DIRECT MEASUREMENT OF THE PROTONS PUMPED INTO THE INNER PHASE OF THE FUNCTIONAL MEMBRANE OF PHOTOSYNTHESIS PER ELECTRON TRANSFER

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1. Introduction

In the primary process of photosynthesis in green plants electrons are transferred from H_2O via different electron-carriers to the terminal electron acceptor $NADP^+$. Electron transport starts with a spontaneous electron shift [1] vectorial across the thylakoid membrane thereby generating a transmembrane electrical potential [2]. The electron transfer is followed by a H^+ uptake from the outer phase [3] and a H^+ release into the inner space of the thylakoid membrane [4,5]. The electron transfer is driven by two light reactions at Chl-a_I (P 700) [6] and at Chl-a_{II} (P 690) [7,8]. It has been shown that each light reaction contributes one half of the electrical potential generation [9]. Furthermore, at each light reaction center one proton is taken up from the outer thylakoid phase for each electron transfer [9]. This has been concluded from the change of pH in the outer aqueous phase of the thylakoids. Because the potential difference $\Delta\varphi$ is proportional to the amount of translocated charges it follows that at each light reaction one proton per transferred electron is released into the inner aqueous phase [9]. This stoichiometry of H^+ release was indirectly checked by the measurement of the pH change in the outer thylakoid phase at varying light reactions with the help of uncouplers which permit the rapid efflux of H^+_{in} [10]. Because these results are the experimental basis for the development of a vectorial zigzag scheme [9], it is desirable to obtain direct evidence for the release of 1 H^+ at each light reaction center into the inner phase of the thylakoid. In this work we have therefore measured directly the change of pH in the inner aqueous phase of the thylakoid.

Abbreviations: AA, 9-aminoacridine; Bv, benzylviologen; Chl-a_I, -a_{II}, chlorophyll-a_I, a_{II}; CR, cresol red; Cyt-f, cytochrome-f; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; $NADP^+$, nicotinamide adenine dinucleotide phosphate; Pc, plastocyanine; PQ, plastoquinone; Pyo, pyocyanine.

2. Materials and methods

Spinach chloroplasts were prepared as described elsewhere [11]. Additionally 10 mM ascorbate was added during grinding. The chloroplasts were either used immediately after preparation or stored under liquid nitrogen until use. The chloroplasts were suspended in 10 ml reaction medium ($2 \cdot 10^{-2}$ M tricine adjusted to pH 8 with NaOH, $5 \cdot 10^{-3}$ M $MgCl_2$, 10^{-2} M KCl, 10^{-2} M sucrose). Chlorophyll concentration was 10^{-5} M. Further additions: $3 \cdot 10^{-5}$ M pyocyanine or $3 \cdot 10^{-5}$ M benzylviologen, $2 \cdot 10^{-7}$ M DCMU, $1.5 \cdot 10^{-5}$ M cresol red, 10^{-6} M 9-aminoacridine.

2.1. *Excitation and registration of optical changes*

Photosynthesis was excited with saturating single turnover flashes (duration 20 μsec or 1 μsec). Optical changes were measured with a repetitive pulse spectrometer with double beams as described earlier [12]. Thus, two events (e.g. the proton release inside the thylakoid and the generation of the electric field) can

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be measured simultaneously. To improve the signal to noise ratio the optical changes induced by the repetitive flashes were summed. Depending on the time resolution 10–8000 signals were averaged. The activity of light reaction I and II was established with benzylviologen (Bv) or pyocyanine (Pyo) as terminal electron acceptors instead of $NADP^+$. The operation of light reaction I alone was realized by inhibition of electron flow from light reaction II with DCMU. In this case reduced pyocyanine (PyoH) acts as the electron donor for Chl-a_I^+ and oxidized pyocyanine (Pyo) as the terminal electron acceptor for Chl-a_I [13].

2.2. *Light reactions*

The activity of light reaction I was measured by the absorption change of Chl-a_I at 705 nm [6] and light reaction II by the absorption change of Chl-a_{II} at 690 nm [7,8].

