Inactivation of Photosynthetic O_2 Evolution in the Cyanobacterium *Anacystis nidulans* PCC 6301: Influence of Nitrogen Metabolites and Divalent Cation Concentration

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An investigation about the *in vivo* inactivation of photosynthetic water oxidation has been carried out in the cyanobacterium *Anacystis nidulans* (*Synechococcus* PCC 6301). Photosystem II and photosystem I activity as well as the relative amount of the D 1 and manganese stabilizing peptide of photosystem II were determined after growing the cells in nutrient media with variations in the nitrogen source and the concentration of the major divalent cations (Mg^{2+} and Ca^{2+}). The results show a rapid inactivation of water oxidation in *A. nidulans* in response to nitrogen deficiency and in response to reduced Mg^{2+} and Ca^{2+} concentrations. The inactivation of water oxidation observed under divalent cation deficiency could be greatly accelerated when L-amino acids instead of ammonia or nitrate were used as nitrogen source. Under these conditions inactivation of water oxidation correlated with a rapid loss of D 1 and with a slower loss of the manganese stabilizing peptide from photosystem II. A possible regulation of the photosystem II activity in *A. nidulans* by nitrogen metabolites is suggested.

Introduction

After CO_2 fixation, the photosynthetic reduction of nitrate to ammonia is quantitatively the most important reductive process in cyanobacteria. The maintenance of a balanced assimilation of these two major inorganic substrates is important for the cells and would require an efficient regulation of both anabolic pathways. While the relationship between photosynthesis and nitrogen metabolism has been well examined with respect to the influence of photosynthesis on nitrogen assimilation, very little information exists about the influence of nitrogen metabolites on photosynthesis [1, 2]. It is well established that ammonia is an inhibitor of water oxidation [3] and an uncoupler of photosynthetic ATP-synthesis [4], but it is unclear how significant the ammonia inhibition might be under *in vivo* conditions. A possible regulatory function of nitrogen metabolites on photosynthetic water oxidation also seems likely if our model of the water oxidizing enzyme is taken into consideration [5, 6]. This hypothetical model suggests that the water oxidizing enzyme has evolved from a substrate dehydrogenase type enzyme which originally had an L-amino acid dehydrogenase (oxidase) activity with high specificity for basic L-amino acids (such as L-arginine) and which could mediate electron flow from L-arginine to the plastoquinone pool of the electron transport chain.

There are several reports in the literature showing a rapid inactivation of photosynthetic water oxidation to occur in nonheterocystous cyanobacteria under various *in vivo* conditions. Allen *et al.* [2] have shown an inactivation of water oxidation in *Synechocystis* PCC 6803 as a consequence of nitrogen deficiency. In *Anacystis nidulans* (*Synechococcus* UTEX 625) an inactivation of O_2 evolution has been reported to occur under Ca^{2+} and Na^+ deficiency (in the presence of a divalent cation chelator, such as glycylglycine) [7], and in *Synechocystis* PCC 6714 inactivation occurs under Na^+ deficiency [8]. In our experiments with *A. nidulans* (PCC 6301) an inactivation of water oxidation was observed to occur as a consequence of Mg^{2+} and Ca^{2+} deficiency (Na^+ ions were present and no chelator for divalent cations was added) [5]. The nature of the inhibition of the water oxidation under these various *in vivo* conditions is unclear.

Abbreviations: Chl, chlorophyll; PS, photosystem; MSP, manganese stabilizing peptide.

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Since *Synechocystis* and *Synechococcus* species show such a fairly rapid *in vivo* inactivation of water oxidation, and since Wallen and Allan [9] have shown that the cyanobacterium *A. nidulans* (PCC 6301) can grow well on various L-amino acids as sole nitrogen source, we selected *A. nidulans* to investigate the influence of nitrogen metabolites on water oxidation. Moreover, the interrelationship between cation concentration and L-amino acid (or ammonia) concentration on the *in vivo* inactivation of water oxidation and on the degradation (or synthesis) of photosystem II peptides has been examined in this paper. We have chosen the psbA gene product (D 1 of 30–32 kDa) and the psbO gene product (the manganese stabilizing peptide (MSP) of 28 kDa) as marker for PS II [6].

