somewhat greater

TABLE I

THE RATIO OF PROTON UPTAKE FROM THE EXTERNAL MEDIUM TO ELECTRON TRANSFER FOR DIFFERENT ELECTRON-TRANSPORT REACTIONS

Flash-induced proton uptake was measured with a glass electrode in 4 ml of reaction medium consisting of 100 mM sorbitol/2 mM $MgCl_2$/20 mM KCl/0.5 mM Hepes-KOH (pH 7.5)/150 units per ml superoxide dismutase/0.5 μM nonactin/thylakoid membranes containing 140 nmol chlorophyll. The concentration of methyl viologen was 100 μM when present. When duroquinol (0.2 mM) was used as the electron donor, DCMU (5 μM) was present to prevent Photosystem II turnover. When 2,5-dimethylquinone (0.2 mM) was used to intercept electrons directly from Photosystem II, DBMIB (0.5 μM) was present to prevent electron flow beyond plastoquinone. Flash-induced electron transfer through Photosystem II alone, or from H_2O through PS II + PS I, was measured using a glass pH-electrode as described in Materials and Methods. Flash-induced electron transfer from duroquinol through PS I was measured using a Clark-type oxygen electrode as described in Materials and Methods. The values given are the average ± standard deviation.

Electron-transfer reaction	H^+/e^-
$H_2O \rightarrow$ methyl viologen	2.7 ± 0.3
$DQH_2 \rightarrow$ methyl viologen	1.7 ± 0.2
$H_2O \rightarrow$ DMQ	1.0 ± 0.3

* Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or the University of Illinois and does not imply its approval to the exclusion of other products or vendors which may be suitable.

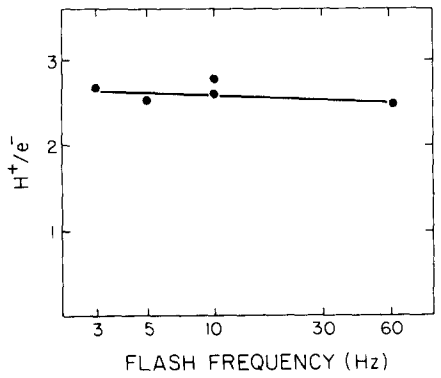

Fig. 1. Ratio of proton uptake from the external medium to electrons transferred from H_2O to methyl viologen as a function of flash frequency. Proton uptake and electron transfer were measured with a glass pH-electrode as described in Table I.

than that estimated from the slope of the line from 50 to 80 flashes shown in Fig. 2. The reason for the underestimation of proton uptake arises from the fact that the system is beginning to enter a steady state, in which the rate of proton uptake is equal to the rate of proton efflux so that additional flashes result in no net proton uptake.

The effects that nonactin- or valinomycin-catalyzed K^+ movements are apt to have on the membrane surface charge raise the possibility of flash-induced proton binding to the external surface of the thylakoid membrane (e.g., Ref. 28). The observation that the initially high H^+/e^- value observed in the presence of nonactin is unaffected by medium pH (Fig. 3) indicates that external proton binding is an unlikely possibility, as do the measurements of proton release into the inner thylakoid space that are presented below.

Since the aerobic oxidation of photoreduced methyl viologen involves the uptake of one proton from the external medium per electron to form H_2O_2, the data in Fig. 2 indicate that about 1.6 protons were taken up from the external medium by the thylakoid membrane for each electron transferred between the photosystems. Flash-induced proton uptake associated with Photosystem I-dependent oxidation of duroquinol by the Cyt *b/f* complex is consistent with this notion. We measured the uptake of 1.7 ± 0.2 protons for each electron transferred to O_2 via photoreduced methyl viologen (Table I). Since in this reaction, as for the one above, one proton is taken up from the external medium for each electron reducing

