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A chloroplast processing enzyme functions as the general stromal processing peptidase

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ABSTRACT **A highly specific stromal processing activity is thought to cleave a large diversity of precursors targeted to the chloroplast, removing an N-terminal transit peptide. The identity of this key component of the import machinery has not been unequivocally established. We have previously characterized a chloroplast processing enzyme (CPE) that cleaves the precursor of the light-harvesting chlorophyll *a*/*b* binding protein of photosystem II (LHCPII). Here we report the overexpression of active CPE in *Escherichia coli*. Examination of the recombinant enzyme *in vitro* revealed that it cleaves not only preLHCPII, but also the precursors for an array of proteins essential for different reactions and destined for different compartments of the organelle. CPE also processes its own precursor in trans. Neither the recombinant CPE nor the native CPE of chloroplasts process a preLHCPII mutant with an altered cleavage site demonstrating that both forms of the enzyme are sensitive to the same structural modification of the substrate. The transit peptide of the precursor of ferredoxin is released by a single cleavage event and found intact after processing by recombinant CPE and a chloroplast extract as well. These results provide the first direct demonstration that CPE is the general stromal processing peptidase that acts as an endopeptidase. Significantly, recombinant CPE cleaves in the absence of other chloroplast proteins, and this activity depends on metal cations, such as zinc.**

The majority of chloroplast proteins are encoded by the nuclear genome and cytosolically synthesized as precursor proteins with an N-terminal transit peptide that directs the import of the polypeptide into chloroplasts. The transit peptide is removed by a stromal processing activity that is sensitive to metal chelators (1–3). Recent studies have uncovered a set of molecular components of the chloroplast import pathway and suggested that a single common apparatus mediates the translocation of most cytosolically translated precursors (4, 5). The molecular structure of the proteolytic machinery needed for the maturation of this large diversity of imported precursors has been unknown.

Two antigenically related proteins, originally estimated to be 145 and 143 kDa, copurify with a soluble chloroplast processing enzyme (CPE) that cleaves the precursor of the light-harvesting chlorophyll *a*/*b* binding protein of photosystem II (preLHCPII) (6). Furthermore, organelle-free processing of the precursors for two stromal proteins, acyl carrier protein (ACP) and ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (RBCS), was inhibited by 75% in immunodepletion experiments by using antibodies against the 145/143-kDa protein doublet. However, the role of CPE in the processing of precursors other than preLHCPII has never been directly tested because purified CPE is not easily attainable from chloroplast extracts for *in vitro* assays. The anti-145/143-kDa antibodies were used to isolate a cDNA from pea (*Pisum sativum*) that encodes a putative metallopeptidase of 1,257 amino acids that was referred to as CPE (7). It contains the zinc-binding motif His-X-X-Glu-His, characteristic of the pitrilysin family that includes *Escherichia coli* protease III, insulin-degrading enzyme, and subunit β of the mitochondrial processing peptidase (8). To identify the range of precursors recognized by CPE, we undertook experiments to express an active enzyme in *E. coli* from the pea cDNA clone. These studies were facilitated by cloning of a CPE homolog from *Arabidopsis thaliana*. Alignment of the amino acid sequences of pea and *Arabidopsis* CPE revealed an N-terminal extension with properties of a chloroplast transit peptide and suggested the start of mature CPE at position Ala-143 of the pea polypeptide. A mature form of pea CPE, lacking the predicted transit peptide, was expressed in *E. coli* and tested for processing activity. Processing of preLHCPII and 10 other precursors destined for different compartments and pathways within the organelle demonstrates that CPE is the general stromal processing peptidase (SPP). We show further that the transit peptide is removed in one endoproteolytic step from a representative precursor.

MATERIALS AND METHODS

Expression of CPE in *E. coli*. Expression vector pBEX5BA (9) contains a promotor inducible by isopropyl-β-D-thiogalactoside and yields a recombinant protein N-terminally fused to a biotin-containing peptide from *Propionibacterium shermanii*. Fragments encoding preCPE (codons 1–1257; GenBank accession no. U25111) and mature CPE (codons 143-1257; accession no. U25111) were generated by PCR and cloned into pBEX5BA. Cultures (100 ml) of *E. coli* WM1704 (9) carrying the new constructs were grown at 30°C, induced with 2 mM isopropyl-β-D-thiogalactoside and cells were lysed by sonication (Branson sonifier) in 5 ml 25 mM Hepes-KOH (pH 7.5), 1 mM phenylmethanesulfonyl fluoride (PMSF). Crude extracts were centrifuged (20 min at 8,500 $\times$ *g*) and the supernatants were used in Western blot analysis and processing reactions. Biotinylation of the mature CPE fusion (recombinant biotinylated CPE; CPE-B) was demonstrated by partial purification by using recombinant biotinylated CPE, affinity chromatography with avidin-resin (Promega).

