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Reconstitution of mature plastocyanin from precursor apo-plastocyanin expressed in *Escherichia coli*

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The precursor plastocyanin from *Silene pratensis* (white campion) has been expressed in *Escherichia coli*. The precursor protein was accumulated in insoluble aggregates and partially purified as an apo-protein. The purified precursor apo-plastocyanin was processed to the mature apo-plastocyanin by chloroplast extracts. N-terminal amino-acid sequencing indicated that the processed protein was identical to the N-terminal amino-acid residues of mature plastocyanin that was deduced from the nucleotide sequence. The copper could be incorporated into the apo-plastocyanin of mature size in vitro, but could not into the precursor apo-plastocyanin under the same conditions. Absorption spectra and reduction potential of the reconstituted mature plastocyanin were indistinguishable from those of the purified spinach plastocyanin. The electron transfer activities of the reconstituted plastocyanin with both the Photosystem I reaction center (P700) and cytochrome *f* were almost the same as those of the purified spinach plastocyanin.

Introduction

Plastocyanin (PC) is a protein with a single polypeptide chain (10.5 kDa) and a single Type I copper atom. It is a mobile protein and takes up electrons from cytochrome *f* in the cyt b_6/f complex and donates electrons to the reaction center chlorophyll (P700) in the Photosystem I (PS I) protein complex [1]. The crystal structures of oxidized [2], reduced [3] and apo-PC [4] have been determined. Recent studies suggested the presence of two areas on the PC surface acting as sites for the electron transfer reactions [5–11]. One is the hydrophobic region of histidine-87, and the other is the region of negatively charged carboxyl residues around 42–45 and 59–61. The importance of negative charges of carboxyl residues around 42–45 and 59–61 on PC for the electron transfer reaction with cytochrome *f* has been demonstrated [5–10], whereas the binding site of PC for P700 is controversial and is not clear [10,11].

It has been shown that higher plant PC is nuclear encoded and synthesized as a precursor in the cytosol and transported into the thylakoid lumen of chloroplasts via two steps [12,13]. First, the precursor protein is transported into the stroma and processed to an intermediate form. Then, the intermediate protein is transferred across the thylakoid membrane, where maturation is completed. In these steps, the involvement of two processing proteinases has been demonstrated [13–16]. During the maturation, a copper atom is incorporated into the apo-PC. It is unknown, however, at which step copper is incorporated into the apo-PC; the precursor apo-PC, the intermediate precursor apo-PC, or the mature apo-PC. In view of the need for unfolding of proteins during membrane transport, the most likely place is the thylakoid lumen.

Recently, the precursor PC was expressed in *Escherichia coli* (*E. coli*) [17]. It was demonstrated that the precursor PC expressed in *E. coli* was after purification imported into isolated chloroplast and processed to the mature size. Large quantities of purified precursor PC are needed to study the molecular mechanisms of incorporation of copper into the apo-PC as well as the physicochemical properties of resulting holo-proteins. Therefore, we have started a systematic study of the construction of holo-PC from the precursor apo-PC. In this paper, we report that the purified precursor apo-PC could be processed to the mature apo-PC and a copper

Abbreviations: P700, Photosystem I reaction center chlorophyll; PS I, Photosystem I; PC, plastocyanin; IPTG, isopropyl 1-thio-β-D-galactopyranoside; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.

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atom could be incorporated into the mature apo-PC in vitro, but not into the precursor apo-protein. We also report that the reconstituted mature PC shows almost the same absorption spectrum, redox potential, and kinetic properties as those of the purified spinach PC.

