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Purification of an acidic plastocyanin from *Microcystis aeruginosa*

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Plastocyanin and cytochrome *c*-553 are two functionally equivalent electron carriers in the photosynthetic chain of cyanobacteria. *Microcystis aeruginosa*, a unicellular cyanobacterium which grows well at a high pH (8.6) and which was not known to possess plastocyanin, has been studied for its ability to synthesize plastocyanin in culture media with and without Cu. In the absence of Cu, an acidic cytochrome *c*-553 alone was isolated. With the inclusion of 2 μM Cu, cytochrome *c*-553 synthesis was partially suppressed and an acidic plastocyanin was isolated. A newly developed procedure, using high concentrations of ammonium sulfate to fractionate water-soluble proteins on Sephacryl S-200 was successfully used to isolate and concentrate the plastocyanin, thus allowing it to be further purified to homogeneity. This protein has an isoelectric point of 4.8 which is similar to the p*I* value reported for other acidic plastocyanins from higher plants and green algae. Its N-terminal sequence of the first 15 amino acids has been determined; 9 of these amino acids are identical to those in the sequence of the basic plastocyanin from *Anabaena variabilis*.

Introduction

Plastocyanin is a small, copper protein which functions as a photosynthetic catalyst in the electron-transport chain between membrane bound cytochrome *f* and P-700, the reaction centre of Photosystem I. While higher plants [1] possess only plastocyanin, many eukaryotic green algae [2] and prokaryotic cyanobacteria [3] possess both plastocyanin and a functional equivalent, cytochrome *c*-553. In the case of cyanobacteria [4], plastocyanin is found only when Cu is present in the growth medium and cytochrome *c*-553 formation appears to be suppressed by Cu. In some species, the suppression of cytochrome *c*-553 synthesis can lead to a complete replacement of cytochrome *c*-553 by plastocyanin.

It has been shown that the isoelectric points of plastocyanin and cytochrome *c*-553 from the same source fall in the same range [5]. Most of the plastocyanins in cyanobacteria have been isolated from mesophilic species that possess a basic cytochrome *c*-553 and are basic. In an isolated case, an acidic plastocyanin has been reported in a thermophilic cyanobacterium, *Phormidium laminosum* [6]. This is significant as higher plants [7] and green algae [5] all yield an acidic plastocyanin. This finding may represent the first evidence for a turning point in the evolution of the prokaryotic basic plastocyanin to the more advanced acidic form, so ubiquitous in eukaryotes.

In cyanobacteria, only the basic plastocyanin in *Anabaena variabilis* has been purified to homogeneity. This and other acidic plastocyanins from higher plants and green algae have been used extensively in comparative studies relating their structures and functions [8–10]. Such studies have provided important insights into not only the mechanism of photosynthetic electron transport but also the evolution of photosynthetic organisms. It is therefore of interest to extend such studies to an acidic plastocyanin in cyanobacteria. The difficulty lies in the purification of this protein. The study on *P. laminosum* has shown that the acidic plastocyanin from this organism binds poorly to ion exchangers and appears to be unstable in low-ionic-strength buffers. In the initial extracts of cyanobacteria, there are also large amounts of colored pigments, particularly the blue phycobiliproteins, which can aggregate into different forms and have a wide range of acidities. Ammonium sulfate precipitation, ion-exchange chromatography and gel filtration chromatrography are usually used for removing phycobiliproteins. Such procedures did not allow a clear separation of phycobiliproteins and plastocyanin in *P. laminosum*. Detection of plastocyanin in the presence of phycobliproteins is also difficult owing to their similarity in spectroscopic properties.

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Recently, we have developed a simple method for fractionation of crude extracts of cyanobacteria on Sephacryl S-200 in the presence of high concentrations of ammonium sulfate [11]. Sephacryl S-200 is a gel matrix for molecular-sieve chromatography, but it adsorbs water-soluble cytochromes and phycobiliproteins in the presence of high concentrations of ammonium sulfate. This adsorption of phycobiliproteins is stronger than that of cytochromes *c*-553 and *c*-550. Taking advantage of these adsorption properties of Sephacryl S-200, we have succeeded in achieving a clear separation of the cytochromes and the phycobiliproteins. It is therefore desirable to extend this procedure to the isolation of a plastocyanin which has very similar properties to those of cytochrome *c*-553 [12].

