Expression of spinach plastocyanin in *E. coli**

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An expression vector designed for overexpression of plastocyanin in the periplasmic space of *E. coli* has been developed. The vector contains the signal peptide sequence of *Pseudomonas aeruginosa* azurin and the mature sequence of spinach plastocyanin. The precursor is efficiently translocated to the periplasmic space and correctly processed to mature plastocyanin. No detectable amount of plastocyanin was present in the cytoplasmic or in the membrane fraction. A large scale preparation of the recombinant plastocyanin in a 20 litre fermentor yielded approximately 30 mg of pure plastocyanin. The recombinant protein obtained from *E. coli* shows CD, EPR and optical properties identical to plastocyanin isolated from spinach.

Plastocyanin; Blue-copper; Expression; *E. coli*; Photosynthesis

1. INTRODUCTION

Plastocyanin is a small ($M_r \approx 10\,500$) electron-transfer protein containing a single blue or type 1 copper ion [1]. It plays a central role in photosynthesis where it carries electrons from the cytochrome b_6/f complex to photosystem I [2].

The participation of plastocyanin in photosynthesis is not the only reason why it has attracted great interest. The type 1 character of the copper atom gives the protein unique spectral properties, such as intense blue color and narrow copper hyperfine splitting in the EPR spectrum [1]. The reduction potential in type 1 copper proteins is unusually high due in part to the binding geometry of the copper atom which is held in a distorted tetrahedral conformation [3]. This geometry stabilizes the Cu(I) state and the reduction potential of Cu(II)/Cu(I) is $E_o' = 370$ mV [4] compared to $Cu^{2+}(aq)/Cu^{+}(aq)$ with $E_o' = 170$ mV.

There have been several attempts to describe the interactions between plastocyanin and its biological redox partners [5,6]. Other experiments have been based on chemical modifications of amino acid residues [7,8] and binding of inorganic complexes [9] to the presumed binding sites. These investigations suggest the possibility of two different binding sites on plastocyanin for its redox partners.

For the complete understanding of the structure and function of plastocyanin, it will be necessary to develop new experimental methods. One important step in this direction is the construction of mutant proteins by site-directed mutagenesis of the plastocyanin structural gene. The first essential requirement for mutant construction is overproduction of proteins in a suitable host. In this work we describe the development of an expression vector designed for overproduction of plastocyanin in the periplasmic space of *E. coli*.

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; RBS, ribosome binding site; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; EPR, electronic paramagnetic resonance; CD, circular dichroism

2. MATERIALS AND METHODS

2.1. *Subcloning into expression vectors*

The cDNA clone encoding mature spinach plastocyanin was isolated from the cDNA fragment subcloned into the *Eco*RI site of pBluescript KS M13$^+$ [10]. From this construct the mature plastocyanin sequence was subcloned into the various expression-vectors. To obtain the precursor with the signal peptide from alkaline phosphatase pHIR21 [11] was used. The β-galactosidase fusion was constructed in pTTQ18 and the protein A fusion in pRIT5.

The hybrid percursor containing the azurin signal peptide and the mature sequence of plastocyanin was constructed from expression vector pUG4 which included the *lac* promoter [12]. Using site-directed mutagenesis an *Hpa*I site was introduced in the 3'-end of the azurin signal peptide. The plastocyanin fragment was isolated with *Eae*I (fill-in) and *Bam*HI. *Eae*I has a recognition site at the 5'-end of the mature plastocyanin sequence. The *Hpa*I site of the azurin signal peptide was ligated to the filled-in *Eae*I site in the plastocyanin sequence. In this way a recognition site for the *E. coli* signal peptidase was generated. For mutant contruction the in vitro-mutagenesis system from Amersham (RPN 1523) was used. All other molecular biology techniques were performed as described in [13].

2.2. *Bacterial strains*

The strains used where all derivatives of *E. coli* K12, MC1000 (F$^-$

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*ara*D139 Δ(*ara* ABC-*leu*) 7679 *gal*U *gal*K Δ(*lac*) X74 *rps*L *thi*) [14], KS 272 (F⁻ Δ*lac*-X74 *gal*E *gal*K *rps*L(Str^r) Δ*pho*A(*Pvu*II)) [15], KS 474 (KS 272 *deg*P) [16] and TG1 (Δ(*lac-pro*), *sup*E,*thi*,*hsd*D5/F' *tra*D36, *pro*A$^+$B$^+$, *lac*^{q},*lac*ZΔM15) [17].

