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Physiological and evolutionary aspects of the O_2/H_2O_2-cycle in cyanobacteria [1]

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Abstract

Various cyanobacteria evolve oxygen upon illumination with the very first flash of a sequence and interact with the oxygen from the surrounding atmosphere. In the present paper I describe recent experiments allowing the discrimination between photosynthetic water splitting and the light induced peroxide decomposition, the latter showing a strong dependence on the oxygen partial pressure in the suspension. Under appropriate conditions a clear period-2 oscillation of a flash sequence is observed. This fits into the interpretation, as the decomposition of hydrogen peroxide requires only two light quanta and S_2 has been shown to be the most reactive redox state within the S-state system. Comparison of the relaxation kinetics of the first two flashes of a sequence with the steady state signals as well as the detailed analysis of the mass spectrometric signals revealed completely different time constants for both water splitting and peroxide decomposition. These observations can be correlated to the suggestion that a peroxide might be involved in the higher oxidation states (Renger, G. (1987) Photosynthetica 21, 203–224). Moreover, the phenomena can be specifically and independently influenced and stimulated e.g. by the addition of various salts which strongly increases the water splitting reaction. In the present discussion on the participation of hydrogen peroxide the reaction sequences described for *Oscillatoria* in this and in previous papers might be the linking condition between oxygenic photosynthesis and the type of anoxygenic photooxidations such as the photooxidation of ferrous or sulfide ions which prevailed earlier. From the evolutionary point of view these two mechanisms might have been linked by the involvement of a *quasi* semireduced component like hydrogen peroxide.

Keywords: Photosynthesis; Oxygen evolution; Peroxide; Mass spectrometry; Flash pattern; Cyanobacterium

1. Introduction

The process of oxygenic photosynthesis has been developed 3–4 billion years ago by cyanobacteria. This procedure occurred in a largely reducing atmosphere most probably containing only carbon dioxide (or hydrogen) at concentrations of some percent beside nitrogen. Thus, the composition of the atmosphere on Earth was completely different from the one of today which is strongly oxidizing with the high oxygen partial pressure of 21%. This means that the transition from anoxygenic to oxygenic photosynthesis took place along with the exchange of the major electron donors for photosynthetic electron transport. In a reducing atmosphere it is speculated that there was no need to oxidize water, as high concentrations of reducing components like ferrous or sulfide ions were available [1]. Moreover, these oxidation reactions required much less energy than does the oxidation of water with 115 kcal mol^{-1}. Oxygenic photosynthesis, on the other hand, makes use of the large amount of water on Earth thus making the electron donor for photosynthesis available in enormous excess. However, at this point an evolutionary gap has to be considered as no continuous development seems to be possible on the way from the pheophytin-quinone and the iron-sulfur reaction centres to the heterodimeric form present in higher plants [2]. A point that also has to be taken into account is the interpretation that oxygenic photosynthesis can have hardly been developed in a completely anoxygenic atmosphere, as e.g. the synthesis of chlorophyll requires

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[1] Dedicated to the memory of my father Dr. Bernhard Bader, deceased on March 15th 1994.

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some oxygen [3]. This relatively low but indispensable oxygen might have been derived from biotic or abiotic reactions. Among the latter, photodissociation of water in the atmosphere and photochemical reactions with seawater are discussed [4,5]. In this context, our observation that the water splitting apparatus in the filamentous cyanobacterium *Oscillatoria chalybea* does not function under completely anaerobic conditions, hence, requires the binding of minimal amounts of oxygen prior to water oxidation might be of particular interest [6]. It is not clear, however, how the substantial and principal transition in evolution to the mechanism of water oxidation had been achieved. One interesting idea in discussion at present is the argument that atmospheric hydrogen peroxide was transitionally involved in the process which took place in cyanobacteria.

