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Study on the properties of the S_3-state by mass spectrometry in the filamentous cyanobacterium *Oscillatoria chalybea*

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In the present paper we analyzed the properties of the S_3-state in the filamentous cyanobacterium *Oscillatoria chalybea* by mass spectrometry. In this organism a substantial O_2-signal due to a single flash is observed even after extensive dark adaptation (20 min). This signal can be measured by mass spectrometry as well as amperometrically on an oxygen electrode and is not due to an interference of respiratory and photosynthetic electron transport in the prokaryotic membrane. The mass spectrometric analysis shows that, if S_3 is generated by two flashes in a medium containing only $H_2{}^{16}O$, addition of $H_2{}^{18}O$ and subsequent firing of a third flash yields O_2 evolution labelled with ^{18}O. It appears that the isotopic composition of the O_2 evolved corresponds to the isotopic composition of the water in the suspension. This experiment shows that water oxidation does not proceed via an oxygen precursor or water derivatives bound to the S_3-state. This conclusion has been reached shortly before ours by Radmer and Ollinger [15] in the reverse marker experiment. From our study with *O. chalybea* it appears that freshly generated S_3 can be distinguished from metastable S_3 by the mass spectrometric method. It looks as if in contrast to freshly generated S_3 metastable S_3 contained bound unexchangeable water or an oxidized water derivative.

Introduction

In recent publications we have analyzed the oxygen evolution pattern of the filamentous cyanobacterium *Oscillatoria chalybea* [1,2]. If oxygen evolution is measured as the consequence of short saturating light flashes in the green alga *Chlorella* or in spinach chloroplasts, a damped oscillation with a periodicity of 4 is observed [3,4]. In all *Chlorella* or chloroplast patterns very little or no oxygen is evolved under the first flash, provided the sample has been sufficiently dark adapted. This is coherent with the assumption that the oxygen yield under the first flash is due to the S_3-state, whose decay time is very rapid in the dark [3,4].

In contrast to *Chlorella* or higher plant chloroplasts, preparations of *O. chalybea* always showed a flash pattern characterized by a substantial positive amperometric signal under the first flash despite extensive dark adaptation [2]. If the amperometric signal is due to photosynthetic oxygen, the existence of metastable S_3 has to be assumed in the filamentous cyanobacterium *O. chalybea*, which is practically not acceptable in the coherent Kok model. Indeed, it had to be taken into account that the first amperometric signal was simply not due to oxygen. The first possibility was that under the first flash the fast three-electrode device used [5] had measured a photoinhibition of respiration

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of the type reported by Verméglio and Carrier [6] or that the amperometric signal was produced by a substance (in the light on our electrode surface) which did not really have the same half-wave potential as oxygen. Both possibilities have been clearly excluded [2], since the positive amperometric signal under the first flash was still observed in *Oscillatoria* preparations with fully inhibited respiration and a polarogram showed the same half-wave potential for the first flash as for the following signals in the flash sequence. An artifactual peroxide-like substance or H_2O_2 produced with our plant material on the electrode surface, incidentally having the same half-wave potential as oxygen, was also excluded, since catalase or peroxidase addition did not change the flash pattern [7]. Consequently, the signal seemed to be due to oxygen which was fully confirmed by the observation that mass spectrometry yielded a substantial mass 32 signal under the first flash of a train of short saturating light flashes [8]. This result encouraged us to study the properties of the S_3-state by mass spectrometry in the filamentous cyanobacterium *Oscillatoria*.

In this study we are able to show that *Oscillatoria* preparations suspended in buffer containing $H_2{}^{18}O$ evolve $^{18}O_2$ under the first flash. If the S_3-state is generated by two preflashes in a suspension of *Oscillatoria* preparations containing only $H_2{}^{16}O$, i.e., ordinary water, the addition of $H_2{}^{18}O$ and a subsequent third flash yield $^{18}O_2$-labelled oxygen evolution.