2.3. *Growth of* E. coli

Growth media used were TB-medium [12] supplemented by 50 μg/ml ampicillin. *E. coli* strains were grown at 37°C except for strains indicated by the KS prefix which were grown at 30°C. Bacterial cultures were induced by IPTG at a final concentration of 0.3 mM when the absorption at 550 nm reached 1.0. After induction the bacterial cells were allowed to grow for an additional 8 h. The strain KS 474 containing pUG101 was grown in a 20 litre Chemap fermentor according to [18]. Cells carrying the plasmid pDR720 were induced by adding 3-β-indole acrylic acid.

2.4. *Fractionation of* E. coli *cells*

The periplasmic protein fraction was isolated according to [12]. From the resulting spheroplasts the cytplasmic and membrane fractions were isolated. From a 2 litre culture this pellet was resuspended in 1/10 vol. of 20 mM Tris-HCl, pH 7.5, 50 mM NaCl. The cells were sonicated and centrifuged for 5 min at 5000 × *g*. The cytoplasmic proteins were separated from the membrane-bound proteins by ultracentrifugation of the supernatant at 120 000 × *g* for 30 min. The pellet, containing the membrane-associated proteins, was dissolved in SDS-PAGE-sample buffer. The total protein fraction was obtained by sonicating intact *E. coli* cells in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.05% Tween-20 and the solution was centrifuged at 10 000 × *g* for 5 min. The supernatant contained the total protein fraction.

2.5. *Protein purification*

The periplasmic fraction was acidified to pH 5.0 and centrifuged. The supernatant was loaded on 300 ml Whatman CM 52 cation exchange cellulose equilibrated with 20 mM Bis-Tris-HCl, pH 6.0. The plastocyanin-containing permeate was directly applied on a DE 32 column (75 ml Whatman anion exchange microgranular cellulose) equilibrated with the same buffer. The permeate contained the bulk proteins. Plastocyanin was eluted with a 600 ml 0–0.5 M NaCl-gradient in the same buffer. Fractions containing plastocyanin were pooled (approx. 0.1–0.2 M NaCl), titrated to pH 8.3 with 1 M Tris-base, diluted with 1 vol. of 20 mM Tris-HCl, pH 8.0, and loaded on a preparative (8 ml) mono-Q FPLC-column (Pharmacia) equilibrated with the pH 8 buffer. The proteins were eluted with a salt gradient (0–0.35 M NaCl in 160 ml). Plastocyanin detected in fractions with 0.14–0.20 M NaCl, was pooled and concentrated through ultrafiltration (Filtron Omega membrane, cut-off 3K). Copper sulphate was added in a 10-fold excess and the intensely blue plastocyanin solution was gel-filtrated on a Superose 12 FPLC-column (Pharmacia). The plastocyanin was homogeneous as judged by SDS-PAGE analysis. The copper-containing protein was purified from the apoprotein by repeating the previous anion (mono-Q) chromatography step. The purified plastocyanin had an A_{278nm}/A_{597nm} ratio of 1.2.

Spinach plastocyanin was purified according to [4].

2.6. *SDS-PAGE and Western blotting*

SDS-PAGE analysis was performed on the Pharmacia PhastSystem with 8–25% gradient gels. Western blotting was performed according to [12] with the exception that the proteins separated by SDS-PAGE were transferred to an Immobilon-P membrane (Millipore) through contact diffusion at 37°C for 2 h. Rabbit anti-plastocyanin serum was obtained by immunization of white rabbits according to [19]. The immunological reactions were performed at 37°C.

2.7. *Recording of CD and EPR spectra*

Optical spectra were recorded on a Shimadzu UV 3000 spectrophotometer. CD spectra were recorded on a Jasco J-500A Spectropolarimeter. X-band EPR spectra were recorded on a Bruker ER 200D-SRC ESR spectrometer equipped with an Oxford Instruments EPR-9 helium-flow cryostat at 20K.

