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ISOLATION OF PHOTOSYNTHETIC CATALYSTS FROM CYANOBACTERIA

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Summary

Methods are described for the isolation of ferredoxins I and II, cytochrome *c*-553, cytochrome *f*, cytochrome *c*-550 and plastocyanin from large quantities of various cyanobacteria. The amino acid composition of cytochrome *c*-550 is reported. There is a variation in the relative amounts of these proteins in different batches of cells which may relate to the nutritional status of the organisms.

Introduction

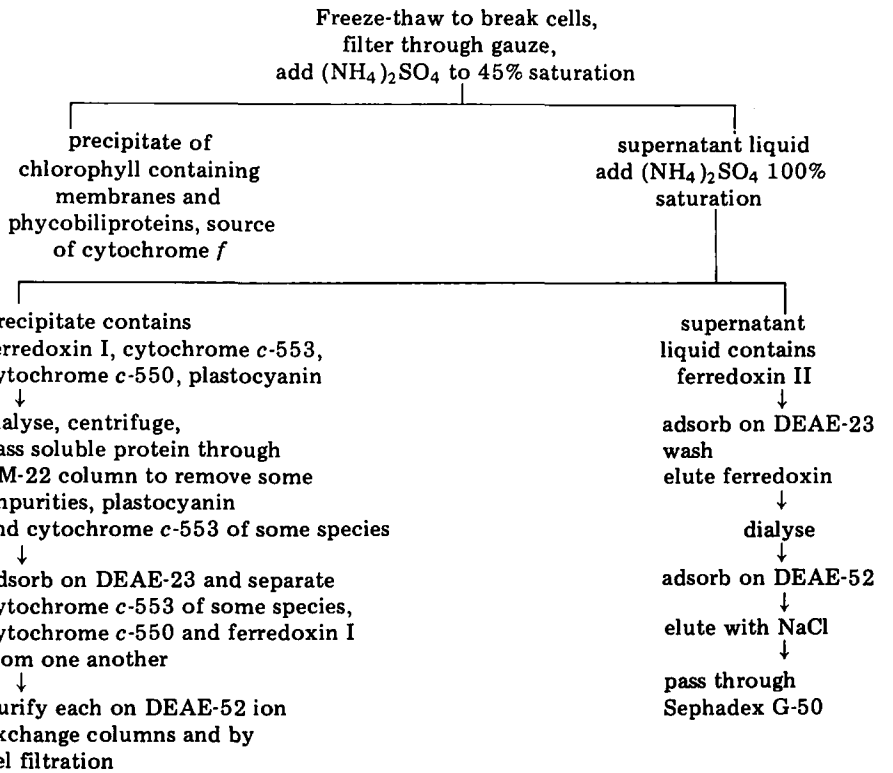
This work began as an effort to isolate large amounts of certain photosynthetic catalysts from cyanobacteria (blue-green algae) in order to provide sufficient amounts of material for nuclear magnetic resonance and other types of structural analysis. Several naturally occurring unialgal blooms were used as a source of cells so that large quantities of starting material could be obtained without the labor needed to grow these organisms in the laboratory. Ferredoxin I and II, cytochrome *c*-553, cytochrome *f*, cytochrome *c*-550 and plastocyanin were sought. These large preparations of cyanobacteria allowed some interesting observations on changes in the concentration of some catalysts and suggest that important changes in metabolism occur in the natural state in response to growth conditions.

Materials and General Methods

Large samples of approx. 50 kg fresh weight of packed cyanobacteria were obtained from the following locations: *Microcystis aeruginosa* was collected

Abbreviations: Mes, 4-morpholinoethanesulfonic acid; SDS, sodium dodecyl sulfate.

from Lake Kegonsa, Wisc., in August, 1974. *Aphanizomenon flos-aquae* was collected from Lake Okoboji, Iowa, in August, 1975 and again in September, 1976. Several samples of *Spirulina maxima* were obtained from the commercial culture of Sosa Texcoco Co., at Lake Texcoco, Mexico, during January and February, 1977. In each case, the cyanobacteria were skimmed from the surface of the lake where they had collected in a dense mass and the excess water was drained off through a fine cloth screen. The cells were poured into steel drums and frozen. Fresh samples were scanned visually with a light microscope and no gross contamination could be detected. Amino acid sequence determinations on several of the proteins are in progress and indicate that the proteins are of uniform primary structure so the cell material appears to be quite homogeneous. *Anabaena variabilis* was grown in the laboratory as previously described [1]. The sample of *Phormidium luridum* was the generous gift of Dr. H.L. Crespi and had been grown in the mass culture facility of the Argonne National Laboratory [2]. Scheme I is a diagrammatic outline of the general procedure for



SCHEME I. Diagrammatic outline of the general procedure for fractionation of proteins from cyanobacteria.

