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Cloning and Correct Expression in *Escherichia coli* of the *pet*E and *pet*J Genes Respectively Encoding Plastocyanin and Cytochrome c_6 from the Cyanobacterium *Anabaena* sp. PCC 7119[1]

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The genes coding for plastocyanin (*pet*E) and cytochrome c_6 (*pet*J) from *Anabaena* sp. PCC 7119 have been cloned and properly expressed in *Escherichia coli.* The recombinant proteins are identical to those purified from the cyanobacterial cells. The products of both the *pet*E and *pet*J genes are correctly processed in *E. coli,* as deduced from their identical N-terminal amino acid sequences as compared with those of the metalloproteins isolated from the cyanobacterium. Physicochemical and functional properties of the native and recombinant protein preparations are also identical, thereby confirming that expression of *pet*E and *pet*J genes in *E. coli* is an adequate tool to address the study of the structure/function relationships in plastocyanin and cytochrome c_6 from *Anabaena* by site-directed mutagenesis.

***Key Words: Anabaena*; cytochrome c_6: *pet*E gene; *pet*J gene; plastocyanin.**

Plastocyanin (Pc)[3] and cytochrome c_6 (Cyt) are two small redox proteins that function as mobile electron carriers between the two membrane-embedded complexes cytochrome b_6f and Photosystem I (PSI) in oxygenic photosynthesis (see refs. 1 and 2, for recent reviews). Some less-evolved cyanobacteria synthesize just Cyt and higher plants produce only Pc, but there are a number of intermediate cyanobacteria and eukaryotic algal species that are able to form either Cyt or Pc depending on copper availability in the culture medium (3). The two proteins are acidic in eukaryotes and either neutral or basic in cyanobacteria, but they both exhibit a similar isoelectric point when isolated from the same organism (3, 4). In filamentous cyanobacteria such as *Anabaena,* Pc and Cyt are positively charged with an isoelectric point of ca. 9 (see below).

Whereas a great deal of structural information exists for Pc from eukaryotic organisms (see ref. 5, for a review), 3D structures of cyanobacterial Pc have been reported only very recently: first, the solution structure of Pc from *Anabaena variabilis* as solved by NMR spectroscopy (6); and second, the X-ray structure of a triple mutant Pc from *Synechocystis* sp. PCC 6803 (7). Regards Cyt, its crystal and solution structure has only been reported in eukaryotic algae (8–11). From all these structural data, it is clear that Pc and Cyt posses primary and secondary structures that are completely different, but they exhibit a number of common surface features that account for their functional interchangeability (2).

Recent laser flash kinetic analyses indicate that the redox interaction between the two metalloproteins and PSI follows three different reaction mechanisms of increasing complexity (4). In *Anabaena,* in particular, PSI reduction kinetics can be well fitted to a simple oriented collisional mechanism with Pc but to a more complex and efficient three-step model with Cyt, the latter involving complex formation, rearrangement of redox partners inside the complex and electron transfer itself. In fact, *Anabaena* Cyt shows the faster electron transfer rate constant up to now reported for any three-step Pc(or Cyt)/PSI system ($t_{1/2}$ = 4 μs). The different kinetic mechanisms presented by Pc and Cyt in *Anabaena* makes this organism an interesting case to investigate by site-directed mutagenesis the specific role played by certain amino acids in both metalloproteins.

[1] EMBL Accession Numbers AJ002361 for the *pet* J gene and AJ002362 for the *pet* E gene.

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[3] Abbreviations: Cyt, cytochrome c_6; k_{bim}, second-order rate constant for PSI reduction; k_{et}, first-order electron transfer rate constant; LB, Luria-Bertani; ORF, open reading frame; Pc, plastocyanin; PCR, polymerase chain reaction; pI, isoelectric point; PSI, photosystem I.

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In cyanobacteria, the *pet*E and *pet*J genes from *Synechocystis* sp. PCC 6803 are the only ones that have been cloned and correctly expressed in *E. coli* (12, 13). This has allowed us to design and produce a number of site-directed Pc mutants that show significant changes in their reaction mechanism with PSI (14). A few other genes coding for Pc (15) and Cyt (16) from cyanobacteria have been cloned, but the recombinant proteins have not further been produced.

