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Interaction of hydroxylamine with the water-oxidizing complex at oxidation states S_1, S_2 and S_3 in etiochloroplasts of oat

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The effect of hydroxylamine on the flash pattern of oxygen evolution was studied in plastids from etiolated oat leaves illuminated for 4 h. Since the etiochloroplasts exhibit unusually long S_2 and S_3 lifetimes (Franck, F. and Schmid, G.H. (1984) Z. Naturforsch. 39c, 1091–1096) it was possible to observe the effect of hydroxylamine on S_1, S_2 and S_3 by adding the inhibitor shortly after two flashes and by recording the oxygen flash sequence thereafter. The results show that S_1, S_2 and S_3 are affected by hydroxylamine at concentrations which only slightly inhibited steady-state oxygen evolution. Analysis of the concentration dependence of these effects reveals that S_2 is much more sensitive than S_1 and S_3.

Introduction

Despite intensive investigations on the mechanism of water decomposition by photosynthesizing organisms, the molecular mechanism of water oxidation is still a matter of speculation. It is well established [1,2] that the water-oxidizing complex of Photosystem II undergoes a light-induced accumulation of 4 oxidizing equivalents before it releases one oxygen molecule by oxidation of two water molecules. Charge accumulation in the water-oxidizing complex can be generated stepwise by flash illumination of chloroplasts, leading to successive S_n states of the complex, where n is the number of positive charges. After a long dark period, the complex is mainly in the S_1 state and oxygen is released at the third flash [1].

At present it is not clear at which of the S_n states water binds to the complex. Although water decomposition was often supposed to involve several oxidation intermediates [3–5], recent mass spectrometric measurements involving water labelling with ^{18}O seem to contradict the hypothesis of the existence of bound water derivatives up to the S_3 state [6,7]. However, observation of a hyperfine broadening of the low-temperature EPR signal due to S_2 in the presence of $H_2{}^{18}O$ was interpreted as the consequence of water binding at oxidation states of the water-oxidizing complex lower than S_2 [8]. On the other hand, the occurrence of intermediate water-oxidation products is also suggested by the proton release pattern obtained under flash excitation [9–12].

In this report, we use hydroxylamine to approach this problem by comparing the affinity of the water-oxidizing complex for hydroxylamine at different S states. This molecule has been shown [13,14] to substitute for water at the reaction site of water oxidation. Its addition at low concentrations to dark-adapted chloroplasts (i.e., under the S_1 state) causes a two-digit shift in the oxygen release pattern [13], whereas N_2 is released upon the first flash as a result of hydroxylamine oxidation [14]. Only few attempts to determine the effect of hydroxylamine on other S-states have been reported up to now. Hanssum and Renger [15] showed a more rapid interaction of hydroxylamine with S_2 and S_3 than with S_0 and S_1 in thylakoids first incubated with the inhibitor at the S_1 state. A rapid interaction of hydroxylamine with S_2 was also deduced by Andreasson and Hansson [16] from EPR measurements.

In order to compare the affinity of hydroxylamine towards the different S states we use here etiochloroplasts from partly greened etiolated oat leaves, which

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Abbreviations: DCMU, *N*,*N*′-3,4-dichlorophenyldimethylurea; Tes, 2([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethane sulfonic acid; Hepes: 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

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were shown to exhibit unusually long S_2 and S_3 lifetimes. This property made the experimental approach easier than in previous studies, since it was now possible to add the inhibitor to a sample enriched in states other than S_1 and to measure the oxygen release pattern within the lifetime of these states.

Materials and Methods

Etiolated oat plants *Avena sativa* were grown on soil for 6 days in complete darkness at 24° C. The seedlings were transferred thereafter to white light (800 lux) for 4 h at 27° C.

Plastid preparation was carried out in Tes-Hepes buffer (pH 7.5) as described earlier [17].

Oxygen measurements were performed by polarography using the rapid and sensitive three-electrode-system described in Ref. 18, assisted by an Apple computer. The assay suspension contained the undisrupted plastids in 25 mM Hepes buffer (pH 7.5) with 0.4 M sucrose and 150 mM KCl. The total chlorophyll content of the sample was approx. 40 μg.

Results

Dark-adapted etiochloroplasts from partially green leaves (4 h under continuous white light) exhibited the well-known damped oscillation of oxygen flash yield with the maximum at the third flash and a periodicity of four flashes (Fig. 1A). As reported in Ref. 17, a substantial although relatively low amount of oxygen was produced under the first flash, in contrast to green chloroplasts where no oxygen can be detected under the first flash. When plastids were preilluminated by two flashes and then kept in darkness for only 2 min, trace B of Fig. 1 was obtained during subsequent illumination. Relatively larger amounts of oxygen were then produced under the first two flashes. This is explained by the slow deactivation rates of S_3 and S_2 after the two pre-illumination flashes. The centers in the S_3 and S_2 states at the end of the 2 min dark period produced oxygen at the first and second flash, respectively.

