Interrelation between Cyanophycin Synthesis, L-Arginine Catabolism and Photosynthesis in the Cyanobacterium *Synechocystis* Sp. Strain PCC 6803

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Z. Naturforsch. **55c,** 927-942 (2000); received October 5, 2000

Dedicated to Professor Dr. Wilhelm Menke on the occasion of his 90th birthday

L-Arginine Catabolism, Cyanophycin, Ultrastructural Analysis

Ultrastructural and immunocytochemical investigations gave evidence that cyanophycin (multi-L-arginyl-poly-L-aspartate) granules accumulate in the cyanobacterium *Synechocystis* sp. strain PCC 6803 under nutrient deficient growth conditions, especially under phosphate limitation. Besides nutrient deficiency, growth of *Synechocystis* PCC 6803 on L-arginine or L-asparagine as sole N-source also led to high increase of cyanophycin synthesis, while growth on the combination of L-arginine or L-asparagine with nitrate only caused minor cyanophycin accumulation. Growth of *Synechocystis* PCC 6803 on L-arginine as sole N-source caused substantial morphological and physiological changes, such as severe thylakoid membrane degradation with partial loss of pigments and photosynthetic activity leading to a phenotype almost like that seen under nutrient deficiency. In contrast to the wild type, the PsbO-free *Synechocystis* PCC 6803 mutant could grow on L-arginine as sole N-source with only minor morphological and physiological changes. Due to its fairly balanced growth, the mutant accumulated only few cyanophycin granules. L-arginine degrading activity (measured as ornithine and ammonium formation) was high in the PsbO-free mutant but not in the wild type when cells were grown on L-arginine as sole N-source. In both cells types the L-arginine degrading activity was high (although in the PsbO-free mutant about twice as high as in wild type), when cells were grown on L-arginine in combination with nitrate, and as expected very low when cells were grown on nitrate as sole N-source. Thus, net cyanophycin accumulation in *Synechocystis* PCC 6803 is regulated by the relative concentration of L-arginine to the total nitrogen pool, and the intracellular L-arginine concentration is greatly influenced by the activity of the L-arginine degrading enzyme system which in part is regulated by the activity status of photosystem II. These results suggest a complex interrelation between cyanophycin synthesis, L-arginine catabolism, and in addition photosynthesis in *Synechocystis* PCC 6803.

Introduction

Cyanophycin, a product of nonribosomal peptide synthesis, is a polymer of 25 to 100 kDa consisting in general of equimolar amounts of L-aspartic acid and L-arginine: A polyaspartic acid backbone is linked with the β-carboxyl groups to the α-amino groups of L-arginine residues through amide bonds (multi-L-arginyl-poly-L-aspartate) (Simon, 1971; Simon and Weathers, 1976 and see reviews: Allen, 1984, 1988; Simon, 1987). The polymer is unique for cyanobacteria and has been reported in almost all species with the exception of some strains of the unicellular group *Synechococcus* (Lawry and Simon, 1982; Simon, 1987; Carr, 1988). Cyanophycin is absent in *Synechococcus cedrorum* (UTEX 1191) and in the two closely related *Synechococcus* species PCC 6301/PCC 7942 (see also Ziegler *et al.*, 1998), but was shown to be present in the thermophilic *Synechococcus* sp. MA19 (Hai *et al.*, 1999) and in the thermophilic *Synechococcus elongatus* (Berg *et al.*, 2000; Stephan, unpublished results). Cyanophycin recently gained interest as a source of polyaspartic acid

Abbreviations: Asc, sodium ascorbate; Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol indophenol; EDTA, ethylenediamine tetraacetic acid; Epps, N-2-hydroxyethyl-piperazine-N'-3-propanesulfonic acid; Hepes, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; MV, methylviologen; PCV, packed cell volume; PS I and PS II, photosystem I and II; PsbO (or MSP), manganese stabilizing protein (*psbO* gene product); Tricine, N-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane.

0939-5075/2000/1100-0927 $ 06.00

that can be used as a biodegradable substitute for polyacrylic acid (Schwamborn, 1996; Joentgen *et al.*, 1998).

Cyanophyin and cyanophycin metabolism have been thoroughly characterized in *Anabaena cylindrica* by Simon and his coworkers (see review: Simon 1987) and in *Aphanocapsa* 6308 (now *Synechocystis* PCC 6308) by Allen and her coworkers (see reviews: Allen 1984, 1988 and Merritt *et al.*, 1994). Recently, Ziegler *et al.* (1998) and Berg *et al.* (2000) purified and characterized the cyanophycin synthetase from *Anabaena variabilis*, and identified and analysed the gene encoding the synthetase (*cphA*) from *Anabaena variabilis* ATCC 29413, *Anabaena* sp. PCC 7120, *Synechocystis* sp. PCC 6803, and *Synechococcus elongatus*. In addition, Lockau's group identified the gene encoding the cyanophycin degrading enzyme in *Synechocystis* PCC 6803, and showed that cyanophycinase hydrolyzes cyanophycin to a dipeptide consisting of aspartic acid and arginine (Richter *et al.*, 1999). Possibly a putative glycoprotease recently characterized by Zuther *et al.* (1998) might also be involved in cyanophycin degradation in *Synechocystis* PCC 6803.

The amount of cyanophycin as a nitrogen storage compound in the cyanobacterial cell varies greatly in dependence of the environmental conditions. Cyanophycin is low in exponentially growing cells, but accumulates in cells of stationary growth phase and may then comprise as much as 16% of the dry weight (Lawry and Simon, 1982; Golecki and Heinrich, 1991). It is rapidly depleted upon growth after dilution into fresh medium when balanced growth resumes. The material was also shown to accumulate in some species following addition of a variety of nitrogen-containing compounds to the medium, including ammoniumchloride, urea, glycine, aspartic acid and arginine. Cyanophycin levels are low during nitrogen starvation whereas levels are high in other starvation conditions, such as starvation for light, phosphorus or sulfur or when cells are grown at low temperature. Induction is also caused by the addition of transcriptional or translational inhibitors (see reviews: Allen, 1984; Simon, 1987).

A great number of experimental evidence exists showing that cyanophycin is not just a relatively inert reserve compound in the stationary phase of growth but has a dynamic metabolism as e.g. during transitions between nitrogen deficiency and nitrogen repletion and vice versa. However, the mechanisms which regulate the synthesis and degradation of cyanophycin during the transient accumulation are still largely unknown (see reviews: Allen, 1984; Simon, 1987; and Carr, 1988; Mackerras *et al.*, 1990a,b). In this respect, results by Sherman *et al.* (1998) are relevant showing that in the unicellular, diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142 that temporarely separates nitrogen fixation and photosynthesis, cyanophycin granules are formed during the period of nitrogenase activity with low photosynthetic activity and degraded during the period when photosynthesis recommences. These results suggest that besides a diurnal rhythm of N_2-fixation and photosynthesis also a diurnal oscillation in cyanophycin accumulation occurs.

