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Mass spectroscopic analysis of N_2 formation by flash-induced oxidation of hydrazine and hydroxylamine in normal and Tris-treated tobacco chloroplasts

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The flash-induced dinitrogen evolution was analyzed in normal and Tris-inhibited tobacco chloroplasts (*Nicotiana tabacum* var. John William's Broadleaf) which were dark-incubated (at least 20 min) in the presence of NH_2NH_2 and/or NH_2OH. (a) The N_2 yield due to a single turnover flash as a function of NH_2NH_2 concentration is practically the same in both normal and Tris-washed chloroplasts, even in a range where the oxygen evolution remains almost unaffected by NH_2NH_2. Illumination with ten flashes leads to higher integral N_2 evolution in Tris-washed samples at limiting NH_2NH_2 concentrations. (b) Under aerobic conditions oxidation of NH_2NH_2 and NH_2OH is coupled with significant O_2 uptake. Both N_2 formation and O_2 uptake remain unaffected by DCMU in a single turnover flash but become drastically inhibited under multiple flash excitation. (c) The N_2 yield per flash due to PS-II-induced NH_2NH_2 oxidation markedly depends on the O_2 content of the suspension. This effect is also observed in the presence of superoxide dismutase (SOD). (d) If chloroplasts are incubated with an equimolar ratio of $^{14}NH_2{}^{14}NH_2$ and $^{15}NH_2{}^{15}NH_2$, hardly any $^{14}N^{15}N$ formation is detected, indicating that the nitrogen–nitrogen bond remains intact during the oxidation process. A totally different isotope distribution is obtained in the case of an equimolar mixture of $^{14}NH_2OH$ and $^{15}NH_2OH$ which reflects a statistical bimolecular reaction between NHOH radicals. In a mixture of $^{14}NH_2OH$ and $^{15}NH_2{}^{15}NH_2$ the ratio of mass 29 to mass 30 increases with increasing $^{14}NH_2OH$ content. Based on the present results, flash-induced N_2 formation is inferred to occur via univalent oxidation of NH_2OH and NH_2NH_2 either by P-680$^+$ or by the functionally connected tyrosine(s) of polypeptide D-1 (Y_Z^{ox}) and/or D-2 (Y_D^{ox}). NH_2OH and NH_2NH_2 do not interact with the S_1 state of the catalytic site of water oxidation in a way that leads to flash-induced N_2 production.

Introduction

Based on the classical work of Joliot, Kok and coworkers (for review see Ref. 1) photosynthetic water oxidation to dioxygen was shown to take place at a manganese-containing catalytic site via a redox cycle of 1-electron oxidation steps referred to as the Kok scheme [2] comprising five different states S_i ($i = 0 \ldots 4$). Here index i denotes the number of oxidizing redox equivalents accumulated at the catalytic site. S_0 and S_4 formally correspond with the redox levels of water and dioxygen, respectively, while S_1, S_2 and S_3 represent differently stabilized intermediary redox states.

Despite intensive research activities, the chemical nature of the redox states S_i is still an unresolved problem (for review see Ref. 3). It has been shown that the reaction pattern of water oxidation can be modified by different chemicals. Of special mechanistic relevance is the interaction of H_2O_2 with the S_i cycle [4–7] because a peroxidic state of unknown electronic structure and nuclear geometry is probably formed as an intermediate of water oxidation to dioxygen (for discussion see Ref. 8). Two other types of compound are also of interest because they induce, at properly selected concentrations, specific effects without impairing the oxygen evolution capacity: (a) ADRY agents [9–11] which selectively shorten the lifetime of S_2 and S_3, and (b) hydroxylamine (NH_2OH) and hydrazine (NH_2NH_2) which cause a two-digit phase shift of the S_i state cycle towards more reduced states in thylakoids and PS II membrane fragments [12–14].

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After sufficiently long dark adaptation practically all catalytic sites of water oxidation are in S_1 [15]. The action of NH_2OH and NH_2NH_2 can be formally explained by an additional state, S_{-1}. There are different lines of evidence for the existence of S_{-1} in algae and higher plants [16–18]. Accordingly, NH_2OH and/or NH_2NH_2 could reduce in the dark S_1 into S_{-1} so that the redox cycle of the dark-adapted samples starts at the level of S_{-1} in the presence of these compounds [19,20]. Alternatively, NH_2OH and/or NH_2NH_2 could bind in the vicinity or directly at the manganese in a way that the S_2 formed by a single turnover flash rapidly reacts with the bound agents in a two-electron transfer, thereby tracking back the system into S_0 [21–23]. Thus far, an unambiguous proof for one or the other mechanism has not been achieved. In addition to the reactions affecting the oscillation pattern of oxygen evolution, NH_2OH and NH_2NH_2 also act as exogenous PS II electron donors in systems deprived of their water oxidizing activity. The latter process leads to N_2 formation [24].