Microcystis aeruginosa and *Spirulina maxima* are known to possess an acidic cytochrome *c*-553 but plastocyanin has not been demonstrated in these mesophilic species [5]. It has been suggested that for cyanobacteria growing in an alkaline medium, the uptake of Cu and therefore the synthesis of plastocyanin are hindered, as most of the Cu is insoluble at a high pH [4]. In this paper, we report not only the suppression of cytochrome *c*-553 synthesis in the presence of Cu, even when grown at a high pH, but also the isolation of an acidic plastocyanin from *M. aeruginosa* using adsorption chromatography on Sephacryl S-200. The plastocyanin isolated by this procedure was further purified to homogeneity and some of its properties determined.

Materials and Methods

Preparation of cell extracts

M. aeruginosa strain NIES-44 was obtained from the Environmental Agency of Japan. Cells were grown in a modified M.A. medium [13] with constant aeration, at 25° C. The light regime was 12 h of darkness and 12 h of light at 20 $\mu E \cdot m^{-2} \cdot s^{-1}$. The cells were grown either with or without 2 μM $CuSO_4$ at pH 8.6 to determine the effect of Cu on cytochrome *c*-553 synthesis at a high pH. The cells, in 4-l batches were grown for 3-, 6- and 9-day periods, at the end of which they were harvested and the cytochrome extracted. All purification procedures were carried out at 4° C. The cells were harvested by centrifuging at $29000 \times g$ for 20 min. The pelleted cells were resuspended in 6 ml 50 mM Tris-HCl (pH 7.5) and disrupted by sonication over ice with a Soniprep 150 (power at 2/5 maximum) for 9 1-min intervals with 1-min cooling periods in between. About 1 ml of the resultant green fluid was withdrawn for the estimation of protein, chlorophyll and phycocyanin. The remaining cell fragment suspension was centrifuged at $125000 \times g$ for 20 min. Solid $(NH_4)_2SO_4$ was added to 45% saturation to the deep blue supernatant and left overnight. After centrifuging at $29000 \times g$ for 10 min, the visible absorption spectrum of the light blue-green supernatant was scanned for cytochromes *c*-550 and *c*-553.

Isolation of plastocyanin

For plastocyanin isolation, cells were grown for 14 days at room temperature in natural light (11 $\mu E \cdot m^{-2} \cdot s^{-1}$) in the presence of 2 μM $CuSO_4$. The cells were harvested and resuspended in 50 mM NaCl-50 mM Tris-HCl (pH 7.5) until cells equivalent to 50 l of medium were accumulated (35 g fresh weight). The cells were disrupted by sonication and then centrifuged at $29000 \times g$ for 10 min. The blue supernatant was kept aside while the green pellet was resuspended in more buffer and the procedure repeated until the supernatant was no longer blue. The pooled supernatants were centrifuged at $125000 \times g$ for 20 min. The resultant supernatant was brought to 1.37 M $(NH_4)_2SO_4$ and centrifuged for 20 min at $29000 \times g$. The pellet was re-extracted with 1.37 M $(NH_4)_2SO_4$-50 mM Tris-HCl (pH 7.5) to ensure that all the cytochromes were solubilized, and centrifuged. The blue supernatant was then brought up to 2.34 M $(NH_4)_2SO_4$ and centrifuged at $29000 \times g$ for 20 min. The resultant light blue supernatant was divided into two batches and each loaded on to a separate Sephacryl S-200 column (1.5 cm × 17.5 cm) equilibrated with 2.34 M $(NH_4)_2SO_4$-50 mM Tris-HCl (pH 7.5) buffer. A 500 ml gradient of $(NH_4)_2SO_4$ decreasing from 2.34 to 0 M in 50 mM Tris-HCl (pH 7.5) was used to elute plastocyanin, cytochromes *c*-553 and $c = 550$.