In the present paper we have analyzed cyanophycin accumulation in *Synechocystis* sp. strain PCC 6803 during growth under nutrient deficiency, especially phosphate, and during growth on L-amino acids, especially L-arginine, which is taken up with high rates by *Synechocystis* PCC 6803 (Montesinos *et al.*, 1997). Thus, this cyanobacterium is extremely well suited to investigate how photosynthesis affects cyanophycin accumulation under conditions of optimal L-arginine supply. For this reason, the previously constructed PsbO-free *Synechocystis* PCC 6803 mutant (Engels *et al.*, 1994) with a limitation on the donor side of PS II was included in the investigations to see how such a limitation in the first reaction of the overall photosynthetic process affects cyanophycin accumulation and L-arginine catabolism.

Materials and Methods

Cyanobacterial strain, growth conditions and cell breakage

Synechocystis sp. strain PCC 6803 was obtained from the Pasteur Culture Collection of Cyanobacterial Strains, Paris, and was grown in gas wash bottles of 250 ml capacity in a stream of 2% CO_2 in air and in BG11 medium according to Rippka *et al.* (1979) with slight modifications: Na_2Mg-EDTA, ferric ammonium citrate and $Co(NO_3)_2$ were replaced by Na_2-EDTA, Fe(III)-citrate and $CoSO_4$, respectively. The pH of the medium was 7.5. When cells were grown on L-amino acids as

sole N-source (5 mM), $NaNO_3$ (17 mM) was omitted, and the medium was additionally buffered with Epps-NaOH (50 mM, pH 7.5). The various L-amino acids were added seperately after sterile filtration through a Minisart single use filter (0.2 μm pore size, Sartorius AG, Göttingen). When cells were grown on the combination of nitrate and L-amino acids, the BG11 medium was buffered as above, and the respective L-amino acid was added in addition to nitrate. Nutrient deficient medium consisted of BG11 medium without $NaNO_3$ or with reduced sulfate (300 μM → 30 μM) or with reduced phosphate (180 μM → 9, 4.5 or 0 μM).

For dilution cells were collected by centrifugation, washed once with distilled water and resuspended in N-free medium under aseptic conditions. The standard inoculum was 0.4 μl packed cell volume (PCV)/ml medium. The bottles were placed in a water bath (size: 75 × 38 × 23 cm) of 30 °C and illuminated from top with six Philips lamps (120 W, PAR 38 EC cool beam) at a distance of 35 cm from the water bath surface (257 $\mu E/m^2 \times sec$ – measuring total quantum flux density between 400 and 740 nm on the water surface with a Quantaspectrometer QSM-2500, Techtum Instrument, Umeå-Sweden).

Growth of the previously constructed and characterized PsbO-free *Synechocystis* PCC 6803 mutant (Engels *et al.*, 1994) was as above, except that the growth medium contained 7.5 mg kanamycin sulfate/l.

Growth was determined by measuring the absorbance of *Synechocystis* PCC 6803 cultures at 750 nm (absorbance at 750 nm of 0.1 (up to a total absorbance of ≤ 1) corresponds to about 0.1 μl PCV/ml) (see also Flores *et al.*, 1982). *Synechocystis* PCC 6803 (inoculation: 0.4 μl PCV/ml) was grown for 18 to 72 h resulting in a cell density of 2 to 5 μl PCV/ml and harvested by centrifugation for 20 min at 2200 × *g*. After washing twice with distilled water, the cells were resuspended in HMCGS (50 mM **H**epes-NaOH, pH 6.5, containing, 10 mM **M**gCl_2, 30 mM **C**aCl_2, 25% (w/v) **g**lycerol, and 1 mM **s**ucrose) to give a cell density of 100 μl PCV/ml (cells suspension used for photosynthetic measurements).

When cell-free extracts were used, cells were broken according to the procedure described by Burnap *et al.* (1989). The above cell suspension (100 μl PCV/ml) was mixed with an equal volume of glass beads (0.17–0.18 mm) and treated in a "Bead Beater" (Biospec. Products, chamber volume 15 or 300 ml) 15 to 20 times for 30 sec with cooling intervals of 5 minutes. After this treatment the cell extract was decanted from the beads, and the beads were washed once with an equal volume of HMCGS buffer. The two supernatants were combined. Thus, the obtained cell extract corresponded to a cell suspension of 50 μl PCV/ml. Subsequently, the cell extract was centrifuged for 10 min at 2000 × *g* to remove the residual glass beads and unbroken cells (cell-free extract for PSI measurements).

Cell-free extracts used for determination of the L-arginine degrading activity were prepared in HMCG buffer (HMCGS buffer without sucrose). Cell breakage was as described above.

Determination of pigment content and photosynthetic activity measurements

Chlorophyll was estimated according to Grimme and Boardman (1972), phycocyanin and allophycocyanin according to Tandeau de Marsac and Houmard (1988).

Photosynthetic O_2 evolution with whole cells or cell free extracts of *Synechocystis* PCC 6803 was measured in a Clark type electrode (Rank Brothers, Bottisham Cambridge, England) at 20 °C at a polarization voltage of 600 mV. Red continuous light was provided by a halogen lamp (24 V, 250 W from Spindler and Hoyer, Göttingen). The light was filtered through a glass cuvette containing 2% (w/v) $CuSO_4$ and a red plexiglas filter (RG1 (610), Schott-Mainz). Light intensity on the water surface corresponded to 1995 $\mu E/m^2 \times sec$ (400 to 740 nm).

O_2 evolution of whole cells was determined in a reaction mixture of 3 ml containing 33.3 mM Hepes-NaOH, pH 6.5, 20 mM $CaCl_2$, 1 mM DCBQ, and cell suspension corresponding to 1–20 μl PCV. When 15 mM $NaHCO_3$ was used as electron acceptor, the measurement was done in BG11 medium.

PS I activity was determined with cell-free extracts. The reaction mixture contained in a total volume of 3 ml: 50 mM Hepes-NaOH, pH 7.0, 0.08 mM DCPIP, 3.33 mM sodium ascorbate, 0.17 mM methylviologen, 0.4 mM KCN, 0.01 mM DCMU, and 20–200 μl cell extract (corresponding to 1 to 10 μl PCV).

L-arginine degrading activity

L-arginine degrading activity was determined in cell-free *Synechocystis* extracts (prepared as described above) by measuring ornithine and ammonium formation. The reaction mixture contained in a total volume of 3 ml: 67 mM Tricine-NaOH, pH 9.0, 20 mM L-arginine-HCl, 0.5 mM $MnCl_2$, 20 µl urease (Jack bean urease S from Roche Molecular Biochemicals: 8.65 mg protein/ml = 500 units/ml), and cell-free extract corresponding to 40 µl PCV. After incubation for 2 h at 30 °C, the reaction was stopped by adding 0.2 ml 1 N H_2SO_4. Ornithine was determined according to Ratner (1962), and after neutralization of the reaction mixture ammonium was determined enzymatically with glutamate dehydrogenase (Gau *et al.*, 1995).

