The Flash Pattern of Photosynthetic Oxygen Evolution after Treatment with Low Concentrations of Hydroxylamine as a Function of the Previous S_1/S_0-Ratio

Further Evidence that NH_2OH Reduces the Water Oxidizing Complex in the Dark

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Flash induced oxygen evolution patterns of isolated PS II complexes from the cyanobacterium Synechococcus were measured with a Joliot-type electrode. By suitable preflash and dark adaptation procedures, samples were prepared in the state S_1 (100%), as well as enriched in S_0 (60% S_0, 40% S_1). After treatment with low concentrations of NH_2OH (≤ 100 μM), the two flash patterns were identical. This is further evidence for a reduction of the water oxidizing complex by hydroxylamine in the dark. Two reduced states (S_{-1} and S_{-2}) below S_0 are formed by this reduction.

Introduction

In Photosystem II, photooxidized Chl-a_{II}^+ ($P680^+$) extracts four electrons in four turnovers out of the water oxidizing complex S. S is also called oxygen evolving complex (OEC). This complex is thereby oxidized to the states S_0, S_1, S_2, S_3 and finally, *via* an intermediary state S_4, 2 H_2O are oxidized to 1 O_2 and the complex returns to the state S_0 [1, 2].

Since only the state S_1 is stable after sufficient dark adaptation [3], the sequence of S states, upon exitation with short, saturating flashes of light, starts with $S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu}(S_4)\longrightarrow S_0 \xrightarrow{h\nu} S_1$ etc. The first maximum of oxygen evolution therefore occurs upon the third flash, the second maximum on the seventh flash and so on. The oscillation of this flash pattern is damped, because some centres do not make a turnover (misses), whilst others make a double turnover (double hits) [2]. An oscillation with a period of four can also be observed in the pattern of proton release [4, 5], UV-absorption changes [6, 7], UV-absorption changes attributed to manganese [8, 9] and electrochromic changes due to surplus charges on S_2 and S_3 [10].

Abbreviations: Chl, chlorophyll; HA, hydroxylamine; HEPES, N-(2-hydroxyethyl) piperazine-N′-2-ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; OEC, oxygen evolving complex; PS II, photosystem II; SB 12, sulfobetaine 12; Y_n, oxygen yield upon the n-th flash.

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Different reducing agents interact with the S-states and have been used to get information on the nature of these states. ADRY-reagents specifically interact with the S_2 and S_3 state [11]. Low concentrations of hydroxylamine [12] and of hydrazine [13] lead to a characteristic two-flash delay of the oxygen evolution pattern. The first maximum of oxygen evolution now occurs on the fifth flash, but besides this shift the usual period four oscillation is observed. Again, also the pattern of proton release [14], UV- and electrochromic absorption changes [9] show this delay.

Obviously the usual sequence of S-state transitions is changed in the presence of these reagents. It can now be described by the formal sequence $S_{-1} \xrightarrow{h\nu} S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu} S_0$. Various proposals have been made for the mechanism through which the formal state S_{-1} is formed.

On the one hand it has been proposed [15, 16] that S_{-1} is the product of a reduction of the OEC by NH_2OH or NH_2NH_2 in the dark. Flash patterns of oxygen yield, UV- and electrochromic absorption changes measured recently in water oxidizing PS II complexes from the cyanobacterium Synechococcus, showed the two-flash delay even 10 h after removal of unreacted hydroxylamine by gel filtration [17, 18]. These results are in favour of a reduction of the OEC by NH_2OH in the dark. This reaction may either be realized by a reduction of two Mn(III) in S_1 which are redox active in the S-cycle within the manganese cluster so that these become two Mn(II) in S_{-1} [15, 18]. Alternatively the reduction of the OEC may be ex-

plained by the reduction of a component C different from these two "active" manganeses [16, 19]. C might be the additional manganese of the cluster or a protein component (see also [27]).

On the other hand it has been proposed ([20], see also [12, 21]) that hydroxylamine and hydrazine are only reversibly bound to the S_1 state in the dark; *i.e.*, in the case of NH_2OH, S_{-1} = ($S_1 \cdot n$ NH_2OH, n = 2, 3, 4), so that the two-flash delay is realized by a transition of $S_1 \cdot n$ NH_2OH to $S_2 \cdot n$ NH_2OH in the first flash and a subsequent fast reduction of the latter to S_0 [20].

