Studies on the O₂ Evolution under Flash Light Illumination in Preparations of *Anacystis nidulans*

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The O₂ yield under flash light illumination in preparations of *Anacystis nidulans* was examined in the presence of redox components such as ferricyanide and ferrocyanide. When ferricyanide (1 mM) was added to the lyophilized, lysozyme treated and EDTA washed cells of *A. nidulans* the familiar Joliot-Kok oscillation with maximum on the third flash and periodicity of four was observed (in the presence of added CaCl₂ and MnCl₂). However, when the ferricyanide concentration was increased to about 8 mM, a reduction of the O₂ evolution under flash illumination was observed, and an O₂ uptake could clearly be seen under the first and second flash. This O₂ uptake was inhibited by DCMU and o-phenanthroline, but was not inhibited by KCN or salicyldihydroxamic acid. As ferrocyanide was progressively added to 1 mM ferricyanide, the oscillations faded out and the steady-state O₂ evolution decreased. Light would progressively increase the O₂ evolution and reduce the damping of the oscillations.

Two patterns of O₂ evolution under short saturating light flashes were observed with these *Anacystis* preparations on first illumination in the presence of cations but in the absence of ferricyanide. One pattern corresponded to the typical pattern which has been repeatedly described in the literature, but the second pattern gave the indication of an initial “over-reduced” state of the photosystem II reaction center. With suitable procedures the two patterns were interchangeable. Moreover, the effect of L-arginine on the flash pattern was examined. The results are discussed in connection with the possible dual function of the previously described flavin protein as an L-amino acid oxidase and as a component of the O₂ evolving reaction center (E. K. Pistorius and H. Voss, Eur. J. Biochemistry 126, 203–209 (1982)).

**Introduction**

Despite a considerable amount of kinetic information about the S-state concept of photosynthetic water oxidation, the biochemical mechanism of O₂ evolution is still not understood. Recently, studies of photosynthetic O₂ evolution have concentrated on 3 membrane-bound polypeptides of approx. 33, 24 and 18 kDa. Moreover, it has been shown that the 24 and 18 kDa polypeptides can be substituted by high Ca²⁺ concentrations [1, 2] and that high Cl⁻ concentrations can replace the 33 kDa polypeptide for preserving the Mn bound to the O₂ evolving reaction center [3]. These experiments seem to favor the conclusion that both cations are directly bound to the reaction center of photosystem II.

We have shown that EDTA-extraction of *Anacystis nidulans* preparations leads to the loss of the O₂ evolution capacity and that the combined addition of Mn²⁺ and Ca²⁺ is required for restoration [4]. This dual requirement for Mn²⁺ and Ca²⁺ could be demonstrated on the O₂ evolution under steady-state conditions as well as on the O₂ evolution under flash illumination [5]. Photosynthetically active particles of the blue-green algae *A. nidulans* contain an L-amino acid oxidase, and this enzyme has a high specificity for the basic L-amino acids and is inhibited by o-phenanthroline, chlorpromazine and cations [6]. Our previous results suggested that this flavin protein might have a dual role: participating in the “water oxidation reaction” in the presence of certain cations (Mn²⁺ and Ca²⁺) and acting as an L-amino acid oxidase in the absence of these. This would imply that the enzyme is able to catalyze O₂ uptake and is also able to participate in the reaction sequence leading to O₂ evolution. Preliminary experiments indicate that the enzyme has two binding sites for divalent cations — one with high affinity and another with low affinity — (7 and un-
published results). The enzyme undergoes a conformational change (and possibly a change in the oxidation state) upon addition of e.g. Ca$^{2+}$ as can be seen by changes in the flavin spectrum of the enzyme [4]. Since the flavin protein is an extremely efficient oxidase with an unusual high turn-over number for an amino acid oxidase (turn-over number of 55 000 with L-arginine as substrate [6], we wanted to investigate whether an O$_2$ uptake could be observed during illumination even under conditions where O$_2$ evolution is normally favored (light and presence of cations). We were interested to see whether "extreme" solution redox conditions could e.g. counteract the cation mediated control of this flavin protein. That this can be demonstrated when the proper solution redox conditions are chosen, is shown in the present paper. We have also examined the effect of L-arginine which is a substrate of the L-amino acid oxidase, on the O$_2$ yield under flash illumination and on the S-state distribution.