2.3. *Electrical potential*

The flash-induced electrical potential generation $\Delta\varphi$ was measured by absorption changes at 515 nm in the msec-range. These changes are due to electrochroism, i.e. to changes of the optical absorption bands of the bulk pigments by the electrical field across the thylakoid membrane [14]. This interpretation has been proved by three types of experiments: kinetic [2], spectroscopic [15,16] and electric [17]. The extent of the absorption change is proportional to the electrical potential change. For details see [18].

2.4. *Proton uptake from the outer phase*

The pH change in the outer aqueous phase of the thylakoids was measured by the pH indicator cresol red (CR) at 583 nm [10]. Besides the pH-indicating absorption change there is a background signal at this wavelength. The non-pH-indicating absorption change was eliminated by summing up the optical signals from a weakly buffered ($2 \cdot 10^{-4}$ M tricine) chloroplast suspension and subtracting the same number of signals from a strongly buffered one ($5 \cdot 10^{-2}$ M tricine). Because the pH value of the suspension medium does not change significantly during the experiment, the buffer capacity is constant and the optical signal is proportional to the amount of protons taken up (ΔH^+_{out}).

2.5. *Proton release into the inner phase*

The pH change in the inner aqueous phase of the thylakoids was calculated from the fluorescence change of the 9-aminoacridine (AA) [19], i.e.

$$H^+_{in} \sim Q/(1-Q) \quad (1)$$

(Q = quenched fluorescence/initial fluorescence). This relationship is applicable only in a limited range [20, 33,34]. The amount of protons released at the inside ΔH^+_{in} is proportional to the change of H^+_{in}:

$$\Delta H^+_{in} \sim H^+_{in} \quad (2)$$

This is valid in steady state flash light (see fig.1, bottom) if the membrane permeability is constant at varying light reaction activity. This was observed under non-phosphorylating conditions by the unchanged kinetics of the potential decay. (It should be remembered that under steady state light conditions a change of the buffer capacity does not influence Eqn. (2).) Under these conditions the fluorescence quenching is proportional to ΔH^+_{in}:

$$Q/(1-Q) \sim \Delta H^+_{in} \quad (3)$$

This relation can be questioned if Eqn. (1) is not applicable. In this case the knowledge of the true relation between H^+_{in} and Q is not necessary if we use a flash frequency balance technique as outlined below. AA fluorescence was excited at 367 nm, $\Delta\lambda$ = 10 nm, with 60 erg/cm² sec. Fluorescence emission was measured at an angle of 180° at 454 nm, $\Delta\lambda$ = 10 nm, through a Balzers 585 nm cut-off filter, a 4 mm GG 385 (Schott) and a 3 mm BG 26 (Schott), shielding the photomultiplier from flash light and from scattered 367 nm light.

3. Results

Fig.1 shows the time course of five different primary events in photosynthesis: the activity of light reaction I and II, the electrical potential difference across the thylakoid membrane, the proton uptake ΔH^+_{out} from the outer phase and the proton release ΔH^+_{in} into the inner phase of the thylakoid. In the first row the events are shown in the presence of Pyo as terminal