Materials and Methods

Growth of cells, preparation of cell suspensions and French press extracts

Anacystis nidulans (*Synechococcus* SAUG 1402-1 or PCC 6301) was grown on a culture medium according to Kratz and Myers [10] with some modifications as described in [5]. The growth conditions were varied with respect to nitrogen source and divalent cation concentration. The various nitrogen sources were: 10 mM KNO_3 or 15 mM NH_4Cl (the medium was buffered with 50 mM Hepes-NaOH, pH 7.6) or various L-amino acids (5 or 15 mM as indicated in the legends to the figures), and the divalent cation concentration was either the regular concentration (1 mM $MgSO_4$ and 0.1 mM $CaCl_2$) or a reduced divalent cation concentration (0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$). The preparation of cell suspensions and French press extracts was the same as described previously [5], except that the cells were resuspended in 10 mM sodium phosphate buffer, pH 7.0 (100 µl cells/ml). Chlorophyll content was determined according to [11].

Activity measurements

Photosynthetic O_2 evolution was determined with cell suspensions in a Clark type electrode (Yellow Springs Instruments Co., Model 53). In a total volume of 3 ml the reaction mixture contained: 54 mM Hepes-NaOH, pH 7, 0.5 mM phenyl-*p*-benzoquinone, and 2.5 to 5 µl *Anacystis* cells.

The PS I activity was determined as O_2 uptake with French press extracts of *A. nidulans* obtained from cell suspensions containing 100 µl *Anacystis* cells/ml 10 mM sodium phosphate buffer, pH 7. The reaction mixture (3 ml) contained: 54 mM Hepes-NaOH, pH 7, 0.4 mM KCN, 0.17 mM methylviologen, 3.3 mM sodium ascorbate, 0.08 mM 2,6-dichlorophenol indophenol, 0.01 mM 3-(3,4-dichloro-phenyl)-1,1-dimethylurea, and 50 to 100 µl French press extract.

The PS I and PS II activity are given as µmol O_2 taken up or evolved/100 µl cells × h in the figures. We did not use the chlorophyll content of the cells as a reference value, but presented the chlorophyll values separately in the figures, since under some of the growth conditions bleaching of the cells was observed due to degradation of phycobiliproteins and reduction of chlorophyll. The values for PS II and PS I activity on chlorophyll basis can be easily calculated from the numbers given in the figures and are not presented in a separate graph for shortness. As can be seen from the figures, under some of the growth conditions the antenna size was altered. Therefore, we always made sure that the light intensity was high enough to obtain maximal rates.

SDS PAGE and immunoblots

Polypeptide composition of French press extracts was examined by SDS PAGE according to Laemmli [12] with slight modifications (gradient gels 8 to 12% polyacrylamide – size 8.5 × 13 cm). The samples were denatured by incubating with denaturating buffer for 1 h at room temperature and in addition for 30 min at 37 °C. Immunoblots were performed as described in [6]. The first antibodies were diluted as follows: antibody raised against D 1 from oat was diluted 1:30, and the antibody raised against the MSP from oat was diluted 1:50. In Fig. 3 B the complete immunoblot is shown, while in the other figures the relevant areas of the blots are only shown.

Results and Discussion

In vivo inactivation of photosynthetic water oxidation: Effect of nitrogen deficiency

Under our growth conditions, *A. nidulans* showed a fairly linear growth rate on nitrate and