**Materials and Methods**

*Anacystis nidulans* (*Synechococcus leopoliensis*) B 1402-1 was obtained from the Sammlung von Algenkulturen, Universität Göttingen. The growth of the cells was the same as in [8] and the preparation of the lyophilized and lysozyme treated cells of *A. nidulans* and the washing with EDTA have been described in [5]. The O$_2$ evolution under short light flashes and the O$_2$ gush was measured as described in [5]. The reaction mixture contained in a total volume of 1.85 ml: 100 μmol Mes-Tricine buffer (pH 7.3), 100 μmol CaCl$_2$ (54 mM), 0.2 μmol MnCl$_2$ (0.1 mM) and the lyophilized, lysozyme treated and EDTA washed *Anacystis* preparation containing 100–120 μg chlorophyll. 0.5 ml of this reaction mixture was used on the electrode.

Ferricyanide, ferrocyanide, L- or D-arginine was added to the sample on the electrode when indicated in the legends to the figures.

Preincubation with L- or D-arginine:

The reaction mixture contained in a total volume of 1.85 ml: 100 μmol Mes-Tricine (pH 7.3), 60 μmol L- or D-arginine and the Anacystis preparation containing 100–120 μg chlorophyll. The mixture was kept for approx. 1 h in ice. Then 100 μmol CaCl$_2$ and 0.2 μmol MnCl$_2$ were added, and 0.5 ml of this reaction mixture was used on the electrode.

**Results**

*Effect of ferricyanide and ferrocyanide*

The results obtained when increasing concentrations of ferricyanide were added to the Anacystis preparation, are given in Fig. 1. When the flash pattern without added ferricyanide (Fig. 1 A) was compared to that in the presence of 1 mM ferricyanide (Fig. 1 B), a general increase in O$_2$ evolution under flash illumination and an increase in the O$_2$ gush was observed. The steady state rate of O$_2$ evolution went up because of the addition of an electron acceptor. The ratio of the O$_2$ yield of the third to fourth flash was increased by ferricyanide and a reversal of the relative magnitude of Y$_5$ and Y$_6$ occurred, the minimum now being under the fifth flash. Moreover, the O$_2$ yield under the first and second flash became bigger. These observations are in agreement with the literature about the effect of ferricyanide on the flash pattern [9, 10].

When the ferricyanide concentration was increased to 4 mM, a reduction of the O$_2$ gush was
observed, although the steady state rate of O₂ evolution still went up because of the higher concentration of electron acceptor (Fig. 1 C). There was also an indication of an O₂ uptake under the first and second flash. Under these conditions the O₂ uptake was properly only transient and disappeared as soon as the suggested light-mediated conformational change of the water oxidation center [11] had occurred. However, the decrease in O₂ evolution under flash illumination became even more significant when the ferricyanide concentration was increased to 8 mM (Fig. 1 D). Now the steady state rate also decreased. Moreover, an O₂ uptake could clearly be observed under the first and second flash. In some preparations, the O₂ uptake exceeded the O₂ evolution. Therefore, an O₂ uptake was observed during the 30 s illumination period instead of the reduced O₂ evolution (Fig. 2B). This O₂ uptake was light-dependent and required in addition to the high ferricyanide concentration, the presence of Mn²⁺ and Ca²⁺. The O₂ uptake – as well as the O₂ evolution – was inhibited by DCMU (Fig. 2 A and B) and by 3 mM α-phenanthroline (not shown) but was not influenced by 1 mM KCN or by 1 mM salicylhydroxamic acid (not shown). The experiments with ferricyanide showed that relatively high ferricyanide concentrations (2 to 5 mM ferricyanide – depending on preparation) were required to obtain maximal rates of O₂ evolution under steady state conditions in the presence of 54 mM CaCl₂ and 0.1 mM MnCl₂. On the other hand, with ferricyanide concentrations of approx. 4 mM or above an O₂ uptake could be observed in addition to the O₂ evolution, and this O₂ uptake became most visible under the first and second flash where the O₂ evolution was low. Since the O₂ uptake only occurred at ferricyanide concentrations above 4 mM, this seemed to indicate that a high solution redox potential (above 550 mV) was required for this O₂ uptake. A sufficiently high solution redox potential could properly only be obtained with the higher ferricyanide concentrations because small amounts of some endogenous compounds or small amounts of ferrocyanide formed during the 5 min preincubation of the Anacystis preparations with ferricyanide on the electrode before the illumination, might have reduced the solution redox potential slightly. This would of course be more evident at the lower ferricyanide concentrations than at the higher ones.

