Nitrogen and oxygen evolution by hydroxylamine-treated chloroplasts

Richard Radmer and Otto Ollinger

Martin Marietta Laboratories, 1450 South Rolling Road, Baltimore, MD 21227, USA

Received 26 May 1982

Photosystem II *Oxygen evolution* *Hydroxylamine* *Nitrogen evolution* *Mass spectrometry* *Spinach chloroplast*

1. INTRODUCTION

Hydroxylamine (NH_2OH) is one of a small group of compounds (along with hydrazine (NH_2NH_2) and hydrogen peroxide) that appear to be competitive inhibitors of photosystem II-mediated H_2O oxidation. These compounds, which are all analogs of (2 molecules of) H_2O, can evidently override H_2O oxidation without destroying the O_2 system.

Earlier studies showed that dark incubation of chloroplasts in the presence of low concentrations of NH_2OH caused a 2-flash delay in oxygen evolution. This finding was interpreted as the irreversible binding of 2 NH_2OH molecules at the substrate site of the H_2O-oxidizing enzyme [1] or alternatively, as the binding of a single NH_2OH molecule following the reduction of S_1 to S_0 [2]. Similar effects were also observed with N_2H_4 [3] and H_2O_2 [4]. Results obtained with H_2O_2 led to the proposal of a reduction of S_1 to an over-reduced 'S_{-1}' state [4].

In [5,6] we found that molecular N_2 was the sole significant stable reaction product of the PS II-mediated photooxidation of hydroxylamine and hydrazine under conditions in which the O_2-evolving system was inoperative. The stoichiometry of these reactions under substrate-saturating conditions suggested that in both cases the N_2 was formed by the combination of 1 e^- oxidation products.

Here, we describe experiments in which we directly monitored the photooxidation of low concentrations of hydroxylamine (as N_2 evolution) in O_2-evolving chloroplasts during a series of flashes. We hoped to thereby distinguish among several possible oxidation mechanisms at low substrate concentrations, i.e.:

(1) N_2 evolution on the first flash, but not significantly thereafter, would imply that one molecule of NH_2OH was bound to the O_2 system; the other 'lost equivalent' would then be ascribed to reduction in the dark.
(2) Equal N_2 evolution on the first 2 flashes would suggest that 2 NH_2OH molecules were bound.
(3) Absence of a NH_2OH oxidation product when there was a delay in the O_2 flash yield pattern would imply that NH_2OH served as a donor but was not bound.

Our results showed that 1 molecule of tightly-bound hydroxylamine is oxidized on the first flash, after which O_2 evolution proceeds normally, starting from S_0 generated in the dark. This unusually tenacious binding suggests that the H_2O oxidation site of the chloroplasts has 2 H_2O binding sites $\sim$1.47 Å apart (the length of the O–N bond [7]).

2. METHODS

The mass spectrometric apparatus and measuring technique used were similar to those in [5]. The heart of this system is a 1-mm-thick silicone rubber membrane (in the bottom of the 1-ml reaction vessel) that admits dissolved gases in the liquid phase to the mass spectrometer vacuum. A chloroplast suspension is carefully layered on the membrane (through a hole in the Plexiglas lid) and illuminated from above. Since the membrane and the chloro-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS II, photosystem II

Published by Elsevier Biomedical Press

plast suspension are in direct contact, the mass spectrometer can effectively monitor chloroplast gas-exchange reactions.

Saturating actinic flashes were provided by a xenon flash tube (FX-101, EG and G, Salem MA) mounted a few millimeters above the vessel. The flash duration at half-maximum was 1 μs, and the frequency was 0.33 Hz. The signal passed through an R–C network with a time constant of 70 ms before being recorded on a fast-running strip chart.

The experiments were performed with spinach chloroplasts [8]. Sample handling was as in [5]. All measurements were made at 15°C. To increase the sensitivity of the analysis, all N_2 evolution studies were conducted using hydroxyl[^{15}N]amine–HCl (99.5 atom% ^{15}N, obtained from Prochem, London and buffer from which the N_2 had been removed by purging with argon.