isolating the proteins. Cells in 5 kg batches were suspended in an equal volume of distilled water and broken by three freezing cycles at −15°C overnight and thawing. The broken cell mass was filtered through a gauze pad. This filtration held back very large amounts of green residue from the blue aqueous extracts

of *M. aerugenosa* and *A. flos-aquae*. The green residue which consisted of broken cells with photosynthetic membranes was re-extracted several times with large volumes of distilled water until all of the soluble protein, identified by the blue color of phycocyanin, had been released. The filtrates were then combined. *P. luridum*, *S. maxima*, and *A. variabilis* preparations pass through the gauze filter, therefore the preparation was diluted with distilled water and frozen and thawed again to get maximum cell disruption. Occasionally, small batches of cells were broken by prolonged sonication or by repeated passage through a Manton Gaulin homogenizer. These treatments increased the yield of soluble protein by as much as 30%. However, it was impractical to process very large volumes of cells by these techniques, so a higher yield was sacrificed for the implicity of the freeze-thawing in disrupting the cells. A 50-kg batch of fresh weight in cells resulted in approximately 150 l aqueous extract. The aqueous extract was brought to 45% saturation by addition of solid $(NH_4)_2SO_4$. The preparation was put in 8-l carboys and allowed to stand for several weeks at 2°C to allow precipitation of green membrane fragments and most of the phycobiliproteins. The yellow-brown soluble phase was siphoned off and the blue-green precipitates were recombined, allowed to settle, siphoned and finally centrifuged at $3000 \times g$ for 5 min to remove the insoluble material. The soluble portion at this stage was returned to the cold room for several weeks and then filtered through a celite pad to remove small particles of green and blue pigments. The soluble material from the 45% $(NH_4)_2SO_4$ fractionation was brought to saturation with solid $(NH_4)_2SO_4$ and returned to the cold for several weeks to allow precipitation of the proteins. The *S. maxima* preparation was unique in that a large layer of yellow material formed at the top of the carboys within 24 h after saturation with $(NH_4)_2SO_4$. If allowed to stand undisturbed this material would precipitate to the bottom of the container. However, it proved convenient to remove this layer by siphoning it off and carefully filtering it through coarse filter paper. This insoluble material was rich in lipid and contained a great deal of carotenoid-protein complex which, if allowed to precipitate into the main protein fraction, would saturate the cellulose ion-exchange columns used in subsequent steps.

All of the protein that could be precipitated by saturating with $(NH_4)_2SO_4$ had collected at the bottom of the carboys within 2 weeks. The soluble phase was carefully siphoned off and the remaining suspensions of insoluble protein in each carboy were combined and allowed to settle for an additional week. The soluble fraction was again siphoned off. By recombining the fractions containing precipitated protein and allowing these to settle, the volume of the 45—100% $(NH_4)_2SO_4$ fraction was reduced from 150 l to approx. 8 l. The suspension was centrifuged at $3000 \times g$ for 5 min to further concentrate the insoluble protein.

The protein precipitated by saturated $(NH_4)_2SO_4$ was resuspended in 1—2 l cold distilled water and dialysed for 48 h against running tap water at 4°C. We subsequently realized that prolonged dialysis against tap or distilled water caused a lowering of ferredoxin (but not cytochrome) yield. If ferredoxin is to be recovered, it is necessary to dialyse against 5 mM phosphate buffer, pH 7.2. The dialysed protein was centrifuged at $8000 \times g$ for 5 min to remove a gray precipitate and, if necessary, filtered through Whatman No. 1 filter paper

to remove the last traces of opalescent material. Samples of this fraction containing approx. 200 mg protein were further fractionated by ion-exchange chromatography. The dialysed protein was brought to 5 mM with Tris-Mes buffer (pH 7.5) and applied to a coarse-grade carboxymethyl cellulose column (5 X 20 cm; Whatman CM-22) which had been equilibrated with the same buffer. This column would adsorb soluble cytochrome *c*-553 and plastocyanin from extracts of some species of cyanobacteria and conveniently removes, by irreversible adsorption, some denatured phycobiliproteins. The protein which did not adsorb on the carboxymethyl cellulose column was loaded onto a coarse grade DEAE cellulose column (5 X 20 cm; Whatman DEAE-23) equilibrated with the same Tris-Mes buffer. The column was then eluted with successive washes of 0.1, 0.2, 0.3, and 0.5 M NaCl containing Tris-Mes buffer, so that the cytochromes and ferredoxin could be separated from one another and from residual blue pigments. The details of purification for each of these proteins are given below.

