Stoichiometry of Proton Release from the Catalytic Center in Photosynthetic Water Oxidation

REEXAMINATION BY A GLASS ELECTRODE STUDY AT pH 5.5–7.2a

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The catalytic center (CC) of water oxidation in photosystem II passes through four stepwise increased oxidized states (S0–S3) before O2 evolution takes place from 2H2O in the S1 transition. The pattern of the release of the four protons from the CC cannot be followed directly in the medium, because proton release from unknown amino acid residues also takes place. However, pH-independent net charge oscillations of 0:0:1:2 in S0:S1:S2:S3 have been considered as an intrinsic indicator for the H+ release from the CC. The net charges have been proposed to be created as the charge difference between electron abstraction and H+ release from the CC. Then the H+ release from the CC is 1:0:1:2 for the transitions S0 → S1 → S2 → S3 → S4. Strong support for this conclusion is given in this work with the analysis of the pH-dependent pattern of H+ release in the medium measured directly by a glass electrode between pH 5.5 and 7.2. Improved and crystallizable photosystem II core complexes from the cyanobacterium Synechococcus elongatus were used as material. The pattern can be explained by protons released from the CC with a stoichiometry of 1:0:1:2 and protons from an amino acid group (pK ~ 5.7) that is deprotonated and reprotonated through electrostatic interaction with the oscillating net charges 0:0:1:1 in S0:S1:S2:S3. Possible water derivatives that circulate through the S states have been named.

In photosystem (PS)II of oxygenic photosynthesis, the primary act starts with an electron transfer from the excited primary donor, chlorophyll a P680 (1), located at the inside of the thylakoid membrane, via a phoephtin (2) to the plastocyanin, Qa, at the membrane outside (3). The extremely high positive redox potential of P680+/P680 (≥ +1.1 eV) is the driving force for water oxidation. P680+ oxidizes tyrosine 161, YZ, located on subunit D1 of PS II (4). YZ, the immediate electron donor to P680+ (5), YZ+, in turn, extracts an electron from the water oxidizing complex and catalytic center (CC), respectively (6). For the oxidation of 2H2O, the CC passes four oxidation states, S0 → S1 (7, 8) through four turnovers of P680 Qa → P680+ Qa:, O2 evolution takes place in the S1 → S0 transition. With regard to the four H+ released from the two

H2O in the CC, a pattern of 1:0:1:2 for the transitions S0 → S1 → S2 → S3 → S4 transitions has been accepted for many years (9–11). Subsequently, it was shown, however, that the stoichiometry of the protons released into the medium is generally noninteger and depends strongly on the pH, the material, and its preparation (12, 13). It was suggested that this H+ release is a composition of protons released from the CC and protons dissociated from unknown amino acid residues by pK shifts because of electrostatic interaction with the charges of the CC. Therefore, no direct conclusions regarding the pattern of the H+ release from the CC can be drawn from pH measurements in the medium. Junge and co-workers (13, 14) have shown that in most if not in all S state transitions H+ release into the medium occurs very rapidly from amino acid residues located at the periphery of PS II possibly because of the electrostatic interaction with the charge of the transiently oxidized YZ. It was supposed that in thylakoids 3–5 unknown bases are thereby created. Water oxidation with the release of 4 H+ from 2H2O should occur in the terminal S4 → S0 transition; the protons should be trapped by the bases produced in the S4 → S0 transitions (13, 16). On the basis of an extensive biochemical consideration, Babcock and coworkers proposed a mechanism in which the tyrosyl radical abstracts a hydrogen atom from the bound water in each S state transition (see e.g. Ref. 17). The preceding oxidation of YZ through P680+ should be accompanied by one H+ release into the medium. This would correspond to a H+ stoichiometry of 1:1:1:1.

A different model has been developed since 1984 based on an observed period of four oscillation of net charges in the CC (18–21). The latter was concluded from two independent experiments: (i) In the S state cycle of water oxidation, a local stable electric field was observed by electrochromic band shifts (18, 19). It was attributed to a positive net charge of the CC in S2 and S3 relative to S0 and S1. (ii) The net charge was also monitored by the strongly retarded reduction kinetics of the oxidized primary electron donor, P680+, in the S states S2 and S3 (20). It was shown that the net charge oscillation 0:1:1:1 in S0:S1:S2:S3 is pH-independent between pH 5.5 and 7.2 (21). This pH independence was measured also for the oxidation of the CC followed at 365 nm and attributed to manganese oxidation (21) as well as for the O2 evolution (22). It was proposed that the net charge is created as the charge difference between electron abstraction and intrinsic proton release from the CC. From this it follows that the pH-independent H+ release from the CC is 1:0:1:2 coupled with the S0 → S1 → S2 → S3 → S4 → S5 transitions (18–21). This stoichiometry differs from the measurements and conclusions outlined in Ref. 12–14 (see above).