3. RESULTS

3.1. *Expression constructs*

The structural gene for spinach plastocyanin is nuclear encoded, and the gene product is transported post-translationally through three different membranes into its final location in the thylakoid lumen of chloroplasts [20]. During this translocation a transit peptide is cleaved off in two steps [21].

The expression of mature spinach plastocyanin was examined in several different *E. coli* vector systems. In these plasmids the plastocyanin gene was placed under control of different promoters: *trc* in pKK233-2 [22], *tac* in pKK223-3 [23], *trp* in pDR720 [24] and λ_{PL} in pCP3 [25] and pPLEX [26]. The expression constructs were transformed into *E. coli* strain TG1. After growth and induction of transformed *E. coli* cells the total cell protein content was released by sonication. Plastocyanin could not be detected by Western blotting in any of these constructs.

To increase the stability of plastocyanin we constructed two different fusion proteins, one being designed for intracellular expression in the cytoplasm and the other for translocation of the fusion protein to the periplasmic space of *E. coli*. The first hybrid protein carried a short segment of the α-region of β-galactosidase [27] at the 5'-end of plastocyanin, whereas the second hybrid was a fusion between *Staphylococcus* protein A [28] and plastocyanin. The intracellularly expressed fusion protein could not be detected by Western blotting. The translocated protein A/plastocyanin fusion was detected in low amounts. However, degradation of the protein A moiety was a problem and there was no sign of copper uptake.

To increase mRNA stability and translation efficiency, we made two expression vectors which contained two different signal peptide sequences. The first contained the signal peptide of alkaline phosphatase and placed the construct in control of the *tac* promoter [11]. After cell growth and induction of the protein production with IPTG the periplasmic fraction was isolated [12]. Plastocyanin could not be detected in this fraction by Western blotting.

In the second construct plastocyanin was put after the signal peptide sequence and ribosome binding site of a related protein of bacterial origin. The gene encoding azurin from *Pseudomonas aerugiinosa* [29] was chosen because of its similarity in secondary structure to plastocyanin. This protein is also a type 1 copper protein and has proved to be efficiently produced and correctly processed in *E. coli* [12]. Using site-directed mutagensis we introduced a *Hpa*I site at the 3'-end of the azurin signal peptide. This site was ligated to a filled-in *Eae*I site which was situated in the 5'-end of the mature plastocyanin sequence. In this way it was possible to construct an expression vector for overproduction of a hybrid precursor with the signal peptide

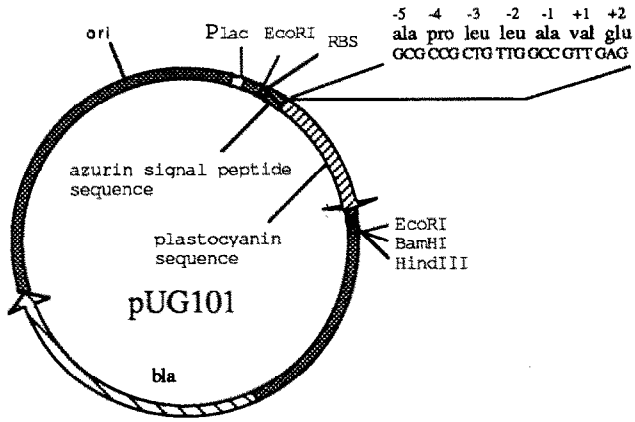

Fig. 1. Expression vector, pUG101, for overproduction of plastocyanin. The amino acid sequence around the cleavage site of the precursor is shown.

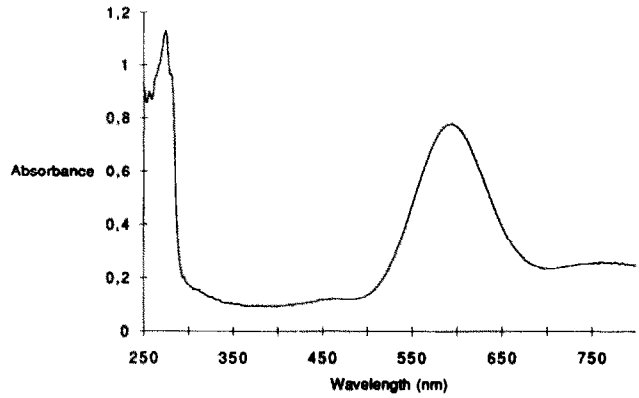

Fig. 3 The optical absorption spectrum of plastocyanin purified from *E. coli*. The tube color of plastocyanin is due to the absorption maximum at 597 nm.

sequence from azurin and the mature protein sequence from plastocyanin. This construct produced a functional recognition site for the signal peptidase of *E. coli* [30] (Fig. 1).