Results

Distribution of specific proteins in various cyanobacteria

The occurrence of several proteins varied with the source of cyanobacteria. Ferredoxin I, a protein which is easily detected by fractionation, could not be detected in two samples of cyanobacteria collected from nature (*M. aeruginosa* and *A. flos-aquae*, 1975) and was markedly diminished in the *S. maxima* from a stationary culture in poor condition. Ferredoxin II [3,4] was found in all samples containing ferredoxin I. Cytochrome *c*-553 was absent from the 1975 *A. flos-aquae* sample and from the 'poor' *S. maxima* sample, both of which were deficient in ferredoxins. However, cytochrome *c*-553 was present in the *M. aeruginosa* sample which lacked ferredoxin so the disappearance of these two proteins is not necessarily linked. Cytochrome *f* was found in all samples examined for this protein. Here we follow Wood's definition of using cytochrome *f* to mean the cytochrome tightly bound to the membrane while cytochrome *c*-553 refers to the smaller, easily solubilized cytochrome [5]. While cytochrome *c*-550 is not known to be a participant in photosynthesis, it appears in these fractionations and shows striking quantitative variations in the different samples of cyanobacteria. Cytochrome *c*-550 was the only protein among those looked for which was present in the 1975 collection of *A. flos-aquae*. This protein was relatively abundant in the soluble protein extracts of *M. aeruginosa* and the *S. maxima* in 'poor' condition. Laboratory grown *A. variabilis* and *P. luridum* gave only 10% of the maximum yield of soluble cytochrome *c*-550 seen in the 1975 *A. flos-aquae*, *M. aeruginosa* and 'poor' *S. maxima*. Cytochrome *c*-550 was not detected in aqueous extracts of the relatively healthy collections of *A. flos-aquae* 1976 and *S. maxima* but traces of this cytochrome were released from the 0—45% $(NH_4)_2SO_4$ pellets from these cyanobacteria after treatment with 80% acetone or 1% Tween 20. Plastocyanin was not found in any of the algae except laboratory grown *A. variabilis* in the late logarithmic phase of growth and the *P. luridum*. If the *A. variabilis* culture was allowed to reach the stationary phase, plastocyanin could no longer be detected.

Isolation and characterization of cytochrome c-550

The *A. flos-aquae* collected in 1975 proved an abundant source of the low potential cytochrome *c*-550. This cytochrome is usually found in the oxidized state in extracts and is easily recognized by its lack of reduction by ascorbic acid and full reduction by sodium dithionite. The dialysed 45—100% $(NH_4)_2SO_4$ fraction of protein was brought to 1 mM with Tris-HCl buffer (pH 7.2) and passed through a CM-22 cellulose ion-exchange column as described in Methods. This step removed some green and blue pigments from the extract. The sample was then loaded onto a DEAE-23 column (5 X 25 cm) which had been equilibrated with 1 mM Tris (pH 7.2). A pink material which came through the column appeared to be a carotenoid protein. The column was first washed with 2 l each of 0.05 M and 0.1 M NaCl which removed a carotenoid protein. Washing with 0.2 M NaCl removed material with blue visible color and red fluorescence which is probably a phycocyanin degradation product. The cytochrome *c*-550 was eluted with 200 ml 0.5 M NaCl. The cytochrome fraction was dialysed against 5 mM phosphate buffer, pH 7.0, and adsorbed onto a microgranular DEAE-cellulose column (2.5 X 20 cm; Whatman DEAE-52) which had been equilibrated with that buffer. The column was washed with 2 l 0.175 M NaCl containing 1 mM phosphate buffer (pH 7.0) to remove completely a yellow pigment. The cytochrome was then eluted in 800 ml 0.2 M NaCl. The cytochrome fraction was again dialysed against 1 mM phosphate buffer (pH 7.0) and adsorbed on a DEAE-52 column (2.5 X 20 cm) equilibrated with this buffer. The column was washed with 2 l 0.1 M NaCl containing 1 mM phosphate buffer, pH 6.0, then developed with a linear gradient from 0.15 to 0.3 M NaCl containing the same buffer. Yellow pigments again appeared both ahead of and behind the cytochrome fraction. The cytochrome fractions were pooled, diluted 4-fold and adsorbed onto a small (1 X 5 cm) DEAE-52 column. Elution of the column by 2 M NaCl released all of the cytochrome in a volume less than 10 ml. The sample was dialysed against 1 mM phosphate buffer, pH 7.0, sucrose was added to 10%, and then the sample was applied to a Sephadex G-50 fine column (2.5 X 90 cm) equilibrated with 1 mM phosphate buffer. Small amounts of yellow pigment emerged ahead of the cytochrome. The peak fractions of cytochrome were pooled, the phosphate concentration raised to 0.1 M, and passed through the same column equilibrated with 0.1 M phosphate buffer, pH 7.0. This preparation, when injected into a rabbit, elicited an antiserum which gave a single precipitin line [6].