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1 The abbreviations used are: PS, photosystem; CC, catalytic center; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

2 Oxidized tyrosine is a neutral radical (15) but acts as a positive charge by the released phenolic proton, which is reversibly bonded to His(190) in its vicinity, i.e. YZ stands for Y2…H+ His190.
This may be due to the varying features of the materials investigated. In Ref. 16, it was reported on thylakoids, PS II-enriched membrane fragments, and PS II core particles from plant material. We used PS II core complexes from cyanobacteria. Our material, provided to Junge’s laboratory, was analyzed with the pH-indicating dye technique. The observed pattern of the H⁺ release in the medium (listed in Ref. 21 with the author’s kind permission) showed between pH 5 and 7 principal differences compared with that which they measured with their PS II core complexes (14) and thylakoids from pea (13). We have explained in Ref. 21 that the H⁺ pattern of our material would be in moderate agreement with the 1:0:1:2 proton release from the CC and protons from an acid group (pK ~ 6), which is deprotonated and reprotonated by electrostatic interaction with the oscillating net charge 0:0:1:1 of the CC. In this work, we give evidence for this prediction. (i) The H⁺ release into the medium was directly followed by pH measurements with a sensitive glass electrode. This technique was used to prevent problems coupled with the use of pH-indicating dyes in biological systems (see Experimental Procedure). (ii) We used improved purified PS II core complexes from cyanobacteria *Synechococcus elongatus* which are highly active in O₂ evolution and crystallizable (23). (iii) The discrepancy between the results obtained with the PS II material of cyanobacteria and the results measured in different materials from plants are discussed. (iv) Possible water derivatives that circulate through the S states have been named.

**EXPERIMENTAL PROCEDURES**

Oxygen-evolving PS II core complexes were prepared from the cyanobacterium *S. elongatus* according to Refs. 23 and 24. The PS II complexes were characterized by about 55 Chl/Qa and 66 Chl/1/4 O₂ estimated from the amount of photo-reducible Qa and by the flat membrane type (SA 9218/2.N from Schott). The reference electrode (B 2830 from Schott) was separated from the reaction medium by an electrolyte bridge filled with the reaction medium. A pH meter (model 645 from Knick) was used to read out the pH-dependent voltage. After compensation of the steady-state level by a constant offset voltage, the output signals were further amplified, filtered by a low pass filter (Dual Hi/Lo Filter model 452 from Wavetek Rockland) and digitized by a storage oscilloscope (Nicolet 1090A). The reaction medium contained 0.5 mM HEPES (pH 7.4), 0.5 mM MgCl₂, 10 mM CaCl₂, 0.02% D-maltoside, 100 μM KCl, 10 mM MgCl₂, 10 mM CaCl₂, 0.02% n-dodecyl-β-D-maltoside, 100 μM 2,5 dichloro-p-benzoquinone, 1 mM K₃[Fe(CN)₆]₃, and PS II complex equivalent to 20–30 μg chlorophyll a. For measurements 3 ml of the reaction medium were filled into the reaction vessel (diameter, 3 cm) and stirred at 17 °C. The dark-adapted samples were illuminated through the vessel bottom with saturating xenon flashes (pulse duration, 15 μs) filtered by a colored glass (RG610 from Schott). The dark time between flashes in each series of flashes was 1 s. For each flash series with a different number of flashes we used a fresh sample. The difference between the proton release after i and i + 1 flashes was calculated to obtain the proton release induced by the ith flash. Calibration of the proton release was achieved by addition of 5 μl of 1 mM HCl to the medium.

The use of a sensitive pH glass electrode to measure pH changes offers the following advantages compared with pH-indicating dye techniques: (i) Excitation of the sample by measuring light that is necessary to follow pH-indicating absorption changes is excluded by glass electrode measurements performed in the dark. (ii) The interpretation of pH-indicating absorption changes is complicated in a suspension of biological particles. The response may depend on the partition of the dye in the different phases (aqueous and hydrophobic regions and their interfaces). The meaning of a multiphasic response has led to controversial conclusions (25).

