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ELECTRON DONORS TO P700 IN CYANOBACTERIA AND ALGAE

AN INSTANCE OF UNUSUAL GENETIC VARIABILITY

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Cytochrome *c*-553 and/or plastocyanin have been isolated from many cyanobacteria and several eukaryotic algae. The isoelectric point for both the cytochrome and plastocyanin varies from that of a basic protein (p*I* 9.3) in the filamentous cyanobacteria to that of an acidic protein (p*I* 3.8) in unicellular cyanobacteria and eukaryotes. The cytochrome from a given genus may show isomeric forms distinguishable in either net charge or nonpolar character. Some of the variation in net charge between the cytochromes from different genera is localized in one region of the primary structure.

Introduction

In cyanobacteria and algae, P700 is reduced by either plastocyanin or cytochrome *c*-553. If copper is available during growth [1] and if the organism is genetically competent to synthesize plastocyanin [2] then the copper protein will catalyze electron transfer to P700. Under conditions of copper deficiency, cytochrome *c*-553 will replace plastocyanin. The isoelectric points of plastocyanin and cytochrome *c*-553 vary greatly in various genera of cyanobacteria [3]. Other proteins in the photosynthetic apparatus – ferredoxin, coupling factor, subunit I of P700, cytochrome *f*, phycobilins – do not show this variation in isoelectric point. Here, we report an extended survey of the cytochrome *c*-553 and/or plastocyanin in cyanobacteria and algae. Variation in isoelectric point does not follow the conventional taxonomic lines. The amino-acid compositions of several cytochromes and one plastocyanin are reported here and compared to an earlier analysis to illustrate further the variability of these proteins.

Materials and Methods

Trichodesmium erythraeum was the generous gift of Dr. C. Van Baalen who had collected it from the Gulf of Mexico. *Fremyella diplosiphon*, *Nostoc muscorum*, *Anacystis nidulans* and *Anabaena variabilis* were obtained from the Culture Collection of Algae, University of Texas and were grown on Kratz and Myers medium C [4] at 30 °C. Dr. S. Berg of the University of Denver gave us a sample of *Anabaena azolla* which had been obtained from the same collection and grown in the same way. *Mastigocladus laminosus* was grown with the help of Dr. A. Binder as described [5]. *Aphanizomenon flos-aquae* was collected from Lake Okoboji, Iowa, and *Microcystis aeruginosa* was collected from Lake Kegonsa, Wisconsin. *Spirulina maxima* was kindly given to us by the Sosa Texcoco Co. from their commercial culture at Lake Texcoco, Mexico. *Oscillatoria princeps* was collected with the help of Dr. Claude Boyd from an apparently homogeneous bloom growing attached to the bottom in the shallow water of a pond at The Auburn Uni-

versity Fishery Station at Auburn, AL. *Oscillatoria submembranacea* and *Schizothrix calcicola* were collected as a dry mat from the surface of a field near Port Aransas, TX. These mats were collected with the help of Dr. C. Van Baalen and Dr. J. Brand of the University of Texas. The mats were washed free of adhering soil, dried in the sun, and brought back to our laboratory for later use. The mat was floated on Kratz and Myers medium C containing 0.2% NaCl under fluorescent lights for 48 h. This gave a fully rehydrated mat whose surface was covered with bubbles, apparently oxygen produced by photosynthesis. The mat was first dispersed in a Polytron homogenizer and then in a large Waring blendor. The mixture was allowed to stand for 5 min and then decanted to separate the still-suspended cyanobacteria from soil particles. On standing for 1 h, most of the *O. submembranacea* had settled to the bottom and the *S. calcicola* remained in suspension. The two were separated by decantation and the *S. calcicola* settled from the suspension in 24 h. Both fractions were resuspended with the Waring blendor in a large volume of 0.2% NaCl and put through the settling cycle again. Microscopic examination of many samples indicated that 90% of each fraction was the single species. *Chlamydomonas eugametous* was a gift of Dr. S. Strachan of Purdue University who grew this organism as described by Hess and Bayer [6]. *Porphyra teneria* was purchased from the local grocery store as Nori. *Porphyridium cruentum* was obtained from the Culture Collection of Algae at the University of Texas and grown in artificial sea water medium [7].