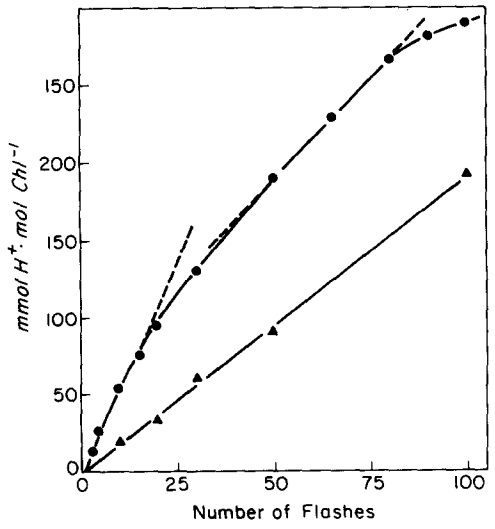

Fig. 2. The dependence of the efficiency of flash-induced proton uptake and electron transport on the number of flashes at a flash frequency of 10 Hz. Proton uptake (●) was measured in a 4 ml reaction mixture consisting of 100 mM sorbitol/2 mM $MgCl_2$/25 mM KCl/0.5 mM Tricine-KOH (pH 8.1)/100 μM methyl viologen/0.4 μM valinomycin/thylakoid membranes containing 175 nmol chlorophyll. Electron transport (▲) was determined as described in Table I using a glass pH electrode.

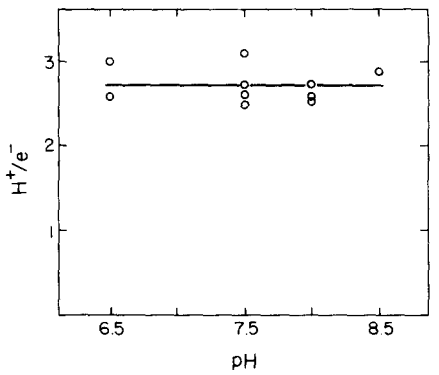

Fig. 3. Ratio of proton uptake from the external medium to electron transferred from H_2O to methyl viologen per flash as a function of pH. Proton uptake was measured with a glass electrode and flashes were given at a frequency of 10 Hz. The reaction conditions were the same as in Table I, except that the buffers used were 0.5 mM Mes-KOH (pH 6.5), Hepes-KOH (pH 7.5), and Tricine-KOH (pH 8.0 and 8.5).

oxygen, about 0.7 protons per electron are taken up by the thylakoid. With either water or duroquinol as the electron donor it is presumed that for each proton consumed in the external medium during H_2O_2 formation one proton is released and retained within the thylakoid vesicle.

Flash-induced proton uptake from the external medium dependent solely on Photosystem II can be measured when dimethylquinone is present to intercept electrons directly from Photosystem II and DBMIB is present to prevent any residual electron flow through Photosystem I [29]. We observed 1.0 ± 0.3 protons taken up from the external medium for each electron transferred from H_2O to dimethylquinone to form dimethylquinol (Table I). The flash-induced proton uptake was entirely reversible after the completion of the flash series indicating that, as with H_2O_2 formation discussed above, one proton is retained within the vesicle for each one consumed externally in dimethylquinone reduction.

Flash-induced proton release into the inner space of thylakoid vesicles

The flash-induced release of protons into the internal space of thylakoid vesicles was monitored using the pH-indicating dye neutral red. Despite the fact that interpreting the neutral red absorbance change is complicated and depends on several simplifying assumptions, Junge and coworkers [24–26] have shown that it can be a useful probe of internal proton release under carefully selected experimental conditions. In order to isolate the portion of the neutral-red signal indicative of this release of protons into the inner space of the thylakoid vesicle, all absorbance changes of neutral red due to proton uptake in the external medium were eliminated by stabilizing the external pH with membrane-impermeant buffers such as Hepes or Tricine. Flash-induced absorbance changes due to components other than neutral red, for example Cyt *f* redox changes, were accounted for by subtracting the absorbance change observed at 545 nm in the absence of neutral red from that measured in its presence. The remaining flash-induced absorbance change observed at 545 nm in the presence of neutral red was sensitive to buffers that penetrate into the thylakoid vesicle and thereby prevent, or substantially decrease, flash-induced pH changes. For example the presence of either 4-(2-hydroxyethyl)-morpholine (1 mM) or imidazole (10 mM) decreases the absorbance change greater than 90%. Furthermore, the neutral red signal was abolished by nigericin (0.33 μM), and diminished more than 85% by FCCP (0.5 μM). High concentrations of gramicidin (5 μM) failed to eliminate the neutral red signal (in some cases more than 30% of the signal remained); it should be noted that gramicidin has been reported to be less effective than other uncouplers in preventing the uptake of basic amines that are used to quantify the transmembrane ΔpH [30].

