Transient and intramembrane trapping of pumped protons in thylakoids

The domains are delocalized and redox-sensitive

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We measured flash-induced rapid proton release into the lumen of thylakoids. We found that the proton-trapping domains which were inducible by low concentrations of gramicidin and at alkaline pH [e.g. (1983) Biochim.Biophys.Acta 723, 294–307] were each not restricted to a particular photosystem II complex. Instead, they were delocalized. Oxidizing conditions increased the buffering capacity of the domains from approx. 5 H^+ per photosystem II more than 2-fold by shifting the pK towards acidity.

Photosynthesis *Photosystem II* *Proton pump* *Chemiosmotic hypothesis* *Localized proton*

1. INTRODUCTION

In the original concept of the chemiosmotic hypothesis, Mitchell [1] discussed energetic coupling between electron transport, proton translocation, and ATP synthesis in terms of only 3 compartments, two aqueous 'bulk' phases and the membrane. The bulk phases were conceived as protonically isopotential, so that proton pumps and ATP synthases sensed the same potential difference. During the last few years, however, several laboratories working on different organisms published observations which were difficult to reconcile with the 'macro-chemiosmotic' concept (reviews [2,3]). Working with chloroplasts, Dilley and co-workers (see [4] for further references) found an intramembrane proton pool with a buffering capacity of 30–40 mmol H^+/mol chlorophyll in dark-adapted membranes. Amine groups with an anomalously low pK_a (7.8) are supposedly involved. Only after brief thermal or uncoupler treatment of thylakoid membranes do protons from this pool leak into the bulk. After equilibration the onset of ATP synthesis upon illumination is delayed as if the buried pool has first to be refilled. Homann's group (see [5] for further references) have reported an uncoupler-induced proton release from dark-adapted chloroplasts. The pool size varies with the experimental conditions (i.e. pH during pre-incubation of the thylakoids, osmolarity of the medium) from 10 to 100 mmol H^+/mol chlorophyll. Both Dilley's and Homann's groups have reported that protons are retained in PS II-related domains.

We studied proton release into the lumen of swollen thylakoids with a membrane-adsorbed indicator dye, neutral red [6–8]. Proton release showed two main kinetic phases: a complex fast phase (half-rise time 100 μs to ~10 ms) which was attributed to the known partial reactions of water oxidation [9] and a slow phase (half-rise time in the range <20 ms) which was attributed to plastoquinol oxidation [6]. Although the lumen of freshly prepared thylakoids lacked the properties of an ex-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, dinitrophenyl ether of iodonitrothymol; PS, photosystem

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tended aqueous bulk phase, the same kinetic phases of internal acidification were observed [8]. After addition of very low concentrations of gramicidin (1 mol/3×10^4 mol chlorophyll) and at alkaline pH the rapid proton release from water oxidation was no longer detected in this type of membrane. After approx. 6 turnovers of PS II the proton-trapping domain which became accessible via gramicidin and alkaline pH was filled and rapid acidification jumps were again observed [10].

Here, we evaluate the following questions. (i) Are these domains each attributable to one particular PS II, or do several photosystems fill one larger and common doamin with protons? (ii) Are the proton-trapping groups sensitive to the redox conditions?

2. MATERIALS AND METHODS

Thylakoids were prepared according to [11] for stacked thylakoids. For measurements of pH changes in the lumen, thylakoids equivalent to 10 μM chlorophyll were suspended at room temperature in a standard assay medium containing NaCl (10 mM), $MgCl_2$ (5 mM), 2.6 mg BSA/ml, pH 7.7, neutral red (13 μM) and gramicidin D (0.2 nM). pH changes in the external phase of thylakoids were measured in the absence of BSA at pH 7.8 and cresol red (15 μM) was used as pH indicator. pH-specific absorption changes were obtained by subtracting signals obtained in the absence of the dye from those obtained in its presence (for details of the method see [6,7,12]). Further additions to the assay medium as electron acceptors and inhibitors are given in the figure legends. Flash-induced absorption changes were detected spectrophotometrically in the same apparatus as in [10].