Purification of plastocyanin

The plastocyanin fraction eluted from the Sephacryl S-200 column was concentrated in an Amicon ultrafiltration unit fitted with a Diaflo nitrocellulose filter ($M_{r\ cut\text{-}off} = 5000$). The concentrated sample was loaded onto a Sephadex G-50 column (110 × 1.5 cm) equilibrated with 200 mM NaCl-50 mM Tris-HCl (pH 7.5). When eluted, the plastocyanin fractions were concentrated by ultrafiltration and the buffer exchanged with 30 mM NaCl-50 mM Tris-HCl (pH 7.5). The concentrated sample was then applied to a DEAE-Sephacel column (17 × 1.5 cm) equilibrated with 30 mM NaCl-50 mM Tris-HCl (pH 7.5). The plastocyanin was eluted in diluted fractions without adsorbing. The diluted fractions were pooled and then concentrated by ultrafiltration. The concentrated plastocyanin sample was made to 0.1% Tween 20 and chromatographed on a Sephadex G-75 column (36.5 × 1.6 cm) washed with 10 mM Tris-Mes (pH 7.8) and 0.1% Tween 20. The eluted plastocyanin fractions were pooled and diluted with distilled water to 0.05% Tween 20. The diluted fractions were oxidized with $K_3Fe(CN)_6$ and then concentrated by ultrafiltration. The plastocyanin was adsorbed on to a DEAE Sephacel column (17 × 1.5 cm) equilibrated with 5 mM Tris-Mes (pH 7.5). The adsorbed plastocyanin was eluted slowly by washing the column exten-

sively with the equilibrating buffer. The eluted plastocyanin was used for SDS-polyacrylamide gel electrophoresis, isoelectric focusing and N-terminal amino acid analysis.

Absorption measurements

Chlorophyll and phycocyanin were assayed by the method of Arnon et al. [14]. Cytochrome was estimated using an extinction coefficient of 20 $mM^{-1} \cdot cm^{-1}$ at 550 nm ($A_{\text{dithionite reduced}} - A_{\text{ascorbate oxidized}}$) for cytochrome *c*-550 and 553 nm ($A_{\text{ascorbate reduced}} - A_{K_3 (Fe(CN)_6 \text{ oxidized}}$) for cytochrome *c*-553 [15]. The isobestic points were taken to be 542 nm and 561 nm [2] for cytochrome *c*-553, and 556 nm and 542.5 nm for cytochrome *c*-550. Plastocyanin was detected using a ($A_{K_3\ Fe(CN)_6 \text{ oxidized}} - A_{\text{ascorbate reduced}}$) spectrum. The amount of plastocyanin was estimated using the extinction coefficient at 597 nm = 4.5 $mM^{-1} \cdot cm^{-1}$ [7]. The presence of plastocyanin was confirmed by adding KCN to a final concentration of 1 mM to an oxidized sample and following the decrease in absorbance at 597 nm over 10 min. Scanning was done on a dual-beam spectrophotometer, Shimadzu UV-160.

Electrophoresis

SDS-Polyacrylamide gel electrophoresis was performed on rod gels using the procedure described by Hames [16]. Isoelectric focusing was conducted according to Righetti and Drysdale [17].

Protein analysis

N-Terminal amino-acid sequence was determined by the automated Edman degradation as described by Mahoney et al. [18]. Protein was assayed by the method of Peterson [19].

Results

Suppression of cytochrome c-553 formation

Experiments on the effect of Cu in the growth medium on cytochrome *c*-553 synthesis were repeated several times. Listed in Table I are some typical results. There is clearly a suppression of cytochrome *c*-553 synthesis when the cells are grown with copper. This suppression is not enhanced when the pH of the growth medium is lowered to 7.5 (data not shown). Experiments were not conducted to study the suppression of cytochrome *c*-553 synthesis in cells grown in media with $CuSO_4$ concentrations exceeding 2 μM or with a pH

