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MASS SPECTROMETRIC DETERMINATION OF HYDROXYLAMINE PHOTOOXIDATION BY ILLUMINATED CHLOROPLASTS

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Summary

A mass spectrometer with a special inlet was used to directly monitor the products evolved when hydroxylamine-treated chloroplasts were exposed to short saturating light flashes. We found that:

1. Molecular dinitrogen was the sole product of hydroxylamine photooxidation, and was formed in an amount equal to twice the O_2 evolved during H_2O photooxidation.
2. This reaction was driven by Photosystem II, and did not involve Photosystem I-generated superoxide or peroxide.
3. In the presence of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, N_2 was evolved only on the first flash.

These results suggested that N_2 was formed by the combination of two single-electron oxidation products of hydroxylamine.

Introduction

It has been recognized for some time that hydroxylamine (NH_2OH) can serve as an electron donor to Photosystem II [1]. At high substrate concentrations (>100 μM) this photooxidation reaction bypasses the O_2 evolution system, which is inactivated by the hydroxylamine (see Refs. 2 and 3). Bennoun and Joliot [4] reported the detection of an amperometric signal which they ascribed to an oxidized form of hydroxylamine. Unlike O_2 evolution, this reaction showed no activation, i.e. the rate was maximal at the onset of illumination, and thus occurred via a one-quantum, one-equivalent process.

In this communication, we describe experiments in which we used a mass

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

spectrometer to monitor the products evolved by hydroxylamine-treated chloroplasts in short saturating light flashes. We found that molecular dinitrogen was the only reaction product. Its formation involves the combination of two one-electron oxidation products of hydroxylamine.

Materials and Methods

Fig. 1 is a schematic diagram of the mass spectrometer 'flash-yield inlet' used for these measurements. The 1.0 ml reaction vessel is enclosed on top by a thin Plexiglass lid with a small centered hole. The bottom of the reaction vessel is a circular window (1.1 cm^2) of 1 mil silicone rubber membrane (MEM 213, General Electric Co.). A chloroplast suspension was carefully layered on to the membrane (through the hole in the lid) and illuminated from above.

Dissolved gases from the liquid phase are admitted to the mass spectrometer vacuum via the silicone rubber membrane (see Refs. 5 and 6). The permeability of this membrane for the gaseous oxidies of nitrogen (NO, N_2O, and NO_2) is at least as high as that of O_2; the N_2/O_2 permeability ratio is one-half [7]. The dissolved gases must diffuse through a liquid boundary layer to reach the membrane surface; since this boundary layer can have a resistance to gas transfer comparable to that of the membrane itself, the rate of gas transport from the liquid to the mass spectrometer vacuum system will not be proportional to membrane permeability.

In this configuration the chloroplast suspension is in direct contact with the membrane, and thus the mass spectrometer can directly monitor chloroplast gas exchange reactions. The time response of this system is limited by the mass spectrometer and its associated vacuum system in addition to the transport characteristics of the membrane. The end-to-end time response, measured as the delay between the firing of the flash and the maximum signal, was 0.7 to 1 s. This value depended, at least partly, on how tightly the membrane was stretched.

A modified Micromass 6 magnetic mass spectrometer (V.G. Micromass Ltd., Winsford, England) was used for gas analysis. The vacuum and gas handling systems and ancillary electronic equipment were built in-house. A compen-

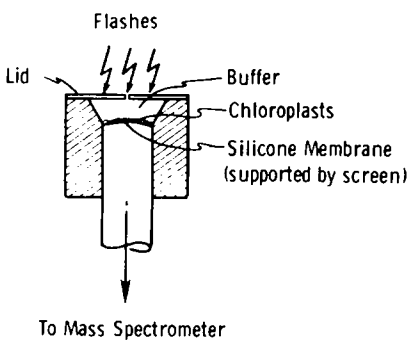


Fig. 1. Schematic diagram of 'flash-yield inlet'.

