Hydroxylamine as an inhibitor between Z and P680 in photosystem II

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Upon addition of hydroxylamine to chloroplasts or photosystem II preparations, the EPR signal of Z^+ disappears and a new signal is observed. From its shape and g-value this signal is identified with the oxidized reaction center chlorophyll, $P680^+$. The decay of $P680^+$ occurs with a halftime of $\lesssim 200\ \mu s$ and apparently is the result of a back reaction with the reduced form of the primary acceptor, Q_A. This mode of hydroxylamine inhibition is reversible. These observations indicate that hydroxylamine, in addition to its well known inhibitory action on the oxygen evolving complex, is also able to disrupt physiological electron flow to P680 itself.

Photosystem II *Electron-transfer inhibition* *Reaction center* *Electron paramagnetic resonance*

1. INTRODUCTION

Exposure of isolated thylakoid membranes to NH_2OH is known to be a mild treatment for inactivation of the oxygen evolving reaction system [1–4]. In [2] it was reported that the mechanism of NH_2OH inactivation of the oxygen evolving complex (OEC) is different from that of Tris-inactivation: inhibition by Tris is accelerated by low light intensities [5,6], whereas NH_2OH produces a rapid inactivation at low concentrations in the dark. In the same work [2], the authors report that NH_2OH-inhibition occurs by two different mechanisms. One is the extraction of Mn from the photosynthetic membrane while the other mechanism, which has an immediate but reversible effect, was not localized. Authors in [7], using NMR relaxation techniques, proposed that higher S-states are immune to attack by NH_2OH and that NH_2OH-induced Mn extraction occurs only from the lower S-states. Authors in [8] studied the fluorescence kinetics in *Chlorella pyrenoidosa* in the presence of NH_2OH and suggested that an inhibition site could be localized between Z, the physiological donor, and the oxidized reaction center pigment, P680. This inhibition was reversed during illumination of a washed sample. These authors also reported that for a series of high frequency flashes in the presence of NH_2OH, $P680^+$ was rereduced by a back reaction with the reduced form of the primary acceptor, Q_A^-.

Here, we have used EPR spectroscopy to monitor the formation of signal IIf and $P680^+$ directly in order to localize the immediate NH_2OH inhibition observed in the past [2,8]. Since the EPR signal which arises from $P680^+$ is reported to have the same shape and g-value as that which arises from $P700^+$, we used PSII preparations free of photosystem I in addition to unfractionated thylakoids in these experiments. In agreement with [8], we find that NH_2OH exerts an immediate and reversible inhibition which results in a blocking of electron flow from Z to P680.

Abbreviations: EPR, electron paramagnetic resonance; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; MES, 4-morpholinoethane sulfonic acid; OEC, oxygen evolving complex; PSI, photosystem I; PSII, photosystem II

2. MATERIALS AND METHODS

Intact thylakoid membranes were prepared as in

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[9]. Subchloroplast membranes, free of photosystem I and capable of oxygen evolution, were prepared as in [10] except that the initial Triton extraction and subsequent washings were carried out at pH 6.0. We have found that by using lower pH values during the isolation, PSII particles with higher O_2 rates result. The buffer system used contained 0.4 M sucrose, 0.01 M NaCl and either 0.05 M HEPES for pH 7.6 or 0.05 M MES for pH 6.0. Chlorophyll concentrations were determined as in [11]. EPR spectroscopy was carried out on a Bruker ER-200D spectrometer operated at X-band and interfaced to a Nicolet 1180 computer. Instrument modifications as well as the xenon flash lamp circuitry and the protocol followed in signal-averaged, flashing-light kinetic experiments are described in [9].