Interactions of Photosystem II (a complex with sufficient oxidizing potential to split molecular water) with hydrogen peroxide and catalase have been repeatedly shown [7–9]. Mass spectrometric analyses of the oxygen gas exchange in a filamentous cyanobacterium (*Oscillatoria chalybea*) revealed a completely unexpected type of oxygen evolution upon addition of oxygen-18 isotope to the gas phase over a thylakoid suspension from *O. chalybea*. Oxygen from the surrounding gas atmosphere is dissolved in the liquid phase up to the respective equilibrium, substantially taken up by the organisms and converted to a component that is immediately reoxidized by light, liberating the very oxygen that has just been taken up. From the isotopic distribution ($^{16}O_2$, $^{16}O^{18}O$, $^{18}O_2$) it was concluded that molecular water can not play a role in this oxidation, as it can easily be calculated that the oxidation of $H_2{}^{18}O$ would result in a considerably higher portion of the $m/e = 34$ signal from $^{16}O^{18}O$ than is observed [10]. Above all, due to the apparent high rate constant of the involved reaction, it was postulated that hydrogen peroxide was the resulting molecule of the uptake mechanism. One of the major implications of the phenomenon is that under actual atmospheric conditions in several filamentous cyanobacteria a considerable portion of oxygen evolved upon illumination comes from this peroxide decomposition. Photolytic water oxidation also takes place in these organisms but to a lesser extent. This observation fits into the idea that photosynthetic electron transport in filamentous cyanobacteria was still substantially based on other oxidation reactions using 'older' electron donors like, e.g., sulfide ions, when oxygenic photosynthesis was eventually developed by these organisms. This interpretation might by substantiated by the observation of evolutionary alterations and adaptations of Photosystem II and the oxygen evolution complex, as higher plants contain two extrinsic polypeptides of 16 kDa and 23 kDa (principally absent in cyanobacteria), evolve oxygen from water at much higher rates than cyanobacteria do and do not show any interaction with atmospheric oxygen leading to O_2 from hydrogen peroxide [11].

2. Materials and methods

The filamentous cyanobacterium O. chalybea was obtained from the algal collection in Göttingen and cultivated on clay plates in large Petri-dishes [12].

Thylakoids from 3–4-week-old cultures were prepared according to the mechanic and enzymic treatment (glucuronidase, cellulase, lysozyme) described by Bader et al [12]. For the experiments aliquots from the thylakoid suspension equivalent to 40 μg chlorophyll in a total volume of 2 ml with 0.06 M tricine and 0.03 M KCl were used.

Mass spectrometric experiments were performed as previously described by Bader and Schmid [13] and Bader et al. [14]. The stable isotope ratio mass spectrometer 'Delta' from Finnigan MAT (Bremen, Germany) is a magnetic sector field instrument and has been substantially modified for our experiments. The modifications have also been described [15]. Calibration of the set-up and calculation of the isotope distribution was carried out by two procedures:

(1) The average of at least 10 determinations of the signals at $m/e = 32$, $m/e = 34$ and $m/e = 36$ for 'normal' air was correlated with the well-known natural atomic abundance of 99.7587% oxygen-16 and 0.2039% oxygen-18.

(2) Various concentrations of exogenously added hydrogen peroxide yield definite signals in the detection system upon decomposition by addition of catalase.

Corrections for the isotope dilution can be made according to the equation given by Peltier and Thibault [16]. The mass spectrometric set-up that we use is a closed system with a unidirectional gas flow towards the ion source (no circuit). Thus, all signals are superimposed on a continuously decreasing negative incline. This slope, however, is asymptote-like shaped and can easily be used as baseline for positive (or negative) changes in the respective recorder tracing. The resulting signals for $^{16}O_2$, $^{16}O^{18}O$ and $^{18}O_2$ were simultaneously detected in Faraday cups and recorded on a SE 130–03 BBC Metrawatt 3-Channel recorder. Flash illumination was performed with the Stroboscope 1539 A of General Radio which yields flashes of 5 μs duration (usually spaced 300 ms apart).

Oxygen isotopes were obtained from CEA-Oris, Bureau des Isotopes Stables, Gif-sur-Yvette, France ($^{18}O_2$) and Euriso-top, Saint-Aubin, France ($H_2{}^{18}O$). Depending on the application of different isotopes together with high or low oxygen partial pressures, different phenomena (water oxidation or hydrogen peroxide decomposition) were detected at $m/e = 32$ or

$m/e = 36$. Therefore, details of the composition of the assay are given in the figure legends or in the corresponding text.