3. RESULTS AND DISCUSSION

3.1. *Effect of NH_2OH concentration*

Table 1 presents the initial fluorescence yields observed after a saturating flash in the presence of DCMU as a function of [NH_2OH]. As shown in [6,9], the period of high fluorescence yield following a saturating flash is markedly extended in the presence of NH_2OH and DCMU. The data of table 1 indicate that this effect becomes saturated at ~ 10 μM NH_2OH. Consequently, above this concentration all O_2-evolving centers are occupied by, or have reacted with, NH_2OH after a long dark period. (A similar conclusion can be drawn from the activation-phase data in [2].)

Table 1

Initial fluorescence yield after a saturating flash in the presence of DCMU as a function of [NH_2OH]

NH_2OH (μM)	Normalized initial fluorescence
0	0
1	0.15
2	0.28
4	0.44
6	0.54
8	0.57
10	0.60
30	0.59

The reaction mixture for each experiment contained 50 mM Tricine (pH 7.4), 5 mM $MgCl_2$ and chloroplasts (6 μg chl/ml), in addition to the NH_2OH. The experimental protocol was as follows: 10^{-4} M DCMU added, 4 min dark time, flash, 1 min dark time, fluorescence rise curve obtained [17]. The tabulated fluorescence was calculated from the expression,

$(F_i - F_i,\text{min})/(F_m - F_i,\text{min})$ where

F_i = initial fluorescence level under the stated conditions,

F_i,min = initial fluorescence level in dark-adapted samples in the absence of DCMU,

F_m = maximum fluorescence level attained after prolonged illumination (> 10 s)

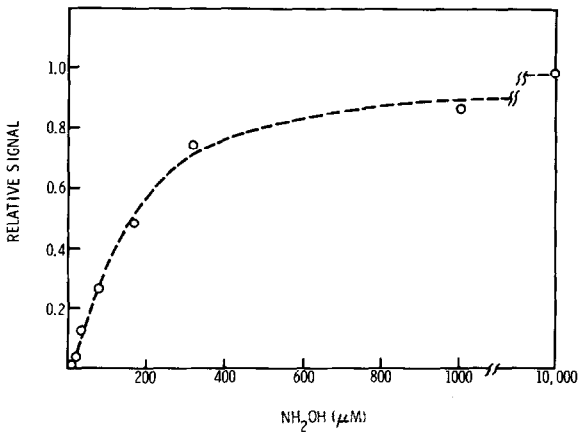

Fig.1. Relative N_2 signal on the first flash as a function of [hydroxylamine]; 10 μl chloroplasts (6 mg chl/ml) were subjected to a series of flashes after a dark time of 10 min.

Fig.1 shows the effect of NH_2OH concentration on the amplitude of the N_2 signal observed on the first flash. Under these conditions, the PS II centers were substrate-saturated down to 10–20 μM, as evidenced by measurements of the initial fluorescence yields (above) and O_2 flash yields (see below). Thus, at >20 μM, the curve reflects the efficiency of NHOH• combination. These data suggest that:

(1) The N_2 flash yield increases with [NH_2OH] up to several hundred μM;

(2) This increase is independent of PS II reactions at > 10–20 μM.

Table 2

Relative flash yields for N_2 and O_2 at different [NH_2OH]

NH_2OH (μM)	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6
			N_2 evolution			
0	0	0	0	0	0	0
5	11	5	2	2	2	2
10	22	12	12	10	10	10
20	47	32	30	26	26	26
30	64	46	46	42	40	40
40	88	72	72	64	60	60
80	176	160	152	144	128	128
160	400	368	360	360	336	328
320	584	528	512	504	504	496
			O_2 evolution			
0	0	80	976	560	272	192
5	0	16	536	520	440	256
10	0	0	288	384	528	336
20	0	0	112	256	608	464
30	0	0	48	112	528	464
40	0	0	16	64	448	448
80	0	0	0	0	192	368
160	0	0	0	0	32	112
320	0	0	0	0	0	16

Experiments were done as in fig.2; at [NH_2OH] >500–600 μM, the N_2 flash yields Y_1-Y_6 were essentially equal