Isolation and determination of amino acid composition of cyanophycin, and preparation of an anti-cyanophycin antiserum

Cyanophycin was isolated from broken *Synechocystis* PCC 6803 cells. The cells were grown for three days either in $NaNO_3$-free BG11 medium supplemented with 5 mM L-arginine-HCl or L-asparagine as sole N-source or on phosphate reduced BG11 medium (reduction of K_2HPO_4 from 180 → 9 µM, nitrate as N-source). Cyanophycin isolation was done according to the procedure described by Dembinska and Allen (1988). After acid hydrolysis (in 10 N HCl for 30 h at 100 °C) and lyophylisation of the hydrolysate (Speed vac), the amino acid composition was determined by HPLC according to Büntemeyer *et al.* (1991).

Cyanophycin isolated from cells grown on L-arginine as sole N-source was resuspended in 0.1 mM HCl (400 mg/ml). 1 ml of this suspension was mixed with 1 ml complete Freud's adjuvans and was injected subcutan into a rabbit. After 24 days an intraveneous booster injection was given with 1 ml of the same cyanophycin suspension (400 mg/ml 0.1 mM HCl). After 9 days the first antiserum sample and subsequently every 8th day further samples were collected.

To reduce non-specific labelling, the anti-cyanophycin antiserum was purified. A cell-free extract of non-cyanophycin-containing *Synechocystis* PCC 6803 cells grown for 36 h on BG11 medium was subjected to SDS-PAGE (10% polyacryl–amide gel) according to Laemmli (1970). Samples were denaturated for 1 min at 100 °C in denaturation buffer (200 mM Tris-HCl, pH 6.8, 1 M sucrose, 5 mM EDTA, 4% (w/v) SDS, and 0.46% DTE). 40 µg protein were applied per lane. Immunoblotting was done as described by Engels *et al.* (1997) by transferring proteins to nitrocellulose membranes (Schleicher & Schüll, BA 85). The sheets (6 × 9.5 cm) were incubated on a shaker with 10 ml of a diluted anti-cyanophycin antiserum solution (dilution 1 to 50 in 20 mM Tris-HCl, pH 7.0, containing 150 mM NaCl and 0.05% (w/v) NaN_3) for 24 h at 4 °C. The supernatant was collected, and used for incubation with a second nitrocellulose membrane under the same conditions. Subsequently, the purified antiserum was collected and used for immunocytochemical investigations.

Ultrastructural and immunocytochemical investigations

Synechocystis PCC 6803 cells were grown and harvested as described above. A cell pellet obtained from 10 ml cell suspension was washed three times with EM buffer (50 mM KH_2PO_4-Na_2HPO_4, pH 7). The ultrastructural and immunocytochemical investigations were performed as previously described (Engels *et al.*, 1997).

Results

Cyanophycin accumulation under selected nutrient deficient growth conditions in Synechocystis PCC 6803

The extent of cyanophycin accumulation in *Synechocystis* PCC 6803 was compared when cells were grown for three days either in nitrate or sulfate or phosphate deficient BG11 medium (Fig. 1 – control cells grown in sufficient medium see later in Fig. 4). These experiments gave clear evidence that phosphate limitation led to the highest yield in cyanophycin granules per cell suggesting that under initial phosphate limiting conditions the nitrogen pool and the energy reserve of the cell are still sufficient to allow a high rate of cyanophycin synthesis. Reduction of the phosphate concentration to 9 µM (regular concentration being 180 µM) was sufficient to obtain maximal yield in granules. Complete omission of phosphate did not further increase the yield per cell. As expected,