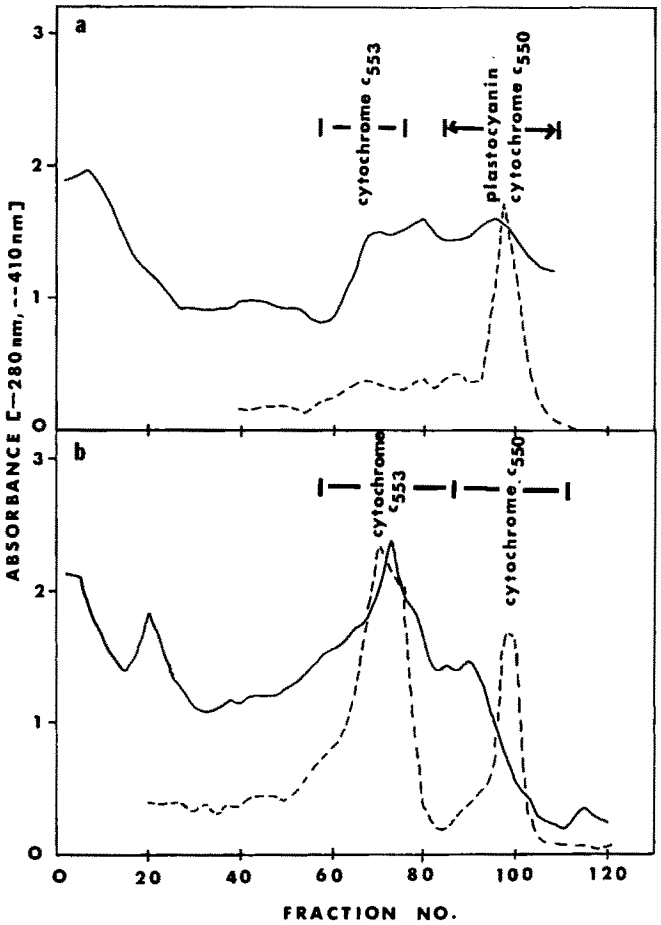

Fig. 1. The elution profile of *Microcystis aeruginosa* proteins from a Sephacryl S-200 column when a salt gradient was run from 2.34 M to 0.0 M $(NH_4)_2SO_4$. Horizontal arrows indicate fractions pooled for further purification. (a) Cells grown in the presence of Cu. (b) Cells grown in the absence of Cu.

TABLE I

The effect of Cu on cytochrome c-553 synthesis in Microcystis aeruginosa grown in pH 8.6

Values in columns 3–5 are expressed in mg cytochrome per mg chlorophyll, phycocyanin and protein respectively. Values in column 6 are expressed in μmol cytochrome *c*-553 per μmol cytochrome *c*-550.

Growth condition	Growth period (days)	Cytochrome *c*-53 / Chlorophyll	Cytochrome *c*-553 / Phycocyanin	Cytochrome *c*-553 / Protein	Cytochrome *c*-553 / Cytochrome *c*-550
Without Cu	3	0.030	0.012	$1.2 \cdot 10^{-3}$	1.29
	6	0.032	0.019	$1.4 \cdot 10^{-3}$	1.30
	9	0.026	0.013	$0.9 \cdot 10^{-3}$	1.23
With Cu	3	0.026	0.009	$1.0 \cdot 10^{-3}$	1.07
	6	0.016	0.001	$0.6 \cdot 10^{-3}$	0.66
	9	0.012	0.005	$0.5 \cdot 10^{-3}$	0.61

TABLE II

Isolation of plastocyanin and cytochromes c-553 and c-550, from Microcystis aeruginosa

	Volume (ml)	Protein (mg)	Absorbance × volume 620 nm	Cytochrome *c*-553 (mM)	Cytochrome *c*-550 (mM)	Plastocyanin (mM)
Crude extract	580	2184.09	>1160	–	–	–
2.34 M $(NH_4)_2SO_4$ supernatant	1000	181.58	2.00	$5 \cdot 10^{-5}$	$1.5 \cdot 10^{-4}$	*
Sephacryl column	55	3.627	0.77	$4.4 \cdot 10^{-5}$	$1.49 \cdot 10^{-4}$	$1.83 \cdot 10^{-4}$

* Not detectable due to interference of phycocyanin.