When the solution redox potential was gradually brought down by increasing ferrocyanide concentrations (at constant ferricyanide concentration), the clear and well defined oscillations as seen under oxidizing conditions with 1 mM ferricyanide alone (Fig. 1B) disappeared and the O₂ evolution under steady-state conditions decreased. This was observed when the solution redox-potential was lower than approx. 440 mV. The results obtained with 1 mM ferricyanide and 6 mM ferrocyanide (solution redox potential approx. 414 mV) are given in Fig. 3. The pattern of O₂ evolution approached a monotonic climb to the steady state with no obvious oscillatory behavior. Light progressively increased the O₂ evolution and reduced the damping of the oscillation, and this effect could also be seen on the O₂ gush. Similar results as those in Fig. 3 (bottom curve) have been obtained when the O₂ yield of Chlorella has been investigated in the presence of
Fig. 3. O₂ yield in the presence of ferricyanide and ferrocyanide. The *Anacystis* preparation was suspended in Mes-Tricine buffer, pH 7.3, CaCl₂ (54 mM) and MnCl₂ (0.1 mM) was added. The ferricyanide concentration was 1 mM and the ferrocyanide concentration was 6 mM. ○—○ First illumination; ○—○ second illumination; ○—○ third illumination. A 5 min dark period was allowed between the various illuminations. A) O₂ evolution under flash illumination and B) O₂ gush.

redox components such as benzoquinone and hydroquinone in an atmosphere of 10 ppm O₂ by Greenbaum [12].

**Effect of L-arginine**

The cation concentration of Mn²⁺ and Ca²⁺ which was required to give a clearly detectable O₂ evolution under flash illumination, would block L-arginine oxidation totally. Therefore, it was not possible to show a direct effect of L-arginine on O₂ evolution under flash illumination under these conditions. However, the experiments with the high ferricyanide concentration indicated that there existed an O₂ uptake associated with photosystem II and that under these conditions the cation mediated conformational change probably was not totally effective. Therefore, we examined the effect of L-arginine (substrate of the L-amino acid oxidase) and D-arginine (inhibitor of L-arginine oxidation) under these conditions. The results are given in Fig. 4. L-arginine slightly stimulated the O₂ evolution but clearly decreased the O₂ uptake under the first and second flash. D-arginine had an inhibitory effect on the O₂ uptake as well as on the O₂ evolution, although the degree of the inhibition of the O₂ evolution varied considerably in various preparations, while the inhibitory effect on the

Fig. 4. Effect of L- or D-arginine addition in the presence of cations and ferricyanide. The *Anacystis* preparation was suspended in Mes-Tricine buffer, pH 7.3, CaCl₂ (54 mM) and MnCl₂ (0.1 mM) was added. A) No further additions, B) ferricyanide (8 mM) was added, C) ferricyanide (8 mM) and L-arginine (30 mM) was added, D) ferricyanide (8 mM) and D-arginine (30 mM) was added.
O₂ uptake was always seen. Therefore, it seemed that the most significant effect of arginine (especially L-arginine) was the reduction of the O₂ uptake under the first and second flash. This could be an indication that L-arginine had a protective effect on the reaction center under these condition — possibly by causing a conformation change and/or a change in the solution redox potential.

