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ELECTRON TRANSFER AND PROTON PUMPING UNDER EXCITATION OF DARK-ADAPTED CHLOROPLASTS WITH FLASHES OF LIGHT

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Proton release inside thylakoids, which is linked to the action of the water-oxidizing enzyme system, was investigated spectrophotometrically with the dye neutral red under conditions when the external phase was buffered. Under excitation of dark-adapted chloroplasts with four short laser flashes in series, the pattern of proton release as a function of the flash number was recorded and interpreted in the light of the generally accepted scheme for consecutive transitions of the water-oxidizing enzyme system: $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_4$, S_0. It was found that the proton yield after the first flash varied in a reproducible manner, being dependent upon the dark pretreatment given. In terms of the proton-electron reaction during these transitions, the pattern was as follows. In strictly dark-adapted chloroplasts (frozen chloroplasts thawed in darkness and kept for at least 7 min in the dark after dilution), it was fitted well by a stoichiometry of 1 : 0 : 1 : 2. In a less stringently dark-adapted preparation (as above but thawed under light), it was fitted by 0 : 1 : 1 : 2. Mechanistically this is not yet understood. However, it is a first step towards resolving controversy over this pattern among different laboratories. Under conditions where the 1 : 0 : 1 : 2 stoichiometry was observed, proton release was time resolved. Components with half-rise times of 500 and 1000 μs could be correlated with the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4$ transitions, respectively. Proton release during the $S_0 \rightarrow S_1$ transition is more rapid, but is less well attributable to the transitions due to error proliferation. A distinct component with a half-rise time of only 100 μs was observed after the second flash. Since it did not fit into the expected kinetics (based on literature data) for the $S_i \rightarrow S_{i+1}$ transitions, we propose that it reflects proton release from a site which is closer to the reaction center of Photosystem (PS) II than the water-splitting enzyme system. This is supported by the observation of rapid proton release under conditions where water oxidation is blocked. Related experiments on the pattern of proton uptake at the reducing side of PS II indicated that protons act as specific counterions for semiquinone anions without binding to them.

Introduction

The linear electron-transport chain in thylakoids interacts with protons at four sites: (1) oxidation of water, (2) reduction of plastoquinone, (3) oxidation of plastohydroquinone and (4) reduction of the terminal electron acceptor. While protons are taken up from the outside at the even-numbered sites, protons are released inside at the odd-numbered ones. Under continuous illumination and also under repetitive flashes, the proton-electron stoichiometry is 1 : 1 at each of the first three sites whereas at the fourth it depends on the nature of artificial acceptors (Refs. 1–3, for further references see Ref. 4). Recently, several laboratories have reported on the existence of a cyclic electron transport around PS I

Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMSO, dimethyl sulfoxide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Chl, chlorophyll; PS, photosystem.

which is probably also proton pumping [5–10]. This complication is usually absent in broken chloroplasts after freeze-thawing and under oxidizing conditions, and therefore it will be neglected in the following.

Under strictly linear electron transport the 1 : 1 H^+/e^- stoichiometry at each of the first three sites seems to indicate that protons are trivially linked to electrons on water and on plastohydroquinone without the need to invoke 'allosteric' coupling of protons with electrons (as, for instance, in cytochrome *c* oxidase [11]). However, water oxidation is a four-electron step and plastohydroquinone formation a two-electron step, whereas the driving reaction center of PS II promotes the transfer of single electrons only. It is worth asking whether the 1 : 1 stoichiometry prevails also when the two- and four-electron carriers are driven through their consecutive redox states. This can be studied by exciting dark-adapted chloroplasts with a series of flashes. Various workers have previously addressed themselves to the stoichiometric pattern of proton release and uptake under such conditions [12–16]. The oscillatory behavior of the electron (a period of four for oxygen production and a period of two for quinone reduction) was already well established [17–19]. Nevertheless, there are the following unsettled problems concerning protons and electrons around PS II.

(1) The stoichiometric pattern of proton release during the transitions of the water-oxidizing system $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_4$, S_0 is controversial even among authors who have applied a similar technique [12,14–16]. While the data favor the pattern 1 : 0 : 1 : 2 [13,14,16] in some experiments, in others the outcome is better described by 0 : 1 : 1 : 2 [12] or 1 : 1 : 1 : 1 [15].

(2) Apart from the controversy over stoichiometry, the kinetics of proton release during the consecutive transitions of the water-oxidizing system ought to be resolved, both (a) to increase the confidence in the stoichiometric pattern and (b) because protons, besides ESR signal IIvf [20], are so far the best diagnostic tool for the kinetic behavior of the water-oxidizing enzyme system.

(3) There is a discrepancy between the semiquinone anion observed after excitation with the first flash [19] and the observation of proton uptake after the formation of this anion [13].

These items are the subject of this communication. We have studied proton uptake from the outside by laser flash spectrophotometry with the dye cresol red as indicator [1] and also proton release into thylakoids with the dye neutral red [2,21], as described previously. In comparison with similar studies using repetitive excitation of a sample, studies with dark-adapted chloroplasts are complicated by the requirement for large quantities of material – each sample is flashed only once – and the necessity to set-off automatically and to record the optical transmission of the new samples with great accuracy.