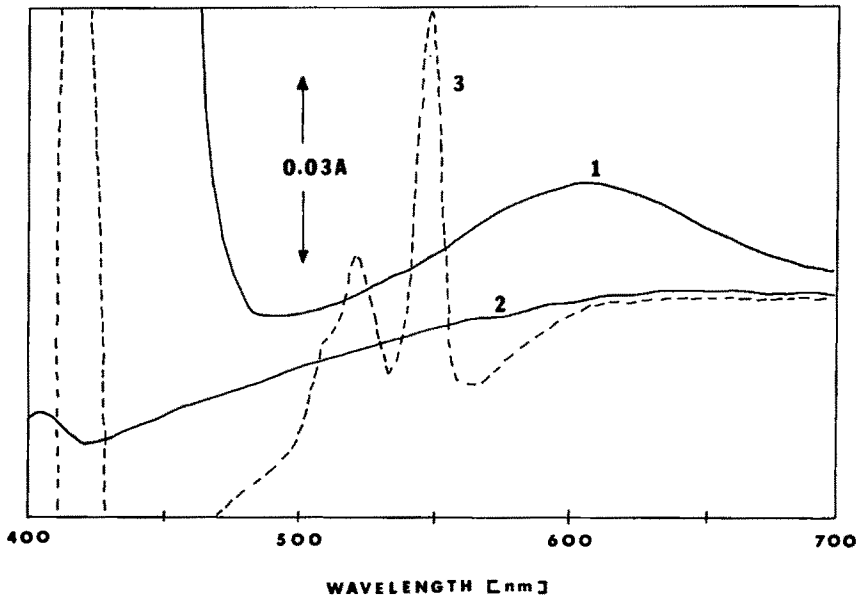

Fig. 2. The difference spectra of the cytochrome *c*-550-plastocyanin sample. The blank in all cases is the sample plus 1 mM ascorbate. (1) Sample plus 1 mM $K_3Fe(CN)_6$. (2) The sample plus 1 mM $K_3Fe(CN)_6$ and 1 mM ascorbate. (3) The sample plus 1 mM sodium dithionite.

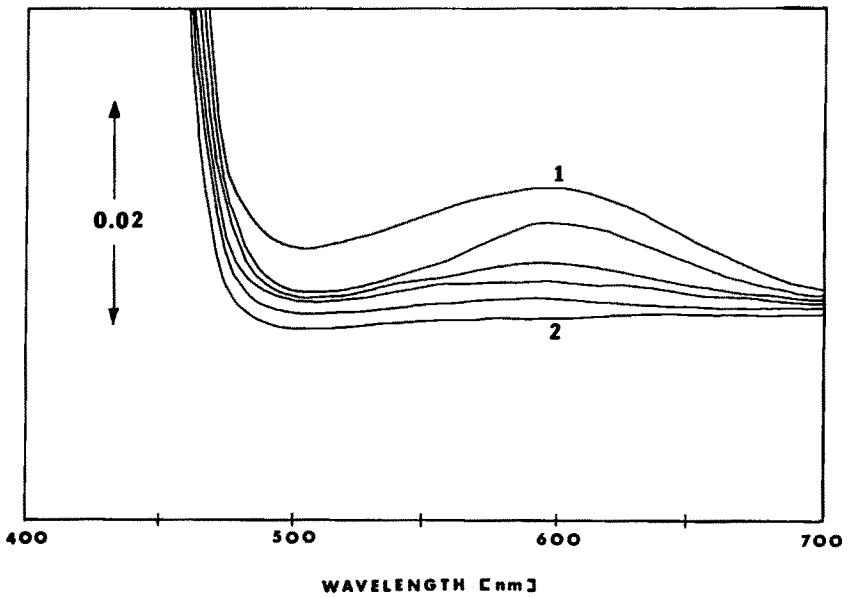

Fig. 3. The denaturation of plastocyanin in the presence of 1 mM $K_3Fe(CN)_6$ and 1 mM KCN. The denaturation is gradual. (1) Time 0. (2) After 10 min.

M. aeruginosa *Glu* *Thr* Phe *Thr* *Val* *Lys* Met *Gly* Gly *Asp* Ala *Gly* Thr *Leu* Gln
A. variabilis *Glu* *Thr* Tyr *Thr* *Val* *Lys* Leu *Gly* Ser *Asp* Lys *Gly* Leu *Leu* Val

Fig. 4. N-Terminal amino-acid sequences of cyanobacterial plastocyanins. Identical residues in the two sequences are set in italics.

below 7.5 because under such conditions *M. aeruginosa* does not grow very well. These growth studies provided the first indirect evidence that *M. aeruginosa* may produce plastocyanin when Cu is made available, although plastocyanin was not detected spectrophotometrically. Spectrophotometric detection of plastocyanin, while the sample is still in a very crude state, is possible in organisms where plastocyanin is relatively abundant but in *M. aeruginosa,* the very high absorbance in the range 600–650 nm due to phycobiliproteins tends to mask the small amounts of plastocyanin.

