Mass Spectrometric Analysis of N₂-FORMATION Induced by the Oxidation of Hydrazine and Hydroxylamine in Flash Illuminated Thylakoid Preparations of the Filamentous Cyanobacterium Oscillatoria chalybea

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In tobacco chloroplasts hydrazine-dependent dinitrogen formation measured by mass spectrometry as the consequence of short saturating light flashes is always linked to a substantial oxygen uptake (G. Renger, K. P. Bader, and G. H. Schmid, Biochim. Biophys. Acta 1015, 288, 1990). However, in thylakoids of the filamentous cyanobacterium Oscillatoria chalybea this dinitrogen formation is not linked to an apparent O₂-uptake, even at the high concentration of 1 mM hydrazine. Whereas in tobacco chloroplasts Tris-treatment does not affect hydrazine-dependent dinitrogen formation up to a concentration of 3 mM hydrazine, Tris-treatment of thylakoids of O. chalybea affects strongly both oxygen evolution and dinitrogen evolution under a single turnover flash as well as under ten flashes. In contrast to tobacco chloroplasts, the presence of hydrazine up to concentrations of 3 mM does not substantially affect photosynthetic O₂-evolution. The observed dinitrogen evolution is affected by DCMU regardless whether induced by a single turnover flash or by ten flashes, whereas in tobacco dinitrogen evolution and the O₂-uptake linked to it (which is not observed in the cyanobacterium) were clearly not affected by DCMU in the single turnover flash. In Oscillatoria the earlier described Photosystem II-mediated H₂O₂-formation and decomposition is influenced by hydrazine. In the presence of 300 µM hydrazine the usually present O₂-uptake leading to H₂O₂ formation appears diminished.

Introduction

In the filamentous cyanobacterium Oscillatoria chalybea Photosystem II exhibits in comparison to that in higher plants a number of essential differences [1–4]. Thus, if photosynthetic oxygen evolution is analyzed as the consequence of short saturating light flashes, the observed oxygen evolution pattern differs from that described manifold in the literature for Chlorella or higher plant chloroplasts [5]. In an Oscillatoria sequence the first flash and the second flash yield oxygen even after an extensive dark adaptation, an observation which was correlated to metastable (long-living) S₃ and also long living S₁ [1, 2]. In general the life time of the S-states seems to be increased in Oscillatoria [6] a property which is also observed in higher plants when the photosynthetic apparatus develops its structure and function during greening [7]. Thus, Franck and Schmid have shown that in the early stage of greening oat etioplasts exhibit photosystem II properties comparable to those of the phylogenetically old filamentous cyanobacterium Oscillatoria chalybea. Due to these properties it was possible to characterize the effect of reducing agents, such as hydroxylamine, on S₁, S₂ and S₃ by adding the inhibitor shortly after two flashes and by recording the oxygen flash sequence thereafter [8]. In Oscillatoria photosynthetic oxygen evolution under normal oxygen partial pressure of air always consists of two portions, one coming from water-splitting and the other from H₂O₂-decomposition [3]. The electrons for the H₂O₂-formation seem to originate from water, with the formation of H₂O₂ being due to the interaction of oxygen with one of the S-states [9]. In the present paper we investigate the effect of hydrazine and to a minor extent that of hydroxylamine, which according to the literature interact with the S-state system [10, 11] on the S-state system of Oscillatoria chalybea.

Abbreviations: Tris, Tris(hydroxymethyl)-aminoethane.
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Materials and Methods

The filamentous cyanobacterium Oscillatoria chalybea was obtained from the algal collection in Göttingen (F.R.G.) and cultured for the assays of the present paper on nitrate as the sole nitrogen source in the medium. Growth conditions and medium compositions have been described earlier [1].

Thylakoid preparations of Oscillatoria chalybea were prepared according to Bader et al. [1]. The mucoid layer on the cells was digested with gluco­ronidase (Boehringer, Mannheim) and the cell walls with lysozyme (Sigma) and cellulase (Kinki Yakult, Japan).

Tobacco chloroplasts were prepared from Nicotiana tabacum var. John William's Broadleaf (JWB) according to Homann and Schmid [12]. The freshly prepared chloroplasts were stored on ice before the measurements in the mass spectrometer.