In this study the flash induced oxidation of NH_2OH and NH_2NH_2 in normal and Tris-treated isolated tobacco thylakoids was analyzed by mass spectroscopic measurements of dinitrogen formation. The implications of the results for the mechanism of NH_2OH and NH_2NH_2 oxidation by the PS II donor side are discussed.

Materials and Methods

Isolated chloroplasts were prepared from *Nicotiana tabacum* var. John William's Broadleaf (IWB) according to a method described previously [25,26]. The freshly prepared chloroplasts were stored on ice before measurement in the mass spectrometer.

The reaction mixture contained: chloroplasts (70 μg chlorophyll/ml), 30 mM KCl and 60 mM Tricine-NaOH (pH 7.0) and different additions of NH_2NH_2 as indicated in the figure legends.

NH_2OH or NH_2NH_2 were added to dark-adapted (about 10 min) samples under very dim green light. The measurements were performed with a modified magnetic sector field mass spectrometer 'type Delta' from Finnigan (Bremen, F.R.G.) which is a stable isotope ratio mass spectrometer equipped with a two-directional focusing device 'Nier type I'. The measuring cell was laboratory-built. The details of the device and of the technique to improve the sensitivity are outlined in Ref. 27. The equipment permits the detection of dioxygen and/or dinitrogen evolved due to excitation of the sample with a single turnover flash.

Saturating excitation flashes of about 8 μs duration were obtained from a xenon flash lamp (Stroboscope 1539 A of General Radio). The time between the flashes was 300 ms.

Results

For the measurements of flash-induced N_2 evolution due to hydrazine oxidation, chloroplasts were kept in the cuvette for 10 min in the dark before addition of different amounts of NH_2NH_2. After a subsequent dark period (> 20 min) to allow a complete equilibration with the substrate [23] and sufficient sedimentation, the samples were illuminated with one flash followed by another dark period of 5 min and illumination with a train of ten flashes (time between the flashes, 300 ms). In order to eliminate base line problems due to $^{14}N_2$ in the gas phase above the samples, the experiments were performed with $^{15}NH_2{}^{15}NH_2$ as substrate, and dinitrogen evolved was monitored at mass 30. The relative amount of $^{15}N_2$ evolved by one or ten flashes was measured in normal and Tris-treated chloroplasts, because the oxidation of NH_2NH_2 does not require an intact oxygen-evolving system [24,28].

The effect of NH_2NH_2 (and of NH_2OH) on the catalytic site of water oxidation is complex. The well-characterized two-digit phase shift in the oscillation pattern of the oxygen yield induced by a flash train in dark-adapted samples [12–14] at low concentrations is accompanied by inhibition of a fraction of centers and by an accelerated decay of S_2 and S_3. At higher concentration, the oxygen evolution capacity is completely eliminated [29]. Therefore, it is necessary to check under the same experimental conditions the functional integrity of the catalytic site of water oxidation in the presence of NH_2NH_2 by measuring the average oxygen yield per flash. An attempt to determine this quantity by measurement of light-induced changes at mass 32 failed because a huge overlapping O_2 uptake was observed which strongly depends on the NH_2NH_2 concentration (vide infra). To circumvent this problem, $H_2{}^{18}O$ was injected into the suspension, and the dioxygen evolved was detected mainly at mass 34. Typical traces of a measurement at 100 μM NH_2NH_2 are depicted in Fig. 1. The left trace at mass 32 shows a huge O_2 uptake which masks any O_2 evolution. In contrast, a clear O_2 evolution is observed at mass 34. The signal at mass 36 is markedly smaller because in this particular case the content of $H_2{}^{18}O$ in the sample was only 6.7%. Generally, the total amount of O_2 evolved can be calculated by using the following formula (specific isotope effects are assumed to be negligibly small):

$$M_{O_2} = [1/2\alpha(1-\alpha)]\, M_{O_2}(\text{mass } 34) \qquad (1)$$

where M_{O_2}(mass 34) represents the measured O_2 evolution at mass 34 and α gives the mole fraction of $H_2{}^{18}O$ of the total water content. Using Eqn. 1, the oxygen-evolution capacity was determined from the signals M_{O_2}(mass 34) measured at different NH_2NH_2 concentrations. The values of O_2 evolution normalized