3. RESULTS

When O_2 evolution is inhibited at the oxygen evolving complex, the lifetime of the intermediate electron carrier, Z^+, is extended into the hundreds of ms time range and becomes detectable as EPR signal IIf. In [12] it was reported that incubation of chloroplasts in the dark with NH_2OH followed by washing of the system with buffer resulted in complete inhibition of O_2 evolution and conversion of signal IIvf to signal IIf. When we incubated chloroplasts with NH_2OH in the dark and then, without removing the inhibitor, transferred them to the EPR spectrometer we found that neither signal IIvf nor signal IIf was present. After washing the system twice with buffer signal IIf could be observed. We repeated the above experiment with Tris-treated chloroplasts and noted the same behavior (i.e., the presence of NH_2OH in the system) inhibited signal IIf formation, whereas its removal allowed full development of signal IIf. PSII subchloroplast preparations showed analogous behavior in the presence of hydroxylamine except that lower concentrations of NH_2OH were required. Indeed when we measured the O_2 rates of PSII preparations immediately following addition of NH_2OH (fig.1), we noticed that low concentrations of NH_2OH were very effective in inhibiting O_2 evolution. Intact chloroplasts required higher concentrations of NH_2OH in order to obtain the same extent of inhibition. For example, under the conditions of fig.1, 50% inhibition of O_2 evolution in PSII preparations occurs at ~1 mM NH_2OH; in chloroplasts under these conditions ~5 mM NH_2OH was required for 50% inhibition.

Reflecting the behavior of O_2 evolution in response to NH_2OH addition, signal IIvf of untreated PSII preparations and signal IIf of Tris-treated preparations disappears and a new signal, which rises within the 50 μs response of our instrument and had a decay halftime of < 200 μs, is present (fig.2). Removal of NH_2OH from Tris-treated PSII preparations restores the system to its initial state (full signal IIf, no new signal, not shown).

The shape of the new signal, as determined by signal-averaged experiments at different field values, is shown in fig.3. Its peak-to-peak width is 7–8 Gauss and its g-value is 2.002; these values are in agreement with those reported for $P680^+$ [13,14]. Quantitation of the new signal, by double integration and correction for the instrument time constant, showed that it is stoichiometric ($\pm$ 20%) with signal IIf. The saturation properties of the radical are shown in the inset to fig.3 which indicates that it saturates at relatively high power. Compared to this radical, signal I (EPR signal of $P700^+$) saturates at somewhat lower power which could be the result of environmental differences between the two species.

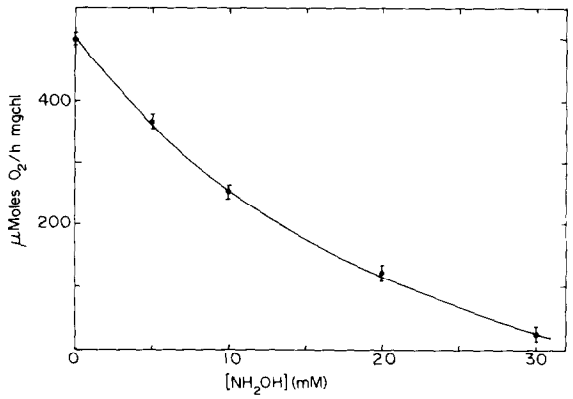

Fig.1. Effect of NH_2OH concentration on the O_2 rate of PSII preparations at pH 6.0. PSII preparations (10 μg chl/ml) were suspended in the polarograph vessel with the exogenous acceptors, $Fe(CN)_6^{3-}$ (3.5 mM) and 2,5-dichloro-p-benzoquinone (250 μM). Addition of NH_2OH was followed by immediate illumination to determine the rate of O_2 evolution.

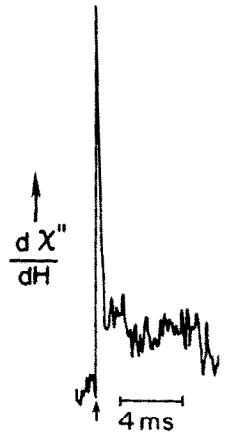

Fig.2. Kinetic transient of the P680$^+$ EPR signal: 1 mM $Fe(CN)_6^{3-}$, 1 mM $Fe(CN)_6^{4-}$ and 2 mM NH_2OH were added to a suspension of PSII particles at pH 6.0. The instrument time constant was 50 μs; 150 flashes were averaged at a frequency of 0.1 Hz.