Isolation of plastocyanin

While working with the cytochromes *c*-553 and *c*-550, we have discovered that these coloured proteins bind to Sephacryl S-200 in the presence of 2.34 M $(NH_4)_2SO_4$ and it is therefore possible to concentrate them by this means [11]. A similar experiment using the supernatant (see Materials and Methods) from the 2.34 M $(NH_4)_2SO_4$ precipitation of the *Microcystis* proteins proved as fruitful for plastocyanin. Most of the proteins, including plastocyanin stuck to the top of the Sephacryl S-200 column forming a thick brownish-blue band at 2.34 M $(NH_4)_2SO_4$ while the eluate was straw coloured and contained neither cytochromes not plastocyanin. When a decreasing salt gradient was run, the cytochromes *c*-553 and *c*-550 were eluted close together followed by a slower moving blue phycobiliprotein band (Fig. 1a). The first pink band contained cytochrome *c*-553 whereas the slower pink band contained cytochrome *c*-550 and plastocyanin. The recovery of cytochromes *c*-553 and *c*-550 and plastocyanin obtained by this technique is given in Table II. As the cytochromes and plastocyanin are eluted at a higher salt concentration than the phycobiliproteins, they are relatively free of the troublesome pigments after this purification step and are easily detected by differential spectroscopy. Fig. 2 shows the difference spectrum of a cytochrome *c*-550-plastocyanin sample. When oxidized with $K_3Fe(CN)_6$, the sample forms a broad peak with a maximum around 597 nm, which indicates the presence of plastocyanin. On reduction with ascorbate this peak disappears but the reduction can be reversed by adding an excess of $K_3Fe(CN)_6$. Phycobiliproteins from *M. aeruginosa* do not show the same redox properties. Further reduction with sodium dithionite reveals the presence of cytochrome *c*-550. Fig. 3 shows that plastocyanin is slowly denatured in the presence of 1 mM KCN. The addition of more $K_3Fe(CN)_6$ cannot reoxidize the denatured plastocyanin. The yield of plastocyanin was estimated to be 4.2 times that of cytochrome *c*-553. Fig. 1b shows that plastocyanin was not found in cells grown in the absence of Cu.

With the isolated cytochrome *c*-550-plastocyanin fractions, we were able to further purify the plastocyanin to homogeneity (see Materials and Methods). We started with a soluble extract equivalent to 2.184 mg protein and recovered about 3 mg of homogeneous plastocyanin.

Properties of homogeneous plastocyanin

The molecular weight of plastocyanin determined by SDS-polyacrylamide gel electrophoresis was 10 500. This is similar to the value obtained for *A. variabilis* basic plastocyanin. Fig. 4 shows that the N-terminal amino acid of *M. aeruginosa* plastocyanin is similar to the sequence of *A. variabilis* plastocyanin. The two N-terminal amino-acid sequences have nine identical residues. The isoelectric point of *M. aeruginosa* is given in Table III. For comparison, p*I* values reported for other acidic and basic plastocyanins are included in the table. The p*I* values for *M. aeruginosa* and *P. laminosum* are quite similar and are closer to those reported for eukaryotic plastocyanins.

TABLE III

Isoelectric points of plastocyanins

	p*I*
Spinach [a]	4.2
Chlamydomonas eugametous [b]	4.49
M. aeruginosa [c]	4.8
P. laminosum [d]	5.1
A. variabilis [b]	7.75
Mastigocladus laminosus [b]	8.78

[a] Ramshaw et al. [7].
[b] Ho and Krogmann [5].
[c] This work.
[d] Stewart and Kaethner [6].