regular cation concentration for the first three days (Fig. 1 A). The cells grew equally well or even better on ammonia under these conditions (not shown: the growth rate was 1.6, 4.4 and 8.4 µl cells/ml growth medium, and the O_2 evolution rate was 286, 340 and 170 µmol O_2 evolved/100 µl cells × h for the 1st, 2nd, and 3rd day, respectively). When the cells were kept in nitrogen deficient medium a very rapid inactivation of photosynthetic O_2 evolution was observed. The activity dropped to less than 20% of the starting activity within 24 h and was zero after 48 h. Only a minor reduction of the PS I activity was observed after 24 h, even after 3 days 40% of the PS I activity could still be detected (Fig. 1 A). Parallel to the inactivation of PS II an almost total loss of MSP and a significant loss of D 1 could be seen after 24 h (Fig. 1 B). During the degradation of D 1 a noticable amount of a transient 25 kDa degradation product could be detected. A similar degradation product has been shown bo be formed during degradation of D 1 in algae and plants [13]. When in the nitrogen deficient medium the cation concentration was reduced in addition, the inactivation rate of PS II was essentially the same (not shown – 80% inactivation of water oxidation after 24 h). However, under these conditions the reduction of D 1 was faster than the reduction of MSP, while under nitrogen deficiency in the presence of the regular cation concentration the degradation of MSP was faster than the D 1 degradation (or about equal) (Fig. 1 B).

We showed previously that a reduced growth rate and a partial inactivation of PS II occurred temporarily when the cells were transferred from a nitrate medium to a medium containing 5 mM L-arginine as sole nitrogen source (regular divalent cation concentration). Under such conditions regular growth started after about 24 h [5]. Since the doubling time of the cells under our growth condi-

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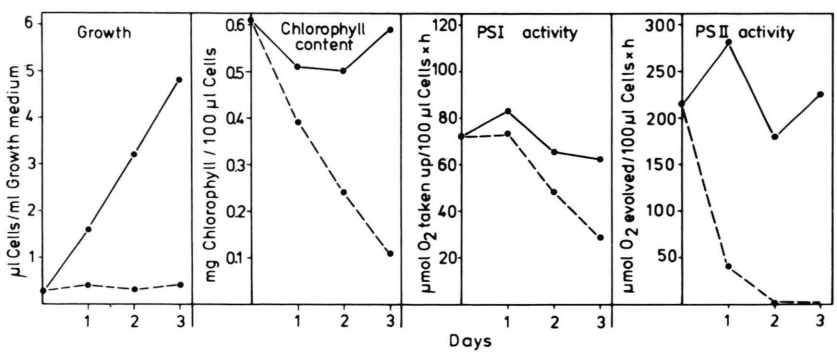

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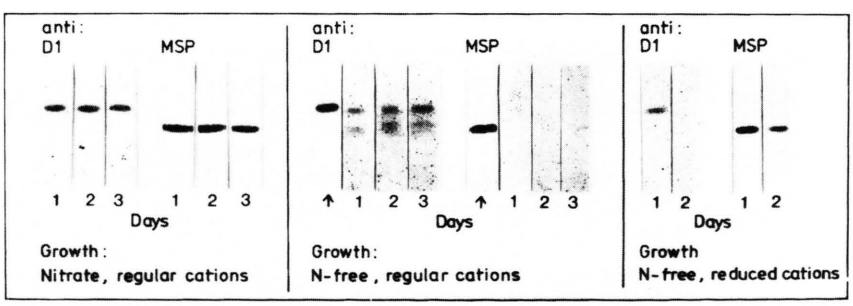
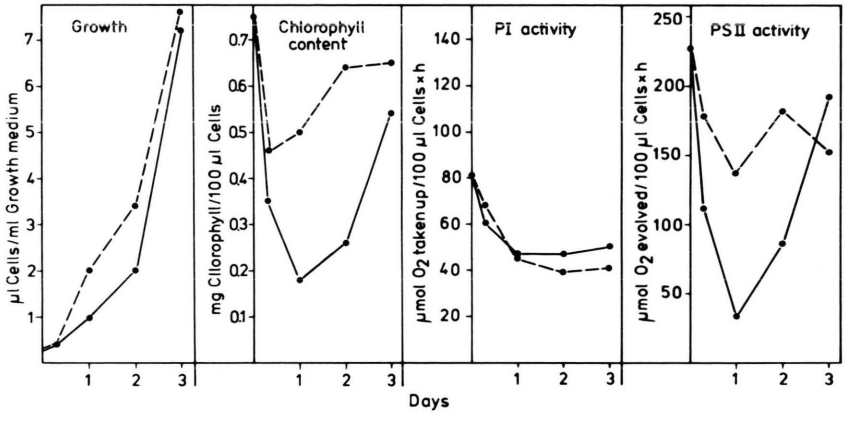