Materials and Methods

Plasmid construction, expression and purification of precursor PC

Details of plasmid construction, expression, and purification of precursor apo-PC will be described in a separate paper (De Boer, A.D., Pilon, M. and Weisbeek, P.J., unpublished data). Briefly, the cDNA of precursor apo-PC (Ref. 13 and the unpublished data) was cloned in the expression plasmid pKK233-2 [18,19], resulting in $pKPAP_{14}$. The *E. coli* strain PC2495[α] was used for the expression of precursor apo-PC from the expression plasmid $pKPAP_{14}$. Cells were grown at 37°C in Luria broth with 50 μg/ml ampicillin until a density of (1–2) $\cdot 10^8$ cells/ml was reached. Then, 0.5 mM IPTG was added to induce the expression of precursor apo-PC. Cells were allowed to grow another 3 h and were collected by the centrifugation at 10000 × *g* for 30 min. The pellets were dissolved in 20 ml of 10 mM Tris-HCl (pH 8.0), 0.12 M NaCl, 0.02% β-mercaptoethanol, 1 mM EDTA and 0.5 mM PMSF (buffer A) per liter of culture. Then 2 mg/ml of lysozyme was added and the suspension was passed through a Yeda press cell two times. Insoluble material was pelleted by spinning for 30 min at 10000 × *g* and suspended in 6 M guanidine-HCl, 25 mM dithiothreitol (DTT), 25 mM Tris-HCl (pH 8.0) for 1 h. The suspension was diluted two times with 25 mM DTT and centrifuged at 200000 × *g* for 1 h. The supernatant containing precursor apo-PC was dialyzed against buffer A overnight and then centrifuged at 200000 × *g* for 1 h. The supernatant was passed through a 0.2 μm membrane filter and applied on a DEAE-Sephacel column (30 × 2 cm) that was equilibrated with the buffer A. Precursor apo-PC was eluted with a linear (0–400 mM NaCl) gradient. The fractions containing precursor apo-PC were applied on a HPLC column of TSKgel DEAE-5PW and eluted with the same conditions.

Preparation of protein

PC and cytochrome *f* were purified from spinach and *Brassica komatsuna* as previously described [5]. The Photosystem I complex, which contains about 180 chlorophyll per P700 (PS I 180), was prepared essentially according to the method of Mullett et al. [20] as previously described [21]. The crude extracts of processing enzymes were prepared from cucumber cotyledons according to the method of Robinson's group [14,15]. The cucumber seedlings (*Cucumis sativus* L.) were grown in darkness for 4 days at 28°C, then illuminated with white fluorescent light for 2 days. Cotyledons were homogenized using 50 mM Tris-HCl (pH 8.0) buffer containing 0.4 M sucrose, 10 mM NaCl, 0.1% NaN_3 and 1 mM PMSF. The homogenate was filtered through two layers of Miracloth (Calbiochem) and centriguged at 1000 × *g* for 3 min. The pellets were dissolved in 20 mM Tris-HCl (pH 8.0), incubated for 30 min, and centrifuged at 10000 × *g* for 30 min. The pellets were used as the thylakoid membrane processing enzyme.

Electrophoresis, immunoblotting and peptide sequencing

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 15.0% acrylamide was done according to Laemmli as previously described [22]. Western blotting analysis was done as described in Ref. 23. An antiserum raised against the spinach PC was prepared as described in Ref. 23. The N-terminal amino-acid sequence was determined with the use of gas phase protein sequencer (Applied Biosystems, model 470A). Proteins on a gel were transferred onto a PVDF membrane (Immobilon, Milipore) in 20 mM Tris-borate buffer (pH 9.5) and 20% methanol. The membrane was stained with 0.01% Coomassie brilliant blue G-250 in methanol/acetic acid (40 : 4, v/v), destained with 90% methanol, and applied to the sequencer.

Spectroscopy

Kinetic experiments on the electron transfer between cytochrome *f* and PC were performed with a Union RA-401 stopped flow spectrometer [5]. The reaction of cytochrome *f* were measured by monitoring the absorbance at 422 nm. The flash-induced absorbance change of P700 was measured at 697 nm using a Union Giken single-beam spectrometer as previously described [22]. The contents of P700, PC, and cytochrome *f* were calculated according to the methods previously described [5,22]. The redox potentials of PC were obtained by a combined spectrophotometric/platinum-calomel electrode method in 50 mM sodium phosphate buffer (pH 7.0) as previously described [5]. The content of copper in PC was determined with a Jarrell-Ash atomic absorption spectrometer AA-845 with a flameless graphite furnace. All these experiments were done at 25°C.

