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Mass spectrometric analysis of a Photosystem-II-mediated oxygen uptake phenomenon in the filamentous cyanobacterium, *Oscillatoria chalybea*

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Flash-induced oxygen production was studied by mass spectrometry in thylakoid particle preparations of the filamentous cyanobacterium *Oscillatoria chalybea*. Essentially, two oxygen uptake phenomena related to Photosystem II were observed. First, photosynthetic oxygen evolution requires the presence of a minimal threshold quantity of oxygen. Under completely anaerobic conditions the photosynthetic water-splitting reaction does not occur. In nitrogen-flushed assays, a small oxygen uptake precedes oxygen evolution induced by a train of short saturating flashes. Second, flash-induced photosynthetic oxygen evolution was measured in the presence of the oxygen isotope, $^{18}O_2$, in the ambient atmosphere of the assay. The oxygen evolved was labelled with $^{18}O_2$, which showed that the evolution reaction included an $^{18}O_2$-uptake phenomenon. The labelling density completely excludes $^{18}O_2$ exchange via $H_2{}^{18}O$ (by respiration or other processes) and subsequent photosynthetic water splitting, since too little mixed ($^{16}O^{18}O$) oxygen label was found. Since most of the label was found to be $^{18}O_2$, the label could come from hydrogen peroxide (or an equivalent) produced in the immediate vicinity of the S-state system. The decomposition of this hydrogen peroxide appears to be managed by the S-state system (S_2) itself. Addition of exogenous hydrogen peroxide or the addition of high amounts of catalase does not affect the behaviour of our preparation. The phenomenon seems to be an inherent property of our cyanobacterium and does not take place in tobacco chloroplasts under identical conditions. The phenomenon seems to play a role under natural conditions, also, and might be the consequence of the absence of two of the extrinsic polypeptides in cyanobacteria. It is enhanced by high concentrations of oxygen in the ambient atmosphere and is diminished by low oxygen tension.

Introduction

In recent years we have studied the properties of the S-state system in the filamentous cyanobacterium *Oscillatoria chalybea* [1,2]. The peculiarity of these cyanobacteria is an appreciable oxygen yield under the first flash, which has been shown to be due to metastable S_3 [2]. Due to the life-time of this metastable S_3-state we were able to demonstrate by mass spectrometry that, in particle preparations of the cyanobacterium which were suspended in $H_2^{16}O$-containing buffer and subjected to two preflashes, the addition of $H_2^{18}O$ to the suspension medium and a subsequent third flash yielded ^{18}O-labelled oxygen [3]. This, together with the isotopic distribution pattern of the evolved oxygen, showed that, after reaching the S_3-state, oxygen is evolved from $H_2^{18}O$ by one single flash [3]. The experiment confirms an experiment by Radmer and Ollinger [4] in which the authors, due

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to the short life-time of S_3 in spinach chloroplasts, had performed the experiment essentially the other way round. They had suspended spinach chloroplasts in $H_2^{18}O$ containing buffer preflashed with two flashes, removed $H_2^{18}O$ and resuspended the system in $H_2^{16}O$. The third analysing flash yielded no ^{18}O-labelled O_2. The conclusions drawn from our experiments and those of Radmer and Ollinger constrain any model of the mechanism of photosynthetic water splitting into a situation in which S_2 and S_3 can seemingly not contain bound unexchangeable water. On the other hand, according to the literature, oxygen evolution should proceed via formation of a 'cryptoperoxide', which would imply that S_3 under certain conditions should not exchange with the surrounding bulk water [5]. And indeed, in our previous articles [2,3,6], we already had indications for a bound 'cryptoperoxide' or an equivalent condition, and came to the conclusion that possible equilibrium situations should not be neglected [3]. Most interestingly, we had observed that particle preparations of the cyanobacterium required a certain threshold oxygen partial pressure in order to be able to evolve oxygen [6]. No oxygen was evolved when the preparation had become too anaerobic. This property of *Oscillatoria* was not due to over-reduction, since addition of ferricyanide had no effect and was not, or to a much lesser extent, observed with higher plant chloroplasts or with 'BBY particles' [6,7]. Our observation might show that a certain minimal amount of oxygen must be present or may be even bound before the oxygen-evolving apparatus becomes functional. This observation has led us to study photosynthetic oxygen evolution in dependence on oxygen partial pressure by means of mass spectrometry. By this technique we were able to demonstrate the presence of another Photosystem-II-mediated O_2-uptake phenomenon in particle preparations of the filamentous cyanobacterium. The uptake phenomenon seems to be connected with the S-state system.