The reaction mixture contained in a total of 2 ml tobacco chloroplasts corresponding to 70 μg chlorophyll and thylakoids of Oscillatoria corresponding to 40 μg chlorophyll, 30 mM KCl and 60 mM Tricine-NaOH (pH 7) and the additions of NH₂NH₂ or NH₂OH indicated in the figures.

Mass spectrometry was carried out with a modified magnetic sector field mass spectrometer type "Delta" from Finnigan MAT (Bremen, F.R.G.), which is an isotope ratio mass spectrometer equipped with a two-directional focussing device "Nier type I" [2]. The experimental set-up with the valve system is described in detail earlier [2].

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

$^{15}$N-Labeled hydrazine and hydroxylamine were purchased from IC-Chemicalien (München, F.R.G.).

$^{18}$O-Labeled water was purchased from Ventron (Karlsruhe, F.R.G.).

Results

The effect of increasing hydrazine concentrations on oxygen evolution and dinitrogen evolution in normal and Tris-washed thylakoid preparations of Oscillatoria chalybea is shown in Fig. 1. Several points are different when compared to tobacco chloroplasts [13]. First the apparent oxygen evolution in Oscillatoria thylakoids is not much affected by hydrazine concentrations up to 1000 μM, whereas in tobacco chloroplasts O₂-evolution is zero around 300 μM. Also in contrast to tobacco chloroplasts dinitrogen evolution under both the single turn-over flash or by a train of saturating flashes is sensitive to Tris-treatment (Fig. 1), whereas in tobacco chloroplasts it is not [13]. On the other hand, O₂-evolution in Oscillatoria thylakoids is sensitive to Tris-treatment (Fig. 1) as it should be and as is the case with tobacco chloroplasts. Dinitrogen evolution from hydrazine oxidation is linked in tobacco chloroplasts to a considerable O₂-uptake [13] whereas in Oscillatoria thylakoids no such uptake is observed (Fig. 2). The assay contained 25% H₂O in its buffer system and was carried out in the presence of 100 μM hydrazine. The assay was in equilibrium with normal air. Under the assumption that the observed O₂-evolution comes exclusively from water-splitting the isotope ratios between masses 32, 34 and 36 should according to equation 32:34:36 = (1−α)^2:2α(1−α):α^3 be for 32:34:36, with α being the atom fraction which is 18O, 56.25:37.5:6.25. Fig 2 clearly shows that the measured mass 32 peak exceeds manyfold the necessary value. Due to the fact that the observed ratio between masses 34 and 36 is 5.9/1, which is close to the theoretical ratio, the observed 18O₂-evolution does not come
from H$_2^{16}$O/H$_2^{18}$O splitting. In fact, the observed $^{16}$O$_2$-evolution is due to the photosystem II mediated H$_2$O$_2$-formation and decomposition described in detail earlier [3, 9]. Indeed, it was shown in this cyanobacterium that photosynthetic oxygen evolution measured as the consequence of a train of short saturating light flashes contains two portions, one coming from water-splitting proper and the other by interference of photosystem II with O$_2$ (e.g. of the gas phase), leading to H$_2$O$_2$ formation which is decomposed by the S-state system to give O$_2$, protons and electrons [3, 9]. Mass spectrometry shows that qualitatively O$_2$-evolution coming from water-splitting proper is in Oscillatoria chalybea inhibited by increasing concentrations of hydrazine (Fig. 3) just as in tobacco [13]. At a concentration of 300 μM hydrazine in Oscillatoria approx. 50% of the water-splitting activity has disappeared (Fig. 3) whereas at the same concentration oxygen evolution in tobacco is totally absent. Despite the first impression given by Fig. 2 dinitrogen evolution in Oscillatoria clearly depends on the oxygen partial pressure in the assay. Decreasing oxygen background in the assay decreases the observed dinitrogen signal (Fig. 4), hence the absence of an apparent oxygen uptake linked to dinitrogen evolution does not exclude the reaction of the HN$_2$–NH$_2$-radical with oxygen [13]. It rather looks as if hydrogen peroxide decomposition was inhibited or its production lesser in the presence of hydrazine (Fig. 2). A close-up scrutiny of the oxygen signal at mass 32 in the presence and absence of 300 μM hydrazine reveals some marked shape

Fig. 2. Dioxygen evolution measured at mass 32 (16O$_2$), 34 (16O18O) and 36 (18O$_2$), respectively, as a consequence of illumination with ten flashes in a thylakoid preparation of Oscillatoria chalybea incubated with 100 μM NH$_2$NH$_2$ in an aqueous suspension containing 25% H$_2$18O. In a control experiment with the same preparation in the absence of hydrazine and 18O-labelled water the di-oxygen evolution at mass 32 (16O$_2$) was measured (dashed line) to show the effect of hydrazine on O$_2$-evolution. The experiments shows that no apparent O$_2$-uptake is linked with hydrazine oxidation.

