Proton Release during the Four Steps of Photosynthetic Water Oxidation: Induction of 1:1:1:1 Pattern due to Lack of Chlorophyll *a*/*b* Binding Proteins†

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ABSTRACT: In photosynthesis of green plants water is oxidized to dioxygen. This four-step process is accompanied by the release of four protons (per molecule of dioxygen) into the lumen of thylakoids. In dark-adapted thylakoids which are excited with a series of short flashes of light, the extent of proton release oscillates with period four as a function of flash number. Noninteger and pH-dependent proton/electron ratios (e.g., 1.1, 0.25, 1.0, and 1.65 at pH 7) have been attributed to a superposition of two reactions: *chemical production* of protons and transient *electrostatic response* of peripheral amino acid side chains. Aiming at the true pattern of proton *production*, we investigated the relative contribution of peripheral proteins. Thylakoids with and without chlorophyll *a*/*b* binding proteins were compared. Thylakoids lacking chlorophyll *a*/*b* binding proteins were prepared from pea seedlings grown under intermittent light [Jahns, P., & Junge, W. (1992) *Biochemistry* (preceding paper in this issue)]. We found no oscillation of proton release in the pH range from 6 to 7.5. These and other results showed that chlorophyll *a*/*b* binding proteins, which primarily serve as light-harvesting antennas, modulate proton release by water oxidation. A non-oscillating pattern of proton release, with proton/electron ratios of 1:1:1:1 more closely represents the events in the catalytic center proper. This implies hydrogen abstraction rather than electron abstraction from water during the oxygen-evolving step $S_3 \rightarrow S_0$.

Higher plants, algae, and cyanobacteria oxidize water to produce dioxygen and protons. The photochemically active pigments and the catalytically active Mn cluster are contained in photosystem II (PS II)[1] (Babcock, 1987; Rutherford, 1989). Photosystem II is embedded in the thylakoid membrane. Photochemically driven electron transfer from water to bound quinones is directed across the membrane. This generates a transmembrane voltage and pumps protons by consecutive protolytic reactions. The oxidation of two molecules of water to yield one molecule of dioxygen and four protons requires four univalent oxidation steps in series, each powered by one quantum of light. The catalytic center thus cycles through four oxidation states (S_0, S_1, S_2, S_3) with dioxygen appearing during the transition between S_3 and S_0 (Kok et al., 1970). Since S_1 is most stable in the dark, excitation of dark-adapted membranes with a series of short (single-turnover) flashes produces an oscillating pattern of oxygen and of proton release with period four and damped. The proton pattern is interesting for identifying those transitions where electron abstraction from (bound) water might occur. On the average, all protons originate from the cleavage of water. During a given transition, $S_i \rightarrow S_{i+1}$, however, it is difficult to decide whether released protons are "chemically produced" or owed to a transient electrostatic relaxation of *peripheral* amino acid side chains in response to the positive charge generated at the oxidizing side of photosystem II (membrane Bohr effect). First evidence for the latter is the observation that proton liberation during one particular transition, $S_2 \rightarrow S_3$, is faster than electron abstraction from the manganese center (Förster & Junge, 1985). Clues for a dichotomy are expected from studies on the pattern of proton release under alteration of the protein periphery. This is the subject of this article. So far, patterns of proton release have been recorded for thylakoids (Fowler, 1977; Saphon & Crofts, 1977; Wille & Lavergne, 1982; Förster & Junge, 1985; Lavergne & Rappaport, 1990; Jahns et al., 1991), photosystem II enriched membrane fragments (so-called BBY-particles) (Wacker et al., 1990; Rappaport & Lavergne, 1991), and oxygen-evolving reaction center preparations (Lübbers & Junge, 1990). The earlier results have favored a pattern of proton/electron ratios of 1:0:1:2 for the four transitions $S_i \rightarrow S_{i+1}$ (i = 0, 1, 2, 3). Work from 1990 on has produced different patterns with noninteger ratios. Some differences between the reported results have been ascribed to methodological deficiencies, as discussed elsewhere (Lavergne & Rappaport, 1990; Jahns et al., 1991; Rappaport & Lavergne, 1991); others may be attributable to different protein peripheries in the above three types of preparations (Wacker et al., 1990; Lübbers & Junge, 1990). We used the membrane-adsorbed dye neutral red in the presence of non-permeating buffer (Junge et al., 1979, Hong & Junge, 1983; Jahns et al., 1991) to measure proton release into the thylakoid lumen. The pattern of proton release was recorded for two types of unstacked thylakoid membranes: (A) *CL-thylakoids*, i.e., thylakoids from pea plants grown under continuous light and unstacked by treatment with EDTA, and (B) *IML-thylakoids*, i.e, thylakoids from pea plants grown under intermittent light. The main difference between these preparations was the absence of chlorophyll *a*/*b* binding proteins (CAB-proteins) in IML-thylakoids. Growth conditions and biochemical and flash-photometrical properties of IML-thylakoids have been given in the accompanying article (Jahns & Junge, 1992). CAB-proteins serve as light-capturing antennas [see, for example, Peter and Thornber (1988), Green et al. (1991), and Kühlbrandt and Wang (1991)]. We have found, however, that covalent modification by *N*,*N*′-dicyc-