Materials and Methods

Mass spectrometry

All assays were performed with a modified magnetic sector field mass spectrometer type 'Delta' from Finnigan (Bremen, F.R.G.). The apparatus, including the valve system used, is described in detail in an earlier publication [3]. All assays were performed in a measuring cell in which 2–3 ml of the assay suspension were separated by a 7 μm thick Teflon membrane from the gas space on the ion-source side. The schematic diagram of our measuring cell was described earlier [3,8]. The measuring cell was equipped with a gas-tight lid, which permitted flushing with different gas mixtures but also injection of solutions as, for example, the addition of $H_2^{18}O$ into normal water (i.e., $H_2^{16}O$-) containing buffer systems. Signals were recorded on an SE 130-03BBC Metrawatt three-channel recorder. Light flashes of 8 μs duration were provided by a Stroboscope (1539A of General Radio) and usually spaced 300 ms apart.

It should be noted that our mass spectrometric assay system in which particle preparations, algae or chloroplasts are sedimented on a Teflon membrane, represents a condition in which the biological system continuously slips towards anaerobiosis. This is because the Teflon membrane seperates the assay from the ionic source space (under high vacuum) and that the assay is in equilibrium with only a closed gas volume. The continuous depletion of the gas space and the assay mixture in equilibrium with it can be permanently measured by monitoring the actual O_2 background in millivolts. Injections of defined gas mixtures into this closed system establish the desired experimental conditions (see Figs. 1 and 4).

Measuring procedure

2 ml of the *Oscillatoria* preparation, equivalent to approx. 70 μg chlorophyll or tobacco chloroplasts [9] corresponding to 130 μg chlorophyll in buffer containing only $H_2^{16}O$ were given on the Teflon membrane in the measuring cell and were allowed to sediment on the membrane. It is important that the membrane be completely covered by the solution and that the sedimentation film itself homogeneously covers the membrane surface. $H_2^{18}O$ was purchased as 99 atom% from Ventron. $^{18}O_2$ (98%) was from CEA-Oris, Bureau des Isotopes Stables, Gif-sur-Yvette, France.

Particle preparations

Preparation of the filamentous cyanobacterium *Oscillatoria chalybea* particles was as described

earlier [2] from 20-day-old cultures grown on nitrate as the sole nitrogen source. Usually the filament digestion included treatments with glucuronidase (Boehringer-Mannheim) and Cellulase (Kinki Yakoult, Japan) as described earlier [2].

Assay suspension

The assay suspension usually contained an aliquot of *Oscillatoria* particle preparations equivalent to approx. 70 μg chlorophyll, or, for comparison, chloroplast preparations of *N. tabacum* var. John William's Broadleaf equivalent to 130 μg chlorophyll in 2 ml 0.06 M Tricine/0.03 M KCl (pH 7.5).

Results

In a previous paper we have shown that *Oscillatoria* particle preparations did not evolve oxygen under anaerobic conditions [6]. The anaerobic condition was easily realized by flushing the measuring cell with nitrogen. After flushing with nitrogen, oxygen evolution was restored only if a minimal amount of oxygen was supplied to the gas space of the measuring cell. Fig. 1 shows this oxygen requirement of O_2 evolution in *Oscillatoria*. The figure shows that a threshold regulation of O_2 evolution exists, since a partial pressure corresponding to 50 mV oxygen background in our assay system does not permit oxygen evolution. The inactivation of oxygen evolution is not due to an overreduction of the acceptor side of Photosystem II, since addition of ferricyanide does not relieve the inhibition under anaerobic conditions. This inactive system is reactivated by a very slight increase in O_2 background, to 53 mV, a condition which yields an appreciable oxygen signal (Fig. 1). The experiment shows that under really anaerobic conditions no oxygen evolution is possible in *Oscillatoria*, which might furthermore imply binding of O_2 prior to oxygen evolution, an assumption which is substantiated by the fact that we observe a rapid O_2 uptake preceding the O_2-evolving signal in nitrogen-flushed assays (Fig. 2).