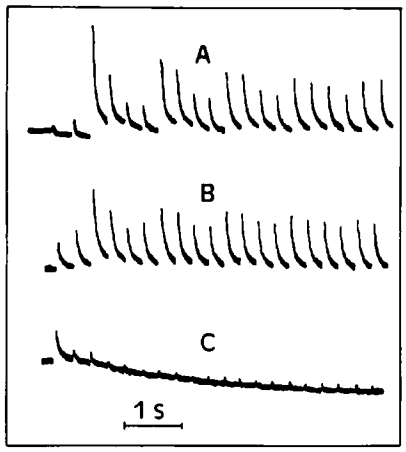
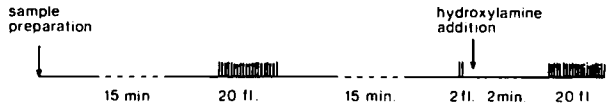


Fig. 1. Traces of the amperometric signal against time during a sequence of 20 flashes in etiochloroplats. (A) Dark-adapted (15 min). (B) Pre-illuminated by two flashes followed by 2 min darkness. (C) As (B), but with $3 \cdot 10^{-6}$ M DCMU added immediately after the two pre-illumination flashes.



Scheme I.

The experiment reported in Fig. 1C shows that it was possible to add a reactant immediately after the two pre-illumination flashes and to observe its effects during a series of flashes given 2 min later. In our case $3 \cdot 10^{-6}$ M DCMU was used. The oxygen evolution under the first flash was unaffected by the presence of this compound, while subsequent flashes produced only very low amounts of oxygen in comparison to the control. This result is in accordance with the generally accepted view following which DCMU acts by inhibiting the reoxidation of the Q_a electron acceptor of PS II. Accordingly, stable charge separation can only take place during the first flash which follows DCMU addition.

A similar procedure was used to study the interaction of hydroxylamine with the water-oxidizing complex under the condition of a mixed S-state population. Care was taken of eliminating fortuitous variations between different samples when comparing the effect of different hydroxylamine concentrations. This could be achieved by normalizing the oxygen data on the steady-state oxygen evolution value calculated at the end of a 'test' sequence given 15 min before the experiment with hydroxylamine. The complete experiment thus followed the protocol:

Fig. 2 shows the calculated Y_N sequences obtained with various concentrations of hydroxylamine added immediately after the two pre-illumination flashes, where Y_N is the normalized oxygen production at flash N, computed from the electrode output signal. At low

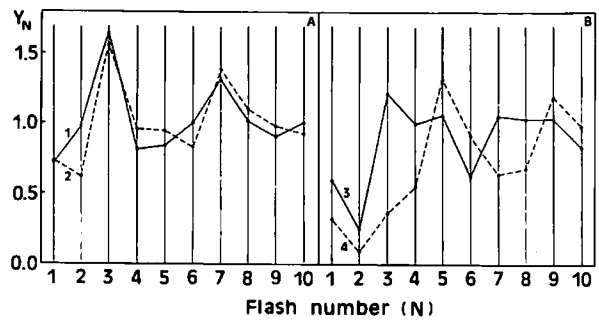


Fig. 2. Y_N sequences (see text for Y_N definition) obtained at various hydroxylamine concentrations. (A,1) Control without NH_2OH. (A,2) $9.5 \cdot 10^{-6}$ M NH_2OH. (B,1) $24 \cdot 10^{-6}$ M NH_2OH. (B,2) $95 \cdot 10^{-6}$ M NH_2OH.

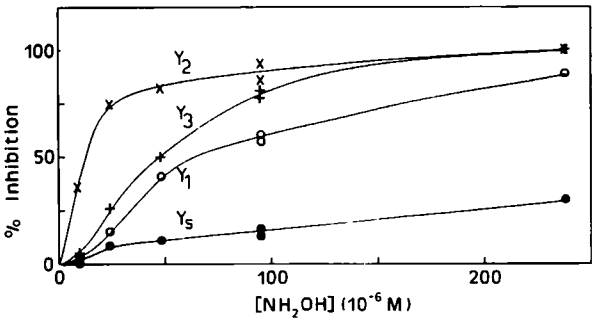


Fig. 3. Effect of hydroxylamine concentration on the extent of inhibition of oxygen evolution upon the first (Y_1), second (Y_2) and third (Y_3) flash and on the steady-state oxygen evolution (Y_S), expressed in percent of the control without hydroxylamine.

hydroxylamine concentrations (such as $9.5 \cdot 10^{-6}$ M; Fig. 2A, pattern 2), a decrease in Y_2 and an increase in Y_4 and Y_5 were observed in comparison to the control (Fig. 2A, pattern 1). This result indicated the formation of S_0 and S_{-1} at the expense of S_2. With increasing hydroxylamine concentrations (Fig. 2B), Y_1, Y_3 and Y_4 were also reduced, while the maximum progressively shifted from Y_3 to Y_5, indicating the accumulation of S_{-1} at the expense of all other states.

