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O_2 evolution and Chl a_{II}^+ (P-680$^+$) nanosecond reduction kinetics in single flashes as a function of pH

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The flash-number-dependence of oxygen flash yield and nanosecond reduction kinetics of Chl a_{II}^+ (P-680$^+$) have been measured as a function of pH between pH 4.0 and 9.0 in oxygen-evolving PS II complexes from *Synechococcus* sp. (1) The oxygen-flash yield pattern has been measured at the optimum of oxygen evolution (pH 7.0) and at pH values where the inhibition of oxygen evolution is about half-maximal (pH 4.5 and pH 8.3, respectively). At pH 4.5, the oscillation pattern is the same as that observed at pH 7.0, but with halved amplitude. At pH 8.3, the oscillation pattern is strongly damped, indicating an increase of misses. The reversible inhibition at low pH already takes place in the dark. For the inhibition at high pH, at least one PS II turnover is needed. (2) The pH-dependence of Chl-a_{II}^+-reduction kinetics measured in single flashes yielded the following results. The fraction of Chl a_{II}^+ decaying in the nanosecond time-range decreases when the pH is lowered from pH 7.5 to 4.0. The nanosecond kinetics after the first flash could be adapted mono-exponentially with a half-life increasing from about 20 ns around pH 7.0 to about 40 ns at pH 4.0. The nanosecond kinetics after the second flash are biphasic around pH 7.0 with half-lives of 40 ns (60% of the total nanosecond decay) and 280 ns (40%) and change to nearly 100% with $t_{1/2} = 280$ ns at pH 4.0. The dependence of the nanosecond Chl-a_{II}^+-reduction kinetics on pH and S states is discussed. The different nanosecond half-lives may be explained by an electrostatic effect of positive excess charges in the O_2-evolving system due to a pH-dependent protonation of the S states.

Introduction

In photosystem II, four consecutive photooxidations of a specialized chlorophyll *a*, Chl a_{II} (P-680), lead to the oxidation of 2 H_2O into 1 O_2 and 4 H^+. The manganese-containing O_2-evolving complex, S, accumulates four oxidizing equivalents, passing thereby through four different redox states (the so-called S-states S_0–S_3) before oxygen is released during the transition $S_3 \rightarrow S_0$ [1–3]. The successive redox transitions $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_0$ are accompanied by proton release. The sequence of proton release measured around pH 7 is 1, 0, 1, 2 [4–7]. The electron transfer from S to Chl a_{II}^+ proceeds through an intermediate electron carrier, Z, probably a tyrosine residue of the D1 polypeptide [8–10]. In O_2-evolving PS II, Chl a_{II}^+ is rereduced with half-lives in the nanosecond time-range [11–13]. Z is oxidized in exact accordance with the reduction kinetics of Chl a_{II}^+ [10]. The half-lives of Z^+ reduction [14] and the oxidation of the S states [15–17] also coincide but occur in the micro–millisecond time-range. The electron transfer rates of $Z^+S_i \rightarrow ZS_{i+1}$ [14–17] and Chl $a_{II}^+Z \rightarrow$ Chl $a_{II}Z^+$ [18] have been shown to be dependent on the oxidation state of S. In the case of Chl a_{II}^+ reduction, the retardation of electron transfer in the higher S states, S_2 and S_3 (compared to S_0 and S_1), was explained by an electrostatic effect of a positive excess charge in the O_2-evolving complex [18]. The pattern of excess charges has been measured through electrochromic absorption changes [19].

Since protolytic reactions are involved in the sequence of redox reactions at the level of the S-state transitions and possibly also at the Z level [20,21], it is of interest to investigate the pH-dependence of water

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Abbreviations: Chl, chlorophyll; D, component in PS II, characterized by EPR signal II_{slow}; FWHM, full width at half-maximum; Mes, 4-morpholineethanesulfonic acid; PS II, Photosystem II; S, water-oxidizing system; Z, intermediate electron carrier in PS II, characterized by EPR signal $II_{very\ fast}$; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; Mops; 4-morpholinepropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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oxidation. In a preceding paper we reported on the pH-dependence of oxygen-flash yield and reduction kinetics of photooxidized Chl a_{II} under repetitive excitation [22]. In this work we have extended these investigations by measuring the flash-number-dependence of the oxygen yield and the nanosecond reduction kinetics of Chl a_{II}^+ in single flashes as a function of pH. The oscillation pattern of flash-induced O_2-yield indicates that the reversible inhibition in the acidic region can be ascribed to PS II centers which are completely blocked; whereas, the inhibition in the alkaline region is caused by an increase of misses. The dependence of Chl-a_{II}^+-reduction kinetics in the nanosecond range with respect to S state and pH value is discussed. The different nanosecond half-lives of Chl a_{II}^+ reduction ($t_{1/2} \approx 20$, 40 and 280 ns) possibly reflect the number of positive excess charges in the O_2-evolving complex, which depends on the S state [18,19] and on the pH value.