In this work, the cloning and correct expression of *Anabaena pet*E and *petJ* genes in *E. coli* is reported. The purification procedure of recombinant proteins is also reported, along with a comparative physicochemical and functional analysis of Pc and Cyt isolated from the two organisms.

MATERIALS AND METHODS

DNA techniques. The direct primer TCT CAA GGA TCC TTA TTT GCA GAG A and the reverse one TTA GTA AGC TTT GCT CTT GAA GTG G for the *petJ* gene, as well as the direct primer TAA AAT GAA TTC AGA AAA ATA AAA C and the reverse one AAA ATC ATC TAC TTG CGT CAG CGT T for the *pet*E gene were designed from the respective known sequences in the cyanobacterium *Anabaena* sp. PCC 7120 (17). These primers were used to amplify the regions containing the genes *pet*E and *pet*J from the genomic DNA of *Anabaena* sp. PCC 7119 by means of the polymerase chain reaction (PCR). The resulting PCR products were cloned using the pGEM-T cloning kit (Promega). The expression vector was pBluescriptII SK(+) (Stratagene). *E. coli* DH5α (Bethesda Research Laboratories) was used for cloning, plasmid construction and expression of the cloned genes. To be sure that the sequences did not contained any mutation introduced by PCR, two independent amplifications were carried out for each strain and two clones of each were sequenced separately. Nucleotide sequence analysis was carried out by the DNA Sequencing Service MediGene. Other molecular biology protocols were standard.

Production of recombinant proteins and purification procedures. *E. coli* DH5α transformed cells were grown in standard Luria-Bertani (LB) medium (18) supplemented with 100 μg/ml ampicillin. For the production of Pc, the medium was supplemented with 200 μM $CuSO_4$. Cells from 10 L cultures were collected, and the periplasmic fraction was extracted according to the method of Hoshino and Kageyama (19) as modified by Eftekhar and Schiller (20). Solid amonium sulfate was added to the periplasmic suspension up to 60% saturation. After centrifugation, ammonium sulfate was again added to the resulting supernatant up to 100% saturation. The final pellet was resuspended in 2 mM potassium phosphate, pH 7.0, extensively dialyzed against the same buffer and applied to a CM-cellulose column equilibrated with 2 mM potassium phosphate, pH 7.0. Proteins were eluted with a 2-30 mM potassium phosphate, pH 7.0, gradient. Protein concentration was determined spectrophotometrically using absorption coefficients of 4.5 mM^{-1} cm^{-1} at 597 nm for oxidized Pc (13) and 26.2 mM^{-1} cm^{-1} at 553 nm for reduced Cyt (21). Purity of the resulting protein fractions was determined using an absorbance ratio A_{553}/A_{275} of ca. 1.0 for pure Cyt (21) and A_{275}/A_{597} of 2.0 for pure Pc (13). Pc and Cyt were purified from *Anabaena* 7119 cells as described previously (22). PSI particles from *Anabaena* 7119 were obtained by β-dodecil maltoside solubilization as described by Rögner *et al.* (23) and modified by Hervás *et al.* (24).

Analytical methods. Molecular mass was determined by SDS-PAGE (25) using a 16% acrylamide running gel. Isoelectric point was determined by electrofocusing (26), with a mixture of ampholite carriers from Bio-Rad, pH range 3-10; the standard proteins used were those of the Sigma isoelectric focusing calibration kit for a pH range 6.8-9.3. The N-terminal amino acid sequences were determined by using an Applied Biosystems automated microsequencer model 477A. Redox titrations were performed in a dual wavelength spectrophotometer as described previously (27). The differential absorbance changes were monitored at 597 minus 500 nm with Pc and at 553 minus 570 nm with Cyt. Menadione, diaminodurol and *p*-benzoquinone, at 20 μM final concentration, were used as redox mediators. Laser flash-induced kinetics of PSI reduction by Pc and Cyt were monitored by following the absorbance changes at 820 nm as reported by Hervás *et al.* (4). Kinetic analyses were carried out according to the reaction mechanisms previously proposed (4, 28).