Immobilization of CPE-B. CPE-B protein from 100 μl of *E. coli* extract was bound to 50 μg of streptavidin-coated paramagnetic particles (Promega) by incubation in 200 μl of 1$\times$

Abbreviations: CPE, chloroplast processing enzyme; CPE-B, recombinant CPE N-terminally linked to a biotin-containing peptide; FD, ferredoxin; LHCPII, light-harvesting chlorophyll *a*/*b* binding protein of photosytem II; RBCA, ribulose-1,5-bisphosphate carboxylase/oxygenase activase; RBCS, ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit; SPP, stromal processing peptidase; ACP, acyl carrier protein; OEE, oxygen evolving enhancer; PMSF, polymethanesulfonyl fluoride; PC, plastocyanin.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF008444).

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binding buffer (10 mM Tris·HCl/250 mM NaCl/0.5% Tween-20, pH 7.5) at 28°C for 30 min. Immobilized CPE-B was intensively washed (three times with 2× binding buffer, twice with 2× binding buffer without Tween-20, and once with 25 mM Hepes-KOH, pH 7.5) and finally resuspended in 20 μl 25 mM Hepes-KOH (pH 7.5) for one processing reaction by using a radiolabeled substrate. For processing of nonlabeled recombinant preRBCA, CPE-B protein from 1 ml of *E. coli* extract was immobilized on 400 μg of streptavidin-coated paramagnetic particles by incubation in 2 ml 1× binding buffer at 28°C for 30 min. Immobilized CPE-B was intensively washed as described above and resuspended in 40 μl of 25 mM Hepes-KOH (pH 7.5) for one processing reaction.

Expression of PreRBCA in *E. coli*. A cDNA encoding preRBCA cloned into plasmid pTZ18U (10) was used as template in a PCR. An *Nco*I site including the start codon was introduced by the sense primer (5′-GGATTGCCATGGCTACTGCTGTCTCGACC-3′). An oligonucleotide that anneals to the *lacZ′* sequence of pTZ18U downstream of the insert was used as the antisense primer (5′-GCTATTACGCCAGCTGGCGAAAGGGGGATGTG-3′). After PCR, the amplified fragment was digested by *Nco*I and *Bam*HI, which have a single site downstream of the coding sequence. The fragment was ligated into the expression vector pET-3d (11) at its *Nco*I and *Bam*HI sites. The resulting expression plasmid, pET-3d::preRBCA, was transformed into *E. coli* strain BL21(DE3)pLysS (11). Expression and protein isolation was carried out as described for expression of preLHCPII in *E. coli* (12).

***In Vitro* Processing Assay Using Radioactive-Labeled Substrates.** Precursors labeled with [^{35}S]methionine or [^{35}S]cysteine were generated by translation in a reticulocyte lysate (Promega) using template RNA synthesized by either SP6 or T7 RNA polymerase (2). Precursors (0.5–4 μl) were incubated with 20 μl of soluble protein extract from *E. coli* or immobilized CPE-B solution in a 25-μl total volume of 20 mM Hepes-KOH (pH 7.5), 1 mM PMSF at 28°C for 1.5 h. Soluble chloroplast extract was prepared as described (3) and used in processing reactions under the same conditions as chosen for recombinant CPE. Substrates and processing products were denatured and analyzed by standard SDS/PAGE (13) and autoradiography. For detection of transit peptides, the processing reaction was modified. The reaction was carried out at room temperature for 10 min in the presence of 2 mM PMSF. Products were analyzed by tricine-SDS/PAGE (14). The precursors used in our experiments are listed in Table 1.