Materials and Methods

Chloroplasts and media

We prepared large quantities of chloroplasts according to a modified protocol by Reeves and Hall [22]. 1.5 kg of spinach leaves were detached from their stalks, washed and precooled. Together with 2 l of isolation medium (400 mM sorbitol, 10 mM NaCl, 4 mM $MgCl_2$, 2 mM isoascorbic acid, 4 g/l bovine serum albumin, 50 mM Mes, pH 6.5), they were chopped up for about 5 s in a Waring Commercial blendor at low speed and the resulting pulp was squeezed through a nylon tissue (mesh size 25 μm). The filtrate was accelerated to 4000 × *g* in a Beckman refrigerated centrifuge J2–21 (rotor JA10) and then slowed down (this procedure lasts about 7 min). The pellet was resuspended in a medium containing 400 mM sorbitol, 10 mM NaCl, 4 mM $MgCl_2$, 4 g/l bovine serum albumin, 50 mM Hepes, 5% DMSO (pH 7.5) and carefully homogenized. The whole preparation was carried out at 0–5°C and yielded essentially complete chloroplasts. Aliquots of 2.5 ml at a chlorophyll content of 2–4 mM were frozen and stored under liquid nitrogen until use.

Chloroplasts were thawed in darkness, i.e., a minimum light intensity at 512 nm (less than 0.5 $\mu W/cm^2$) was allowed for 5–10 s only, to handle them, and then transferred into an optically sealed reservoir which contained the standard medium: 25 mM KCl, 3 mM $MgCl_2$, 0.7 g/l bovine serum albumin, 10 μM benzyl viologen or 200 μM $K_3Fe(CN)_6$ (2 mM, when DBMIB was present), pH 7.1, 21°C. 13 μM neutral red, 30 μM cresol red, 6 μM DBMIB and 5 μM DCMU were added where indicated. Chloroplasts treated in this manner will be referred to as 'completely' or 'strictly' dark-adapted chloroplasts.

Measurements with completely dark-adapted chloroplasts will be compared with others on just 'dark-adapted' chloroplasts which were exposed to dim light while thawing and then dark-adapted for at least 7 min.

Sampling and averaging

In nearly all experiments we measured time-resolved absorption changes at 20 or 100 μs per channel of the averaging computer (Tracor TN 1500). Signals of 50 or 100 samples were averaged. In measurements with indicator dyes the same number of signals were taken with and without the indicator dye: the latter to be subtracted from the former to yield the 'pH-indicating absorption change of neutral red/cresol red . . .', as described previously [21]. To accomplish averaging of such high numbers of dark-adapted samples, 0.5 l of chloroplast suspension (20 μM Chl) was prepared and pumped through an automatic flow system upon triggering. The sequence of events is illustrated in Fig. 1. A peristaltic pump (Perifill) was used to deliver a new sample into the absorption cell before each flash group. After sampling, the flow-through cell (1 X 1 X 3 cm, optical path-length 1 cm) required a pulse of 7.5 ml to be refilled again at 97% purity with dark-adapted material (tested optically). The whole arrangement

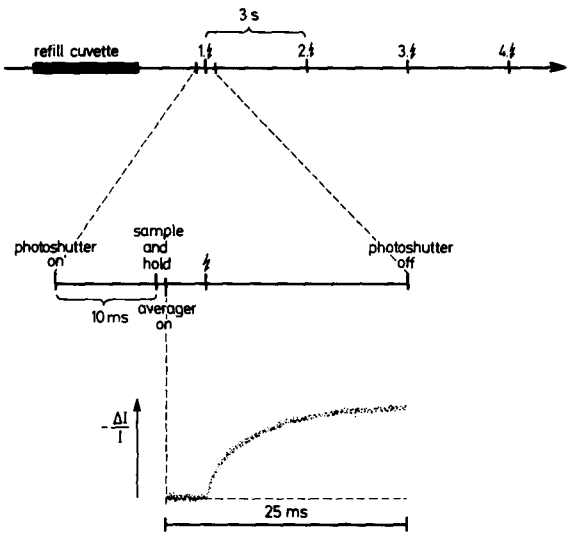

Fig. 1. Time regime for flash photometric measurements of absorption changes under excitation of dark-adapted chloroplasts with a series of four flashes (one cycle).

was kept in total darkness and the measuring light was only applied for 35-ms intervals. The energy of the measuring light pulse (λ = 548 nm for neutral red, 575 nm for cresol red and 522 nm for electrochromic absorption changes; $\Delta\lambda$ = 8 nm) was kept below 3 μJ/cm^2 which excited less than 5% of the reaction centers. (This was calculated as well as experimentally verified by optical detection of the reduction of hexacyanoferrate(III) by chloroplasts exposed to different light intensities.) The photomultiplier (EMI 9558 QB with 10 kΩ load and supplied via a Nucletron NU 1250 B) plus amplifier (Tektronix AM 502) required less than 10 ms in order to reach a sufficiently stable plateau. About 1 ms before triggering the averaging computer, a sample and hold amplifier was activated which had two functions (see Fig. 2). The hold voltage was fed into the negative input of the differential amplifier to compensate for the d.c voltage and fed via an A/D converter into a single-channel averager. Averaging of the d.c. voltages was particularly important, since measurements of pH changes with the neutral red technique require the subtraction of digitized transient voltage changes obtained in the absence from those obtained in the presence of the dye [21]. That is why slow fluctuations of the measuring light and the photomultiplier sensitivity are to be compensated accurately during the whole experiment. The absorption change is proportional to the voltage change divided by the d.c. voltage at the photomultiplier load:

$$2.3\Delta A = -\frac{\Delta I}{I} = \frac{\Delta U}{U}$$

If there are only slight fluctuations in the d.c. voltage

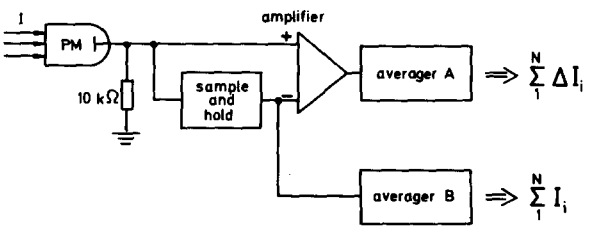

Fig. 2. Experimental setup for the d.c. voltage offset and for averaging both changes of transmitted intensity ΔI_i and transmitted intensity I_i of a sample. PM, photomultiplier.

the absorption change ΔA is given by:

$$2.3\Delta A \simeq \frac{\sum_{i=1}^{N} \Delta U_i}{\sum_{i=1}^{N} U_i}$$

and the pH-indicating absorption change of neutral red (NR) by:

$$2.3\Delta A_{pH_{in}} = \frac{\sum \Delta U_i(+NR)}{\sum U_i(+NR)} - \frac{\sum \Delta U_i(-NR)}{\sum U_i(-NR)}$$

where N is the total number of averaged samples. Hence, we averaged both the voltage changes ΔU_i and the d.c. voltages U_i.

After triggering of the averaging computer the sample was excited with four saturating flashes from a Q-switched ruby laser (λ = 694 nm, 40 ns duration (full width at half maximum), energy on the sample about 5 mJ) spaced 3 s apart. The photoshutter was shut usually 25 ms after each flash to be opened again 10 ms before the following flash in order to allow the d.c. output voltage of the photomultiplier plus supply to reach a stable output voltage again. The computer was instructed to average the signals of the four consecutive flashes in four different memory sections. In measurements with a time resolution of 20 μs per channel the effective 10–90% rise time of the analog circuit was 30 μs which was tested by measuring only one point in the apparent rise of the electrochromic absorption change at 522 nm.

Neutral red and cresol red as pH indicators

We have previously shown that neutral red can be used as a pH indicator for the internal phase of thylakoids provided that the external phase is strongly buffered with impermeant bovine serum albumin. Absorption changes were calibrated with respect to pH changes and artefacts were shown to be negligible [21]. Cresol red, on the other hand, seems to be specific for the external phase [1,2]. The usefulness of neutral red as an indicator for pH changes inside thylakoids is mainly due to its high lipid solubility. Although it is enriched to a large extent in the membrane (the partition coefficient $NR_{membrane\ bound}/NR_{water}$ is about $3 \cdot 10^5$; Hong and Junge, unpublished data) it was proved to be a clear indicator of pH changes which occurred in the internal bulk phase (defined as being accessible to hydrophilic buffers and also as an osmotically variable space) within less than 100 μs [23] or even more rapidly (diffusion-controlled protonation of neutral red adsorbed to detergent micelles [24]). Our recent studies have shown that the apparent pK of neutral red which resides in the surface regions of the thylakoid membrane depends on the surface potential (Hong and Junge, unpublished data).

The specificity of cresol red for the outer phase can be explained not so much by the fact that is does not penetrate the membrane but by the high external phase-to-internal phase ratio which is of the order of 10^3 in a typical chloroplast suspension. Since the buffering capacity, which is mainly due to the membrane proteins, is similar at both sides of the membrane, the pH changes are of similar magnitude as well, but pH changes inside hardly contribute to the apparent total absorption changes of a water-soluble indicator.

Infrared and electrochromic absorption changes

In this study we have also monitored the electrochromic absorption changes at 522 nm as indicators of the activity of both photosystems [25,26]. Absorption changes in the near infrared at 819 nm were measured to indicate the activity of PS I, i.e., the formation of $P\text{-}700^+$. $P\text{-}680^+$ also absorbs at 819 nm but cannot be seen at a time resolution of 20 μs, the shortest dwell time we used in our experiments, because of its more rapid rereduction [27,28].

Results and Discussion

The stoichiometry of proton release into thylakoids

Fig. 3 shows the patterns of proton release into thylakoids which were obtained by excitation of completely dark-adapted chloroplasts in the absence and presence of DBMIB, measured with the neutral red technique. In the upper pattern, hexacyanoferrate(III) was used as terminal electron acceptor. At a concentration of 200 μM it was shown to accept electrons only from PS I and protons are released inside from both sites, from the water-splitting enzyme and from plastohydroquinone, as reflected by the kinetic behavior. The rapid rise of the absorp-

