Volume 319, number 3, 257–260 FEBS 12245 March 1993


# *Synechocystis* 6803 plastocyanin isolated from both the cyanobacterium and *E. coli* transformed cells are identical

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Received 4 January 1993; revised version received 4 February 1993

Native plastocyanin from *Synechocystis* 6803 has been isolated and purified to electrophoretic homogeneity. The corresponding gene (*petE*) has been cloned and expressed in *E. coli*, thus leading to a protein completely identical to plastocyanin purified from the cyanobacterial cells. The *petE* gene product is correctly processed in *E. coli* as deduced from the N-terminal amino acid sequences. These results, along with the identical physicochemical and kinetic properties of the two protein preparations, confirm that expression of *petE* in *E. coli* is an adequate tool to address the study of *Synechocystis* plastocyanin by site-directed mutagenesis.

*petE* gene; Plastocyanin; Protein translocation, *Synechocystis*

## 1. INTRODUCTION

Plastocyanin (Pc) is a blue copper-protein which functions as an electron carrier between the cytochrome (cyt) $b_6f$ complex and photosystem I (PSI) in the thylakoidal lumen [1,2]. Pc is constitutively synthesized in higher plants, but can be replaced by soluble cytochrome $c_6$ in some green algae and cyanobacteria depending on the relative availability of Cu and Fe in the medium [3–5]. Elucidation of the fine structure and reaction mechanism of both proteins may allow to explain why Pc has evolutively been chosen over the ancient heme-containing molecule.

In this respect, we have recently used laser flash absorption spectroscopy to carry out a comparative study of the reaction mechanism of electron transfer from Pc and cyt $c_6$ from the green alga *Monoraphidium braunii* [6] and from the cyanobacterium *Anabaena* 7119 (Medina et al., submitted) to PSI particles. In order to extend such studies to proteins modified by site-directed mutagenesis to evaluate the specific role played by a number of amino acids, it is necessary to use a genetically well-known organism as is the case of *Synechocystis* 6803. In spite of the great deal of work carried out with this cyanobacterium, the only references in the literature to molecular properties of Pc were derived from the nucleotide sequence of the gene [7] until Zhang et al. [8] have recently achieved its partial purification and characterization. The Pc gene (*petE*) has been previously cloned from the cyanobacteria *Anabaena* 7937 [9] and *Synechocystis* 6803 [7], the latter one encoding a precursor protein of 126 amino acids that contains a signal peptide with substantial homologies to that of *Anabaena* 7937 [7,9].

In this work we describe an improved procedure for the isolation and purification to electrophoretic homogeneity of Pc from *Synechocystis* 6803, as well as the cloning and correct expression of the *petE* gene in *E. coli*. The purification procedure of the expressed protein is also reported, along with a comparative physicochemical study of native Pc isolated from the two sources.

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*Abbreviations:* cyt, cytochrome; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria broth; Pc, plastocyanin; PCR, polymerase chain reaction; pI, isoelectric point; PSI, photosystem I; X-gal, 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside.

## 2. MATERIALS AND METHODS

### 2.1. *Purification of plastocyanin from* Synechocystis *6803*

Cells were grown autotrophically in a standard BG-11 medium [10] enriched with 4 μM copper so as to induce the synthesis of Pc. 150 g of wet algal paste were washed, suspended in 1 liter of 50 mM Tris-HCl, pH 7.9, 50 mM NaCl, and disrupted in a Manton-Gaulin homogenizer set at 7,000 psi (three cycles). After centrifugation at 3,000 × *g* for 2 min to remove unbroken cells, the supernatant was sonicated with a Branson 250 sonifier for 1 min at 90 W in 150-ml aliquots to extract Pc associated to membranes. After centrifugation at 12,000 × *g* for 20 min, the supernatant was treated with streptomycin sulfate to remove most pigments and nucleic acids [11]. After centrifugation at 12,000 × *g* for 20 min, ammonium sulfate was added to the supernatant to 60% of saturation and the precipitate was removed by centrifugation as before. Ammonium sulfate was again added to the resulting supernatant to 97% of saturation, and the solution was centrifuged to

concentrate the proteins. The final pellet was suspended in 100 ml of 5 mM Tris-HCl, pH 7.9, dialyzed extensively and applied to a DEAE-cellulose (DE-52) column. Proteins were eluted by a 0–0.2 M NaCl gradient, the fractions containing Pc being pooled, dialyzed and charged on a DEAE-Sephacel column. Proteins were eluted as before, the Pc fractions being pooled (discarding those with a $A_{275}/A_{597}$ ratio higher than 5), concentrated in an Amicon pressure cell, and subjected to chromatofocusing in the pH range 4–7. Pure protein preparations were concentrated and stored at −80°C.