The cytochromes or plastocyanins were isolated by the procedure of Ho et al. [8]. Acidic proteins were purified on DEAE-cellulose columns and basic proteins on CM-cellulose columns. Those proteins used in amino-acid composition analysis were further purified by high performance liquid chromatography. For amino-acid composition analyses, samples of protein were hydrolyzed in distilled 6 M HCl in evacuated sealed tubes for 24, 48 and 96 h. All analyses were performed on a Durrum D-500 amino-acid analyzer according to the manufacturer's instructions.

Isoelectric points were determined using the procedure of Righetti and Drysdale [9]. The proteins were first electrophoresed on an LKB 3-10 ampholyte and then eluted and refocused on a narrower range ampholyte suitable to their p*I*. A 50–100 μg sample of the cytochrome or plastocyanin purified by cellulose ion-exchange chromatography was used for each gel. The cytochrome could be recognized as a pink band after electrophoresis. In each case, the identity was confirmed by eluting the cytochrome and measuring the absorption spectrum between 600 and 360 nm. The plastocyanin became reduced during electrophoresis. At the end of the 4 h focusing period, the gel was sliced into sections, the pH of each section measured, and then 50 μl $1 \cdot 10^{-3}$ M $K_4Fe(CN)_6$ was added to oxidize the plastocyanin and so locate it on the gel. Samples of cytochromes and plastocyanin from the isoelectric focusing gels were eluted and then subjected to reversed-phase HPLC.

Reduction of P700 by the various cytochromes was measured as described by Davis et al. [10]. Antiserum to cytochrome *c*-553 of *A. flos-aquae* was prepared according to Evans and Krogmann [11].

Results

Cytochrome *c*-553 from 13 genera of cyanobacteria, two genera of red algae and one genus of green alga, as well as plastocyanin from three genera of cyanobacteria and one genus of green alga were isolated. Their isoelectric points are shown in Table I. The spectra of all of the cytochromes were similar to published spectra [8,12]. The isoelectric points of plastocyanin and cytochrome *c*-553, when they were both isolated from the same organism, were in the same range. These data support the general observation that the isoelectric point of cytochromes *c*-553 or plastocyanins of eukaryotic organisms are acidic, while those from cyanobacteria vary. In four genera of cyanobacteria, isoelectric focusing revealed minor cytochrome bands clearly separated from the main cytochrome band. The spectra of the cytochrome eluted from these minor bands were always identical to those of the major component. The minor component was present in 5–20% of the amount of the major component. Smaller amounts of cytochrome would go undetected.

Where sufficient cytochrome was available, we

TABLE I

ISOELECTRIC POINTS OF DONORS TO P700

	Cytochrome *c*-553	Plastocyanin
O. princeps	9.22–9.49	
A. flos-aquae	9.33	
T. erythraeum	8.99	
A. variabilis	8.86 (minor 8.58, 8.29)	7.75
A. azolla	8.75 (minor 8.29)	7.50
N. muscorum	8.78	
F. diplosiphon	8.78	
M. laminosus	8.31 (minor 8.79–8.95)	8.78
C. eugametous	5.76–5.90	4.49
M. aeruginosa	5.50 (minor 6.53)	
S. maxima	5.19	
O. submembranacea	5.01	
S. calcicola	4.74 (minor 5.06)	
P. cruentum	4.10	
P. teneria	3.75	
A. nidulans	3.84–3.87	