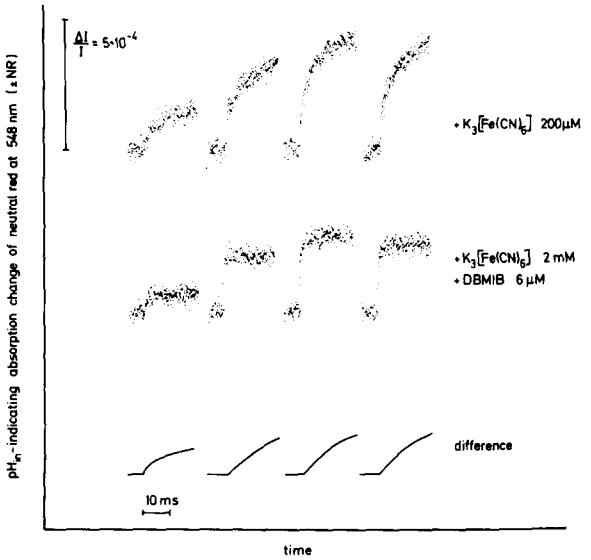

Fig. 3. Absorption changes of neutral red (NR) at 548 nm indicating proton release into thylakoids caused by excitation of strictly dark-adapted chloroplasts with four laser flashes. Measurements were carried out as described in Materials and Methods. The upper pattern shows proton release inside thylakoids from both sites, from the water-splitting enzyme and from plastohydroquinone. The latter is absent in the pattern in the middle because of the addition of the inhibitor DBMIB. The lower pattern shows the subtraction (no noise) of the signals obtained in the presence of DBMIB (middle pattern) from those obtained in its absence (upper pattern). The lower pattern represents proton release during plastohydroquinone oxidation.

tion change was interpreted to be due to proton release by the water-splitting enzyme and a fast phase could be isolated by adding the inhibitor DBMIB (middle pattern). In previous work using repetitive flash excitation at low frequency, it was shown that 6 μM DBMIB plus 2 mM hexacyanoferrate(III) abolishes proton release during plastohydroquinone oxidation, although PS I (*P*-700) is still reduced by some nonproton-donating donor [29]. The missing slow phases in the presence of DBMIB which were attributed to the deprotonation of plastohydroquinone can be seen as the difference in the signals in the upper and middle patterns in Fig. 3, shown in the lower drawings.

Oscillations of the oxygen evolution of dark-adapted chloroplasts [30] led to the model put forward by Kok et al. [31]. It was proposed that the water-splitting enzyme accumulated four oxidizing equivalents before liberating one molecule of oxygen. The consecutive states of accumulation were named S_0, S_1 . . ., according to the number of oxidizing equivalents, with S_1 most stable in the dark. Oxygen release occurs after input of three quanta of light into PS II, i.e., after the third flash. The detailed oscillatory pattern including its damping behavior was interpreted in terms of three parameters: the S_0/S_1 ratio in the dark and the proportion of double hits and misses.

There is still controversy about the pattern of proton release which does not follow that of oxygen evolution. This controversy persists among authors who have used different techniques as well as among those who have used essentially the same technique. Their results are listed in Table I. Experimentally, the most drastic difference concerned the proton yield after the first flash. While it was greater than 85% of the average of the first four flashes in the study of Hope and Morland [15] and in our previous

TABLE I

STOICHIOMETRIC PATTERN OF PROTON RELEASE BY THE WATER-SPLITTING ENZYME DURING THE TRANSITIONS $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_4$, S_0

Survey of results obtained by several authors using different techniques.

Authors	Experimental technique	Proton release stoichiometry
Fowler [13]	glass electrode technique	0.75 : 0 : 1.25 : 2
Junge et al. [12]	neutral red	0 : 1 : 1 : 2
Saphon and Crofts [14]	neutral red	1 : 1 : 1 : 2
Bowes and Crofts [32]	fluorescence	1 : 0 : 1 : 2
Hope and Morland [15]	neutral red	between 0 : 1 : 1 : 2 and 1 : 1 : 1 : 1
Velthuys [16]	neutral red	1 : 0 : 1 : 2

work [12,33], it was down to about 20% in the studies of other authors [13,14,16,32]. As Hope and Morland [15] have pointed out and as we find for our own work, neither excitation by the measuring light nor double turnover by excessively long light flashes caused the higher yield after the first flash [34]. Neither was it caused by the presence of ADRY agents as apparent from a comparison of Fig. 7 in Ref. 33 and Fig. 4 (see below), or by the presence of DBMIB. We found that we could consistently reproduce either pattern when applying two different techniques of dark adaptation.

(1) Low proton yields after the first flash were obtained with completely dark-adapted chloroplasts, i.e., when they were thawed in the dark and not illuminated before sampling (see Fig. 3, middle pattern).

(2) High proton yields were obtained when chloroplasts were thawed and suspended in the light and then dark adapted for at least 7 min. Since the measurements lasted about 20 min, the majority of the chloroplasts were dark adapted for even much longer than 7 min (see Fig. 4).

In the following we evaluate the possible consequences of incomplete dark adaptation based on literature data. From oxygen-evolution patterns of chloroplasts which had been preilluminated by one or two flashes given prior to dark adaptation, Joliot et al. [17] and Forbush et al. [35] have calculated the kinetics of relaxation of the states S_2 and S_3 to S_1. Complete relaxation to S_1 required about 15 min. After 7 min of dark adaptation the residual population in S_2 was less than 20% of the steady-state population. This could have increased the proton yield after the first flash. However, considering that the majority of the chloroplasts were kept in the reservoir much longer than 7 min (typically 17 min), we have estimated that the incomplete dark adaptation could not have raised the proton yield in the first flash by more than 10% with respect to the average yield from four flashes.