Fig. 3. Dioxygen evolution measured at mass 32 (16O$_2$), at mass 34 (16O18O) and at mass 36 (18O$_2$) as a consequence of illumination with ten flashes in a thylakoid preparation of Oscillatoria chalybea. The aqueous suspension contained 25% H$_2$18O. Control experiment (a) and (b) assay incubated with 300 μM NH$_2$NH$_2$.

Fig. 4. $^{15}$O$_2$-Evolution in a thylakoid preparation of Oscillatoria chalybea, incubated with 100 μM NH$_2$NH$_2$ and illuminated with a train of ten flashes, in dependence on the O$_2$-content of the assay.
differences (Fig. 5). Oxygen evolution measured by mass spectrometry as the consequence of a train of 10 flashes (300 ms between flashes) in a single (cumulated) signal shows a respectable portion of delayed oxygen evolution seen as a signal shoulder in Fig. 5, which should correspond to the two digit shift of flash patterns in the presence of hydrazine or hydroxylamine [11, 14]. This delayed oxygen evolution is DCMU sensitive and it looks as if this portion of O_2 evolution was more sensitive than the not delayed one (Fig. 6). This means that the interaction of hydrazine with the donor side of photosystem II in Oscillatoria might be two-fold: part of the compound reacts with the S-state which decomposes in Oscillatoria H_2O_2 (probably with S_2, less probably S_3) or it reacts with H_2O_2 or the oxygen resulting from its decomposition in the way discussed earlier [9]. Formally hydrazine just takes the place of H_2O_2 (Fig. 2). The major effect of the compound consists in an interaction with the S-state system namely with that particular state which is the most reactive one to hydrazine, leading to a reduction of the S-state system and to the otherwise observed two digit shift of the O_2 evolution pattern in a train of saturating light flashes. As the peculiarity of the dark adapted S-state system in Oscillatoria is, in comparison to higher plants, the longer half-life time of all S-states, in particular that of the S_2 and S_3 states, the effect and the difference to tobacco chloroplasts [13] lies probably in this region.

The mechanism of dinitrogen formation from hydrazine and hydroxylamine oxidation of photosystem II of Oscillatoria clearly is the same as in tobacco. If equimolar amounts of^{14}N-labelled and^{15}N-labelled hydrazine are presented to the system, only masses 28 and 30 are observed (Fig. 7), the
mixed label is absent. If the same experiment is made with an equimolar mixture of $^{15}$NH$_2$OH and $^{14}$NH$_2$OH, the mixed labeled ($^{15}$N·:$^{14}$N) nitrogen is the predominant species (Fig. 7). The mechanism therefore should be that of a univalent oxidation of NH$_2$OH and NH$_2$NH$_2$ as described for tobacco chloroplasts [13]. However, in Oscillatoria the major difference within this mechanism type is the fact that here the water oxidase seems to be involved or necessary for this type of oxidation, since dinitrogen evolution under the single turnover flash as well as under a train of 10 saturating light flashes is sensitive to Tris-treatment (Fig. 8).

Discussion

According to Joliot [15] and Kok [16] photosynthetic water splitting takes place via a sequence of 4 oxidation steps in which a manganese containing enzyme complex successively goes through 4 redox states, eventually leading to the splitting of water and the evolution of oxygen. This system of redox states has been termed S-state system [19]. In the transition from