3. Results

Filamentous cyanobacteria contain Photosystem II, a complex capable of oxidizing molecular water by a process called oxygenic photosynthesis. Dioxygen is evolved upon illumination of such organisms with continuous light or short saturating actinic flashes of 5 μs duration. Consequently, part of the light induced oxygen evolution is based on photolytic water oxidation. This process takes place in a concerted reaction where 4 different oxidation states (S-states) are successively reached by the successive absorption of 4 light quanta [17,18]. The phenomenon is described and explained by the so-called Kok model and easily measured on rapid and sensitive, large-surface electrodes like the Joliot-electrode or the one described by Schmid and Thibault [19]. One of the peculiarities of such flash patterns as the one obtained with *O. chalybea* (Fig. 1) is the substantial signal under the first flash. This signal which has been described several years ago [12] was clearly correlated with metastable S_3-redox state. Similar results have been obtained and described for *Synechocystis* PCC 6803, *Synechococcus* PCC 6301 and *Synechococcus* PCC 7942. Moreover, various mutants from cyanobacteria also showed the described phenomena, e.g., mutant A5 from *Synechococcus* PCC 8942, whereas the thermophilic cyanobacterium *Synechococcus sp.* does not. Mutant A5 is a MSP-deficient strain in which the water oxidase requires photoactivation after extensive dark adaptation. It should be stressed that under non-activated conditions (with virtually no photolysis of water) Y_1 and Y_2 of a flash sequence appear completely unaffected in comparison to the activated condition (compare also Fig. 7) [20]. Analysis of this signal by our mass spectrometric device where a teflon membrane is used to separate the reaction assay from the detection side of the set-up shows that the signal (Fig. 2) is clearly independent and distinct from any type of artefact observed at the surface of a rate electrode. When we analyzed the light-induced oxygen evolution from *O. chalybea* in a mass spectrometric set-up allowing the discrimination between different oxygen isotopes, we observed oxygen evolution signals from the oxidation of $H_2{}^{18}O$ which were detected at $m/e = 36$ and signals from the decomposition of the peroxide which is formed depending on the oxygen partial pressure in such organisms [21] and Fig. 3. The process of water oxidation is largely independent from the surrounding oxygen gas atmosphere (Fig. 3) apart from the fact that the phenomenon requires a minimal amount of oxygen to be present. This effect has been described [6] and will be

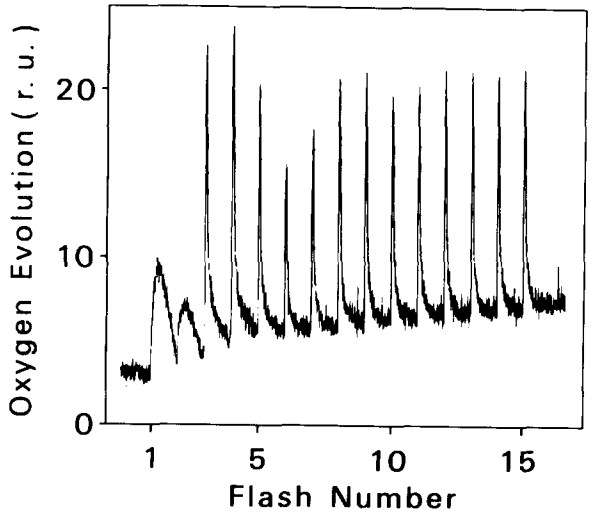

Fig. 1. Polarograhic recording of photosynthetic oxygen evolution measured as the consequence of short (5 μs) saturating light flashes (spaced 300 ms apart) in thylakoids of *O. chalybea* (25 μg chlorophyll). Dark adaptation time was 30 min before the first flash was fired.