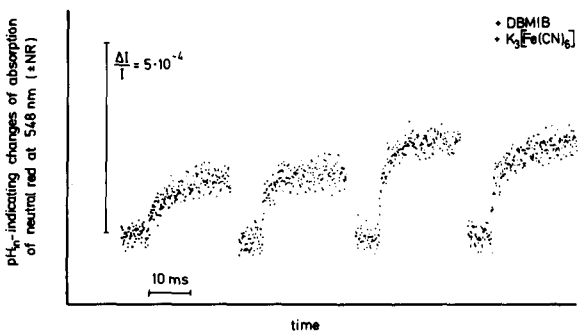

Fig. 4. Absorption changes of neutral red (NR) at 548 nm in dark-adapted chloroplasts as in Fig. 3 (middle) except that chloroplasts were thawed under room illumination and the diluted suspension was then dark adapted for at least 7 min.

On the other hand, a slow relaxation from S_1 to S_0 was observed by Forbush et al. [35] which became noticeable after about half an hour of dark adaptation. Apart from a slight intrinsic shift of the S_0/S_1 partition to S_0, Bouges-Bocquet [38] showed that the S_0/S_1 partition in the dark could be influenced by oxidants. The percentage of S_1 could be increased to 100% by addition of 0.1 mM hexacyanoferrate(III) [38]. Since 2 mM hexacyanoferrate(III) was present in either type of our measurements (in the ones presented here as well as in those we have previously carrier out in the presence of ADRY agents [33]), we have to deal with 75–100% S_1 rather than assuming a relaxation from S_1 to S_0 in either case. We conclude that these possible sources of excessively high proton yield after the first flash cannot account for the one we observed under conditions of less stringent dark adaptation.

The present data were fitted well by a pattern calculated under the assumptions of an equilibrium partition of 25/75 (S_0/S_1), 10% double hits and 10% misses and a proton release stoichiometry of 1 : 0 : 1 : 2 (Fig. 5). However, it is still an open question as to what caused the high proton yield in the first flash under the less stringent conditions of dark adaptation. As a control of the activity of both photosystems we measured the absorption changes at 522 nm, indicative of the activity of both photosystems, and at 819 nm, here indicative of PS I only (Fig. 6). Both patterns were measured in the presence of DBMIB on completely dark-adapted chloroplasts. It is evident that both photosystems are about equally active throughout the four flashes apart from a slight surplus in the first flash at 522 nm.

Kinetics of proton release during the transitions $S_i \rightarrow S_{i+1}$

We have also attempted to obtain high time resolution of proton release by the water-oxidizing com-

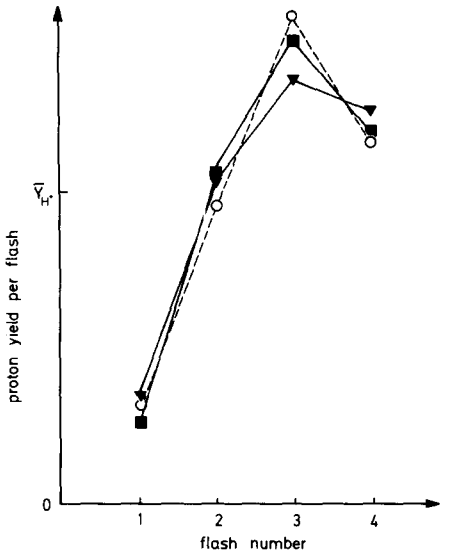

Fig. 5. Amounts of proton release after excitation of strictly dark-adapted chloroplasts with four laser flashes in the presence of 6 μM DBMIB and 2 mM potassium hexacyanoferrate(III) normalized to the average proton yield Y_{H^+} of the four flashes given to the strictly dark-adapted suspension. (■, ▲) Experimental pattern obtained from measurements as shown in Fig. 3, middle (■). (○) Pattern calculated using the following assumptions: dark equilibrium 25 (S_0) : 75 (S_1), 10% double hits and 10% misses, proton release stoichiometry 1 : 0 : 1 : 2.

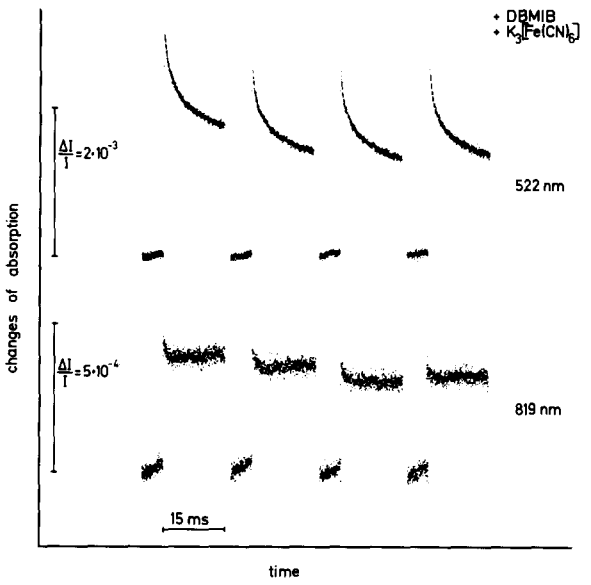

Fig. 6. Electrochromic and infrared absorption changes induced by a group of four short laser flashes in strictly dark-adapted chloroplasts indicating the activity of both photosystems. Both patterns were measured under conditions when proton release from plastohydroquinone oxidation was abolished, i.e., in the presence of 6 μM DBMIB and 2 mM potassium hexacyanoferrate(III) [29]. Average of 50 samples.