purified the material eluted from the isoelectric-focusing gel by passage through a reversed-phase HPLC column. While this procedure denatures the protein, it yields a polypeptide suitable for amino-acid composition and sequence analysis. Protein subjected to reversed-phase HPLC undergoes a change in its characteristic red color to a brown. We were surprised to find in preparations from several genera of cyanobacteria that two distinct, brown peaks emerged from the HPLC column. Fig. 1 illustrates the elution behavior of cytochrome *c*-553 from *O. princeps*. The peaks labelled 1 and 2 were brown in color. When each peak was put through the column a second time, it emerged as a single, apparently homogeneous entity. Thus, a cytochrome which had been brought to near homogeneity in its ionic character by isoelectric focusing was found to be heterogeneous in its binding to the nonpolar matrix of the HPLC column. The catalysts of P700 reduction from various cyanobacteria are eluted from the HPLC column at acetonitrile concentrations that reflect the hydrophobic character of the protein surface. The percentage of acetonitrile at the elution peak of each of the cytochromes is as follows: *O. princeps*, 35 and 37%; *M. laminosus*, 43%; *O. submembranacea*, 40 and 42%; *S. calcicola*, 41 and 42%; and *A. flos-aquae*, 50%. *M. laminosus* plastocyanin eluted at 33% acetonitrile. Note that three of the cytochromes gave two peaks, and in these cases, the amounts of material in each of the two peaks were nearly equal.

The amino acid composition of cytochromes *c*-553 from four genera of cyanobacteria and of one plastocyanin is shown in Table II. The amino-acid composition of *M. laminosus* plastocyanin is not very different from that of *A. variabilis* [13]. These two basic proteins differ from the acidic plastocyanins from eukaryotes which have been analyzed [14], in that they have more lysine and arginine (12 or 10, respectively, cf. 4–6) and fewer glutamics and aspartics (Glx + Asx = 17 or 16, respectively, cf. 21–24 in the eukaryotes). The cyanobacterial plastocyanins contain 8 or 9 prolines, while the eukaryotic plastocyanins have only 4–6 prolines. The total number of amino acids ranges from 85 to 89 in five other cyanobacterial cytochromes *c*-553 [15–17]. The composi-

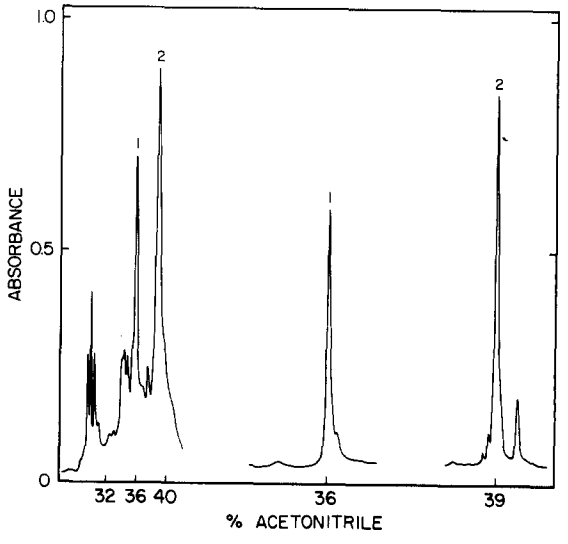

Fig. 1. Elution profiles of cytochrome *c*-553 of *O. princeps* from reversed-phase HPLC. This cytochrome had been purified by chromatography on a carboxymethyl-cellulose column and by isoelectric focusing. The protein was loaded on a SynChropak RP-P C-18 reversed-phase HPLC column of 250×401 mm. A Varian 5000 Liquid Chromatograph with a built-in UV-100 Varian Aerograph detector was used. The column was eluted at a flow-rate of 0.7 ml/min with a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. In the diagram on the left, the peaks marked 1 and 2 both had the characteristic brown color of a cytochrome in the trifluoroacetic acid-acetonitrile solvent used for elution. Each of the peaks was rechromatographed with the results shown on the right.