Fig. 1. Influence of nitrogen deficiency on PS I and PS II activity. A: *Anacystis* cells were grown on nitrate and regular cation concentration (●—●) or kept in a nitrogen free medium with the regular cation concentration (●– – –●) for 3 days. The growth rate, chlorophyll content of the cells and the PS I and PS II activity are given for the various growth conditions. B: The corresponding immunoblots to Fig. 1 A with the anti-D 1 and anti-MSP. In addition the immunoblots for cells kept in nitrogen free medium with the reduced cation concentration is shown. The immunoblot with the French press extract of the starting cells is marked with an arrow.

tions is about 12 h, this is a fairly long delay of the linear growth rate. Here we show that the lag phase was due to an initial degradation of D 1 and MSP (to about the same extent) (Fig. 2 A and B). These results indicate that the cells temporarily suffered from nitrogen deficiency after the transfer, and as shown above this led to degradation of D 1 and MSP. Linear growth started after the cells had adjusted to the new nitrogen source and after PS II had been repaired.

A. nidulans contains a highly active constitutive L-amino acid oxidase which we believe to be the precursor form of the water oxidizing enzyme [5, 6]. This enzyme has a high specificity for basic L-amino acids (L-arginine is the best substrate) and is inhibited by divalent cations (such as Mg^{2+} and Ca^{2+}) in a partly competitive manner which means that the L-amino acid substrates and divalent cations have an antagonistic effect on the L-amino acid oxidase activity of this flavoprotein [5]. Therefore, we were interested to investigate under which conditions this enzyme could supply sufficient NH_4^+ from L-arginine to prevent the initial damage of PS II. As the results of Fig. 2 A and 3 A show, this enzyme is indeed able to provide the cells with sufficient NH_4^+ from L-arginine initially after the transfer from nitrate to L-arginine (before new enzyme synthesis occurs), but only under conditions if either the divalent cation concentration in the medium was reduced (Fig. 3 A, curve ●– – –●) or the L-arginine concentration was increased above 5 mM (Fig. 2 A, curve ●– – –●), while at low L-arginine concentration and regular cation concentration (Fig. 2 A, curve ●—●) the cells initially suffered from nitrogen deficiency. These results indicate that a complex correlation

A



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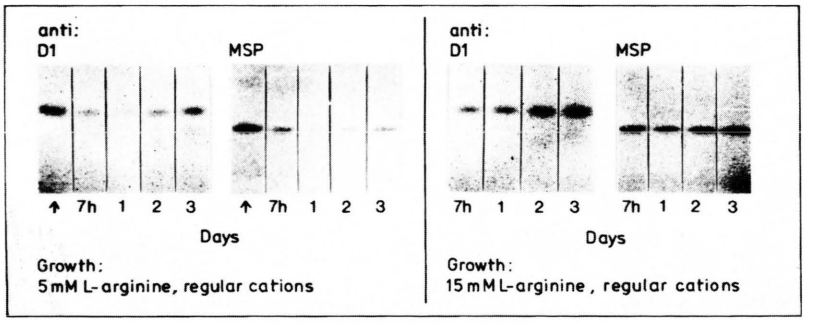
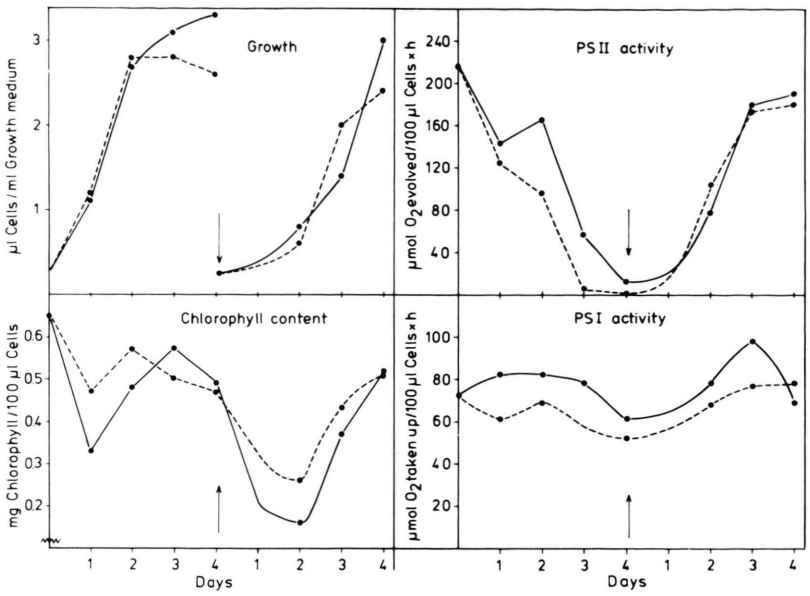