plex. The proton-release pattern measured at 20 μs per channel of the averaging computer is represented in the expanded plots in Fig. 7. It should be emphasized that there are no flash-burst artefacts, that the instrumental time resolution is fast (equivalent to one channel on the rise) compared with the rapid rise of the adsorption changes of neutral red and that the subtraction of the intrinsic absorption changes from those in the presence of neutral red left no electrochromic artefacts [21]. The final amplitudes of absorption changes in the presence of DBMIB which are reached after 15 ms are indicated by dashed lines. Whereas the signal of the first flash is too small to be analyzed kinetically, the following ones are composed of different and clearly distinguishable components which are characterized by their half-rise times in the following. A rapid rise of 100 μs plus a slow rise in the second flash, a distinct slow phase in the third and a predominating slow phase in the fourth are conspicuous. We have analyzed the kinetic pattern on the assumption that protons are released with a single characteristic exponential during each of the transitions $S_i \rightarrow S_{i+1}$. As can be seen in the last columns of Table II, a 500 μs phase in the second flash can be attributed to the transition $S_2 \rightarrow S_3$ and a 1000 μs phase to $S_3 \rightarrow S_4$. These results agree broadly with the data from the literature, for instance, those obtained by ESR measurements on signal IIvf by Babcock et al. [20], listed in column 4 of Table II, but also with work on fluorescence lifetimes and oxygen release [31,36–38]. The decay times of ESR signal IIvf were proposed to reflect the rereduction of an electron acceptor Z which is intermediate between the water-splitting enzyme and the reaction center of PS II. The 150 and 250 μs phases appearing in the third and fourth flash, respectively, are not in contradiction to the literature data although we cannot attribute them unequivocally to distinct transitions. If the above assumptions hold, the 100 μs component

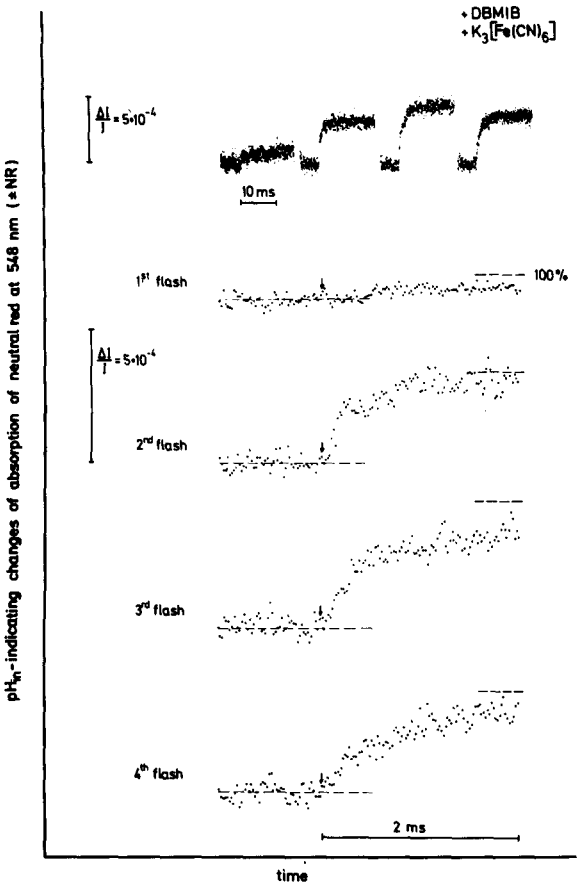

Fig. 7. Time-resolved measurement of the kinetics of proton release by four short laser flashes given to strictly dark-adapted chloroplasts. 6 μM DBMIB and 2 mM potassium hexacyanoferrate(III) were present. The expanded plots show the rapid rise of proton release within 2 ms after the flash. The time resolution is 20 μs per point. The maximum amplitudes of absorption change reached after 15 ms (upper pattern) are indicated by dashed lines. 100 signals were averaged. The instrument rise time was equivalent to one point within the 10–90% range.

in the second flash, however, must be due to $S_3 \rightarrow S_4$. This is not consistent with the attribution of 1000 μs to this transition from the third and fourth flash. Possibly, this phase is caused by a proteolytic reaction preceding water splitting which in turn would cause the analysis to fail because the signal of the second flash should be essentially composed of two phases due to the transitions $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4$.

Fig. 8 summarizes our present view of electron

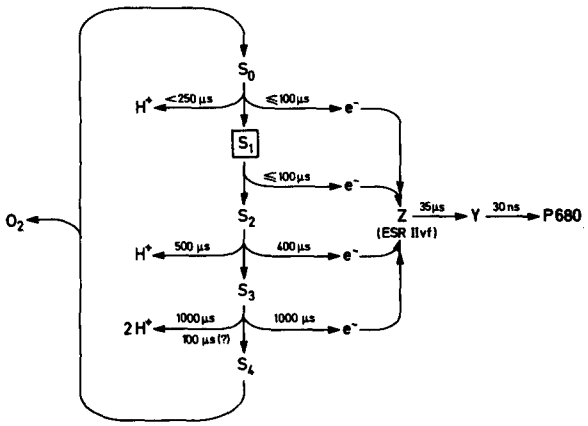

Fig. 8. Tentative scheme of electron transfer and water splitting at the oxidizing side of PS II. Kinetic data on H^+ release are taken from the present work, those on electron transfer from ESR measurements of signal IIvf by Babcock et al. [20] and on work by Van Best and Mathis [27] and Eckert and Renger [47].

transport, proton and oxygen release at the oxidizing side of PS II. Half-rise times of proton release are taken from the present work, those of the ESR-observable acceptor Z from the work of Babcock et al. [20].