Materials and Methods

Oxygen-evolving PS II complexes were prepared from the thermophilic cyanobacterium *Synechococcus* sp. according to Schatz and Witt [23] and further purified by sucrose density-gradient centrifugation as described by Rögner et al. [24] in order to remove most of the phycobiline pigments. The PS II complexes were finally obtained in $2 \cdot 10^{-2}$ M Mes/NaOH (pH 6.5), 10^{-2} M $MgCl_2$, $2 \cdot 10^{-2}$ M $CaCl_2$ and about 1 M sucrose and stored at $-80\,^{\circ}C$. The chlorophyll content was about $2 \cdot 10^{-4}$ M in the stock suspension. The PS II complexes were characterized by a PS II/PS I ratio of more than 20, an O_2 flash yield of about $2.8 \cdot 10^{-3}$ O_2 per flash and Chl corresponding to about 90 Chl per Chl a_{II} active in O_2 evolution. Estimation of the antenna size based on the amount of photo-reducible Q_A yielded about 60 Chl/PS II indicating that approx. 30% of the reaction centers were inactive in O_2 evolution. For the measurements, the stock suspensions were diluted with the reaction medium containing $5 \cdot 10^{-2}$ M buffer, 10^{-2} M $MgCl_2/2 \cdot 10^{-3}$ M KH_2PO_4/0.5 M mannitol.

The following buffers were used:

pH 4.0	glycylglycine (pK 3.1)
pH 4.0–5.0	succinic acid (pK_1 4.19, pK_2 5.57)
pH 5.5–6.5	Mes (pK 6.15)
pH 6.5–7.0	Mops (pK 7.2)
pH 7.0–7.5	Hepes (pK 7.55)
pH 7.5–9.0	Tricine (pK 8.15)

The chlorophyll concentration and the acceptors used are given in the figure legends. Flash-induced oxygen-evolution was measured with a zirconia oxygen sensor (Programm-Electronic, Dornach, Switzerland). Purified N_2 gas (50 ppb O_2) was used as a carrier gas. Oxygen produced in the reaction cuvette was swept out by the carrier gas and transported to the zirconium dioxide high-temperature oxygen electrode. The oxygen content of the gas stream was recorded with a microcomputer (Commodore 64) and the peak due to oxygen evolution was integrated to give the absolute amount of oxygen produced. Calibration of the apparatus was achieved by injecting 50 μl of air-saturated water. Dark-adapted samples were illuminated with saturating Xe flashes of approx. 20 μs (FWHM) spaced 1 s apart. The duration of the dark-adaptation was longer than 40 min. For the measurement of the O_2-evolution pattern in flash series the O_2 yield of the ith flash was obtained as the difference between the O_2 yield after i and after $i-1$ flashes. A detailed description is given elsewhere [25]. Absorption changes at 824 nm were measured as described previously [18,22].

Results

Oxygen yield in single flashes in dependence on the pH

Fig. 1 shows the oxygen yield after excitation of dark-adapted PS II complexes from *Synechococcus* at pH 7.0 with 1, 2, 3 or 4 flashes. There is no oxygen detectable after the first flash. After two flashes the oxygen yield is 60 pmol, after three flashes 320 pmol, and after four flashes 422 pmol. The difference between the oxygen yield after i and $i-1$ flashes was calculated in order to obtain the oxygen yield induced by the ith flash.

Fig. 2 shows the oxygen yield as a function of the flash number and pH normalized to the amount per flash under repetitive excitation ($= Y_{ss}$) at pH 7.0, the optimal pH for oxygen evolution in the *Synechococcus* PS II preparations [22]. The accuracy decreases with higher flash numbers because of the difference formation (see error bars in Fig. 2). At pH 7.0, there is a typical oscillation of the oxygen yield [3] with a maximum after the third and a minimum after the fifth flash. The pattern could be fitted according to the Kok model [2,3] with 10% S_0 and 90% S_1 in the dark, 10% misses and 7% double hits upon each flash. According to the results of Vermaas et al. [26], the oscillation pattern could alternatively be adapted with 100% S_1 in the dark, 10% misses, 7% double hits and the assumption that in 19% of the PS II centres the higher S states, S_2 or S_3, can be reduced once by the donor D (characterized by EPR signal II_{slow} [27]) with a half-life of 0.9 s. This kind of adaptation is supported by EPR measurements with PS II complexes from *Synechococcus* (Bock, C.H. and Meyer, B., unpublished data). It was found that approx. 20% of D is reduced in the dark and becomes oxidized after flash excitation with a half-life of about 0.9 s.

