Proton release from water oxidation by photosystem II: similar stoichiometries are stabilized in thylakoids and PSII core particles by glycerol

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Abstract During the four-stepped catalytic cycle of water oxidation by photosystem II (PSII) molecular oxygen is released in only one of the four reaction steps whereas the release of four protons is distributed over all steps. In principle, the pattern of proton production could be taken as indicative of the partial reactions with bound water. In thylakoids the extent and rate of proton release varies as function of the redox transition and of the pH without concomitant variations of the redox pattern. The variation has allowed to discriminate between deprotonation events of peripheral amino acids (Bohr effects) as opposed to the chemical deprotonation of a particular redox cofactor, and of water. In contrast, in thylakoids grown under intermittent light, as well as in PSII core particles the pattern of proton release is flat and independent of the pH. This has been attributed to the lack in these materials of the chlorophyll a,b-binding (CAB) proteins. We now found that a thylakoid-like, oscillatory pattern of proton release was restored simply by the addition of glycerol which modifies the protein–protein interaction. Being a further proof for the electrostatic origin of the greater portion of proton release, this effect will serve as an important tool in further studies of water oxidation.

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Key words: Photosystem II; Water oxidation; Proton release; Electron transfer; Stoichiometry; Glycerol

1. Introduction

Photosystem II (PSII) of green plants and cyanobacteria produces dioxygen from two water molecules at the expense of light energy. PSII is a multisubunit intrinsic protein of the thylakoid membrane. The minimal O\textsubscript{2}-evolving preparation contains the core proteins D1 and D2 which probably carry the cofactors, the core antennae CP47 and CP43, cytochrome b\textsubscript{580}, and an extrinsic luminal protein of 33 kDa which stabilizes the Mn-binding center [1]. Depending on the species further proteins are present [1]. (1) In plants the light energy is funneled to the PSII core by the chlorophyll a,b-carrying antenna (CAB) proteins: CP24, CP26, CP29, and the mobile LHII-type antennae. At the luminal side, two extrinsic proteins of 17 and 23 kDa stabilize O\textsubscript{2} evolution. Several proteins of smaller size and unknown function are also present. (2) In cyanobacteria [2] proteins of the CAB family are absent. Phycobilisomes serve as antennae instead. Of the extrinsic proteins only the 33 kDa is homologous to plant material but other small extrinsic polypeptides are also present [34].

The catalytic site of oxygen evolution is located at the luminal side of PSII. It contains a tetra-manganese cluster (Mn\textsubscript{4}) [3], probably another (amino acid) redox cofactor (X) [4], and a tyrosine, D1-tyr\textsuperscript{101} (Y\textsubscript{z}) [5]. Each of four absorbed quanta of light oxidizes P\textsubscript{680} and reduces Q\textsubscript{A}, P\textsubscript{680}\textsuperscript{+} sequentially abstracts four electrons from Mn\textsubscript{4}XY\textsubscript{z} which is thereby driven through the five increasingly oxidized states of the catalytic cycle, S\textsubscript{0} to S\textsubscript{4}. The release of O\textsubscript{2} occurs in the dark and is concentrated on only one step, S\textsubscript{3} \rightarrow S\textsubscript{4} [6]. The release of the complement of four protons, on the other hand, is spread over all five transitions S\textsubscript{0} \rightarrow S\textsubscript{1} to S\textsubscript{4} \rightarrow S\textsubscript{5}.

The rates and extents of proton release have been studied in various types of O\textsubscript{2}-evolving PSII preparations. The aim was to detect which portion of proton release was a direct consequence of the redox chemistry of water and to disclose the chemical nature of intermediates [7]. In thylakoids and BBY membranes, which contain the full protein complement of PSII, the extent of proton release as function of the S-transition oscillate with period of four in the catalytic cycle [8,9]. The strong pH dependence of these oscillations is not accompanied by a similar variation of the redox reactions [7]. The decoupling of proton release from the electron transfer between Mn\textsubscript{4}X and Y\textsubscript{z} is also kinetically apparent. Under certain conditions protons are released in 10 µs after a flash of light, i.e. before the electron hole is transferred from Y\textsubscript{z} to Mn\textsubscript{4}X [9]. The variable extents and the kinetic properties of proton release have been used to discriminate between electrostatically driven proton release from the periphery and 'chemical' proton production/uptake due to oxidoreduction [4,7,10]. Chemical' proton production that followed electron transfer from Mn\textsubscript{4}X to Y\textsubscript{z} has been detected on two transitions, namely S\textsubscript{0} \rightarrow S\textsubscript{1} and S\textsubscript{3} \rightarrow S\textsubscript{4} (for review see [7]). In the former case protons resulted from the oxidation of the cofactor X, in the latter from the oxidation of bound water.