Recently, De Wolf et al. [28] have shown that under certain circumstances neutral red can undergo flash-induced reduction by Photosystem I, resulting in an absorbance decrease. Using Photosystem I vesicles and ferredoxin and NADP as the terminal electron acceptor De Wolf et al. show that neutral-red absorbance changes can be caused by the reduction of neutral red as well as by pH changes. In our study, using methyl viologen as an efficient Photosystem I electron acceptor, we find no evidence for a redox indicator function for neutral red. We base this conclusion on the following: (1) there is no rapid absorbance decrease indicative of neutral-red reduction; (2) the neutral-red signal is sensitive to the uncouplers nigericin and FCCP; and (3) the neutral-red signal is abolished by permeant buffers. Additional support for this conclusion is provided by the observation that the absorbance change due to neutral red in the electron-transfer reaction from duroquinol to methyl viologen (Fig. 4, trace E) is inhibited 90% by 1.5 μM DBMIB (data not shown). Under these conditions we have shown that at the low flash frequency of 0.2 Hz used, Photosystem I continues to turn over, even though the rapid oxidation of quinol by the Cyt *b/f* complex is inhibited. The sensitivity of a flash-induced neutral red absorbance change to DBMIB indicates that the signal is associated with quinol oxidation by the Cyt *b/f* complex and is not due to the Photosystem I reduction of neutral red under our experimental conditions.

The neutral red-dependent absorbance change associated with flash-induced water oxidation is shown in trace A of Fig. 4. In order to improve

the precision of the measurements the results of 64 flashes given at a frequency of 0.2 Hz were summed for signal averaging. Even at this low repetition frequency the ΔpH does not entirely relax between successive flashes, and as a result the internal pH becomes progressively lower during the flash series. This is an important issue, since the differential absorption coefficient of the protonated and unprotonated forms of neutral red is a sensitive function of pH. The extent to which the internal pH is lowered during the series of 64 flashes will depend upon the number of protons released per electron transferred. In order to evaluate the effect that a lower internal pH brought on by higher H^+/e^- ratios has on the size of the neutral-red absorbance change, we mimicked, with

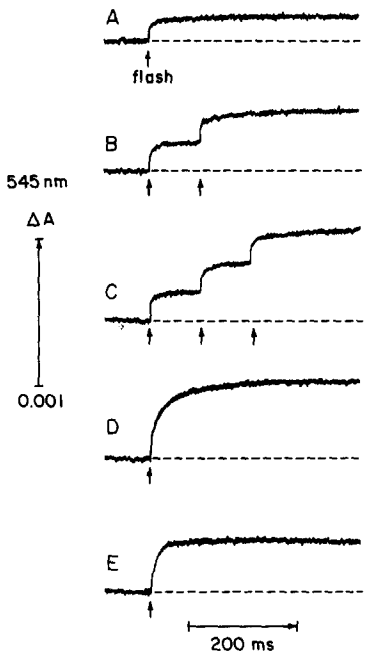

Fig. 4. Flash-induced absorbance changes of neutral red measured at 545 nm. The traces shown are absorbance changes of thylakoids in the presence of 10 μM neutral red minus the absorbance change of thylakoids in the absence of neutral red. The 3 ml reaction mixture consisted of 100 mM sorbitol, 20 mM KCl, 2 mM $MgCl_2$, 2 mM Hepes-KOH (pH 7.5), 0.5 μM nonactin, 100 μM methyl viologen, thylakoid membranes containing to 90 nmol chlorophyll, and the following additions: (A–C) H_2O to dimethylquinone, 500 μM dimethylquinone and 0.5 μM DBMIB; (d) H_2O to methyl viologen; and (E) DQH_2 to methyl viologen, 250 μM duroquinol and 10 μM DCMU. The flashes are indicated by the arrows. The traces shown are the average of 64 flashes given at a frequency of 0.2 Hz.

Photosystem II turnovers, reactions of higher H^+/e^- ratios by delivering groups of two (Fig. 4, trace B) or three (Fig. 4, trace C) flashes spaced 100 ms apart at a flash group frequency of 0.2 Hz. The results show that there is an increase in the average absorbance per flash of about 20% for the 2- and 3-flash groups compared to the train of individual flashes. Thus, in making a quantitative comparison to the Photosystem II-dependent absorbance change with absorbance change dependent on Photosystem I turnover it is important that this feature of neutral-red behavior be accommodated. Additionally, any such comparison must be concerned with the relative abundance of Photosystem II centers relative to the Cyt *b/f* complex. We have shown previously [22] that the number of Photosystem II centers able to oxidize water in the presence of dimethylquinone is very nearly identical to the number of Photosystem I centers and Cyt *b/f* complexes (that is, each is present at about 1.7 mmol per mol Chl). There are numerous indications of Photosystem II centers that under normal conditions have negligible turnover rates [31–34]. While these normally inactive centers can be induced to oxidize water rapidly by the introduction of halogenated benzoquinones, they remain inactive with methyl-substituted benzoquinones such as 2,5-dimethylbenzoquinone used in these experiments at flash frequencies greater than 1 Hz [33].