RESULTS AND DISCUSSION

On the basis of the known sequence of the *pet*E and *pet*J genes from *Anabaena* sp. PCC 7120 (17), we have designed a couple of oligonucleotides for each gene outside the ORF. This allowed us to clone the complete genes by PCR amplification, with just one DNA band

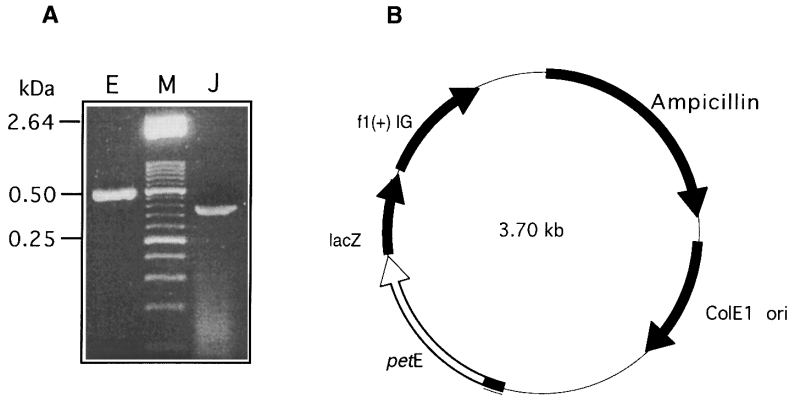

FIG. 1. (A) Agarose gel electrophoresis of PCR products of the genes *pet*E (lane E) and *pet*J (lane J); lane M corresponds to DNA molecular weight markers. (B) A plasmid construct (3.7 kb) showing insertion of the *pet*E gene under control of the *lac* promoter. The fragment was inserted at the multiple cloning site of the plasmid pBluescriptII SK(+[1]). A similar plasmid (3.6 kb) was constructed for inserting the *pet*J gene.

A

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    -16                                                                  36
AAT ACA GGT TAG GAG AAC GCC ATG AAA TTG ATT GCG GCA AGC TTG CGA CGC TTA AGT TTA
                           met lys leu ile ala ala ser leu arg arg leu ser leu
     45                                                                  96
GCT GTG TTA ACT GTT CTT TTA GTT GTT AGC AGC TTT GCT GTG TTC ACA CCT TCT GCA TCG
ala val leu thr val leu leu val val ser ser phe ala val phe thr pro ser ala ser
   ↓105                                                                 156
GCT GAA ACA TAC ACA GTA AAA CTA GGT AGC GAT AAA GGA CTG TTA GTA TTT GAA CCA GCA
ala glu thr tyr thr val lys leu gly ser asp lys gly leu leu val phe glu pro ala
    165                                                                 216
AAA TTA ACA ATC AAG CCA GGT GAC ACG GTT GAA TTT TTA AAC AAC AAA GTT CCT CCC CAT
lys leu thr ile lys pro gly asp thr val glu phe leu asn asn lys val pro pro his
    225                                                                 276
AAT GTT GTG TTT GAT GCT GCT CTA AAC CCG GCT AAG AGT GCT GAT TTA GCT AAG TCT TTA
asn val val phe asp ala ala leu asn pro ala lys ser ala asp leu ala lys ser leu
    285                                                                 336
TCT CAC AAA CAG TTG TTA ATG AGT CCT GGC CAA AGC ACC AGC ACT ACT TTC CCA GCA GAT
ser his lys gln leu leu met ser pro gly gln ser thr ser thr thr phe pro ala asp
    345                                                                 496
GCA CCC GCA GGT GAG TAC ACC TTC TAC TGC GAA CCT CAC CGT GGT GCT GGT ATG GTT GGT
ala pro ala gly glu tyr thr phe tyr cys glu pro his arg gly ala gly met val gly
    405
AAA ATC ACT GTC GCC GGC TAG AAA
lys ile thr val ala gly
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B