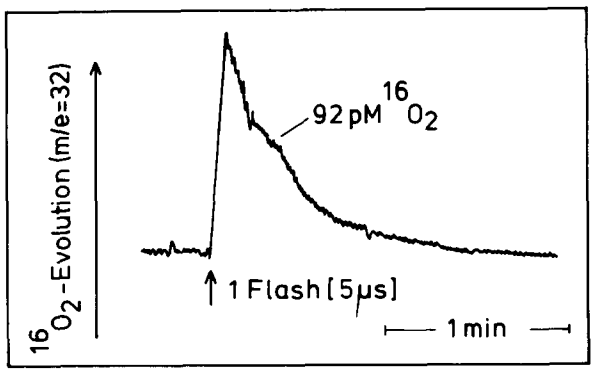

Fig. 2. Mass spectrometric measurement of photosynthetic oxygen evolution induced by a single saturating flash after extensive dark adaptation (30 min).

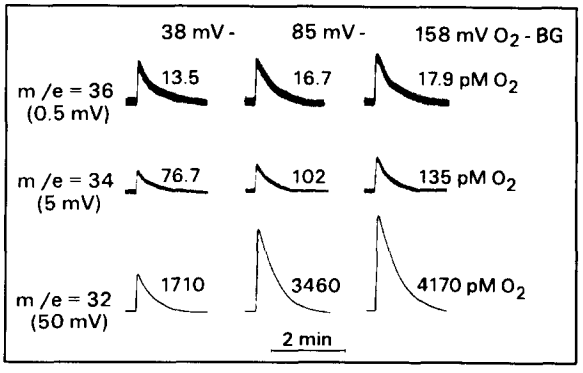

Fig. 3. Isotope distribution of oxygen evolved from *Oscillatoria*-thylakoids (40 μg chlorophyll) in a reaction assay containing 32% $H_2{}^{18}O$ at different oxygen partial pressures of $^{16}O_2$. (100 mV background signal corresponds to 26.3 μM O_2.) The mass spectrometric signals represent the cumulated signals after 50 light flashes.

further analyzed in a forthcoming paper [22]. The oxygen-16 detected at $m/e = 32$ in Fig. 1, however, consists of the photolytic water oxidation of the $H_2{}^{16}O$ portion of the assay together with a relatively low level of oxygen from peroxide decomposition. Under these conditions the low oxygen partial pressure of the assay permits only small amounts of peroxide to be built up. Under conditions with increased oxygen partial pressure the isotopic distribution of the evolved dioxygen changes impressingly (Fig. 3). Oxygen evolution from the water splitting reaction remains low, hence is apparently completely unaffected, whereas oxygen evolution connected with the peroxide decomposition increases drastically with the oxygen partial pressure (Fig. 4). The increased O_2-partial pressure under these conditions leads to an increased oxygen uptake, apparently an enhanced formation of peroxide and an increased liberation of $^{16}O_2$ upon illumination. This observation seems principally independent from the water splitting reaction although a connection between the two phenomena is discussed (vide infra) showing a strong dependence on the oxygen content of the surrounding gas atmosphere. Among all S-states, S_2 seems to be the predominantly reacting one e.g., towards agents like hydroxylamine. This has been investigated by Franck and Schmid using etiochloroplasts of oat [23] and confirmed by Messinger et al. [24] for normal chloroplasts. If this interpretation was correct and transferable to cyanobacteria, one might speculate that a periodicity of two should be observable under appropriate conditions. In fact, this can be observed (Fig. 5). This hints at the preponderant involvement of S_2 and

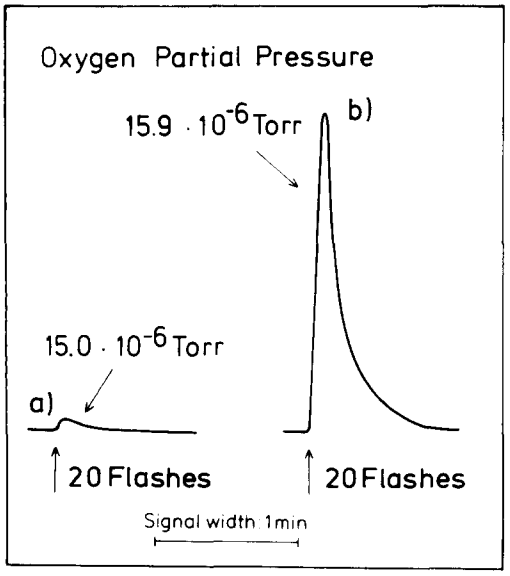

Fig. 4. Oxygen evolution detected at $m/e = 32$ with thylakoids from *O. chalybea*. Note the minimal difference in oxygen partial pressure between the two tracings recorded with identical sensitivity. The flash signals were recorded after 20 light flashes.