Fig. 2 also shows the patterns of oxygen-flash yields at pH 4.5 and pH 8.3. At these pH values, the inhibition of oxygen evolution at the respective low and high pH regions is about half-maximal [22]. At pH 4.5, the oxygen yield after 40 flashes amounts to about 48% of the value at pH 7.0 (see Fig. 2). The O_2-yield oscillation

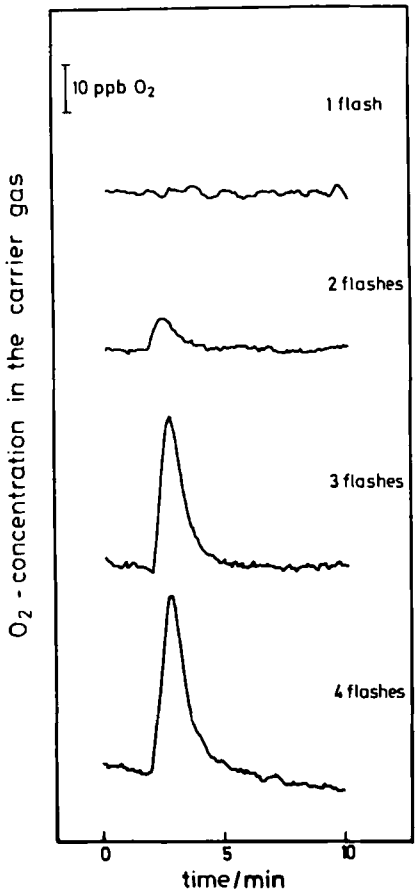


Fig. 1. Time-course of the O_2 concentration in the carrier gas measured with a Zirconia oxygen sensor (see Materials and Methods) after illumination of dark-adapted (longer than 40 min) PS II complexes from *Synechococcus* at pH 7.0 with 1, 2, 3 and 4 saturating xenon flashes of approx. 20 μs FWHM. The flashes were spaced 1 s apart within each series of flashes. The area under the curves represents the respective oxygen yield taking into account the volume flow of the carrier gas (4.1, 3.9, 4.3 and 4.3 ml/s from top to bottom). Volume of the samples 1.2 ml; $3 \cdot 10^{-5}$ M Chl; $2 \cdot 10^{-4}$ M 2,5-dichloro-*p*-phenyl-*p*-benzoquinone.

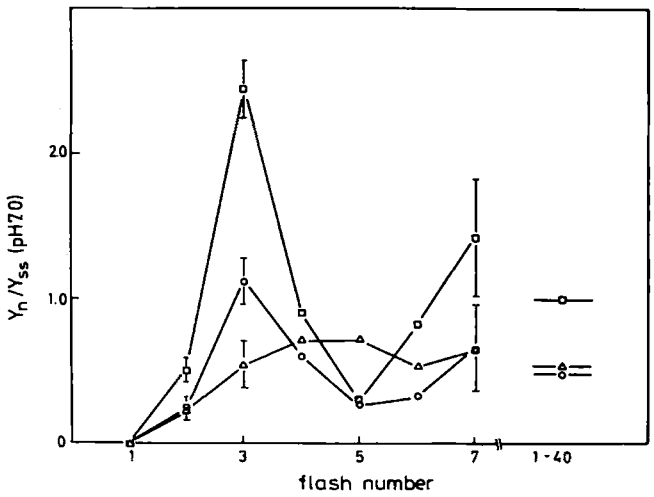


Fig. 2. Flash-number-dependent oxygen yield at pH 4.5 (○), pH 7.0 (□) and pH 8.3 (△), standardized at the average value, Y_{ss}, determined at pH 7.0 with 40 flashes. Measurement conditions as in Fig. 1.

shows about the same pattern at pH 4.5 as at pH 7.0 (only the yields are about halved) and can be fitted with approximately the same parameters. Apparently, half of the centers are functioning normally and half of them are completely blocked.

At pH 8.3, the oxygen yield after 40 flashes is about 54% of the control value at pH 7.0 (see Fig. 2). The oscillation pattern is very different from those at the lower pH values. A rather flat maximum is located at the fourth and fifth flashes. The pattern could be fitted with a dark S-state distribution of 100% S_1 and 19% D (see above), 49% misses and 7% double hits. These data suggest that the inhibition of O_2 evolution at alkaline pH is mainly due to an increase of the miss parameter.

Chl a_{II}^+ reduction in single flashes in dependence on the pH

The reduction kinetics of Chl a_{II}^+ (P-680$^+$) were analyzed by measuring the absorbance changes at 824 nm. Previously, we reported the pH-dependence of Chl-a_{II}^+ decay kinetics under repeptitive flash excitation [22]. In this work we studied the Chl-a_{II}^+-reduction kinetics in dark-adapted PS II preparations after excitation with one or two flashes and, for a comparison, under repetitive excitation.