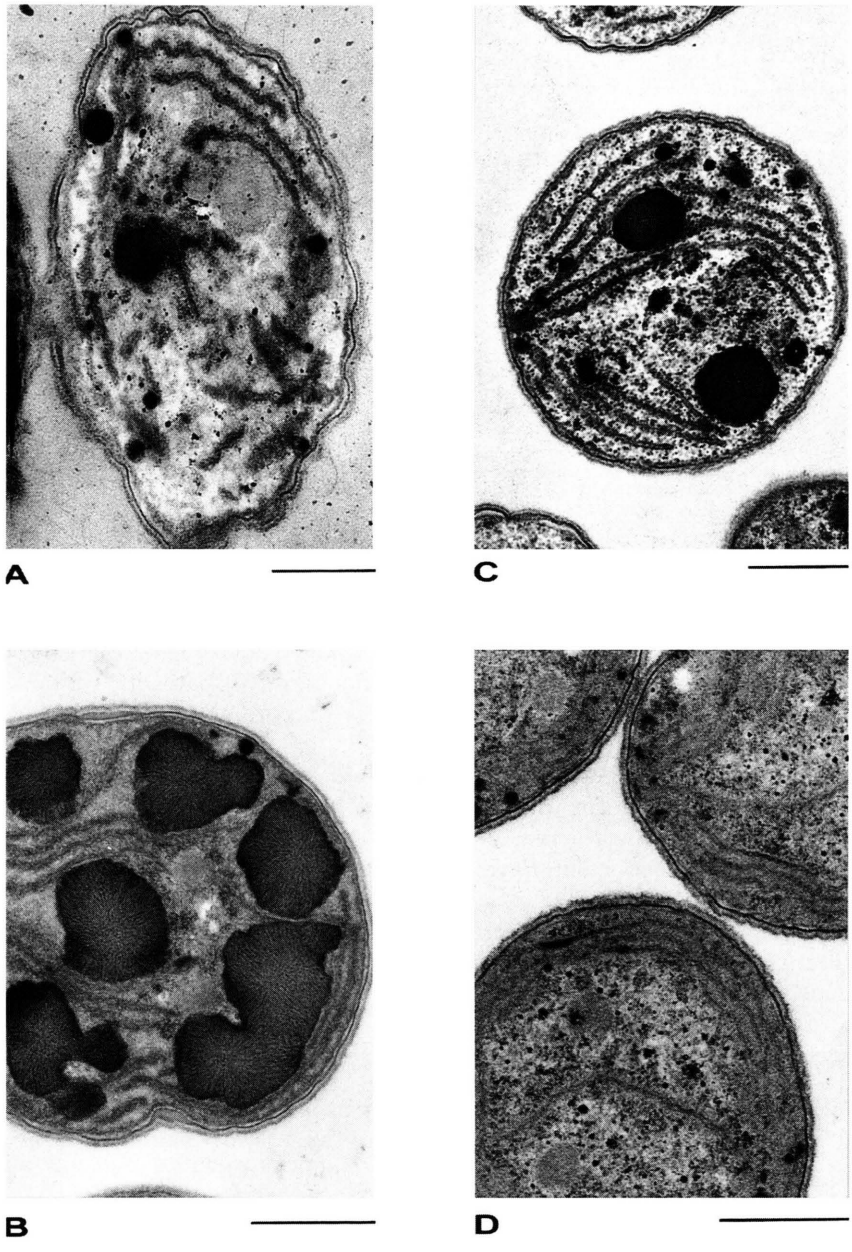

Fig. 1. Electron micrographs of *Synechocystis* PCC 6803 cells grown under nitrate, sulfate or phosphate deficient conditions and cells transferred from phosphate deficient to phosphate sufficient medium.
Synechocystis PCC 6803 wild type cells were grown for three days in medium in which nitrate was omitted (**A**), or sulfate was reduced from 300 μM to 30 μM (**B**) or phosphate was reduced from 180 μM to 4.5 μM (**C**). In (**D**) cells are shown that were grown for two days under phosphate deficient conditions (9 μM phosphate), thereafter transferred with dilution into phosphate sufficient medium (180 μM phosphate) and grown for another 24 h. Fixation of cells was performed with glutaraldehyde and OsO_4. Bar = 0.5 μm.
The structured, mainly large granules that are preferentially located in the thylakoid membrane area are cyanophycin granules. The granules with low electron density, located in the center of the cell, are carboxysomes. The small granules with very high electron density are polyphosphate granules (see also Figs 3 and 4).

Table I. Growth, chlorophyll content and photosynthetic O_2 evolution of *Synechocystis* PCC 6803 wild type cells cultivated under phosphate sufficient or deficient conditions.
Synechocystis PCC 6803 wild type cells were cultivated for one, two or three days in phosphate sufficient (180 µM phosphate) or phosphate deficient (9 µM phosphate) medium. In addition, cells grown for two days under phosphate deficient condition were thereafter transferred with dilution into phosphate sufficient medium and cultivated for another 24 h. Growth was determined as absorbance at 750 nm of the cell culture (absorbance at 750 nm at inoculation time was 0.4). Photosynthetic O_2 evolution was determined in cell suspensions with DCBQ as electron acceptor. Values were calculated on cell and chlorophyll basis.

Growth period	**Growth** (Absorbance at 750 nm)		**Chlorophyll content** [mg / 100 µl PCV]		**Photosynthetic O_2 evolution** (with DCBQ as electon acceptor) [µmol O_2 / 100 µl PCV × h]		[µmol O_2 / mg Chl × h]	
	Phosphate		Phosphate		Phosphate		Phosphate	
	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
24 h	1.17	1.27	0.31	0.14	88.9	36.9	286.8	263.6
48 h	3.47	3.43	0.32	0.07	70.4	16.4	220.0	234.3
72 h	4.44	3.91	0.40	0.07	102.0	8.5	255.0	121.4
48 h (−) → 24 h (+)	0.41		0.11		34.0		310.0	

phosphate limitation led to substantial morphological changes – most clearly seen by the reduced number of thylakoid membranes in the cell. As a consequence, the pigment content and the photosynthetic activities were greatly reduced on cell basis (Table I and Fig. 1).

When *Synechocystis* cells grown for three days in phosphate limiting medium, were transferred with dilution into phosphate sufficient medium, growth only slowly started after a rather long lag phase of about 48 h (not shown). As seen in Fig. 1D, already after 24 h cyanophycin granules had been degraded completely or almost completely, and substantial resynthesis of thylakoid membranes had occurred being followed by regaining normal photosynthetic activity (Table I). These results imply that balanced growth after transfer of cells from deficient into sufficient medium only recommences after cyanophycin granules had been degraded.

Synthesis of cyanophycin granules in Synechocystis PCC 6803 grown on selected L-*amino acids as N-source*

Synechocystis PCC 6803 has been shown to be able to take up a broad range of L-amino acids. Especially L-arginine is taken up extremely well (Labarre *et al.*, 1987; Flores and Muro-Pastor, 1990; Montesinos *et al.*, 1997). Therefore, *Synechocystis* PCC 6803 is well suited to investigate whether growth on L-amino acids will elevate cyanophycin synthesis. Growth of *Synechocystis* on L-arginine or L-asparagine (both taken up effectively - according to Montesinos *et al.*, 1997) during the first three day period after transfer from a nitrate containing medium was practically identical to growth on nitrate as N-source, while L-aspartic acid (poor uptake – according to Montesinos *et al.*, 1997) only supported a reduced growth relative to growth on nitrate. For comparison growth on L-alanine and cultivation in N-free medium are also given (Fig. 2).

As shown in Fig. 3, *Synechocystis* PCC 6803 cells contain a high amount of cyanophycin granules when grown on L-arginine or L-asparagine as sole N-source (the latter amino acid can be converted to L-aspartic acid by asparaginase - Sll0422: Kaneko *et al.*, 1996; CyanoBase). The combination of L-arginine with L-asparagine did not further increase the cyanophycin yield. In case of L-aspartic acid none or occasionally one or two small granules per cells were detected (most likely due to its poor uptake) (not shown). None of the other tested L-amino acids, such as L-alanine or L-glutamine, led to any significant cyanophycin accumulation. Thus, only growth of *Synechocystis* on L-arginine or L-asparagine as sole N-source resulted in highly increased cyanophycin production. However, in contrast to the more round shaped granules in cells grown on L-arginine as sole N-source (being comparable to granules obtained in cells

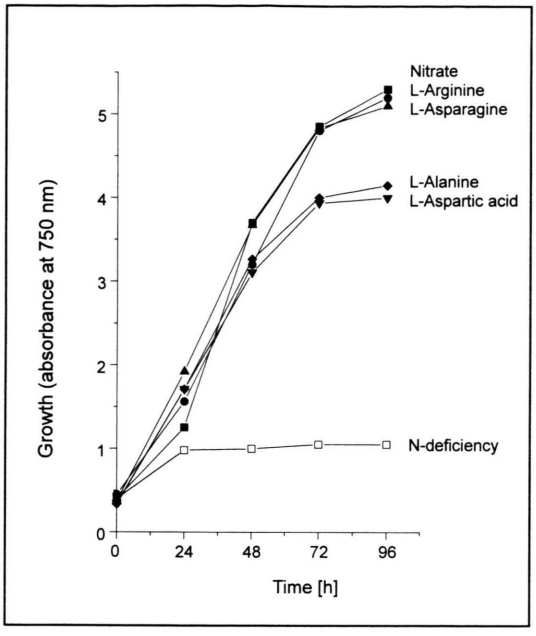

Fig. 2. Growth of *Synechocystis* PCC 6803 on various N-sources.
Synechocystis PCC 6803 wild type was grown for four days on the N-sources indicated in the figure: 17 mM sodium nitrate or 5 mM L-amino acids (no nitrate present) or nitrogen-free medium.

grown under phosphate limitation), growth on L-asparagine led to granules that bulge out sharply (Fig. 3). When cells were grown on the combination of L-arginine and L-asparagine, then again basically round granules were obtained (not shown) comparable to those in cells grown on L-arginine alone.