The absorption spectra of the oxidized and reduced cytochrome *c*-550 are seen in Fig. 1. The cytochrome is not reduced by sodium ascorbate, but it is reduced by sodium hydrosulfide so it can be characterized as a low potential cytochrome. The cytochrome is not reduced by NADH, NADPH, NO_2^- or NH_2OH even when a crude extract of *A. flos-aquae* is included in the reaction mixture. The cytochrome is reduced by excess NaHS. The cytochrome shows absorption maxima at 530, 408, and 350 nm in the oxidized form and at 550, 522, and 418 nm in the reduced form which is virtually identical to the low potential *c*-type cytochrome from *Anacystis nidulans* described by Holton and Myers [7]. The absorption spectrum of the protein at a concentration of 2 mg per ml was examined carefully, but showed no 695 nm peak which is attributed to a methionine ligand on the heme iron.

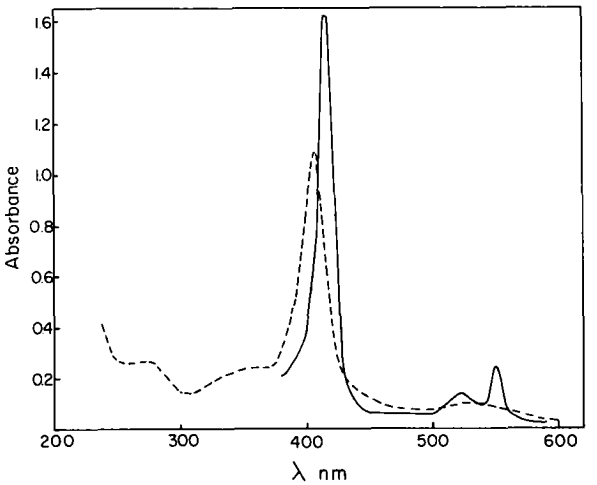


Fig. 1. Absorption spectra of cytochrome *c*-550 from *A. flos-aquae*. The cytochrome was purified as described in the text. The cytochrome was isolated in the oxidized state in 0.1 M phosphate buffer (pH 7.0) and the spectrum of the oxidized form (dashed line) measured with a Cary 14 spectrophotometer. The excess solid potassium hydrosulfide was added and the spectrum of the reduced cytochrome (solid line) was measured.

The amino acid composition of the cytochrome *c*-550 was determined using samples containing 1 mg protein in 1 mM potassium phosphate buffer, pH 7.2, diluted with an equal volume concentrated HCl in a hydrolysis tube. The samples were frozen, evacuated, and hydrolysed as described by Moore and Stein [8]. Hydrolysis was carried out for 24, 48, and 72 h. Amino acid analysis was done in a Durrum amino acid analyzer. Duplicate samples were subjected to the procedure of Ambler and Wynn [9] to remove the heme and were then oxidized by the procedure of Hirs [10] for determination of cysteine and methionine as their performic acid oxidation products. The amino acid composition of cytochrome *c*-550 is given in Scheme II. A minimal molecular

Lys 6	Thr 8	Gly 8	Met 2
His 5	Ser 6	Ala 8	Ile 4
Arg 4	Glu 13	Cys 6	Leu 16
Asp 17	Pro 8	Val 6	Tyr 4
			Phe 4
			Trp —
			(not determined)

SCHEME II. Amino acid composition (as number of residues per molecule) of cytochrome *c*-550 from *Aphanizomenon flos-aquae*.

weight of approx. 15 000 gave the best integral fit of the amino acid analyses data and this value was confirmed by molecular weight measurement of the cytochrome in the ultracentrifuge. The molecular weight was calculated by the method of Yphantis [11]. The partial specific volume was estimated from the amino acid composition by the procedure of McMeeken et al. [12]. The ultracentrifuge measurements were made in 5 mM phosphate buffer, pH 7.2.

Occurrence of cytochrome c-550

Cytochrome *c*-550 was not found in the easily extracted proteins from the 1976 collection of *A. flos-aquae*. However, when 45% $(NH_4)_2SO_4$ precipitate, which contains the chlorophyll-rich membrane, was treated with acetone or Tween 20, cytochrome *c*-550 was released. For the acetone extraction, 400 g precipitate from the ammonium sulfate fractionation were suspended in 1 l distilled water, and an equal volume of cold acetone (—15°C) was slowly added with vigorous stirring. The mixture was filtered through Whatman No. 1 filter paper and the soluble phase was discarded. The insoluble residue was resuspended in 250 ml 0.1 M NaCl and dialysed against cold distilled water. After dialysis, the preparation was centrifuged at $4000 \times g$ for 10 min to remove a large mass of insoluble material. The soluble protein was brought to 55% saturation with $(NH_4)_2SO_4$ and the precipitated protein removed. The soluble protein was dialysed against cold distilled water, centrifuged to remove insoluble material, loaded onto a DEAE-52 column (2.5 × 20 cm) and equilibrated with 5 mM phosphate buffer (pH 7.0). The column was eluted with a 0.1—0.5 M NaCl gradient and the cytochrome *c*-550 was easily identified as a red band emerging in the first third of the gradient. Recognition of this cytochrome is even easier when the membrane fraction is treated with Tween 20. A sample of the 45% $(NH_4)_2SO_4$ precipitate of similar size was resuspended in distilled water and made 1% with Tween 20 and then stirred for 15 min. The preparation was centrifuged at $10\,000 \times g$ for 20 min and the precipitate discarded. The soluble proteins were dialysed over night against cold distilled water, centrifuged as before, and loaded onto a DEAE-52 column (2.5 × 20 cm) equilibrated with 5 mM phosphate buffer (pH 7.0). The gradient elution with 0.1—0.5 M NaCl again yielded cytochrome *c*-550. In these experiments the yield of cytochrome *c*-550 was low, approx. 5% of the amount of cytochrome *c*-553 from the same algae or 5% of the amount of cytochrome *c*-550 in the 1975 collection of *A. flos-aquae* on a fresh weight basis.

