pH dependence of the $S_2 \rightarrow S_3$ transition associated with O_2 evolution in inside-out thylakoids

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In inside-out vesicles, a large difference is observed in oxygen yield activity between pH 6.5 and 7.6 which is in contrast to right-side-out vesicles. The light saturation behaviour of the transition $S_2 \rightarrow S_3$ also shows a pH dependence. In inside-out thylakoids, at pH 6.5, the light saturation curve of the $S_2 \rightarrow S_3$ transitions exhibits 2 different light saturation processes whereas at pH 7.6 it resembles the light saturation behaviour of the other S-state transitions. This result is explained by the existence of a second donor in the S_2 state (different from the main donor), which contributes to about 30% of the $S_2 \rightarrow S_3$ transitions at pH 6.5. This second donor needs to be protonated and thus, is not active at pH 7.6 in inside-out thylakoids.

pH effect   Oxygen evolution   Photosystem II   Inside-out thylakoid   Secondary donor

1. INTRODUCTION

The ability to isolate PS II complexes from thylakoid membranes provides a means of studying the oxygen-evolving reactions more directly. The components needed for water oxidation appear to be located at the lumenal side of the membrane [1]. Thus, the isolation of inside-out thylakoids which expose the oxygen-evolving system to the external medium has opened up possibilities for new experimental approaches to the water oxidation system (e.g., biochemical studies [1,2], proton release [3]). In PS II particles isolated by detergent methods, the oxygen evolution activity is optimum in a small pH range (5.5–6.5) and falls off rapidly at more alkaline pH values [4,5], in contrast to the constant oxygen activity of intact thylakoid membranes between pH 6.5 and 8.5 [5]. Inside-out thylakoids appear to behave like PS II particles with regard to pH. Here we investigate the causes of the pH dependence of the oxygen yield. Our results show a specific pH

Abbreviations: PS, photosystem; DCBQ, 2,5-dichloro-*p*-benzoquinone

dependence of the $S_2 \longrightarrow S_3$ transition. At pH 6.5, the quantum efficiency of the $S_2 \longrightarrow S_3$ transition is greatly enhanced with respect to that at pH 7.6, suggesting that a protonated electron carrier is involved in this reaction in one third of the centers.

2. MATERIALS AND METHODS

Inside-out and right-side-out vesicles were prepared according to Åkerlund and Andersson [6]. The 2 membrane populations were tested for sidedness by measuring the direction of light-induced proton translocation as in [6]. A rate electrode was used for oxygen flash yield measurements as described [7]. Flash excitation was provided by Stroboslave General Radio flash lamps (half rise time 3 μs). Uniformity of illumination was measured with a photodiode (HP 2-4207) and estimated to be homogeneous within ± 5%. The fluorescence yield of chlorophyll *a* was measured 25 ms after each flash of a sequence as in [8]. Signals were recorded using a transient waveform recorder (Physical Data Incorporation 523-a) and stored in a microcomputer (Apple II plus). Vesicles were used the same day as their

preparation, as the right-side-out vesicles, in particular, become more sensitive to pH after several days of storage, due to the degradation of their membranes.

3. RESULTS

Fig.1 shows oxygen yields induced by a series of high-energy flashes in inside-out vesicles at both pH 6.5 and pH 7.6 (each series was preceded by 25 flashes followed by a dark adaptation of 10 min). The small change in pH from 6.5 to 7.6 induces an inhibitory effect as indicated by a decrease in the mean oxygen yield amplitude, and an increase in the damping of the oscillations. External acceptors which can accept electrons at the level of Q_B such as DCBQ (0.1 mM) or ferricyanide (0.5 mM) were added to the circulating medium of the upper chamber of the electrode in order to increase the steady-state level of the oxygen yields. As shown in fig.1, DCBQ increased the oxygen yields after the 10th flash but did not change significantly the beginning of the oxygen yield pattern. However, the modifications due to these acceptors were often less significant than those indicated in fig.1 and variable from one sample to another.

