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Rapid Report

Photosynthetic water oxidation under flashing light. Oxygen release, proton release and absorption transients in the near ultraviolet – a comparison between thylakoids and a reaction-centre core preparation

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In dark-adapted thylakoids the pattern of proton release as a function of flash number oscillated with period four, whereas it was featureless, close to 1:1:1:1, over a wide pH range (5.5–7.5) in a reaction centre core preparation. The patterns of oxygen release and of UV-absorption transients, on the other hand, barely differed between both types of material.

The oxidation of water by Photosystem II of green plants is a four-step process which is catalysed by an Mn cluster (see Ref. 1 for a recent review). When, by dark adaptation, the catalytic centres are synchronised typically in redox state S_1, excitation with a series of short flashes of light steps the centres to higher oxidation states, S_2 and S_3, until mainly on flash number 3 dioxygen is liberated during the transition $S_3 \rightarrow S_4,S_0$. Oxygen release as function of flash number shows damped oscillation with period of four. This is interpreted in terms of the Kok scheme with only three parameters: the initial population of state S_1, the proportion of misses (α) and the proportion of double hits (β) [2]. Four protons are necessarily produced for any molecule of dioxygen which is liberated. The pattern of proton release as function of flash number is more complicated than the pattern of oxygen release. Proton release is distributed over several transitions. Previous studies by several authors with different methods have favoured an integer pattern of proton release with different stoichiometries [3–8]. More recent studies, however, have revealed a non-integer and pH-dependent pattern [9–11]. While this pattern reveals marked oscillations with period of four in thylakoids [11] and in Photosystem-II-enriched membrane fragments (BBY) [10], the oscillations are weaker in thylakoids with diminished contents of light-harvesting pigment proteins [12,13] and virtually absent in oxygen-evolving reaction centre core preparations [14,15].

The abstraction of an electron from the catalytic centre and the concomitant release of protons alters the electrostatic charge of the centre, if the ratio is not exactly 1 H^+/1 e^-. Certain absorption changes in the blue and the red spectral regions have been interpreted as a local electrochromic response to changes of the net charge [16–20]. The oscillations of the rate constant for the reduction of $P680^+$ by Y_z have also been interpreted as indicative of net charge transients [21,22]. A critical reevaluation of the featureless pattern of proton release in core preparations is desirable, as Van Leeuwen et al. [23] have reported that the pattern of the blue electrochromic bandshift *in their core preparation* is oscillatory and its pH dependence is similar as reported for BBY membrane fragments [10], whereas we found a 1:1:1:1 pattern of proton release, implying no changes of the net charge in the same preparation [24]. This has been challenged by Van Leeuwen et al. [25] on the grounds that the more rapid deactivation of higher S-states between successive flashes in core preparations might have damped out an intrinsically oscillatory pattern of proton release. This,

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Abbreviations: BSA, bovine serum albumin; Chl, chlorophyll; DCBQ, dichlorobenzoquinone; CR, Cresol red; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; DNP-INT, dinitrophenylether of iodonitrothymol; Mes, 2*N*-morpholinoethanesulfonic acid; NR, Neutral red; PR, Phenol red; PS II, Photosystem II; $S_i (i = 0–4)$, redox states of the water oxidase according to the Kok model; Q_B, secondary quinone in PS II; Y_z, primary donor to P_{680}.

in turn, has prompted us to reinvestigate the pattern of proton release in a core preparation after Ghanotakis et al. [26], which differs from the one by Van Leeuwen et al. [27] by a much slower deactivation of higher oxidation states. To evaluate the stepping through the oxidation states under flashing light we recorded in parallel the patterns of oxygen evolution and of certain UV-absorption transients which have been attributed to valence changes of the manganese cluster and/or neighbouring amino acids [16–18]. At each pH, the Kok parameters, S_1, α and β, were determined from the pattern of oxygen evolution. The respective parameter triples were then used to calculate expected patterns of UV-absorption transients as function of flash number. We thereby used the relative extinction coefficients of the four oxidation steps $S_i \rightarrow S_{i+1}$, which have been derived by Lavergne [18]. Patterns of pH transients as function of flash number were also calculated.

