FEBS Letters 347 (1994) 173–177

FEBS 14143

# Cloning and correct expression in *E. coli* of the *petJ* gene encoding cytochrome $c_6$ from *Synechocystis* 6803

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Received 25 April 1994; revised version received 10 May 1994

**Abstract**

Cytochrome $c_6$ from the cyanobacterium *Synechocystis* 6803 has been isolated and purified to electrophoretic homogeneity. The gene coding for such a heme protein (*petJ*) has been cloned and properly expressed in *E. coli*. This procedure yields a protein preparation completely identical to that obtained from the cyanobacterial cells. The N-terminal amino acid sequences of cytochrome $c_6$ synthesized in both organisms are the same, thus allowing us to conclude that the *petJ* gene product is correctly processed in *E. coli*. To the best of our knowledge, this is the first time that any cytochrome $c_6$ is produced in the enterobacterium. The identical physicochemical and kinetic properties of the proteins isolated from both sources confirm that expression of the *petJ* gene in *E. coli* is an adequate tool to address the study of *Synechocystis* cytochrome $c_6$ by site-directed mutagenesis in a parallel way to that carried out with plastocyanin from the same organism.

*Key words:* Cytochrome $c_6$; *petJ* gene; Protein translocation; *Synechocystis*

## 1. Introduction

Cytochrome $c_6$ is a class I *c*-type cytochrome which functions as an electron carrier between the cytochrome $b_6f$ complex and photosystem I (PSI) in the thylakoidal lumen [1]. In several green algae and cyanobacteria, this heme protein does act as a physiological substitute for the blue copper protein, plastocyanin, depending on the relative levels of iron and copper in the medium [1,2]. Elucidation of the fine structure and reaction mechanism of these two functionally equivalent proteins may then allow us to explain why higher plants have chosen plastocyanin over the ancient cytochrome $c_6$.

The structure of plastocyanin from a number of photosynthetic organisms is very well known, but unfortunately a detailed structural analysis of cytochrome $c_6$ has not been performed as yet. We have recently carried out a study by EPR, NMR and Mössbauer spectroscopies of cytochrome $c_6$ from the green alga *Monoraphidium braunii*, and have shown that such a cytochrome exhibits an unusual histidine–methionine heme axial coordination [3]. Crystallization of cytochrome $c_6$ from this same alga, as well as a preliminary diffraction analysis have been recently achieved [4].

Laser flash absorption spectroscopy has been used to carry out a comparative kinetic study of the reaction mechanism of PSI reduction by plastocyanin and cytochrome $c_6$ from the green alga *Monoraphidium braunii* [5,6], as well as from the cyanobacteria *Anabaena* 7119 [7] and *Synechocystis* 6803 [8]. In order to extend such studies to metalloproteins modified by site-directed mutagenesis and evaluate the specific role played by a number of amino acids, it is necessary to use an organism amenable to genetic manipulation, easily transformable and able to incorporate DNA into its genome by homologous recombination, as is the case with *Synechocystis* 6803.

The *petE* gene coding for plastocyanin from *Synechocystis* 6803 has recently been cloned and correctly expressed in *E. coli*, the resulting protein being purified and physicochemically characterized [9]. In contrast, the data in the literature concerning cytochrome $c_6$ from this cyanobacterium are rather scarce [10]. The *cytA* gene coding for cytochrome $c_6$ from the cyanobacterium *Synechococcus* has been isolated by Laudenbach et al. [11], whereas the *petJ* gene coding for cytochrome $c_6$ from *Synechocystis* 6803 has been isolated by Zhang *et al.* [12], who have reported that neither cytochrome $c_6$ nor plastocyanin are required for photosynthesis or respiration.

As a complement to our previous work with plastocyanin from *Synechocystis* 6803 [9], this paper describes the cloning and correct expression of the *petJ* gene in *E. coli*, as well as an improved procedure for the isolation and purification to electrophoretic homogeneity of cytochrome $c_6$ isolated both from the cyanobacterium and from *E. coli* transformed cells. A comparative physicochemical study of the heme protein synthesized in the two organisms is also reported.

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*Abbreviations:* $E_m$, midpoint redox potential; PCR, polymerase chain reaction; pI, isoelectric point; PSI, photosystem I.


*SSDI* 0014-5793(94)00529-5

## 2. Materials and methods

*2.1. Purification of cytochrome $c_6$ from* Synechocystis *6803*

In order to favour the synthesis of cytochrome $c_6$ over that of plastocyanin, cyanobacterial cells were grown as previously described [9] except that 4 mM copper was omitted from the BG-11 medium [13]. Cytochrome $c_6$ was purified following the method described in [9] for plastocyanin, which included ammonium sulfate fractionation, DEAE-cellulose chromatography, DEAE-Sephacel chromatography and chromatofocusing. The heme protein remained in its reduced state all along the purification procedure.