According to the calculations of the 'misses' by the least-square fit carried out on the flash patterns in fig.1 (in the presence of DCBQ), fewer misses were found at pH 6.5 than at pH 7.6, when using either the equal [9] or unequal miss model [10] (see fit values given in figure legends). The numerical fitting on the first 10 values of flash yield, assuming a theoretical constant level of centers after each flash, is incorrect when the mean value of oxygen yield steadily decreases as a function of flash number as observed in fig.1. A preliminary study assuming a decrease of the order of 10% of the total number of centers after each flash indicates that the classical analysis overestimates miss values and underestimates the ratio $S_0/S_0 + S_1$ if applied to such decreasing patterns. Nevertheless, the large difference between the miss values obtained at pH 6.5 and 7.6 is in agreement with the qualitative observation of the patterns. At pH 6.5, the period of 4 is sustained over 4 oscillations, whereas there are 2 oscillations at pH 7.6 (fig.1).

The large effect of pH on oxygen evolution observed in inverted vesicles is, in contrast, attenuated in right-side-out vesicles. Fig.2 shows on-
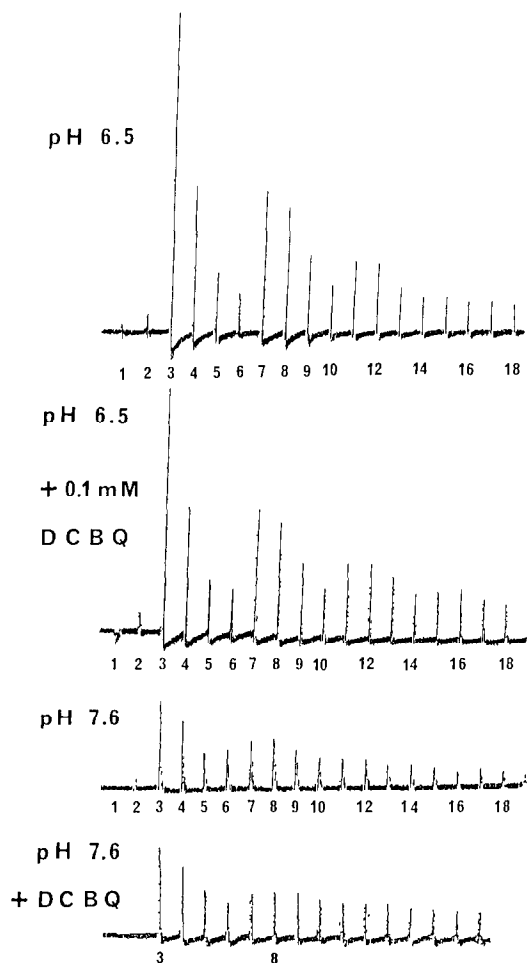

Fig.1. Oxygen oscillation patterns in inside-out thylakoids at pH 6.5 and 7.6 with or without 0.1 mM DCBQ. Dark adaptation, 10 min, time between flashes, 400 ms; circulation medium, 300 mM sorbitol, 10 mM NaCl, 5 mM MgCl$_2$, 40 mM buffer [Mes-NaOH, pH 6.5, N-Tris(hydroxymethyl)methylglycine (Tricine), pH 7.6]. Calculation of the miss and double hit percentage by the least-square fit applied on the patterns with DCBQ: according to the equal miss and double hit model [9] and a constant number of centers after each flash, $\alpha = 0.16$, $\beta = 0.04$ at pH 6.5 and $\alpha = 0.37$, $\beta = 0.30$ at pH 7.6. According to the unequal miss model [10], $\beta = 0$ and the miss distribution: 0, 0, 0.50, 0 at pH 6.5 and 0, 0, 0.76, 0 at pH 7.6.