If S_{-1} is the lowest oxidation state of the OEC and formed by a reduction of the higher S-states, the flash patterns of oxygen evolution, UV- and electrochromic absorption changes should be independent of the S-state distribution present before incubation with NH_2OH or NH_2NH_2. It was, however, shown with hydrazine that the oxygen evolution pattern of spinach thylakoids depends on the previous S_1/S_0-ratio [16]. These patterns have been described by the formation of formal states S_{-1} and S_{-2} [22] through reduction of S_1 and S_0, respectively. This result does not exclude a mere binding of NH_2NH_2 to S_1 and S_0, however.

Because of earlier results [23], which indicated differences in the interaction of NH_2OH *vs.* NH_2NH_2 with the OEC, and the evidence presented in [17, 18] for a reduction of the OEC by NH_2OH in the dark, we have investigated the oxygen evolution patterns after different incubation times with NH_2OH as a function of the previous S_1/S_0-ratio. In the case of a dark reduction of the OEC by NH_2OH (see above) the pattern should be independent of this ratio. Different ratios of S_1/S_0 were adjusted in PS II complexes from Synechococcus by a suitable preflash and dark adaptation procedure as in [16, 22].

Materials and Methods

O_2-evolving PS II complexes from the Cyanobacterium Synechococcus sp. have been prepared according to [24] and [25]. They were stored at −80 °C in 20 mM MES-Na pH 6.5, 20 mM $CaCl_2$, 10 mM $MgCl_2$, 1 M sucrose and 0.06% SB 12.

Flash induced O_2-oscillation patterns were measured with a modified Joliot type electrode as in [22] at 20 °C. The samples with a volume of 10 µl contained 230 µM Chl and had about 100 Chl per OEC. The flow buffer contained 50 mM HEPES-Na pH 7.2, 20 mM $CaCl_2$, 10 mM $MgCl_2$, 0.3 M mannitol. The electrode was polarized with −750 mV. Flashes were given with a frequency of 2 Hz. The measured oxygen yields per flash were normalized for presentation to the sum of the yield of flashes 3 to 6 (without NH_2OH) or flashes 5 to 8 (with NH_2OH).

In order to avoid a redox reaction of reduced donor D (a special tyrosine not active in the electron transport chain of PS II, Tyr-160 of the D2 protein) with the S states, the PS II complexes were given one saturating flash (Xenon flash lamp) and dark adapted for at least 15 min at room temperature. Some samples were then given three preflashes to populate the S_0 state, followed by another dark incubation for 15 min. For measurements in the presence of hydroxylamine, samples were incubated with 50 µM or 100 µM NH_2OH at room temperature after the preflash procedure described above. For further details see text and figure legends.

Results

Fig. 1, top, shows the oxygen oscillation patterns of control samples without preflashes and

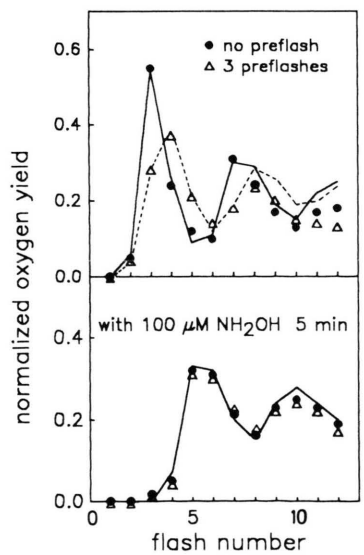

Fig. 1. Flash pattern of oxygen evolution without and with 3 preflashes in the absence (top) and presence of 100 µM NH_2OH (5 min incubation time) (bottom). Curves represent simulations with 16% misses, 4% double hits and S-state distributions: 100% S_1 (top, solid line), 60% S_0, 40% S_1 (top, dashed line), 25% S_{-2}, 75% S_{-1} (bottom, solid line).

after three preflashes (see Materials and Methods). The patterns are distinctly different. The former shows maxima on the third and seventh flash, whereas the maxima occur on the fourth and eighth flash with the latter. Both patterns are markedly damped. This may be due to the limited capacity of the acceptorside of these PS II preparations. Exogeneous acceptors cannot be used on this Joliot-type electrode.