Cytochrome *c*-550 and *c*-553 were present in equal concentrations in aqueous extracts of *M. aeruginosa*. *M. aeruginosa* cytochrome *c*-550 was more easily purified than this protein from *A. flos-aquae* since it was held more tightly to DEAE columns and eluted at 0.5 M NaCl. Otherwise purification proceeded in the same fashion as for *A. flos-aquae*. We failed to observe this cytochrome *c*-550 among the soluble, easily extractable proteins obtained from *S. maxima* collected from the Lake Texcoco culture when the culture was judged to be in optimum condition by the Sosa Texcoco S. A. staff. These samples contained ferredoxin and cytochrome *c*-553. However, cytochrome *c*-550 was found in a sample of *S. maxima* collected when the algal harvesting process had been shut down for several days and the culture was judged to be in poor condition. It is interesting to note that this sample gave a strong odor of H_2S when the cells were broken open by sonication. Recently one of us has detected traces of cytochrome *c*-550 in apparently healthy samples of *S. maxima* [13]. The *S. maxima* cytochrome *c*-550 is bound to DEAE columns and elutes in 0.3 M NaCl either with or immediately after any blue phycocyanin which had failed to precipitate in the $(NH_4)_2SO_4$ fractionation.

Cytochrome *c*-550 is regularly found in the soluble protein fraction extracted from *A. variabilis* at a concentration of about 10% that of cyto-

chrome *c*-553 in this organism. *P. luridum* also yielded small amounts of this cytochrome in an easily extractable form. In both of these cyanobacteria, the cytochrome was adsorbed to DEAE and eluted with 0.3 M NaCl.

Cytochrome c-553 and plastocyanin

The procedures for the isolation and purification of these proteins have been described elsewhere [14,15]. Plastocyanin was found only in laboratory-grown cells, *A. variabilis* and *P. luridum*. Its absence from the other samples could reflect the depletion of copper from the environment of the dense natural blooms [16]. The cytochrome *c*-553 which is the functional replacement for plastocyanin in copper-deficient cultures, was found to be present with plastocyanin in the laboratory-grown cells and was present in the other material except in *A. flos-aquae* 1975 and in the poor sample of *S. maxima*, both of which were also lacking ferredoxin. There was relatively more cytochrome *c*-553 in *S. maxima* (1 mg/10 mg chlorophyll) than in *A. variabilis* (1 mg/33 mg chlorophyll) in which some of its function was being fulfilled by plastocyanin.

It is interesting to note that cytochrome *c*-553 can exist in three forms with identical primary structure [17]. Large samples of this cytochrome from *A. variabilis*, *S. maxima* and *A. flos-aquae* were chromatographed on ion-exchange cellulose and careful elution revealed three distinct bands of cytochrome with identical absorption spectra. Table I summarizes these data. When the major band of cytochrome is re-chromatographed under the same conditions, three bands result in approximately the same proportions.

Cytochrome f

In a variety of photosynthetic organisms, a *c*-type cytochrome commonly referred to as cytochrome *f* is found to be tightly bound to the chloroplast membrane. Wood [5] achieved the isolation of cytochrome *f* from a cyanobacterium, *A. nidulans*, and clearly distinguished it from the soluble cytochrome *c*-553. We sought cytochrome *f* in the membrane fraction of the three genera of cyanobacteria, *A. variabilis*, *A. flos-aquae*, and *S. maxima*, and found it in all three. Since Wood's method allows the isolation of cytochrome *f* in