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[1] Abbreviations: PS II, I, photosystem II, I; CL, continuous light; IML, intermittent light; EDTA, ethylenediaminetetraacetic acid; CAB, chlorophyll *a*/*b* binding; NR, neutral red; DNP-INT, dinitrophenyl ether of iodonitrothymol; BSA, bovine serum albumin.

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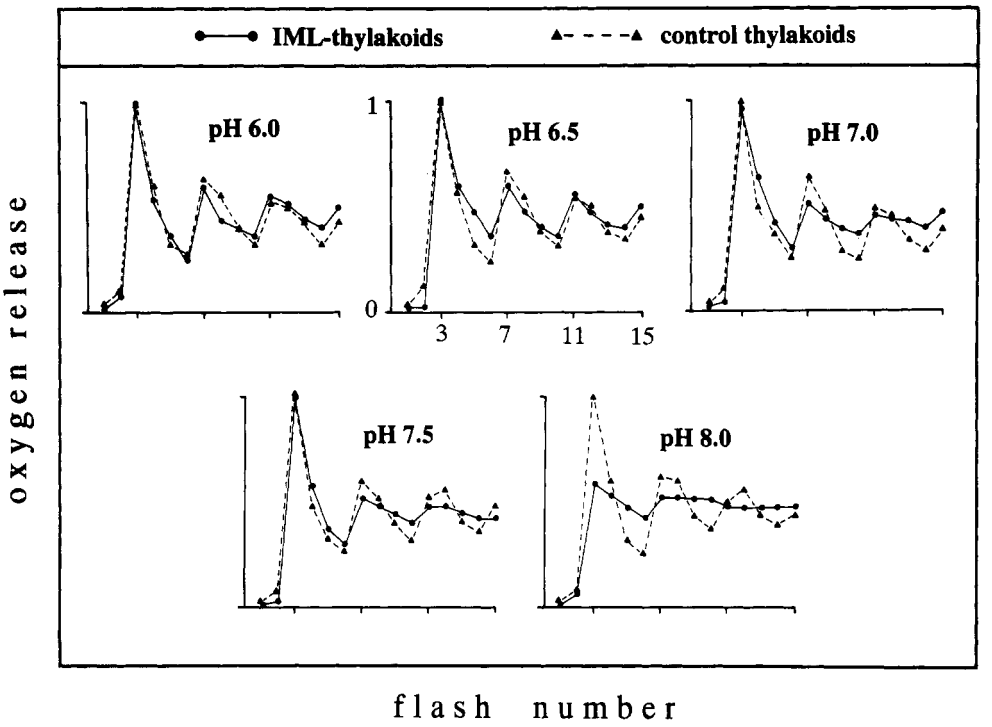

FIGURE 1: Oxygen yield as function of flash number at various pH values in the medium. Dark-adapted IML-thylakoids (circles) and control thylakoids (triangles) were compared at each pH. Samples were excited with 15 short (5 μs) saturating flashes, spaced 300 ms apart. The data were normalized to the oxygen yield after the third flash (exception pH 8; see text).

lohexylcarbodiimide (DCCD) of acidic amino acids in CAB-proteins is paralleled by a short circuit of the proton-pumping activity of photosystem II (Jahns et al., 1988; Jahns & Junge, 1990). This has been taken as evidence for an additional role of CAB-proteins as the outlet for protons from the catalytic center of water oxidation.

