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PROTON TRANSLOCATION IN CHLOROPLASTS AND ITS RELATIONSHIP TO ELECTRON TRANSPORT BETWEEN THE PHOTOSYSTEMS

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SUMMARY

Using dark adapted isolated spinach chloroplasts and sequences of brief saturating flashes the correlation of the uptake and release of protons with electron transport from Photosystem II to Photosystem I were studied. The following observations and conclusions are reported:

(1) Flash-induced proton uptake shows a weak, damped binary oscillation, with maxima occurring after the 2nd, 4th, etc. flashes. The damping factor is comparable to that observed in the O_2 flash yield oscillation and therefore explained by misses in Photosystem II.

(2) On the average and after a steady state is reached, each flash (i.e. each reduction of Q) induces the uptake of $2H^+$ from outside the chloroplasts.

(3) Flash induced proton release inside the chloroplast membrane shows a strong damped binary oscillation with maximum release occurring also after the 2nd, 4th, etc. flashes.

(4) This phenomenon is correlated with the earlier reported binary oscillations of electron transport [2] and shows that both electrons and protons are transported in pairs between the photosystems.

(5) In two sequential flashes $4H^+$ from the outside of the thylakoid and $2e^-$ from water are accumulated at a binding site B. Subsequently, the two electrons are transferred to non-protonated acceptors in Photosystem I (probably plastocyanin and cytochrome *f*) and the $4H^+$ are released inside the thylakoid.

(6) It is concluded that a primary proton transporting site and/or energy conserving step located between the photosystems is being observed.

INTRODUCTION

In a previous paper, results were presented which suggested that the H^+/e ratio in spinach chloroplast with ferricyanide as the acceptor is 3 [1]. Two of these protons were translocated via electron transport in the chain connecting the photosystem and one was released from the O_2 evolution system.

In this paper, details of the coupling between electrons and protons in the chain connecting the photosystem are presented. For discussion purposes, some information

about the proton release associated with O_2 evolution is also presented. A complete description of the latter is deferred until the next paper of this series.

MATERIALS AND METHODS

Spinach chloroplasts were isolated as previously described [2]. Flash induced ΔpH changes were measured by an electrode technique as discussed in previous papers [1, 3]. Measurements of the oxidation and reduction of *P*-700 were made according to Marsho and Kok, [4]. Other experimental procedures are described in the text. O_2 flash yields were measured by a method described by Kok, Forbush and McGloin [5].

RESULTS

In most of the reported experiments, we measured the pH changes induced by a sequence of intensity saturated flashes spaced 1 s apart in a chloroplast suspension; the samples were dark adapted 5 min prior to measurement. When ferricyanide is used as an acceptor the following overall reaction takes place:

$$\tfrac{1}{2}H_2O + Fe(CN)_6^{4-} \rightarrow \tfrac{1}{4}O_2 + H^+ + Fe(CN)_6^{3-}$$

On the average, $1/4\,O_2$ and one proton are released per Photosystem II trapping center