Proton uptake from the external phase of thylakoids

An electron from PS II is transferred to two special plastoquinone molecules termed Q and R which are distinct from plastoquinone of the 'pool'. In the dark, the nonreduced form of both Q and R is most stable [18,39]. Their reduction can be followed by the appearence of an absorption band at 320 nm which was attributed to formation of the semiquinone anion [19,39] by analogy with the respective difference spectrum in vitro [41]. In contrast to the reduced acceptor Q^-, of which the reoxidation by R occurs very rapidly, R^- is stable in the dark for at least a few minutes. It reduces the plastoquinone pool only after having accepted a second electron. While the spectroscopic evidence suggests that the singly reduced quinone R^- is nonprotonated, and while an oscillation of period two for the proton uptake should be expected, very little oscillation was observed experimentally [42]. The pattern of absorption changes of cresol red indicating a pH increase in the external phase shows no oscillation at all (Fig. 9).

TABLE II

EXPERIMENTAL RESULTS AND EXPECTATION FOR THE KINETICS OF PROTON RELEASE INSIDE THYLAKOIDS

Column 2 lists the transition $S_i \rightarrow S_{i+1}$ which are expected to occur after the given flash (column 1). (Only those transitions are listed which are assumed to be coupled with proton release in a stoichiometric pattern of 1 : 0 : 1 : 2.) Column 3 shows the expected proton yield which was calculated on the assumptions of the stoichiometric pattern $1H^+$ ($S_0 \rightarrow S_1$) : OH^+ ($S_1 \rightarrow S_2$) : $1H^+$ ($S_2 \rightarrow S_3$) : $2H^+$ ($S_3 \rightarrow S_4, S_0$), an equilibrium partition of $25(S_0) : 75(S_1)$ in the dark and 10% double hits and 10% misses. The values are normalized to the average proton yield per flash over the four flashes. The expected half-rise times are based on ESR signal IIvf (column 4). (The 100 μs components of signal IIvf decay had not been time resolved by Babcock et al. [20]. They might be more rapid.) In columns 5 and 6 the results of the analysis of the time-resolved proton-release pattern (Fig. 7) are listed for comparison with the expectation (see Ref. 20) in columns 3 and 4. Phases which are not clearly attributable to the transitions are indicated by an asterisk.

Flash number	Expectation			Experimental results (H^+ release)	
	Transition type	H^+ yield (% of average calculated)	Expected half-rise time (μs)	Yield (% of average)	Half-rise time (μs)
(1)	(2)	(3)	(4)	(5)	(6)
1	0–1	24.5	100		
	2–3	7.5	400		
2	0–1	3.2	100		
	2–3	63.7	400	65	500
	3–4	28.1	1 000	41*	100*
3	0–1	18.3	100	67*	150*
	2–3	28.0	400		
	3–4	109.7	1 000	82	1 000
4	0–1	48.3	100	42*	250*
	2–3	9.7	400		
	3–4	58.0	1 000	77	1 000

In particular, full proton uptake is observed after the first flash in the presence of DCMU, which allows only formation of Q^- but prevents the electron transfer from Q^- to R. In addition to the spectroscopic line of evidence for the nonprotonation of the semiquinonoid forms of Q and R, there is kinetic evidence: Haehnel [43] has studied the relaxation of the absorption changes at 335 nm, which represents PQ^- rather than any other form of plastosemiquinone. He observed a more rapid decay at more alkaline pH (see Fig. 4 in Ref. 43), which excludes the possibility of protonation of Q^- in the time domain from 200 μs upward. This together with the failure to find oscillations in the proton uptake leaves room to speculate that the proton taken up serves as a specific counterion for the semiquinone anion, without binding to the latter. This may occur by either of two ways, by specific binding to a proton-storage site in the host protein of R, as illustrated in Fig. 10, or by uptake into a matrix of negative fixed charges which is accessible to protons only.

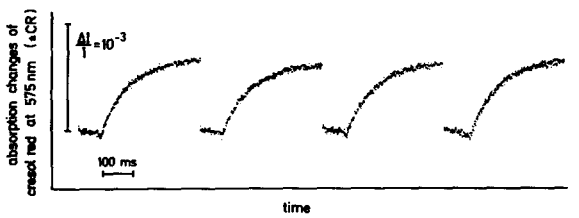

Fig. 9. Pattern of proton uptake from the outer aqueous phase represented by the absorption changes of cresol red (CR) at 575 nm following four consecutive flashes. 10 μM benzyl viologen was used as electron acceptor; the pH of the suspension was adjusted to 7.9. Average of 50 samples.