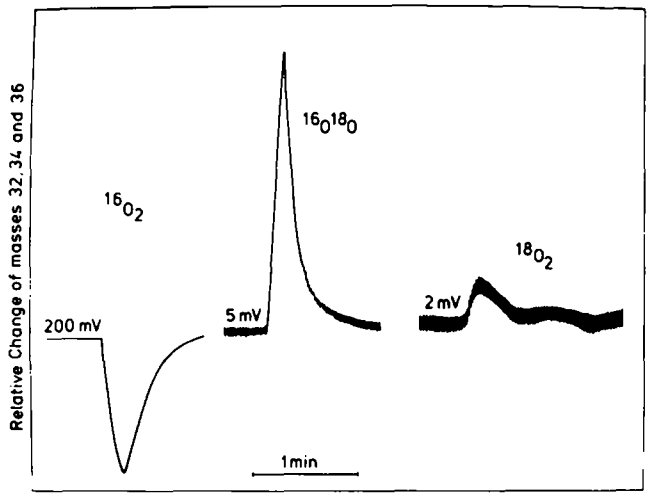

Fig. 1. Dioxygen uptake measured at mass 32 ($^{16}O_2$) and dioxygen evolution measured at mass 34 ($^{16}O^{18}O$) and 36 ($^{18}O_2$), respectively, as consequence of illumination with a train of ten flashes in normal tobacco chloroplasts incubated with 100 μM NH_2NH_2 in an aqueous suspension containing 6.67% $H_2{}^{18}O$.

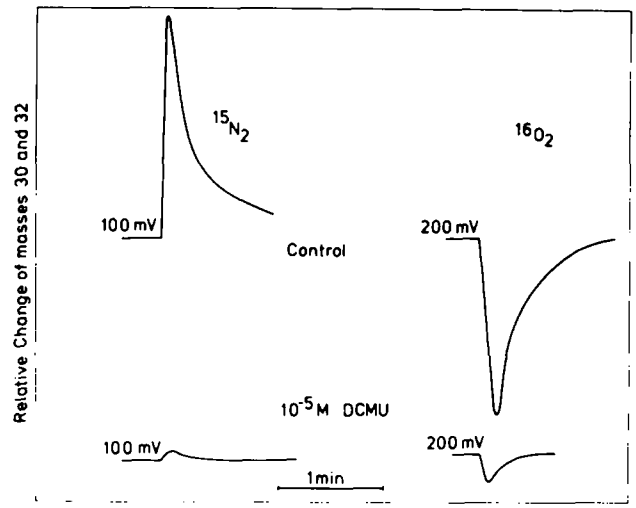

Fig. 3. Dinitrogen evolution measured at mass 30 ($^{15}N_2$), dioxygen uptake measured at mass 32 ($^{16}O_2$) as a consequence of illumination with a train of ten flashes in normal tobacco chloroplasts incubated with 1 mM $^{15}NH_2{}^{15}NH_2$ in the absence (top traces) or presence of 10 μM DCMU (bottom traces).

to that of the control without NH_2NH_2 and the relative $^{15}N_2$ yield in normal and Tris-washed chloroplasts are depicted in Fig. 2 as a function of NH_2NH_2 concentration.

Fig. 2 reveals a number of interesting features: (a) the extent of dinitrogen formation in a single flash and its dependence on NH_2NH_2 concentration are very similar in Tris-treated samples completely deprived of their oxygen-evolution capacity and in normal chloroplasts; (b) after a long incubation time (30 min), the oxygen-evolution capacity markedly decreases, even at comparatively small NH_2NH_2 concentrations of less than 100 μM; and (c) the average N_2 formation per flash of a ten-flash group is markedly smaller than the extent upon a single flash, and significant differences are observed between normal and Tris-washed chloroplasts at subsaturating NH_2NH_2 concentrations. The differences in the N_2 yield obtained under single and flash group excitation, respectively, are caused by rate-limiting step(s) because the amount of N_2 evolution