Materials and Methods

Mass spectrometry. We used 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 focussing device 'Nier type I'. The measuring set-up is shown in Fig. 1a. In order to bring the mass-spectrometer to the sensitivity required for the measurement of photosynthetic oxygen evolution under single light flashes the spectrometer was equipped with a home-made valve system permitting direct connection of the measuring cell, via a cooling trap, to the ion source, at the same time short-circuiting the voluminous ordinary inlet system of the mass spectrometer. As a measuring cell we used a home-made stainless steel block in which 2–3 ml of assay suspension were separated from the gas space on the ion source side by a teflon membrane (Fig. 1b). The measuring cell was equipped with a gas-tight lid permitting flushing with different gas mixtures and permitting injection of solutions, for example the addition of $H_2{}^{18}O$ to a suspension originally containing only $H_2{}^{16}O$, i.e., ordinary water. The teflon membrane was mounted gas-tightly on a supporting device of teflon with a porous or foamy consistence representing no gas barrier. In our assay system, the sensitivity of the set-up depended on the geometry of the measuring cell, i.e., on the diameter of the teflon surface on which we gave our resting 2–3 ml of the assay

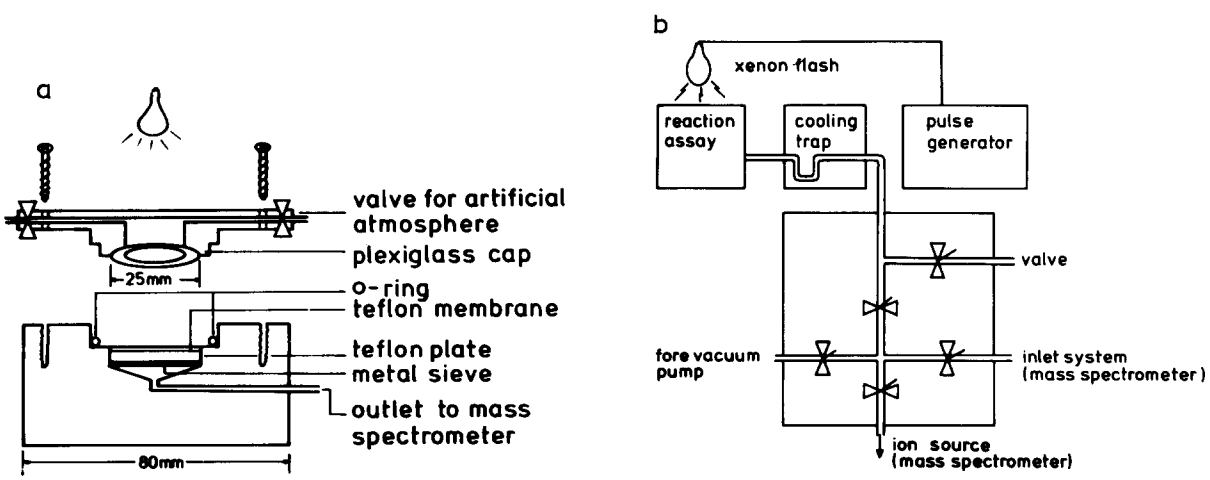

Fig. 1. (a) Schematic diagram of the measuring cell. (b) Schematic diagram of the experimental set-up.

solution. Sensitivity was best when the diameter was 25–40 mm. The second requirement which was crucial for high sensitivity was to keep the tube volume between the measuring cell and the ion source minimal. In our set-up 20 cm (cooling trap included) of a stainless steel tube with a diameter of approx. 0.5 cm linked the measuring cell with the ion source. In this set-up the sensitivity concerning the signal amplitude recorded on a SE 130-03 BBC Metrawatt three-channel recorder equalled our polarographic three-electrode device described earlier [5]. The performance of our set-up is shown in Fig. 2 in which the *Chlorella* signal pattern of 30 s continuous light recorded with our polarographic system [5] is compared to the effect of 20 saturating xenon light flashes (Stroboscope 1539 A of General Radio) of 8 μs duration spaced 300 ms apart in our mass spectrometric system. The general shape of both patterns is comparable, showing the classical fast oxygen gush followed by a slower phenomenon [9].