A comparison of the number of protons released internally during electron transfer from H_2O to methyl viologen, and from duroquinol to methyl viologen is compiled in Table II. The extent of the neutral red absorbance increase due to a single flash given at 0.2 Hz for the reaction H_2O to methyl viologen (Fig. 4, trace D) is approximately the same as that observed for the group of three flashes given to Photosystem II (trace C); duroquinol to methyl viologen (trace E) is about the same as the two flash group (trace B). Thus, these measurements indicate that approx. twice as many protons are released internally for each electron transferred through the Cyt *b/f* complex than are released due to water oxidation. Using faster time resolution than shown here we found that the half-time for the absorbance rise for proton release associated with the Cyt *b/f* complex (approx. 15 ms) was much slower than for

TABLE II

A COMPARISON OF THE NUMBER OF PROTONS RELEASED INTO THE THYLAKOID VESICLE DURING ELECTRON TRANSPORT FROM WATER TO METHYL VIOLOGEN AND FROM DUROQUINOL TO METHYL VIOLOGEN

Flash-induced proton release into the inner space of thylakoid vesicles was estimated from absorbance changes observed in the presence of neutral red. Internal proton release due solely to Photosystem II-dependent water oxidation was measured using dimethylquinone (0.2 mM) to intercept electrons directly from Photosystem II and DBMIB (0.5 μM) to prevent any residual electron flow beyond plastoquinone. The reactions H_2O to methyl viologen, which includes PS II, Cyt *b/f* and PS I, and duroquinol to methyl viologen, which includes Cyt *b/f* and PS I, were measured as described in Fig. 4. Internal proton release due solely to PS II was assumed to be $1H^+$/PS II per flash.

pH	$H_2O \rightarrow$ methyl viologen	$DQH_2 \rightarrow$ methyl viologen
7.0	2.8±0.2	2.0±0.2
7.5	2.7±0.3	1.9±0.3
8.0	2.8±0.2	1.9±0.2

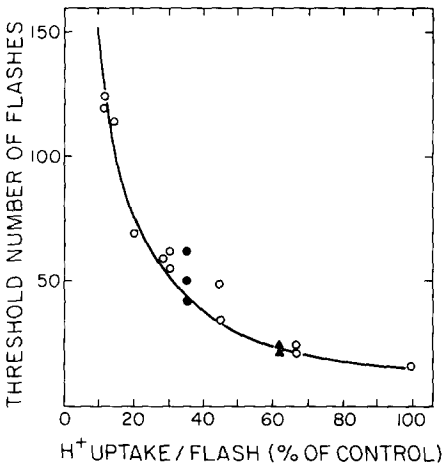

Fig. 5. The relationship between the onset of ATP synthesis and the amount of proton uptake per flash. The threshold number of flashes required for the onset of ATP synthesis was determined as described under Materials and Methods. Proton uptake per flash was varied in the H_2O to methyl viologen (open circles) by changing the intensity of the flashes with neutral density filters. The maximum H^+ uptake observed with saturating flash intensities was 5.4 mmol H^+ per mol Chl per flash. Proton uptake in PS II (solid circles) and PS I (triangles) reactions was measured using saturating flashes. The electron donor and acceptor systems are the same as in Table I, except for the addition of 0.1 mM ADP and 0.5 mM $Na_2H^{32}PO_4$ (10 μCi) in the reaction mixture used to measure ATP synthesis. The flash frequency was 10 Hz. The solid line is the relationship predicted for the filling of a threshold proton pool by the translocated protons (details in the text and Ref. 20).

water oxidation (equal to or faster than 1 ms). Analysis of the proton release kinetics associated with quinol oxidation will be discussed elsewhere (Jones and Whitmarsh, unpublished results).