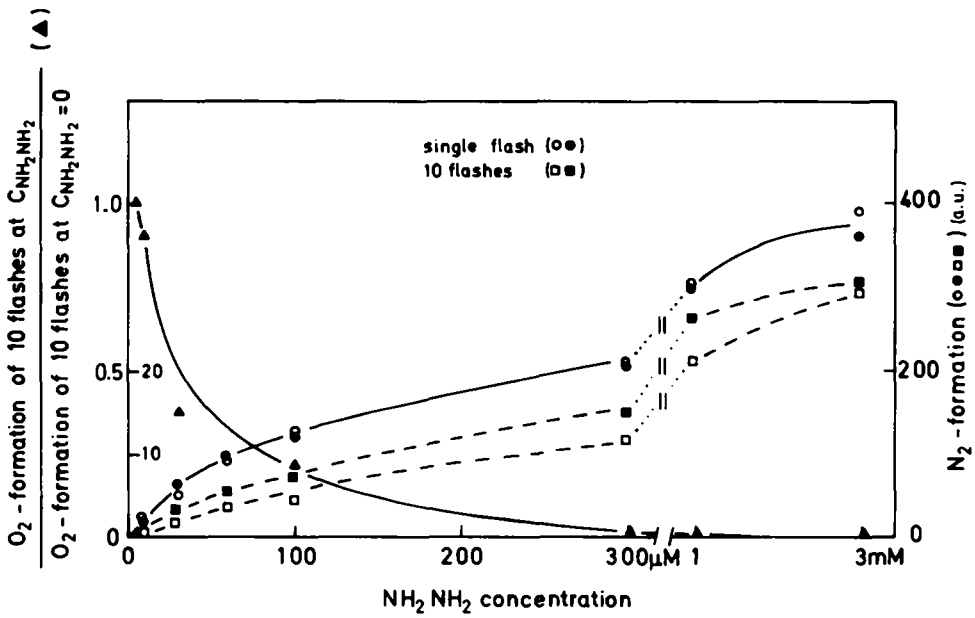

Fig. 2. Extent of flash-induced $^{15}N_2$ formation as a function of $^{15}NH_2{}^{15}NH_2$ concentration in normal (open symbols) and Tris-treated (closed symbols) tobacco chloroplasts (*Nicotiana tabacum* var. John Williams' Broadleaf). Dark-adapted samples were illuminated with one flash (circles) or a train of ten flashes (squares). The relative O_2 yield induced by ten flashes in normal chloroplasts is symbolized by closed triangles.

induced by a train of ten flashes exhibits a strong dependence on the time, t_d, between the flashes, with half-time values of about 300 ms at 100 μM NH_2NH_2 (data not shown).

A mechanistically interesting phenomenon of NH_2NH_2 oxidation is its coupling with a strong oxygen uptake under aerobic conditions (see Fig. 1). This finding corresponds to previous results reported by Radmer and Ollinger [30]. Two questions arise about the origin of this effect: (a) Is the oxygen uptake dominated by a PS I Mehler-type reaction with NH_2NH_2 as exogenous donor which feeds electrons either directly or indirectly via another endogenous redox component (e.g., plastocyanin) into P-700$^+$? (b) Does the extent of flash-induced dinitrogen evolution depend on the oxygen content of the suspension? To answer the first question, experiments were performed in the presence of 10 μM DCMU. The results obtained are depicted in Fig. 3. The left traces show that the N_2 formation is drastically diminished by DCMU. This confirms previous data [30] indicating that NH_2NH_2 oxidation to N_2 in thylakoids is a PS II reaction. Likewise, the O_2 uptake becomes also severely inhibited by DCMU. Therefore, the NH_2NH_2-induced process(es) leading to the O_2 consumption is (are) also strongly dependent on PS II activity.

The relation between N_2 formation and O_2 uptake was analyzed as a function of the O_2 concentration in the suspension. It was found that the N_2 formation induced by a train of ten flashes in a suspension containing 1 mM NH_2NH_2 increased by a factor of 2–4, when the O_2 content was raised from about 2 μM to 100 μM. In the same range the O_2 uptake exhibited a much more pronounced stimulation (15–20-fold). At 100 μM O_2 the ratio of O_2 uptake to N_2 evolution was 1.3–1.5 (data not shown). These findings suggest that the O_2 yield is able to oxidize $NH_2\dot{N}H$-radicals formed at PS II, thereby increasing the average N_2 yield per flash.