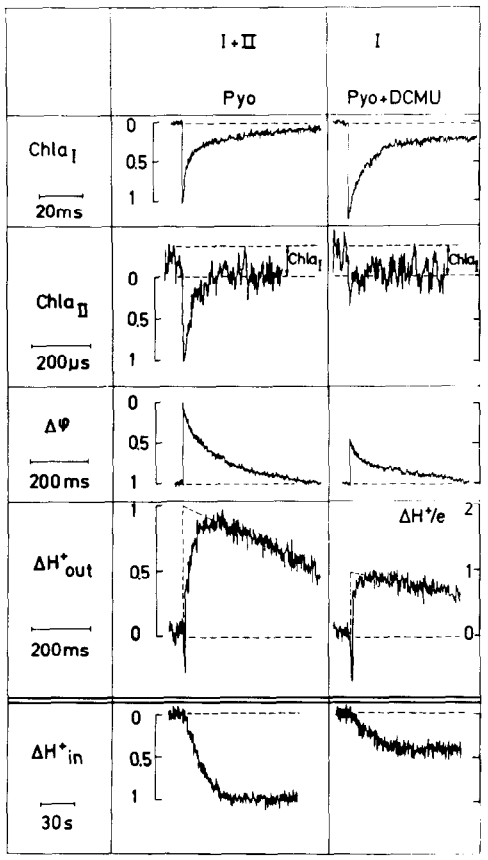

Fig.1. Time course of five primary events in photosynthesis at varying light reactions indicated by optical changes. First line: activity of light reaction I measured by the absorption change of Chl-a_I at 705 nm. Frequency 2 Hz, 500 flashes are sampled. Second line: activity of light reaction II measured by the absorption change of Chl-a_{II} at 690 nm. Frequency 2 Hz, 8000 flashes are sampled. The slow kinetic change is due to a super-imposed absorption change of Chl-a_I at 690 nm. Third line: generation of the electrical potential difference measured by electrochromic absorption changes at 515 nm. Frequency 2 Hz, 200 flashes are sampled. Fourth line: proton uptake measured by the absorption change of cresol red at 583 nm. Frequency 2 Hz, 200 flashes are sampled. On the right hand side the absolute values are depicted. Fifth line: proton release into the inner aqueous thylakoid space measured by the fluorescence quenching of 9-aminoacridine at 454 nm. Flash frequency 2 Hz. About 100 flashes are fired until a steady state is reached. All signals are proportional to the indicated event with the exception of the fluorescence quenching which is related to ΔH^+_{in} by $\Delta H^+_{in} \sim Q/(1-Q)$. However, for the measurement depicted in fig.1 fifth line it is $Q \ll 1$ and in this case it follows that $\Delta H^+_{in} \sim Q$. Reaction medium as described in Materials and methods.

electron acceptor. Light reaction I and II are both operating so that the subsequent reactions, electric potential generation ($\Delta\varphi$), proton uptake (ΔH^+_{out}) and proton release (ΔH^+_{in}) are all maximal.

The second row shows the events in the presence of Pyo and DCMU. In this case light reaction II is blocked whereas light reaction I is unaffected. Under these conditions the signal due to the potential generation $\Delta\varphi$, proton uptake ΔH^+_{out} and proton release ΔH^+_{in} are all halved. Because the signals are proportional to the indicated events (see Materials and methods), the results above the double line in fig.1 indicate the following: using Pyo as electron acceptor, our earlier results [9] are confirmed in that each light reaction generates one half of the electrical potential $\Delta\varphi$ and results in one half of the total proton uptake ΔH^+_{out} from the outer phase. These results are the basis for the interpretation of the new measurements on the H^+ release depicted on the bottom of fig.1: if either both light reactions (I + II) or only light reaction I are operating, the difference of the fluorescence change indicates that each light reaction releases about one half of the translocated protons into the inner phase. This is correct if the fluorescence change is proportional to ΔH^+_{in}.

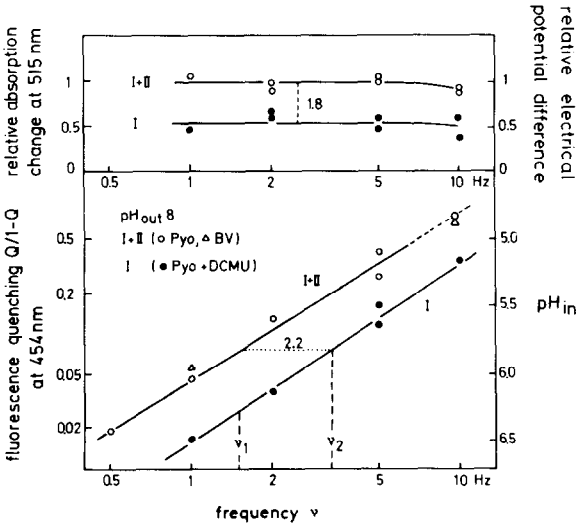

Fig.2. Top: field indicating absorption change at 515 nm as a function of the flash frequency ν at varying light reactions. Conditions as described in Materials and methods. Bottom: fluorescence quenching of 9-aminoacridine as a function of flash frequency ν at varying light reactions. On the right hand side the calculated pH_{in} values are depicted.