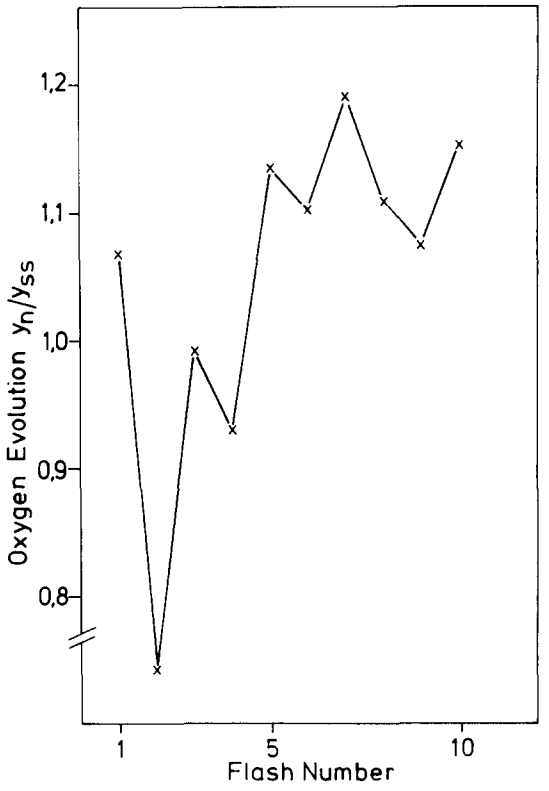

Fig. 5. Flash induced oxygen evolution pattern with thylakoids from *O. chalybea* in the presence of 0.01% H_2O_2. Flashes were spaced 300 ms apart after dark adaptation of 30 min.

at an intimate interrelationship between hydrogen decomposition and the S-state system of photosynthesis.

Although we discussed here a close relationship between the two phenomena, we also observed significant differences which might allow the distinction between S_3 and/or S_2 (peroxide?) on the one hand and states S_1 and S_0 on the other hand. The well-known general sensitivity of the water-splitting system was taken as parameter and confirmed for our conditions in a system with no $^{18}O_2$-gas atmosphere and 32% $H_2{}^{18}O$ in the liquid phase. Under such conditions the $m/e = 36$ signal comes exclusively from the photolysis of water ($H_2{}^{18}O$) and looses virtually all activity within 6–7 h (Fig. 6). Due to a relatively high $^{16}O_2$-partial pressure in the assay, oxygen evolution at $m/e = 32$ reflects here exclusively peroxide decomposition (minimal oxygen evolution derived from $H_2{}^{16}O$-splitting and contained in this signal can be neglected under such conditions) and its activity did not change during 6 h (Fig. 6). This might fit to the interpretation that the resulting O_2/H_2O_2-cycle, although linked to the S-state system of photosynthesis, comprises only few components around the light reaction of Photosystem II, thus making the phenomenon relatively unsensitive to deactivation and inhibitors.

When thylakoid preparations from several cyanobacteria are investigated on a rapid flash electrode, oxygen evolution patterns like the own shown in Fig. 1