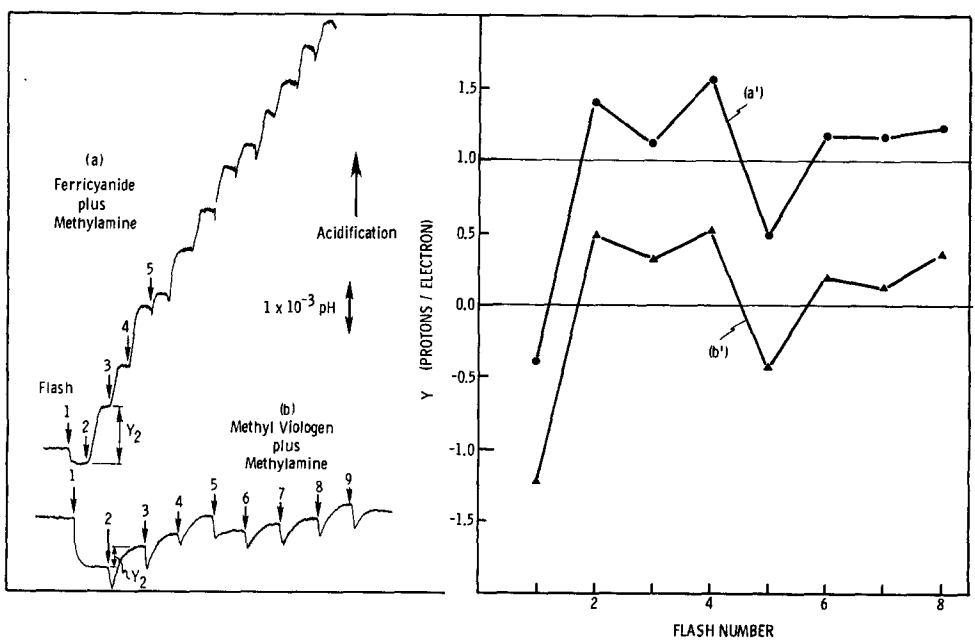

Fig. 1. Changes of pH induced by short saturating flashes in a spinach chloroplast suspension. Actual data obtained in presence of 1.0 mM $K_3Fe(CN)_6$. (a') plot of the proton yields Y (computed as indicated for Y_2) as a function of flash number. (b) and (b'), the same for a sample containing 100 μM methylviologen. The suspension medium contained in addition 0.4 M sucrose, and 0.05 M NaCl and 0.03 M methylamine. Chlorophyll concentration was between 10–20 μg/ml. Flashing rate was 1/s.

as a flash moves one electron from water to ferricyanide. The acidification which occurs can then be used to calibrate the system in protons per electron [3]. In Fig. 1a we see such an experiment where the protons are allowed to move freely due to the presence of an uncoupler. With the exception of the first one, all flashes induce a net acidification which is completed in approx. 0.3 s. The size of the pH shift (Y_n) however, varies with the flash number, as can be seen more clearly in Fig. 1a', where the individual flash yields of Fig. 1a are plotted. Each data point is the net pH change measured from the pH level just prior to each flash to the level just before the next flash (as shown for Y_2 in Fig. 1a) divided by the average pH change in 12 or more flashes. This average, defined as Y_{ss}, reflects 1 H^+/e^- and is used to normalize all succeeding data.

The proton flash yields oscillate with a period of 4, but the pattern is considerably more complicated than a typical O_2 flash yield pattern. Yields Y_2, Y_4, and Y_6 are high, Y_1, Y_5 and Y_9 are minimal and Y_3, Y_7, etc. are intermediate. A damping of the oscillation is observed similar to that measured with O_2 flash yield. Therefore, after a sufficient number of flashes, all yields approach one H^+/e^-. The experiment was repeated with methylviologen as the acceptor; the actual data are presented in Fig. 1b and the individual flash yields are plotted in 1b'. Almost identical oscillations (both amplitude and phase) are obtained. The rapid initial uptake is neglected and only the steady state pH level at the end of each dark period is considered. As was expected, however, with methylviologen the yields oscillate about zero $Y_{ss} = 0$. The reoxidation

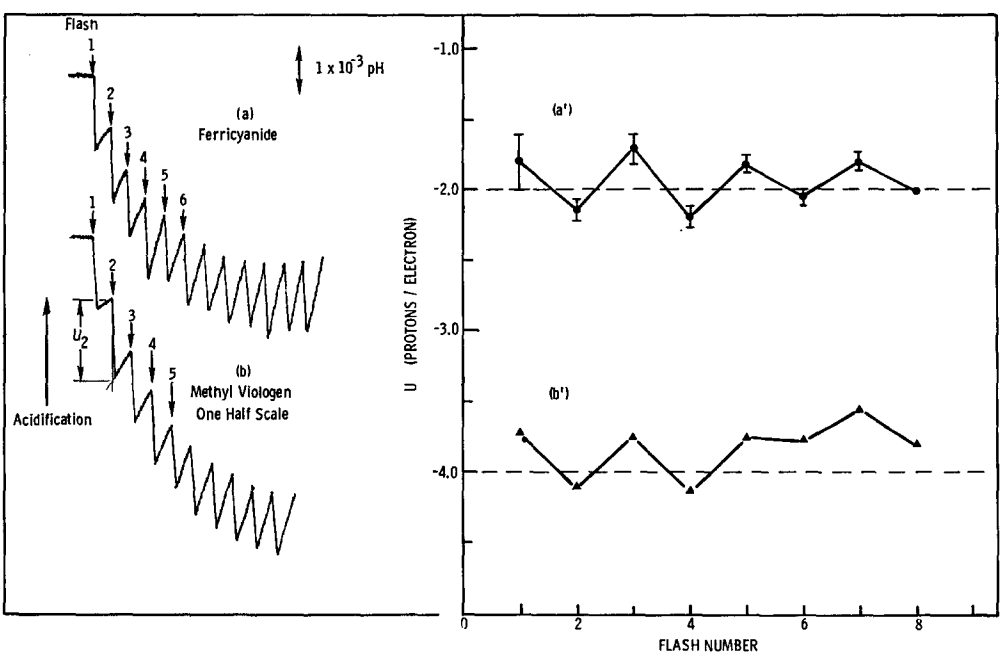

Fig. 2. pH changes induced by short saturating flashes in a chloroplast suspension containing no uncoupler. Conditions were otherwise identical to those in Fig. 1. (a) and (a'), actual data and computed uptake yields U as a function of flash number respectively, observed in the presence of 1.0 mM ferricyanide. (b) and (b') the same except for $K_3Fe(CN)_6$ being replaced by 100 μM methylviologen.

of methylviologen via hydrogen peroxide takes up one proton per electron, exactly offsetting the H^+ liberation from O_2 evolution.