Therefore, the measurements were refined by the following experiment. In fig.2 bottom (curve I + II) the fluorescence quenching in the steady state is shown as a function of the flash frequency under conditions where both light reactions are operating. The fluorescence quenching is proportional to the flash frequency. If only light reaction I is operating, ΔH^+_{in} and hence the fluorescence quenching is decreased at all frequencies. This is shown in fig.2 bottom (curve I). The pH_{in} values calculated from the fluorescence quenching assuming an internal value of 50 l/mol chlorophyll are shown on the right hand side. Under conditions of light reaction I activity alone we can achieve the same fluorescence quenching, i.e. the same pH_{in} value and proton flux as with both light reaction I + II activity, by increasing the frequency, e.g. from ν_1 to ν_2. The factor by which the frequency has to be increased at constant proton flux corresponds to the ratio of protons released by light reaction I + II and I respectively:

$$\frac{\nu_2}{\nu_1} = \frac{\Delta H^+_{in}\ (I + II)}{\Delta H^+_{in}\quad (I)} \qquad (4)$$

Fig.2. indicates that $\nu_2/\nu_1 = 2.2$. This relation is correct if the same number of protons is translocated per flash across the membrane at all frequencies. Because at constant membrane capacity the transmembrane potential difference $\Delta\varphi$ is proportional to the amount of translocated charges (protons) this can be checked by measuring the dependence of $\Delta\varphi$ on ν. The constancy of $\Delta\varphi$ is realized for both, light reaction I + II, and for light reaction I alone for frequencies up to 20 Hz as shown in fig.2 (top). Therefore, we can accept the validity of Eqn. (4). (The results confirm again that each light reaction contributes to about one half of the electrical potential generation.) The frequency factor 2.2 in fig.2 (bottom) means that the relative amount of proton release ΔH^+_{in} caused by light reaction I is 0.45 and that caused by light reaction II 0.55. This is in fair agreement with the expected value of 0.5 (see Introduction). The advantage of the experiment in fig.2 is that the result is obtained at constant pH_{in} which allows the application of Eqn. (4). It is even unnecessary to know the absolute value of pH_{in}. This avoids the use of Eqn. (1) which may be incorrect (see 2.5.) and exempts us from the knowledge of the true relation between H^+_{in} and Q. The absolute number of protons released per electron by each light reaction follows from the calibrated proton uptake (titration with acids) [9,10]. 2 H^+ are taken up per electron when both light reactions I + II are active (see fig.1). Because in one turnover the H^+ uptake is identical with the H^+ release, it follows from the above results that about 1 H^+ per electron is released at each light reaction. The results are summarized in table 1. Additionally, measurements are shown with Bv as electron acceptor and with Bv + DCMU. With Bv the same results are obtained as with Pyo whereas with Bv + DCMU all reactions are blocked.

Table 1
Observed activity of five primary events in photosynthesis (Results from fig.1 and fig.2 and additional measurements with Bv and Bv + DCMU). Expected values are derived on the basis that each light reaction contributes one half of the translocated protons and one half of $\Delta\varphi$. Details see text.

Varying light reaction	Relative activity of Chl-a_I		Relative activity of Chl-a_{II}		Relative extent of the electric potential $\Delta\varphi$		Proton uptake outside ΔH^+_{out} per electron		Proton release inside ΔH^+_{in} per electron	
	obs.	expect.	obs.	expect.	obs.	expect.	obs.	expect.	obs.	expect.
I + II (+BV)	1	1	1	1	1	1	2	2	2	2
0 + 0 (+ BV / + DCMU)	0.1	0	0.1	0	0.1	0	0.2	0	0	0
I + II (+Pyo)	0.95	1	0.9	1	0.95	1	2.2	2	2	2
I + 0 (+ Pyo / + DCMU)	1.1	1	0.1	0	0.5	0.55	1.1	1	0.9	1