TABLE I

MULTIPLE FORMS OF CYTOCHROME *c*-553

Organism	Chromatogram	Cytochrome (%)		
		I	II	III
Anabaena variabilis	0.1—5 mM phosphate, pH 7, gradient elution of CM-22 column	2	97	1
Aphanizomenon flos-aquae	50—100 mM phosphate, pH 8, gradient of CM-Sephadex column	2	14	84
Spirulina maxima	1—100 mM NaCl, pH 6, gradient of DEAE-52 column	4	68	28

amounts too small to be practical for further purification and characterization studies, an attempt to find a large scale purification procedure has been made. A sample of the membrane-rich 45% ammonium sulfate precipitate from broken cells of *A. flos-aquae* was first subjected to lyophilization and 25 g dry membrane were resuspended in about 1 l distilled water. To this suspension, 20 g of each sodium cholate, sodium deoxycholate and Triton X-100 were added. The suspension was stirred in the cold for 3 h, then centrifuged at 48 000 X *g* for 10 min. This detergent washing step removed most of the phycocyanin and other coloured pigments which tend to burden subsequent chromatographic steps and which, by virtue of their colors, interfere with spectrophotometric detection of the cytochrome. The pellet was resuspended in 1 l 0.1 M phosphate buffer solution, pH 7.6, containing 1 mM EDTA. Cold acetone (—20°C) was added to the resuspended membrane fraction to a final concentration of 80%. After standing for 30 min at 0°C, most of the acetone could be removed by decantation and the remaining suspension was centrifuged briefly to remove the rest of the acetone extract. This acetone extraction step is important for the removal of cartenoids and lipophilic materials which otherwise would contaminate the cytochrome *f* fraction at a later stage. The acetone precipitate was resuspended in 0.1 M phosphate buffer, pH 7.6, containing 1 mM EDTA and 1 mM dithiothreitol in a final volume of 250 ml. An equal volume of cold ethanol (—20°C) containing 0.5% NH_4OH was added and the mixture stirred for 5 min. The preparation was centrifuged at 48 000 X *g* for 10 min and the precipitate was discarded. The supernatant liquid was loaded onto a DEAE-23 column (3.5 X 10 cm) which had been equilibrated with 0.01 M phosphate buffer (pH 7.6). This chromatographic step is essential for the removal of materials which tend to aggregate with cytochrome *f*. The column was washed with 300-ml volumes each of the following: ethanol containing 0.5% NH_4OH, 0.1 M phosphate buffer (pH 7.6), 0.3 M phosphate buffer (pH 7.6), 1 M phosphate buffer (pH 7.6), and 1 M Tris-HCl buffer (pH 8). These washings removed blue pigments which seem to be phycocyanin in various stages of denaturation. The cytochrome was eluted as a brown band using 150 ml of a solution containing 1% deoxycholate and 2% cholate in 1 M Tris-HCl buffer (pH 8). The cytochrome fraction was dialysed extensively against 10 mM Tris-HCl buffer containing 1 mM dithiothreitol and then adsorbed on a DEAE-23 column (3.5 X 10 cm) which had been equilibrated with the dialysis buffer. The column was washed with 2 l 5 mM phosphate buffer (pH 7.6) to remove blue pigments. Cytochrome *f* was eluted as a red band with 0.5 M phosphate buffer (pH 7.6). Additional cytochrome *f* could be eluted from the column with the deoxycholate/cholate mixture used to elute the previous column and this detergent-eluted material was recycled through dialysis and DEAE-chromatography to render it in a phosphate-elutable form. The cytochrome *f* that had been eluted with phosphate buffer was almost free of other colored contaminants as shown in Fig. 2. Using other known proteins as markers, the molecular weight of this cytochrome was estimated to be 30 000 by SDS-gel electrophoresis.

Ferredoxins

Ferredoxin was isolated from the extracts of cyanobacteria in two ways.

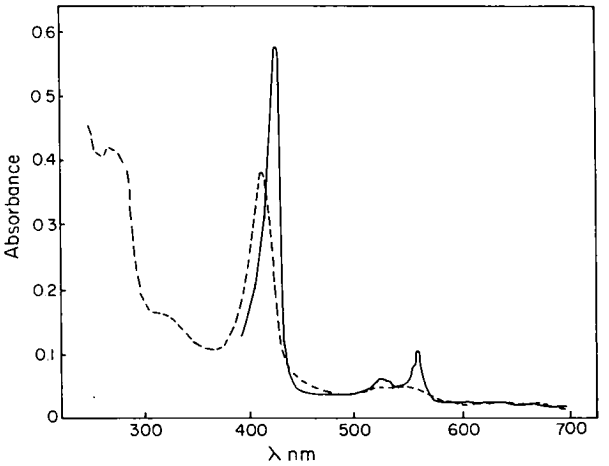


Fig. 2. Absorption spectra of cytochrome *f* from *A. flos-aquae*. The cytochrome was prepared as described in the text and sufficient potassium ferricyanide was added to completely oxidize the cytochrome. The sample was then dialysed against 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.2 M NaCl. The absorption spectrum of the oxidized cytochrome (dashed line) was measured with a Cary 14 spectrophotometer. Then solid sodium ascorbate was added in excess and the spectrum of the reduced cytochrome (solid line) was measured.