The experiments of Fig. 1 were repeated minus the uncoupler and are presented in Fig. 2. In this case, each flash induced a rapid proton uptake ($t_{\frac{1}{2}} = 30$ ms) (U_n) followed by a slow recovery ($t_{\frac{1}{2}} \cong 2$ s). The details of this measurement were reported previously [3]. The actual data obtained with ferricyanide and methylviologen are shown in Figs. 2a and 2b, respectively. The amount taken up (U_n) on each flash was obtained as shown for flash 2 in Fig. 2a and plotted as a function of flash number in Figs. 2a′ and 2b′, respectively. Each data point in a′ and b′ represent the average of 10 separate measurements with different samples. In each experiment, the data were normalized to the average steady state yield Y_{ss} observed with a parallel sample containing methylamine and ferricyanide.

Note that the uptake (U_n) again oscillates, but in contrast to the case with uncoupler, only a periodicity of two is evident. The extent of the oscillation is the same with both acceptors, but is a small fraction of the total uptake ($\pm 10\,\%$). A damping is observed similar to that obtained with the uncoupled chloroplasts. Uptake is maximal after the 2nd, 4th, etc. flashes exactly in phase with the maximum yields of release in the uncoupled chloroplasts.

The average amounts of proton uptake following each flash with ferricyanide ($2\ H^+/e^-$) and methylviologen ($4\ H^+/e^-$) and its implication for energy conservation were described previously, [1]. This earlier paper showed that when ferricyanide is the acceptor, the only pH changes that occur are associated with the O_2 evolving system and with the electron transport between the photosystems. On the average, one H^+/e^- per flash is released by the O_2 evolution system and $2\ H^+/e^-$ per flash are taken up in electron transport between the photosystems and translocated to the inside of the thylakoid. This implies that the oscillation observed with ferricyanide as the acceptor must originate either in the O_2 system or between the photoacts. With methylviologen as the acceptor both photosystems produce proton exchange. However, the oscillation (of release Y as well as uptake U) are identical with the two acceptors and therefore are not related to Photosystem I.

Analyses of pH changes

In the following analyses, it is assumed that Photosystems II and I and associated electron transport components are located vectorally in the thylakoid membrane in a manner facilitating proton accumulations inside the thylakoid. (See review by Trebst for complete discussion [6]).

The electrode measures only the pH outside the chloroplasts. When the equilibration across the membrane is much faster than the darktime between the flashes, such as when uncoupler is present, a sum of the events occurring outside and inside are observed (1a′). The rate of equilibration is in fact too rapid to separate these processes. However, when chloroplasts are not uncoupled, the uptake rate ($t_{\frac{1}{2}} \cong 30$ ms) is much faster than the leak rate ($t_{\frac{1}{2}} > 1$ s), thus allowing an accurate determination of the extent of proton uptake as plotted in Figs. 2a′ and 2b′. From these two measured quantities, the total number of protons released inside H_n on each flash can be obtained by subtracting the uptake U_n in Fig. 2a′ from the net change Y_n observed in Fig. 1a. The result of this calculation is shown in Fig. 3. Since the oscillation of U_n is clearly binary but weak, and the oscillation of Y_n is large and complex,

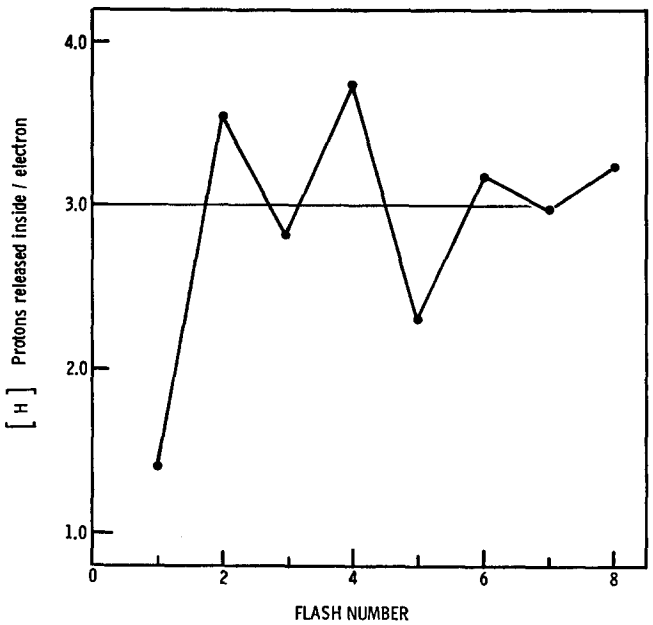

Fig. 3. Number of protons per electron released inside the chloroplast membrane plotted as a function of flash number. H_n was computed by subtracting the proton uptake U_n (Fig. 2a') seen in absence of uncoupler from the change in proton concentration (Y_n) also protons/electron measured with uncoupler (1a').