TABLE II

AMINO-ACID COMPOSITION OF CYTOCHROMES *c*-553 AND PLASTOCYANIN

	Cytochrome *c*-553					Plastocyanin
	O. princeps (peak 2)	*M. laminosus*	*O. submembranacea* (peak 1)	*S. calcicola* (peak 1)	*A. variabilis* (Ref. 12)	*M. laminosus*
Asx	8	9	13	11	9 (5 Asp, 4 Asn)	8
Glx	11	6	8	10	7 (5 Glu, 2 Gln)	9
Thr	3	1	3	4	9	5
Ser	5	5	4	4	7	6
Pro	3	3	1	3	9	8
Gly	9	6	10	11	9	8
Ala	19	9	14	14	11	7
Cys	2	2	2	2	1	1
Val	7	4	6	6	8	7
Met	3	2	4	3	2	3
Ile	6	2	4	4	2	3
Leu	6	5	5	4	10	9
Tyr	2	2	3	3	3	3
Phe	2	1	2	2	5	4
Lys	17	7	7	8	9	11
His	1	1	1	1	3	3
Arg	1	2	1	0	1	1
Total + Lys, Arg	18	9	8	8	10	12
Total Glx, Asx	19	15	21	21	16	17
Total amino acids	105	67	88	90	105	96

tion data in Table II indicate that the cytochrome from *O. princeps* is larger than the others.

Table III was constructed from the published sequences of the cytochromes *c*-553. The isoelectric points of several of these cytochromes are shown. These data show that the differences in the net charge of the cytochrome are due to both a reduction in the number of positively charged residues (lysine and arginine) and an increase in the number of negatively charged residues (glutamic and aspartic acids). Amino-acid sequences of some of the cytochromes *c*-553 have been determined, and Fig. 2 shows the available data for cytochromes from cyanobacteria. The cytochromes are arranged in the order of decreasing isoelectric point. One region in the primary structure, from residue 62 to 69, appears as a site of major change in ionic charge.

We had earlier described differences in the reactivities of these cytochromes in the reduction of

TABLE III

NET CHARGE AND p*I* OF CYTOCHROMES *c*-553

The amino-acid sequences can be found in the following references: *A. flos-aquae* [14]; *A. nidulans* [17]; *S. maxima*, *Synechococcus* 6312, *P. boryanum*, *Monochrysis lutherii*, *Porphyra teneria*, *Euglena gracilis* and *Alaria esculenta* [15], and *Petalonia fascia* [27].

Source	Lys + Arg	Glu + Asp	Net	p*I*
Cyanobacteria				
A. flos-aquae	13	7	+6	9.3
P. boryanum	10	7	+3	
S. maxima	9	9	0	5.19
Synechococcus 6312	7	8	−1	
A. nidulans	6	12	−6	3.84
Eukaryotic algae				
P. teneria	7	13	−6	3.75
P. fascia	7	11	−4	4.1
M. lutherii	5	9	−4	
A. esculenta	7	13	−6	
E. gracilis	5	12	−7	