As starting material we used 12-day-old pea seedlings. Thylakoids were prepared according to Förster and Junge [8]. For the preparation of PS II core complexes we used the method by Ghanotakis et al. [26], but slightly modified [15]. Preparations, stored frozen at −80°C, were thawed and diluted into a medium with 5 mM $CaCl_2$. The chlorophyll concentration was determined according to Porra et al. [28].

The *oxygen yield* per exciting flash was determined by a commercial Clark-type electrode with increased platinum surface to increase the sensitivity. It was Teflon-covered for avoidance of artefacts caused by added electron acceptors (100 μM hexacyanoferrate(III) and 200 μM DCBQ). Since we were interested in a comparison of oxygen production with absorption transients, we used comparable conditions in the polarographic and spectrophotometric experiments. The electrical bandwidth was 10 Hz in all oxygen measurements. The sample volume was 100–200 μl. The chlorophyll concentration was 200 μM for thylakoids and 80 μM for reaction centre core preparations, accounting for the different antenna sizes (approx. 400 Chl/PS II in thylakoids, approx. 100 Chl/PS II in reaction centre core preparations). Actinic, saturating xenon flashes (wavelength ≥ 610 nm, duration ≈ 8 μs fwhm) were supplied through fibre optics. The sum of the oxygen release after the first four flashes was set as 100%.

Absorption changes at 320 nm were recorded flash spectrophotometrically [29]. The chlorophyll concentration in all spectrophotometric measurements was 20 μM for thylakoids and 4 μM for reaction centre core preparations. The optical path of the cuvette was 2 cm. Each dark-adapted sample was excited with a set of 9 short flashes. The binary oscillations resulting from the semiquinone $\Rightarrow$ quinol(quinone) transitions at the acceptor side of PS II were eliminated by adding 200 μM DCBQ in addition to 100 μM hexacyanoferrate(III)

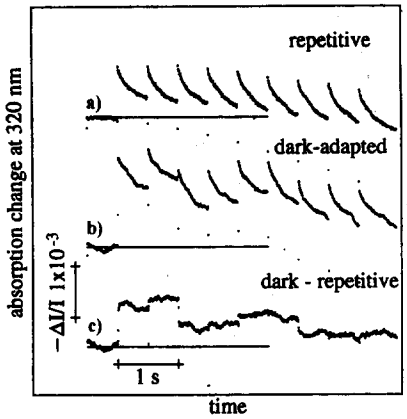

Fig. 1. Original traces from thylakoids at 320 nm under repetitive and dark-adapted conditions and their difference. 20 μM Chl (thylakoids) in 5 mM $CaCl_2$, 20 mM Mes (pH 6.5), 100 μM hexacyanoferrate(III), 200 μM DCBQ. Flashes spaced 500 ms apart, electrical bandwidth 0.1 kHz. (a) Under repetitive conditions; (b) 20 min dark-adapted; (c) difference.

[18]. The non-oscillating absorption transients which were due to the reduction of DCBQ were determined in a parallel experiment under repetitive excitation (10 groups of 9 flashes per fresh sample) and then subtracted from the traces which were obtained with dark-adapted material. To increase the signal to noise ratio, 30 signals were averaged, each representing a new, dark adapted sample excited by a series of 9 flashes. The original traces from repetitively excited material (top), from dark-adapted material (middle) and their difference (bottom) are given in Fig. 1. The sum of the changes over the first four flashes was set to 100%.