The patterns can be simulated, for the case of no preflashes, with an initial S-state distribution of 100% S_1 and, after 3 preflashes, with 60% S_0, 40% S_1 (solid and dashed line, respectively, in Fig. 1, top). The values of misses (16%) and double hits (4%) were chosen to simulate both patterns consistently.

After 5 min incubation with 100 μM NH_2OH, the oxygen evolution patterns are the same with and without 3 preflashes (Fig. 1, bottom). Practically no oxygen is evolved in the first three flashes, and the maximum of oxygen evolution occurs on the fifth flash, but this maximum is not pronounced.

We found that if the incubation time is extended, the ratio of the yield upon the fifth flash (Y_5) to the yield upon the sixth flash (Y_6) becomes even less than one (see inset of Fig. 2). After 20 min incubation, the maximum of oxygen evolution occurs upon the sixth flash (Fig. 2), *i.e.*, a three-flash

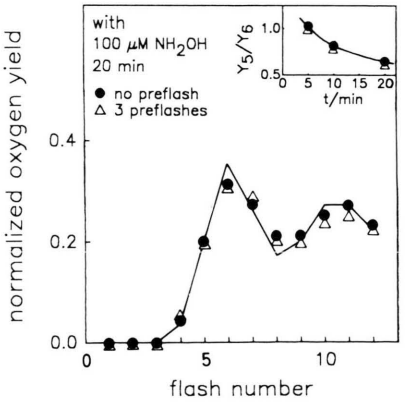

Fig. 2. Flash pattern of oxygen evolution without and with three preflashes, incubated with 100 μM NH_2OH for 20 min. Parameters of simulation (solid line): 65% S_{-2}, 35% S_{-1}, 16% misses, 4% double hits. Inset: Ratio of the oxygen yield upon the fifth flash to that upon the sixth flash as a function of incubation time.

delay has taken place. Again, the oxygen evolution patterns are the same without and with three preflashes.

The three-flash delay, which has also been observed by Bouges [12] as well as Hanssum and Renger [23] leads one to consider the formation of a state S_{-2} besides S_{-1} (see also 26). The formation of this state through reaction of NH_2NH_2 with S_0 has been shown in [16, 22]. For the case of NH_2OH this is supported in [27] and by observation of an additional step of UV- as well as electrochromic absorption changes prior to S_{-1} (H. Kretschmann, personal communication).

In order to obtain information on the time course of the development of the different S-states, we measured oxygen evolution patterns in the presence of 50 μM NH_2OH after different incubation times. This was done without and with 3 preflashes. As an example, Fig. 3 shows the patterns

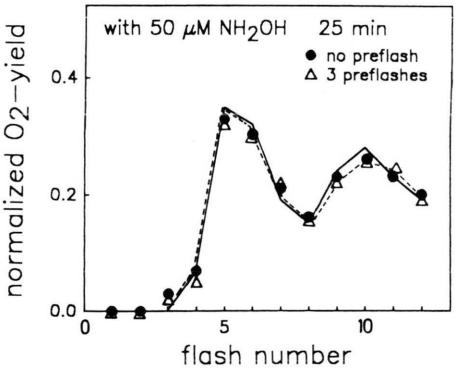

Fig. 3. Flash pattern of oxygen evolution without and with 3 preflashes, incubated with 50 μM NH_2OH for 25 min. Curves represent simulations with 20% S_{-2}, 80% S_{-1}, 16% misses, 4% double hits (solid line) or 100% S_{-1}, 20% misses, 4% double hits (dashed line).

measured after 25 min. These can be simulated with 20% S_{-2}, 80% S_{-1}, 16% misses and 4% double hits. The initial S-state distributions which were used for simulating the patterns measured after the different incubation times are plotted in Fig. 4. In these simulations, constant miss (16%) and double hit (4%) parameters were assumed. Three points should be noted:

1. After 25 min incubation, again the same S-state distribution (mainly S_{-1}) is reached, regardless of the preflash procedure.