ATP synthesis associated with the flash-induced uptake and release of protons

We investigated the relationship between the threshold number of flashes necessary to initiate ATP synthesis and the amount of proton uptake from the external medium. In the absence of a transmembrane electric field about 80 mmol. H^+ per mol Chl need to be accumulated by thylakoid membrane vesicles for the initiation of net ATP synthesis [20]. This was found to be true even when the amount of H^+ uptake per flash was varied over a wide range and is documented again by the open circles in Fig. 5, where we measured the number of flashes required for the onset of ATP synthesis for flashes of different intensities. The solid line is the predicted relationship for a uniform threshold pool of protons that must be filled in order for net ATP synthesis to commence [20]. Based on the H^+/e^- ratios reported in Table I, it is anticipated that for the reaction DQH_2 to methyl viologen ($H^+/e^- = 1.7$) about 1.5-times more flashes should be required than for the reaction H_2O to methyl viologen ($H^+/e^- = 2.7$). Similarly, for the reaction H_2O to DMQ ($H^+/e^- = 1$) it is anticipated that about 3-times more flashes should be necessary to initiate net ATP synthesis than for H_2O to methyl viologen. In Fig. 5 the triangles represent the reaction DQH_2 to methyl viologen and the solid circles represent the reaction from H_2O to DMQ. About 22 flashes were required for the onset of ATP synthesis for the DQH_2 to methyl viologen reaction, about 50 for the H_2O to DMQ reaction and about 15 for the H_2O to methyl viologen reaction. Clearly these data points correspond closely to the predicted relationship (solid curve) indicating that the additional proton translocated by the Cyt *b/f* complex contributes in proportion to the measured

H^+/e^- ratios to the energetic proton pool that drives ATP synthesis.

Discussion

The mechanism by which electron transfer from Photosystem II to Photosystem I transports protons across the thylakoid membrane has yet to be determined. A central element in efforts to understand this process is determining the number of protons that are translocated for each electron transferred through the Cyt *b/f* complex. While several previous studies support H^+/e^- values greater than 1 these have measured either proton uptake from the external medium [9,14,15] or release into the thylakoid vesicle [35], but not both. Thus, in these earlier studies the possibility that a fraction of the proton uptake or release was due to light-induced proton binding or debinding due to changes in the membrane surface charge could not be excluded. In the work presented here we measured proton uptake from the external medium and proton release into the internal aqueous phase under similar conditions. The data show that for each electron transferred through the Cyt *b/f* complex to Photosystem I that 1.7–1.8 protons are taken up by the thylakoid membrane, and that 1.7–2.0 protons are released into the inner aqueous space of the thylakoid membrane vesicle. These results demonstrate that, for each electron transferred through the Cyt *b/f* complex to Photosystem I an additional proton can be transferred across the thylakoid membrane. Moreover, these high H^+/e^- values for the flash-induced turnover of the Cyt *b/f* complex were not detectably affected by the pH of the external medium (Fig. 3 and Table II).

Independent evidence for the translocation of an extra proton by intersystem electron transfer is found in the onset behavior of ATP synthesis. In an earlier paper Hangarter and Ort [20] studied the filling of the threshold proton pool that drives ATP synthesis and found that the pool became sufficiently energized for ATP synthesis after about 80 mmol H^+ per mol Chl had been accumulated by the membrane vesicles regardless of the rate of accumulation. This was true when the rate of proton accumulation was varied by any of several different treatments that lowered the number of protons taken up per flash including the inhibition of either Photosystem II-dependent or Photosystem I-dependent proton uptake. Our data presented here (Fig. 5) show that Photosystem II-driven proton accumulation required approx. twice the number of flashes to fill the threshold pool as did the Photosystem I reaction from DQH_2 to methyl viologen that includes the Cyt *b/f* complex. The most straightforward explanation of these data is that intersystem electron transport translocates two protons for each proton released by PS II and that the additional proton translocation contributes to the electrochemical proton potential that drives the synthesis of ATP.

While the data shown here demonstrate that oxidation of quinol by the Cyt *b/f* complex results in the translocation of an additional proton, it must be emphasized that in electron transport under conditions of continuous high light intensity the question of additional proton translocation by the Cyt *b/f* complex remains unresolved. Several investigators support the suggestion that the extra proton translocation associated with the electrogenic steps of the Q- or *b*-cycle may be energetically feasible only when the transmembrane electrochemical potential is small [8,9,16]. In fact, our results indicate that even with single-turnover flashes given at relatively low frequency (10 Hz) and in the presence of an ionophore that prevents the formation of an electric potential, the extra proton translocation associated with the Cyt *b/f* complex is fully present during the first 20–30 turnovers after which the H^+/e^- probably declines gradually toward the steady-state value.