ly small changes in oxygen activity and also a smaller increase in damping on going from pH 6.5 to pH 7.6 in right-side-out vesicles. Only when the pH increased to higher basic pH of the order of 8.5 did we observe an increase in the damping of the
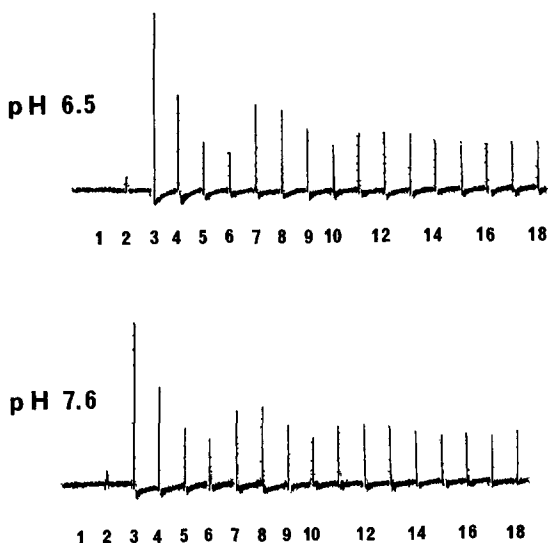
Fig.2. Oxygen oscillation patterns in right-side-out thylakoids at pH 6.5 and 7.6. Dark adaptation, 10 min, spacing between flashes, 400 ms; same circulation medium as in fig.1. According to the equal miss and double hit model [9], at pH 6.5, $\alpha = 0.20$, $\beta = 0.06$ and at pH 7.6, $\alpha = 0.23$, $\beta = 0.07$, whereas according to the unequal miss model [10] $\beta = 0$ and the miss distribution is 0, 0, 0.58, 0 at pH 6.5 and 0, 0, 0.62, 0 at pH 7.6.
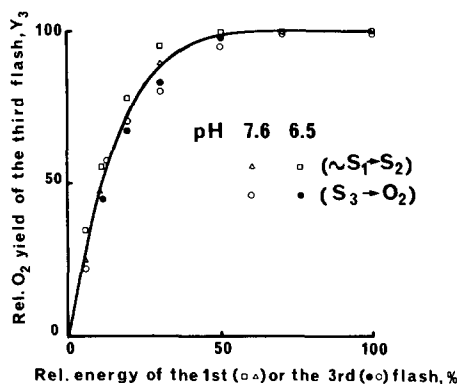

Fig.3. Light saturation curves of the transitions $S_1 \longrightarrow S_2$ and $S_3 \longrightarrow O_2$ at pH 7.6 and 6.5 in inside-out vesicles. Oxygen evolved on the third flash (Y_3) of a series of flashes as a function of the light energy of the first flash I_1 (for $S_1 \longrightarrow S_2$), and of the third flash I_3 (for $S_3 \longrightarrow O_2$). All these light saturation curves are normalized at a 100% flash energy, dark adaptation 10 min, time between flashes 400 ms, 0.1 mM DCBQ in the circulating medium.

sequences in right-side-out vesicles comparable to that observed in inverted vesicles at pH 7.6. In right-side-out vesicles, no external acceptor was added, nevertheless the oxygen yield was measurable at large flash numbers ($n > 20$) in agreement with the observation of Andersson et al. [11] showing that right-side-out vesicles contain PS I which is in contrast to inside-out vesicles.