the oscillations of the latter must originate, for the most part, inside the chloroplast during the release of protons. This release (see Fig. 3) is due to two processes: (a) proton liberations during O_2 evolution (1 H^+/e^-); and (b) proton release coupled to electron transport to *P*-700 (2 H^+/e^-). As discussed in a previous paper [1] the average number released inside is 3 H^+/e.

Bouges-Bocquet showed in a previous paper that electrons are transported in pairs between the photosystems [7]. Therefore, if there is coupling between proton transport and electron transport a binary oscillation in the proton released should also be observed. An analysis of the patterns of Figs. 1a' and 3 show that they consist of the linear combinations of a period of 4 and a period of 2.

Location of sources of oscillation

In order to analyze the respective contributions of those possible sites, the following experiments designed to isolate the different parts of the electron transport system were carried out.

In Fig. 4a and 4a' data are presented which show what happens when O_2 evolution is inhibited and the normal electron transport bypassed by the Photosystem II donor semicarbazide [8]. The conditions of the experiment are otherwise identical to that of Fig. 1a and 1a'. The advantage of this donor is that it reacts only slowly with the acceptor ferricyanide, allowing both to be used in the same reaction mixture. Semicarbazide was reported previously to act only in Tris-treated chloroplasts [8]. However, we noticed that when used with a sufficiently high concentration (approx.

100 mM), it will inhibit O_2 evolution and act at the same time as a Photosystem II donor.

The resulting pattern, presented in Fig. 4a', is devoid of the periodicity of 4 but a period of two remains. This suggests that the period of 4 and the period of two originate in different parts of the electron transport system. The damping factor remains unchanged, however, implying a dependence upon Photosystem II function. The average net proton release was also 1 H^+/e^- which is assumed to come from the oxidation of semicarbazide. Parallel experiments with the O_2 polarograph showed that no O_2 was evolved during flash illumination with this concentration of semicarbazide.

It has been established that the site of action of ferricyanide is strongly dependent upon chloroplast membrane integrity. In freshly prepared chloroplasts, it acts mainly as a Photosystem I acceptor, implying a fully functional electron transport chain [9]. In fragmented (e.g., sonicated or osmotically shocked) chloroplast, the site of action is much closer to Photosystem II and Photosystem I is bypassed [10]. A previous paper in this series also showed that the link between the two photosystems is functional (*P*-700 totally reduced in 1/s flashes) in freshly prepared chloroplasts with ferricyanide as the acceptor [1].

Osmotically shocked chloroplasts were prepared and the effect on the flash induced pH changes were measured. These structurally degraded chloroplasts were