Isolation and amino acid composition of cyanophycin

Cyanophycin was isolated from cells grown under phosphate limitation (with nitrate as N-source) as well as from cells grown with L-arginine or L-asparagine as sole N-source. The isolated cyanophycin from all three cell types consisted of aspartic acid and arginine. Other amino acids when present represented less than 2%. In cyanophycin isolated from cells grown on L-arginine or L-asparagine the ratio of the two amino acids was close to 1 (aspartic acid to arginine: 1 to 1.07 or 1 to 0.93, respectively). However, in cyanophycin isolated from cells grown under phosphate limitation (with nitrate as sole N-source), the arginine content exceeded that of aspartic acid (aspartic acid to arginine: 1 to 1.3–1.6). At the present time no explanation can be given, since from a structural point of view aspartic acid could exceed arginine but arginine not aspartic acid.

Immunocytochemical detection of cyanophycin granules

A polyclonal antiserum was raised in a rabbit against cyanophycin isolated from *Synechocystis* PCC 6803 grown on L-arginine as sole N-source. This antiserum recognized the cyanophycin granules formed under phosphate limitation (not shown) as well as those formed in cells grown on L-arginine or L-asparagine (Fig. 3). This is an additional support that the differently shaped cyanophycin granules (Fig. 1 and 3) are very similar, not only with respect to amino acid composition but also with respect to their surface properties. In addition to the strong labeling of cyanophycin granules, the anti-cyanophycin antiserum gave a slight labeling of the cell membrane that was also seen in control cells not containing cyanophycin granules. Thus, the labelling of the cell membrane by this antiserum is most likely due to a non-specific binding.

Comparative analysis of cyanophycin production, photosynthetic activity and L-arginine degrading enzyme activity in Synechocystis PCC 6803 wild type and PsbO-free mutant

Synechocystis PCC 6803 can grow on L-arginine as sole N-source as shown in Fig. 2, but only in the light and in presence of CO_2. Cells can not grow on L-arginine in the dark or in the light in presence of DCMU or in the absence of CO_2 (not shown). These results imply that *Synechocystis* can grow on L-arginine as sole N-source but that L-arginine is not sufficient as sole C-source, since photosynthesis is required. Flores' group (Montesinos *et al.*, 1997) has shown that *Synechocystis* PCC 6803 can take up L-arginine extremely well, and the above presented results give evidence that growth on L-arginine leads to high accumulation of cyanophycin granules (Fig. 3). Therefore, *Synechocystis* PCC 6803 is especially well suited to investigate how a limitation in PS II, catalyzing the first reaction of the overall photosynthesis process, will affect cya-

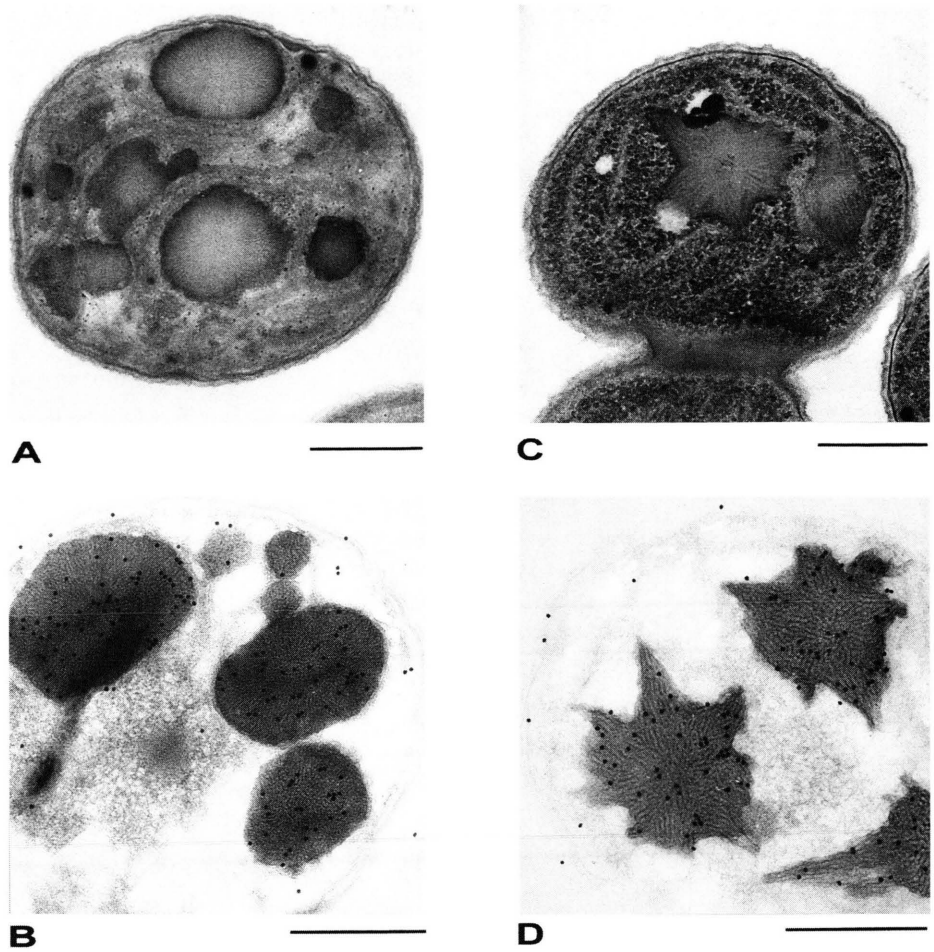

Fig. 3. Electron micrographs of *Synechocystis* PCC 6803 cells grown on L-arginine or L-asparagine. *Synechocystis* PCC 6803 wild type was grown for three days on L-arginine or on L-asparagine as sole N-source. Fixation of cells was performed with glutaraldehyde and OsO_4: (**A**) Cell grown on L-arginine and (**C**) cell grown on L-asparagine. In addition, immunocytochemical detection of cyanophycin granules with the anti-cyanophycin antiserum in combination with gold-labeled anti-rabbit IgG is shown (Fixation with glutaraldehyde): (**B**) Cell grown on L-arginine and (**D**) cell grown on L-asparagine. Bar = 0.5 μm.

nophycin synthesis under conditions of optimal L-arginine supply. For this reason the previously constructed PsbO-free *Synechocystis* PCC 6803 mutant (Engels *et al.*, 1994) was included in our investigations. PsbO is the manganese (and calcium) stabilizing peptid on the donor side of PS II. It is not absolutely essential for photoautotrophic growth in cyanobacteria, but its absence leads to a reduced water oxidizing capacity (see reviews: Barry *et al.*, 1994; Bricker and Ghanotakis, 1996).