Measuring procedure. 2 ml of the *Oscillatoria* preparation containing approx. 70 μg chlorophyll in buffer containing only $H_2{}^{16}O$ were placed on the teflon membrane in the measuring cell described above. Care was taken to cover the entire membrane surface with the solution. The solution surface was covered with an exactly fitting plexiglass disk (diameter, 4 cm) which was covered with 1-mm large holes. The disk permitted practically free gas exchange with the surface but limited perturbation due to the addition of degassed $H_2{}^{18}O$. The cell was closed with the lid and flushed with N_2 in the dark for 5 min. Since under our conditions the sample in the absence of O_2 would not evolve any oxygen [7,10] we added 5 ml 98% $^{18}O_2$ from CEA-Oris, Bureau des Isotopes Stables, Gif-sur-Yvette, to the 21 ml gas space between the suspension surface and the lid. This addition of $^{18}O_2$ to the gas phase did not introduce an O_2-concentration-dependent signal portion of the type reported by Peltier et al. [16], as shown by our Table I or Fig. 7. The fact that no ^{18}O-signal is observed under the two preflashes before $H_2{}^{18}O$ addition, despite the presence of $^{18}O_2$ in the gas phase clearly excludes an uptake phenomenon in our preparation under the conditions used. *O. chalybea* does not show this photoinhibited O_2-uptake [2]. However, we clearly observe this phenomenon in *Chlorella* cells (see also Fig. 2; results to be published in a forthcoming paper). The thus conditioned *Oscillatoria* preparations were illuminated with two xenon light flashes as for example shown in Fig. 6. Immediately upon these preflashes 1 ml 99 atom% $H_2{}^{18}O$ from Ventron (flushed before addition for 10 min with N_2 in order to remove $^{16}O_2$) was carefully and slowly added through the lid with a microsyringe. The addition procedure required 15–20 s. The ensuing perturbation of the system was the major experimental problem. It usually took 2–3 min until the original base line was restored. As soon as this was the case the analysing third flash was fired. A second and important problem was the above-mentioned observation that the surface oxygen tension had to be high enough to avoid anaerobiosis. Usually we recorded our signals with an O_2-tension corresponding to approx. 340 mV. All experiments were carried out at room temperature.

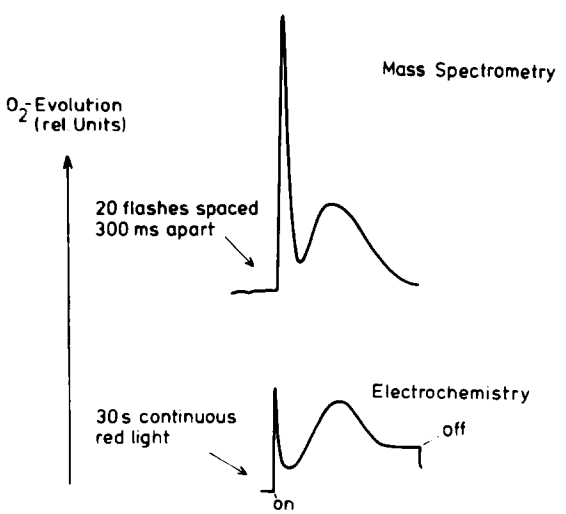

Fig. 2. Comparison of the sensitivity of our mass spectrometric set-up with that of our three-electrode device [5]. *Chlorella* cells corresponded to 40 μg Chl suspended in 3 ml water. O_2-evolution measurement in the mass spectrometric assay as $^{16}O_2$.

Particle preparations of the filamentous cyanobacterium *O. chalybea* were prepared as described earlier [2] from 20-day-old cultures grown on nitrate as the sole nitrogen source. Cells were centrifuged from the medium and suspended in 0.1% glucuronidase (Merck) and 0.6 M mannitol and incubated for 30 min at 37°C in order to

remove the mucoids. The cells were spun down at 2000 × *g* and suspended in 0.1% cellulase (Kinki Yakult) and 0.6 M mannitol and passed through the French Press at 34.5 MPa. The French pressate was incubated for 30 min at 37 °C in order to remove the cell walls. The resulting protoplasts were centrifuged at 2400 × *g* for 10 min, washed twice with 0.6 M mannitol and suspended in the respective buffer. Disruption of the protoplasts in dilute buffers of any kind yielded thylakoids [2].