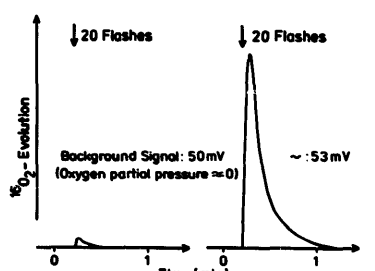

Fig. 1. Photosynthetic oxygen evolution measured as mass 32 in a particle preparation of *O. chalybea*. (a) Anaerobic conditions represented by a minimal oxygen background of 50 mV. (b) reactivation of (a) by increasing the oxygen tension to a background value of 53 mV. Normal oxygen content of air (21% O_2) corresponds to a background value beyond the upper limit of our device, which lies at 12000 mV.

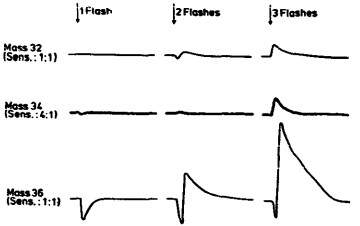

Fig. 2. Oxygen evolution measured as mass 32 ($^{16}O_2$), as the consequence of one, two and three short saturating light flashes in a particle preparation of *O. chalybea*. The assay has been flushed with nitrogen to remove $^{16}O_2$ from the assay. Thereafter the gas phase was supplemented with a trace of $^{18}O_2$ (98%) from the assay. The experiment shows that the evolution signal is preceded by a small uptake signal.

In the following experiment we analysed photosynthetic oxygen evolution measured as the consequence of two or five saturating xenon flashes in a medium containing only $H_2^{16}O$ by monitoring the appearance of masses 32, 34 and 36 (Fig. 3a). The signal amplitudes of mass 34 and 36 correspond to the theoretical natural abundance of 0.2% of $^{18}O_2$ in natural air or water [10]. The suspension was in equilibrium with normal air, i.e., 21% O_2 and 79% N_2. Fig. 3b shows the same experiment in which half of the $^{16}O_2$ content of the gas phase was replaced by $^{18}O_2$ and Fig. 3c the corresponding experiment in which all the oxygen of the gas

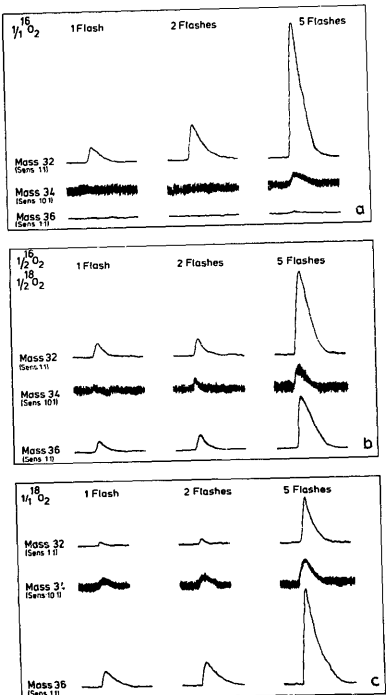

Fig. 3. Photosynthetic oxygen evolution measured in a particle preparation of *O. chalybea* as the consequence of one, two and five short saturating light flashes in an ambient atmosphere of 21% O_2/79% N_2 in equilibrium with the assay mixture composed of normal $H_2^{16}O$ containing buffer (no $H_2^{18}O$!). Simultaneous recording of mass 32 ($^{16}O^{16}O$), mass 34 ($^{16}O^{18}O$) and mass 36 ($^{18}O^{18}O$). Signal amplitudes measured in an assay in equilibrium with a gas phase: (a) of normal air; (b) in which half of the $^{16}O_2$ has been replaced by $^{18}O_2$; (c) in which 98% of the oxygen is $^{18}O_2$.