The construct was transformed into *E. coli* strains: MC1000, KS272 and KS474. After cell growth and induction of the protein production with IPTG, the periplasmic fractions were isolated and the protein content was analysed by SDS-PAGE followed by Western blotting (Fig. 2A,B). Expressed plastocyanin was detected in all the transformed strains at different concentrations.

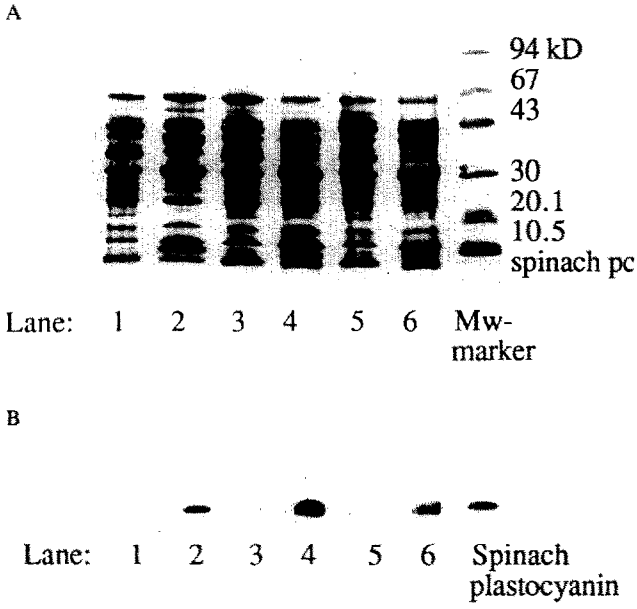

Fig. 2.(A) SDS-PAGE for detection of plastocyanin (pc) in the periplasmic fractions of MC1000, KS272 and KS474, transformed with pUG101. The parental strains were included as controls. (Lane 1) MC1000; (lane 2) MC1000/pUG101; (lane 3) KS272; (lane 4), KS272/pUG101; (lane 5) KS 474; (lane 6) KS474/pUG101. Spinach plastocyanin added to the M_r-marker is seen as the band just below 10.5 kDa. (B) Western blot of the above SDS-PAGE.

3.2. *Large scale preparation*

The transformed strain KS474 was grown in a fermentor [18]. Plastocyanin was isolated from the periplasmic fraction using anion and cation exchange chromatography and gel filtration. Native protein could be separated from apoprotein by anion exchange chromatography. Approximately one third of the amount of plastocyanin expressed contained copper. The purified plastocyanin has an A_{278nm}/A_{597nm} ratio of 1.2. The ratio for plastocyanin isolated from spinach is 1.2–1.4 [7]. From a 15 litre culture 30 mg of pure plastocyanin was obtained. The precursor was correctly processed as determined by amino-acid analysis of the first 20 N-terminal residues.

3.3. *Subcellular distribution of plastocyanin*

In order to investigate the subcellular location of the expressed plastocyanin a fractionation of the *E. coli* cells was made. The amount of plastocyanin in the periplasmic, cytoplasmic and membrane fractions was investigated by SDS-PAGE and Western blotting (not shown). No detectable amount of plastocyanin was present either in the cytoplasmic or in the membrane fraction. This indicates that the cleavage of the precursor and the translocation of the protein to the periplasmic space of *E. coli* occur efficiently.

3.4. *Spectroscopic measurements*

The optical, CD and EPR spectra of the recombinant plastocyanin were measured. The resulting spectral parameters are identical to the parameters obtained for spinach plastocyanin (Figs 3 and 4, the CD-spectrum is not shown).