When dark-adapted samples of either type of preparation were excited with a series of light flashes, they produced the *same pattern of oxygen release* as a function of flash number but a *different pattern of proton release*. While CL-thylakoids again showed damped oscillations, IML-thylakoids did not. This corroborated our previous suggestion that CAB-proteins are involved, directly or as modulators of p*K*, in the protolytic reactions at the oxidizing side of photosystem II.

MATERIALS AND METHODS

Pea seedlings (*Pisum sativum*, var. Kleine Rheinländerin) were grown under intermittent light (2000 lux, 2-min light/118-min dark) for 10 days or under continuous light (10 000 lux, 16-h light, 8-h dark) for 12–14 days. Thylakoid membranes from seedlings of both growing conditions were isolated as described previously (Polle & Junge, 1986; Jahns & Junge, 1992). pH transients in the thylakoid lumen were detected by absorption changes of neutral red (NR) under selective buffering of the suspending medium by nonpermeant buffer (bovine serum albumin, BSA). To ensure rapid access of externally added buffer to the sites of proton uptake from the medium, it is necessary that the membranes are totally unstacked. Only under this condition is the selectivity of NR for pH transients in the thylakoid lumen guaranteed even at short times (Lavergne & Rappaport, 1990; Jahns et al., 1991). The rate of proton uptake at the reducing side of PS II as seen by a hydrophilic pH-indicating dye was used as a sensitive measure for the degree of unstacking, as described previously (Polle & Junge, 1986; Junge & Polle, 1986). Due to the absence of CAB-proteins, IML-thylakoids are intrinsically unstacked. This was experimentally obvious from the very fast rate of proton uptake [half-rise time 1.1 ms; see Figure 6 in Jahns and Junge (1992)]. Control thylakoids grown under continuous light were treated with 1 mM EDTA (for 10 min on ice, at a Chl concentration between 0.5 and 1 mM) to induce complete unstacking.

Flash-spectrophotometric measurements were performed as described previously [for technical setup and methodological details, see Junge (1982)]. The sample was contained in an optical cell with 2-cm path length, both for the interrogating beam and the exciting flash of light. It was determined that the energy of the exciting xenon flash (1.8 mJ/cm^2, half-time duration 5 μs, wavelength >610 nm) was saturating both photosystems. From a shielded stock vessel with a dark-adapted thylakoid suspension, 10-mL samples were automatically filled into the absorption cell for each of typically 20–60 repetitive recordings of absorption transients. These were averaged for improved signal-to-noise ratios. Thylakoids (stored at −80 °C) were suspended in 10 mM NaCl and 2.6 mg/mL BSA; 2 mM hexacyanoferrate III (Fecy) was added as electron acceptor. The chlorophyll concentration was equivalent to 5 (IML-thylakoids) and 10 μM (CL-thylakoids). pH transients in the thylakoid lumen were determined by absorption changes of the amphiphilic dye neutral red (NR). pH-specific absorption changes were obtained by subtracting a signal detected in the absence of the dye from a second one recorded in its presence [see also, Junge et al. (1979) and Junge (1982)]. In these unstacked membranes, BSA quenched all pH changes in the medium within much less than 1 ms. This left only pH transients in the thylakoid lumen to be picked up by NR. The dinitrophenyl ether of iodonitrothymol (DNP-INT, 20 μM) was added to abolish proton release from plastoquinol oxidation [Trebst et al. (1978); see also Jahns and Junge (1992)]. The medium pH was adjusted between 6 and 8 as indicated in the figure legends. Thylakoids were dark adapted for 15 min at room temperature. Samples were excited by a series of eight short flashes of light with 150 ms between them.

Table I: Relative Extent (%) of NR Absorption Transients at pH 7.8 as a Function of Flash Number[a]

	flash number			
flash distance	1	2	3	4
30 ms	100	60	53	50
100 ms	100	71	64	60
300 ms	100	77	74	72
1 s	100	97	97	97

[a] Dark-adapted IML-thylakoids were excited with a series of four short flashes. The interval between flashes was varied. Other conditions as in Figure 2. For each row the extent after the first flash was set to 100%.

Oxygen evolution under single-flash excitation was measured with an electrode as described by Schmidt and Thibault (1979). For these experiments, thylakoids equivalent to 30 μM chlorophyll were suspended in 10 mM NaCl, 15 mM $MgCl_2$ plus buffer (30 mM) as indicated.