The diagnostically important oscillations of the extent and rate of proton release are absent in thylakoids which were grown under intermittent light (IML) [11] and in PSII core
2.5. Protein compositions

2.4. Rate of electron transfer

2.3. Proton release

2.2. Flash spectrophotometry

2.1. PSII preparations

...omitted proteins [14]. Measurements in our laboratory with peripheral proton release [11]. Alternatively, the removal of most of the antenna (CAB) proteins it has been proposed that CAB proteins may contribute to the pronounced variations of peripheral proton release [11]. Alternatively, the removal of CAB proteins may expose additional amino acids of the remaining proteins [14]. Measurements in our laboratory with core particles of Synechococcus shed first doubt on these interpretations. We found that proton release oscillated in a similar fashion as in thylakoids from pea (the data are unpublished but cited by Kretschmann et al. in [15]).

In this work we solved the apparent discrepancy between thylakoids and core preparations. We compared the patterns of proton release with pH-indicating dyes in various types of PSII preparations from pea, spinach and cyanobacteria. In the presence of glycerol thylakoid-like oscillations of proton release were detected in all materials.

3. Materials and methods

2.1. PSII preparations

Thylakoids (THY) [9], PSII-enriched membrane fragments (BBY) [16], IML thylakoids (IML) [11] and O2-evolving PSII core particles of the octylglycoside-type (OGC) [12,17], and of the β-dodecylmaltoside-type (DMC) [13,18] were prepared from pea seedings. Core particles of the hemege-type (HMC) were prepared from spinach [19]. From the latter the extrinsic 17 and 23 kDa proteins were removed by NaCl washing [20] (HNC).

2.2. Flash spectrophotometry

Flash spectrophotometry [21] was done with saturating flashes from a Xenon lamp (FWHM 10 μs, > 610 nm). With dark-adapted material every flash train was recorded with a fresh sample filled into the cuvette (optical path, 1 cm) from a light-shielded reservoir. Transients were digitized, averaged (Nicolet Pro30), and stored (MicroVax).

2.3. Proton release

Proton release into the lumen of thylakoids (chl., 40 μM) and IML thylakoids (chl., 5 μM) was recorded with the amphiphilic pH-indicating dye neutral red (20 μM) as previously [9]. The medium contained BSA (2.6 mg/ml), DNP-INT (10 μM), NaCl (20 mM) and DCBQ (200 μM) plus hexacyanoferrate(III) (2 mM) as electron acceptors. With the non-vesicular membrane preparation BBY (chl., 10 μM) and with core particles (chl., 5 μM) proton release into the medium was recorded with phenol red (30 μM, pH 7) and bromocresol purple (30 μM, pH 6·5) [13]. From transients obtained in the presence of these dyes, transients obtained in their absence under the same conditions were subtracted (± dye). With non-vesicular material the medium contained no buffer in the proton measurements (otherwise 10 mM Bis-Tris was added), 10 mM CaCl₂ and hexacyanoferrate(III) (200 μM) plus DCBQ (100 μM). From the raw pH transients the numbers of protons per active PSII reaction center were obtained by setting the average over transients from flashes 2 to 8 to one proton. The first flash was neglected in the analysis because it contained variable extents of proton uptake at the PSII acceptor side due to the reduction of the non-heme-iron by Qₐ⁻ [12,22,23].

2.4. Rate of electron transfer

The rate of electron transfer during the O₂-evolving step S₄→S₀ was resolved by recording the decay of the absorption transient at 360 nm on flash number three in a series given to dark-adapted material (data here not shown; for examples of raw transients see [24,25]).