4. DISCUSSION

Earlier attempts to express plastocyanin in *E. coli* have been unsuccessful [31] and a new approach for construction of an expression system was considered.

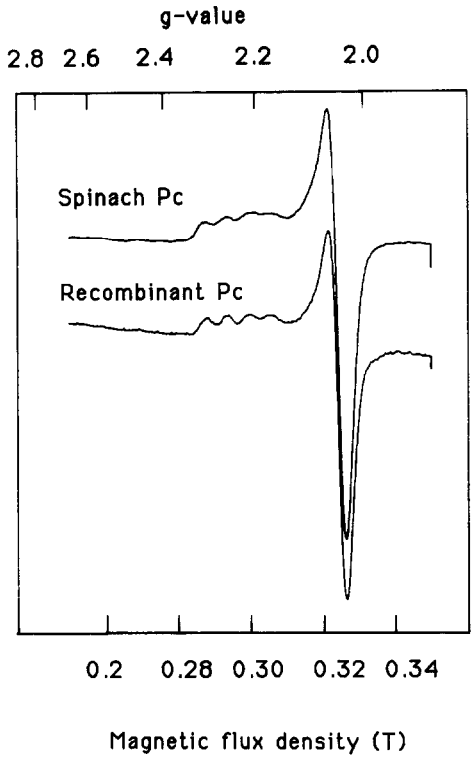

Fig. 4. The EPR spectra of spinach and recombinant plastocyanin.

The expression vector, pUG4, consisting of the signal peptide from *Pseudomonas aeruginosa* azurin was chosen partly because of the similarity of the proteins, both being members of the family of small blue copper proteins, and partly because of the success in expressing azurin in *E. coli* [12].

The precursor resulting from the expression vector inherits the characteristics of a signal peptide as postulated [32] and is correctly processed as shown by determination of the N-terminal amino acid sequence.

The recombinant plastocyanin and plastocyanin obtained from spinach are spectroscopically indistinguishable. The conformation around the copper center is reflected both in the optical absorption spectrum and in the EPR spectrum (Figs 3,4), whereas the CD spectrum indicates the similarity in β-structure.

The varying amount of plastocyanin obtained in the different constructs described in this paper, depend among others on the mRNA. The sequence and conformation in the 5'-end of the mRNA is important for efficient transcription and translation [33]. This structure is also important for the stability of the mRNA [34]. The absence of detectable amounts of plastocyanin in the intracellular constructs might be due to incorrect folding, proteolytic breakdown of the protein or to instability of the mRNA.

In the fusion protein constructs and when plastocyanin is placed after the signal peptide of alkaline phosphatase, the mRNA is probably stabilised by the sequence introduced in the 5'-end. Incorrect folding or proteolytic breakdown is the most likely explanation for the failure in obtaining a functional plastocyanin in these constructs.

The signal peptide of *Pseudomonas aeruginosa* azurin functions very well in *E. coli*. This might be one reason for the high level of expressed azurin. The efficient translocation and processing of the precursor also verifies the advantage of this signal peptide. There are good reasons to expect the azurin signal peptide to fit well in with the mature plastocyanin protein because of the similarity in three-dimensional structure between the proteins [35]. This might protect the precursor from attack of proteases and improve the translocation.

When transforming the expression vector, pUG101, in *E. coli* strains MC1000, KS272 and KS474 the amount of plastocyanin expressed varied unpredictably. The expected results would have suggested the strain KS474 to be the most efficient for plastocyanin expression since this strain is a *deg*P mutation of strain KS272. Mutations in *deg*P are known to prevent degradation of certain periplasmic proteins [15]. Thus the amount of expressed plastocyanin was expected to be higher in this strain. However, strains MC1000 and KS474 seemed to produce approximately the same amount of plastocyanin while the yield in KS272 appeared to be higher. The reason for this remains to be clarified.

With this expression system it is now possible to produce a functional copper-containing plastocyanin, the prerequisite for mutant construction with the aid of site-directed mutagenesis. In this way we hope to elucidate the structural basis of the unique properties of the copper center in plastocyanin and reveal the binding sites between plastocyanin and its redox partners in the photosynthetic electron transfer chain. Studies in this direction are in progress.

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