*2.2. DNA techniques*

On the basis of the amino acid sequence of cytochrome $c_6$ from the cyanobacteria *Synechococcus* and *Anabaena* and the green alga *Chlamydomonas* previously reported [11,14,15], the direct primer TT (T/C)AGNGC(T/C)AA(T/C)TG(T/C)GC(PheSerAlaAsnCysAla) and the reverse one GGCATNGC(A/G)TT(T/C)TTNCC(A/G)TT (AsnGlyLysAsnAlaMetPro) were synthesized to amplify an internal region of the *Synechocystis* 6803 *petJ* gene by means of the polymerase chain reaction (PCR). A gene ATAQ Controller from Pharmacia was utilized. The strains used for cloning and plasmid construction were *E. coli* DH5α (Bethesda Research Laboratories) and *E. coli* MC1061 [16]. The plasmids used for chromosomal DNA libraries and *petJ* gene expression were pBluescriptII (SK+) (Stratagene). DEAE-cellulose membranes were used to isolate fragments from agarose gels as described in [16]. Southern blot hybridization was performed at 65°C in 5 × SSC buffer (SSC: 0.15 M NaCl in 1.5 mM sodium citrate) according to [17]. Colony hybridization was carried out at 65°C in 5 × SSC buffer as described in [16]. Nucleotide sequence analysis was carried out by the dideoxy chain termination method, using a Tac-Track sequencing kit (Promega). Other molecular biology protocols were standard.

*2.3. Purification of cytochrome $c_6$ expressed in* E. coli

*E. coli* DH5α transformed cells were grown in standard Luria–Bertani (LB) medium [16]. Cells from a 9 liter culture were collected and suspended in 400 ml of 20 mM Tris-HCl buffer, pH 7, supplemented with 50 mM EDTA and 0.5 M sucrose. Hereinafter, the purification procedure was the same as described previously for plastocyanin expressed in *E. coli* [9].

*2.4. Analytical methods*

Molecular mass was determined by SDS-PAGE using a 16% acrylamide running gel [18]. The isoelectric point (pI) was determined by electrofocusing [19] with a mixture of ampholite carriers from Pharmacia, pH range 4–6.5; other conditions were as reported in [9]. Redox titrations were performed in a dual wavelength spectrophotometer as described previously [20]; the differential absorbance changes at 552 *minus* 561 nm were monitored in the presence of the following redox mediators at 20 μM final concentration: menadione, diaminodurol and *p*-benzoquinone. Laser flash-induced kinetics of cytochrome $c_6$ photooxidation by flavins were monitored by following the flavin triplet state decay at 640 nm [21], whereas the kinetics of cytochrome $c_6$ photooxidation by PSI particles were determined at 697 nm by following the re-reduction of $P700^+$ after the laser flash [5]. *Synechocystis* 6803 PSI particles were obtained as described in [8]. The laser flash photolysis apparatus and data analysis have been previously described [5,22].

## 3. Results and discussion

About 2 μmol pure cytochrome $c_6$ were extracted from 150 g of *Synechocystis* cell paste, the final net yield of heme protein (ca. 65% from the crude extract) being more than three times higher than that of plastocyanin (0.6 μmol) purified by the same procedure [9]. Such differences in the two protein yields can be due to the higher stability of cytochrome $c_6$ as compared to that of plastocyanin. As shown in Fig. 1, the UV/visible absorption spectrum of purified cytochrome $c_6$ in its reduced state

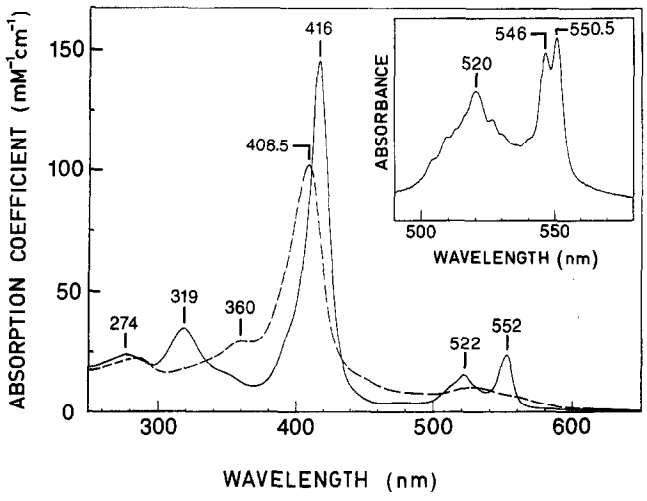


Fig. 1. UV/visible absorption spectra of cytochrome $c_6$ from *Synechocystis* 6803 in its native reduced state (continuous line) and after oxidation by ammonium persulfate (dashed line). The inset shows the electronic absorption spectrum of the α–β region of the reduced form at 77 K.