We studied the light saturation behaviour of each S_i state in inside-out thylakoids and found a pH dependence of the light saturation behaviour of only the S_3 state centers. The oxygen yield after the third flash Y_3 (i.e. the first high oxygen yield of the series) was measured as a function of the flash energy of the first flash I_1 for the saturation behaviour of the $S_1 \longrightarrow S_2$ transition, or the third flash I_3, for $S_3 \longrightarrow O_2$. In fig.3, the light saturation curves $Y_3(I_1)$ and $Y_3(I_3)$ are approximately exponential and completely saturated at light intensities above one half the maximum intensity used in our experiments. This result shows that very few or no double hits of the type $S_0 \longrightarrow S_2$ contribute to the curve $Y_3(I_1)$ or $S_2 \longrightarrow O_2$ to the curve $Y_3(I_3)$. The light saturation behaviour of the transitions $S_1 \longrightarrow S_2$ and $S_3 \longrightarrow O_2$ is identical at pH 6.5 and 7.6 (fig.3) and cannot explain the pH dependence of the oxygen yield. In contrast, a significant change with pH is observed in the shape of the light saturation curve of the S_3 centers. The oxygen yield on the third flash, Y_3, was measured as a function of the light energy of the second flash I_2. In fig.4, the curve $Y_3(I_2)$ shows 2 different light saturation processes at pH 6.5, whereas at pH 7.6, $Y_3(I_2)$ resembles the light saturation behaviour of the other transitions $S_1 \longrightarrow S_2$ and $S_3 \longrightarrow O_2$, with only one saturation intensity. At pH 6.5, the experimental curve $Y_3(I_2)$ can be theoretically described by the sum of 2 exponential functions of flash energy. One of the saturation intensities is nearly identical to that at pH 7.6 and the other is found at much higher flash energies (10 times higher) (fig.4). As the higher intensity saturation process does not exist at pH 7.6 but at pH 6.5, it may reflect a modification, due to the pH, of the centers in the $S_2 \longrightarrow S_3$ transition. At pH 7.6 a homogeneous population of centers is observed. At pH 6.5, in addition to this population, active pH-dependent centers appear which saturate at a much higher light intensity than the others. The proportion of this pH-dependent population evaluated from the
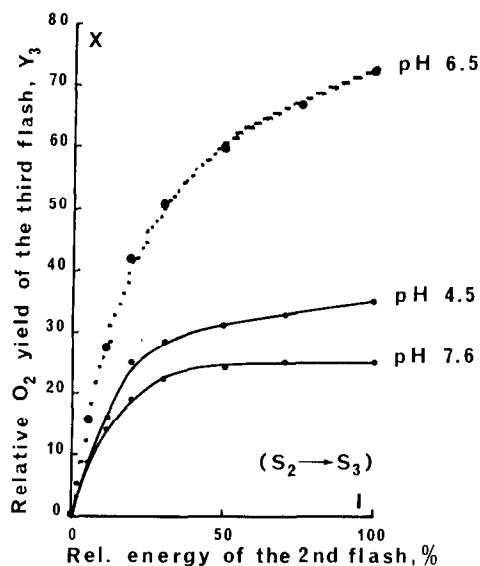
Fig.4. Light saturation curve of the transition $S_2 \longrightarrow S_3$ in inside-out thylakoids. Oxygen evolved on the third flash (Y_3) of a series of flashes as a function of the energy of the second flash (I_2), $Y_3(I_2)$, in inside-out thylakoids at pH 7.6, 6.5 and 4.5. Same experimental procedure as in figs 1 and 3. The circulation medium for pH 4.5 was 300 mM sorbitol, 10 mM NaCl, 5 mM MgCl$_2$, 50 mM succinic acid. The complementary part of the light saturation curve $Y_2(I_2)$ has been subtracted from $Y_3(I_2)$. (– – –) Theoretical curve which fits the experimental curve $Y_3(I_2)$ at pH 6.5. The formula used is

$$X = X_0(1 - e^{-I/I_0}) + X_1(1 - e^{-I/I_1})$$

with $X_0 = X_1 = 49$, $I_0 = 15$, $I_1 = 150$ (I_0 and I_1 are the saturation intensities of the 2 kinds of centers).

theoretical simulation of $Y_3(I_2)$ is of the order of 30% of the S$_3$ centers (at 100% flash energy corresponding to 6–7 photons/center).