Ferredoxin I was isolated by the same procedures which work well with higher plant chloroplast ferredoxins. This protein precipitates from saturated $(NH_4)_2SO_4$ solutions and is tightly held on DEAE columns. Ferredoxin I was eluted from DEAE columns with 0.5 M NaCl after all cytochromes, phycobilins and other proteins had been washed off with 0.3 M NaCl. This ferredoxin can be further purified by rechromatography on DEAE cellulose and Sephadex gel filtration columns [11]. In contrast, ferredoxin II is not precipitated by saturating $(NH_4)_2SO_4$ and is held less tightly on DEAE cellulose, it is eluted by 0.2 M NaCl in samples from the genera we have examined.

For the isolation of ferredoxin II, the supernatant liquid from the saturated $(NH_4)_2SO_4$ solution was passed through a large DEAE-23 column (6 X 20 cm) which had been equilibrated with saturated $(NH_4)_2SO_4$. A brownish-red band collected in the column and approx. 30 l extract saturated the column with brown pigment. The column was washed with 5 l saturated $(NH_4)_2SO_4$ to remove all visible pigment from the washings. The column was then washed with 10 l cold distilled water. This causes loss of ferredoxin but efficiently removes a yellow-green pigment which otherwise is hard to separate in gradient elutions. The ferredoxin was then eluted with 2 M NaCl and dialysed against 1 mM phosphate buffer (pH 7.6). The ferredoxin sample was centrifuged at 8000 X *g* for 5 min to remove insoluble material and was then adsorbed onto a DEAE-52 column (2.5 X 30 cm) which had been equilibrated with the dialysis buffer. The column was washed with 0.1 M NaCl until all of the pale yellow color was removed. Ferredoxin II was then eluted with 0.2 M NaCl. Elution at this salt concentration required a large volume of eluant but improves the separation from ferredoxin I. Although most had been precipitated, small amounts of ferredoxin I are present in the saturated $(NH_4)_2SO_4$ supernatant fraction and this ferredoxin is eluted from the DEAE column with 0.4—0.5 M NaCl.

Discussion

The variations in amount of photosynthetic catalysts in a cyanobacterium has been well documented. The most obvious variable is the concentration of the phycobiliproteins which normally serve as light-harvesting pigments but, in conditions of nitrogen deficiency, are consumed as a nitrogen reserve by the cells [18]. The disappearance of ferredoxin in response to iron deficiency has been well documented with laboratory cultures of cyanobacteria [19,20]. Photosynthetic function is maintained by the substitution of a flavoprotein (flavodoxin) for the ferredoxin which may be sacrificed for the sake of conserving iron. This may occur in nature since the *A. flos-aquae* 1975 and the *M. aeruginosa* were both devoid of ferredoxin. Both samples came from dense blooms of cyanobacteria which had been at the surface of the lake for several days. In addition, ferredoxin had nearly disappeared from the *S. maxima* in the controlled lake culture of Sosa Texcoco when the culture ponds were allowed to stand unharvested for several days. In all samples of obviously healthy organisms, ferredoxin was present, and in all of these samples that were properly examined for them, both ferredoxin I and ferredoxin II were found.

Plastocyanin content of cyanobacteria has been shown to vary with the availability of copper and its photosynthetic function is taken over by cytochrome *c*-553 in copper deficient cells [15]. We found plastocyanin only in laboratory grown cells, and noted that it seems to disappear from older cultures of *A. variabilis* with a concomitant rise in the cytochrome *c*-553 level.

Much credit is due to Dr. P. Wood for clearly distinguishing between cytochrome *c*-553 and cytochrome *f*. While these two cytochromes are similar with respect to the position of the α-band in the absorption spectrum and similar with respect to their redox potential, they are distinct in their membrane-binding properties, distinct in their functional roles in the electron transport chain, and distinct in their sizes. Cytochrome *c*-553 might be classified as a peripheral membrane protein since it is easily released from the photosynthetic membranes by sonication [13] or freeze-thawing. This is in sharp contrast to cytochrome *f*, an intrinsic membrane protein which is not even released by detergent treatment but is only solubilized after harsh extraction with alkaline organic solvents. Cytochrome *c*-553 was present in all but two of the samples examined. Cytochrome *c*-553 was not found in the *A. flos-aquae* 1975 or the *S. maxima* from the shut down culture. One might imagine that the small proteins like this cytochrome and ferredoxin had leaked out of these cells, although the large phycobiliproteins had clearly remained inside the cells. The leakage of small proteins seems unlikely since these cells contained cytochrome *c*-550 (M_r = 15 000) in a soluble and readily extractable form. It may be that in the terminal stages of iron deficiency, iron is salvaged from cytochrome *c*-553 to sustain some more critical process.

Cytochrome *f* was found in each of those samples where it was properly looked for. It will be of interest to see if this protein ever disappears from a viable bacterium or alga since one might expect an intrinsic membrane protein of apparently critical function to be exempt from self-cannibalism.