For these experiments we used purified PS II preparations from *Synechococcus* which are depleted for the most part of the (allo)phycocyanine pigments [24]. The fast peak ($t_{1/2}$ less than 5 ns) observed otherwise in 824 nm measurements [22] was strongly reduced by this purification.

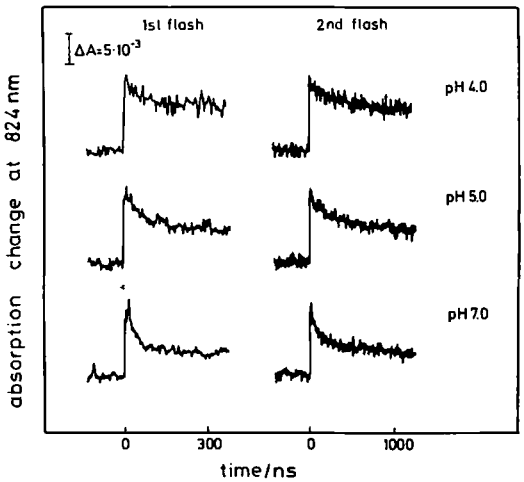


Fig. 3. Time-course of absorption change at 824 nm after the first and second flash measured in dark-adapted PS II complexes from *Synechococcus* at pH 4.0, pH 5.0 and pH 7.0; $3 \cdot 10^{-5}$ M Chl, $2 \cdot 10^{-4}$ M 2,5-dichloro-*p*-benzoquinone; optical path of the measuring beam 50 mm, electrical bandwidth 100 Hz–50 MHz; digitized with 2 ns/point; flash distance between the first and second flash 1 s; excitation energy of the 3 ms laser flashes at 532 nm corresponds to about 95% saturation. The signals are an average of n independent measurements ($n = 4$ at pH 4.0, $n = 7$ at pH 5.0 and $n = 6$ at pH 7.0).

Fig. 3 shows the time-course of the absorption changes at 824 nm after the first and second flash at pH 4.0, pH 5.0 and pH 7.0 in the nanosecond time-range. Lowering the pH, we found that (a) the fraction of Chl a_{II}^{+} decaying in the nanosecond time-range decreases and (b) the kinetics of the remaining nanosecond fraction are retarded.

The Chl a_{II}^{+} portion that is reduced in the nanosecond time-range after the first flash, after the second flash and under repetitive excitation is depicted in Fig. 4 as a function of the pH value. Very similar pH-dependencies are observed. It has been shown before [22] that the amplitude of nanosecond decay phases of Chl a_{II}^{+} and the O_2-flash yield follow the same pH-dependence under repetitive excitation. At pH 4.0, the relative nanosecond amplitude is slightly larger after the first and second flash than under repeptitive excitation. This deviation is probably due to the scattering of the data observed at pH 4.0, which was also found for the flash-induced oxygen yield.

The solid line in Fig. 4 has been calculated according to the assumption that the inhibition in the acidic region is caused by a single protonation with pK 4.5. The inhibition must already have taken place in the dark, since already for the first flash the nanosecond portion of the signal decreases upon lowering of the pH.

In order to analyze the change of the kinetics of the remaining nanosecond fraction, the signals were fitted by either a monophasic or biphasic exponential decay. Contributions from phases decaying in the micro- and millisecond range were taken into account by addition of a slightly declining straight line.

The decay after the first flash in the time-range up to 400 ns can be satisfactorily fitted by one exponential. The resulting half-lives as a function of pH are shown in Fig. 5. Above pH 6.0, the Chl a_{II}^{+} reduction proceeds

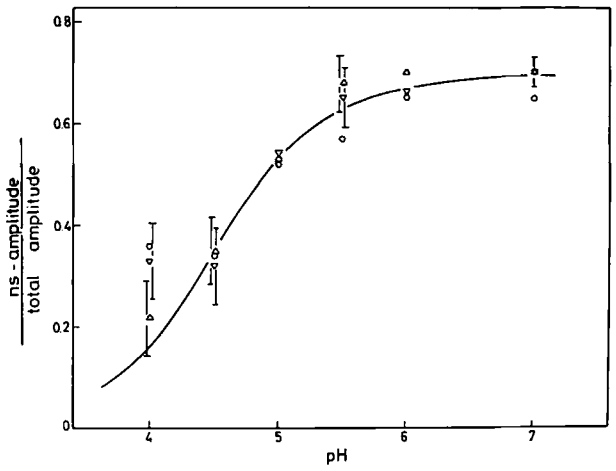


Fig. 4. Nanosecond amplitude of the Chl a_{II}^{+} reduction related to the total initial amplitude at 824 nm after the first (▽) and the second (○) flash and during repetitive excitation (△) in dependence on the pH value. Experimental conditions as in Fig. 3.