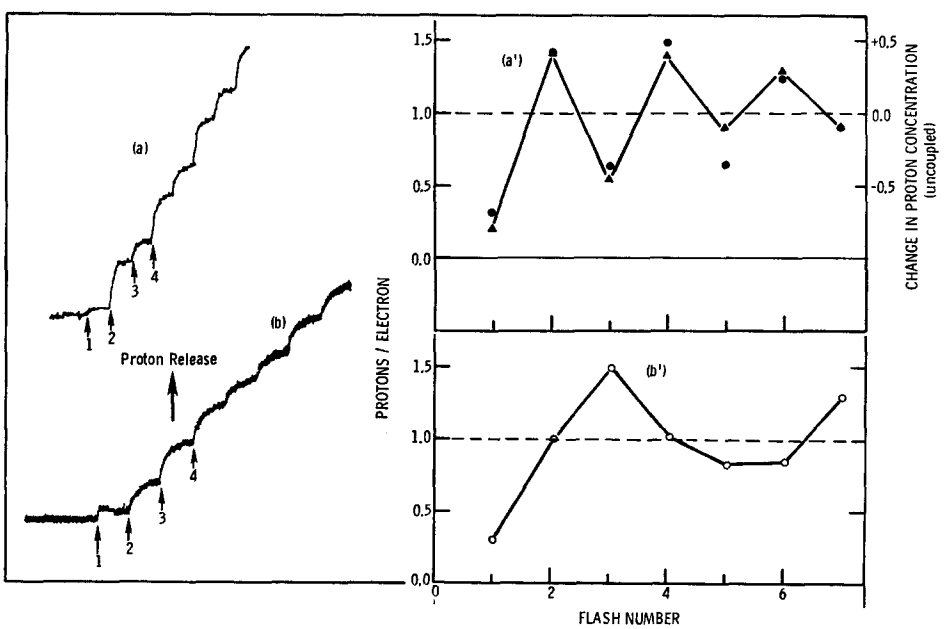

Fig. 4. pH changes induced by flashes in chloroplast suspensions containing semicarbazide (100 mM) (a and a') and in chloroplasts osmotically shocked prior to measurement (b and b'). Both samples were dark adapted 5 min prior to measurement and contained 1.0 mM $K_3Fe(CN)_6$. The suspension medium containing semicarbizide was otherwise identical to that of Fig. 1a. Chloroplasts were osmotically shocked in a suspension medium containing only 10 mM NaCl and 1.0 mM $K_3Fe(CN)_6$. The closed circles on Fig. 4a' were computed by subtracting the yields 4b' from the uptake U_n in 1a'.

obtained by suspension in a medium containing only 10 mM NaCl and 1 mM ferricyanide. As is evident in Figs. 4b and 4b', and in contrast to 4a and 4a', the period of two is lost, implying that ferricyanide is now able to bypass the source of the binary oscillations. Parallel experiments were carried out which showed that *P*-700 was totally oxidized in the dark by ferricyanide and did not become re-reduced in the light. In addition, the O_2 yield pattern was normal [5]. Chloroplasts were uncoupled by the treatment making the addition of uncoupler unnecessary. The pattern shown in Fig. 4b' which is dominated by a periodicity of 4, probably represents the release of protons from the O_2 evolution system. A complete description of the characteristics of the oscillations and a discussion of their significance in the mechanism of O_2 evolutions is deferred until the succeeding paper of this series.

Based upon the results of Fig. 4, it is clear that the oscillatory pattern in Fig. 1a' can be interpreted as a linear combination of a period of 4 and a period of two oscillation. If the oscillations in Fig. 4a' and 4b' actually represent the isolated components, it follows that the period of two pattern (4a') should also be obtainable by subtracting 4b' from 1a'. This procedure was carried out and the result plotted on Fig. 4a' (closed circles). The resulting pattern as anticipated does oscillate with a period of two and has both amplitude and phase very similar to that obtained with semicarbazide. It oscillates about zero as an average, meaning that no net protons are being released in the process. Thus it follows that the procedures described above and reported in Fig. 4 do clearly separate the sources of the oscillations.

The protons released inside the thylakoid as a result of electron transport between the photosystems can also be calculated. In Fig. 3, the total protons released inside during a flash sequence due to both O_2 evolution and electron transport between the photosystems have already been presented. By subtracting the protons released during O_2 evolution (Fig. 4b') from this data, the required information can be obtained. The result of this calculation is presented in Fig. 5. As described earlier, the average number released is 2 H^+/e^-. Maximum yields of proton release occur on the 2nd, 4th, etc. flashes in phase with the electrons transported from Photosystem II to Photosystem I during flash illumination, implying a common origin [7].

In order that a quantitative determination of the electron transport between the

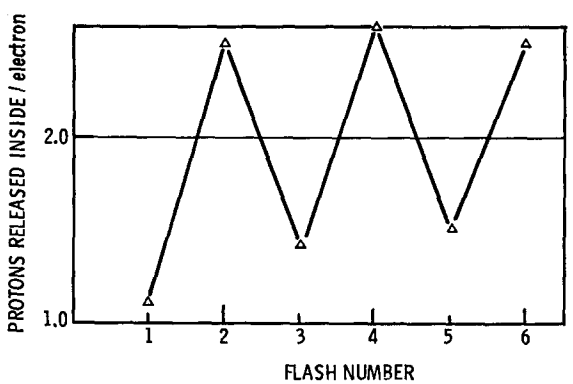

Fig. 5. Number of protons per electron released inside the chloroplast membrane due only to electron transport between the photosystems (see text for discussion).

photosystems could be made under similar conditions of the pH measurements, part of the experiments of Bouges-Bocquet were repeated [7]. In contrast to those measurements the transport of electrons was observed more directly by measuring spectrophotometrically changes in the redox level of *P*-700. Since the spectrophotometer has a response time of approximately 1/4 s, only the steady state level attained before and after each flash (net change) could be measured accurately. The full extent of $P_{red}-P_{ox}$ was measured before and after each flash sequence by totally oxidizing *P*-700 with white light followed by its total reduction in the succeeding dark period. This deflection defined as 100 % *P* was subsequently used to normalize the level of *P* during sequences of flashing light. 100 % *P* was assumed to be equal to one equivalent. Flash intensity was adjusted to produce maximum response of the flash induced pH change shown in Fig. 2a. Although not measured, it was further assumed that *P*-700 was at least 90 % oxidized on each flash.