**Preincubation with L-arginine**

When the O₂ evolution with a sequence of short saturating light flashes in suitably dark adapted Anacystis preparations (EDTA-washed preparation in the presence of added Mn²⁺ and Ca²⁺ but without ferricyanide) was measured, two distinct patterns of O₂ evolution could be observed on first illumination (Fig. 5, curve A and curve B). Pattern A was the typical pattern which has been repeatedly described in the literature. The maximum O₂ was evolved under the third flash and the periodicity of the oscillation was four. The only difference of this sequence to that of e.g. Chlorella was an occasional small O₂ evolution under the first flash. Pattern B (Fig. 5, curve B) showed a reduced overall O₂ evolution when compared to curve A. The O₂ evolution under the first four flashes was low, but detectable and almost equal, the maximum O₂ evolution was observed under the fifth or sixth flash and then a damped oscillation with a periodicity of four followed. The two patterns of O₂ evolution were interchangeable. Firing a second flash series about two minutes after the first flash sequence (sometimes more than one flash series was required to obtain maximum O₂ evolution) would change the O₂ evolution pattern B to pattern A. On the other hand, preparations with a flash pattern A changed occasionally (but not always) to a pattern B when standing in ice for one or two hours.

Since the O₂ evolution pattern of whole cells of *A. nidulans* also gave the impression of an “over-reduced” form with the maximal O₂ yield under the fourth or fifth flash (not shown), the pattern B did not seem to be a consequence of the preparation procedure. Moreover, patterns of the B-type which gave the impression of a more reduced form of the S-states, have been described for other algae [13, 14].

Since the modification of pattern B might be caused by an endogenous substance, we have preincubated both types of preparations with L-arginine in the absence of added cations. After the preincubation, Ca²⁺ and Mn²⁺ was added and the sample was used on the electrode. In those preparations which would develop a pattern of the B-type, L-arginine would speed up this development. On the other hand, in those preparations which kept a pattern of the A-type when standing in ice, L-arginine had the effect as shown in Fig. 6. We used D-arginine as a control. In these preparations L-arginine altered the ratio of the O₂ yield under the third to the fourth flash from 1.77 with D-arginine (as control) to 1.53 with L-arginine. This indicated that L-arginine caused a partial reduction of the S₁-state to the S₀-state. These results show that in both types of preparation L-arginine changed the distribution of the S-states in favor of the more reduced states — only the degree to which this happened, depended on the preparation. This properly indicated that the small peptides (and/or residual cations) were more or less tightly bound to the reaction center and therefore made the L-arginine effect more or less evident. Since the Anacystis preparation contained
Fig. 6. Effect of preincubation with L- or D-arginine on the flash pattern. The *Anacystis* preparation was suspended in Mes-Tricine buffer, pH 7.3, and preincubated with L-arginine (○—○) or with D-arginine (●—●) for about 1 h. Then CaCl₂ (54 mM) and MnCl₂ (0.1 mM) were added and the sample was used on the electrode.

the L-amino acid oxidase, addition of L-arginine (in the absence of added cations) would cause a reduction of the O₂ concentration in the solution, since the O₂ was consumed in the oxidation of the L-arginine. Whether the effect of L-arginine on the O₂ evolution pattern was a consequence of the reduced O₂ concentration in the solution or a direct effect of L-arginine on the reaction center can not be decided at the present time.