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     -4                                                                  48
GAA CGC GAC ATG AAA AAG ATT TTT TCC CTA GTA CTG TTA GGC ATA GCA CTC TTC ACT TTT
          met lys lys ile phe ser leu val leu leu gly ile ala leu phe thr phe
     57                       ↓                                          108
GCC TTC AGT AGC CCT GCA TTA GCA GCA GAC AGT GTA AAT GGA GCC AAG ATA TTC AGT GCT
ala phe ser ser pro ala leu ala ala asp ser val asn gly ala lys ile phe ser ala
    117                                                                 168
AAC TGC GCT TCT TGC CAT GCA GGT GGC AAG AAT TTG GTT CAA GCA CAG AAA ACT CTG AAG
asn cys ala ser cys his ala gly gly lys asn leu val gln ala gln lys thr leu lys
    177                                                                 128
AAA GCC GAT TTG GAA AAA TAT GGG ATG TAC TCA GCA GAG GCA ATC ATT GCC CAG GTA ACA
lys ala asp leu glu lys tyr gly met tyr ser ala glu ala ile ile ala gln val thr
    237                                                                 288
AAC GGT AAG AAC GCC ATG CCT GCT TTC AAA GGT CGC TTA AAA CCT GAA CAA ATT GAA GAT
asn gly lys asn ala met pro ala phe lys gly arg leu lys pro glu gln ile glu asp
    297
GTA GCT GCT TAC GTG CTA GGA AAA GCC GAT GCA GAT TGG AAG TAA ATT
val ala ala tyr val leu gly lys ala asp ala asp trp lys
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FIG. 2. Nucleotide sequence (coding strand) and deduced amino acid sequence of pre-apoplastocyanin (A) and pre-apocytochrome c_6 (B). Residues 1 to 25 of pre-apocytochrome c_6 and 1 to 34 of pre-apoplastocyanin constitute the respective putative transit sequences, as indicated by arrows.

amplified in every case (Fig. 1). In order to avoid amplification of undesired DNA bands, a first PCR cycle using an annealing temperature of 43°C was extremely critical, the following cycles being carried at 46°C. DNA bands were recovered and ligated using the pGEM-T cloning kit. SacII/HindIII for Cyt and BamHI/SalI for Pc were used as the cloning sites in pBluescript SK(+). The genes *pet*E and *pet*J were sequenced, the corresponding amino acid sequences for Pc and Cyt being thus deduced (Fig. 2). The nucleotide sequences of cloned genes were identical to those previously reported in *Anabaena* 7120 for *pet*J but differed in one base for *pet*E, even though such a difference does not induce any change in the amino acid sequence of Pc.

E. coli DH5α transformed cells were able to produce both Pc and Cyt from *Anabaena* 7119. The purification procedure yielded ca. 5 mg of Pc and 2 mg of Cyt from the periplasmic fraction of *E. coli* 10-L cultures. Figure 3 shows the UV/vis absorption spectra of recombinant Pc and Cyt, which are identical to those of the native proteins obtained from *Anabaena* 7119 and similar to proteins from other sources. In the reduced state, *Anabaena* Cyt exhibits characteristic absorbance maxima at 553 (α), 521 (β), 416 (γ, or Soret), 318 (δ) and 274 nm (protein); upon oxidation, the α and β peaks are replaced by a broader band with a maximum at 528 nm, the Soret band shifts to 409.5 nm, the δ band disappears, and a new band at 359 nm as well as a shoulder