Fig.5 shows the pH titration of the increase of the light saturation curve $Y_3(I_2)$ at high light intensity as measured by the ratio $Y_3(I_2 = 100\%)/Y_3(I_2 = 30\%)$, and also the pH dependence of Y_3, from pH 6.3 to pH 7.6 [Y_3 is compared with Y_8, O$_2$ yield on the eighth flash, that we considered as a better estimate of the mean value of the amount of centers than Y_n ($n > 10$) which depends on samples even in the presence of DCBQ]. In fig.5, both measurements follow the same pH titration curve. The quantum efficiency of the O$_2$ yield after the third flash, as well as S$_3$ centers converted in the higher intensity saturation process, depends on one protonated species. The pH titra-
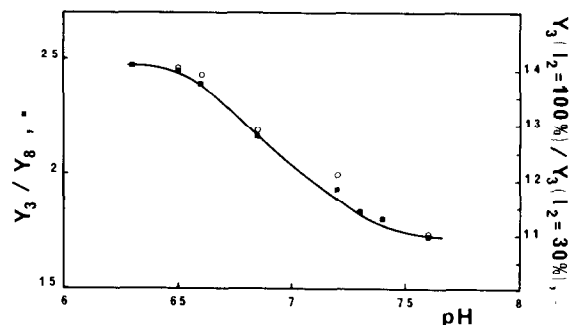

Fig.5. pH titration curve of the O$_2$ yield on the third flash compared with the O$_2$ yield on the eighth flash Y_3/Y_8 and also on the ratio $Y_3(I_2 = 100\%)/Y_3(I_2 = 30\%)$ which is a measurement of the number of S$_3$ centers appearing in the higher light saturation process of the $Y_3(I_2)$ curve (fig.4). Same experimental procedure as in figs 1 and 4; circulating medium: 300 mM sorbitol, 40 mM buffer (Mes, pH 6.0–6.6; Hepes, pH 6.7–7.2; Tricine, pH 7.3–7.6), 10 mM NaCl, 5 mM MgCl$_2$.

tion curve shows a maximum at pH 6.3 and a minimum at pH 7.6. At a more acidic pH (lower than pH 6.3), the curve diminishes (not shown). An example of this can be seen in fig.4, where at pH 4.5 the ratio $Y_3(I_2 = 100\%)/Y_3(I_2 = 30\%)$ is lower than at pH 6.5. At a pH higher than 7.6, all centers (of any kind) are progressively inactivated. Half reaction of protonation occurs at neutral pH, pH 6.95 (fig.5).