Results

Preparation of precursor apo-PC and its processing

The precursor protein of plastocyanin was partially purified as a main band on SDS-polyacrylamide gels as shown in Fig. 1A, lane 3. Although the molecular mass of precursor apo-PC calculated from the nucleotide sequence was predicted as 16622 Da [12], the antibody raised against spinach PC cross-reacted with a 23 kDa polypeptide. The difference between calculated and experimentally estimated sizes of protein was also observed in the case of mature PC. The spinach PC

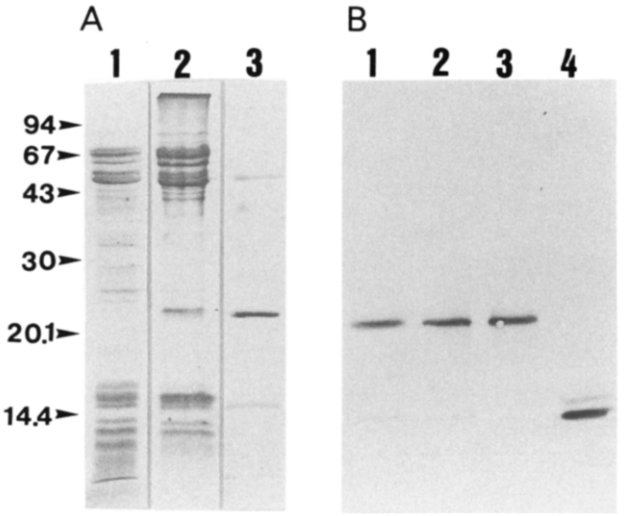

Fig. 1. Purification of precursor PC. Samples (25 μg) taken up from the purification stages of the precursor PC expressed in *E. coli* (pKPAP$_{14}$/PC2495[α]) were analyzed by 15.0% SDS-PAGE. (A), Coomassie brilliant blue staining; (B), immunological detection with an antibody raised against the spinach PC. Lane 1, lysozyme lysate; lane 2, 200000 × *g* supernatant; lane 3, DEAE-Sephacel eluate; lane 4, spinach PC. The minor band above 14 kDa of spinach PC (Fig. 1B, lane 4) may be an artefact of SDS-PAGE.

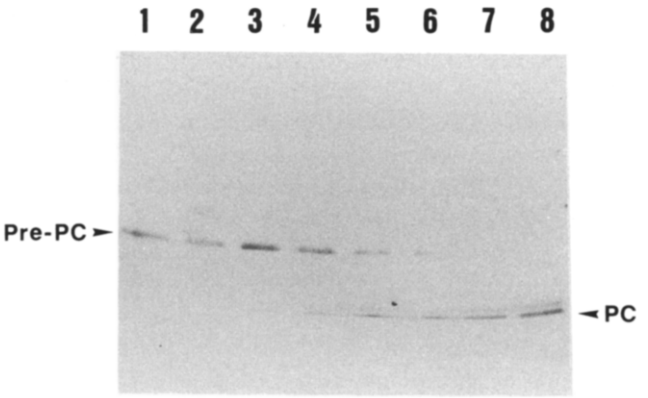

Fig. 2. Processing of the precursor PC by the thylakoid membrane fractions. The partially purified precursor PC was incubated for the various times with the stroma processing enzyme fraction. The reaction mixtures (25 μg) were analyzed by 15.0% SDS-PAGE and detected by the immunological method. Lane 1, partially purified precursor PC; lane 2, incubated time of zero; lane 3, incubated for 5 min; lane 4, incubated for 30 min; lane 5, incubated for 1 h; lane 6, incubated for 3 h; lane 7, incubated for 12 h; lane 8, purified spinach PC. The minor band above 14 kDa may be an artefact of SDS-PAGE.

migrated as a 14 kDa protein (Fig. 1B, lane 4), although its calculated molecular mass is 10415 Da [25]. The minor band at 14.5-kDa of spinach PC (Fig. 1B, lane 4) may be an artefact of SDS-PAGE. The N-terminal amino-acid sequence of the 23 kDa polypeptide was determined as shown in Table I. The 17 N-terminal amino-acid residues were sequenced. The sequence was consistent with the N-terminal amino-acid sequence deduced from the cDNA for the precursor apo-PC, except for the initiating methionine, which was absent in the 23 kDa protein. Copper was absent from the partially purified precursor PC.