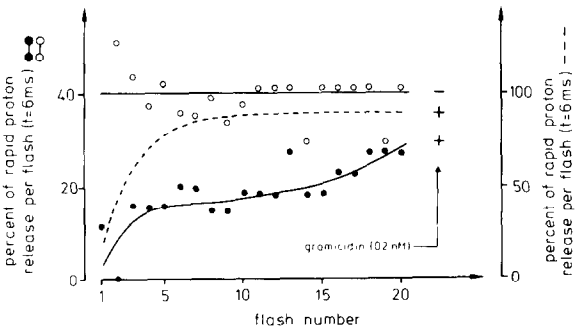

Fig.1. Relative extent of rapid proton release to the lumen of the thylakoids as a function of flash number. Absorption changes of neutral red were measured in the presence of DCMU (20 nM) and in the absence (○—○) or presence of gramicidin (0.2 nM) (●—●). (- - -) Proton recovery in the absence of DCMU and presence of gramicidin. Methyl viologen (10 μM) was used as electron acceptor. The flashes were spaced 150 ms apart and 20 sequences were averaged. Dwell time 3 ms/point, electrical bandwidth 100 Hz.

Thylakoids were excited by a train of 20 saturating flashes (xenon lamp, PRA610B, $\lambda \geqslant 610$ nm, 3 μs FWHM, 1 mJ/cm^2 spaced 150 ms apart. The energy of the gated measuring beam was 3 μJ/cm^2. The sample was kept in the dark for 40 s before the next series of flashes was delivered. Typically 10 or 20 trains were averaged. The time resolution was 2 or 3 ms/point. In some experiments with single flashes at low flash frequency (0.2 Hz) a xenon flashlamp ($\lambda \geqslant 610$ nm, 15 μs FWHM, 1 mJ/cm^2) was used.

3. RESULTS

3.1. *Are proton-trapping domains each attributable to one particular PS II or are they larger and common units?*

We measured the acidification of the thylakoid lumen under excitation of thylakoids with a train of 20 light flashes. Fig. 1 represents a plot of the extent of the rapid acidification, 6 ms after each flash, as a function of the flash number. In agreement with [10] the results shown by the dashed line were obtained upon addition of gramicidin (0.2 nM) at pH 7.8. After the very first flashes there was only slight acidification, but a recovery after 6 flashes had been fired. The circles in fig. 1 resulted from experiments where approximately one half of PS II was blocked by DCMU. This decreased the rapid acidification to approx. 40% with little variation in the dependence of the flash number in the absence of gramicidin (fig.1, ○). In the presence of gramicidin (and still of DCMU, ●), there was little rapid acidification after the first flashes, and the recovery was delayed beyond flash no.15. This indicated that the proportion of proton-buffering groups to proton-pumping photosystems was increased. In other words, protons released by one particular PS II could be trapped by a domain which was unused by another, DCMU-poisoned centre.

As a control for the partial inhibition by DCMU

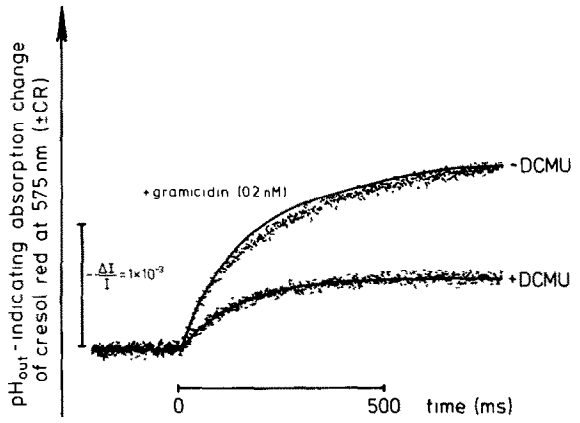

Fig.2. Proton uptake from the external phase at the reducing site of PS II in the presence of gramicidin D (0.2 nM) (both traces) and additionally DCMU (20 nM) (lower trace only). Ferricyanide (0.2 mM) was used as electron acceptor. The lines show the respective signals obtained in the absence of gramicidin. Flash frequency 0.2 Hz, 40 samples averaged, dwell time 1 ms/point, electrical bandwidth 3 kHz.

and the very slight uncoupling by gramicidin (0.2 nM), we measured proton uptake from the suspending medium and by PS II. The results are given in fig.2. In comparison with the control, where gramicidin was absent (fig.2, smooth lines), the kinetics and magnitude of proton uptake were barely affected by gramicidin. DCMU (20 nM) inhibited proton uptake at the reducing site of PS II by 50%. This deviates from the 40% remaining rapid proton release inside (fig.1). This small difference in the extent of inhibition was probably caused by the different excitation conditions in the experiments shown in figs 1 and 2. These are known to affect the extent of inhibition of PS II by DCMU [13,14].

The foregoing showed that the proton-trapping domains were not restricted to each PS II complex but, instead, were delocalized. The extent of delocalization could not be inferred from these experiments.