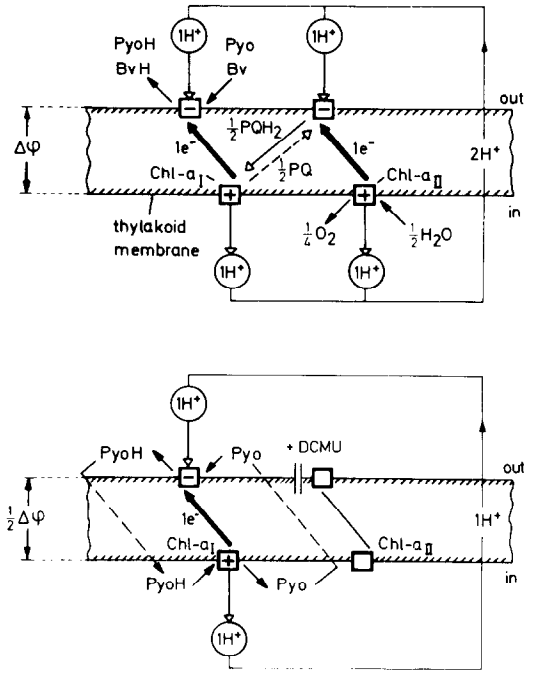

Fig.3. Zigzag scheme of the vectorial electron-, proton- and hydrogen-transfer in the functional membrane of photosynthesis derived from pulse spectroscopic results. Top: both light reactions I + II are operating. Bottom: light reaction II is blocked with DCMU and Pyo acts as hydrogen carrier. Details see text.

4. Discussion

Direct experimental evidence is given for the release of 1 H^+ per electron into the inner phase at each light reaction. This is in agreement with prior conclusions drawn from indirect measurements (see Introduction). Our earlier results have been explained by a zigzag scheme with a vectorial flow of the electron and an opposite vectorial flow of one hydrogen (coupled to PQ) as first outlined in the chemiosmotic hypothesis of Mitchell [21]. Together with the results of this work, in which we used Pyo as carrier for hydrogen, the experiments may be explained by the schemes shown in fig.3.

In the case of Pyo (or Bv) as electron acceptor, the dye picks up the electron from the photooxidized Chl-a_I and reacts in its reduced state with 1 H^+ taken up from the outer phase. The primary acceptor for the electron from the photooxidized Chl-a_{II} was shown to be plastoquinone PQ [22–24]. Plastosemiquinone can react in its reduced state with 1 H^+ which we assumed to be the proton taken up at light reaction II from the outer phase [9]. The oxidized Chl-a^+_{II} is finally reduced by the ultimate electron donor 1/2 H_2O. Thereby 1/4 O_2 and 1 H^+ are released. Because Chl-a^+_{II} is localized close to the membrane interior [25] the H^+ released with the oxidation of H_2O must be the proton released into the inner phase at light reaction II. That the oxidizing site of light reaction II is located at the inside of the thylakoid membrane has also been shown by other types of experiments [9,10,26,27]. It was shown that the oxidized Chl-a^+_I is finally reduced by 1/2 PQH_2 [23] via intermediate electron carriers (Pc, Cyt f) [28,29]. Because Chl-a^+_I is also located on the inner side of the membrane [25,30], the H^+ released with the oxidation of 1/2 PQH_2 is assumed to be the proton released into the inner phase at light reaction I. In toto these processes are equivalent to a pumping of 2 H^+ from the outer phase across the membrane into the inner phase. (In the presence of Pyo the dye may serve as an artificial shuttle for H^+ and electrons between light reaction I and II in addition to the endogenous PQ.) By addition of DCMU H_2O oxidation is blocked. In the presence of both, DCMU and Pyo, there are no reducing substances present other than PyoH. It must be therefore assumed that PyoH reduces Chl-a^+_I. Because Chl-a^+_I is located at the membrane inside, the H^+ released by the oxidation of PyoH is assumed to be the proton released into the inner phase at light reaction I [31,32]. The reduction of Chl-a^+_I requires that PyoH diffuse through the membrane from outside to inside with Pyo moving in the opposite direction (see fig.3). This is due to the lipophilicity of this substance. This is not the case for Bv. Therefore, in the case of Bv + DCMU the total process is blocked (see table 1). It should be pointed out that in the presence of Pyo and DCMU the protons released inside the thylakoid at light reaction I are in general less than 1 ΔH^+_{in} per electron. In chloroplasts frozen in liquid nitrogen before use the value is maximal 0.9 ΔH^+_{in}. In freshly prepared chloroplasts the value is 0.7 ΔH^+_{in}. This difference and the deviation of ΔH^+_{in} from the expected value 1 may be due to different permeabilities of the membranes for Pyo or PyoH and a partial release of H^+

in the membrane. However, a satisfying explanation cannot be given as yet.

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