The results of the comparative analysis of *Synechocystis* PCC 6803 wild type and PsbO-free mutant are presented in Fig. 4 and Tables II to IV. Growth of *Synechocystis* PCC 6803 wild type on L-arginine as sole N-source for three days (after transfer from nitrate containing medium) led to a high amount of cyanophycin granules per cell (Fig. 3 and 4). In addition, severe morphological and physiological changes occurred. Cells became a yellowish appearance and contained a greatly reduced amount of thylakoid membranes. As a consequence, the pigment content and the photosynthetic activity on cell basis were greatly reduced (Tables II and III). Thus, these cells had an appearance almost like cells grown under nutrient deficient conditions (compare Fig. 1 with 3 and 4).

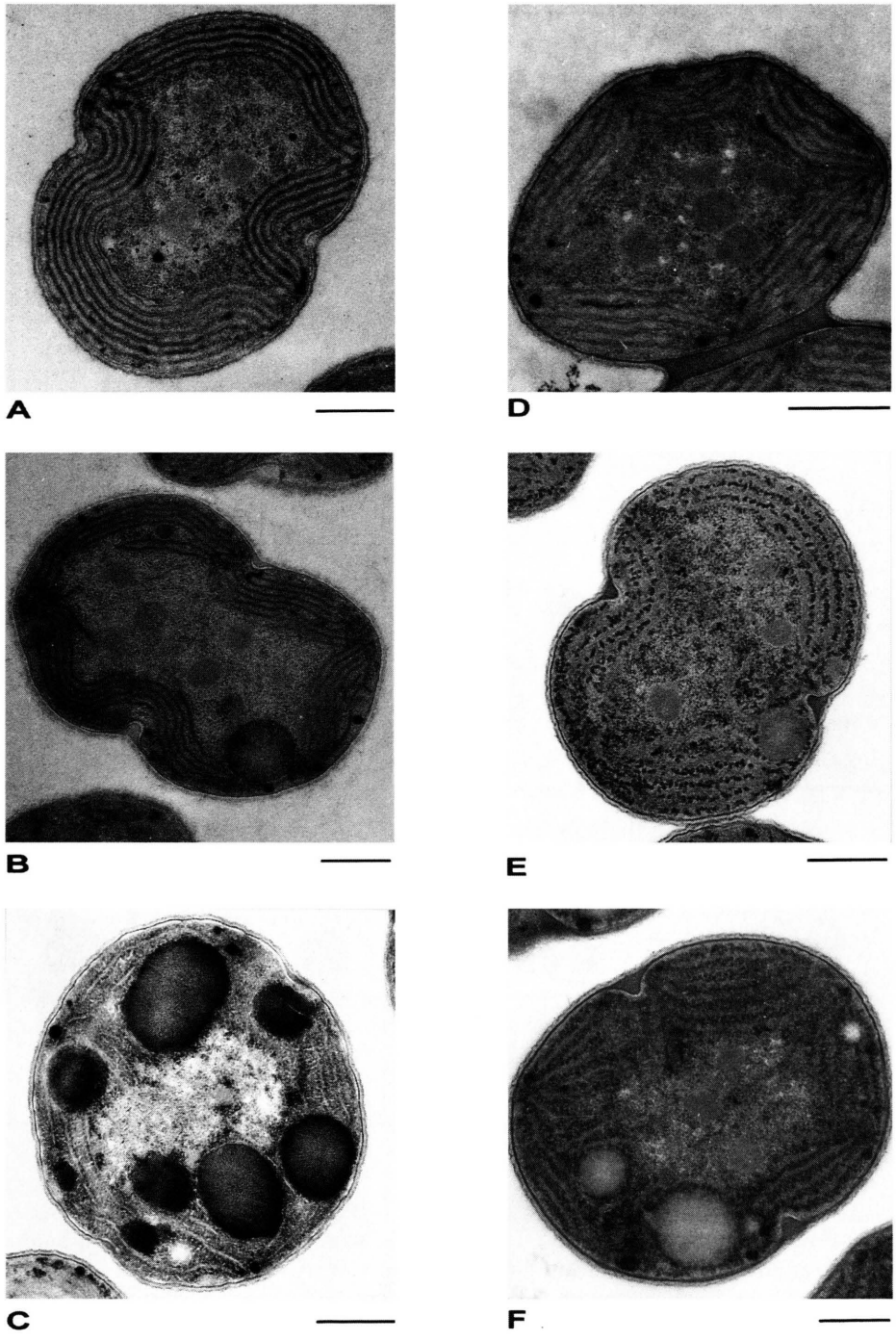

Fig. 4. Electronmicrographs of *Synechocystis* PCC 6803 wild type and PsbO-free mutant cells grown on various N-sources.
Synechocystis PCC 6803 wild type and PsbO-free mutant cells were grown for three days on various N-sources. Fixation of cells was performed with glutaraldehyde and OsO_4. (**A**) Wild type grown on nitrate, (**B**) wild type grown on L-arginine in combination with nitrate, (**C**) wild type grown on L-arginine; (**D**) PsbO-free mutant grown on nitrate, (**E**) PsbO-free mutant grown on L-arginine in combination with nitrate, (**F**) PsbO-free mutant grown on L-arginine.

Table II. Appearance and growth of cell culture and chlorophyll content of cell suspension of *Synechocystis* PCC 6803 wild type and PsbO-free mutant grown on various N-sources.
Synechocystis PCC 6803 wild type and PsbO-free mutant were grown for three days (one growth cycle) on various N-sources: nitrate or L-arginine in combination with nitrate or L-arginine. In addition, cells were grown on L-arginine as sole N-source for two and for three growth cycles. For comparison, cells cultivated on N-free medium for three days were included. Appearance and growth of cell culture are presented (absorbance at 750 nm at inoculation time was 0.4). The chlorophyll content is given per 100 µl PCV.

		N-source during growth					
		NO_3^-	**L-Arg + NO_3^-**	**L-Arg**	**L-Arg ↓ L-Arg**	**L-Arg ↓ L-Arg ↓ L-Arg**	**N-free**
Wild type	**Growth (Absorbance at 750 nm)**	6.3	7.5	5.9	2.3	2.1	1.4
	Appearance of cell culture						
	Chlorophyll content [mg Chl / 100 µl PCV]	0.32	0.23	0.06	0.05	0.04	0.08
PsbO-free mutant	**Growth (Absorbance at 750 nm)**	4.9	5.9	5.6	5.5	5.1	0.9
	Appearance of cell culture						
	Chlorophyll content [mg Chl / 100 µl PCV]	0.32	0.26	0.16	0.19	0.20	0.10

When such cells that were grown for three days on L-arginine as sole N-source, were diluted into fresh L-arginine containing medium, cyanophycin granules became completely degraded and partial thylakoid membrane resynthesis occurred (not shown). However, growth was significantly reduced during this second growth cycle on L-arginine and eventually ceased when cells were further cultivated on L-arginine (Table II). Thus, *Synechocystis* PCC 6803 wild type cells, when continuously cultivated on L-arginine as sole N-source with dilutions, were not able to maintain balanced growth.