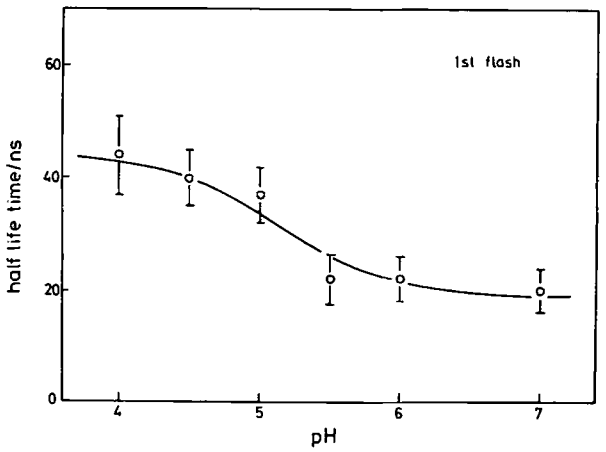


Fig. 5. pH-dependence of the half-life of the Chl a_{II}^{+} reduction in the nanosecond time-range after excitation of dark-adapted PS II preparations after the first flash. The half-lives are results of a one-exponential fit with slightly declining slope. Experimental conditions as in Fig. 3.

in about 20 ns as reported before [18], but below pH 6.0 the kinetics are slowed down to about 40–50 ns. The depicted calculated curve is based on a single protonation with pK = 5.3.

The nanosecond decay kinetics of the second flash is biphasic and has been fitted by two exponentials. In accordance with earlier data [18] half-life times of 40 and 280 ns were found above pH 6.0. For the sake of simplicity, these half-life times were fixed, analyzing the kinetics of the electron transfer to Chl a_{II}^{+} upon lowering the pH. Using this fitting procedure, the amplitude portions $a_{40\,ns}$ and $a_{280\,ns}$ are pH-dependent. Fig. 6 shows the ratio of the amplitude portion of the 40 ns phase and the total nanosecond amplitude ($a_{40\,ns} + a_{280\,ns}$). The portion of the 40 ns reduction phase increases from 17% at pH 4.0 to 63% at pH 7.6. At pH 7.6, the analysis is more complicated due to the similarity of the 280 ns kinetics in O_2-evolving centers and the reduction kinetics of Chl a_{II}^{+} in PS II centers inhibited in oxygen evolution [22]. For an unequivocal determination of the amplitude portion of the 280 ns phase belonging to PS II centers active in oxygen evolution, the donor Z in the inhibited PS II centers was kept oxidized before the second flash by shortening of the flash distance to 10 ms between the first and second flash. For the measurements at pH 7.6 the first flash was delivered by a Xenon flash lamp, the second flash by the 3 ns laser. The amplitude portions of the 40 ns phase (approx. 60%) and the 280 ns phase (approx. 40%) determined in this way were found unchanged between pH 7.6 and 6.0. Lowering the pH to pH 4.0, the amplitude portion of the 40 ns phase decreases from 60 to about 17%. The remaining 17% might be caused by PS II centers which are in the state S_1 prior to the second flash. In these centers, Chl a_{II}^{+} would be reduced by 44 ns as observed after the first flash. This assump-

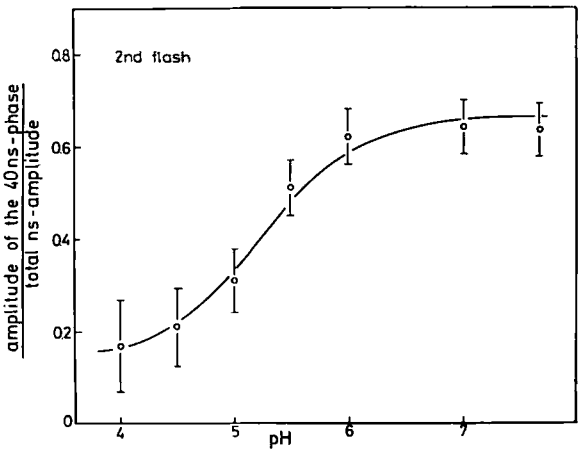


Fig. 6. pH-dependence of the amplitude of the 40 ns phase related to the total amplitude of the Chl a_{II}^{+} reduction (40 and 280 ns phase) after the second flash. The amplitude portions, a_{40} and a_{280}, are the results of a two-exponential fit with fixed half-lives (40 ns and 280 ns) and slightly declining slope. Experimental conditions as in Fig. 3.