Actual data obtained under optimal conditions for producing the flash induced oscillation of *P*-700 are presented in Fig. 6. This particular sample contained methylviologen as the acceptor and the flash spacing was 1.5 s. *P*-700 was initially 90 % oxidized by preilluminating with 720 nm light for 1 min, then allowed to recover to different levels before initiating the flash sequence. When *P*-700 was $\leqslant$ 60 % reduced (60 % in Fig. 6), but with sufficient dark time ($>$ 10 s) it became only 5–15 % more oxidized following the first flash. After the second flash, it was considerably more reduced (approx. 80 %) and following the third flash, *P*-700 again was more oxidized. When the redox level of *P*-700 was subsequently plotted as a function of flash number

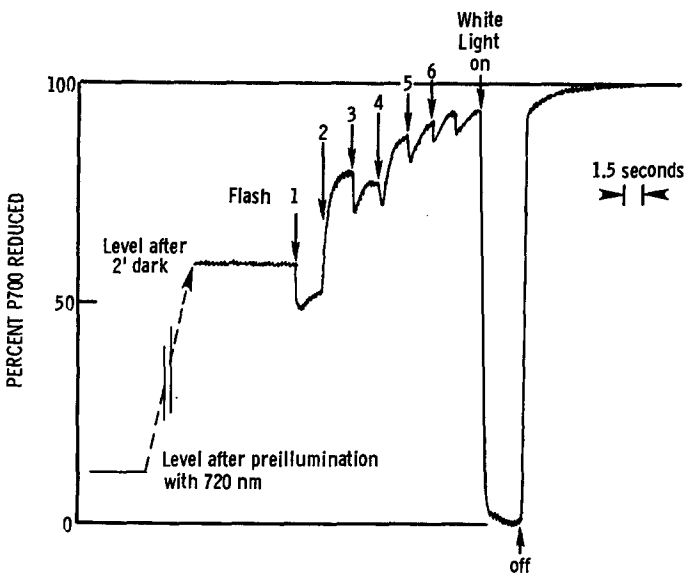

Fig. 6. Flash-induced changes in the oxidation-reduction level of *P*-700 in a chloroplast suspension containing 100 μM methylviologen as the acceptor. The suspension medium was identical to that of Fig. 2b. The chlorophyll concentration was approximately 100 μg/ml in a 1-mm pathlength cuvette. The sample was preilluminated with 720 nm light for 1 min followed by a 2 min dark recovery period. Flashing rate was 1 per 2 s. Maximum deflection of *P*-700 (100 %) was obtained by total oxidation in strong white light followed by total reduction in a subsequent dark period.

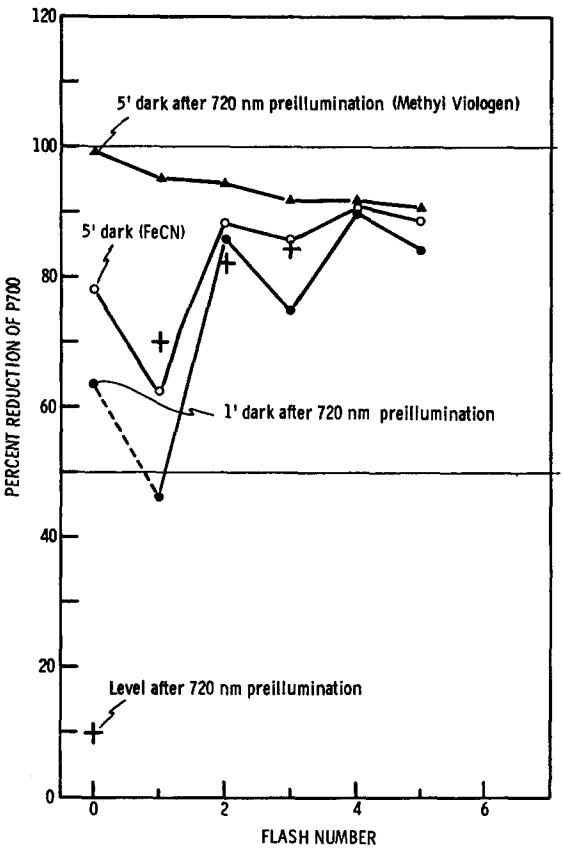

Fig. 7. Flash-induced changes of the oxidation-reduction level of *P*-700 in chloroplasts with several different measuring conditions. All samples were preilluminated with 720 nm light for 1 min prior to dark recovery and subsequent flash illumination, ▲—▲, sample containing methylviologen 100 μM) and was dark adapted 5 min after preillumination. ○—○, sample containing $K_3Fe(CN)_6$ (1.0 mM) and dark adapted 5 min after preillumination. ●—●, sample containing methylviologen and dark adapted 1 min prior to measurement. +, sample containing methylviologen (100 μM). Flashes were initiated immediately after shutting off the 720 nm light.