$$S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4 \rightarrow O_2$$

no intermediate (such as an “oxygen precursor” or partially oxidized water) has been detected so far [2, 17] although some experimental evidence hints at a peroxidic state [18]. The observation that no reaction intermediate or even binding of bulk water to one of the S-states up to the S$_3$-condition should occur is disturbing and not easily acceptable from the thermodynamic point of view. As pointed out in earlier publications the water-splitting complex of the filamentous cyanobacterium Oscillatoria chalybea exhibits a number of peculiarities in comparison to that of higher plant chloroplasts [1–3]. The observed properties of the S-state system in Oscillatoria compare only in a defined state of chloroplast development, namely in an early greening stage to that of higher plants. In both conditions the S-state system is characterized by a longer life-time of the S-states measurable as a much slower S-state deactivation than usual. This is particularly striking with respect to the S$_2$- and S$_3$-states which are states that in the normal higher plant situation decay within a few seconds whereas corresponding states in Oscillatoria or greening oat chloroplasts are much longer living. Thus, it is observed that the S$_2$-state has a half-life time of 1 min and a portion of the S$_3$-state is stable for many minutes (i.e. metastable). There is no doubt that the observed O$_2$-evolution pattern in Oscillatoria as the measured consequence of short saturating light flashes fits all requirements of the Kok model, with the peculiarity that an Oscillatoria pattern gives much better fits in the four state Kok model than a Chlorella pattern does [1]. This is to say that the measured O$_2$-signals in Oscillatoria surely measure the S$_2$- and S$_3$-state and nothing else. Already in this situation it is striking that S$_3$ might be a condition which is very little reactive and does certainly not react with oxygen, otherwise it would not be metastable. As the search for intermediates has not been successful so far [2, 17] a comparison of the endogenous reactivity of the S-states in Oscillatoria and higher plants, together with the reactivities of the S$_3$-state towards exogenous reagents like hydrazine or hydroxylamine promises some interesting insights. If one starts out from the point of view that the Oscillatoria properties are also seen in higher plant chloro-
plasts under special conditions such as greening [7] or at low temperatures [19], the comparison of the endogenous S-state properties within Oscillatoria to a certain extent might reflect the regular situation amongst the S-states in the much faster higher plant system: In all systems S1 is the least reactive state as it is dark stable; S0 in the dark slowly oxidizes to S1 and the major part of S1 decays directly to S0 [20]. The S2 decay kinetics in relation to the other S-states are alike in higher plant chloroplasts and in Oscillatoria, with the difference, as said above, that S2 is much longer lived in Oscillatoria. However, the reactivity of the S-state towards endogenous agents is very big in the two systems. Thus, in Oscillatoria the S2-state reacts with exogenous oxygen [3], whereas in tobacco chloroplasts it does not [3]. This reaction leads in Oscillatoria thylakoids to the production of free hydrogen peroxide which upon the successive absorption of two photons is decomposed to give oxygen and protons (and electrons). The Oscillatoria system thus seems accessible to oxygen or more reactive by principle. It should be noted that in higher plants H2O2 is electron donor to photosystem II under certain conditions of chloride and/or calcium deficiency [21, 22]. Clearly under the oxygen partial pressure of normal air, the water-oxidizing complex of Oscillatoria interacts with oxygen. Addition of hydrazine to the Oscillatoria thylakoids leads to an interaction of this compound with the hydrogen peroxide forming and decomposing reaction (Fig. 2). It appears that in Oscillatoria hydrazine is oxidized by photosystem II in an univalent oxidation reaction leading to dinitrogen evolution (Fig. 3). The mechanism of this oxidation reaction corresponds to the earlier described one in tobacco chloroplasts [13]. By applying equimolar quantities of 15NH3-/15NH3 and 14NH3-/14NH3 and measuring the observed masses of the nitrogen evolved it is clearly seen that in this oxidation reaction the N−N-bond is not split (Fig. 7). Oxidation should therefore, as described earlier, go via the HN−NH2-radical [13]. As a difference to the tobacco chloroplast system dinitrogen evolution requires the water oxidase which is demonstrated by the fact that dinitrogen evolution is eliminated by Tris-washing (Figs. 1 and 8). Fig. 2 and 5 clearly show that hydrazine interacts with the hydrogen peroxide forming and decomposing system, which is to say that hydrazine preponderantly interacts with the S2-state. The presented data shows that hydrazine oxidation deactivates the S-state system into a condition which is more reduced than S0, thus leading to the two digit shift of a flash pattern reported in the literature [10] or the delayed oxygen evolution of a cumulated signal of a train of ten flashes as shown in Fig. 5.

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