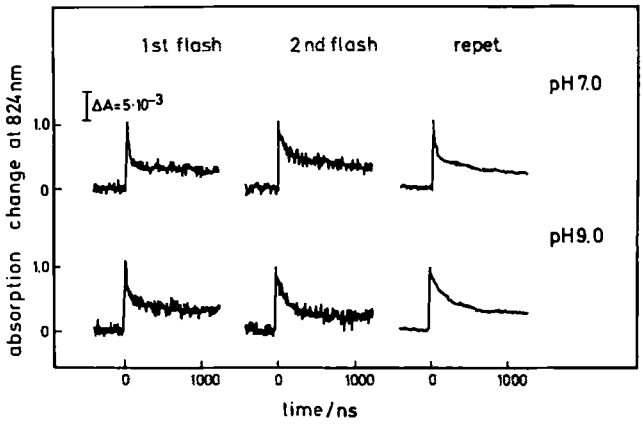


Fig. 7. Time-course of the absorption change at 824 nm after the first and second flash after dark-adaptation and after repetitive excitation with 64 flashes, measured in PS II complexes from *Synechococcus* at pH 7.0 and 9.0. The signals at pH 7.0 are the same as those shown in Fig. 3. The signals after the first and second flash at pH 9.0 are the average of eight independent measurements. Other experimental conditions as in Fig. 3.

tion is in accordance with the relative populations of the S states, which was calculated with the fitting parameter for the oscillation pattern of the O_2 flash yield.

The calculated curve depicted in Fig. 6 reflects a single protonation with pH 5.3, the same as that for the retardation of fast nanosecond kinetics after the first flash. These data suggest that the retardation of nanosecond kinetics after the first and second flash in the acidic pH region is caused by the same protonation.

The following is to analyze whether the inhibition of oxygen evolution in the alkaline pH region takes place in the dark or only in the higher S states as proposed earlier [28]. If the inhibition takes place in the dark, then Chl a_{II}^{+} should be reduced with a half-life of 180 ns after the first flash. This half-life was found under repetitive excitation after inhibition by alkaline pH (pH 9) [22]. Fig. 7 shows the absorption changes at 824 nm in a dark-adapted PS II preparation after the first and second flash and under repetitive excitation. The signals are shown for pH 7.0 and pH 9.0. Under repetitive excitation an almost single-phased nanosecond decay with a half-life of 180 ns is observed after inhibition at pH 9. The nanosecond decay kinetics in the first flash were fitted by two exponentials.

Table I lists the half-lives and amplitude portions for the first flash at pH 7.0, 8.6 and 9.0, resulting from the fitting procedure. After the first flash, Chl a_{II}^{+} is reduced in the nanosecond time-range mainly with a half-life of 20 ns, even at pH 9.0. This indicates that the inhibition in the alkaline pH region does not take place mainly in the dark. The 140 ns phase might reflect a small fraction of PS II centers inhibited prior to the first flash. After the second flash, the decay kinetics are most probably a mixture of the slow nanosecond kinetics (50 and 280 ns) observed normally after the second flash in O_2-evolving centers and the 180 ns kinetics observed in inhibited centers. Therefore, it is not possible to evaluate the degree of inhibition prior to the second flash.

TABLE I

Results of the two-exponential fit for the signals after the first flash shown in Fig. 7 and at pH 8.6

$t_{1/2}$, half-life; a_1, a_2, relative initial amplitudes of the fast and the slow nanosecond phase; a_3, relative initial amplitude of the micro- and millisecond phases.

pH	$t_{1/2}$ (ns)	a_1	$t_{1/2}$ (ns)	a_2	a_3
7.0	18	0.62	200	0.06	0.32
8.6	20	0.56	120	0.16	0.28
9.0	21	0.45	140	0.24	0.31

Discussion

Inhibition of oxygen evolution at extremes of pH

The oscillation pattern of flash-induced oxygen yield has been measured at the pH optimum and at pH values where the inhibition is about half-maximal (pH 4.5 and pH 8.3) (see Fig. 2). Two different reasons have been found fo the inhibition at acidic and alkaline pH.

The oscillation pattern observed at pH 4.5 resembles very much that at the pH optimum, only the yields are about halved. We conclude that a protonation at low pH ($pK_a = 4.5$) results in an all-or-none type inhibition, i.e., a fraction of the PS II centers is completely blocked. This inhibition has been shown to be reversible [22]. Fig. 4 shows that the fraction of Chl a_{II}^{+} decaying in the nanosecond time-range after the first flash and, under repetitive excitation, decreases in the same way as a function of pH. This indicates that the inhibition occurs

in the dark. In order to explain the all-or-none type inhibition, we assume that the transition between the inhibited and the non-inhibited state and vice-versa is slow compared to the duration of the measurement (approx. 10 s).