(Fig. 7, closed circles) its level was observed to oscillate with a period of two, but with an apparently severe damping factor. After a sufficient number of flashes, a steady state level was approached amounting to approx. 90 % reduction.

When *P*-700 was allowed to become more reduced (above 60 %) preceding a flash-sequence, the extent of the oscillation became smaller finally becoming negligible when fully reduced. The level of *P*-700 after the first flash is particularly sensitive to its initial preflash level. Both of these points are demonstrated in Fig. 7 (closed triangles and open circles) for 5 min dark-adapted chloroplast suspensions containing methylviologen and ferricyanide, respectively. In the former, *P*-700 became totally reduced in the dark after 5 min. Little or no net change in the redox level of *P*-700 occurred during a subsequent flash sequence, except for a gradual oxidation to the 90 % reduced level. In the latter, where *P*-700 became only 80 % reduced during a 5 min or longer dark period the oscillations occurred with diminished amplitude.

Also, no oscillations occurred when a flash sequence was initiated simultaneously with the shutting off of the 720 nm illumination. *P*-700 was approx. 10 % reduced before the first flash, became 80 % reduced following the first flash and approached 90 % reduction after 4 flashes. This result is shown in Fig. 7 (crosses).

Starting from the same 90 % oxidized level, full oscillatory behavior could be measured after a dark period of 5–10 s (not shown), even though *P*-700 had recovered to only 30 % reduced. This shows that the capacity to observe the oscillation is time dependent as well as dependent upon the initial redox level of *P*-700.

The results described above are consistent with a model which connects *P*-700 to two secondary donors who are in turn connected to Photosystem II through a common electron transport pool. According to Marsho and Kok, these donors are probably cytochrome *f* and plastocyanin and have equilibrium constants in the dark of $K_{\text{cyt}\,f}$ *P*-700 = 50–100 and K_{pc}-*P*-700 ≅ 20 or 100–120 mV and 80 mV, respectively [4].

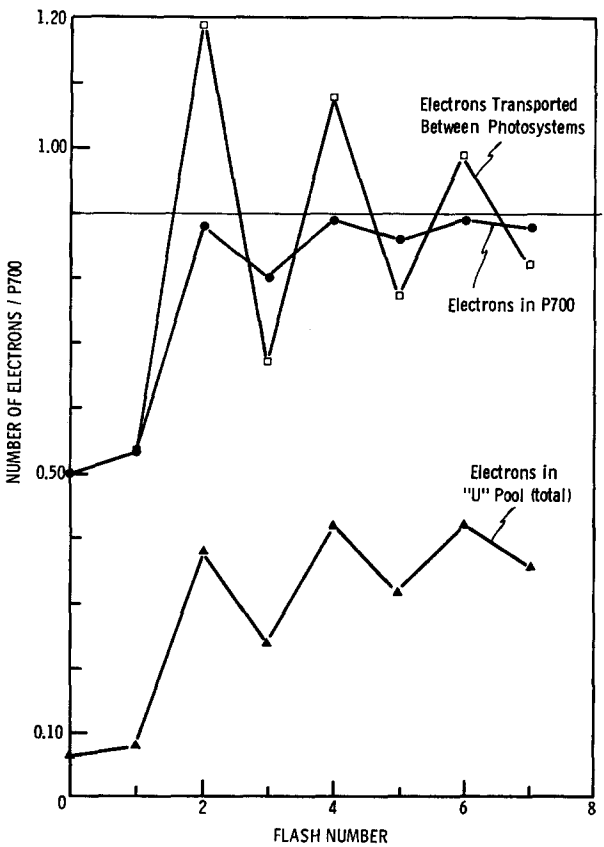

Fig. 8. Average number of electrons in *P*-700 (closed circles) and in the *U* pool (plastocyanin and cytochrome *f*) (triangles) after each flash and the number of electrons transported between the photosystems (open squares) as a function of flash number. Calculations were based on the model described in the text. Assumed initial conditions for electron transport from Photosystem II to Photosystem I. B, 70 % and B^-, 30 %; *P*-700, 50 % reduced.

Therefore, when *P*-700 is totally reduced, enough equivalents are available in the secondary pool (in addition to those coming from Photosystem II) to totally reduce it following oxidation by the first or any subsequent flash. No oscillations of *P*-700 would occur. On the other hand when *P*-700 is $\leqslant$ 50 % reduced, only a small fraction of an equivalent would be available from secondary pool. Hence, when *P* is reduced by 50 % or less, its level after the first flash is a good measure of the number of electrons transferred from Photosystem II following the flash.