Polarographic measurements of oxygen evolution with the rapid and sensitive three-electrode device described by Schmid and Thibault [5] were made for comparison.

The assay suspension usually containing *Oscillatoria* particle preparations equivalent to approx. 70 μg chlorophyll, was composed of 0.06 mM Tricine and 0.03 M KCl (pH 7.5) in a total volume of 2 ml.

Results

If oxygen evolution was measured electrochemically in extensively dark adapted *O. chalybea* as the consequence of a train of short light flashes, the evolution pattern corresponded to that of *Chlorella* cells or chloroplasts often reported in the literature [3,4,11], with the major difference being that the first flash always yielded a substantial positive amperometric signal [2]. Moreover, the *Oscillatoria* patterns usually showed maximal flash yield under the fourth flash [2]. An analysis of the S-state distribution of such a pattern yields in a dark-adapted preparation 50% S_0 40% S_1 and 10% S_3 [2]. If the first signal was indeed due to photosynthetic oxygen, and oxygen evolution in particular, it would imply that metastable S_3 exists in *Oscillatoria.* Fig. 3 compared a flash pattern for oxygen evolution in a particle preparation of *O. chalybea* obtained electrochemically with a pattern obtained by mass spectrometry. Both flash patterns are fully comparable, a fact which definitely settles the question whether the positive amperometric signal under the first flash is oxgyen.

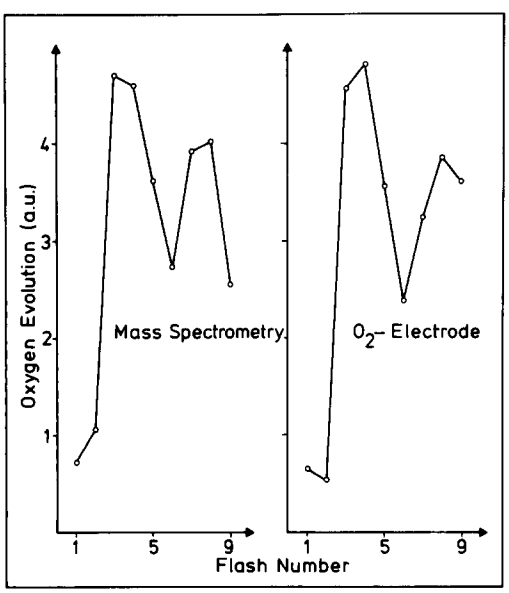

Fig. 3. Oxygen evolution measured as consequence of short saturating flashes in a thylakoid preparation of *Oscillatoria chalybea* by mass spectrometry ($^{18}O_2$) and by amperometry.

In view of discussions on the active and resting states of the O_2-evolving complex of Photosystem II [12,13] we compared the signal amplitudes obtained by mass spectrometry in extensively dark adapted samples (20 min) and in just sufficiently dark adapted samples (3 min) and noted no appreciable differences (Fig. 4). However, in the course of these analyses we became aware of the fact that the oxygen evolving complex must be able to bind or to consume a certain quantity of oxygen before being active or capable to evolve oxygen. The *Oscillatoria* preparations as we use them in our mass spectrometric analysis have a remarkable tendency to become anaerobic under a variety of conditions. This tendency can be sustained by flushing with nitrogen. If a sample is sufficiently flushed with nitrogen, absolutely no flash-induced oxygen evolution is observed (Fig. 4a). Measurements of fluorescence induction in anaerobic *Scenedesmus* have led to the conclusion that the oxidizing side of Photosystem II becomes inactivated [10]. The preparation which is inactive after flushing with nitrogen (Fig. 4a) is fully reactivated by flushing with oxygen. This experiment can be easily brought in line with experiments with *Anacystis nidulans* Photosystem II particles in which the oxygen-evolving complex under defined conditions has the properties of an oxidase [14] or experiments based on EPR-studies in which an active state of the O_2-evolving complex in spinach preparations exhibits an O_2-uptake phe-