Fig. 2. Growth of *A. nidulans* on low and high L-arginine concentrations. A: *Anacystis* cells were grown for 3 days on 5 mM L-arginine and regular cation concentration (●—●) or on 15 mM L-arginine and regular cation concentration (●– – –●). B: The corresponding immunoblots to Fig. 2 A with the anti-D 1 and anti-MSP. The immunoblot with the French press extract of the starting cells is marked with an arrow.

A



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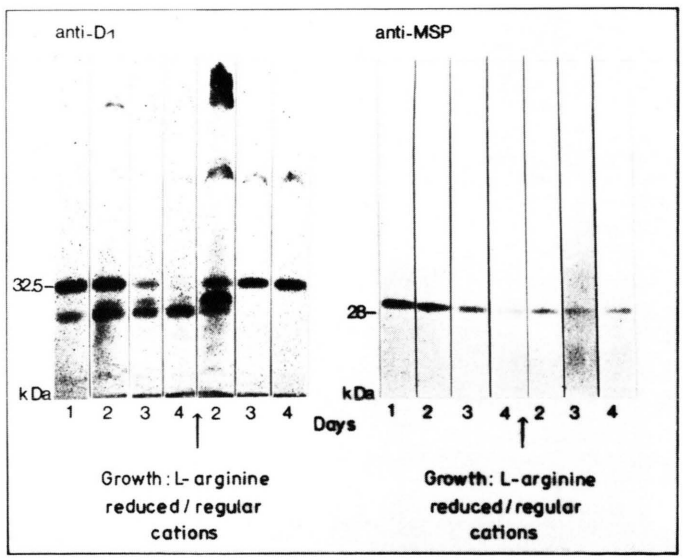


Fig. 3. Influence of cation deficiency on PS I and PS II activity. A: *Anacystis* cells were grown for 4 days in a medium with reduced cation concentration (0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$) either on 10 mM KNO_3 (●—●), or on 5 mM L-arginine as nitrogen source (●– – –●). After 4 days the cation concentration was changed to the regular concentration (1 mM $MgSO_4$ and 0.1 mM $CaCl_2$) indicated by the arrow. B: The corresponding immunoblots to Fig. 3A with the anti-D1 and anti-MSP.

exists between L-arginine and cation concentration with respect to the participation of the constitutive L-amino acid oxidase in L-arginine degradation in *A. nidulans*. Qualitatively similar results for a temporary inactivation of PS II after transfer from a nitrate to an L-amino acid containing medium was seen with all L-amino acids tested. The damage of PS II varied relatively to the effectiveness with which the cells could adjust to the new nitrogen source.

In vivo inactivation of water oxidation: Influence of cation and L-amino acid concentration

In Fig. 3A the inactivation of water oxidation as a consequence of Mg^{2+} and Ca^{2+} deficiency is shown. Transfer of the cells to a medium with re-

duced divalent cations led to an initial reduction of the chlorophyll content per cell (for about 24 h), but no significant decrease in O_2 evolution – on the contrary on chlorophyll basis the PS II activity increased in some experiments after 24 h (Fig. 3A). Since the linear growth rate was not greatly affected for the first 48 h, it seemed that the relative rate of synthesis of the antenna pigments was slower than that of the PS II reaction center under Mg^{2+} deficiency. The inactivation of water oxidation due to cation deficiency started after about 48 h, and almost complete inactivation was reached after 4 days. No significant change in the PS I activity was observed during the whole period of 4 days. This total inactivation of O_2 evolution was not reversed by just adding Ca^{2+} (and/or Mg^{2+}) to the cell suspension. Under the conditions here described, the reactivation of O_2 evolution required *de novo* protein synthesis (see later). When L-arginine instead of nitrate was used as nitrogen source, the inactivation rate was faster, since total inactivation was reached after 3 days (Fig. 3A).