Proton release was also spectrophotometrically detected. To avoid complications due to proton uptake by the quinoid electron acceptor DCBQ as used in the UV experiments, 100 μM hexacyanoferrate(III) served as the only electron acceptor. With *thylakoids* we used the amphiphilic dye Neutral red and recorded absorption transients at 548 nm. In the presence of bovine serum albumin (2.6 mg/ml) as non-permeating buffer, pH transients in the medium were buffered and the difference of two recordings with/without added neutral red represented pH transients in the lumen [30–32]. High time resolution of the luminal pH transients was here not attempted. Therefore, the kinetic complications with this indicator in stacked thylakoids, which have been discussed elsewhere [9,11], did not matter in these experiments. DNP-INT (10 μM) was added to inhibit the reoxidation of plastoquinol at the cytochrome b_6/f complex and the concomitant proton release into the thylakoid lumen. It was checked that transient signals which were attributed to pH transients were sensitive to the addition of buffers.

Proton release from *reaction centre core preparations* was spectrophotometrically recorded by absorption

transients of hydrophilic pH-indicating dyes (Bromocresol purple, Cresol red, Phenol red) at 575 nm. The buffering capacity of the standard preparation was lowered by dilution in buffer-free medium containing 5 mM $CaCl_2$ and centrifugation (60 min, $31000 \times g$). Protolytic events at the acceptor side were quenched by using only 100 μM hexacyanoferrate(III) as electron acceptor.

Oxygen data were fitted to yield the parameter triple S_1, α and β. With this parameter set and the published extinction coefficients at 320 nm for the different S-state transitions [18] we calculated the expected absorption transients at 320 nm which compared well with the experimental ones. Using the thus cross-checked parameter triples we simulated the expected pattern of proton release per flash under the assumption of a given proton pattern per transition $S_i \rightarrow S_{i+1}$.

Fig. 2 summarises original traces of oxygen release (top), UV-absorption transients (middle) and proton release (bottom), as recorded for thylakoids (left) and reaction centre core preparations (right) at pH 6.5. The oxygen yield patterns of the reaction centre core preparation (Fig. 2, right upper trace, Fig. 4, lower part) were much improved compared to the ones in the literature [15,33]. From the oscillations of oxygen release the Kok parameter triples were calculated –they are given in percent and in the order (S_1, α, β): for thylakoids (100%, 12.5%, 7.7%) and the reaction centre core preparation (68.1%, 13.4%, 2.3%) (see also Table I). The chemical conditions were similar except for the absence of DCBQ in the experiments on proton release (bottom). We checked whether the addition of DCBQ changed the parameter triple by recording the pattern of oxygen yield with and without 200 μM DCBQ added. The result is given in Fig. 3. The respective parameter triples with and without DCBQ were 100%, 12.5%, 7.7% vs. 100%, 13.3%, 8.3% in thylakoids and 68.1%, 13.4%, 2.3% vs. 87.3%, 13.1%, 2.9% in the reaction centre core preparation. We considered these differences in thylakoids as small enough to justify the comparison of proton patterns which were recorded without DCBQ with O_2 and UV patterns which were recorded with DCBQ added, respectively. In the core preparation the increased S_1 population without DCBQ could imply an even more pronounced proton pattern.