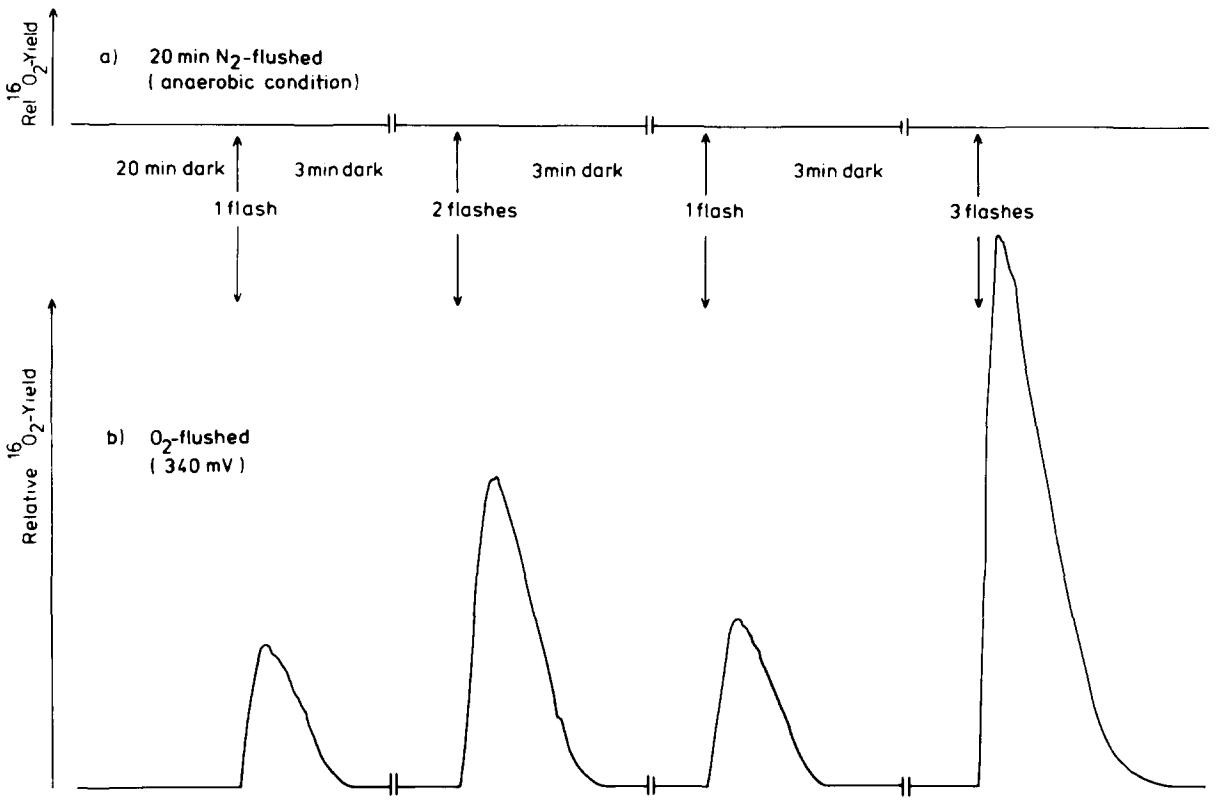

Fig. 4. O_2-evolution as the consequence of short light flashes measured as mass 32 by mass spectrometry in preparations of *Oscillatoria chalybea*. (a) Anaerobic conditions; (b) as in the preparation of (a), but reactivated by a short $^{16}O_2$ flushing.

nomenon [12] which is strictly correlated with oxygen evolution. The question whether the tendency of our *Oscillatoria* preparations to become anaerobic and thus inactive is an inherent property of the organism, eventually characterized by our observed metastable S_3 condition, or whether it is artifactual due to the conditions produced in our measuring cell is not answered in this paper. At any rate the experiment shown in Fig. 4 might demonstrate the requirement of oxygen binding onto the oxygen-evolving complex.