In Fig. 3, the extent of inhibition of Y_N in comparison to the control is plotted against hydroxylamine concentration. The initial slope of the inhibition curves decreases in the order $Y_2 > Y_3 > Y_1$, which suggests a decreasing reactivity of hydroxylamine with S_2, S_1, S_3, respectively. The extent of inhibition of the steady-state oxygen evolution (Y_S) was also calculated from the average Y_N value at the last four flashes of the 20-flash sequence. In the concentration range and for the incubation time used here, the inhibition of Y_S was low. This shows that only a small portion of the oxygen evolving centers were destroyed by hydroxylamine treatment.

More precisely determined S-state distributions are presented in Table I. The calculations take into account the occurrence of misses (10%) in the S-state transitions at each flash. The proportions are expressed relatively to the sum of centers which were still functional, as calculated from the Y_S values. The two pre-illumination flashes brought the oxygen evolving centers essentially to the S_3 and S_2 states. After 2 min darkness, more than 70% of the initially present S_3 population had deactivated while the S_2 population, which deactivates more slowly [17], was not much decreased at that time. Hydroxylamine at $24 \cdot 10^{-6}$ M had neglectable effects on S_3 and S_1 after the 2 min dark period, while S_2 was strongly decreased. At higher concentrations ($95 \cdot 10^{-6}$ M), all S states were largely affected.

TABLE I

Calculated S-state distributions, assuming 10% misses (double-hits not taken into account)

Pretreatment	S_0	S_1	S_2	S_3	Σ
2 $h\nu$	0.10	0.10	0.28	0.61	1.09
2 $h\nu$ + 2 min	0.17	0.42	0.24	0.17	1.00
2 $h\nu$ + 24 μM hydrox. + 2 min	0.25	0.42	0.06	0.16	0.89
2 $h\nu$ + 95 μM hydrox. + 2 min	0.17	0.12	0.02	0.10	0.41

Discussion

In dark-adapted chloroplasts, the water-oxidizing complex is mostly in the S_1 state [1]. Therefore, all previous studies on its interactions with hydroxylamine are relevant to this particular state. At low concentrations ($< 50 \cdot 10^{-6}$ M) and for an incubation time of 10 min [13], hydroxylamine binds irreversibly to S_1. The two-digit shift of the oscillatory oxygen flash pattern is explained by assuming that bound hydroxylamine is oxidized by the first flash, while the complex is restored to the S_0 state. The second flash then oxidizes S_0 into S_1 and the next transitions are those normally found in untreated, dark-adapted chloroplasts.

We show here that hydroxylamine interacts not only with S_1 but also with S_2 and S_3 when it is added to plastids containing a mixed S-state population. However, the extent of inhibition shows pronounced differences in its dependence towards hydroxylamine concentration depending on the considered S state. State S_2 is much more sensitive to the inhibitor than states S_1 and S_3, the latter being the less sensitive. The high sensitivity of S_2 is in agreement with the results of Andreasson and Hansson [16], who found a rapid decline of the EPR signal of S_2 upon addition of NH_2OH to PS II membranes blocked at the S_2 state by preillumination in the presence of DCMU. NH_3 was found previously by Velthuys [19] to bind rapidly to S_2. Other attempts to compare the reactivity of the S states with NH_2OH have been reported by Hanssum and Renger [15]. In this case, both S_2 and S_3 were found to react rapidly in comparison with S_0 and S_1. The discrepancy concerning the reactivity of S_3 between these results and ours may arise from the fact that, in the work of Hanssum and Renger, NH_2OH is first added to dark-adapted thylakoids and S_3 is reached by subsequent flash illumination, while we add NH_2OH to plastids in which the S_3 state has first been populated in the absence of the inhibitor. A different behavior of S_3 in etiochloroplasts or in mature chloroplasts may also be responsible for this discrepancy.

The fact that S_3 is also sensitive to hydroxylamine, although here at higher concentrations than the other states, tallies well with the hypothesis of a concerted final oxidation of the two water molecules taking place after the $S_3 \rightarrow S_4$ transition. This hypothesis implies that no bound water derivative occurs up to the S_3 state, which was suggested by mass-spectrometric measure-

ments using ^{18}O-labelled water [6,7], although it should be also mentioned that this hypothesis has to be further verified. The involvement of redox equilibria with possible exchange reactions in different time-scale regions are actually under investigation. The different sensitivity of the S states to hydroxylamine might arise either from differences in the accessibility of the water-oxidizing complex to the inhibitor due to protein conformational modifications or from differences in the affinity for hydroxylamine molecules which bind to the complex, and may vary according to the state of the complex. Although it was initially believed that one [6,13] or two [20] hydroxylamine molecules could bind to the complex in dark-adapted chloroplasts (i.e., mostly under the S_1 state), recent experiments [21] suggest this number to be rather 3 or 4.

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