phase is $^{18}O_2$. It should be emphasized again that the suspension of thylakoids contains only $H_2^{16}O$. The overall conclusion from Fig. 3 is that the signal which measures photosynthetic oxygen evolution includes an O_2-uptake phenomenon. In Fig. 3a the signal caused by five flashes yields an integrated value of 146 relative units, in Fig. 3b of 100 and in Fig. 3c of 40 units. In the same sense, the integrated mass 36 signal increases from Fig. 3a to 3c. Hence, $^{36}O_2$ evolution competes with $^{32}O_2$ evolution. The sum of the integrated signals of the registered masses 32, 34 and 36 seems to remain constant, around approx. 160 units throughout all three experiments. The experiment of Fig. 3 shows that O_2 uptake very close to the S-state system must have occurred in which the oxygen taken up is immediately and exclusively funnelled into oxygen evolution. An uptake phenomenon due to a respiratory process yielding $H_2^{18}O$, which then would be decomposed photosynthetically, can be fully excluded, since the 5 ml $^{18}O_2$ used, for example, in the assay of Fig. 3c would yield, if quantitatively converted to $H_2^{18}O$, approx. 8 μl $H_2^{18}O$. However, in order to obtain a labeling level like that shown in Fig. 3c, approx. 200-times that amount would be necessary, fully disregarding the aspect that, if the $^{18}O_2$ evolution observed proceeds via the splitting of $H_2^{18}O$, the main label should be found as $^{16}O^{18}O$ in mass 34, which is obviously not the case. The lack of mixed label also excludes the obvious and spontaneous interpretation that the effect might be due to photoinhibition of uptake phenomena, such as inhibition of respiration in the same cyanobacterial membrane [11], besides the unequivocal argument that our measuring device permits the simultaneous measurement of O_2 uptake and evolution (Fig. 2). A further alternative interpretation to be tested is $^{18}O_2$ exchange between water and the O_2 gas phase in all S-states. This implies binding of H_2O also to S_2 and S_3, a possibility which has been excluded by the earlier experiments of Radmer and Ollinger [4] and ourselves [3]. Since no or little $^{16}O^{18}O$ has been evolved, $^{18}O_2$ evolution could come from $H_2^{18}O_2$ or an equivalent and this H_2O_2 production seems to be contained in regular O_2 evolution in *O. chalybea* (Fig. 3a). At this point it should be noted that Renger [5] has postulated that a cryptoperoxide should be involved in the water-splitting process. In the context of our experiment, this would imply that this peroxide should be able to exchange its

oxygen to a large extent with $^{18}O_2$ of the gas phase. A difficulty in this interpretation is that the $^{18}O_2$ evolution observed in Fig. 3 seems not to be restricted to a certain flash number. The literature describes an anomalous oxygen evolution due to H_2O_2 under the first two flashes in $CaCl_2$-washed Photosystem II particles of spinach, and this anomalous oxygen evolution appears to be enhanced upon addition of H_2O_2 [12]. However, if H_2O_2 is added to our *Oscillatoria* preparation (experiment not shown), no effect on the oxygen evolution pattern is observed, which clearly shows that, if the $^{18}O_2$ production originates from $H_2^{18}O_2$, its production location is precisely defined. In other words, the sites of production and its decomposition must be very close together and should, according to our analysis, be in the region of the S-state system itself. The observed evolution of $^{16}O_2$ and $^{18}O_2$ is sensitive to DCMU (experiment not shown), which demonstrates that H_2O_2 serves, like water, as electron donor to the photosynthetic electron-transport chain. In work by Berg and Seibert on $CaCl_2$-washed Photosystem II particles of spinach [12], it appeared that the H_2O_2-stimulated O_2 production in these particles could be fully abolished by the addition of catalase. Addition of catalase in our assay system, even in high amounts (experiment not shown), has no effect on the $^{18}O_2$-evolution pattern, which confirms our earlier data obtained by electrochemistry [6]. The interpretation must be that the added catalase either does not have access to the site of H_2O_2 production and/or that the decomposition is not handled by catalase but rather by a catalatic activity of the S-state system of the kind described by Mano et al. [13].

The phenomenon described in Fig. 3 has a strong dependence on the partial pressure of oxygen in the ambient atmosphere (experiment not shown). We know that $^{18}O_2$ evolution is strongly dependent on the O_2 dissolved in the assay mixture, whereas appearance of mass 32 measured as the consequence of ten saturating xenon flashes depends hardly at all on the oxygen content of the assay solution. The dependence of oxygen gas exchange on the $^{18}O_2$ background in the assay is shown in Fig. 4. With a normal $H_2^{16}O$-containing buffer system, hence no $H_2^{18}O$ labelling, and with a normal $^{16}O_2$/nitrogen atmosphere in the gas phase being in equilibrium with the assay mixture, 5 ml $^{18}O_2$ were injected and permitted to diffuse into the assay mixture. The signals of oxygen evolved of masses 32 ($^{16}O^{16}O$), 34 ($^{16}O^{18}O$) and 36 ($^{18}O^{18}O$) were plotted against the developing oxygen 36 background. The experiment shows the following. Evolution of oxygen 32 ($^{16}O^{16}O$) is barely affected by the increasing background of oxygen 36 ($^{18}O^{18}O$) in the region measured. Oxygen 36 evolution begins immediately. This experiment clearly shows that, taking together all exchange reactions in which oxygen 36 would be transformed to water ($H_2^{18}O$), e.g., via respiratory reactions which take place together with photosynthetic reactions in the same membrane in cyanobacteria [11], these play only a minor role under our conditions in comparison to the described phenomenon, since oxygen-34 ($^{16}O^{18}O$) increases with increasing $^{18}O_2$ partial pressure only very slowly.