is quite similar to those previously reported for cytochrome $c_6$ from other organisms [3], thus exhibiting the characteristic absorbance maxima at 552 (α), 522 (β), 416 (γ or Soret band), 319 (δ) and 274 (protein) nm. Upon oxidation by ammonium persulfate, the α and β peaks are replaced by a broader absorbance band with a maximum at 528 nm, whereas the Soret band shifts to 408.5 nm; the band at 319 nm disappears and a new band at 360 nm is observed. When the spectrum of the reduced form is recorded at 77 K (Fig. 1, inset), the α peak is splitted into two distinct peaks at 550.5 and 546 nm, and the β peak shows a fine structure. The absorbance ratios $A_{552}/A_{274}$ and $A_{416}/A_{552}$ of reduced cytochrome $c_6$ are equal to 1.14 and 6.03, respectively, the former ratio actually reaching the highest value up to now reported for this type of cytochromes. The absorption coefficient (ε) of reduced cytochrome $c_6$ at 552 nm was found to be 24.1 $mM^{-1} \cdot cm^{-1}$. The heme group was not extractable from the protein with acidic ethylmethyl ketone, as is was the case with *Monoraphidium braunii* cytochrome $c_6$ [3], thus suggesting that the heme is covalently bound to the peptide.

The physicochemical properties of *Synechocystis* cytochrome $c_6$ are summarized in Table 1. Purified cytochrome $c_6$ was electrophoretically homogeneous, with one major band corresponding to an apparent molecular mass of 8.12 kDa. Isoelectric focusing of reduced cytochrome $c_6$ gave one single band with a pI of 5.6, which is the same as those previously reported by Zhang et al. [10] for this protein and by Hervás et al. [9] for plastocyanin from this cyanobacterium, but is less acidic than that of cytochrome $c_6$ and plastocyanin from green algae (pI = 3.6) [3]. The midpoint redox potential ($E_m$) of

*Synechocystis* cytochrome $c_6$ was determined to be 324 mV at pH 7.0, with a $n$ value of 1. Such a redox potential value is somewhat lower than those previously reported for cytochrome $c_6$ from other organisms (around 350 mV) and for plastocyanin from *Synechocystis* (360 mV) [9].

On the basis of the already known amino acid sequence of cytochrome $c_6$ isolated from several photosynthetic organisms [11,14,15], an internal fragment of the *petJ* gene from *Synechocystis* 6803 was amplified by PCR as indicated in section 2. The amplified 130 bp DNA fragment was sequenced. Analysis of the deduced amino acid sequence revealed a high homology with cytochrome $c_6$ from other organisms, thus confirming that the above mentioned fragment is an internal region of the *petJ* gene from *Synechocystis*. Hence, the amplified fragment was used as a DNA probe for Southern blot hybridization of *Synechocystis* genomic DNA (Fig. 2A); only one copy of the *petJ* gene in genomic DNA was found. According to the results obtained, the 3 kb *Hin*dIII fragment was used for cloning of the *petJ* gene. Chromosomal DNA libraries of *Hin*dIII fragments of this size were constructed in pBluescript plasmids, which were further screened by colony hybridization in *E. coli* MC1061 using the same DNA probe as above. Out of the 1000 colonies tested, only one clone was found to be positive, from which the plasmids were extracted; an internal 1.1 kb *Hin*cII fragment (Fig. 2B) cloned into the pBluescript plasmid in *E. coli* was partially sequenced. The amino acid sequences corresponding to some internal DNA regions in the 1.1 kb *Hin*cII fragment were deduced and compared to that of the protein reported by Zhang et al. [12], thus confirming that such a DNA fragment contained the whole *petJ* gene. In consequence, the plasmid with the 1.1 kb *Hin*cII fragment was further used to express cytochrome $c_6$ in *E. coli* DH5α. The *petJ* gene, which was found to be in opposite direction to the *lacZ* promoter in the pBluescript plasmid, appears to be expressed from its own promoter.