A rather different oscillation pattern is observed at pH 8.3 (see Fig. 2). The strong damping indicates an increase of misses during the water-cleavage cycle. The high-miss factor obtained by the fit (approx. 49%) corresponds to a flash-induced oxygen yield of 60% under repetitive excitation. This is near the experimental value (approx. 54%). The high-miss factor might be explained by the assumption that the transition between the inhibited and the non-inhibited state and vice-versa occurs on a time-scale which is rapid compared to the duration of the measurement.

From measurements of ultraviolet absorbance changes in BBY PS II preparations it has been recently concluded that at pH 8.3 mainly the S_0 state is populated in the dark [29]. Our data (see Fig. 2) cannot be fitted with a dark S-state distribution of 100% S_0, 10% misses and 7% double hits. However, based on the oscillation pattern of the O_2 yield, it cannot be excluded that both an increase of misses and an increased S_0 population appear at alkaline pH.

Since the Chl-a_{II}^+-reduction kinetics for the first flash are nearly unchanged between pH 7.0 and 9.0 (see Fig. 7 and Table I) we conclude that the inhibition in the alkaline pH region does not take place in the dark. This finding is in accordance with the earlier finding that probably the higher S states are sensitive to alkalinization [28].

Nanosecond reduction kinetics of Chl a_{II}^+ at low pH

Lowering the pH, the fraction of inhibited PS II centers increases, but the still active centers show an O_2-yield oscillation pattern that looks very similar to the normal pattern at the pH optimum (see Fig. 2). This indicates that S_1 is predominantly present prior to the first flash and S_2 predominantly prior to the second flash, independent of pH between pH 4.0 and 7.0. Despite that, the Chl-a_{II}^+-reduction kinetics change between pH 4.0 and 7.0. In state S_1 (monitored by the first flash in dark-adapted preparations), the kinetics are retarded from 20 ns around pH 7.0 to 40–50 ns at pH 4.0. In state S_2 (monitored by the second flash) the amplitude of the phase with $t_{1/2} = 280$ ns increased from about 40% relative to the total nanosecond amplitude at pH 7.0 to approx. 100% at pH 4.0, whereby the amplitude of the 40 ns phase decreases. The pH-dependence of kinetics after the first and second flash indicates a monoprotic binding site with an equilibrium constant corresponding to a pK_a value of about 5.3 (see Figs. 5 and 6).

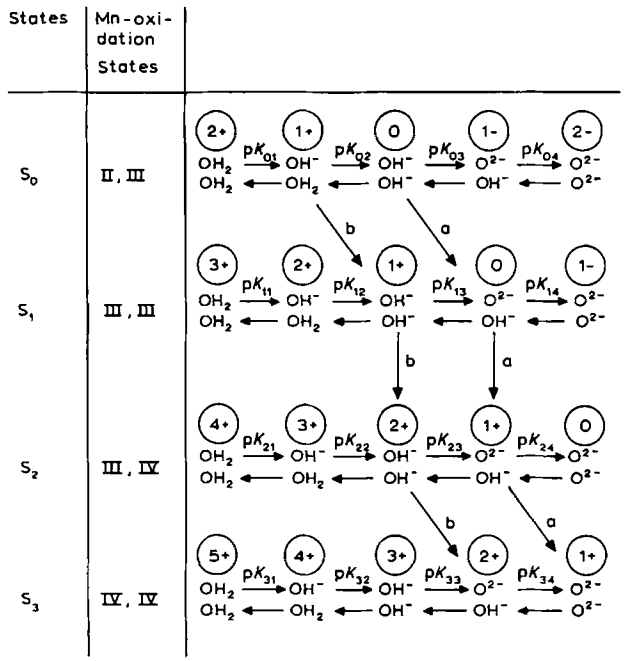
The rate constant of deprotonation, k_D, of an acidic group with pK_a of 5.3 can be estimated to be approx. $5 \cdot 10^5$ s^{-1} ($= K_a \cdot k_A$), assuming a diffusion-controlled rate of protonation, k_A, of 10^{11} $M^{-1} \cdot s^{-1}$. Since the electron transfer, Chl a_{II}^+ $Z \rightarrow$ Chl a_{II} Z^+, is much more rapid [10–14], the observed pH effects on the nanosecond kinetics do not reflect a protolytic reaction coupled with the oxidation of the intermediate electron carrier, Z. Instead, it has to be a protonation that takes places before the flash, i.e., in the dark.

With regard to recent evidence that Z is a tyrosine [8–10] the pK_a value of oxidized Z would be expected to be less than 0 [30] and that of reduced Z should be around 10. This would also exclude a protolytic reaction at the level of Z as the origin of the pH effect.