Acknowledgments

We thank Colin Wraight for his critical comments. This research was supported in part by grants from NSF (PCM 83-14461) (D.R.O.) and USDA/CRGO (AG82-CRCR-101044) (J.W.).

References

1 Crofts, A.R. and Wraight, C.A. (1983) Biochim. Biophys. Acta 726, 149–185
2 Polle, A. and Junge, W. (1986) Biochim. Biophys. Acta 840, 257–264
3 Mitchell, P. (1976) J. Theor. Biol. 62, 327–367

4 Wikström, M.K.F. and Krab, K. (1980) Curr. Top. Bioenerg. 10, 51–101
5 Cramer, W.A., Widger, W.R., Black, M.T. and Girvin, M.E. (1986) Topics in Photosynthesis, Vol. 8: Electron Transfer Mechanisms and Oxygen Evolution (Barber, J., ed.), in the press
6 Joliot, P. and Joliot, A. (1986) Photosynth. Res. 9, 113–124
7 Joliot, P. and Delosme, R. (1974) Biochim. Biophys. Acta 357, 267–284
8 Bouges-Bocquet, B. (1981) Biochim. Biophys. Acta 635, 327–340
9 Graan, T. and Ort, D.R. (1983) J. Biol. Chem. 258, 2831–2836
10 Velthuys, B.R. (1978) Proc. Natl. Acad. Sci. USA 75, 6031–6034
11 Jones, R.W. and Whitmarsh, J. (1985) Photobiochem. Photobiophys. 9, 119–127
12 Jones, R.W. and Whitmarsh, J. (1987) in Proceedings of the VIIth International Congress on Photosynthesis (Biggins, J., ed.), Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, in the press
13 Joliot, P. and Joliot, A. (1986) Biochim. Biophys. Acta 849, 211–222
14 Fowler, C.F. and Kok, B. (1976) Biochim. Biophys. Acta 423, 510–523
15 Olsen, L.F. and Cox, R. (1979) Eur. J. Biochem. 95, 427–437
16 Rathenow, M. and Rumberg, B. (1980) Ber. Bunsenges. Phys. Chem. 84, 1059–1062
17 Velthuys, B.R. (1980) FEBS Lett. 115, 167–170
18 Olsen, L.F., Telfer, A. and Barber, J. (1980) FEBS Lett. 118, 11–17
19 Hope, A.B. and Matthews, D.B. (1983) Aust. J. Plant Physiol. 10, 363–372
20 Hangarter, R. and Ort, D.R. (1985) Eur. J. Biochem. 149, 503–510
21 Ziegler, R. and Egle, K. (1965) Beitr. Biol. Pflanz. 4, 11–37
22 Whitmarsh, J. and Ort, D.R. (1984) Arch. Biochem. Biophys. 231, 378–389
23 Bevington, P. (1969) in Data Reduction and Error Analysis for the Physical Sciences, pp. 134–186, McGraw-Hill, New York
24 Junge, W., Ausländer, W., McGeer, A.J. and Runge, T. (1979) Biochim. Biophys. Acta 546, 121–141
25 Hong, Y.Q. and Junge, W. (1983) Biochim. Biophys. Acta 722, 197–208
26 Theg, S.M. and Junge, W. (1983) Biochim. Biophys. Acta 723, 294–307
27 Smith, D.J., Stokes, B.O. and Boyer, P.D. (1976) J. Biol. Chem. 251, 4165–4171
28 De Wolf, F.A., Groen, B.H., Van Houte, L.P.A., Peters, F.A.L.J., Krab, K. and Kraayenhof, R. (1985) Biochim. Biophys. Acta 809, 204–214
29 Flores, S. and Ort, D.R. (1984) Biochim. Biophys. Acta 766, 289–302
30 Portis, A.R. and McCarty, R.E. (1976) J. Biol. Chem. 251, 1610–1617
31 Lavergne, J. (1982) Photobiochem. Photobiophys. 3, 273–285
32 Melis, A. (1985) Biochim. Biophys. Acta 808, 174–180
33 Graan, T. and Ort, D.R. (1986) Biochim. Biophys. Acta 852, 320–330
34 Chylla, R., Garab, G. and Whitmarsh, J. (1987) in Proceedings of the VIIth International Congress on Photosynthesis (Biggins, J., ed.), Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, in the press
35 Hope, A.B., Handley, L. and Matthews, D.B. (1985) Aust. J. Plant Physiol. 12, 387–394