Under cation deficient growth conditions the D1 content in PS II was reduced (Fig. 3B), and the inactivation of water oxidation essentially paralleled the disappearance of D1. Again a transient 25 kDa degradation product of D1 was observed. Moreover, aggregation products of D1 were present in some gels. Eventually the MSP content was also decreased, but much slower than the D1 content. When the cation concentration in the nutrient medium was increased to the regular level, D1 and MSP were resynthesized, and O_2 evolution increased to normal values after a lag period of about 24 h required for repairing PS II under these conditions.

Since *in vivo* inactivation of water oxidation due to divalent cation deficiency was faster with L-arginine than with nitrate as nitrogen source (Fig. 3A), various L-amino acids were tested under these conditions. The results with L-glutamine and L-alanine as well as with ammonia as control (reduced cations) are shown in Fig. 4A and 4B. The inactivation rate with ammonia as N-source was

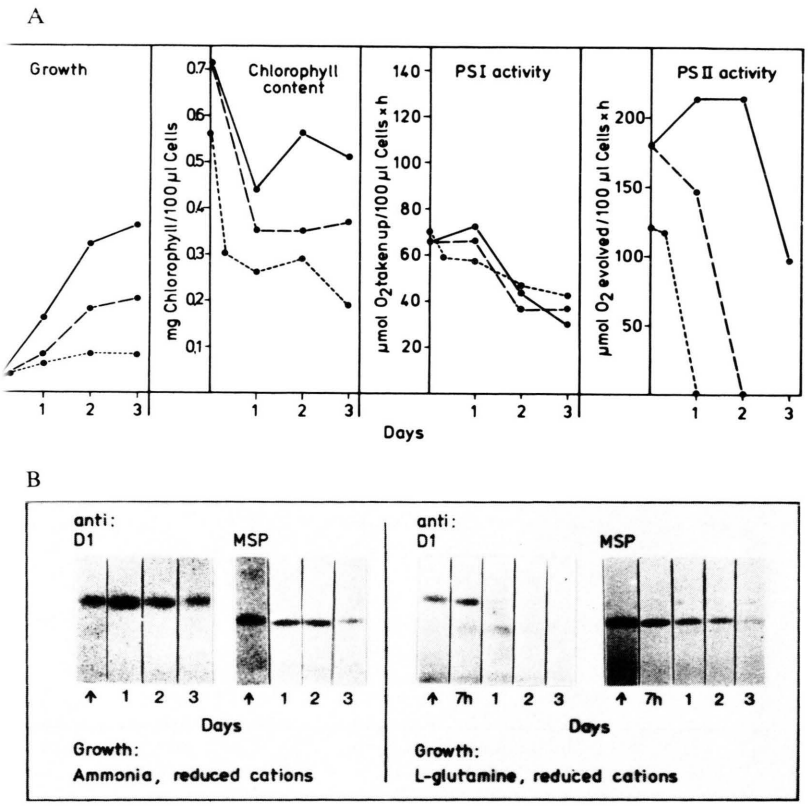
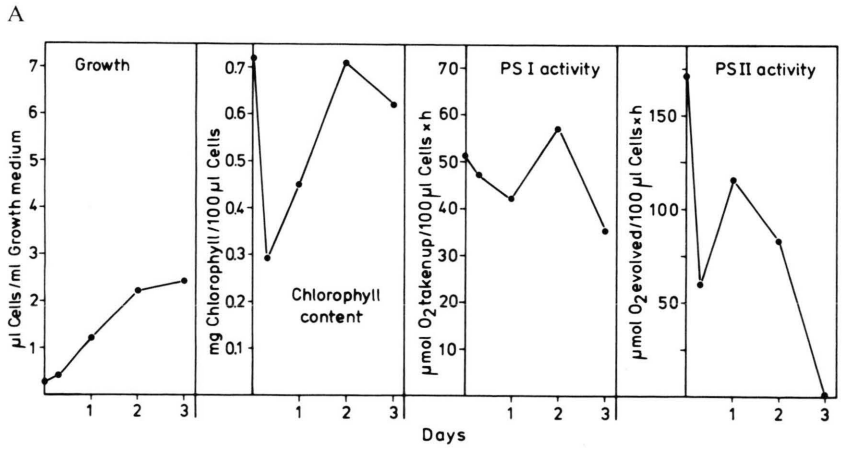