In comparison to *Chlorella* cells or spinach chloroplasts, the deactivation of S-states in *Oscillatoria* preparations shows considerably slower relaxation times for all S-states with the peculiarity that although the deactivation of S_3 at short times after steady-state condition is faster than that of S_2 (which is the normal situation), the deactivation at longer relaxation times reaches a stationary level above the simultaneously measurable S_2-condition (Fig. 5). This is valid for all our preparations without addition of an exogenous reductant. Under all our conditions we observe approx. 10–12% of S_3 in the dark population of S-states [2], perfectly capable of evolving oxygen upon firing a single flash, even after dark adaptations of 20 min or more.

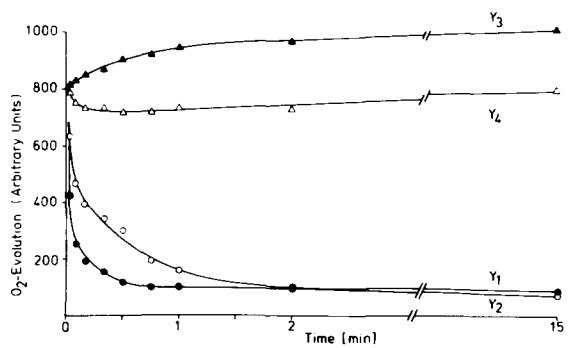

Fig. 5. Amperometrically measured O_2-yield of the first four flashes Y_1, Y_2, Y_3, Y_4 of a train of short saturating xenon flashes in dependence on the dark time between flash sequences.

TABLE I

ISOTOPIC DISTRIBUTION OF THE FLASH YIELD OF ONE SINGLE FLASH FIRED AFTER THE ADDITION OF $H_2{}^{18}O$ TO THE ASSAY CONTAINING $H_2{}^{16}O$

The assay was preflashed with two flashes before the $H_2{}^{18}O$ addition. *Oscillatoria chalybea* preparation corresponding to 80 μg chlorophyll was suspended in 2 ml $H_2{}^{16}O$ containing buffer and illuminated with two 8 μs xenon flashes spaced 300 ms apart. 15 s after flashing 0.95 ml $H_2{}^{18}O$ (99 atom% yielding a final α-value of 0.32) were added and a third xenon flash was fired. The isotopic distribution of the O_2 evolved is related to the isotopic composition of the water. The theoretical distribution of O_2 evolved was calculated according to $36:34:32 = \alpha^2 : 2\alpha(1-\alpha) : (1-\alpha)^2$. α is the atom fraction of ^{18}O and depends on the measured m/e ratio according to $\alpha = (34+2\cdot36)/\{2(32+34+36)\}$ [17].

Isotope	Mass spectrometric signal measured (relative integrated units)	α-Measured fraction of oxygen atoms that are ^{18}O	Measured isotopic distribution of oxygen evolved	Isotopic distribution of oxygen evolved, expected from the composition of H_2O
$^{16}O^{16}O$	270		0.475	0.462
$^{16}O^{18}O$	250	0.3045	0.440	0.435
$^{18}O^{18}O$	48		0.084	0.102

In the following experiment we generated S_3 by giving two flashes onto our dark-adapted preparation. According to our previous analysis [2] the S-state population of dark adapted *Oscillatoria* contains approx. 50% S_1. The generation of S_3 was performed in buffers containing only ordinary water, i.e., $H_2{}^{16}O$. Immediately upon these two flashes we injected $H_2{}^{18}O$ which, as described in Materials and Methods, required approx. 20 s, and looked at the isotopic composition of the O_2 evolved in a subsequent single flash. Two types of experiment were carried out which should permit the distinction between freshly generated S_3 and metastable or resting S_3. In the first type the third flash was given 3 min after the $H_2{}^{18}O$ addition, whereas in the second type the third flash was given 10–15 min after the $H_2{}^{18}O$ addition and after the preilluminating two flashes. From the life-time of the S_3-state (Fig. 5) it follows that at the moment of $H_2{}^{18}O$ addition and also at the time of firing the third analyzing flash a substantial amount of S_3 was still present in both assays. Fig. 6 shows the qualitative experiment in which the time-course of the signal amplitudes was registered as mass 36. It shows that generation of S_3 in $H_2{}^{16}O$, subsequent addition of $H_2{}^{18}O$, and firing a third flash leads to the evolution of oxygen labelled by $^{18}O_2$. If S_3 was bound to unexchangeable water or if partially oxidized water molecules were bound onto S_3, generated in $H_2{}^{16}O$, the O_2-evolution in the third flash should only be composed of $^{16}O_2$ and contain strictly no $^{18}O_2$.