3.2. *Redox dependence of the buffering domains*

Fig.3 in [10] shows a pH titration of the proton-trapping capacity. The steepness of the titration curves suggested the concerted trapping of two protons ($n=2$). The pK varied between 7.2 and 7.7 depending on the chloroplast sample and on the terminal electron acceptor. This hinted as to a possible redox sensitivity of the proton-trapping groups. We investigated this matter in detail. We poisoned slow proton release at the cytochrome f/b_6 complex by DNP-INT (2 μM) [15] to eliminate slow proton release due to plastoquinol oxidation. To overcome the limited electron storage capacity of the plastoquinone pool, an artificial quinone (dimethylquinone (DMQ) 5 μM) was added to increase the pool size. Reoxidation of the pool in the dark was accelerated by ferricyanide (200 μM–2 mM). The upper traces in fig.3 show the internal acidification. In the absence of gramicidin acidification increased rapidly as is characteristic of proton release from water oxidation. In the presence of gramicidin, proton release was virtually abolished during the first flashes (lower trace in upper half of fig.3). The plot of the extent as a function of flash number (fig.3, lower half) differs from that observed with methyl viologen as elec-

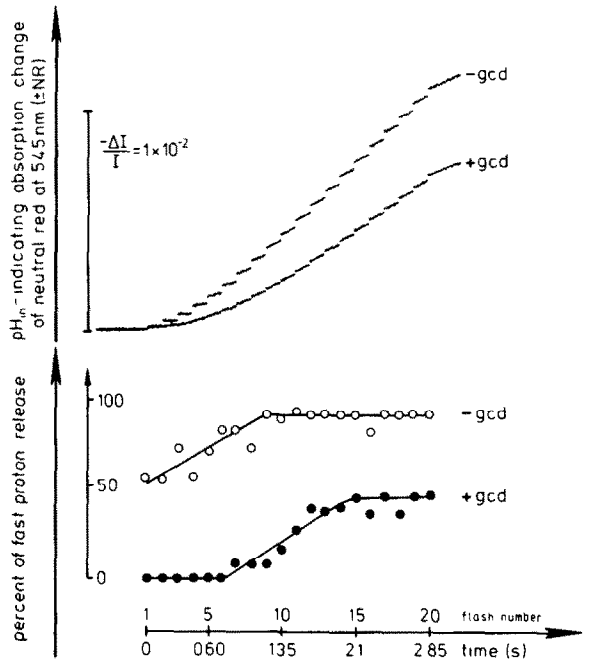

Fig.3 (Upper half) Rapid proton release into the lumen of the thylakoids in the absence (upper trace) and presence of gramicidin (0.2 nM) (lower trace). Further additions to the assay medium were DNP-INT (2 μM), DMQ (5 μM) and ferricyanide (200 μM). (Lower half) Relative extent of rapid alkalinization obtained from the pH jumps shown in the upper half. Excitation conditions as in fig.1.

tron acceptor (fig.1, dashed line) in both the absence and presence of gramicidin. With gramicidin, in particular, fast proton release was totally abolished during the first 6 flashes (fig.3) and not only diminished as in fig.1 (dashed line), reaching a maximum of only 50% of the control after 15 flashes.

The difference in these two types of behaviour could be interpreted in two ways. (i) The ratio of pool size to active pumps was doubled, when only water-derived protons were released. This would imply that plastoquinol-related protons contributed to the filling of the proton-trapping domains in contradiction with previous findings [10]. (ii) Addition of DMQ or/and ferricyanide enhanced the capacity of the proton-trapping domains. We investigated the effect of each component (ferricyanide/DMQ) separately and in the absence of the inhibitor DNP-INT.

Fig.4 shows proton release into the lumen in the presence of ferricyanide for the first 5 flashes of the sequence. In the absence of gramicidin water protons (half-rise time 6 ms) were released during all flashes while plastoquinol protons appeared only after each even-number flash (fig.4, upper). The binary oscillation of the latter reflected the oxidation of the plastoquinol pool and of the two bound quinones (Q_A, Q_B) by ferricyanide before firing of flash no.1. Only after input of two electrons, i.e. after two flashes, was plastoquinol formed, released into the mobile pool and finally oxidized by the cytochrome f/b_6 complex. In the presence of gramicidin and with the trapping domains open (fig.4, lower trace) water protons were virtually absent, but the binary oscillation of proton release from plastoquinol oxidation was still seen. This proved the selectivity of the domain under oxidizing conditions for protons from water oxidation.