It has been reported that the precursor apo-PC, which was synthesized by transcription using SP6 RNA polymerase followed by translation in a wheat-germ system, is processed in two steps, first to an intermediate form by a stroma-processing enzyme, and then to a mature form by a thylakoid membrane enzyme [13–16]. Therefore, it was interesting to examine the processing of purified precursor apo-PC expressed in *E. coli*. The precursor apo-PC was incubated for various times with the crude extract of membrane fraction of processing enzymes, which were prepared as described in Materials and Methods. After incubation, samples were subjected to immmunoblot analysis using an antibody raised against spinach PC and the results are shown in Fig. 2. Upon incubation, the amount of the precursor apo-PC (23 kDa polypeptide) decreased, and a new band of 14 kDa polypeptide was observed. During the incubation time, essentially no intermediate band could be detected. The minor band above the 14 kDa polypeptide probably represents an artefact of SDS-PAGE as described above. Since the above results suggest that the polypeptides at 14 kDa corresponded to the mature apo-PC, we examined the N-terminal amino-acid sequence of this polypeptide. The 14 kDa polypeptide was isolated and sequenced (14 steps). As

TABLE I

N-terminal amino-acid sequence of the precursor apo-PC (A) and the processed apo-PC (B)

(A) N-terminus of the precursor apo-PC															
	1				5					10				15	
Expressed in *E. coli*		Ala	Thr	Val	Thr	Ser	X [a]	X	ALA	Val	Ala	Ile	Pro	X	Phe	Ala	X	Leu
Deduced from the cDNA [b]	Met	Ala	Thr	Val	Thr	Ser	Ser	Ala	Ala	Val	Ala	Ile	Pro	Ser	Phe	Ala	Gly	Leu
(B) N-terminus of the processed apo-PC															
	67			70					75					80	
Expressed in *E. coli*.	Ala	X	Val	X	Leu	Gly	X	X	X	Gly	Gly	Leu	Ala	Phe	
Deduced from the cDNA [b]	Ala	Glu	Val	Leu	Leu	Gly	Ser	Ser	Asp	Gly	Gly	Leu	Ala	Phe	

[a] X, not determined.
[b] Ref. 12.

shown in Table I, the N-terminal amino-acid residues, as determined by protein sequencing, correspond to the amino-acid residues starting from Ala-67 to Phe-80 of the precursor apo-PC, as deduced from the cDNA for Silene PC [12]. The N-terminal amino-acid residue of mature PC from Silene was not determined, but processing between two alanine residues Ala-66 and Ala-67 was proposed [12], indicating the same N-terminal amino-acid residue as with the processed PC. Previously it was shown that the mature PC from tomato has a somewhat higher mobility than that from Silene in transgenic tomato plants on a SDS-PAGE [26]. In this paper, it was confirmed that the spinach PC migrates at the same rate as the processed PC (14 kDa protein) and has a somewhat lower mobility than the PC from tomato leaves (data not shown). From these results, we conclude that the precursor apo-PC could be processed in vitro at the same site as in vivo and the 14 kDa protein is a mature apo-PC.

Incorporation of copper into apo-plastocyanin

So far, it has not been reported at which step the copper atom is incorporated into the apo-PC and whether or not an enzyme-catalyzed reaction is involved in the incorporation of this copper atom. We therefore tested whether or not the apo-PC expressed in *E. coli* can take up copper atoms in vitro. To do this, a method for the reconstitution of copper into apo-PC was developed. That was the denaturation of apo-PC by a chaotropic reagent such as guanidine-HCl and the incorporation of copper into apo-PC during the folding of apo-PC due to the removal of guanidine-HCl.