Herbicides such as DCMU seem to render the redox potential of R more negative relative to Q [18]. The possibility that this could be due to the prevention of access for protons to the proteinaceous

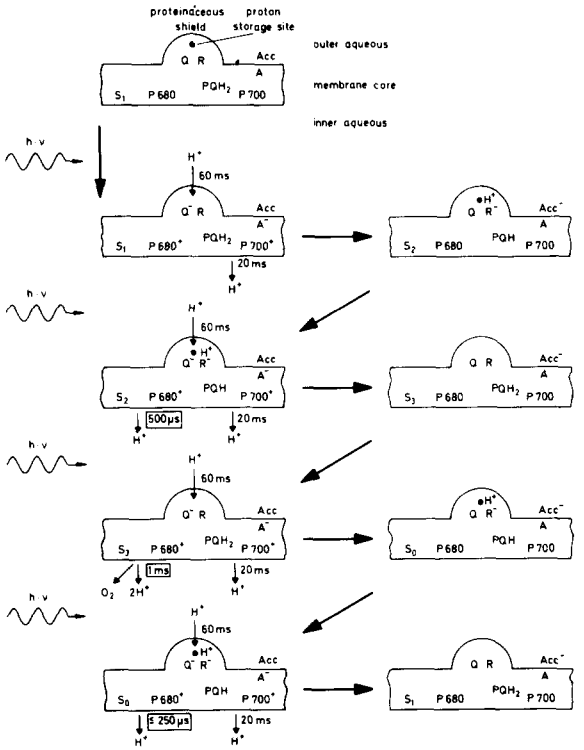

Fig. 10. Simplified scheme of proton and electron transport in the thylakoid membrane during one cycle of the water-splitting enzyme system.

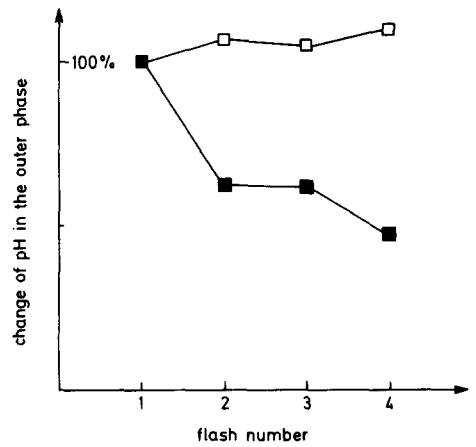

Fig. 11. Pattern of proton uptake from the external phase of thylakoids in the absence (□) and in the presence (■) of DCMU, measured on strictly dark-adapted chloroplasts. Cresol red was used as pH indicator. 10 µM benzyl viologen and 5 µM DCMU were present.

sites can be excluded by inspection of Fig. 11. There is proton uptake after the first flash in the presence of DCMU. A possible regulatory and/or stabilizing role of 'allosteric' proton uptake from outside is debatable.

Conclusions and general discussion

The sequence of proteolytic reactions associated with PS II activity under excitation of dark-adapted chloroplasts with a series of four short flashes is illustrated in Fig. 10. Proton release inside is oscillatory. The pattern of oscillation, mainly of how many protons are released on the first flash, depends reproducibly on the way of adaptation, but so far is not well understood mechanistically. The kinetics of proton release inside after each flash in a row were resolved under conditions when the total stoichiometry seemed to be 1 : 0 : 1 : 2 during $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_4$, S_0. One phase at $\tau_{1/2}$ = 1000 µs correlated well with the transition $S_3 \rightarrow S_4$, S_0 and another at $\tau_{1/2}$ = 500 µs with $S_2 \rightarrow S_3$. These half-rise times matched those predicted for the respective transitions [20] between the S_i states very well. It seems as if proton release follows electron abstraction (Fig. 8) which was experimentally accessible via the ESR signal IIvf [20]. In addition to the above-mentioned two kinetic phases there is one 150 (250) µs half-rise time, which is less well attributable, possibly due to $S_0 \rightarrow S_1$. On the second flash, however, there is a distinct component rising at 100 µs under the given conditions, which could not be fitted into the expected kinetic pattern of electron transfer. We do not know how to locate this proton release. It is conceivable that it occurs prior to the water-oxidizing enzyme system and closer to the reaction center of PS II. If so, the good stoichiometric fit with an assumed proton pattern of 1 : 0 : 1 : 2 would be impaired. It is apparent from experiments with the PS II blocker DCMU (presented elsewhere) that there may be sites for rapid proton release closer to the reaction center under conditions when no oxygen is evolved. This deserves further consideration.

The results of the kinetic analysis of proton release inside thylakoids in the present work should be compared with those previously obtained in the presence of ADRY agents [33]. Although the higher proton yield after the first flash in the presence of

ADRY agents suggested the pattern 0 : 1 : 1 : 2, using the same assumptions of dark equilibrium, double hits and misses as in this study and since ADRY agents were shown not to alter the expected pattern of oxygen evolution significantly, we can assume that S_1 was most stable in the dark and that the absorption changes of neutral red after the first flashes broadly reflected proton release during the transitions $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, ... in either measurement. The half-rise times of the kinetic phases in the presence of ADRY agents (300–600 μs $S_1 \rightarrow S_2$, 100 μs $S_2 \rightarrow S_3$, 1000 μs $S_3 \rightarrow S_4$) were of the same order of magnitude as in the present study. The different stoichiometric pattern makes a comparison of these kinetics difficult. The only invariant component seems to be the 1000 μs rise during $S_3 \rightarrow S_4$, S_0.

Proton uptake during the stepwise reduction of the special bound plastoquinones shows no oscillation although the semiquinone stage seems to be anionic. This has to be reconciled with the existence of a proteinaceous diffusion barrier for protons covering the site of proton consumption which we have demonstrated in earlier studies [2]. This proteinaceous barrier may be partially the one impaired by trypsin treatment of the chloroplasts [44] and holding the sites of herbicide action [45]. It is likely that the freshly produced semiquinone causes the protonation of a neighboring proteinaceous group. This conclusion was reached earlier by Wraight [46] for the protonation of the bound ubiquinone in bacterial reaction centers.

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