In contrast, when besides L-arginine also nitrate was present in the medium, the cyanophycin production was low (only one or two granules per cell), and the morphological and physiological changes were minor. Cells could grow continuously on the combination of L-arginine and nitrate as N-sources (Fig. 4 and Tables II and III) implying that fairly balanced growth was maintained.

Table III. Pigment content and photosynthetic activity of *Synechocystis* PCC 6803 wild type and PsbO-free mutant grown on various N-sources.
Synechocystis PCC 6803 wild type and PsbO-free mutant cells were grown for three days on nitrate or on L-arginine in combination with nitrate or on L-arginine as sole N-source. Pigment contents were determined as described under Materials and methods. Photosynthetic activity was either determined in cell suspensions ($H_2O \rightarrow NaHCO_3$ and $H_2O \rightarrow$DCBQ) or in cell-free extracts (DCPIP/Asc → MV). Values were calculated on the basis of 100 µl PCV and photosynthetic activity values were additonally calculated on chlorophyll basis.

	Wild type			**PsbO-free mutant**		
	NO_3^-	L-Arg + NO_3^-	L-Arg	NO_3^-	L-Arg + NO_3^-	L-Arg
Pigment content: [mg / 100 µl cells]						
Chlorophyll	0.39	0.35	0.06	0.30	0.25	0.14
Phycocyanin	1.57	1.33	0.05	0.82	1.05	0.61
Allophycocyanin	0.71	0.69	0.08	0.42	0.50	0.33
Photosynthetic activity: [µmol O_2 evolved or taken up / 100 µl PCV × h]						
$H_2O \rightarrow NaHCO_3$	25.5	7.1	8.4	17.7	5.1	7.3
$H_2O \rightarrow$ DCBQ	94.6	71.2	7.9	19.9	12.4	9.1
DCPIP/Asc → MV	56.7	98.4	8.2	20.6	13.4	15.0
Photosynthetic activity: [µmol O_2 evolved or taken up / 100 µg Chl × h]						
$H_2O \rightarrow NaHCO_3$	65.4	20.3	140.0	59.0	20.4	52.1
$H_2O \rightarrow$ DCBQ	242.6	203.4	131.7	66.3	49.6	65.0
DCPIP/Asc → MV	145.4	281.1	136.7	68.7	53.6	107.1

The same type of experiments were performed with the PsbO-free *Synechocystis* PCC 6803 mutant (Fig. 4 and Tables II and III). The most significant difference was seen when mutant cells were grown on L-arginine as sole N-source. In contrast to wild type, the PsbO-free mutant contained only few cyanophycin granules per cell. Moreover, the mutant cells kept an almost normal amount of thylakoid membranes and as a consequence had an almost normal pigment content and photosynthetic activity. The mutant cells could be cultivated on L-arginine as sole N-source continuously implying that the PsbO-free mutant cells were able to maintain balanced growth when cultivated on L-arginine as sole N-source in contrast to wild type cells.

Since the above results suggest that L-arginine metabolism is substantially different in *Synechocystis* wild type and PsbO-free mutant, the L-arginine degrading enzyme actvity was investigated. Flores and coworkers (Quintero *et al.*, 2000) have recently shown that in *Synechocystis* PCC 6803 the initial main product of L-arginine degradation is ornithine. However, the enzyme catalyzing this ornithine formation has remained unidentified. We determined ornithine and ammonium formation from L-arginine (in presence of added $MnCl_2$) in cell-free extracts. Under our assay conditions, ornithine is not further metabolized. Urease was added to the reaction mixture to completely degrade the produced urea to ammonium, and $MnCl_2$ was added because ornithine and ammonium production were higher in presence of added $MnCl_2$ as in absence. As the results of Table IV show, extracts of *Synechocystis* cells grown on nitrate as sole N-source had a low ornithine (and ammonium) production. This was the same whether wild type or PsbO-free mutant extracts were examined. In both cell types the ornithine producing activity was high when cells were grown on L-arginine in combination with nitrate, although the activity in the mutant was about twice as high as in wild type. However, the major difference was observed when cells were grown on L-arginine as sole N-source. The ornithine producing activity was low in wild type cell extracts, but substantially elevated in mutant cell extracts.

Presently, the nature of this enzyme activity leading to ornithine and ammonium formation from L-arginine is unclear. Superficially, it can be classified as an arginase in combination with urease (urease is present in *Synechocystis* PCC 6803), but our experiments show that there is a substantial deviation of the expected 1 to 2 ratio of ornithine to ammonium formation (products of the combination of arginase and urease). The ammonium value exceeded the expected value by a factor of about two under the assay conditions of Table IV. Moreover, our results gave evidence that the ratio of ornithine to ammonium was variable being in the range of 1 : 2.5 to 5 depending on the L-arginine and $MnCl_2$ concentration in the reaction mixture and on the reaction temperature (not shown). No other L-arginine degrading activity of any significance was detected.

Discussion

The ultrastructural investigations here presented gave evidence that phosphate limitation led to very high accumulation of cyanophycin in *Synechocystis* PCC 6803, as e. g. previously shown for *Agmenellum* (Stevens *et al.*, 1981). As a consequence of nutrient deficiency, a yellowing of cells occurred due to severe thylakoid membrane degradation with partial loss of pigments and photosynthetic activity. After diluting cells into phosphate sufficient medium, balanced growth only started after a fairly long lag phase of about 48 h. At that time cyanophycin granules had completely been degraded, and substantial thylakoid membrane resynthesis had occurred. This suggests that cyanophycin was not kept as a storage compound after transfer to sufficient growth conditions and that during the period of cyanophycin degradation balanced growth could not proceed. This observation is somewhat similar to results with nitrogen-starved cyanobacterial cells which are given a nitrogen source. Growth did not recommence until the transient accumulated cyanophycin had been degraded despite the availability of a N-source in the medium (see e.g. Allen and Hutchison, 1980).