2.5. Protein compositions

The protein compositions of the PSII preparations were analyzed by SDS-gel electrophoresis followed by Western blots as described in [26]. Antibodies against CP29 (monoclonal), CP26 and CP24 (polyclonal), and the extrinsic 23 kDa protein (polyclonal) were kind gifts of Drs. S. Berg (Winona State University, Minnesota, MN, USA), A. Staehelin (University of Boulder, Denver, CO, USA) and D. Steinemann (Osnabrück/Kiel).

3. Results

At pH 7 proton release at the oxidizing side of PSII was induced by trains of nine flashes and followed by absorption transients of various pH-indicating dyes (Fig. 1, left column). The contents of glycerol and detergent (βDM) in the medium were systematically varied. We studied the effects on the rate of electron transfer upon the O₂-evolving step S₄→S₀.

3.1. Thylakoids

Fig. 1 (left) shows pH-indicating transients at pH 7 in thylakoids (THY) in the absence of glycerol and βDM (conditions listed in Table 1). The extent of proton release as function of the flash number (Fig. 1, right, THY, circles) oscillated with period of four. The oscillations were well described (line) under the assumptions that protons were distributed as 0.8:0.15:1.15:1.9 over the transitions S₀→S₁ to S₀→S₄, that 20/80% of centers were in states S₀/S₁ in the dark, and that the miss (a) and double-hit (β) factors were 5% and 7% (Table 1) as previously [9]. These properties were unchanged in a glycerol-containing medium. The half-rise time of the O₂-evolving step S₄→S₀ was 1.2 ms (Table 1).

3.2. BBY membranes

Thylakoids and BBY membranes (BBY) each contain the full set of PSII proteins (Table 1). We obtained similar oscillations of proton release as in thylakoids in the absence of βDM (Fig. 1, left, BBY) both plus or minus glycerol. Proton release as function of the flash number (Fig. 1, right, BBY, circles) was well described (line) with the same (fixed) basic stoichiometry of proton release and with the same distribution of centers over states S₀ and S₄ as in thylakoids. Only the double-hit and miss factors were slightly adjusted (Table 1). It should be noted that a fit of the observed patterns of proton release with free-running parameters produced a result (not shown) which was still very close to the stoichiometry found in thylakoids. The addition of βDM (0.02%) and a subsequent incubation for 15 min of BBY membranes (chl., 10 μM) in the absence of glycerol abolished the oscillation of proton release and about one proton was released on every flash (not shown). This behavior was accompanied by the solubilization of most of the LHC proteins and of the 17/23 kDa extrinsic proteins (Table 1). They were found in the supernatant (checked for the extrinsic proteins by immunoblotting, not shown) if the βDM-treated BBY were pelleted by centrifugation (the solubilization of LHC proteins was obvious from the about doubled rate of oxygen evolution on a per chlorophyll basis of PSII in the pellet). Concomitant with the loss of the protoytic oscillation was the down-slowing of transition S₄→S₀ to a half-time of 4 ms (Table 1) and the destabilization of the higher S-states (see below).

3.3. IML thylakoids

IML thylakoids (IML) contain no CAB proteins except for CP26, but contain all three extrinsic proteins of PSII (Table
Fig. 1. Proton release from the donor side of PSII as function of the preparation (for abbreviations see Table 1). Left column: Raw pH transients on flashes 1–9. Various pH-indicating dyes as given under Section 2 were used. Note the different time scales. The extents of the transients were normalized for comparison, up to 50 transients were averaged to improve the signal-to-noise ratio. Right column: •, oscillating proton release as function of the flash number taken from the raw data in the left of Fig. 1; ▲, non-oscillating proton release under different conditions (see text). For the parameters of the calculated lines see Table 1 and text.

1). Oscillations of proton release were very weak (Fig. 1, right, IML, triangles, for pH transients see [11]). This situation was changed in the presence of glycerol. Pronounced quarternary oscillations were apparent (Fig. 1, left, IML) which were described by about the same parameter set as in thylakoids (line). The larger miss factor reflected the reduced antenna size of PSII [11, 27]. The half-time of $S_4 \rightarrow S_0$ was normal, 1.3 ms (Table 1).
3.4. PSII core particles from plant material

PSII core particles from plant material lack the LHCII proteins.