Discussion

Stewart and Kaethner [6] were the first to demonstrate an acidic plastocyanin in *P. laminosum*. These authors reported that the plastocyanin, although acidic, adsorbed very poorly on to DEAE-cellulose even in 10 mM phosphate buffer (pH 7.0). Moreover, the protein proved unstable when dialyzed against low-ionic-strength buffers. We encountered similar difficulties in our previous attempts to separate plastocyanin in the crude extracts of *M. aeruginosa*. The present procedure,

using high concentrations of ammonium sulfate to fractionate plastocyanin on Sephacryl S-200, provides a major advantage. This procedure avoids the use of low-ionic-strength buffers. In addition, this procedure allows the separation of plastocyanin from the phycobiliproteins. At the early stages of purification, we were unable to detect plastocyanin even in the 2.34 M ammonium sulfate supernatant, because of these interfering phycobiliproteins. After the Sephacryl S-200 column, plastocyanin and cytochrome *c*-550 were recovered in a concentrated fraction, thus making them more accessible to spectrophotometric studies. Our data indicate that when oxidized, plastocyanin has an absorption maximum at 597 nm and is sensitive to KCN. These are properties common to plastocyanin molecules from other organisms [4,20,21].

In a separate experiment, we have demonstrated that plastocyanin and cytochrome *c*-553 from *M. aeruginosa* are of similar charge and size (data not shown). It is therefore very difficult to resolve the two proteins by ion-exchange or gel-filtration chromatography. In the present study, cytochrome *c*-553 was found to bind less tightly on Sephacryl S-200 and was eluted well before plastocyanin.

It is worth mentioning that Sandmann [4] had examined the Cu-induced exchange of plastocyanin and cytochrome *c*-553 in different cyanobacterial species by means of spectrophotometric procedures. This author concluded that some species synthesized only cytochrome *c*-553, while others could exchange plastocyanin for cytochrome *c*-553 in Cu media. The exchange was complete in some species but not in *Pseudoanabaena catenata* and *Calothrix membranacea*, where a small amount of cytochrome *c*-553 was always present with plastocyanin. *Spirulina platensis* and *Spirulina maxima* are among those organisms in which plastocyanin has not been detected. These organisms, like *M. aeruginosa*, grow well in an alkaline medium (pH 8.5) and *S. maxima* is known to possess an acidic cytochrome *c*-553. Under such an alkaline condition, Cu is thought to be highly insoluble and is not freely available for cellular uptake. Sandmann [4] suggested this may in turn restrict the biosynthesis of plastocyanin. In view of the present finding on *M. aeruginosa*, it will be of interest to re-examine the formation of plastocyanin in *Spirulina* species using the biochemical procedures described here.

M. aeruginosa grows very well at pH 8.6 with 2 μM Cu, a concentration which is comparable to those used in the induction of plastocyanin in other species [4]. This concentration, however, is much lower than the 50 μM used in culturing *P. laminosum* for plastocyanin production [6]. For *M. aeruginosa*, there was a clear suppression of cytochrome *c*-553 synthesis in Cu-grown cultures during the log as well as stationary phases, when compared to those grown without Cu. This behavior parallels that observed in cyanobacteria that can partially exchange cytochrome *c*-553 for PC, although we were unable to detect plastocyanin in the small-scale-growth studies. When *M. aeruginosa* was grown at different pH values within its tolerance range, lowering the pH did not seem to enhance the Cu-induced suppression of cytochrome *c*-553. In many cases, the cells grew very poorly. When Cu concentrations in the growth medium exceeded 2 μM the cells also responded by growing poorly. It is therefore not possible to determine whether a higher Cu concentration will totally suppress cytochrome *c*-553 synthesis in *M. aeruginosa*.

In this study, we have demonstrated not only the occurrence of an acidic plastocyanin in a mesophilic cyanobacterium but also its purification to a state suitable for structural or functional studies. An important characteristic of acidic plastocyanins from higher plants is the existence of a region of acidic amino acids around the centre of the molecule [8]. Other acidic plastocyanins from green algae appear to possess a similar but less acidic region [10,22–24]. In the basic plastocyanin, most of the acidic amino acids that form the acidic region in higher plant plastocyanins are not conserved [25]. For instance, there are nine acidic amino acids that form the acidic region in spinach plastocyanin and only one is conserved in *Anabaena* plastocyanin. We are now working on the entire sequence of *Microcystis* plastocyanin in order to find out how many of the acidic amino acids that form the acidic region in higher plant plastocyanin may persist in this cyanobacterial plastocyanin.

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