RESULTS

Oxygen release was recorded under flashing light, and the yield was plotted as a function of flash number. This served as control for the distribution over the four oxidation states, S_i, in the dark and for the synchronization of stepped transitions between them. Recordings were made at five pH values in the medium, ranging from 6 to 8. Figure 1 shows the patterns obtained with IML-thylakoids (circles) and with CL-thylakoids (triangles). Except for pH 8, the oxygen yields were normalized to the one at the third flash. Only at pH 8, the yield for IML-thylakoids was arbitrarily set to match the average extent during flashes 11–15 in CL-thylakoids. Control thylakoids exhibited the usual oxygen release pattern over the whole pH range: a damped period-of-four oscillation with the maximum at the third, seventh, etc., flash. A similar pattern was observed with IML-thylakoids at the four more acid pH values (6.0–7.5) though with stronger damping. Independent of the type of material, IML- or CL-thylakoids, and in the pH range from 6.0 to 7.5, there was little difference between the yields during the first seven flashes. At pH 8, however, the behavior diverged. While the periodical pattern of CL-thylakoids persisted over more than 15 flashes, in IML-thylakoids it was practically damped out already after 3 flashes. This damping was caused by a dramatic deceleration of the recovery of photosystem II activity in the narrow pH range from 7.5 to 8 (Table I; see below). This was paralleled by a reduction of the rate of oxygen evolution under continuous light (Jahns & Junge, 1992).

For the pH range from 6.0 to 7.5, and considering the first six flashes only, we note that the patterns of oxygen release are similar in CL- and IML-thylakoids.

Proton release was measured by absorption changes of neutral red (NR) in the presence of bovine serum albumin (BSA) and the dinitrophenyl ether of iodonitrothymol (DNP-INT) (Junge et al. 1979; Jahns & Junge, 1992). The *pattern of proton release* in dark-adapted material is described in the following terms: The average extent over the first four flashes is arbitrarily taken as unity, and the stoichiometric ratios are written in sequence starting from the transition $S_0 \rightarrow S_1$.

Figure 2 shows transient absorption changes of NR under excitation with a series of eight flashes in IML-thylakoids (left) and in CL-thylakoids (right). The pH in the medium was adjusted to 7.2. The upward-directed jumps were evaluated. Drifts, which were due to the slow redistribution of NR in response to ΔpH, (Junge et al., 1979) were neglected. For CL-thylakoids, the recently published S-state and pH-dependent proton release pattern from water oxidation (Lavergne & Rappaport, 1990; Jahns et al., 1991; Rappaport & Lavergne, 1991) was confirmed, when account was taken of an initial S-state distribution of 25% S_0 and 75% S_1 (as estimated from the oxygen release patterns). At pH 7 the proton/electron ratios were noninteger, about 1.1:0.25:1:1.65 (Rappaport & Lavergne, 1991). The damped oscillation with period four in CL-thylakoids was directly apparent by inspection of the difference (Figure 2, bottom right) between the pattern of proton release in dark-adapted (Figure 2, dark) and light-adapted (rep) CL-thylakoids. With IML-thylakoids, the oscillation was so weak that the difference between patterns from dark-adapted and light-adapted material almost vanished (Figure 2, left). It was notable that IML-thylakoids and CL-thylakoids revealed the same pattern of oxygen release (at least for the first six flashes) but different patterns of proton release.

The pH dependence of the oscillating pattern of proton release has been characterized by Rappaport and Lavergne (1991) for photosystem II enriched membrane fragments. It showed considerable variation. When one goes from alkaline toward more acid pH there is more emphasis on the first flash and less on the third. We asked whether or not the absence of any pattern in IML-thylakoids at pH 7.2 was accidental. Figure 3 shows original traces recorded at various pH from 6.2 to 7.8. The oscillations were very weak at any pH.

Only at a pH greater than 7.5 did a monotonous decrease of the extent of proton release become apparent. This was paralleled by a monotonous decrease of the oxygen yield both under flashing light and under continuous light (see Figure 1, above, and Figure 2 of the preceding article). We investigated the relaxation behavior of this deactivation as a function of flash number by varying the time interval between consecutive flashes in a series. The results are given in Table I. At pH 7.8 there was a slow relaxation of centers. About 1 s was required for full recovery of the activity. Excitation with more closely spaced flashes caused misses of photochemical activity that could account for the stronger damping of the periodical pattern of oxygen and proton release which was observed at alkaline pH (see Figures 1 and 3). The nature of this slow process is under investigation.