2.2. *DNA techniques*

On the basis of the DNA sequence of the *Synechocystis petE* gene previously reported [7], the two oligonucleotides used for PCR amplification were GAATTCGCCTTTTTGGGCACG as the direct primer and GAATTCAACGACACACAGATT as the inverse primer. *E. coli* strain DH5α (Bethesda Research Laboratories) was used for plasmid construction and amplification [12]. The plasmids used were pBluescriptII (SK+) (Stratagen) and pChN1 (this work). The *petE* gene from *Synechocystis* 6803 was amplified from genomic DNA by PCR techniques, using a Gene ATAQ controller from Pharmacia. DNA fragments were purified from low-melting agarose gels. Nucleotide sequence analysis was carried out by the dideoxy chain termination method [13], using a sequenase version 2.0 T7 DNA polymerase kit (USB). Other molecular biology protocols were standard [12].

2.3. *Purification of plastocyanin expressed in* E. coli

*E. coli* transformed cells were grown in standard Luria broth (LB) medium [12] supplemented with 100 μM $CuSO_4$ to optimize Cu incorporation into Pc apoprotein. Cells from a 2 litre culture were collected and resuspended in 100 ml of 20 mM Tris-HCl, pH 7, supplemented with 50 mM EDTA and 0.5 M sucrose. Lysozyme (2 mg · $ml^{-1}$) was added to the homogenized cell suspension, followed by 1-h incubation under shaking at 37°C. The spheroplasts were sedimented by centrifugation, and ammonium sulfate was added to the supernatant to 60% of saturation. After centrifugation at 12,000 × *g* for 20 min, the pellet was suspended and dialyzed against 2 mM HCl-Tris, pH 8. The dialyzate was diluted four fold and incubated for 48 h at 0°C in order to allow lysozyme to precipitate. Hereinafter, the purification procedure was the same as that described above for Pc synthesized in *Synechocystis*, except that the adsorption step on DEAE-Sephacel was omitted.

2.4. *Analytical methods*

Molecular mass was determined by SDS-PAGE [14] using a 16% acrylamide running gel. The isoelectric point (pI) was determined by electrofocusing [15] with a mixture of ampholite carriers from Pharmacia, pH range 4–6.5; the standard proteins used were those of the isoelectric focusing calibration kit for pH range 2.5–6.5 from Pharmacia. Redox titrations were performed in a dual wavelength spectrophotometer as described previously [16]; the differential absorbance changes at 597 *minus* 500 nm were monitored in the presence of redox mediators such as menadione, *p*-benzoquinone and diaminodurol at 20 μM final concentration. The N-terminal amino acid sequence was determined using an Applied Biosystems automated microsequencer model 477A. Laser flash-induced kinetics of Pc oxidation by flavin triplet state were followed at 640 nm [17]. The laser flash photolysis apparatus and data analysis have been previously described [18].

## 3. RESULTS AND DISCUSSION

About 0.6 μmol pure Pc were extracted from 150 g of *Synechocystis* cell paste, the net yield of the purification method herein reported being more than four times higher than that recently described by Zhang et al. [8]. Purified Pc was electrophoretically homogeneous, with one major band on SDS-PAGE corresponding to an apparent molecular mass of 9.5 kDa (Fig. 1), which is significantly lower than that (12 kDa) reported by Zhang et al. [8]; a 20-kDa minor band, probably arising from Pc dimerization [19], could occasionally be observed. Isoelectric focusing of reduced Pc gave one single band with a pI of 5.5 (Fig. 1), which is in good