According to the data of Bouges-Bocquet, electrons may be transported only in pairs between the photosystems [7]. The model presented to explain the data assumes that the electrons are paired on an unspecified secondary acceptor B of Photosystem II which can exist in three states: completely oxidized B, singly reduced B^- and double reduced B^{2-}. The B^{2-} state is unstable and transfers both its electrons to Photosystem I.

Because electrons are transported to Photosystem I on the first flash after long dark, some of B^- is assumed to be stable in the dark. In the data presented in Fig. 7 (closed circles) around "1/2 electron/*P*-700" was transported on the first flash. If one assumes a 10 % damping factor (misses) then about 27–30 % of the Photosystem II traps are initially in the B^- state and capable of transporting electrons on the first flash. Thus it follows that approx. 70 % of the traps are in the B state.

By combining the model for *P*-700 and its associated donors with the constraints on electron transport just described, the expected behavior of *P*-700 and the donor system during flash excitation can be calculated. It was assumed that the Photosystem II acceptor Q is equal to *P*-700 on an electron basis and that *P*-700 was initially 50 % reduced and totally oxidized by every flash. In Fig. 8, the results of such calculations are presented. It shows the number of electrons/*P*-700 which are (1) transferred from Photosystem II to Photosystem I according to the B model, (2) present in the secondary "*U*" pool and (3) present in *P*-700 as a function of flash number.

There is a remarkable flash by flash resemblance of the calculated level of *P*-700 to the actual data shown in Fig. 6. In addition, the model predicts, in agreement with experimental data, that the steady state reduction level of *P*-700 should be around 90 % due to the 10 % damping factor. This model quantitatively accounts for most, if not all, of the data in Fig. 6.

DISCUSSION

In Figs. 5 and 8, the number of protons transferred to the inside of the chloroplast membrane and the electrons transferred to Photosystem I, respectively, are presented. Firstly, as already mentioned, there are on the average $2H^+$ translocated per electron released to Photosystem I. Second, a comparison of these figures shows that every flash induces the release of two protons per electron transported to the inside. Therefore, at the site of release stoichiometry between electron and proton transport is always maintained. This implies that the B^{2-} state is associated with $2H^+$ per electron (i.e., $4H^+$ altogether). When the electrons are released from B^{2-} they must go to a non-protonated acceptor in Photosystem I in order that protons be released on the inside of the chloroplast membrane.

On the average, $2H^+/e^-$ are also taken up on each flash. The amplitude of the

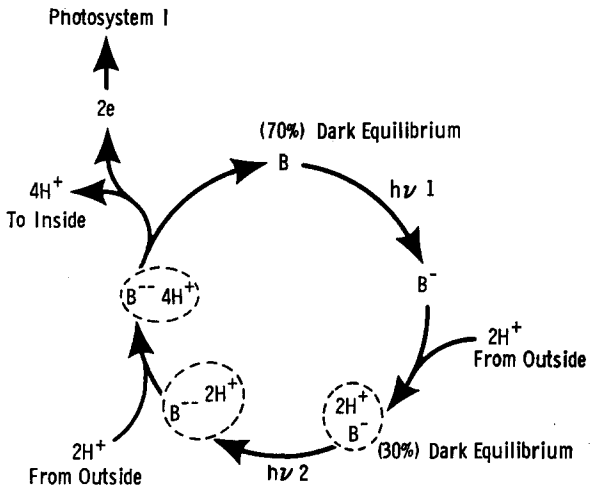

Fig. 9. Scheme summarizing how protons and electrons are coupled in the chain connecting the two photosystems (see text for details).

binary oscillations observed in this proton uptake is considerably smaller than that occurring in proton release. Thus the B^- state must also be associated with $2H^+/e^-$ (i.e., $2H^+$ altogether). In Fig. 9, a scheme is presented which summarizes these results.

There are at least two questions which the scheme does not attempt to explain. The first, which has no adequate answer, is how to explain mechanistically the transport of $2H^+/e^-$. The second question is how to explain the oscillations in uptake. One possibility is that the two reduced states of B have slightly different p*K*. However, the oscillation in proton uptake or release exhibited no pH dependence between 6.0 and 8.0.

The variance of the H^+/e^- ratio with those in the recent literature and the implications to the ATP/2e ratios of photophosphorylation were discussed earlier [1].

Since the release of protons to the inside of the chloroplast membrane is so closely correlated with the binary release of electrons to Photosystem I, B must be a primary site of proton translocation and energy conservation.

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