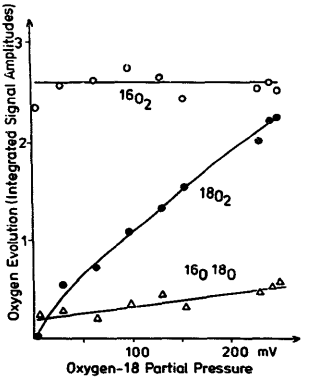

Fig. 4. O_2 evolution in a particle preparation of *O. chalybea* induced by ten saturating xenon flashes spaced 300 ms apart. The assay contained buffer made up with only $H_2^{16}O$ and was in equilibrium with a normal gas atmosphere. Upon this equilibrium $^{18}O_2$ was superimposed and permitted to diffuse into the system. The evolution of the mass signals 32, 34 and 36 is plotted in dependence on the increasing $^{18}O_2$ background.

If the phenomenon shown in Fig. 3 for *O. chalybea* preparations is analyzed with tobacco

TABLE I

FLASH YIELD WITH NORMAL ATMOSPHERE, *N. TABACUM*

Distribution of the flash yield of two, three and ten flashes fired on an assay containing chloroplasts of *N. tabacum* var. John William's Broadleaf, an $H_2^{18}O$-labelled buffer system in equilibrium with a normal gas atmosphere of $^{16}O_2$ (21%) and N_2 (79%). The assay contained chloroplasts corresponding to 135 μg chlorophyll in a total of 3.5 ml $H_2^{18}O$-labelled buffer containing 0.06 M Tricine/0.03 M KCl (pH 7.5), yielding a α-value of 0.274. Xenon flashes of 8 μs duration were fired 300 ms apart. The isotopic distribution of the O_2 evolved depends on the isotopic composition of the water. The theoretical distribution of O_2 evolved was calculated according to $36:34:32 = \alpha^2 : 2\alpha(1-\alpha) : (1-\alpha)^2$ with α being the atom fraction of ^{18}O depending on the measured m/e ratio according to $\alpha = 34+2\times36/2\times(32+34+36)$ [14].

Isotope	α (measured fraction)	Isotopic distribution of oxygen evolved			
		expected from the composition of H_2O	measured		
			2 flashes	3 flashes	10 flashes
$^{16}O^{16}O$		0.527	0.524	0.549	0.555
$^{16}O^{18}O$	0.26±0.008	0.398	0.410	0.396	0.384
$^{18}O^{18}O$		0.075	0.066	0.055	0.062

chloroplasts, the type of $^{18}O_2$ evolution connected with an O_2-uptake phenomenon is absolutely not observed. Table I and Table III show for tobacco chloroplasts that in an assay with $H_2^{18}O$ labelling in which the regular oxygen atmosphere has been substituted by $^{18}O_2$ in much the same way as in Fig. 2, isotopic distributions of the flash yields are observed which correspond to near-theoretical values of the water-splitting reaction. There is no indication of any interference by another O_2-producing process of the kind shown in Fig. 3. It should be noted that the data of Tables I and II have been obtained in an assay solution of ambient O_2 content, which corresponds to 1000 mV oxygen background. At this oxygen content of the gas phase or the measuring solution, oxygen evolution in *Oscillatoria* always consists of two components, one originating from water and one from hydrogen peroxide. At O_2 partial pressures considerably lower than those used in Tables I and II, namely at around 100 mV, the isotopic distributions of oxygen evolved in *Oscillatoria* preparations approach those shown for tobacco chloroplasts in Tables I and II. This means that the oxidation of the component producing the $H_2^{18}O_2$ is largely suppressed at this O_2 partial pressure, permitting the water-splitting reaction to occur exclusively.

Discussion

Photosynthetic oxygen evolution in the filamentous cyanobacterium, *O. chalybea*, exhibits several peculiarities on which we have previously reported [1,2]. One observation was that, if oxygen evolution is measured as the consequence of short

TABLE II

FLASH YIELD WITH $^{18}O_2$ ATMOSPHERE, *N. TABACUM*

The assay conditions, including the $H_2^{18}O$ label, correspond to those in Table I with the difference that the gas space between the suspension surface and the lid of the measuring cell was flushed with N_2, and 5 ml 98% $^{18}O_2$ was added.