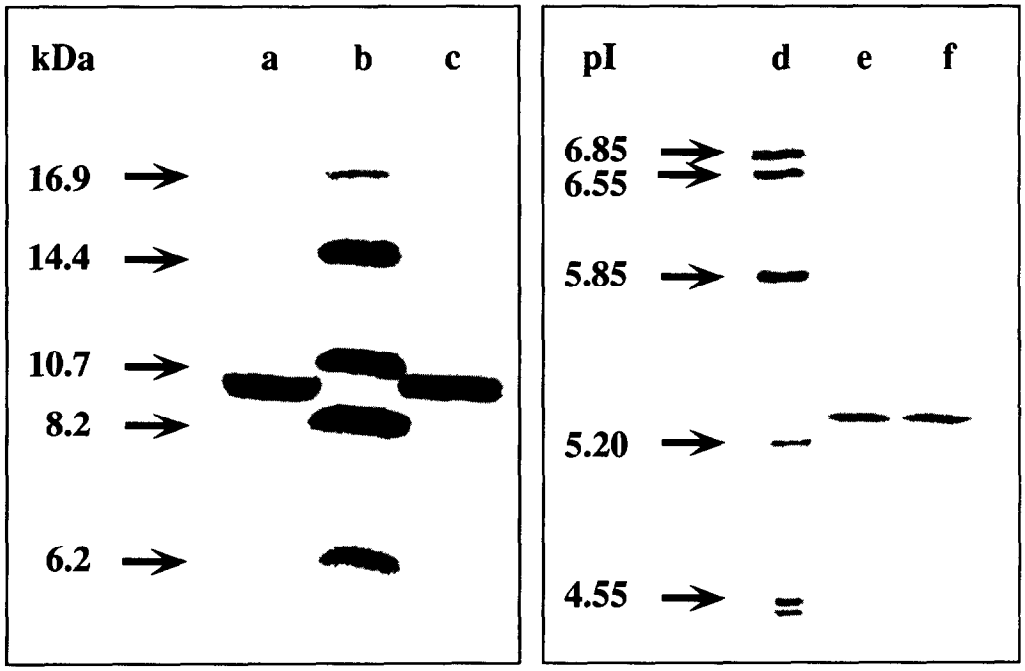


Fig. 1. Molecular weight determination on SDS-PAGE (left) and isoelectric focusing (right) of *Synechocystis* 6803 plastocyanin. (a and e) Pc synthesized by the cyanobacterium; (b and d) standard calibration proteins; (c and f) Pc expressed in *E. coli* transformed cells.

agreement with that estimated from the gene sequence by Briggs et al. [7] (5.6), but is quite lower than that reported by Zhang et al. [8] (6.2).

Fig. 2 shows the UV/visible absorption spectra of purified Pc. The oxidized protein exhibits the characteristic absorption peak at 597 nm, where the extinction coefficient ($\varepsilon$) is found to be 4.5 $mM^{-1} \cdot cm^{-1}$. The $A_{275}/A_{597}$ ratio is 2.2, very close to that reported for parsley Pc [20] (2.1). The midpoint redox potential ($E_m$) of *Synechocystis* Pc was determined to be ca. +360 mV at pH 7, with a $n$ value of 1, in good agreement with those obtained for Pc from other organisms [20].

On the basis of the already known sequence of the *Synechocystis petE* gene [7], we proceeded to clone it by PCR amplification. The only amplified DNA band was recovered from the gel, digested with *Eco*RI and ligated to *Eco*RI-linearized, phosphatase-treated pBluescriptII (SK+) plasmids. *E. coli* DH5α cells were transformed with the ligation mixture. Positive clones were detected by searching white colonies in LB agar plate containing X-gal and IPTG. Sequencing of the pChN1 plasmid isolated from the clone was found to contain the complete *petE* gene, whose sequence was identical to that reported by Briggs et al. [7]. *E. coli* transformed cells containing a high number of copies of the *Synechocystis petE* gene were able to synthesize Pc, 20% of which being detected in the cytoplasmic fraction whereas the bulk was found in the periplasmic space. This suggests a maturation process similar to that of cyanobacteria, which involves translocation of proteins into the thylakoidal lumen.

4 mg Pc was obtained by periplasmic extraction from a 2 litre culture of *E. coli* transformed cells. The molecular mass, pI, redox potential and spectral properties of Pc expressed in *E. coli* were shown to be practically identical to those of Pc isolated from *Synechocystis* (Table I). The expressed Pc was correctly maturated in *E. coli*, as deduced from the identical N-terminal amino acid sequences of Pc synthesized in both organisms (Table I). The molecular mass estimated from the nucleotide sequence of the gene was 10.4 kDa, which is somewhat higher than that obtained by SDS-PAGE (ca. 9.5 kDa) for the two Pc preparations (Table I). Similar discrepancies have recently been reported for other small charged proteins such as ferredoxin [21] and cyt $c_6$ (Campos et al., manuscript in preparation).

The functional integrity of Pc expressed in *E. coli* was tested by comparing its reactivity towards photoexcited flavins with that of Pc synthesized in *Synechocystis*, according to the protocol we have developed [17,22]. Lumiflavin and FMN triplets were selected as probes since both molecules have different sizes (lumiflavin is smaller than FMN) and charges (lumiflavin is neutral and FMN is negatively charged). From the results presented in Table I, it is clear that the two Pc populations exhibit identical reactivity towards lumiflavin and FMN, thus suggesting a unique functional identity.