The proton release measured in dependence on the flash number is not correlated directly to the individual S state transitions. Therefore the experimental pattern of proton release was deconvoluted with the Kok parameters (S/β, ratio of dark-adapted samples, misses and double hits) to obtain the amount of protons released on every S state transition.

**RESULTS**

Fig. 1 shows the time course of pH changes measured with a glass electrode after excitation of dark-adapted PS II core complexes from *Synechococcus* at pH 6 with one, two, three, and four flashes. The kinetics of the proton release were determined by the instrumental response time of ~10 s. Dichloro-p-benzoquinone (100 μM) and K₃[Fe(CN)₆]₃ (1 mM) were used as electron acceptors (see “Experimental Procedures”). Measurements without ferricyanide show the uptake of about 1 proton/flash and PS II in addition to the proton release at the donor side of PS II (not shown). This result gives evidence that ferricyanide oxidizes the reduced dichloro-p-benzoquinone acceptor fast enough to prevent a proton uptake by reduced dichloro-p-benzoquinone at the acceptor side of PS II. Without any electron acceptor the pH change induced by the first flash was below the detection limit, and the signal amplitude induced by 100 flashes was less than 1% of the control. To obtain the proton release induced by the nth flash, the difference between the proton release after n and n − 1 flashes was calculated. Fig. 2 shows the extent of the proton release as a function of the flash number for pH 5.5, 6.0, 7.0, and 7.2 normalized to the average proton yield of flashes 1–9. The measured patterns (see data points in Fig. 2) were fitted (see solid lines in Fig. 2) according to the Kok parameters with 25% S₀ and 75% S₁ in the dark-adapted state, 10% misses, 7% double hits as well as with proton release stoichiometries for the S₀ → S₁ → S₂ → S₃ → S₅ → S₆ transitions as indicated in the legend of Fig. 2. The proton release evaluated for the four S state transitions (Fig. 2) is depicted in Fig. 3 (see data points) as function of pH.

In Ref. 21 and in the introduction it was outlined that the pH-independent H⁺ release 1:0:1:2 from the CC concluded from the net charge oscillation 0:0:1:1 is modified by protons disso-
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**Fig. 2.** Proton release per PS II into the medium measured with a glass electrode at pH 5.5, 6.0, 7.0, and 7.2 as a function of the flash number. The curves are calculated with the Kok parameters 25% S0 and 75% S1 in the dark-adapted state, 10% misses, and 7% double hits and the following proton stoichiometries for the S0 → S1, S1 → S2, S2 → S3, and S3 → S0 transitions: 1.0:0.0:1.0:2.0 at pH 7.0, 7.2, 1.0:0.2:1.0:1.8 at pH 6.0, and 1.0:0.8:1.0:1.2 at pH 5.5.

Calculated from amino acid groups to a pH-dependent H+ release into the medium. The electrostatic interaction between the net charge in S0 and an amino acid residue, AH, might result in a stable base, A−, and a proton release into the medium. If one AH exists per PS II, then m in mAH indicates the fraction of AH that is in the protonated state in S0 (Scheme 1). The base, mA−, is reprotonated when the net charge disappears in Sn → (Sn−1) → S0 through the release of two H+ from the CC. Therefore, the H+ release in S3 → (S4) → S0 is (2 − m), and in the S0 → S1 → S2 → S3 → (S4) → S0 transitions 1:m:1:(2 − m) for nBH in Scheme 1; see legend). In cyanobacteria and PS II-enriched membrane fragments from spinach an amino acid residue with a pK between 5.3 and 6.0 has been discussed (22, 26). For an optimal fit of the data in Fig. 3, we have to assume in the considered pH 5.5–7.2 range. This is the case if a ΔpK ≥ 2 is induced. Neglecting secondary effects, ΔpK ≥ 2 is consistent with the result of a simple calculation in which the reasonable values of ε ~ 10 and d ~ 12 Å are assumed for the unknown dielectric constant ε and distance d between the net charge and the acid. For a weak acid it is log (1 − m)/m = pH − pK. With this relation and a pH-independent 1:0:1:2 proton release from the CC as concluded from the net charge oscillation 0:1:1:1, it results a stoichiometry of the H+ being released into the medium between pH 5 and pH 7 as shown by the three curves in Fig. 3. This calculation fairly agrees with the glass electrode measurements in the medium (data points in Fig. 3). This agreement supports the assumption made in the calculation, especially the prediction that the pH-independent H+ release from the CC is 1:0:1:2. The stoichiometry of the proton release into the medium at pH 7.0 and 7.2 is identical to the predicted H+ release from the CC, because at this pH the acid mAH is practically deprotonated and cannot contribute to the proton release into the medium. The possible pathways of the protons released from the CC and mAH are depicted in Scheme 1.