Figure 4 is a summary of the patterns of proton release as a function of pH in IML-thylakoids (this work) and in photosystem II enriched membranes (Rappaport & Lavergne, 1991). The latter agreed well with the pattern we observed in CL-thylakoids (data not shown). We replotted the data from Rappaport and Lavergne (1991) as calculated from their Figure 4. While a 1:1:1:1 pattern was well approximated for IML-thylakoids at any pH, the nonuniformity and pH-dependent variations of the pattern in the other material was apparent.

For IML-thylakoids, the data points were taken directly from the upward-directed steps of absorption transients of NR. Transients after the first, second, third, and fourth flashes *mainly* represented proton release from the transition $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_0$, and $S_0 \rightarrow S_1$, respectively. An accurate recalculation of the proton stoichiometry under consideration of the S-state distribution of the data (75% S_1 and 25% S_0), double hits, and misses was not necessary, because of the nearly equal extent of the steps (see Figures 2 and 3).

DISCUSSION

Thylakoids were dark adapted to synchronize the catalytic centers of water oxidation mainly in state S_1. Oxygen and proton release under flashing light was recorded for thyla-

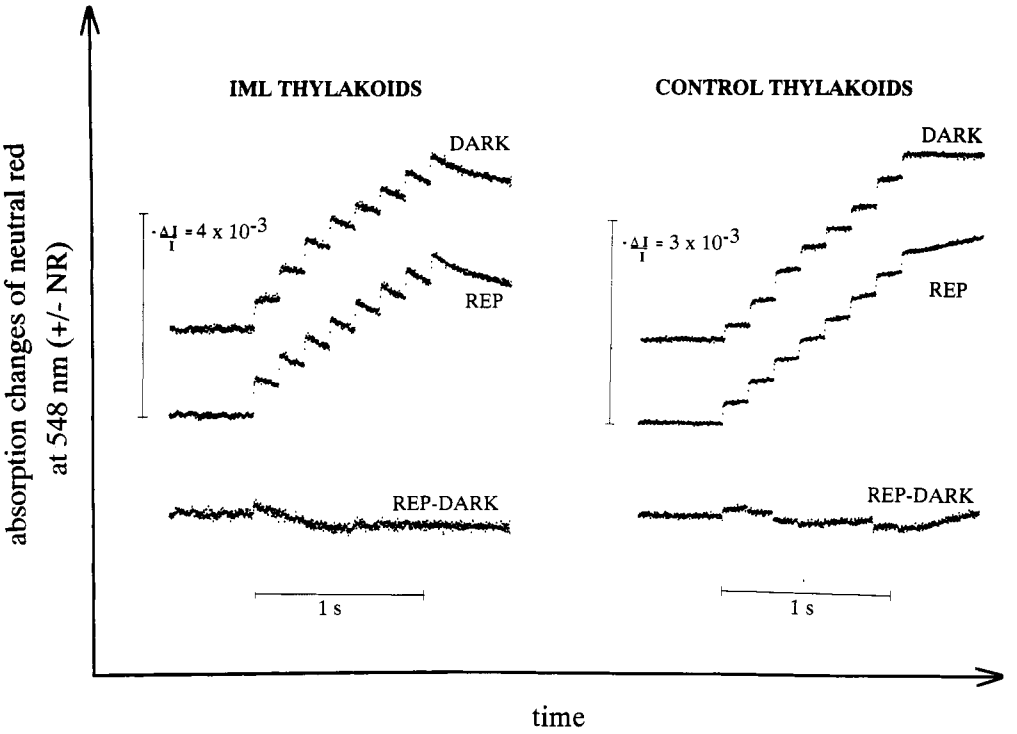

FIGURE 2: Transient absorption of neutral red (NR). Upward-directed signal indicates acidification of the thylakoid lumen. pH changes in the medium were selectively buffered by added bovine serum albumin (BSA, 2.6 mg/mL). All traces were recorded in the presence of DNP-INT, so that proton release was only due to water oxidation. For IML-thylakoids (left side) 26 μM NR was used, and for controls 13 μM NR (right side). Excitation with a series of eight flashes. Top traces (dark) samples dark adapted for 15 min; middle traces (rep) repetitive excitation; and lower traces (rep-dark) the respective differences. The only small difference between dark-adapted membranes and repetitively excited samples for IML-thylakoids (rep-dark, left side) indicates a more strongly damped proton release pattern in IML-thylakoids.