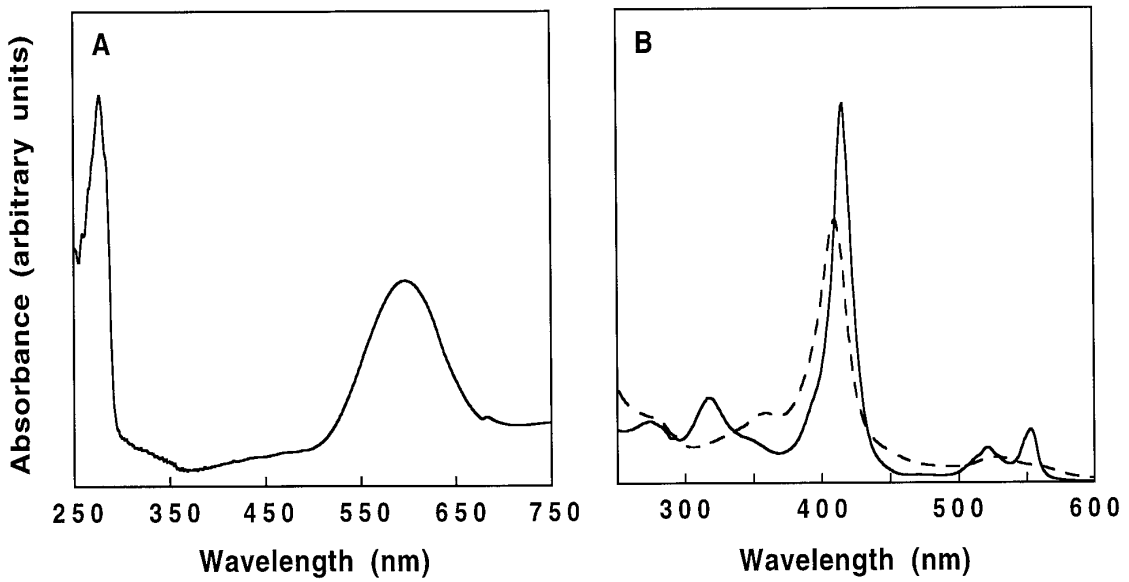

FIG. 3. UV/visible absorption spectra of plastocyanin (**A**) and cytochrome c_6 (**B**) from *Anabaena* 7119 expressed in *E. coli* cells. The spectrum of the copper protein corresponds to that of the oxidized form, whereas the heme protein is shown both in its native reduced state (continuous line) and after oxidation by ammonium persulfate (dashed line).

at 556.5 nm are observed (Fig. 3). Pc in its turn exhibits the characteristic absorption peak at 597 nm in its oxidized form. Other physicochemical properties of recombinant Pc and Cyt were practically identical to those of the proteins purified from *Anabaena* cells, with values for molecular mass, isoelectric point and redox potential that are the same in both organisms (Table I) and similar to values described previously for the metalloproteins from other sources. Recombinant Pc and Cyt were correctly processed and matured in *E. coli* cells, as deduced from their identical N-end amino acid sequences as compared with those from the proteins synthesized in the cyanobacterium (Table I).

Functional integrity of recombinant Pc and Cyt were tested by comparing their reactivity towards PSI, which is their physiological electron acceptor. PSI reduction by Pc and Cyt, monitored by following absorbance changes at 820 nm after laser-flash excitation, showed similar kinetics with both recombinant and cyanobacterial proteins, i.e., monophasic kinetics for the Pc/PSI system and biphasic traces for the Cyt/PSI system (data not shown) (4). Table I shows the experimental values for the second-order rate constants of Pc and Cyt interaction with PSI (k_{bim}), as well as the first-order electron transfer rate constants (k_{et}) and fast phase amplitude in the Cyt/PSI couple. From the presented data, it is clearly inferred that the kinetic behaviour of the cloned proteins is just the same than that of the proteins isolated from the cyanobacterium.

To summarize, we can say that the structural and functional features of both *Anabaena* Pc and Cyt expressed in *E. coli* are identical to those of the native proteins purified from the cianobacterium. This is the second report of a Cyt being properly produced in *E. coli*, a crucial step for the design and production of site-directed mutant metalloproteins. This will allow us to

TABLE I

Physicochemical Properties of *Anabaena* Cytochrome c_6 and Plastocyanin Isolated Both from the Cyanobacterium and from *E. coli* Cells Transformed by the *pet*J and *pet*E Genes, Respectively

Protein	Molecular mass (kDa)		pI	Em, pH 7.0 (mV)	N-terminal sequence	k_{bim} (M^{-1} s^{-1})	k_{et} (s^{-1})	Amplitude of fast phase (%)
	SDS-PAGE	Gene sequence						
Native Cyt	8.6	9.7	9.0	+339	ADSVN	11.6×10^7	1.7×10^5	35
Cloned Cyt	8.6		9.0	+335	ADSVN	12.6×10^7	1.7×10^5	36
Native Pc	10.5	11.1	8.8	+355	ETYTV	7.6×10^7		
Cloned Pc	10.5		8.8	+360	ETYTV	7.6×10^7		

Note. See text for further details.

analyze the role played by certain residues in the structure/function relationships of the two metalloproteins, as well as to unreveal the common structural features that lead them to play the same physiological function with similar efficiency.

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