3.4.1. OGC. Octylglycoside-type core (OGC) particles from pea contained the three core antenna (CP) and extrinsic proteins. OGC showed no oscillations of proton release (Fig. 1, triangles, OGC) without glycerol and detergent (Table 1, for pH transients see [28]). Again, glycerol induced strong oscillations (Fig. 1, left, OGC) which were fitted (right, OGC) by the thylakoid parameters (Table 1). Addition of 1 M sucrose had no effect. When βDM was added (0.02%, w/v) in the presence of glycerol oscillations were detectable after 5 min but disappeared at later times (not shown) which again was paralleled by the solubilization of the 17/23 kDa proteins. In the absence of glycerol and βDM the half-rise time of $S_1 \rightarrow S_0$ was somewhat larger than in thylakoids, 2.2 ms, and reduced to 1.8 ms (Table 1) when glycerol was added.

3.4.2. HMC, HNC. Core particles of the hecameg-type were prepared from spinach. They contain the core antenna proteins (CP). Without glycerol and βDM no oscillations of proton release were detected both in the presence (HMC) and absence (HNC) of the 17/23 kDa extrinsic proteins [19]. Again, with glycerol marked oscillations were apparent on the raw pH transients even in the presence of βDM (0.02%, w/v) in both HMC (not shown) and HNC (Fig. 1, left). Our thylakoid parameters about fitted these patterns (Fig. 1, right, HNC, line). In the presence of glycerol and βDM the half-rise time of $S_1 \rightarrow S_0$ in HMC was fast, $t_{1/2} = 1.1$ ms (Table 1). A protolytic component with this half-rise time was observed on the third flash and attributed to the final deprotonation of bound water [9]. In the absence of either the 17/23 kDa proteins in HNC or of glycerol and βDM in HMC the half-rise time increased, to 1.4 ms and 2 ms (Table 1).

3.4.3. DMC. This preparation lacks the 17/23 kDa extrinsic proteins [18] and in part CP29 (immunoblot not shown). With and without glycerol, and with βDM (Table 1), oscillations of proton release were absent (Fig. 1, right, DMC, triangles) as previously [13]. DMC were washed detergent-free and resuspended with glycerol. The time course of proton release in this material (Fig. 1, left, DMC) was slow due to particle aggregation [12] so that the flash distance was increased to 500 ms to allow for full relaxation of the pH transients. The weak oscillations of the extents (Fig. 1, right, DMC, circles) were described with our thylakoid parameters only if we assumed 30% of misses (Table 1). The patterns of proton release in DMC were similar if they were long-time dark-adapted for 15 min or repetitively dark-adapted [13] for 30 s. This result indicated that in the presence of glycerol the decay of the higher S-states into $S_1$ (likely due to the reduction of $\text{Mn}_4\text{X}$ by ferrocyanide) was still faster than in thylakoids and in the seconds range [29]. The large miss factor of 30% thus likely indicated the partial decay of S-states between the widely (500 ms) spaced flashes. We conclude that proton release in DMC oscillated similarly as in thylakoids although the 17/23 kDa proteins were lacking. Without glycerol and with βDM $S_1 \rightarrow S_0$ occurred with $t_{1/2}$ of 4.6 ms. This figure was reduced to 2.8 ms with glycerol and in the absence of βDM (Table 1).

Table 1. Various O$_2$-evolving PSII preparations, their protein composition, suspending medium and activity

<table>
<thead>
<tr>
<th>Material</th>
<th>THY</th>
<th>BBY</th>
<th>IML</th>
<th>OGC</th>
<th>HMC</th>
<th>HNC</th>
<th>DMC</th>
<th>CYC</th>
<th>COC</th>
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<tr>
<td>Sample conditions</td>
<td>Glycerol (30% w/v)</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td>+</td>
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<tr>
<td></td>
<td>βDM (0.02% w/v)</td>
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<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Protein content</td>
<td>LHCl</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>24 kDa CAB prot.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>26 kDa CAB prot.</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>29 kDa CAB prot.</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Features of the OEC</td>
<td>$t_{1/2}$</td>
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<td>1.4</td>
<td>4.0</td>
<td>1.3</td>
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<td>2.2</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Stability of S-states</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>ε (%)</td>
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<td>$5/10$</td>
<td>$5/10$</td>
<td>$15/2$</td>
<td>$13/5$</td>
<td>$3/6$</td>
<td>$30/5$</td>
<td>$1/5$</td>
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<td></td>
<td>H$^+$ time resolvable</td>
<td>+</td>
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<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td></td>
<td>Oscillating H$^+$ release$^b$</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
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</tbody>
</table>