Fig. 4. Growth of *A. nidulans* on various L-amino acids and ammonia as nitrogen source. A: *Anacystis* cells were grown for 3 days in a medium with reduced cation concentration. The nitrogen source was 15 mM NH_4Cl ●—●, 15 mM L-alanine ●– – –●, or 15 mM L-glutamine ●-----●. When the cells were grown in a medium with regular cation concentration and 15 mM L-alanine as N-source the growth rate was 1.2, 4.0 and 5.4 µl cells/ml and the PS II activity was 164, 178, and 182 µmol O_2 evolved/100 µl cells × h for the 1st, 2nd, and 3rd day, respectively. With L-glutamine as N-source the values were: Growth rate 1.2, 2.8, and 4.0 µl cells/ml and PS II activity 85, 159, and 132 µmol O_2 evolved/100 µl cells × h for 1st, 2nd, and 3rd day, respectively. For the corresponding values with NH_4Cl see text. B: The corresponding immunoblots to Fig. 4A with the anti-D1 and anti-MSP for the experiments with ammonia and L-glutamine as nitrogen source. The immunoblot with the French press extract of the starting cells is marked with an arrow.

basically the same as with nitrate, while the inactivation of PS II was greatly accelerated in the presence of L-alanine or L-glutamine. Glutamine was more effective than any of the other tested L-amino acids (except glycine – see below). The inactivation rate was dependent on the intitial L-amino acid concentration in the growth medium, *e.g.* within 24 h 20% and 75 to 100% inactivation was reached with 5 and 15 mM L-glutamine, respectively.

In the presence of all tested L-amino acids, the cells excreted substantial amounts of ammonia into the medium (not shown). These results indicate that the L-amino acids were taken up by *A. nidulans* rapidly and that they were effectively metabolized by the cells under NH_4^+ production. This might temporarily lead to a high NH_4^+ concentration in the cells. This effect of L-amino acids on the inactivation rate of water oxidation was most pronounced in the presence of the reduced cation concentration in the nutrient medium, while no or only minor effects of L-amino acids on water oxidation were observed in the presence of the regular cation concentration (values are given in the legend to Fig. 4A). Possibly divalent cations could serve as exchange cations for NH_4^+ and thus help to detoxify the cells. This detoxification might be impaired under cation deficiency. As the results clearly show, the degree of PS II inactivation depended on the relative L-amino acid to divalent cation concentration in the nutrient medium.

In Fig. 5 the influence of glycine on O_2 evolution is shown. From all tested amino acids, glycine was most effective in causing inactivation of PS II, because glycine was rapidly metabolized by the cells under NH_4^+ production and in addition glycine is a chelator for divalent cations. This inactivation rate is comparable to that described by Becker and Brand [7] for *Anacystis* in the presence of glycylglycine as a chelator for divalent cations. The results also show that as soon as glycine was metabolized by the cells, a rapid reactivation of PS II is observed for a short time. Eventually the subsequent inactivation rate observed in the experiment of Fig. 5 is similar to that with NH_4^+ as nitrogen source.