About 2 mg cytochrome $c_6$ were obtained by periplasmic extraction from a 9 liter culture of *E. coli* transformed cells (that is, a final yield of 80% from the crude *E. coli* periplasmic fraction). The molecular mass, pI and redox potential of the heme protein expressed in *E. coli* were shown to be practically identical to those of cytochrome $c_6$ isolated from *Synechocystis* (Table 1), as also were its spectral properties (see above). The expressed cytochrome $c_6$ was correctly matured in *E. coli*, as deduced from the identical N-terminal amino acid se-

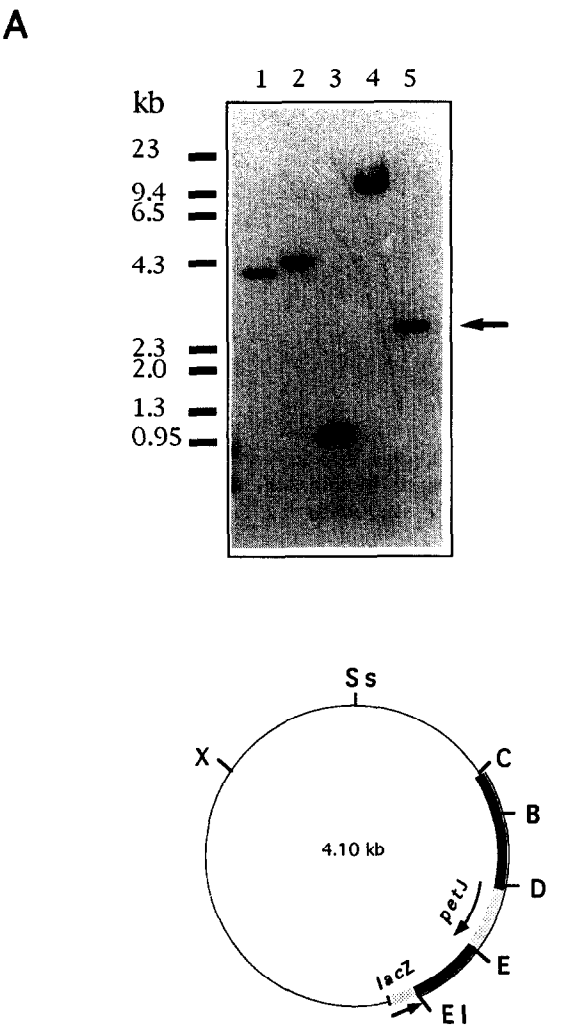


Fig. 2. (A) Southern blot hybridization of *Synechocystis* genomic DNA digested with *Sma*I (lane 1), *Dra*I (lane 2), *Hin*cII (lane 3), *Xmn*I (lane 4) and *Hin*dIII (lane 5). The 3 kb *Hin*dIII fragment cloned is indicated by the arrow. The probe used was the PCR product (130 bp). (B) Plasmid containing the 1.1 kb *Hin*cII DNA fragment (thick black lines) and localization of the *petJ* gene from *Synechocystis* 6803. Arrows stand for the gene transcriptional direction. B, *Bst*XI; C, *Cla*I; D, *Dra*I; E, *Eco*RV; EI, *Eco*RI; Ss, *Ssp*I; and X, *Xmn*I.

Table 1
Physicochemical properties of *Synechocystis* 6803 cytochrome $c_6$ isolated from both the cyanobacterium and *E. coli* cells transformed by the *petJ* gene

| Organism | Molecular mass (kDa) | | pI | $E_m$, pH 7 (mV) | N-terminal sequence | Photo-oxidation rate constant ($M^{-1} \cdot s^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|
| | SDS-PAGE | Gene sequence | | | | PSI | Lumiflavin | FMN |
| *Synechocystis* | 8.12 | 8.74[a] | 5.6 | 324 | ADLA | $10 \times 10^6$ | $3.7 \times 10^9$ | $2.5 \times 10^9$ |
| *E. coli* | 8.15 | – | 5.6 | 323 | ADLA | $9 \times 10^6$ | $3.4 \times 10^9$ | $2.2 \times 10^9$ |

[a]as reported in [12].