koids that were lacking chlorophyll *a*/*b* binding proteins, IML-thylakoids. The results differed from those obtained with normal thylakoids and PS II enriched membrane fragments (Lavergne & Rappaport, 1991). This is discussed below.

Pattern of Oxygen Evolution. IML-thylakoids showed the characteristic pattern of oxygen evolution with period four and a maximum at the third flash. From the seventh flash on a stronger damping than in control chloroplasts was apparent. At pH 8 oscillations were virtually absent (Figure 1). The reason for the stronger damping in IML-thylakoids at alkaline pH was a longer recovery time (about 1 s) of PS II activity after each flash (see Table I). The origin of enhanced damping is still unknown. Slower reactions on the donor side of photosystem II, a back-reaction between its donor and acceptor side, or a slower reoxidation of Q_A^- are possible candidates. The latter has been reported by Chylla et al. (1987) for *inactive* PS II complexes that may be identical with PS II_β centers. Indeed, PS II_β centers seemed to be the dominant type of PS II in IML-thylakoids [for a review, see Melis (1991)]. It was conceivable, but has still to be tested, that chlorophyll *a*/*b* binding proteins are required to support a fast rate of electron transfer from Q_A to Q_B, in particular around the physiological pH of the stroma (about 8).

At less alkaline pH (<7.5) the pattern of oxygen evolution was quite similar in thylakoids with and without chlorophyll *a*/*b* binding proteins.

Stoichiometry of Proton Release. The stoichiometry of proton release during the successive transitions of the water oxidase has been debated for a long time. Starting from the S_0 state, an integer stoichiometry of 1:0:1:2 has resulted from earlier work by various authors using different techniques (Fowler, 1977; Saphon & Crofts, 1977; Bowes & Crofts, 1978; Wille & Lavergne, 1982). Time-resolved measurements of proton release with *stacked thylakoid membranes* seemed to corroborate this stoichiometry at least for the fast components

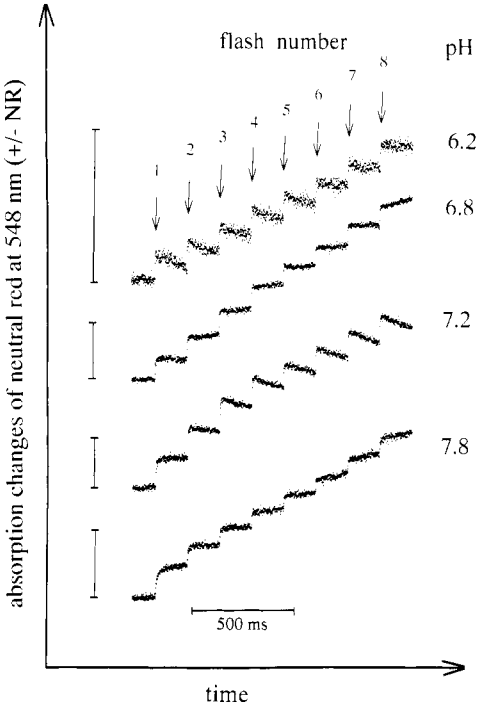

FIGURE 3: Transient absorption of neutral red at four different pH values. Same conditions as in Figure 2. Only dark-adapted IML-thylakoids were used. Ordinate bars each represent an extent of absorption changes corresponding to $-\Delta I/I = 1 \times 10^{-3}$. The pH dependence of the average extent over several flashes reflected the pH-dependent sensitivity of the indicator dye (apparent p*K* 7.1) [see Hong and Junge (1983)].