It was previously argued that the stimulation of N_2 evolution by O_2 is predominantly due to the oxidation of NH_2NH_2 by superoxide rather than an oxidation of $NH_2\dot{N}H$ by O_2 [30]. To test this idea, experiments were performed in the absence and presence of superoxide dismutase (SOD). The results obtained are depicted in Fig. 4. A reduction of the O_2 content by a factor of 5 caused practically the same decrease of N_2 formation, regardless of the presence of SOD in the suspension. On the other hand, the ratio of O_2 uptake to N_2 evolution decreased to levels of 0.8–0.9 in the SOD-containing samples (data not shown). The results of Fig. 4 support the idea of an oxidative attack on $NH_2\dot{N}H$ radicals by O_2. In this case, at constant O_2 content of the suspension, both activities (N_2 evolution and O_2 uptake) should exhibit a similar deppendence on NH_2NH_2 concentration. This was found to be the case for different samples and excitation conditions (data not shown). The above

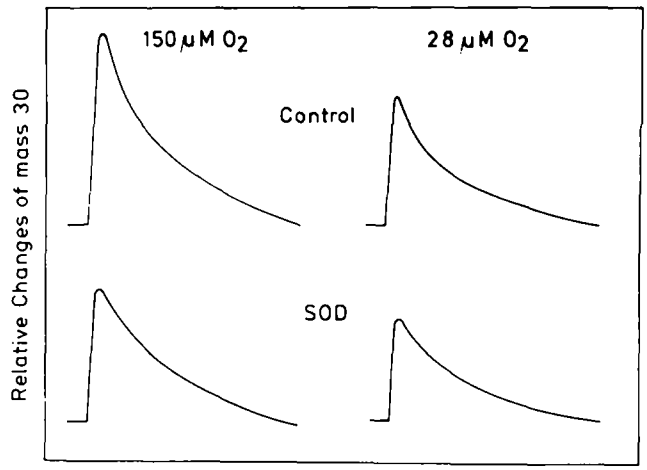

Fig. 4. Dinitrogen ($^{15}N_2$) evolution caused by a ten-flash group at 150 μM (left) or 28 μM (right) O_2 in tobacco chloroplasts incubated with 1 mM $^{15}NH_2NH_2$ in the absence (top) or presence (bottom) of SOD (10000 units). Dark time between the flashes of the ten-flash group: 300 ms.

results can be explained by the assumption that O_2 efficiently competes with the bimolecular dismutation reaction of $NH_2\dot{N}H$ radicals (see Discussion). In order to analyze in more detail the mode of interaction of flash-induced $NH_2\dot{N}H$ radicals, comparative measurements were performed with NH_2OH, which similarly affects the PS II reaction pattern.

One of the major differences between NH_2NH_2 and NH_2OH is that the former substrate already contains two nitrogen atoms which could give rise to an intramolecular N_2 formation; whereas in the latter case (NH_2OH) dinitrogen formation necessarily implies an intermolecular reaction mechanism. In order to address this problem, experiments were performed with ^{15}N-labeled NH_2NH_2 and NH_2OH, respectively. The experimental results are depicted in Fig. 5. The top traces were obtained after the excitation with ten flashes of samples containing 100 μM of a mixture of 50% $^{14}NH_2{}^{14}NH_2$ and 50% $^{15}NH_2{}^{15}NH_2$. The data show that practically the same amounts of $^{14}N_2$ and $^{15}N_2$ are evolved; whereas $^{14}N^{15}N$ evolution is negligibly small. Therefore, it can be concluded that the oxidative N_2 formation from NH_2NH_2 at PS II occurs exclusively via an intramolecular reaction mechanism. The absence of isotopic scrambling might be simply due to the oxidation of $NH_2\dot{N}H$ radicals by O_2 which could be much more efficient than the dismutation reaction of $NH_2\dot{N}H$. Therefore, experiments were performed under strongly anaerobic conditions. A small increase in the relative amount of mass 29 was observed that could indicate a low probability for breaking the nitrogen–nitrogen bond by dismutative interactions of $NH_2\dot{N}H$ radicals.