Isotope	α (measured fraction)	Isotopic distribution of oxygen evolved			
		expected from the composition of H_2O	measured		
			2 flashes	3 flashes	10 flashes
$^{16}O^{16}O$		0.527	0.514	0.559	0.541
$^{16}O^{18}O$	0.265±0.026	0.398	0.379	0.362	0.391
$^{18}O^{18}O$		0.075	0.106	0.078	0.068

saturating flashes, the first flash always yields a substantial amperometric signal with the maximal flash yield usually appearing under the fourth flash [2]. This interesting sequence, when analysed in the 'four-state Kok model' [2], fits much better the prerequisites of the coherent Kok model than, for example, sequences of the green alga *Chlorella* reported many-fold in the literature. Such an *Oscillatoria* sequence, for example, does not seem to contain any abnormality under the first flash [15], a phenomenon which, however, complicates the well-known *Chlorella* sequences [16]. A signal under the first flash and maximal flash yield under the fourth flash is immediately reminiscent of work by Åkerlund [17] on salt-washed inside-out spinach thylakoids which have lost their two extrinsic 16 and 23 kDa peptides. In these particles an amperometric signal appears, just as in *Oscillatoria*, under the first flash and seems to be due to hydrogen peroxide. Just as in experiments by Berg and Seibert [12], Åkerlund observes that addition of H_2O_2 increases the signal and addition of catalase abolishes it under the first and also under the second flash [17]. In our system this is clearly not the case. Addition of high amounts of H_2O_2 or of high amounts of catalase, even in very disrupted particle preparations, never affected the phenomenon [6]. Nevertheless, we pursued the idea contained in the Åkerlund paper, above all, since cyanobacteria like *Oscillatoria* or *Anacystis nidulans* principally lack these two extrinsic peptides and thus under a variety of experimental conditions show calcium and chloride deficiency [18]. Our conclusion that the first amperometric signal was due to metastable S_3 and was inherent to *Oscillatoria* was supported by the observation that a system comparable to that in *Oscillatoria* was found in greening oat etioplasts [19].

The longevity of our S_3 permitted us to show that populating the S_3 condition, addition of $H_2^{18}O$ and a subsequent analyzing flash led to evolution of $^{18}O_2$-labelled oxygen [3]. Hence, our oxygen signal under a first flash is until now not necessarily related to the observation of Berg and Seibert [12] or Åkerlund [17]. However, we also observed in *O. chalybea* two distinct oxygen uptake phenomena which are both inherent properties of the filamentous cyanobacterium and which are both related to Photosystem II. In the first, we observe that photosynthetic oxygen evolution cannot occur under strictly anaerobic conditions. As Fig. 1 clearly shows, a threshold concentration of oxygen is necessary for the water-splitting reaction to occur. This observation might be of interest in the context of observations of Pistorius and Gau [20] who show that a 47 kDa flavoprotein which is a constituent of the Photosystem II complex in *Anacystis nidulans* probably has redox properties on the donor side of Photosystem II. They show that the protein has binding affinities for Ca^{2+}, Mn^{2+} and Cl^- and exhibits under in vitro conditions the properties of an amino-acid oxidase. In comparison to this, the second oxygen uptake phenomenon, the one observed in the present paper, is a phenomenon which might be due to the fact that, on the one hand, cyanobacteria lack the 16 kDa and 23 kDa protein and, on the other, generally contain an unsufficient coupling of electron transport between the two photosystems [21]. Moreover, nitrate-grown *Oscillatoria* exhibits an excess of Photosystem II activity in comparison to Photosystem I activity. Due to these circumstances, *Oscillatoria* thylakoids return part of their Photosystem-II-generated reducing power to oxygen, forming hydrogen peroxide or an equivalent which is decomposed by S_2 under oxygen evolution much in the way described by Åkerlund [17,22] or by Johansen [23]. At any rate, the generation of this H_2O_2 seems to take place in the immediate vicinity of the S-state system. We think that this oxygen-evolving reaction also occurs under natural conditions in *Oscillatoria* and therefore will always be associated with oxygen evolution due to the photosynthetic water-splitting reaction. Under experimental conditions this oxygen evolution is for obvious reasons most pronounced under flash illumination and is observed to a much lesser extent under continuous illumination. The phenomenon is enhanced by high oxygen partial pressure and suppressed or diminished by lower oxygen concentrations, dissolved in the reaction medium.

Acknowledgement

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