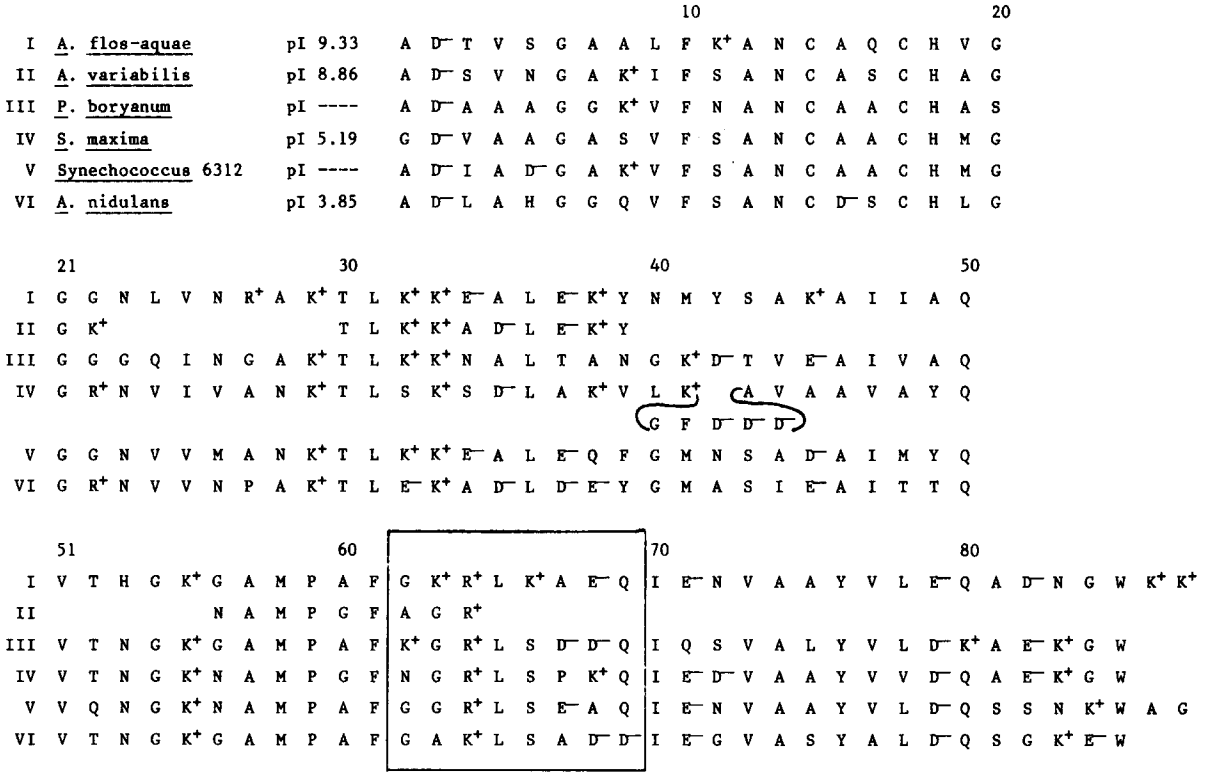

Fig. 2. A comparison of the distribution of charged residues in the primary structures of cytochrome *c*-553 from cyanobacteria. The enclosed area between residues 62 and 69 shows a large shift from positive to negative charge.

P700 in a purified Photosystem I particle preparation from spinach chloroplasts [10]. This assay uses the cytochrome as a catalyst of P700 reduction and provides a sensitive system in which one can measure the susceptibility of cytochromes to inhibition by antiserum prepared against one of them. Antiserum to cytochrome *c*-553 of *A. flos-aquae* was produced in rabbits, and this antiserum is very potent in inhibiting the reduction of P700 by *A. flos-aquae* cytochrome *c*-553. More antiserum (20 μl vs. 1 μl) is required to inhibit the reaction with the cytochrome from *A. variabilis* than that of *A. flos-aquae*, and even more antiserum (75 μl for that of *M. aeruginosa*, 150 μl for that of *S. maxima*) is needed with the other cytochromes. Susceptibility to antiserum inhibition does not strictly parallel change in isoelectric point in these experiments.

Discussion

Both electron donors to Photosystem I, plastocyanin and cytochrome *c*-553, are unusual in that their isoelectric points vary in different genera of cyanobacteria. In contrast, the protein participants in photosynthetic processes in eukaryotes seem quite uniform with respect to their net charge. Among the cyanobacteria, we have observed that the p*I* values of phycobiliproteins, ferredoxin, cytochrome *c*-553 and cytochrome *f* are constant when isolated from genera in which the p*I* of cytochrome *c*-553 varies widely. That the isoelectric points of plastocyanin and cytochrome *c*-553 vary in a parallel fashion is of considerable interest. Both proteins are acidic in all eukaryotic organisms and are basic in the filamentous cyanobacteria described here. Cytochrome *c*-553 and plastocyanin function interchangeably as reductants of P700 and we, and others, have documented the importance of the net charge to their