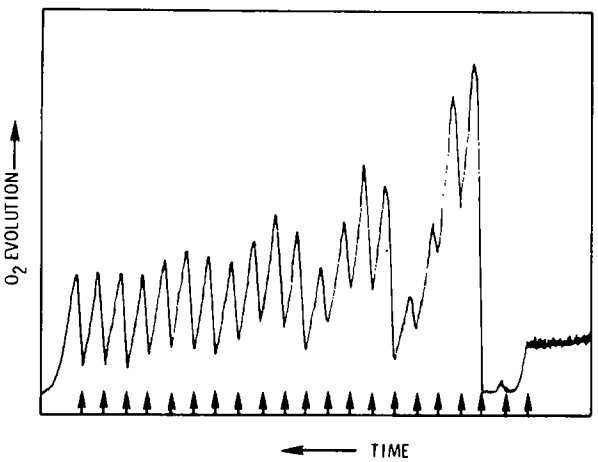


Fig. 2. Flash yields of O_2 (m/e = 32) observed when dark-adapted chloroplasts were subjected to a train of saturating flashes. Dark adaptation time prior to the first flash was 5 min. The vertical arrows in this and subsequent figures indicate the firing of the individual flashes. See text for other details.

sating 'bucking' voltage was applied to the mass spectrometer signals to null out most of the undesired background signal. A mass-stepper-bucking system was used to tune the mass spectrometer and compensate the background signal of the appropriate m/e values. The signals were directly recorded on a fast-running strip chart. In most of the experiments the signal passed through an R-C network with a time constant of 100 ms.

Actinic flashes were provided by a xenon flash tube (FX-6A, EG & G, Salem, Mass.) mounted a few millimeters above the vessel. The flash intensity was 4 J (1000 V, 8 μF), the duration at half-maximum was 17 μs, and the frequency was 0.33 Hz. The rather broad flash envelope was due to the large storage capacitors and long cables used in these experiments. This flash duration was adequate for our purposes (see below and Fig. 2).

The experiments were performed with chloroplasts prepared from greenhouse spinach [8] in a medium containg 0.4 M sucrose, 0.05 M Tris-HCl and 0.01 M NaCl, at pH 7.4. For each experiment appropriate additions were made both to the medium (Tricine buffer 0.05 M, pH 7.5) and to an aliquot of the concentrated (approx. 4 mg chl/ml) chloroplast stock suspension. After the reaction vessel was filled with the medium, a 10 μl portion of this suspension was admitted to the reaction vessel through the small hole in the lid; because of the high density of this preparation (it contained 0.4 M sucrose) it fell to the bottom and formed a thin homogeneous layer over the membrane [9]. All measurements were made at room temperature.

[^{15}N]Hydroxylamine hydrochloride (97 atom% ^{15}N) was obtained from Prochem, London, U.K.

Fig. 2 shows the results obtained when the apparatus was used to determine the flash yields of O_2 evolved from a dark-adapted chloroplast suspension. The characteristic oscillation and the low second flash yield indicate that the flash was saturating, and that its duration was short enough so that little double-