(<1 ms) of absorption transients of the pH indicator neutral red (Förster & Junge, 1985). Only this component had been attributed to proton release by water oxidation. A then unexplained much slower signal, mainly on the first flash,

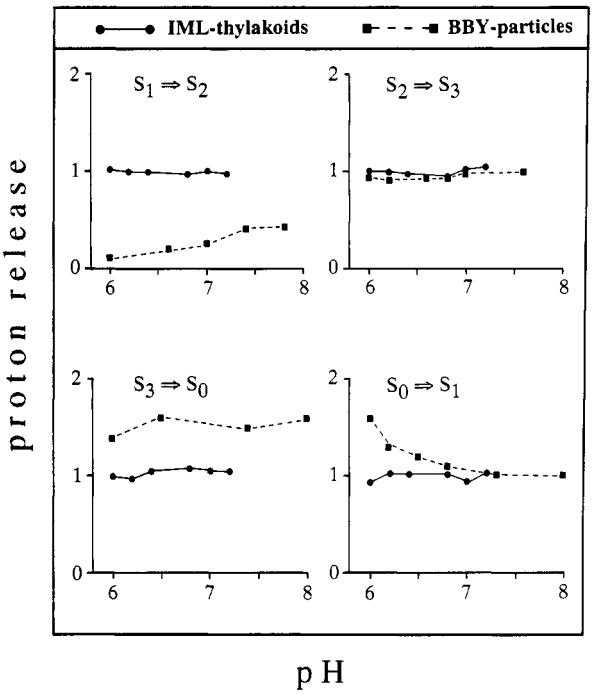

FIGURE 4: Proton/electron ratio as a function of pH. The data points for IML-thylakoids (circles) were taken directly from the extent of transients of neutral red as function of flash number (see Figure 3). The average extent of the first four flashes was normalized to 1. The extent after the first, second, third, and fourth flashes was taken as representative for proton release from $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_0$, and $S_0 \rightarrow S_1$ transitions, respectively (see text). Values for PS II membrane fragments (BBY-particles, squares) were replotted from Rappaport and Lavergne (1991). We observed the same behavior in CL-thylakoids. This is not documented.

however, had been (wrongly) neglected. Later it has been argued that this slow component is due to a superposition of two signals, namely, the response to pH transients in the lumen and the response of neutral red to the transient alkalinization at the outer side of the thylakoid membrane (Lavergne & Rappaport, 1990). In the very narrow partition region between *stacked thylakoids*, pH transients relax very slowly for known reasons (Polle & Junge, 1986; Junge & Polle, 1986), and macromolecular buffers like BSA have no access to this domain. This has been proven responsible for the slow NR response (Jahns et al., 1991). Rappaport and Lavergne (1991) have overcome this difficulty by studying proton release in open PS II membrane fragments. They have found pH-dependent stoichiometric patterns ranging from about 1.55:0.05:0.95:1.45 at pH 6, over 1.1:0.25:1:1.65 at pH 7, to 1:0.5:1:1.5 at pH 8. With correction for the slow component, a reinterpretation of the data of Förster and Junge (1985) leads to a similar result. We recorded the same pattern at pH 7 in *unstacked* CL-thylakoids in this work. Because of this agreement, we did not document our data on CL-thylakoids over the whole pH range but used the ones of Rappaport and Lavergne (1991). Noninteger proton/electron ratios are common to membranes with normal contents of CAB-proteins.

The noninteger stoichiometrical pattern and its pH dependence are interpreted as follows: Several protonable groups are electrostatically influenced by the electron carriers at the donor side of PS II. Electron abstraction from one particular carrier causes a decrease, electron donation an increase of the p*K* of groups in its vicinity. A similar situation has been characterized for the quinone-binding pocket of photosynthetic reaction centers from purple bacteria (Maroti & Wraight 1988; McPherson et al., 1988) where the structure is known to atomic resolution. Depending on the p*K* and the dielectrically weighed distance of particular groups from the location of the generated extra charge in the protein this model allows a wide range of stoichiometries of proton release/uptake per electron abstracted/deposited. Over- or understoichiometries of 1.65 or 0.25 are feasible without need to invoke chemical production/consumption of protons at the redox cofactors.

The observation in this work, that the stoichiometric pattern of proton release caused by water oxidation differs between thylakoid preparations with and without CAB-proteins, is compatible with the notion that alteration of the protein environment (other groups with different p*K* and distance, different local dielectric constant) changes the electrostatic response to electron abstraction from the catalytic center. Still, the presence of CAB-proteins is not necessary for oxygen evolution (Ghanotakis et al., 1987; Enami et al., 1989).