First, we tested the incorporation of copper into the mature spinach apo-PC. The copper atom was removed from the mature PC by dialysis against buffer A which contained 6 M guanidine-HCl. The formation of mature apo-PC was confirmed by a separate experiment, by subsequent dialysis against buffer A without copper and guanidine-HCl and measuring the absorption at 597 nm even in the presence of potassium ferricyanide (Fig. 3B). We did not observe a peak at 597 nm, which indicates the removal of copper from the protein. To the denatured mature apo-PC copper was added by dialysis against buffer A, which contains copper but not guanidine-HCl. Excess of copper was removed by further dialysis against the buffer without both copper and guanidine-HCl. The resulting solution gave a strong absorption peak at 597 nm and showed almost the same absoption shape as the purified spinach PC as shown in Fig. 3C. This means that we have incorporate a copper atom into the apo-PC prepared from the purified spinach PC.

The method described above was also applied for the incorporation of copper into the processed Silene mature apo-PC. The results are shown in Fig. 3D; the in vitro processed mature PC showed almost the same

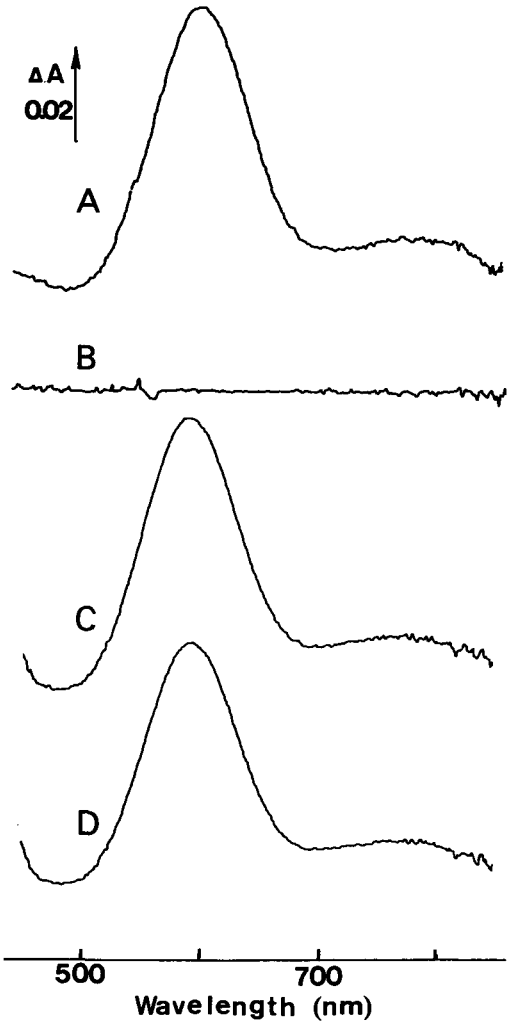

Fig. 3. Absorption spectra of the reconstituted PC. (A) Purified spinach PC; (B) after incubation of spinach PC with 6 M guanidine-HCl, guanidine-HCl was removed; (C) reconstituted spinach PC; (D), reconstituted PC expressed in *E. coli*.

absorption spectrum as the purified spinach PC, indicating the incorporation of a copper atom into the processed mature PC. The same procedures were applied for the reconstitution of a copper atom into the precursor apo-PC, but were unsuccessful.

Physicochemical and kinetic properties of the reconstituted PC

Optical characteristics of the purified and reconstituted PC are compared in Table II. The reconstituted mature PCs prepared from both the precursor apo-PC expressed in *E. coli* and the spinach apo-PC contained approximately one copper atom per polypeptide. The molar absorption coefficients at the absorption maximum (597 nm) and oxidation reduction potentials of the reconstituted spinach PC and processed PC were also almost identical to the native spinach PC. These results indicate the incorporation of copper into the correct site of apo-PC expressed in *E. coli*.