The evaluated stoichiometry of the proton release from the CC may be a basis for obtaining information about the possible water derivatives in the different S states. In this regard, we have to take into account that the light-induced S0 → S1 state cycling can be turned backwards in the dark with reducing agents such as hydroxylamine NH2OH (27). We have shown that with NH2OH S0 can be reversibly overreduced by one electron into a state S−1 identified by a corresponding overreduced manganese state (28). In addition, we observed a local electrochromic absorption change and net charge, respectively, in state S−1 (28) (Table I). These results were supported in Ref. 29 and extended in Ref. 30. In the light-induced forward S−1 → S0 transition, the net charge disappears. This is only possible when in S−1, together with the electron abstraction, two H+ are released from the CC. Then in toto four H+ are released between S−1 and S0. With the assumption that reversibly bound water within the CC is the source of these protons, this is possible if S−1 contains 2H2O. With this “calibration” the possible water derivatives in the native S states should be those depicted in Table I and Scheme 1. Because S0 does not contain any more water protons, the S0 → S1 transition is not accompanied by a proton release from the CC. Therefore, in this transition a second net charge is created in state S1.
the conclusion that 2OH\(^-\) is the substrate in state S\(_0\); it follows that in the S\(_3 \rightarrow S\(_4\) transition, 2H\(_2\)O (not 2OH\(^-\)) are taken up with the subsequent release of two protons.

Because manganese oxidation probably takes place in each S state up to state S\(_3\) (see below) and Y\(_Z\)\(^\text{ox}\) is proposed to be the fourth oxidized state in S\(_4\) (see below), it follows that water is oxidized only in the S\(_3 \rightarrow S\(_4\) transition. The complete proton depletion from water prior to the S\(_3 \rightarrow S\(_4\) transition (Table I and Scheme 1) is in line with an energetic consideration, showing that deprotonated water is possibly the most favorable state for oxidation (31).

In Ref. 32, a slow and a fast exchanging water derivative was measured in the S\(_3\) state, a result that may exclude symmetric water ligands in S\(_3\), e.g., as predicted in Schemes 1 and 2. But, if Y\(_Z\)\(^\text{ox}\) represents the fourth oxidizing equivalent (see below), Y\(_Z\) is possibly in the vicinity of only one of the two active Mn\(^{4+}\) = O couples, because the other ligands of the two manganese may be also different, this could account for different exchange rates. Different limiting water channels between the two manganese sites and the solvent phase could also be responsible for the biphasic behavior (33).

With regard to the oxidized equivalents in the different S states, these have been analyzed by UV absorption changes attributed to oxidation of manganese (34). Manganese oxidation states derived from our measurements in the UV are depicted in Scheme 2. These were obtained inter alia under improved conditions in which the S state sequences from S\(_3\) up to S\(_4\) were before the opposite reaction S\(_3 \rightarrow S\(_0\) (35, 36). Only a manganese dimer was considered to be functional. The mixed valence states in S\(_3\) and S\(_4\), (spin = ½) are in agreement with an EPR multilinear signal in S\(_4\) (41, 42) and S\(_3\) (43). Valence states of the manganesees have also been derived from x-ray absorption shifts at the manganese-K-edge (37–40) indicating manganese oxidations up to S\(_4\). In Ref. 37 a manganese oxidation has, however, not been observed in the S\(_3 \rightarrow S\(_4\) transition. With respect to the S\(_3 \rightarrow S\(_4\) transition, a signal indicating the creation of a fourth oxidizing equivalent has not been found by any of the methods. We have proposed in Ref. 35 that the immediate electron donor to P680\(^-\), i.e., oxidized tyrosine Y\(_Z\)\(^\text{ox}\), itself, represents the fourth oxidizing equivalent, i.e., S\(_3 = Y\(_Z\)\(^\text{ox}\) = S\(_4\).