$^b$ Data taken from [19]; (f) not determined.

peripheral event. The variations of the extents and the rates as

4. Discussion

Proton release from the oxidizing side of PSII is largely a peripheral event. The variations of the extents and the rates as

function of the S-transition, the pH and the type of preparation are not accompanied by similar variations of the rates or the progression of the redox states of oxygen evolution [7]. Bohr effects are present on all transitions. They result as response to an electrostatic field of peripheral amino acids of yet unknown chemical nature and location [7].

Based on the absence of an oscillating pattern of proton release in core particles and intermittent light thylakoids it has been argued that some of these acid residues are located on those CAB proteins that are only present in thylakoids [11,14]. In this work we showed that oscillations similar to the ones of thylakoids and BBY membranes could be induced in IML thylakoids and core particles. As thylakoids and BBY membranes contain the full set of CAB proteins whereas core particles from pea and spinach and IML thylakoids lack most, and cyanobacterial material all, these proteins are not the source of Bohr effect protons. That the latter was also true for the 17/23 kDa extrinsic proteins was apparent from a thylakoid-like behavior in such core particles in which they were absent. It follows that the acid groups are located on proteins that belong to the minimal core of O₂-evolving PSII. It incorporates the 33 kDa protein aside from the essential components, D1, D2, CP43, CP47 and cyt b559.

A thylakoid-like oscillation of proton release in core particles implies that, at least at more alkaline pH, nearly no protons are released on transition $S_1 \rightarrow S_2$. This observation is inconsistent with the hypothesis that $Y_g$ transiently releases its hydroxyl proton into the lumen. It argues against the proposal [35,36] (that has been based on the previous observations of a 1:1:1:1 stoichiometry of proton release in core particles) that a proton from bound water is transferred to the lumen on every transition.

It has been shown that when the pattern of proton release varies as function of the material and of the pH, the rate of the reduction of $P_{680}^+$ and the extents of local electrochromism due to charges on Mn€X are constant [15,37]. The latter parameters thus do not reflect the peripheral protolytic events but rather refer to the intrinsic reactions of charge transfer at the catalytic site (for review see [7]). The latter holds true in the most native material, thylakoids, and also under conditions where the native structure of the proteins at the donor side is pertubed or is restored by the addition of glycerol (in core particles and IML thylakoids).

Three explanations have been proposed to account for a flat (1:1:1:1) pattern of proton release in core particles and IML thylakoids as opposed to an oscillating one in thylakoids. (1) The removal of the CAB proteins exposes additional acid groups that respond to a charge in the catalytic center [8,14]. (2) The electrostatic interactions of the acid groups are stronger in core particles than in thylakoids. As a consequence, the deprotonation of one group suppresses the deprotonations of all other groups in the former material [13]. (3) The pKs of the same (few) groups as in thylakoids are shifted relative to each other due to alterations of the protein environment and/or the dielectric permittivity at the protein/water boundary [32]. The first explanation seems unlikely as the (experimentally observed) restoration of the native (thylakoid-like) protolytic oscillations by the addition of glycerol is not expected. The second one seems also unlikely as a stronger electrostatic interaction implies a decrease of the dielectric permittivity which is hard to reconcile with the easier access of external reductants to the catalytic site in core par-

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**Fig. 2. Hypothetical scheme which explains the effects of (1) the absence of CAB proteins, (2) the arrangement of the extrinsic proteins, and (3) the presence of glycerol and detergent ($\beta$DM) in the medium on the ability of various PSII preparations to generate thylakoid-like oscillations of proton release from the donor side. +, oscillations present; −, oscillations absent (≈1:1:1:1 pattern). Oscillations are initially present but disappear as function of the incubation time with $\beta$DM (see text). A decrease in the protein–protein interactions at the lumenal side of PSII (depicted by the size of the gaps between the proteins) eliminates protolytic oscillations, the latter are restored by glycerol. The curved arrows denote for the increased accessibility of the catalytic site to external reductants when the 17/23 kDa proteins are absent. For further details see Section 4.**
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