TABLE II

Absorption maximum, absorption coefficient, and reduction potential of reconstituted PC

Protein	Absorption maximum (nm)	Absorption coefficient ($M^{-1}cm^{-1}$)	Reduction potential (mV)
Expressed in *E. coli.*	597	4400	379
Reconstituted spinach PC	597	4400	378
Native spinach PC	597	4500	382

Next, we examined the reactivities of the reconstituted PC with the photooxidized P700. As shown in Fig. 4A, after the flash illumination, the photooxidized P700 in the spinach PS I 180 complex was reduced by the reduced spinach PC with a half-time of about 1.6 ms, monitored by the increase in absorbance at 697 nm. The reconstituted mature PC prepared from the precursor apo-PC (Fig. 4C) as well as the spinach apo-PC (Fig. 4B) also showed almost the same reactivities for the photooxidized P700 with that of the purified spinach PC. In vivo, the photooxidation of PC by P700 is known to occur in the microsecond time range [27,28], whereas in this work, a millisecond oxidation was observed. This might be due to the use of low concentrations of PC and/or the resolution time of our instruments. The electron transfer reaction from reduced cytochrome *f* to oxidized PC was also examined using a

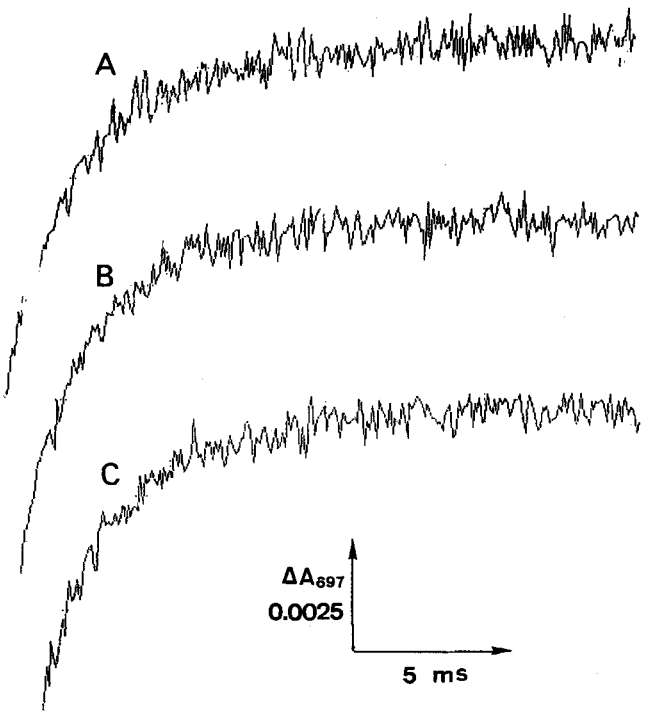

Fig. 4. Electron transfer from PC to photooxidized P700. (A) Purified spinach PC; (B) reconstituted spinach PC; (C) reconstituted PC expressed in *E. coli*. The reaction mixture contained 0.25 μM P700, 2 μM PC, 2.5 mM ascorbate, 0.1 mM methyl viologen, 0.05% Triton X-100, 5 mM $MgCl_2$ and 10 mM Tris-HCl (pN 8.0).