A possible model to explain the observed ns kinetics of Chl a_{II}^+ reduction is offered by the assumption that the three half-lives of Chl a_{II}^+ reduction ($t_{1/2} \approx 20$, 40 and 280 ns) reflect the number of positive excess charges in the O_2-evolving complex. The lowest charge state '0' would be responsible for the 20 ns kinetics. In the state '+1', the kinetics would be retarded to 40 ns as a result of Coulomb attraction, and in the state '+2' the kinetics would be retarded to about 280 ns as a result of an even higher Coulomb attraction. It is assumed that the number of positive excess charges is regulated through a pH-dependent protonation of special acidic group with a pK value of 5.3 (see Figs. 5 and 6). In S_1, monitored by the first flash, lowering of the pH below 5.3 changes the excess charge from $0 \rightarrow +1$. Thereby, the kinetics slow down from $20 \rightarrow 40$ ns. The transition $S_1 \rightarrow S_2$ furthermore gives an increase of '+1' regardless of pH. Hence, in S_2, monitored by the second flash, a change to the acidic pH region results in $+1 \rightarrow +2$. Thereby, the kinetics slow down from $40 \rightarrow 280$ ns.

The nature of the acidic group with $pK_a = 5.3$ is not known. An interesting possibility arises when the charge states reflect protonation states of the H_2O ligands bound to the Mn clusters as depicted in Scheme I. It is based on the 2-Mn model of the water-cleavage cycle proposed by Saygin and Witt [17]. Positive excess charges result from Mn oxidation or by protonation. The number of excess charges is given relative to the S_0 state, Mn(II)Mn(III) $(OH^-)_2$.

Pathway (a) represents the reaction sequence proposed for the pH range around 7.0 [17] that takes into account a proton-release pattern of 1 : 0 : 1 : 2 for the S state transitions ($S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_0$). With the assumption that the pK values, pK_{O2}, pK_{13}, pK_{23} and pK_{34} are similar and about 5.5, protonation at acidic pH leads to an increase of one positive excess charge in all states and reaction pathway (b). Thereby, the proton release pattern would be unchanged. In this simplified model it follows that at acidic pH Chl a_{II}^+ should be reduced with $t_{1/2} \approx 40$ ns after the first flash (one positive excess charge in S_1) and with $t_{1/2} \approx 280$ ns after the second flash (two positive excess charges in S_2). A small amplitude of a 40 ns phase is expected due to



Scheme I. Simplified scheme of possible protonation states of H_2O ligands bound to a 2-Mn cluster (after Saygin and Witt [17]).

centers still in S_1 prior to the second flash. Around neutral pH, Chl a_{II}^+ should be reduced with $t_{1/2} \approx 20$ ns after the first flash (no positive excess charge in S_1) and with $t_{1/2} \approx 40$ ns after the second flash (one positive excess charge in S_2).

The charge-state model explains the observed nanosecond kinetics in the acidic pH region relatively well. A significant discrepancy concerning the second flash (i.e., the S_2 state) arises above pH 6.5, where a portion of Chl a_{II}^+ is still reduced with $t_{1/2} \approx 280$ ns. According to a single protonation with a pK_a of 5.3, only the deprotonated S_2 state (approx. 95%), i.e., the 40 ns phase after the second flash, should be present above pH 6.5. But even at pH 7.6, the portion of the slower 280 ns phase is still approx. 40% of the total nanosecond amplitude. For an explanation, we suggest that the local pH at the proton binding site may be different from the medium pH. The local pH can be modified due to a surface potential as discussed by Conjeaud and Mathis [31]. It might also be suggested that the proton-binding site is accessible to the medium pH only below pH 6.5. Buried proton-interacting groups located in the protein have been discussed to occur in PS II [32]. The observation that the oxygen -flash yield decreases below pH 6.0 [22] may indicate structural changes that increase the accessibility.

The outlined model would also affect the analysis of the kinetics after the first flash above pH 6.5. When the second flash represents a mixture of two phases with half-lives of 40 and 280 ns, reflecting the charge states '+1' and '+2', then a mixture of '0' and '+1' should be present prior to the first flash. Maybe the charge state '0' gives rise to a somewhat shorter half-life, for instance 15 ns. This, with some mixture of charge state '+1' (40 ns), would result in the observed apparent monophasic kinetics of 20–25 ns rather well. The difference between mono- and biphasic kinetics is experimentally hard to distinguish since both half-lives, 15 and 40 ns, are too close together.

An alternative interpretation [25] of the different nanosecond kinetics of Chl a_{II}^+ reduction is based on the assumption that two intermediate electron carriers are located between Chl a_{II} and the oxygen-evolving complex in series. The existence of two carriers has been proposed earlier as an explanation for the biphasic nanosecond reduction kinetics of Chl a_{II}^+ in states S_2 and S_3 around neutral pH [18]. The lack of evidence for existence of a secondary intermediate [10], however, suggests that the charge-state model is a more likely explanation for the different nanosecond half-lives of Chl a_{II}^+ reduction.

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