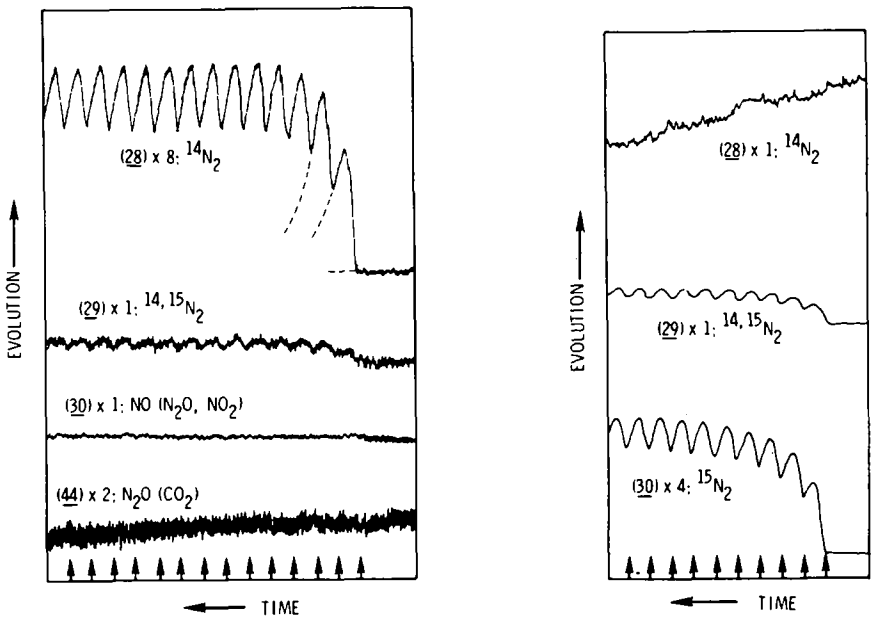


Fig. 3. Relative flash yields at different *m/e* values observed when chloroplasts were subjected to a series of flashes in the presence of 1 mM NH_2OH. Numbers given after the *m/e* values refer to different attenuations; e.g. *m/e* = 28 was attenuated eight-fold compared to *m/e* = 29 and 30. In this figure, and others which display more than one trace, the different traces were sequentially obtained on the sample after identical dark times (3 or 5 min). Measurements of the different *m/e* values were interlaced in different orders to ensure that the identity of the evolved products did not change with time or illumination regimen. See Fig. 2 and text for other details.

Fig. 4. Relative flash yields at different *m/e* values observed when chloroplasts were subjected to a series of flashes in the presence of $^{15}NH_2OH$ (1 mM).

hitting occurred (cf. Ref. 10). The steady-state flash yields of Fig. 2 correspond to about 25 pmol O_2/flash *.

Results and Discussion

Products of hydroxylamine photooxidation

Fig. 3 shows the products evolved when chloroplasts were subjected to a train of saturating flashes (3-s spacing) in the presence of 1 mM NH_2OH. Note that molecular N_2 was formed exclusively in this reaction; the small flash yields observed at *m/e* = 29 were due to the oxidation of naturally occurring $^{15}NH_2OH$, which resulted in the evolution of small amounts of $^{14,15}N_2$. There was no apparent evolution of nitrogen oxides, which, under many conditions, are the major products of NH_2OH oxidation [11].

To demonstrate that we were indeed looking at N_2 evolution, we ran the same experiment using [^{15}N]hydroxylamine. As shown in Fig. 4, the major

* Assume a unit size of 400 chlorophylls/System II trap. 40 μg chlorophyll ≃ 40 nmol chlorophyll. (40 nmol chlorophyll)/(400 nmol chlorophyll/nmoles Photosystem II trap) = 0.1 nmol Photosystem II trap (100 pmol). Since O_2 evolution requires $4e^-/O_2$, each flash releases 25 pmol O_2.

product now appeared at $m/e = 30$, the spectral position of $^{15}N_2$. We also observed an amount of $^{14,15}N_2$ at $m/e = 29$ which was in accord with the isotopic composition of the labeled hydroxylamine. We therefore feel quite certain that the product of this reaction is molecular dinitrogen.

Stoichiometry

In order to roughly quantitate the individual relative N_2 flash yields, companion experiments to those of Fig. 3 were run in which the flash yield sequence was interrupted after one, two, three, etc. flashes, so that the flash yield decay kinetics of a particular N_2 flash yield could be determined. This flash-yield envelope was then used to extrapolate the n-th flash so that the N_2 yield of the $(n+1)$th flash could be computed (dashed lines in top trace of Fig. 3). These experiments and computations showed that all N_2 flash yields, including the first, were equal.

A relative N_2-vs.-O_2 calibration of this system was made by comparing the measured ratio of N_2 and O_2 signals in air-saturated H_2O to those available in the literature [12,13]. Calibration data obtained using a reaction vessel (with a membrane inlet) equipped with a magnetic stirrer [5,6] and with the flash-yield inlet both showed that there was very little end-to-end mass discrimination in our system (i.e. the N_2/O_2 detection sensitivity was about unity).