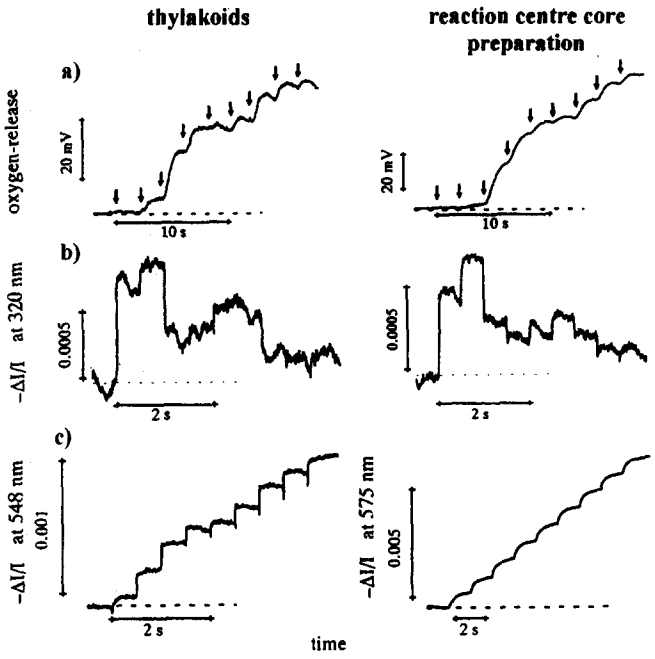

Fig. 2. Oxygen yield, absorption transient at 320 nm and proton release in thylakoids and a reaction centre core preparation at pH 6.5. Left traces: thylakoids, right traces: reaction centre core preparation. (a) 200 μM Chl (thylakoids)/80 μM Chl (reaction centre core preparation) in 5 mM $CaCl_2$, 20 mM Mes (pH 6.5), 100 μM hexacyanoferrate(III), 200 μM DCBQ. Flashes spaced 2 s apart; electrical bandwidth, 10 Hz; 20 min dark-adaptation. (b) 20 μM Chl (thylakoids)/4 μM Chl (reaction centre core preparation) in 5 mM $CaCl_2$, 20 mM Mes (pH 6.5), 100 μM hexacyanoferrate (III), 200 μM DCBQ. Flashes spaced 500 ms apart, electrical bandwidth, 100 Hz; 20 min dark-adapted minus repetitive. (c) In thylakoids: 20 μM Chl in 5 mM $CaCl_2$, 2.6 g/l BSA, 10 μM DNP-INT, 100 μM hexacyanoferrate(III), 15 μM NR (548 nm). In the reaction centre core preparation: 4 μM Chl in 5 mM $CaCl_2$, 100 μM hexacyanoferrate (III), 15 μM PR (575 nm). Flashes spaced 500 ms apart; electrical bandwidth, 100 Hz; 20 min dark adaptation.

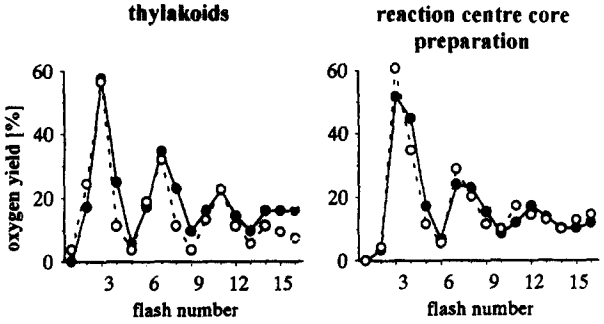

Fig. 3. Oxygen evolution in thylakoids and the reaction centre core preparation at pH 6.5 with and without DCBQ added. For measuring conditions see Fig. 2a. 200 μM DCBQ (+: full symbols, −: open symbols).

TABLE I

S-state distribution in the dark in thylakoids and the reaction centre core preparation (core prep.) as deduced from the oxygen yields

All values are given in %. S_2, S_3 were set to 0.

	pH 5.5		pH 6.5		pH 7.5	
	thylakoids	core prep.	thylakoids	core prep.	thylakoids	core prep.
α	8.6	13.6	12.5	13.4	14.4	9.2
β	9.9	2.5	7.7	2.3	6.9	4.9
S_0	0	15.7	0	31.9	0	84.9
S_1	100	84.3	100	68.1	100	15.5