ability to catalyze P700 reduction [10,18,19]. The parallel evolution of both proteins suggests that their charged character must evolve in response to a change in some critical reaction partner. The P700-containing subunit I of the reaction center from the cyanobacterium *M. laminosus* and from higher plants are very similar in electrophoretic and antigenic properties [20]. However, small patches of altered charge might not be detected in these analyses. Alternatively, a binding protein may have been inserted between P700 and its electron donors which forced the change in net charge of both plastocyanin and cytochrome *c*-553.

One might anticipate a relation between the isoelectric point of a cytochrome and the phylogenetic position of the organism from which the cytochrome is isolated. Ripka et al. [21] have recognized five major subgroups of cyanobacteria based on differences in structure and development of cells maintained in pure culture on defined media. *A. nidulans* and *M. aeruginosa* are placed in Section I and the cytochromes from both of these organisms are acidic. *A. flos-aquae*, *A. variabilis*, *A. azolla* and *N. muscorum* of Section IV and *M. laminosus* of Section V all contain basic cytochrome *c*-553 or basic plastocyanin. The representatives of Section III are most diverse. The cytochromes *c*-553 of *S. calcicola*, *O. submembranacea* and *S. maxima* are acidic while those of *T. erythraeum* and *O. princeps* are basic. Complete primary structure data would allow comparison of all positions in the proteins among the various genera and would give more details of their relatedness.

The variation of cytochrome *c*-553 within a single species is a point of some interest. Our data indicate that five of the organisms examined contain electrophoretically distinct species of cytochrome *c*-553. Ambler and Bartsch [22] have shown that these variants are not the result of differences in primary structure. We now find a variation in the non-polar character of the polypeptide as it interacts with a reversed-phase HPLC column. Cytochrome *c*-553 from four genera of cyanobacteria could be resolved into two distinct peaks by this technique. These results are similar to those of Strahler et al. [23] who used a similar reversed-phase HPLC to detect a substitution of alanine for valine in position 126 of the beta chain of human hemoglobin. Amino-acid composition analysis of the two forms of cytochrome from *O. princeps* did not show a convincing difference in content of nonpolar residues but a small difference, such as reported for hemoglobin [23], would not be detected by this method. Amino-acid sequence analyses of the two peaks must be done to see if a nonionic residue substitution is occurring. The possibility of multiple forms of primary structure in these proteins implies multiple genes, and this is made reasonable by the observations of Herdman et al. [24] that the genomes of cyanobacteria vary in size integrals. Alternatively, these samples collected from nature may contain two strains of the same species that differ slightly in the cytochrome *c*-553 gene. This point will be checked with laboratory-grown cells from a single clone.

Böhme and Pelzer [25] have published an immunological comparison of cytochromes *c*-553 from several prokaryotic and eukaryotic algae. They found that the cytochromes from *A. variabilis*, *N. muscorum*, *Calothrix membranacea* and *S. maxima* showed large differences in cross-reactivity, but the cytochrome of *S. maxima* was closely related to cytochromes isolated from two eukaryotic algae. Our data on the antibody to the cytochrome *c*-553 from *A. flos-aquae* show that it cross-reacts best to the cytochrome from *A. variabilis* and less well with the negatively charged cytochromes of *M. aeruginosa* and *S. maxima*. These observations provide an independent indication of the divergence in cytochrome *c*-553 structure that has occurred within the cyanobacteria.

The amino-acid composition data for the proteins from the thermophile *M. laminosus* were contrasted to similar composition data from mesophiles. Argos et al. [26] suggested that in thermophiles, Ala, Ala, Thr, Arg and Glu would replace Gly, Ser, Ser, Lys and Asp in the proteins of mesophiles. This does not seem to be the case in either cytochrome *c*-553 or plastocyanin.

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