High cyanophycin accumulation also occurred when *Synechocystis* PCC 6803 cells were grown on L-arginine (taken up effectively according to Montesinos *et al.*, 1997) or L-asparagine (taken up effectively and being degraded to L-aspartic acid by an asparaginase: Sll0422), but not when grown on L-aspartic acid (taken up poorly) or any of the other tested L-amino acids (such as L-alanine or L-glutamine). The results suggest that a high intracellular concentration of the two amino acid constituents of cyanophycin, L-arginine and L-aspartic acid, led to highly increased cyanophycin synthesis. However, this elevated cyanophycin accumulation was not obtained when cells were grown on L-arginine (or L-asparagine) in combination with nitrate. Therefore, it can be concluded that one condition leading to high cyanophycin synthesis is an elevated intracellular L-arginine concentration in combination with a low total N-pool. This is also supported by the observation that cyanophycin granule accumulation in cells grown on L-arginine as sole N-source only started when the major N-reserve of the cell (besides cyanophycin), the phycobili proteins, were largely degraded. Under those conditions other metabolic usage of L-argi-

Table IV. L-arginine degrading activity of *Synechocystis* PCC 6803 wild type and PsbO-free mutant grown on various N-sources.
Synechocystis cells were grown as described in legend to Table III. L-arginine degrading activity was determined as ornithine and ammonium formation from L-arginine in presence of $MnCl_2$ and urease in cell-free extracts as described under Materials and methods.

Synechocystis PCC 6803	N-source during growth	Ornithine formation [μmol / 100 μl PCV × h]	Ammonium formation [μmol / 100 μl PCV × h]	Ratio Orn. : Amm.
Wild type	NO_3^-	0.48	2.25	1 : 4.7
	L-Arg + NO_3^-	1.06	4.52	1 : 4.3
	L-Arg	0.29	1.58	1 : 5.4
PsbO-free mutant	NO_3^-	0.54	2.54	1 : 4.7
	L-Arg + NO_3^-	2.40	10.01	1 : 4.2
	L-Arg	1.69	6.96	1 : 4.1

nine besides incorporation into cyanophycin seems to be almost completely prevented resulting in a "nutrient deficient-like phenotype". However, the high cyanophycin accumulation is not exclusively regulated by the ratio of free L-arginine to total N-pool, but also in dependence of the photosynthetic activity.

Namely in contrast to *Synechocystis* PCC 6803 wild type, the PsbO-free *Synechocystis* PCC 6803 mutant (Engels *et al.*, 1994) possessing a limitation on the donor side of PS II due to lack of the manganese (and calcium) stabilizing peptide, was able to grow on L-arginine as sole N-source without developing the "nutrient deficient-like phenotype", and accumulated only few cyanophycin granules per cell. This implies that the PsbO-free mutant when grown on L-arginine as sole N-source was able to degrade L-arginine effectively and to use it as a metabolizable N-source and in part also as a C-source. In agreement with this conclusion, we could show that in the PsbO-free mutant an L-arginine degrading enzyme activity leading to ornithine and ammonium (urease activity being present in *Synechocystis* PCC 6803) was substantially higher in mutant than in wild type cells. Obviously, due to this elevated activity, L-arginine could be effectively degraded, and fairly balanced growth was achieved preventing the almost complete diversion of L-arginine into cyanophycin synthesis as seen in wild type (see summary of results in Table V).

The only condition leading to an elevated L-arginine degrading activity in wild type was cultivation of cells on L-arginine in combination with nitrate. It has to be assumed that under those conditions the upregulation of cyanophycin synthetase activity was slowed down because the intracellular L-arginine concentration relative to total N-pool never reached a value that was high enough to initiate the upregulation of cyanophycin synthesis. Therefore, cells were able to metabolize L-arginine effectively by inducing and/or activating the L-arginine degrading enzyme system, and as a consequence stress was prevented.

L-arginine catabolism in bacteria is rather complex and can proceed via a number of different pathways (see reviews: Stalon, 1985; Cunin *et al.*, 1986). Some of these pathways or enzymes have been described to be present in cyanobacteria (Hood and Carr, 1971; Weathers *et al.*, 1978; Gupta and Carr, 1981; Bednarz and Schmid, 1991; Martel *et al.*, 1993; Singh and Bisen, 1994; Bockholt *et al.*, 1996). For *Synechocystis* PCC 6803, Flores and coworkers (Quintero *et al.*, 2000) have shown that an arginase like pathway in combination with a sort of urea cycle is functioning. The initial main product of L-arginine catabolism was identified to be ornithine. Although two genes *sll1077* and *sll0228* (CyanoBase; Kaneko *et al.*, 1996) encoding arginase related enzymes (Quintero *et al.*, 2000; Perozich *et al.*, 1998) are present in *Synechocystis* PCC 6803, these enzymes are not involved in ornithine formation as shown by Quintero *et al.* (2000). Our results support the conclusion that the enzyme system leading to ornithine and urea/ammonium formation is not a typical arginase, since a substantial deviation of the expected 1 to 2 ratio of ornithine to ammonium formation was observed (see Table IV). Therefore, the true nature of this enzyme system ramains presently unknown and awaits purification for its identification.

In conclusion, it can be stated that although the exact nature of the L-arginine degrading enzyme system is presently uncertain, our results give clear evidence that L-arginine catabolism and the activ-

Table V. Comparative representation of cyanophycin granule content, thylakoid membrane content and L-arginine degrading activity of *Synechocystis* PCC 6803 wild type and PsbO-free mutant grown on various N-sources.

***Synechocystis* PCC 6803**	**N-source during growth**	**Cyanophycin granule content**	**Thylakoid membrane content**	**L-Arginine degrading activity**
Wild type	NO_3^-	– (+)	+ + + +	+
	L-Arg + NO_3^-	+	+ + +	+ +
	L-Arg	+ + + +	+	+
PsbO-free mutant	NO_3^-	– (+)	+ + +	+
	L-Arg + NO_3^-	+	+ +	+ + + +
	L-Arg	+	+ +	+ + +

ity status of PS II are closely interrelated in a yet unknown way in *Synechocystis* PCC 6803, since optimal water oxidizing conditions prevent upregulation of the L-arginine degrading enzyme activity, when cells are grown on L-arginine as sole N-source. Under such conditions L-arginine is diverted almost exclusively into cyanophycin synthesis, and this eventually leads to stress. However, when due to a limitation on the donor side of PS II (PsbO-free mutant) the excitation pressure on PS II is reduced, upregulation of the L-arginine degrading enzyme activity is possible. Thus, in contrast to wild type, balanced growth is achieved in the PsbO-free mutant when grown on L-arginine as sole N-source. Therefore, it can be concluded that net cyanophycin accumulation is dependent on the actual intracellular free L-arginine concentration (relative to total N-pool), and the free L-arginine concentration is a function of L-arginine uptake and degradation. As our results show, the latter activity is regulated in dependence of the activity status of PS II either in a direct or indirect way. This points to an interrelation between L-arginine metabolism and cyanophycin synthesis as expected, but also points to a close interrelation with photosynthesis. Elucidation of this interrelation of L-arginine degradation with PS II activity requires identification of the L-arginine degrading enzyme system.

Acknowledgements

We thank Dr. Heino Büntemeyer, Institute for Cell Culture Technique, University of Bielefeld for performing the HPLC analysis of cyanophycin. The financial support of the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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