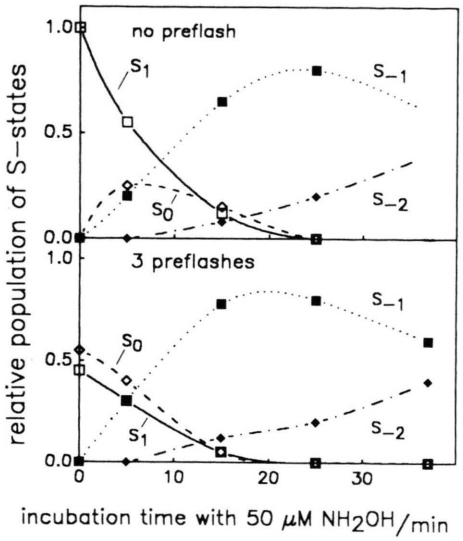

Fig. 4. S-state distribution as a function of incubation time with 50 μM NH_2OH. Top: without preflashes: bottom: with three preflashes. The S-state populations were obtained by simulations of the corresponding oxygen flash patterns with constant misses (16%) and double hits (4%) (Fig. 3 shows the patterns after 25 min).

2. The formation of the S_{-2}-state is slow compared to the formation of S_{-1}.

3. One can see the intermediate formation of an S_0-population in Fig. 3 top. This indicates a stepwise one-electron-reduction of the OEC, in line with the observation of a transient increase of Y_4 in a NH_2OH-concentration dependence measured in [23].

It should be noted that the S-state distribution which had to be used to simulate the patterns in principle depends on the miss parameter chosen, which must not necessarily be the same with and without NH_2OH-treatment. In fact, the pattern in Fig. 3 can be simulated also with 100% S_{-1}, 20% misses and 4% double hits, *i.e.* without S_{-2}, although this state clearly exists (see above). By simulating also the other patterns used in Fig. 4 with a higher miss parameter (not shown), we found that almost only the S_{-2} population was affected, *i.e.* more S_{-1} was formed instead of S_{-2}. Practically the same S_1 and S_0 populations were obtained. Therefore the points made above are valid regardless of the exact value of the miss parameter.

Discussion

When all oxygen evolving centres have been modified by hydroxylamine in the dark, the oxygen evolution patterns are independent of the previous S_1/S_0 ratio. This result is in agreement with a reduction of the OEC by hydroxylamine in the dark. This reduction leads *via* S_0 to a state S_{-1} and to a further reduced state, S_{-2}. Whether these states are due to a reduction of manganese [15, 18] and/or another component of the OEC [16, 19, 27] cannot be decided by our present experiments.

Models which propose only a binding of NH_2OH (see Introduction) cannot convincingly be reconciled with our results. To explain the formation of S_{-1}, one has to assume that S_0 binds only one NH_2OH whereas S_1 binds two. But the formation of the S_{-2} state requires the assumption that S_0 binds two NH_2OH whereas S_1 binds three. A mere binding model therefore seems no longer tenable.

In contrast to NH_2OH, the reducing agent NH_2NH_2 (see Introduction) does not lead to an O_2-evolution pattern that is independent of the previous S_1/S_0-ratio, as shown in [16]. This difference can be explained by the fact that NH_2NH_2 reacts as a two-electron-donor [22, 23], whereas NH_2OH is a one-electron-donor ([23] and this work). After reaction of NH_2OH with S_1 or S_0, the reduction product is the same, S_{-1} or after longer incubation times even S_{-2}: $S_0 \xrightarrow{HA} S_{-1} \xrightarrow{HA} S_{-2}$ and $S_1 \xrightarrow{HA} S_0 \xrightarrow{HA} S_{-1} \xrightarrow{HA} S_{-2}$ (see also [27]). Therefore the oxygen evolution pattern is independent of the previous S_1/S_0-ratio when all centres have been modified by hydroxylamine. NH_2NH_2 as a two-electron-donor reduces, however, S_1 in a single step to S_{-1} and S_0 to S_{-2}. Therefore the populations of S_{-1} and S_{-2} after treatment with hydrazine still reflect the previous S_1/S_0-ratio as observed in [16].

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