One might expect to approach the intrinsic stoichiometric pattern of *proton production* at the redox center by peeling off the electrostatically responding but unnecessary protein periphery. In this respect the pattern of proton release in IML-thylakoids (namely, 1:1:1:1) appears more relevant than the one in normal thylakoids or PS II enriched membrane fragments that contain the full dowry of CAB-proteins. In tendency, a featureless pattern was also observed in oxygen-evolving PS II core preparations (Lübbers & Junge, 1990) and in PS II enriched membrane fragments after treatment with trypsin (Wacker et al., 1990).

Which members of the large family of CAB-proteins are responsible for the electrostatic masking of the intrinsic pattern of proton release? Certain CAB-proteins are more tightly bound to PS II than others [reviewed, for example in Anderson and Andersson (1988) and Green et al. (1991)]. In the direct vicinity of PS II are CP 29 (Machold & Meister, 1979; Camm & Green, 1980) and CP 26 (Bassi et al., 1987). They are supposed to connect the Chl *a* containing reaction center core with peripheral Chl *a*/*b* containing antennas (Camm & Green, 1989; Barbato et al., 1990). The latter are subdivided into two classes, depending on their mobility in the membrane. The nonmobile subset contains mainly a 27-kDa CAB-protein and the mobile subset a 27- and a 25-kDa protein (Larsson et al., 1987; Spangfort & Andersson, 1989; Morissey et al., 1989). For further discussion we will name CP26 and CP29 "core antennas", the nonmobile population "inner LHC II", and the mobile population "outer LHC II".

For stacked thylakoids we have shown that DCCD binding to CAB-proteins is responsible for the protonic short circuit in PS II (Jahns & Junge, 1990). The DCCD-binding CAB-proteins of PS II (25 and 27 kDa) belong to the inner or outer LHC II proteins. With dark-adapted membranes we have found that protons from all redox transitions of the water oxidase were short-circuited in the presence of DCCD (Jahns & Junge, 1989). This implies one common way for all protons from water oxidation into the lumen. This locates the DCCD-binding site rather more closely to the Mn center than to the periphery, most likely on inner LHC II proteins. The same work has demonstrated that the oscillating pattern of proton release is determined *before* the protons "pass" the DCCD-sensitive site. If the DCCD-binding site were located on inner LHC II proteins, the conversion of the intrinsic 1:1:1:1 pattern into an oscillating one may be due to CAB core antennas. This will be investigated by a comparative study with the chlorophyll *b* deficient mutant chlorina f2 of barley that has CP29 but no LHCII and IML-thylakoids that are lacking both (Morrissey et al., 1989).

The already featureless pattern of proton release in IML-thylakoids would have been even flatter, if CAB-proteins were *totally* absent in 10-day-old pea seedlings. How to explain the release of exactly one proton upon deposition of one electron hole in the catalytic Mn center, during each of the four successive oxidation steps? At least for the first two transitions ($S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$) the hole is stored on one of the manganese atoms [see, for example, Guiles et al. (1990a,b)]. Proton release from this redox cofactor is not expected. Is the release of one proton per electron then owed to one particular ligand to this manganese atom? Not necessarily, as a rather precise stoichiometric ratio of 1 can also be owed to the more indirect electrostatic response of several remote groups. Our electrostatic model calculations predicted a proton/electron ratio near unity with less than 10% variation over 2 pH units, for two otherwise identical acid groups in the right configuration relative to the redox cofactor and the boundary between protein and electrolyte solution (not documented). Still it is tempting to associate the ratio of unity with one particular acid group in the direct vicinity of each of the cofactors that carry the newly acquired positive charge. *Each of the oxidation steps would then contribute a* (±) *charge pair in the catalytic center.* Time-resolved studies on proton release are underway to kinetically tag different sources of protons, if existing.

During the transition $S_3 \rightarrow S_0$ water enters the reaction cycle to yield dioxygen. It is obvious that three out of the four protons, which are inevitably produced during this step, are accepted by groups that have been deprotonated during the foregoing steps, since they are not detectable in the lumen. The oxidized catalytic center, in other words the (±) charge pairs, thus abstracts hydrogen atoms (or electrons and protons sequentially) from water rather than electrons. It is likely that the deprotonation of *special groups* during the foregoing steps contributes free energy to the final reaction with water.

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