Table I shows the isotopic composition of the oxygen evolved under the third flash in per cent of masses 32, 34 and 36. The fact that $^{18}O_2$ is evolved under these experimental conditions implies that after the accumulation of three positive charges water is split in a concerted reaction excluding the possibility that S_3 contains unexchangeably bound water or 'oxygen precursors'. This experiment fully agrees with that by Radmer and Ollinger [15], in which the authors have made the experiment presented in Fig. 6 practically the other way round by generating S_3 in spinach chloroplasts in $H_2{}^{18}O$ by two preflashes, washing the sample and resuspending it in $H_2{}^{16}O$ which led to absolutely no $^{18}O_2$ labelling of the O_2 evolved under a third flash. The conclusions reached earlier by these authors are the same as ours.

Fig. 7a shows the experiment in which two

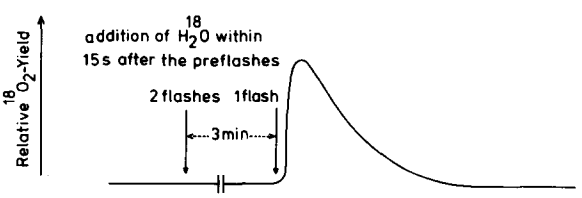

FIg. 6. Flash yield in a preparation of *Oscillatoria chalybea*. Generation of S_3 by two flashes in a medium containing only $H_2{}^{16}O$ with subsequent addition of $H_2{}^{18}O$ and analysis of the $^{18}O_2$-yield (mass 36) of a single flash fired 3 min after the two preflashes.

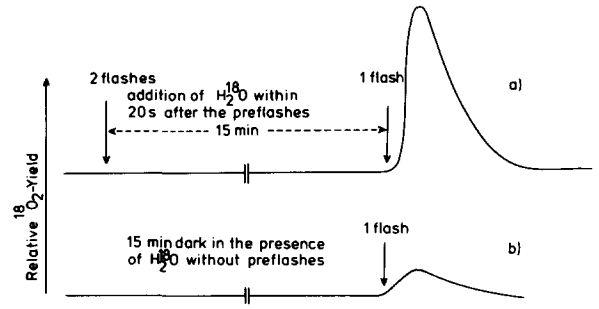

Fig. 7. Flash yield in a preparation of *Oscillatoria chalybea*. (a) Generation of S_3 by two flashes in a medium containing only $H_2{}^{16}O$ with addition of $H_2{}^{18}O$ immediately after the preflashes and analysis of the flash yield in a subsequent third flash (fired 15 min after the preflashes!) by measuring oxygen evolution as mass 36. (b) Same preparation as (a) but addition of $H_2{}^{18}O$ to a dark-adapted sample and dark incubation with $H_2{}^{18}O$ for 15 min as in (a).