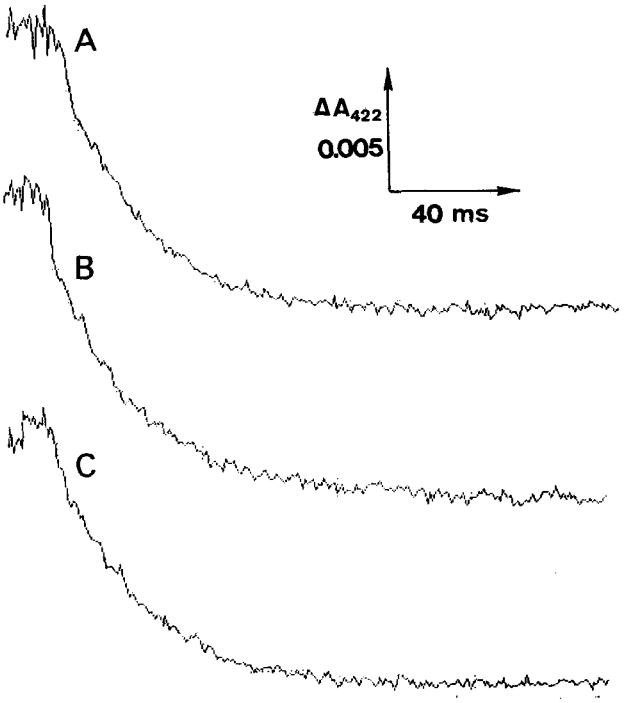

Fig. 5. Electron transfer from reduced cytochrome *f* to oxidized PC. (A) Purified spinach PC; (B) reconstituted spinach PC; (C) reconstituted PC expressed in *E. coli*. The reaction mixture contained 0.3 μM cytochrome *f*, 1 μM PC, 0.15 M NaCl and 20 mM potassium phosphate (pN 7.0).

stopped-flow experiment. The oxidation of cytochrome *f* was monitored by the absorbance change at 422 nm. It is known that the rate of this reaction decreases rapidly upon the increase of ionic strength [5]. The reaction was measured at pH 7.0 and ionic strength of 0.15 M. After the rapid mixing, the purified cytochrome *f* from *Brassica komatsuna* was oxidized by the purified spinach PC with a half-time of about 16 ms as shown in Fig. 5A. Essentially the same rate constants were obtained for the reactions between the reconstituted PCs prepared from the spinach apo-PC (Fig. 5B) and the precursor apo-PC expressed in *E. coli* (Fig. 5C). These results clearly indicate the construction of active mature PC from the precursor apo-PC expressed in *E. coli.*

Discussion

The present results demonstrate that the precursor apo-PC could be expressed in *E coli*, processed to the mature apo-PC in vitro, and reconstituted with a copper atom. The mature PC constructed showed the same reactivities with P700 and cytochrome *f*. This is to our knowledge the first paper describing the construction of an acitive mature PC from the precursor apo-PC expressed in *E. coli*. Copper was absent in the partially purified precursor protein. Due to the use of a denaturation reagent (guanidine-HCl) for dissolving the insoluble aggregate of precursor PC, we could not de-

termine whether nor not the precursor PC binds copper atom in *E. coli*. The fact that we can incorporate a copper atom into mature apo-PC, but not into precursor apo-PC suggests the absence of copper in the precursor PC in *E. coli*. A recent paper appeared which suggests that only the mature apo-PC generated in vitro is competent for the incorporation of copper [29]. Their results are consistent with our observations.

The precursor apo-PC expressed in *E. coli* was processed to the mature apo-PC by the thylakoid membrane fractions and essentially no intermediate form of precursor apo-PC could be detected as shown in Fig. 2. Processing of the precursor apo-PC with the thylakoid membrane fractions suggests the contamination of stroma fractions [16]. The absence of detectable amount of intermediate form of precursor apo-PC is probably due to the rapid processing of the intermediate apo-PC to the mature apo-PC. This might be related to the use of denaturation reagent of guanidine-HCl for the purification of precursor apo-PC expressed in *E. coli*. At this point, it is worthwhile noting that precursor apo-PC, purified using the anionic detergent SDS, was incubated with the crude extract of thylakoid-processing enzyme, the processed mature apo-PC was further degraded and essentially nothing could be seen on the filter (data not shown). These results could be explained by the conformational change of the precursor apo-PC due to the binding of denaturation reagents such as SDS and guanidine-HCl on the protein even after dialysis, that may cause exposure of the amino-acid residues of apo-PC to the processing enzymes. Further studies are necessary to clarify this.

As will be described elsewhere, the expression in *E. coli* of mature PC could not be achieved due to the rapid degradation of synthesized protein. Recently, the expression of azurin from *Pseudomonas* bacteria strain in *E. coli* has been reported [30]. In contrast to the case of PC, accumulation of the mature azurin with a copper atom has been reported. Although both PC and azurin are proteins with a single polypeptide chain and a single type I copper atom, quite different results were obtained for the expression in *E. coli*. Preparation of large quantities of purified precursor PC is especially useful for the study of transport, experiments as well as the assembly of the mature PC. Moreover, the success of reconstitution of mature PC from the precursor apo-PC expressed in *E. coli* will allow us to investigate the structure and function of mature PC by site-directed mutagenesis.

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