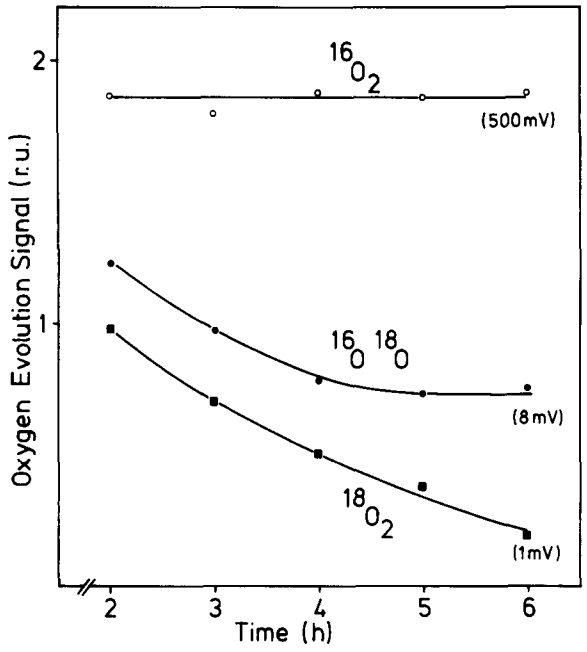

Fig. 6. Time dependence of the oxygen evolution in *Oscillatoria* thylakoids. The signals were recorded as the consequence of 20 light flashes in an air-saturated assay. Under this condition the mass-32 signal represents the peroxide decomposition. The assay was supplemented with $H_2{}^{18}O$ (32%) so that the signal at $m/e = 36$ reflects only photolytic water oxidation.

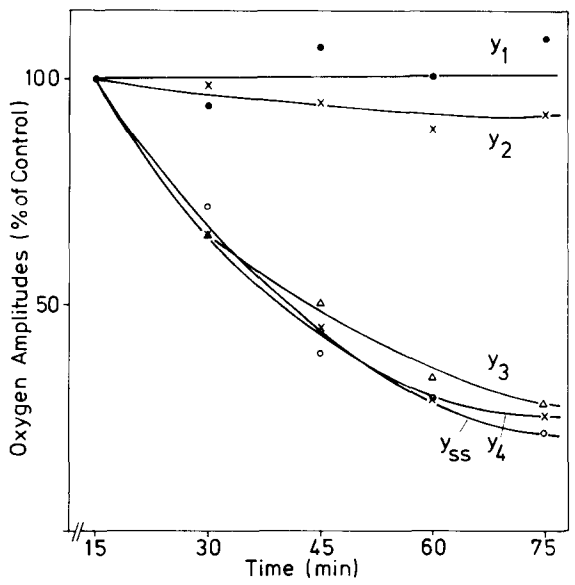

Fig. 7. Stability of the cyanobacterial preparation in respect of Photosystem II activity: decay of the oxygen evolution amplitudes under the first flash (Y_1), second flash (Y_2), third flash (Y_3), fourth flash (Y_4) and the steady state amplitudes (Y_{ss}). Dark time between flashes: 15 min.

are observed. The peculiarities consist mainly of the O_2-yield under the first two flashes and the very strong damping of the oscillation. Time deactivation curves clearly show that the Y_3–Y_{15} signals quickly decay with time, whereas the first two signals (Y_1 and Y_2) are completely unaffected (Fig. 7). The first two signals, however, are extremely sensitive to reagents that reoxidize the reduced primary acceptor of Photosystem II. It should be noted that Styring and Rutherford [25] have shown that if the acceptor side is oxidized, the stability of S_2 and S_3 is increased.

Cyanobacteria like *O. chalybea* proved to be specifically accessible to exogenously added substances. This has been shown for quaternary ammonium salts like ABDAC which interact with the membrane complexes leading to an improved functioning e.g., of the water splitting reaction [26]. One of the most obvious specificities in the structural organization of Photosystem II of cyanobacteria is the principal lack of the two extrinsic polypeptides of 16 and 23 kDa within the membrane region responsible for water oxidation. According to the membrane model developed by Homann [27] this lack would result in an increased and facilitated accessibility of this membrane complex. As the 23 kDa polypeptide in particular is supposed to regulate the