Parallel experiments in which the steady-state flash yields for O_2 (cf. Fig. 2) and N_2 (cf. Fig. 3) were compared showed that the N_2 flash yield was about twice the O_2 flash yield in the steady state. If we assume that, in the steady state, 1/4 O_2 per trap is evolved per flash when H_2O is the electron donor, we can then conclude that 1/2 N_2 per trap per flash (±10—20%) is evolved in the presence of NH_2OH.

Is the observed N_2 evolution a System II reaction?

One aspect of these results that initially concerned us was that we might be indirectly monitoring a Photosystem I reaction. Isolated chloroplasts are known to generate peroxide [14] and superoxide [15] via Photosystem I (PSI). These species could conceivably react with hydroxylamine, e.g.

$$O_2 + e^- \xrightarrow{PSI} O_2^-$$

$$H^+ + O_2^- + NH_2OH \rightarrow 1/2N_2 + H_2O + H_2O_2$$

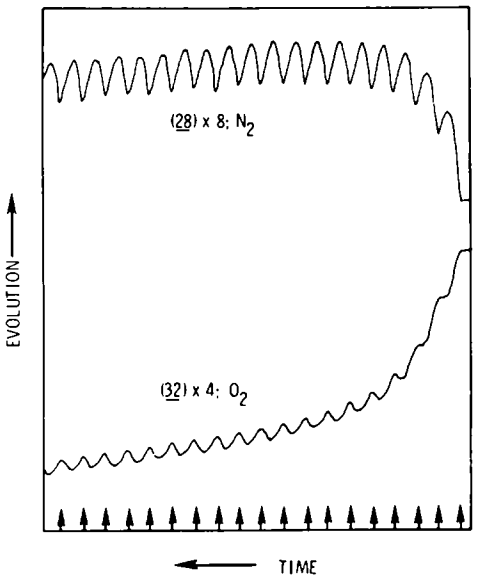
or

$$O_2 + 2H^+ + 2e^- \xrightarrow{PSI} H_2O_2$$

$$2NH_2OH + H_2O_2 \rightarrow N_2 + 4H_2O$$

One would predict that, if the N_2 evolution occurred according to the above reaction sequence, the N_2/O_2 ratio would be either one-half or one. Fig. 5 illustrates that there was indeed a substantial O_2 uptake occurring in synchrony with N_2 evolution. However, there was no apparent simple stoichiometry: N_2 evolution greatly exceeded O_2 uptake, indicating that the N_2 was not formed via O_2^- or H_2O_2.

Further proof that the observed N_2 evolution did not occur via reduced



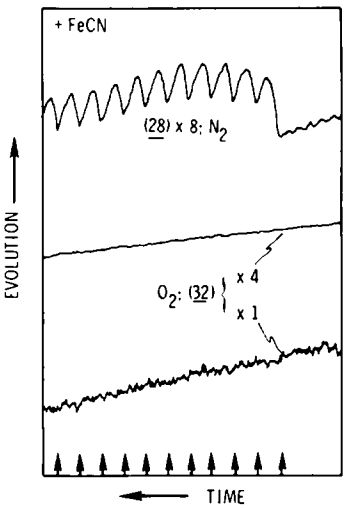


Fig. 5. Relative flash yields of O_2 uptake and N_2 evolution observed in the presence of 1 mM NH_2OH.

Fig. 6. Relative flash yields of O_2 uptake and N_2 evolution observed in the presence of 1 mM NH_2OH and 1 mM potassium ferricyanide.

oxygen intermediates is that O_2 uptake could be suppressed without decreasing the magnitude of N_2 evolution. Fig. 6 shows an experiment in which NH_2OH oxidation was measured in the presence of 1 mM ferricyanide, a System I acceptor. The top two traces show the N_2 evolution and (lack of) O_2 uptake under these conditions at the same relative signal attenuations as those of Fig. 5. Even when the O_2 signal was blown up (bottom trace) no flash-induced O_2 uptake was apparent.