Robinson and Crofts [12] have shown that Q$_B$ is directly or indirectly protonated in chloroplasts. Therefore we wondered whether the effect of pH on inside-out thylakoids could be partly related to the protonation of Q$_B$. Thus, we looked at the binary oscillation of fluorescence yields measured 25 ms after each of a series of flashes in hydroxylamine-treated inside-out thylakoids in the presence of ferricyanide at pH 6.5 and 7.6 (fig.6a,b). At both pH 6.5 and 7.6, the fluorescence as a function of flash number showed binary oscillations of great amplitude (synchronized with the slow (25 ms) absorption change at 320 nm observed in the presence of Tris and artificial donors [13] or hydroxylamine [14]). Ferricyanide fully oxidized Q$_B$ in the dark at either pH 6.5 or 7.6, so that in inside-out thylakoids the PS II centers were mainly in the state Q$_B^-$, 25 ms after the first flash. In contrast, in right-side-out vesicles there appeared to be no oscillations at pH 6.5 (fig.6c), but there were oscillations at pH 7.6
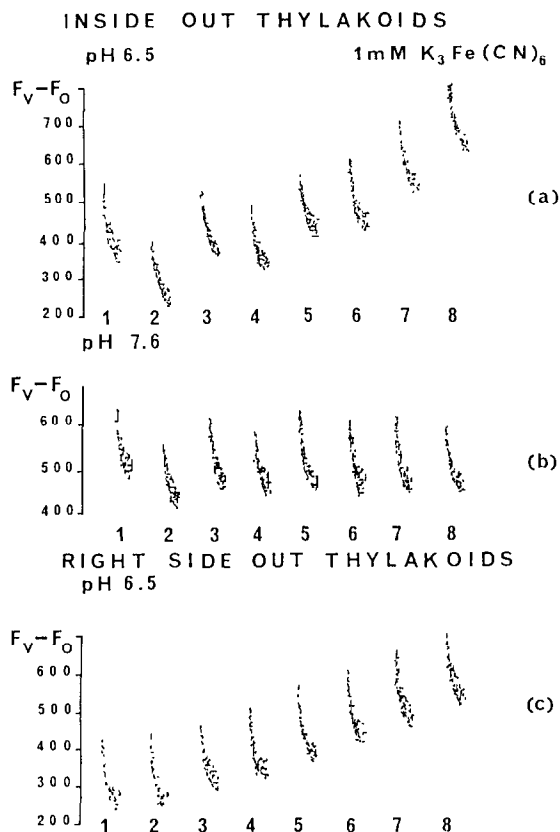
Fig.6. Fluorescence yield induced by high-energy flashes (100%) in the presence of 10 mM hydroxylamine plus 1 mM ferricyanide. Measurement after each flash of the series, 25 ms; spacing between flashes, 500 ms; dark adaptation, 10 min; (a) in inside-out thylakoids buffered at pH 6.5; (b) the same, buffered at pH 7.6; (c) in right-side-out thylakoids at pH 6.5.

(not shown), as is also seen in chloroplasts [12]. The results indicate that the proportion of oxidized Q_B in the dark does not depend on pH in inside-out vesicles, in contrast to right-side-out thylakoids and chloroplast. To explain this, we conclude that without PS I, no proton release coupled to plastohydroquinone oxidation can occur. Thus, in inside-out thylakoids which contain very little PS I, Q_B does not show a pH dependency.

4. DISCUSSION

The reaction center complexes are assumed to be inserted in the thylakoid membrane with the primary electron donors near the inner side and the corresponding primary electron acceptors at the outer side. As seen in figs 1 and 2, oxygen yield is much less dependent on the aqueous phase pH in right-side-out vesicles than in inside-out vesicles. So, the pH of the inner thylakoid surface supporting the PS II donors changes much more the damping and oxygen efficiency than the pH of the outer surface. Secondly, we have shown (fig.6) that there is no pH dependence of Q_B in inside-out thylakoids. These results imply that the variations observed on Y_3 in inside-out thylakoids are only due to a pH dependence of the water-oxidizing system located on the inner surface.

The pH dependence of the light saturation curve $Y_3(I_2)$ (fig.4) shows that some centers are heterogeneous in the $S_2 \longrightarrow S_3$ transition. Two types of centers are involved in the $S_2 \longrightarrow S_3$ transition: stable pH-independent centers and pH-dependent centers which exhibit different light saturation properties (fig.4). At the optimum pH (6.5), only for the $S_2 \longrightarrow S_3$ transition do the pH-dependent centers remain far from saturation at the maximum energy of our flash (6–7 photons/centers). Mathematically this incomplete saturation is described by a miss on $S_2 \longrightarrow S_3$. Thus, at pH 6.5, the incomplete saturation leads to the existence of a miss on $S_2 \longrightarrow S_3$. At pH 7.6, the unsaturated centers are no longer active, therefore the number of misses on $S_2 \longrightarrow S_3$ must have increased. In agreement with this prediction the overall oscillation pattern of O_2 yields is damped much less at pH 6.5 than at pH 7.6. The miss on $S_2 \longrightarrow S_3$ is decreased at pH 6.5 with regard to pH 7.6, when the pH-dependent centers show their maximum activity. Double advancement such as $S_1 \longrightarrow S_2$ does not appear to be the reason as it should have drastically changed the periodicity of the oscillation pattern (periodicity of 3 instead of 4) at pH 6.5 with regards to pH 7.6. Our interpretation is summarized in scheme 1, which is similar, in part, to that described in [8]. At pH 7.6, the same donor Y participates in the formation of each S_i state transition of the Kok cycle (scheme 1, cycle 1), but there is a large intrinsic miss on $S_2 \longrightarrow S_3$ [8]. Thus, at pH 6.5, another donor termed D, which is protonated, can efficiently transfer an oxidizing equivalent in the S_2 state and therefore increase the amount of $S_2 \longrightarrow S_3$ transitions and decrease the number of misses. In this model, Y is the only donor in the $S_0 \longrightarrow S_1$ and $S_1 \longrightarrow S_2$ transitions whereas Y or D are both possi-
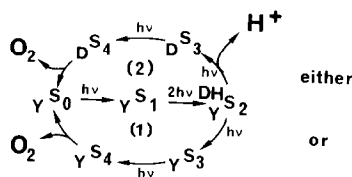
Scheme 1. Proposed model. The indicated donor (Y or D) is the donor which donates electron to P^+680. The donor D only participates in the $S_2 \longrightarrow S_3$ and $S_3 \longrightarrow O_2$ transitions.