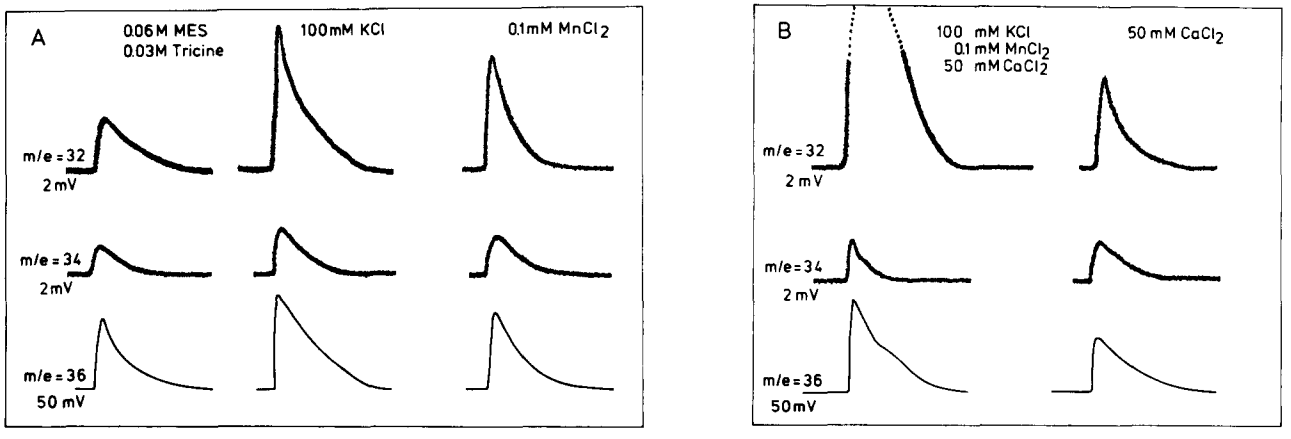

Fig. 8. Influence of various salts on the isotope distribution of the oxygen evolution measured at $m/e = 32$ ($^{16}O_2$), $m/e = 34$ ($^{16}O^{18}O$) and $m/e = 36$ ($^{18}O_2$). The reaction assay was flushed with nitrogen to remove the atmospheric oxygen. Then, $^{18}O_2$ was added to yield an $^{18}O_2$-partial pressure equivalent to the $^{16}O_2$-partial pressure of normal air saturated water. Under such conditions the signal at $m/e = 32$ represents only water-splitting reaction due to the absence of $^{16}O_2$; the signal at $m/e = 36$ is exclusively correlated to the peroxide decomposition, as the $^{18}O_2$-partial pressure is high and no $H_2{}^{18}O$ present in the system. (Salts were added at different concentrations to yield optimal effects.)

binding of chloride ions necessary for an optimal functioning of the water splitting process, one might suggest a strong sensitivity of cyanobacterial preparations towards low or high concentrations of chloride salts. (In its natural habitat this is surely no 'problem' for organisms like *Oscillatoria*, as this species usually lives in a salt water containing environment.) Fig. 8 shows that in fact the oxygen evolution signals from water oxidation can be largely increased by addition of various salts to *Oscillatoria* thylakoids. The different salts were added at different concentrations to yield optimal effects. Under conditions with a high $^{18}O_2$-background most of the light induced oxygen evolution comes from the peroxide decomposition and consequently the oxygen evolution at $m/e = 36$ remains almost unchanged upon salt addition.

Untreated chloroplasts from higher plants do not show any interaction with the oxygen from the atmosphere [11]. The isotopic composition of the evolved oxygen proved to be exactly the one that can be mathematically calculated under the assumption that the $H_2{}^{16}O/H_2{}^{18}O$-ratio in the aquaeous phase is known and only the process of water photolysis contributes to the oxygen evolution. Moreover, the ideal agreement between calculated and observed isotopic distribution of the oxygen evolution was independent of whether the gas atmosphere contained $^{18}O_2$, $^{16}O_2$ or nearly no oxygen at all. In the frame of the above mentioned Homann scheme this might be correlated with the evolutionary younger development of the 16 kDa and 23 kDa polypeptides of the oxygen evolving complex of higher plants. This might have resulted in a decreased accessibility of the complex to substances from outside. At least within very small and definite membrane regions like the OEC this might also hold true for gases like oxygen. If this speculation and interpretation was correct, salt washed inside-out particles from higher plants should be well suited to further investigate the phenomenon. As this treatment is known to remove the 16 kDa and 23 kDa polypeptides, an artificial cyanobacteria-like condition can be produced and should show a similar behaviour. When we examined such preparations in our mass spectrometric set-up, an interaction of the membrane complexes with oxygen was observed. This interaction manifested itself as $^{18}O_2$ uptake which was sensitive to DCMU, hence has to be correlated with Photosystem II [28]. Interaction of this type could never be observed with chloroplast- or untreated inside-out particle preparations. When cyanobacteria 'invented' oxygenic photosynthesis, they were confronted also with an increased oxygen concentration in the membrane-system. Thus, one of the possible speculations has been the idea that the described oxygen uptake mechanism provided useful means to keep oxygen partial pressure low in the respective membrane region e.g., in order to avoid 'uncontrolled' oxidation reactions. Moreover, hydrogen peroxide was used as an electron donor as the decomposition requires only two light quanta. Later in evolution the two extrinsic polypeptides were developed in order to protect more efficiently the inner regions of the OEC. Beside other effects oxygen uptake to produce peroxidic components as electron donors was no longer required, as molecular water had been developed as the sole electron donor for photosynthesis.