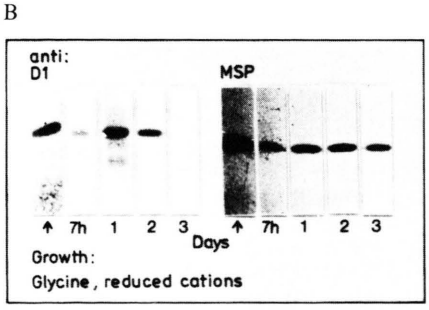


Fig. 5. Influence of glycine on the PS I and PS II activity. A: *Anacystis* cells were grown in a medium with reduced cation concentration and with 15 mM glycine as nitrogen source. B: The corresponding immunoblots to Fig. 5A with the anti-D1 and anti-MSP. The immunoblot with the French press extract of the starting cells is marked with an arrow.

As the corresponding immunoblots (Fig. 3B, 4B, and 5B) show, under cation deficiency the most pronounced effect was on D1. The inactivation of water oxidation paralleled the decrease of D1, while the reduction of MSP was much slower than that of D1 under these conditions. These results again demonstrate that the switch off/switch on mechanism for PS II activity *via* D1 degradation/synthesis is a relatively fast process (Fig. 5A and B – compare 7 and 24 h values).

Concluding remarks

Several groups [14–19] have shown that the D1 polypeptide exhibits a high rate of light dependent turnover relative to other PS II proteins, *e.g.* the granal D1 of *Spirodela* cultures has been reported to have a half-life of 6 to 12 h [14, 15]. The rapid turnover of D1 is observed in all plants, algae and cyanobacteria studied to date [15–19]. Several studies have provided evidence that PS II is the site of photoinhibition and that recovery from photoinhibition damage may rely on turnover and synthesis of D1 [16]. Whether chemical modification of the Q_B protein initiates the proteolytic degradation of the damaged electron carrier or whether the rapid turnover of D1 may be related to a control mechanism for the PS II function is unclear [20]. In this respect, it is interesting that cyanobacteria possess small psbA multigene families, *e.g.* the cyanobacterium *Synechococcos* PCC 7942 contains 3 distinct individual copies of psbA encoding two different forms of D1 [21, 22]. Schaefer and Golden [22] have shown that light availability influences the ratio of two forms of D1 in cyanobacteria thylakoids.

Our results with the cyanobacterium *Anacystis nidulans* PCC 6301 show that inactivation of water oxidation occurs under nitrogen deficiency and also under divalent cation deficiency (mainly Mg^{2+} deficiency). The inactivation seen under divalent cation deficiency is greatly accelerated when amino acids are present, such as L-glutamine, which seems to be rapidly metabolized by the cells under NH_4^+ production [23]. Possibly the inhibition of PS II seen under nitrogen deficiency and under high concentrations of L-amino acids might be related, since under nitrogen deficiency *Anacystis* cells will mobilize their nitrogen reserves (such as phycobiliproteins), and this might temporarily also lead to an increased L-amino acid concentration in the cells and the above described consequences for PS II.

Especially under cation deficiency as well as under cation deficiency in the presence of L-amino acids in the nutrient medium the inactivation of O_2 evolution correlates well with the disappearance of the D1 peptide indicating that the process of degradation and synthesis of D1 is out of balance. We think that under our experimental conditions new synthesis of D1 is most likely prevented due to lack of Mg^{2+} which is required for ATP synthesis. This could be due to the fact that amino acids by themselves (as glycine) or amino acid metabolites might chelate divalent cations or that the degradation of the L-amino acids might temporarily lead to a high NH_4^+ concentration in the cells. As a consequence, the water oxidizing enzyme might become inhibited by NH_4^+, or NH_4^+ might cause uncoupling of photosynthetic phosphorylation. As a consequence ATP becomes deficient and protein synthesis will be impaired. Because of the high turnover of D1, this peptide will be more affected than any of the other PS II peptides when protein synthesis is impaired.

In conclusion it can be said that *A. nidulans* seems to be a good model system to investigate the influence of nitrogen metabolites on photosynthesis. It is quite obvious that the effects of different nitrogen sources and different concentrations of divalent cations on O_2 evolution are rather complex and not completely understood. Nevertheless, we think that it is justified to consider a possible regulatory function of the rapid turnover of D1 in coordinating nitrogen metabolisms with photosynthetic water oxidation.

Acknowledgements

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