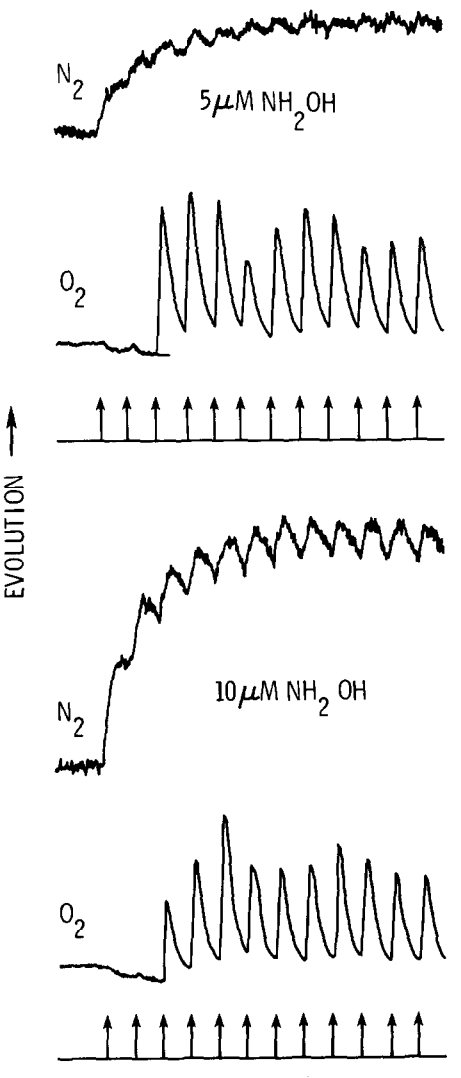

Fig.2. Relative flash yields for N_2 and O_2 observed when 10 μl chloroplasts (3.5 mg chl/ml) were subjected to a series of flashes (arrows) in the presence of 5 μM NH_2OH (top) and 10 μM NH_2OH (bottom). Dark time before the first flash was 10 min.

3.2. *N_2 flash yield sequence at low NH_2OH concentrations*

Fig.2 shows the flash yield pattern of N_2 evolution and O_2 evolution observed with chloroplasts in the presence of 5 μM (top), and 10 μM (bottom) NH_2OH. Note that N_2 was evolved primarily on the first flash, suggesting that NH_2OH was bound to the O_2-evolving site in the dark. The results of a more extensive set of similar experiments are given in table 2.

It is probably futile to attempt to quantitatively describe the interaction of the *S*-states with these low concentrations of NH_2OH, due to the limited stability of the substrate, the occurrence of side reactions, and the well-documented extraction of the O_2 centers by NH_2OH. Nevertheless, the data do suggest that the O_2-evolving site has a very high affinity for NH_2OH, particularly the S_0 state. The absence of significant loss of O_2 evolution capability at low NH_2OH concentrations, coupled with the pronounced shift in the flash yield maximum from Y_3–Y_5, suggests that we are observing N_2 evolution by competent O_2-evolving centers on the first flash.

3.3. *Proposed mechanism*

The salient reactions, and the complexity of the

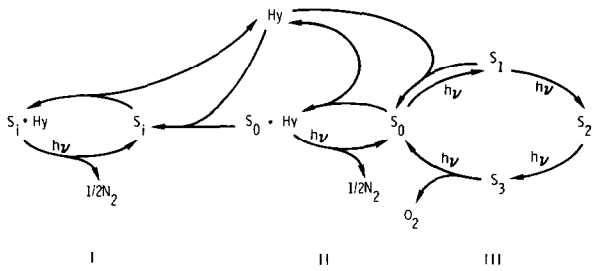

Fig.3. Pathways of NH_2OH interaction with the O_2-evolving system. Hy ≡ NH_2OH.

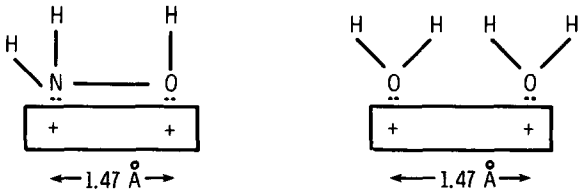

Fig.4. Model of the O_2-evolving system and its interaction with H_2O and NH_2OH.

overall process, can be discussed with reference to fig.3, which comprises 3 relevant cycles:

Cycle I, representing the series of reactions between NH_2OH and centers incapable of O_2 evolution. The production of these centers (from competent O_2 centers) may be unrelated to the reducing ability of NH_2OH [10–12].