**Discussion**

*Anacystis nidulans* has some peculiarities of the O₂ metabolism in the light. It has previously been shown that in whole cells of *A. nidulans* and in a number of other blue-green algae a substantial amount of H₂O₂ is formed during the initial period of illumination. Patterson and Myers [15] concluded that the H₂O₂ is derived from photosynthetic electron transport and that a substance at the reducing site of photosystem I is the most likely site of H₂O₂ production. On the other hand, there are reports on O₂ uptake which seems to be associated with photosystem II. Margolina *et al.* [16] reported on an O₂ uptake in thylakoid preparation of *A. nidulans* in the presence of silicomolybdic acid and DCMU, and they believe that the O₂ uptake under those conditions occurred in photosystem II. Remy [17] has studied the development of photosynthetic activities in etiolated wheat seedlings. At the very early stages of greening he observed a high rate of O₂ uptake which he believed to be associated with photosystem II. This O₂ uptake eventually disappeared with increasing illumination. Franck and Schmid [18] recently showed a similar phenomenon with preparations of oat. These observations should be seen in connection with the effect of extraction and re-addition of Mn²⁺ on the light reactions of photosystem II preparations of peas examined by Klimov *et al.* [19]. Although in these preparations the photoreduction of Q was observed after readdition of Mn²⁺, the O₂ evolution with ferricyanide was small. They believed that this was due to interference with photoinduced O₂ consumption. A connection to the O₂ uptake which we observe in the *Anacystis* preparations in the presence of high ferricyanide concentrations, seems to be obvious. The O₂ uptake which we observe, had the same requirements as the O₂ evolution (light and cations) and also the same inhibitors (DCMU and α-phenanthroline), but was only seen at relatively high ferricyanide concentrations. The DCMU (and α-phenanthroline) inhibition showed that the O₂ uptake was inhibited in the same way as the O₂ evolution when the electron flow was prevented through photosystem II. On the other hand, the DCMU inhibition of the O₂ uptake could also be interpreted that the compound which reacted with O₂, was located between the two photosystems or at the reducing site of photosystem I. However, the required high ferricyanide concentration which we believe to be an indication of a requirement for a high solution redox potential, made the association of the O₂ uptake with photosystem II more likely.

It has been long known that changes in the redox conditions of the environment could significantly influence the O₂ yield and the distribution of the S-states [10]. Since blue-green algae have a more loosely organized thylakoid structure [20] and since in *Anacystis* the cations (Mn²⁺ and Ca²⁺) can be extracted relatively easily [4], the redox control of the O₂ evolution in the *Anacystis* preparation might be more unmasked than in photosynthetic eucaryotes. This could have increased the access of ferricyanide or ferrocyanide to the reaction center of photosystem II. A direct interaction of ferricyanide with "Q₅" has recently been shown by Ghanotykis *et al.*
[21] in Tris-treated and DCMU-inhibited chloroplasts. It seems very likely that the flavin protein which is a very efficient oxidase, actually is the enzyme which reacts with \( \text{O}_2 \) under certain conditions even in the light and in the presence of cations. However, to be absolutely sure further experiments are required and especially the redox properties of this protein (in the presence and absence of cations) should be studied.

Finally, the reason why this relationship between the oxidase activity and water splitting function of the flavin enzyme becomes evident in \textit{A. nidulans} might be an evolutionary one. Introduction of photosystem II in the electron transport of blue-green algae might have completed the oxygenic photosynthesis by replacing substrate dehydrogenases or by modifying substrate dehydrogenases in a way that the enzyme can function as a "water-dehydrogenase" as well as a substrate dehydrogenase. The latter seems to be the case in \textit{A. nidulans}. This is supported by the observation by Flores et al. [22] who studied the production of ammonia from basic amino acids catalyzed by whole cells of \textit{A. nidulans}. They observed that the L-arginine dependent \( \text{O}_2 \) uptake in the light in whole cells is lower than the concomittent ammonia production which suggests that part of the reducing power resulting from the oxidation of the amino acid would be channeled to acceptors other than molecular \( \text{O}_2 \). It should also be mentioned that the metabolism of L-arginine in cyanobacteria is of particular interest since the urea cycle contributes significantly to \( \text{CO}_2 \) fixation of these organisms [23] and also because they characteristically contain a polymer of L-aspartate and L-arginine called cyanophycin [24].

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