In contrast to the products of NH_2NH_2 oxidation, a totally different pattern arises when thylakoids are in-

cubated with a mixture of 50% $^{14}NH_2OH$ and 50% $^{15}NH_2OH$, as shown by the bottom traces in Fig. 5. In this case, a large amount of $^{14}N^{15}N$ is detected; whereas, the formation of $^{14}N_2$ and $^{15}N_2$ is much smaller. The ratio of 0.23 : 0.49 : 0.28 of the integral emission at mass 28, 29, and 30, respectively, corresponds – within the experimental error – to the theoretically calculated isotope distribution for N_2 formation via a bimolecular reaction of NH_2OH oxidation products. However, a closer inspection of the data reveals that under all conditions the contribution of $^{15}N_2$ to the overall dinitrogen evolution exceeds that of $^{14}N_2$. The possible mechanistic implications of such an isotope effect will not be discussed here. Our data on the isotope distribution of flash-induced dinitrogen evolution basically correspond to previous findings by Radmer and Ollinger [30].

The data show that the nitrogen–nitrogen bond in the radical formed by oxidative one-electron abstraction of NH_2NH_2 is highly stable, while the nitrogen–oxygen bond is much less stable and breaks apart under N_2 formation, when two ṄHOH radicals interact. The small effect of strong aerobiosis on the isotope distribution of NH_2NH_2 oxidation (vide supra) raised the question of the reaction mechanism in the case of the interaction

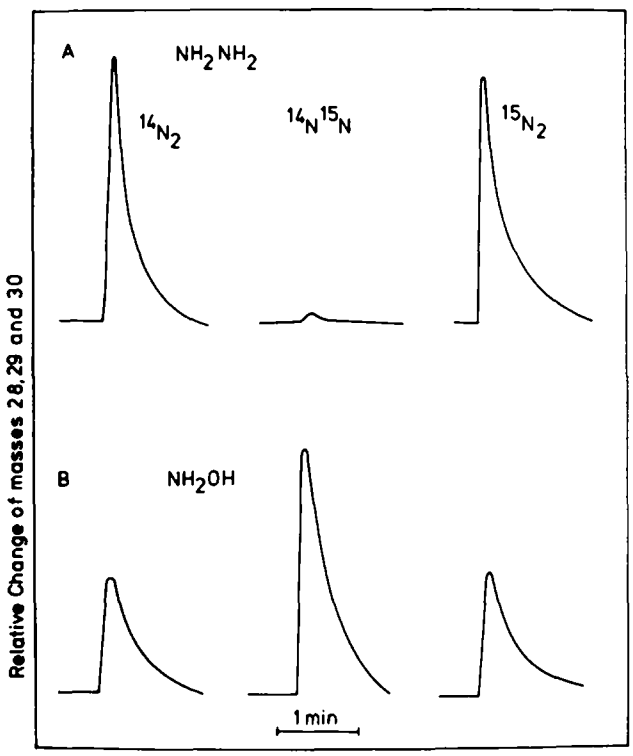

Fig. 5. Dinitrogen formation measured at mass 28 ($^{14}N_2$), 29 ($^{14}N^{15}N$) and 30 ($^{15}N_2$) as consequence of illumination with a train of ten flashes in normal tobacco chloroplasts incubated with an equimolar mixture of $^{14}NH_2{}^{14}NH_2$ and $^{15}NH_2{}^{15}NH_2$ (top traces) and of $^{14}NH_2OH$ and $^{15}NH_2OH$ (bottom traces) at a total concentration of 1 mM.

TABLE I

Dinitrogen isotopic distribution in tobacco chloroplasts

The assay containing chloroplasts of *N. tabacum* var. John William's Broadleaf was incubated with mixtures of $^{14}NH_2OH$ and $^{15}NH_2{}^{15}NH_2$ in the dark (≥ 20 min) and illuminated with ten flashes.

Isotope	Isotopic distribution of dinitrogen evolved	
substrate:	100 μM $^{14}NH_2OH$ 1 mM $^{15}NH_2{}^{15}NH_2$	500 μM $^{14}NH_2OH$: 500 μM $^{15}NH_2{}^{15}NH_2$
$^{14}N^{14}N$	0.14	0.52
$^{14}N^{15}N$	0.04	0.07
$^{15}N^{15}N$	0.80	0.41

between ṄHOH and ṄHNH$_2$ radicals. To address this problem, experiments were performed with mixtures of $^{14}NH_2OH$ and $^{15}NH_2NH_2$. The data obtained are summarized in Table I. Two interesting features are observed: (a) at equimolar ratio, mass 28 dominates, reflecting the higher affinity of NH_2OH with the PS II-donor side, and (b) the ratio of $^{14}N^{15}N$ to $^{15}N^{15}N$ increases if the relative content of $^{14}NH_2OH$ is enhanced. This supports the idea that the reaction between ṄHOH and NH_2ṄH radicals can lead – with a certain probability – to a breakage of the nitrogen–nitrogen bond.