The oxidation of Y\(_Z\) in S\(_3 \rightarrow S\(_4\) was shown to take place biphasically with 50 and 260 ns (20). From this follows that the transition S\(_3 \rightarrow S\(_4\) should take place within this time (Schemes 1 and 2). We have outlined that in S\(_4\) the electrostatic field of Y\(_Z\)\(^\text{ox}\) is the actual promoter giving rise to a two-electron transfer from the two o xo-atoms O\(^2-\) to the active Mn\(^{4+}\) ions resulting in a pero xo- intermediate Mn\(^{3+}\) = O-Mn\(^{3+}\). In a further twoelectron oxidation, this may be followed by the O\(_2\) evolution within milliseconds, coupled with the formation of Y\(_Z\) and Mn\(^{3+}\) -Mn\(^{3+}\), uptake of 2H\(_2\)O, and the subsequent release of 2H\(^+\) (35). The function of Y\(_Z\)\(^\text{ox}\) as electrostastical promoter in S\(_4\) has recently also been discussed in Ref. 44.

**DISCUSSION**

For the clarification of the mechanism of water cleavage, straightforward measurement of protons released from the CC into the medium is not possible because protons released from...
Schematic 2. Model of the period four oscillation of manganese oxidation, net charge formation, and water deprotonation. The $S$ state cycle $S_0 \rightarrow S_2$ is driven by quaternary, light-induced transmembrane electron transfers from P680 to $Q_A$. $P680^+$ oxidizes step by step via tyrosine $Y_Z$, a manganese dimer up to $S_2$. The oxidized $Y_Z$ has been hypothesized in Ref. 35 to be the fourth oxidizing equivalent in $S_0$ that triggers the electron transfer from the two oxo-atoms to the four oxidizing equivalents by its electric field. For clarity the charges of the water derivatives (Scheme 1) have been omitted.

amino acid residues are superimposed. $H^+$ release from these residues is induced by electrostatic interaction between the charges in the CC and amino acid groups. In the medium very different proton stoichiometries were observed, depending on the material (thylakoids, PS II-enriched membrane fragments, and PS II core complexes) and its isolation procedure (12–14, 16). This indicates that at least part of such proton dissociation cannot have a function in connection with the events leading to water oxidation. The treatments during isolation and purification of the material may induce increased contact of hydrophobic protein domains with water and partial protein unfolding. This may be coupled with a decrease of pK values of otherwise “silent” acids, whereby these become deprotonatable. The $H^+$ release relevant to the water oxidation may be masked by interference with such nonspecific protolytic reactions. These reasons may also account for the nonoscillating proton release observed with PS II core complexes from plants (14), because different proton stoichiometries were observed, depending on the pH (5.1–8). In this work, however, we used PS II core complexes from cyanobacteria that are crystallizable. This feature indicates that unfolded protein domains or other defects of the protein matrix are most likely negligible because otherwise with this material crystallization would not be possible. This conclusion is consistent with the observation that added glycerol has no effect on the crystal formation.

Regarding the oscillating $H^+$ release in thylakoids coupled with the $S$ state transition (13), its pH dependence strongly deviates from that of our PS II core complexes shown in Fig. 3. (Recently, stoichiometries for thylakoids were reported in Ref. 14 that are different from the results detailed in Ref. 13.) From the variable extent and kinetics of the proton release at pH 6.5 and 7.5, it was concluded in (13) that 3–5 bases are created by deprotonation of different amino acid residues in the transitions from $S_0 \rightarrow S_0$. The bases have been supposed to trap the 2 $H^+$ suggested to be released upon oxidation of 2H$_2$O in the terminal $S_0 \rightarrow S_0$ transition (13, 16). This concept is, however, not consistent with the two results discussed in this work: (i) Here, the proton release of PS II complexes from cyanobacteria measured in the medium with a glass electrode can be simulated satisfactorily with the assumption of only one deprotonatable acid group $AH$ (pK1 = 5.7) and a pH-independent 1:0:1:2 proton release from the CC (Fig. 3). (ii) The result is in agreement with the net charge oscillation of 0:0:1:1 in $S_0:S_1:S_2:S_3$ (18–20). This observation was explained by a $H^+$ release from the CC with a stoichiometry of 1:0:1:2. This consistently supports the conclusion drawn from the proton measurement in the medium.

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