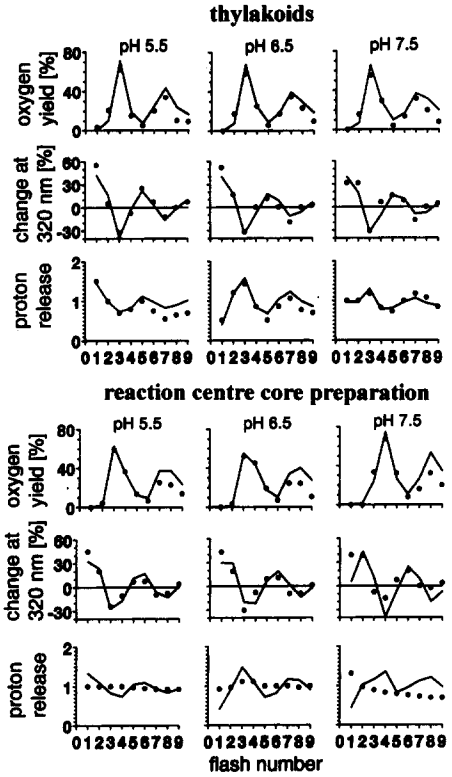

Fig. 4. Oxygen evolution, absorption transient at 320 nm and proton release in thylakoids and the reaction centre core preparation as a function of pH. Conditions as given in Fig. 3. At pH 7.5 instead of 20 mM Mes, 20 mM Hepes was used for oxygen and UV measurements. For proton measurements the pH was adjusted before dark adaptation by addition of NaOH. Data sets are given by filled circles, all simulations by straight lines. Proton release patterns, for thylakoids and the reaction centre core preparation were calculated by assuming the following stoichiometry of protons over electrons for the four transitions (starting with $S_0 \rightarrow S_1$): 0.7: 1.5: 1.1: 0.7 (pH 5.5), 0.5: 0.4: 1.3: 2 (pH 6.5), 0.3: 1: 1: 1.7 (pH 7.5).

The patterns of oxygen release and of the UV-transients were rather similar in both types of preparations, whereas the patterns of proton release drastically differed (Fig. 2). Normalising the sum of proton release over the first four flashes as 4 protons, we experimentally obtained at pH 6.5 the following distribution of proton release as function of flash number: 0.5: 1.17: 1.4: 0.93 in thylakoids and 1: 0.89: 1.06: 1.04 in the reaction centre core preparation.

We extended experiments as underlying to Fig. 3 (pH 6.5) to other pH levels, namely pH 5.5 and 7.5. Fig. 4 summarises patterns of oxygen yield, UV transients and proton release as function of flash number for three pH values. Table I shows the Kok-parameter triples which resulted from a fit of patterns of oxygen release.

Our data on oxygen evolution revealed that the dark synchronisation of catalytic centres (with dominance of state S_1) was fair in both types of material. Moreover, there was fair agreement between the experimental and expected patterns of the UV transients, with the latter based on the extinction coefficients as determined for the four transitions, $S_i \rightarrow S_{i+1}$, from experiments with BBY [18]. This extended the validity of these relative extinction coefficients to thylakoids and the reaction centre core preparation.

The patterns of oxygen evolution and of UV transients were similarly oscillating with period four in thylakoids and in the reaction centre core preparation. The patterns of proton release, on the other hand, differed considerably. A period-four oscillation was seemingly absent in the reaction centre core preparation. For illustrative purpose and to demonstrate that the deviation was real, we calculated the expected pattern as function of flash number. For thylakoids (and at each pH) we used a computer aided choice of the H^+/e^- stoichiometric quadruple over the transitions $S_i \rightarrow S_{i+1}$ $(i = 0–3)$ to yield a viable fit of the data. For the core preparation we used the same quadruple as before. The deviation in the latter case clearly exceeded noise limits.

It was obvious, though, that both the dark synchronisation and the flash induced progression through the oxidation states was similar in both types of material, whereas the pattern of proton release substantially differed. This strengthens the view that proton release barely reflects the chemical production of protons during transitions between successive oxidation states but rather the electrostatic response of peripheral amino-acid side-chains [10,11], in thylakoids with contributions from light-harvesting complexes [12]. On the other hand, this work has corroborated the featureless 1:1:1:1 pattern of proton release in this core preparation. Whether or not the same pattern also holds for the core preparation after Van Leeuwen et al. [27] has still to be corroborated. If so, the reduction rate of $P680^+$ and local electrochromism (see above for references) do not reflect the net charge, but are sensitive to geometrical details of the positioning of a positive charge on one or the other manganese atom and of a negative charge on one or the other amino acid.

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