Discussion

The mode of interaction between PS II and reducing compounds such as NH_2OH and NH_2NH_2 implies a number of mechanistically interesting problems. With respect to the reaction pattern of the donor side, two questions are of special relevance: (a) What is the mechanism for the two-digit phase shift of the period-four oscillation pattern of flash-induced oxygen evolution in dark-adapted samples incubated with low concentrations of NH_2OH or NH_2NH_2, and (b) what is the mechanism of flash-induced N_2 formation due to oxidation of NH_2OH and NH_2NH_2, respectively?

The most interesting observation with regard to the two-digit phase shift is the finding that the N_2 yield due to single flash excitation depends practically in the same way on NH_2NH_2 concentration in normal and Tris-washed chloroplasts. Table II clearly shows that this close similarity also exists at low NH_2NH_2 concentrations (10 μM), where the oxygen-evolution capacity is only marginally affected. These results suggest that the catalytic site of water oxidation is not involved in the flash-induced oxidative N_2 formation from NH_2NH_2. An analogous conclusion for NH_2OH is consistent with the data of Radmer and Ollinger [24], if one assumes that the higher N_2 yield of the first flash is due to the sufficiently long dark equilibration rather than a specific

TABLE II

Nitrogen, oxygen evolution, oxygen uptake in tobacco chloroplasts

N_2 evolution (mass 30) in normal and Tris-treated chloroplasts, O_2 evolution (mass 34 and 36) in normal and O_2 uptake (mass 32) in Tris-treated tobacco chloroplasts at 10 μM $^{15}NH_2{}^{15}NH_2$. The suspension of the normal chloroplasts contained 24.5% $H_2{}^{18}O$ (v/v). n.d., not determined.

Actinic flashes	Signal magnitude (a.u.)				
	normal			Tris-treated	
mass:	30	34	36	30	32
1	29	–	–	31	n.d.
10	102	115	24	146	−50

contribution by a reaction at the catalytic site of water oxidation. This idea is supported by the finding that the extra N_2 evolution of the first flash which exceeds that of the subsequent flashes is rather small compared with the total N_2 yield per flash at saturating NH_2OH concentration [24]. Its relative extent markedly decreases with increasing NH_2OH concentration in a range where the two-digit phase shift is observed.

Based on the results of this study and taking into account the previous data of Radmer and Ollinger [24], it appears reasonable to assume that N_2 formation takes place via a radical mechanism starting with $NH_2\dot{N}H$ and $\dot{N}HOH$, produced either directly by P-680$^+$ or by the functionally connected tyrosine(s) of polypeptides D-1 (Y_Z) and/or D-2 (Y_D) [31,32]. If one accepts that no main products other than dinitrogen are formed by flash-induced reactions of NH_2NH_2 and NH_2OH, the above considerations imply: (a) in agreement with Beck and Brudvig [20], NH_2OH and NH_2NH_2 should not be considered as water substrate analogues, and (b) the two-digit phase shift of the oxygen-yield pattern [12,14,24], of H^+ release [22] and of formation of the S_2 multi-line signal [33] induced by NH_2OH/NH_2NH_2 in dark-adapted samples in the state S_1 is caused by the formation of a state that can be formally symbolized by S_{-1}. The dark conversion of S_1 into S_{-1} is probably coupled to N_2 formation because we observed an increase of the background signal at mass 30 after addition of $^{15}NH_2{}^{15}NH_2$ to the samples.

The assumption of S_{-1} formation in the dark raises questions about the nature of this state. At a first glance, the manganese of the catalytic site of water oxidation appears to be an attractive candidate for direct and slow reduction by NH_2NH_2 and NH_2OH. Different models have been proposed for the manganese cluster. In a tetranuclear model, redox state S_2 was proposed to be a $Mn(IV)_3Mn(III)$ complex [34]. In this case, the reactions $S_2 \rightarrow S_1$, $S_1 \rightarrow S_0$ and $S_0 \rightarrow S_{-1}$ would all comprise a $Mn(IV) \rightarrow Mn(III)$ reduction. This idea, however, is not easily reconcilable with the much faster (two orders of magnitude) NH_2OH/NH_2NH_2 induced S_2 reduction compared to the slow transformation of S_1 [23,35]. In an alternative model, a binuclear cluster is assumed to attain the redox state Mn(III)Mn(III) in S_1 which is postulated to become reduced to Mn(II)Mn(II) in S_{-1} by NH_2OH and NH_2NH_2 [36,37].