Similar results, leading to the same conclusions, were also obtained when O_2 uptake was suppressed by other means. Complete removal of oxygen by use of glucose (0.1 M) and glucose oxidase (100 units) did not affect N_2 evolution (data not shown). We also observed N_2 evolution in particles prepared [16] from *Scenedesmus* mutant No. 8, which is known to lack Photosystem I [17].

Our conclusion that NH_2OH serves as a System II electron donor corroborates several earlier reports (see citations in Refs. 2 and 18). However, it is not in agreement with that of Elstner et al. [18], who argue that NH_2OH oxidation occurs via the superoxide anion, and that NH_2OH is not oxidized by Photosystem II. Their experiments were longterm incubations (15 min) in the presence of low concentrations of NH_2OH and the auto-oxidizable System I acceptor methylviologen; under these conditions the NH_2OH oxidation product was nitrite, and H_2O was presumably the System II donor. Our finding that N_2 evolution occurred with a high yield in the absence of O_2 uptake is strong evidence that the N_2 evolved in our experiments was not produced via superoxide, and that NH_2OH can serve as an electron donor to System II. This conclusion was further corroborated by the observed effect of DCMU (see below).

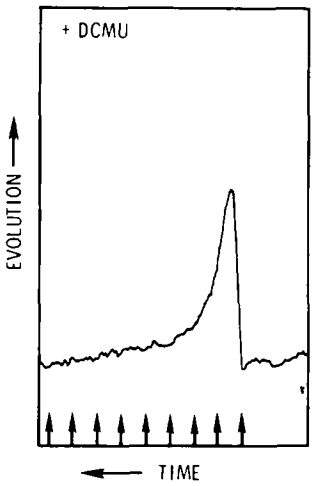


Fig. 7. Relative flash yields of N_2 evolution observed in the presence of 1 mM NH_2OH and 10 μM DCMU.

Effect of DCMU

In the presence of DCMU, NH_2OH inhibits the backreaction between P^+-690, the oxidized primary Photosystem II donor, and Q^-, the reduced primary acceptor [19]. This suggests that the reduction of P^+-690 by NH_2OH is irreversible, in contrast to the reduction of P^+-690 by the O_2 system [20]. Fig. 7 shows that in the presence of NH_2OH and DCMU, N_2 was evolved on the first flash (provided that no light, other than the actinic flashes, had reached the sample after the addition of DCMU) but not on subsequent flashes. The irreversibility of the NH_2OH-DCMU system, compared to the normal DCMU-inhibited system, therefore, can be attributed to the ability of the NH_2OH system to immediately discharge its oxidizing equivalents as N_2. In the normal DCMU-inhibited system the oxidizing equivalents are retained (mainly as S_2) and the system can subsequently backreact (deactivate) to restore the initial conditions.

Proposed mechanism

Our data show that equal amounts of N_2 are evolved on each flash, i.e. there is no binary oscillation of N_2 yields, and that the N_2/O_2 flash yield ratio for NH_2OH vs. H_2O photooxidation is $2N_2/O_2$. These results suggest a mechanism for NH_2OH photooxidation involving the abstraction of a single electron from a single NH_2OH molecule at each Photosystem II (PSII) center, and the subsequent combination of these oxidized products to form molecular dinitrogen (a 'two-electron' product), e.g.

$$NH_2OH \xrightarrow{PSII} NHOH^{\cdot} + H^+ + e^-$$

$$2\ NHOH^{\cdot} \rightarrow N_2 + 2H_2O$$

This oxidation product ($NHOH^{\cdot}$) is probably the same entity as that detected polarographically by Bennoun and Joliot [4]. Their measurements were done using a bare electrode (i.e., it had no membrane covering so that the active

surface was exposed to the solution) that could intercept at least some of the oxidation product before it dimerized. This oxidation product is not detected in our experiments presumably because, like NH_2OH, it has a negligible vapor pressure and therefore is not transmitted by the membrane.

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