Correct maturation of *Synechocystis* Pc in *E. coli* indicates that the cellular machinery of the enterobacteria properly recognizes the cyanobacterial signal peptide. Expression and processing of a cyanobacterial protein (the Mn-stabilizing protein) in *E. coli* have al-

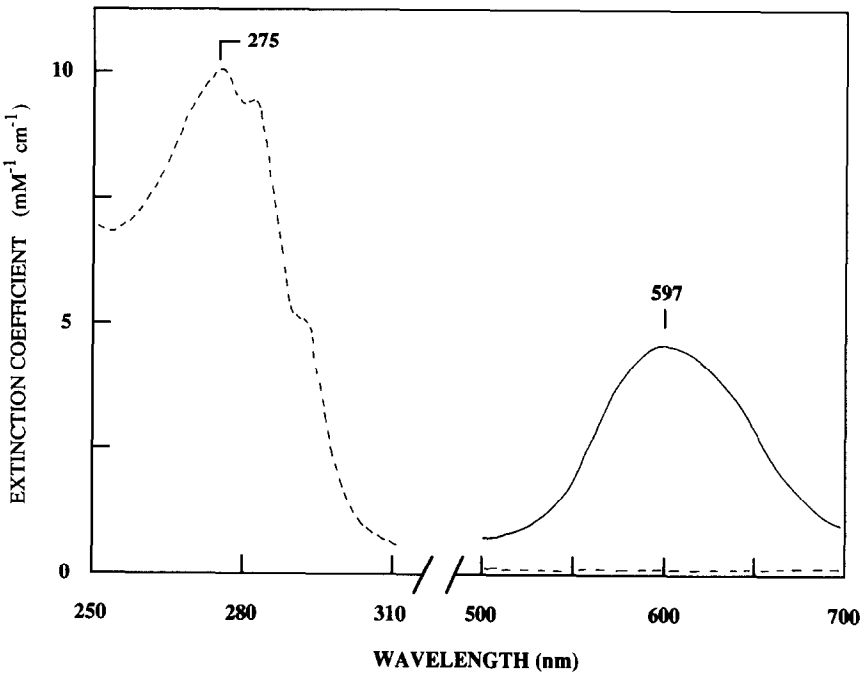


Fig. 2. Electronic absorption spectra of *Synechocystis* 6803 plastocyanin in its native reduced state (dashed line), and upon oxidation by ferricyanide (continuous line). Protein concentration was 0.2 mg · $ml^{-1}$. Optical path length was 1 cm.

Table I

Physicochemical properties of *Synechocystis* 6803 plastocyanin isolated from both the cyanobacterium and *E. coli* cells transformed by the *petE* gene

| Organism | Molecular mass (kDa) | | pI | $\varepsilon_{597}$ ($mM^{-1} \cdot cm^{-1}$) | $E_m$, pH 7 (mV) | N-terminal sequence | Reactivity with flavins[a] $k \times 10^{-9}$ ($M^{-1} \cdot s^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | SDS-PAGE | Gene sequence | | | | | | |
| *Synechocystis* | 9.50 | 10.4 | 5.5 | 4.5 | +358 | ANATV | 10.0[b] | 6.2[c] |
| *E. coli* | 9.55 | | 5.5 | 4.5 | +363 | ANATV | 11.1[b] | 7.0[c] |

[a] The reactivity with flavins is expressed as a second-order rate constant ($k$) for plastocyanin photooxidation by lumiflavin[b] or FMN[c].

ready been achieved, the 30-kDa expression product being reported to be indistinguishable from the authentic mature protein on SDS-PAGE [23]. In this paper, however, correct maturation of *Synechocystis* Pc in *E. coli* is demonstrated by comparing the N-end amino acid sequence of the expressed protein with that of Pc synthesized in the cyanobacterium. A more complicated procedure for overexpression of spinach Pc in *E. coli* – which is based on the use of an expression vector containing the signal peptide sequence of *Pseudomonas aeruginosa* azurin – has been developed by Nordling et al. [24].

The N-terminal amino acid sequence of *Synechocystis* Pc (ANATVKMG) was compared with those of the copper-proteins from other cyanobacteria such as *Microcystis aeruginosa* (ETFTVKMG) and *Anabaena variabilis* (ETYTVKLG). The three Pc sequences are somewhat different from one another but show the same length, which is one residue longer than most Pc sequences in green algae and two more than those in higher plants. This fact unequivocally indicates an evolutionary shortening of the $NH_2$-end.

To conclude, the identical structural and kinetic features of *Synechocystis* Pc purified from the *E. coli* recombinant clone as compared to that isolated from the same cyanobacterium indicate that this is an optimum system for constructing Pc modified by site-directed mutagenesis so as to study the corresponding structure-function relationships.

*Acknowledgements:* This work has been supported by grants from the Dirección General de Investigación Científica y Técnica (PB90-0099, PB91-0085, PB91-0127) and Junta de Andalucía.

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