Regardless of the detailed mechanisms, however, the assumption of manganese reduction at the catalytic site bears some problems: (i) it was recently shown that Y_D^{ox}, identified as Tyr-160 of polypeptide D-2 in *Synechocystis* sp. PCCC 6803 [32] remains oxidized at NH_2OH concentrations that largely convert S_1 to S_{-1} [33]. As S_0 was found to reduce Y_D^{ox} slowly [38], one should expect that the more reduced form, S_{-1}, is even more appropriate for reducing Y_D^{ox}; (ii) X-ray K-edge absorption data do not reveal any effect of NH_2OH on the valence state of manganese in S_1 [39]; (iii) the oscillation pattern of the oxygen yield induced by a flash train depends on the ratio of $[S_0]:[S_1]$ population before NH_2NH_2 addition in the dark [40]. Therefore, we conclude that the formal redox state S_{-1} is due to the double reduction of a redox buffer, X, other than the binuclear manganese center of the catalytic site of water oxidation. X might be the redox component C proposed previously [41] but it cannot be excluded at the present state of knowledge that X represents a special form of bound NH_2NH_2 (NH_2OH) [42], which is oxidized to an as yet not identified product.

With regard to the mechanism of N_2 formation by oxidation of NH_2OH or NH_2NH_2, it is assumed that the reaction sequence starts with radicals of the type $\dot{N}HX$ (where X = OH or NH_2) that are formed through univalent electron abstraction by P-680$^+$ or the oxidized tyrosines Y_z^{ox} and/or Y_D^{ox}. In the case of $NH_2\dot{N}H$ radicals generally three types of reaction have to be considered for N_2 formation:

(a) dismutation according to

$$4\,{}^{15}NH_2{}^{15}\dot{N}H \rightarrow {}^{15}N_2 + 3\,{}^{15}NH_2{}^{15}NH_2 \qquad (3)$$

(b) reaction with O_2

$$2\,NH_2\dot{N}H + 2\,O_2 \rightarrow N_2 + NH_2NH_2 + 2\,O_2^- + 2\,H^+ \qquad (4a)$$

$$NH_2\dot{N}H + 3\,O_2 \rightarrow N_2 + 3\,O_2^- + 3\,H^+ \qquad (4b)$$

(c) reaction of O_2^- formed by Mehler-type reaction with NH_2NH_2:

$$NH_2NH_2 + O_2^- + H^+ \rightarrow NH_2\dot{N}H + H_2O_2 \qquad (5)$$

The strong stimulation of N_2 yield by O_2 is in line with previous findings [30]. Based on their results, Radmer and Ollinger assumed that reaction 5 dominates the O_2-stimulated N_2 formation. However, the data of the present study show that the effect of O_2 on N_2 yield is almost independent of the presence of SOD. Therefore,

we conclude that reactions 4a,b also significantly contribute to N_2 formation under our experimental conditions. The marked difference concerning the O_2 effect between the results of Radmer and Ollinger [30] and those reported in this study can be explained by the quite different excitation conditions (continuous light versus ten-flash group). Under the flash excitation used in this study, rate limitations by the acceptor side of PS II can be assumed to be rather small, due to the plastoquinone pool, while such effects probably become dominating under strong light. In contrast to NH_2NH_2, reactions analogous to 4a,b cannot stimulate the N_2 yield with NH_2OH as substrate.

The results presented in this study show that NH_2OH and NH_2NH_2 undergo two different reaction sequences with the donor side of PS II: (1) univalent radical formation by either P-680$^+$ or Y_z^{ox} and/or Y_D^{ox} eventually leading to N_2 formation via different possible pathways (dismutation, oxidation by O_2 in case of NH_2NH_2) and (2) interference with the catalytic site of water oxidation via a mechanism that involves an unknown two-electron redox buffer system, thereby giving rise to a two-digit phase shift of the period-four oscillation of manganese oxidation [33], O_2 evolution [12–14] and H^+ release [43]. The nature of this redox buffer system remains to be clarified, but the reaction sequence leading to the two-digit phase shift does not lead to flash-induced N_2 formation.

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