preflashes were given onto a suspension of an *Oscillatoria* particle preparation suspended in buffer containing only $H_2{}^{16}O$. As in Fig. 6, $H_2{}^{18}O$ was added immediately after the preflashes but the third flash was given only 15 min later. In Fig. 7 no appreciable qualitative difference is seen to the experiment in Fig. 6, as the oxygen evolved is labelled with $^{18}O_2$. If the two preflashes are omitted the labelling is much lower if not to say negligible (Fig. 7b). In principle we had expected that after a time as long as 15 min the two preflashes would not matter to this extent, but Fig. 7 clearly shows that the oxygen-evolving complex 'remembers' the two preflashes. We feel that this is an observation which complicated the simple and obvious conclusions which must be drawn from experiments like that shown in Fig. 6 or those reported by Radmer and Ollinger [15]. The experiments presented here are limited by the considerable cost of $H_2{}^{18}O$ and cannot be carried out under illimited modified conditions as one would obviously like to do it. The experiment of Fig. 7a and b fits and fully confirms our earlier observation that if a dark-adapted *Oscillatoria* preparation, suspended in buffer containing $H_2{}^{16}O$, is transferred in the dark into buffer containing $H_2{}^{18}O$, the first flash yields strictly no $^{18}O_2$-labelled oxygen [2] although our measuring system easily permitted the detection of even minimal amounts of $^{18}O_2$ [2], as shown by the control experiment. The implication could be that metastable' is a kind of resting condition or state of the oxygen evolving complex in the sense of the diction of Ref. 12 which would contain bound water or oxygen. This S_3-state is perfectly capable of evolving O_2, but in this case the oxygen evolved is unlabelled $^{16}O_2$ [2]. In our opinion the experiment shows that things, at least in *Oscillatoria*, are not necessarily as simple as they seem at first glance in the experiment in Fig. 6 or in those by Radmer and Ollinger [15]. Contribution of chemical equilibrium situations must absolutely be taken into account in future experiments of this kind. The conclusion that one can draw at this point is that metastable S_3 can be distinguished from freshly generated S_3 (Figs. 6 and 7).

Discussion

In the present paper we make a contribution to the understanding of the mechanism of photosynthetic water splitting. The experiments show that the positive charge accumulation proceeds to the condition of S_3 without production of an oxygen precursor or a complex which contains unexchangeable water bound to the S_3-state. This is concluded from the following experiment: two preflashes populate the S_3-condition of the S-state system; addition of $H_2{}^{18}O$ to the suspension medium containing only $H_2{}^{16}O$ leads in a subsequent flash to ^{18}O-labelled O_2-evolution.

The isotopic composition of the O_2 evolved (Table I) corresponds very closely to the isotopic composition of the water in the assay, which means that the O_2 gas analyzed was in the condition of isotopic equilibrium [17]. Since we obtain these nearly theoretical values no substantial O_2-uptake phenomenon of the type described by Peltier et al. [16] can be contained in the oxygen balance of our single analyzing flash. In this context Fig. 7 is at the same time the control and the answer to the question whether $^{18}O_2$ in the gas phase alone (see Materials and Methods) would yield a substantial positive $^{18}O_2$-signal in the sense of Ref. 16. It should be noted that nitrate-grown *O. chalybea* anyway exhibits in comparison to *Chlorella* or other organisms very little conventional NADH-dependent respiration [2]. However, under certain conditions, for example, under mixotrophic conditions or under conditions when the preparation of

the autotrophically grown culture has deteriorated to very low photosynthetic activity, *O. chalybea* may exhibit substantial rates of respiration. Here mass spectrometry indicates that in the light or under a light flash electrons originating from water are partially diverted from the photosynthetic electron path via the *c*-cytochrome and cytochrome *c*-oxidase. In our organism with $^{18}O_2$ as an acceptor this phenomenon contributes mainly to the evolution of $^{34}O_2$ ($^{16}O^{18}O$) and practically not to $^{18}O_2$ or a positive mass spectrometric $^{18}O_2$ signal (Bader, K.P. and Schmid, G.H., unpublished data).

In the course of these studies we made the interesting observation that, if our preparation became anaerobic (by flushing with N_2 in our measuring cell according to Materials and Methods) absolutely no oxygen evolution was observed (Fig. 4a). Although we cannot exclude that this observation might be due to an overreduction of the plastoquinone pool, we favor the possibility that oxygen evolution is dependent on the presence of oxygen in the sense that oxygen binding to the O_2-evolving complex is necessary before the system really evolves oxygen. In this context we would like to note that earlier experiments have shown that β-D-glucose oxidase, which is a very effective oxygen scavenger, would completely abolish any amperometric O_2-signal in a train of short saturating xenon flashes [2] in our assay. These experiments would fit fluorescence measurements in anaerobic *Scenedesmus* from which the authors concluded that the oxidizing side of Photosystem II becomes inactivated [10], or findings of our own laboratory by Pistorius and Gau with Photosystem-II particles of *A. nidulans* in which the O_2-evolving complex under certain conditions exhibits the properties of an L-amino-acid oxidase [14].

Acknowledgements

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