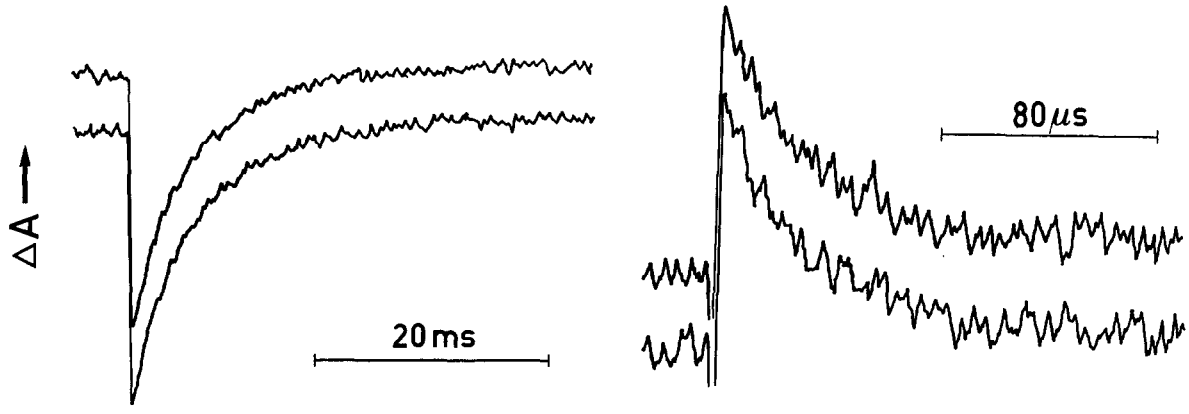


Fig. 3. Transient kinetics showing laser flash-induced cytochrome $c_6$ oxidation either by PSI particles at 697 nm (left) or by the lumiflavin triplet state at 640 nm (right). In both cases, the upper traces correspond to native cytochrome $c_6$ from *Synechocystis*, while the lower traces correspond to the heme protein synthesized in *E. coli*. Assay conditions were as described in section 2. Final cytochrome $c_6$ concentration was 10 μM.

quences of cytochrome $c_6$ synthesized in both organisms. The molecular mass estimated from the nucleotide sequence of the gene is 8.74 kDa [12], which is somewhat higher than that obtained by SDS-PAGE (ca. 8.1 kDa). This is a common characteristic of small charged proteins [3,9].

The functional integrity of cytochrome $c_6$ expressed in *E. coli* was tested by comparing its reactivity towards both its physiological electron acceptor PSI and photo-excited flavins with that of cytochrome $c_6$ synthesized in *Synechocystis* (Fig. 3 and Table 1). Photo-oxidation of the two cytochrome $c_6$ populations by PSI, monitored by following the re-reduction of $P700^+$ at 697 nm, gave similar kinetics at pH 7.5. It is worth noting that the second-order rate constants obtained ($9–10 \times 10^6$ $M^{-1} \cdot s^{-1}$) are in good agreement with that recently reported ($13 \times 10^6$ $M^{-1} \cdot s^{-1}$, pH 7.0) for the same reaction when monitoring $P700^+$ reduction at 820 nm [8]. Such rate constants are also very similar to that of plastocyanin photo-oxidation by PSI [8], in close agreement also with the interchangeable role that plastocyanin and cytochrome $c_6$ play inside the cells. In addition, when comparing the kinetics of flavin-photosensitized oxidation of cytochrome $c_6$ isolated both from *Synechocystis* and from *E. coli*, very similar rate constants were obtained with either lumiflavin or FMN (see Table 1 and Fig. 3). It must be noted, however, that the so calculated rate constants ($2–4 \times 10^9$ $M^{-1} \cdot s^{-1}$) were significantly smaller than those previously reported for *Synechocystis* plastocyanin oxidation by flavins ($6–11 \times 10^9$ $M^{-1} \cdot s^{-1}$) [9]. Such a different kinetic behaviour between plastocyanin and cytochrome $c_6$ can be attributed to the distinct accessibility and nature of their respective redox centers. Putting these kinetic results all together, it is clear that the two cytochrome $c_6$ populations exhibit a practically identical reactivity, both with PSI and with flavins, thus suggesting a unique functional identity.

To summarize, we can say, as was the case for plastocyanin [9], that the structural and kinetic features of *Synechocystis* cytochrome $c_6$ purified from the *E. coli* recombinant clone are identical to those of the protein isolated from the cyanobacterium. It should be emphasized the fact that this is the first report, as far as we know, of cytochrome $c_6$ being produced in *E. coli* and the second report of *c*-type cytochromes being produced correctly in the enterobacterium [23]. Such findings indicate that this is an optimum system for designing cytochrome $c_6$ modified by site-directed mutagenesis and study the corresponding structure–function relationships. A detailed comparison of the structural characteristics of plastocyanin and cytochrome $c_6$ will thus allow us to gain more complete information on how they interact with the reaction partners they have in common. Actually, plastocyanin and cytochrome $c_6$ must exhibit a number of common structural features that surely play a critical role in recognition and binding of their physiological electron acceptor and donor PSI and cytochrome *f*, respectively.

*Acknowledgements:* This work has been supported by grants from the Dirección General de Investigación Científica y Técnica (PB90–0099, PB91–0127) and Junta de Andalucía (PAI 3182).

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