ble donors in the $S_2 \longrightarrow S_3$ and $S_3 \longrightarrow O_2$ transitions.

Renger and Voelker [3] have shown that in Tris-washed inside-out thylakoids, completely deprived of their oxygen-evolving ability, a donor is protonated in the reduced state with a $pK_a(D_1^{ox}) < 5$ and becomes deprotonated in the oxidized state with a $pK_a(D_1^{red}) \geqslant 8$. If the donor D_1 described by Renger and Voelker is identical to donor D, which needs to be protonated in order to advance the state S_2 to S_3, their properties should correspond whatever the circumstances. According to these authors, the optimum pH conditions for both the protonation of the reduced donor and the deprotonation of the same oxidized donor (D_1) must be between the 2 pK_a values. We have found for $S_2 \longrightarrow S_3$ transitions an optimum pH of 6.3–6.5. This pH being an intermediary value between the 2 pK_a values of reduced and oxidized D_1, so D is likely to be the donor D_1 of Renger and Voelker. In agreement with this assumption, at alkaline pH (pH 7.6–8), donor D is unable to be protonated in the reduced state and, thus, cannot take part in the $S_2 \longrightarrow S_3$ transition (fig.5). At a pH more acidic than 6.5, oxidized donor D is less easily deprotonated, which hinders the formation of the S_3 state (fig.4, curve pH 4.5). This interpretation is also in agreement with the results of Förster and Junge [15]. They have observed that during the $S_2 \longrightarrow S_3$ transition, the proton release is biphasic with a rapid first phase ($t_{1/2} = 100 \, \mu s$) which is faster than the turnover time of the $S_2 \longrightarrow S_3$ transition ($t_{1/2} = 400 \, \mu s$) [16]. This additional transient proton release, different from that coupled to water oxidation, seems a natural explanation for this unexpected rapid proton release in the $S_2 \longrightarrow S_3$ transition [15]. According to our results it would correspond to 0.3 H^+/P-680 at a flash energy of 6–7 photons/center, assuming that

one proton is released from D at pH 6.5 in 30% of the centers. This amount of proton release is less than that found in Tris-treated chloroplasts (0.5 H^+/P-680 [15]) or Tris-treated inside-out thylakoids (0.85 H^+/600 Chl [3]). This may be due to differences in flash energy.

Scheme 1 accounts for a proton release coupled with the redox change of an electron carrier (D) observed only during the $S_2 \longrightarrow S_3$ transition. Consequently, the assumption that there are 2 different configurations of the water-oxidizing system in the S_2 state [8] including either Y or protonated D could explain both the pH dependence of O_2-evolution activity and the rapid rise of proton release in the $S_2 \longrightarrow S_3$ transition.

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