Cytochrome *c*-550 is the least known of the proteins examined in this study. It is easily extracted and recognized in those samples which are otherwise

lacking one or more photosynthetic catalysts. Yet this cytochrome is hard to detect in cyanobacteria with a full complement of photosynthetic catalysts. Cytochrome *c*-550 was the only cytochrome present in the *A. flos-aquae* 1975 sample and its concentration was approx. 1 mg per 10 mg chlorophyll, which is about the level of cytochrome *c*-553 in *S. maxima*, *A. variabilis*, and the *A. flos-aquae* 1976. Cytochrome *c*-550 was found in the soluble proteins at about the same concentration in *M. aeruginosa* and was present simultaneous with a similar concentration of cytochrome *c*-553. The two samples of *S. maxima* showed cytochrome *c*-550 only in the unhealthy cells. We have seen traces of this cytochrome in many preparations from *A. variabilis* and in the sample of *P. luridum* but it is present at 5% or less of the concentration of cytochrome *c*-553. This concentration level is considerably below that reported for the analogous cytochrome *c*-549 in *Anacystis nidulans*, in which the concentration of *c*-549 was 60—80% that of *c*-553 [21]. Izawa and Vernon had found cytochrome *c*-550 in *A. variabilis* in a ratio of one for three cytochrome *c*-553 [22]. We have observed that small amounts of cytochrome *c*-550 can be released from well-washed photosynthetic membranes following treatment with 80% acetone or by 1% Tween 20. It may be that this cytochrome is present in a tightly bound state in all cyanobacteria and is released into the extractable protein fraction in deteriorating cells.

Cytochrome *c*-550 is unique with respect to its low redox potential (—0.26 *V*) and its amino acid composition. The strong odor of H_2S released on disruption of the cytochrome *c*-550 containing cells of *S. maxima* prompted us to test for the reduction of this cytochrome by sulfide. The work of Padan et al. [23,24] has shown that cyanobacteria can adapt to H_2S utilization so the low potential cytochrome might function in this pathway.

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References

1 Kratz, W.A. and Myers, J. (1945) Am. J. Bot. 42, 282—295
2 Taecker, R.G., Crespi, H.L., DaBoll, H.F. and Katz, J.J. (1971) Biotechnol. Bioeng. 13, 779—793
3 Hase, T., Wada, K. and Matsubara, H. (1975) J. Biochem. Tokyo 78, 605—608
4 Hutson, R.G. and Rogers, L.J. (1975) Biochem. Soc. Trans. 3, 377—378
5 Wood, P.M. (1977) Eur. J. Biochem. 72, 605—612
6 Ouchterlony, O. (1953) Acta Pathol. Microbiol. Scand. 32, 231—236
7 Holton, R.W. and Myers, J. (1967) Biochim. Biophys. Acta 131, 362—374
8 Moore, S. and Stein, W. (1963) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 6, pp. 819—830, Academic Press, New York

9 Ambler, R.P. and Wynn, M. (1973) Biochem. J. 131, 485—497
10 Hirs, C.H.W. (1967) in Methods in Enzymology (Hirs, C.H.C., ed.), Vol. 11, pp. 59—62, Academic Press, New York
11 Yphantis, D.A. (1964) Biochemistry 3, 297—317
12 McMeekin, T.L., Groves, M.L. and Hipp, N.J. (1949) J. Am. Chem. Soc. 71, 3298—3300
13 Gomez-Lojero, C. and Perez-Gomez, B. (1978) in Temas Bioquimicos de Actualidad (Chagoya, V., Martuscelli, J., Pena, A. and Pina, E., eds.), p. 321, UNAM Publicaciones, Mexico
14 Susor, W.A. and Krogmann, D.W. (1966) Biochim. Biophys. Acta 120, 65—72
15 Lightbody, J.L. and Krogmann, D.W. (1967) Biochim. Biophys. Acta 131, 508—515
16 Wood, P.M. (1977) Abstracts 4th Int. Congr. Photosynthesis, Reading, U.K. (Hall, D.O. and Coombs, V., eds.), p. 415, U.K. Science Committee, London
17 Ambler, R.P. and Bartsch, R.G. (1975) Nature 235, 285—288
18 Allen, M.M. and Smith, A. (1969) Arch. Microbiol. 69, 114—120
19 Smillie, R.M. (1966) Plant Physiol. 40, 1124—1128
20 Trebst, A. and Bothe, H. (1966) Ber. Dtsch. Bot. Ges. 79, 44—47
21 Holton, R.W. and Myers, J. (1963) Science 142, 234—235
22 Izawa, T. and Vernon, L.P. (1971) Biochim. Biophys. Acta 226, 88—97
23 Cohen, Y., Jorgensen, B.B., Padan, E. and Shilo, M. (1975) Nature 257, 489—492
24 Gorlick, S., Oren, A. and Padan, E. (1977) J. Bacteriol. 129, 623—629