Cycle II, the reactions of primary interest in this communication, represent the slow, high-affinity binding of NH_2OH by S_0, and the light-driven discharge with the production of N_2.

Cycle III, the familiar O_2 clock [13], may involve NH_2OH as a mediator of deactivation, particularly from S_1 to S_0.

Our data suggest that one molecule of tightly bound hydroxylamine is oxidized on the first flash by competent O_2-evolving centers, after which O_2 evolution proceeds normally starting from S_0 generated in the dark, i.e.:

$$S_1 + NH_2OH \rightarrow S_0 + 1/2\ N_2 \text{ (dark reaction)}$$

$$S_0 + NH_2OH \rightarrow S_0[NH_2OH]_{bound}$$

$$S_0[NH_2OH]_{bound} \xrightarrow{h\nu} S_0 + 1/2\ N_2$$

This reaction sequence is similar to one proposed in [12] but later withdrawn [1].

The observed steady-state N_2 evolution probably arises from the occurrence of cycle II (and possibly cycle III) back-reactions between flashes. This interpretation is supported by experiments in which the flash spacing was varied (unpublished). At higher [NH_2OH], cycle I becomes increasingly significant.

The reaction sequence shown suggests that hydroxylamine must bind to the H_2O-oxidation site much more strongly than does H_2O itself, since NH_2OH oxidation occurs at 10–20 μM in the presence of 55 M H_2O. The higher binding constant of the artificial donor does not reflect differences in the binding affinities of N and O since PS II reacts with NH_2OH, NH_2NH_2, and H_2O_2 at about the same concentrations [3,4,14]. A more likely possibility is that the efficient NH_2OH binding reflects a decreased dissociation probability attributable to the simultaneous binding of > 1 group, possibly in a hindered environment. This supposition is supported by preliminary data obtained using NH_2OH analogs. (A similar argument has been used to rationalize the stability of chelates; see, e.g. [15,16].) If this inference is correct, it suggests that the H_2O-oxidation site of the chloroplasts has 2 H_2O binding sites ~1.47 Å apart (the length of the O–N bond) (fig.4).

ACKNOWLEDGEMENTS

This work was supported by the Department of Energy, Contract DE-AC02-82ER12015. We thank B. Velthuys for helpful discussions and critically reading the manuscript.

REFERENCES

[1] Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 292, 772–785.

[2] Bouges, B. (1971) Biochim. Biophys. Acta 234, 103–112.

[3] Kok, B. and Velthuys, B.R. (1977) in: Research in Photobiology (Castellani, A. ed) pp. 111–119, Plenum, New York.

[4] Velthuys, B.R. and Kok, B. (1978) Biochim. Biophys. Acta 502, 211–221.
[5] Radmer, R. (1979) Biochim. Biophys. Acta 546, 418–425.
[6] Radmer, R. and Ollinger, O. (1981) Biochim. Biophys. Acta 637, 80–87.
[7] Jones, K. (1973) in: Comprehensive Inorganic Chemistry (Trotman-Dickenson, A.F. ed) vol. 2, pp. 147–388, Pergamon, New York.
[8] Schwartz, M. (1966) Biochim. Biophys. Acta 112, 204–212.
[9] Bennoun, P. (1970) Biochim. Biophys. Acta 216, 357–363.
[10] Cheniae, G.M. and Martin, I.F. (1971) Plant Physiol. 47, 568–575.
[11] Robinson, H.H., Sharp, R.R. and Yocum, C.F. (1980) Biochim. Biophys. Acta 593, 414–426.
[12] Bennoun, P. and Bouges, B. (1972) in: Proc. 2nd Int. Cong. Photosynthesis (Forti, G. et al. eds) pp. 569–576, Junk, The Hague.
[13] Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475.
[14] Diner, B. and Joliot, P. (1977) in: Encyclopedia of Plant Physiology (Trebst, A. and Avron, M. eds) vol. 5, pp. 187–205, Springer-Verlag, Berlin, New York.
[15] Parry, R.W. (1956) in: Chemistry of Coordination Compounds (Bailar, J. ed) pp. 220–252, Reinhold, New York.
[16] Spike, C. (1952) Ph.D. Dissertation, Univ. of Michigan.
[17] Doschek, W. and Kok, B. (1972) Biophys. J. 12, 832–838.