4. Discussion

Oxygenic photosynthesis confronts science with several peculiarities forming a paradox. Continuously running oxygenic photosynthesis supplied oxygen to a largely reducing atmosphere, a substance which is deleterious in many respects for a well-regulated metabolism. Furthermore, the question arises, how filamentous cyanobacteria 'invented' this process in the Precambrian environment. At that time, these organisms were most probably oxidizing preponderantly ferrous and sulfide ions. Thus, a mechanism for the effective lowering of the oxygen partial pressure at least in certain partitions and a strong pressure for the 'transition' from reductants like Fe^{2+} and S^{2-} to the more energy consuming molecular water have to be postulated. In context with this, hydrogen peroxide might have played a substantial role. In a series of papers we have described the interaction of filamentous cyanobacteria like *O. chalybea* with molecular oxygen. A substantial fast uptake is observed leading most probably to the formation of a peroxidic component (hydrogen peroxide) that is immediately re-oxidized by flash or low-light illumination. The result presents itself as a continuously turning-over O_2/H_2O_2-cycle, as the evolved oxygen is taken up again as part of the surrounding gas atmosphere. Under appropriate experimental conditions this process may largely exceed the water oxidation capacity of such organisms and can amount up to 90% of the oxygen evolved in the light. This might fit the idea that filamentous cyanobacteria were originally based on the oxidation of other reductants as were chemolitotrophs. All electron donors that are imaginable in this context, however, have one fundamental disadvantage in comparison to molecular water: they were exhausted with time and not ubiquitously available as is molecular water. Thus, at a given moment, selection pressure might have been sufficiently high to develop such an energy consuming process like the water splitting reaction. There, another problem arises. Nowadays, it is assumed that oxygenic photosynthesis can not have been developed in a completely anaerobic atmosphere; there must have been molecular oxygen already and an oxygen uptake mechanism is needed (e.g., the biosynthesis of chlorophyll *a*

requires molecular oxygen). The source of such *quasi* catalytical oxygen can easily be found by the assumption that hydrogen peroxide can be built even in a reducing atmosphere, e.g., by photolytic dissociation of water [29,30]. Thus, a primary oxidant would have been present. This hydrogen peroxide might have been accumulated by rainfall in suited surroundings like lakes with conditions favorable for cyanobacteria. This in turn would result in limited but substantially oxidizing conditions while in the overall atmosphere the anaerobic situation still prevailed. In such specific 'niches' oxygenic photosynthesis seems to have been evolved under the substantial participation of hydrogen peroxide. In this context it should also be mentioned that a significant oxygen uptake phenomenon in *O. chalybea* **prior** to oxygen evolution has been described by Bader and Schmid [13]. This process can be looked at as the obligatory uptake of small amounts of oxygen that is needed for photosynthesis and can be supplied by photolytic reactions. Oxygen uptake that leads to the formation of hydrogen peroxide might well represent the evolutionary link between mechanisms suited for the oxidation of ferrous and sulfide ions on one hand and the water oxidation reaction of Photosystem II on the other. In this context it should be noted that *O. limnetica* can photooxidize sulfide [31] and that higher plant chloroplasts do not show any interaction with oxygen from the surrounding gas atmosphere [15]. Moreover, a recent theoretical approach using a semi-empirical one-electron molecular orbital procedure, the extended Huckel method revealed that the predicted oxidation to dioxygen is energetically unfavorable and would require two-electron steps forming bound peroxide as an intermediate [32].

Acknowledgements

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