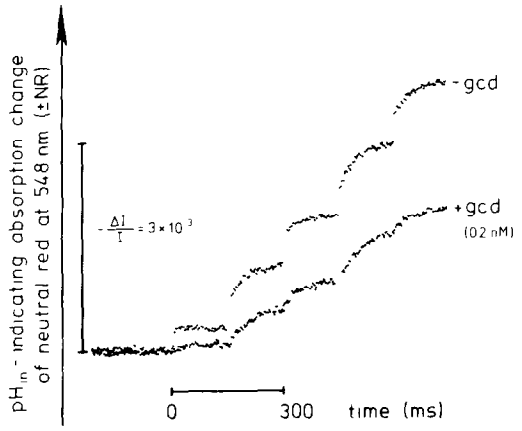

Fig.4. Proton release into the lumen of thylakoids induced by 5 successive flashes after a dark time of 40 s in the presence of ferricyanide (2 mM). Upper trace, gramicidin absent; lower trace, gramicidin (0.2 nM) present.

Fig.5 shows the relative extent of fast proton release for the whole sequence of 20 flashes. In the control (ferricyanide present, gramicidin absent) and with the domain accessible (gramicidin present) the pattern of proton recovery was very similar to that in thylakoids inhibited by DNP-INT. With the open domain, proton release reached a maximal extent of only 50% after 10 flashes. This was caused by a pK shift towards pH 7.0, similar to that shown by Theg and Junge [10]. Addition of DMQ alone enhanced the capacity of the proton-trapping domain, but in contrast to the action of ferricyanide, did not abolish proton release after flash nos 3–6 nor decrease the maximal extent of apparent proton release.

We investigated the reappearance of the fast phase of proton release under reducing conditions, i.e. in the presence of the reduced iron complex, $[Fe(CN)_6]^{4-}$ (fig.5, ▲) or of dithioerythritol, ascorbate, or reduced glutathione (not shown). Under reducing conditions and independent of the reductant the disappearance of protons during the first flashes was incomplete and the recovery after 7 flashes was complete. These findings cor-

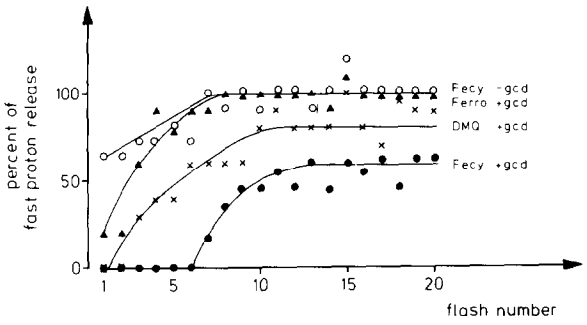

Fig.5. Magnitude of rapid proton release as a function of flash number and with different electron acceptors. Control (gramicidin absent): ferricyanide (2 mM) (○—○). Gramicidin (0.2 nM) present: ferricyanide (2 mM) (●—●), dimethylquinone (DMQ) (5 μM) (×—×), $K_4[Fe(CN)_6]$ (ferro) (2 mM) (▲—▲).

roborated the above proposal that the domain size increased under oxidizing conditions.

4. DISCUSSION

Here, we studied the redox dependence of the proton-trapping groups. We found that oxidizing conditions were responsible for the observed pK shift [10] towards acidity. The simplest way to visualize the shift is to assume that sensitive groups are located in the vicinity of an oxidizable group, which, after being oxidized to its positively charged form, electrostatically repels protons from neighbouring buffers. With evidence for the involvement of amine groups [4] and with the selectivity of proton trapping for PS II we assumed that proton trapping is due to amine groups neighbouring one redox component in PS II. The manganese centre of the water-oxidizing complex and the secondary electron donor to P680 are too positive to be oxidized by oxidants such as ferricyanide. The only known component at the donor side of PS II with a sufficiently low midpoint potential is cytochrome *b*-559 (variable between 350 and 0 mV, see [16]). Redox titrations of the respective oxidizable component are currently underway.

Our experiments using partial poisoning of PS II revealed that the proton-trapping domains were not restricted to one PS II complex each. Instead, domains which belonged to dead centres could be used by those photosystems which remained active. During the transfer to unused domains water protons were not indicated by neutral red which is an indicator of the surface pH of the membrane [7,8]. This suggested that transfer and trapping occurred via the membrane core.

The physiological role of the proton-trapping domains remains unclear. We still question their relevance for steady photophosphorylation since they are saturated at acidic lumenal pH.

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