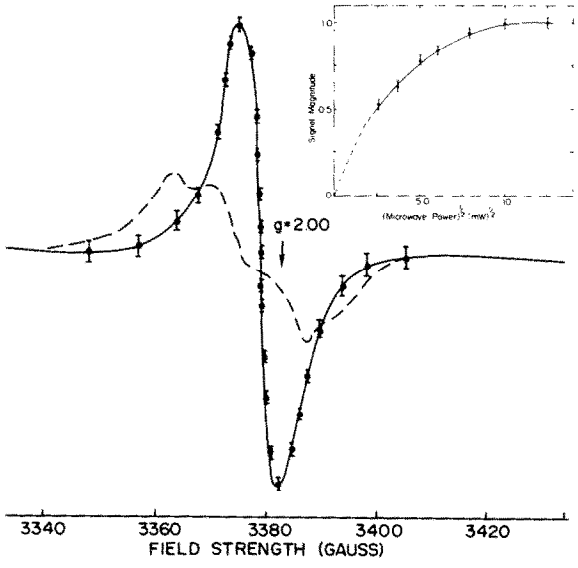

Fig.3. Shape of signal P680$^+$ (——) in PSII preparations obtained by kinetic experiments at the indicated field values. The shape of signal IIs is also shown (– – –). Insert: Saturation properties of signal P680$^+$ in PSII preparations at pH 6.0. Each point represents the amplitude of the P680$^+$ EPR signal at the indicated microwave power. The conditions were the same as those in fig.2.

4. DISCUSSION

The most common inhibitory site on the oxidizing side of photosystem II is at the oxygen evolving complex itself (e.g., [12]) and NH_2OH has a well known inhibition at this site [2,7]. Fluorescence data indicate, however, that NH_2OH also produces an inhibition in PSII which is probably localized between Z, the physiological donor, and the oxidized reaction center pigment, P680 [8]. In the work reported here, we have used EPR to show that NH_2OH does block the flow of electrons from Z to P680 in a way which is completely reversible. In the absence of NH_2OH, Z, which can be identified with D_1 [15], the immediate electron donor to P680$^+$ in a variety of chloroplast preparations [16], reduces P680$^+$ in a reaction with a halftime $\leq$ 15 μs. Under these conditions we observe the EPR signal of Z^+, produced after reaction of Z with P680$^+$, but we are not able to follow the EPR signal of P680$^+$ because of the response time of our instrument. Upon addition of hydroxylamine, electron flow between Z and P680$^+$ is interrupted and P680$^+$ is reduced by back reaction with the reduced form of the primary acceptor, Q_A^-. This reaction is much slower compared to the reduction of P680$^+$ by Z [16] and we can follow it with our experimental apparatus.

Thus, we can explain the NH_2OH inhibition of PSII with the scheme shown in fig.4. According to that scheme, NH_2OH has two effects. In the first, NH_2OH interrupts the flow of electrons from the S-states to Z by extracting Mn [2,7] which results in the conversion of signal IIvf to signal IIf. In the second mode of inhibition, which appears to be

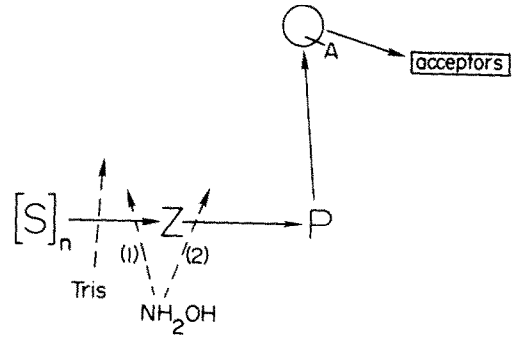

Fig.4. A model for the inhibitory role of NH_2OH in photosystem II: see text for details.

unique to NH_2OH, the flow of electrons from Z to P680 is interrupted in a completely reversible manner. The mechanism of the second inhibition is not clear and it could be a direct effect on Z, for example formation of a Z–NH_2 OH complex, or an effect on the membrane environment of Z. That $P680^+$ is rereduced by Q_A^- in the inhibited centers, however, implies that the chemical and not the redox properties of NH_2OH are responsible for this inhibition.

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