***In Vitro* Processing of Nonlabeled PreRBCA for N-Terminal Amino Acid Sequencing.** Thirty micrograms of preRBCA were incubated with immobilized CPE-B solution in a 60-μl total volume of 20 mM Hepes-KOH (pH 7.5), 2 mM PMSF at 28°C for 4 h. As a control, paramagnetic particles were first incubated with extract of *E. coli* cells carrying pBEX5BA and then with 30 μg of preRBCA under the same conditions. Products were separated by SDS/PAGE, electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P^{SQ}, Millipore), and stained with Coomassie brilliant blue R-250 as described (24). A 45-kDa protein band was generated by immobilized CPE-B. No cleavage product was detectable in the control reaction. The protein band of the processing reaction was cut out of the membrane and subjected to N-terminal amino acid sequencing that was carried out by the Howard Hughes Medical Institute Biopolymer Laboratory and W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT).

Table 1. Precursors.

No.	Chloroplast protein	Organism	Ref. no.	Accession no.
1.	LHCPII	*Triticum aestivum*	15	P04784*
2.	RBCS	*Pisum sativum*	16	P00869*
3.	RBCA	*Spinacia oleracea*	10	P10871*
4.	ATP synthase γ-subunit (CF_1 γ-subunit)	*Spinacia oleracea*	17	P05435*
5.	OEE1	*Arabidopsis thaliana*	18	P23321*
6.	PC	*Silene pratensis*	19	P07030*
7.	ACP1	*Spinacia oleracea*	20	P07854*
8.	3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase	*Arabidopsis thaliana*	21	P29976
9.	HSP21	*Pisum sativum*	22	P09886*
10.	CPE	*Pisum sativum*	7	U25111†
11.	FD	*Silene pratensis*	23	P04669*

*SwissProt.
†GenBank.

RESULTS AND DISCUSSION

Structure of CPE. If CPE is an essential component of the chloroplast processing activity, homologous genes should be present in all higher plants. To test this hypothesis, we screened a genomic phage library of *A. thaliana* (25) with a pea CPE cDNA. Sequence analysis of a positive phage insert revealed a gene encoding CPE in *Arabidopsis.* Assignment of its intron/exon structure was based on comparison of predicted reading frames with the amino acid sequence of pea CPE and on 5′ and 3′ splice consensus sequences (26, 27). A total of 24 exons encode a protein of 1,265 amino acids with a calculated molecular mass of 140,631 Da.

To identify structural features that may be functionally important, pea and *Arabidopsis* polypeptides were aligned by using the University of Wisconsin Genetics Computer Group program BESTFIT (26) (Fig. 1 *A* and *B*). Four highly conserved regions were found. The conserved region I, extending for 209 amino acids, contains the hallmark zinc-binding motif. A conserved stretch of acidic amino acids and prolines found between the regions II and III is predicted to be a flexible spacer (29), which often links functionally and structurally defined domains within both eukaryotic and prokaryotic proteins (30, 31). A significant switch from 26% sequence identity between the N-terminal segments of both CPE to an average of 80% identity occurs at Ala-143 of the pea amino acid sequence, suggesting the start of the mature protein (Fig. 1*A*). The N-terminal region of 142 amino acids, despite its unusual length, shows features found in chloroplast transit peptides (32–34), i.e., it is enriched for the polar amino acids serine and threonine as well as the small hydrophobic amino acids alanine and valine, the overall charge is basic, and 75% of the peptide is predicted to be devoid of regular secondary structure (29). A full-length cDNA of pea CPE has been shown by *in vitro* transcription and translation to code for a polypeptide that can be imported into chloroplasts and cleaved (not shown), and thus it codes for a precursor, preCPE, with a functional transit peptide. Mature CPE has a calculated molecular mass of 123,752 Da.

Expression of Recombinant CPE in *E. coli*. To determine if CPE is sufficient to confer chloroplast processing activity in the absence of other chloroplast proteins, preCPE and an N-terminally truncated version lacking the predicted transit peptide, i.e., mature CPE, were expressed in *E. coli* and analyzed by Western blot analysis with the antibody against the 145/143-kDa chloroplast protein doublet. Both polypeptide versions were barely detectable and insoluble. Subsequently, a biotin-containing peptide of 12.5 kDa was N-terminally linked to both preCPE and its mature form (called preCPE-B and CPE-B, respectively), and expression in *E. coli* was determined by Western blot analysis. Approximately 10% of both expressed fusion proteins was soluble. The biotin-containing peptide seems to have partially stabilized CPE in *E. coli* and prevented complete sequestration in inclusion bodies. The

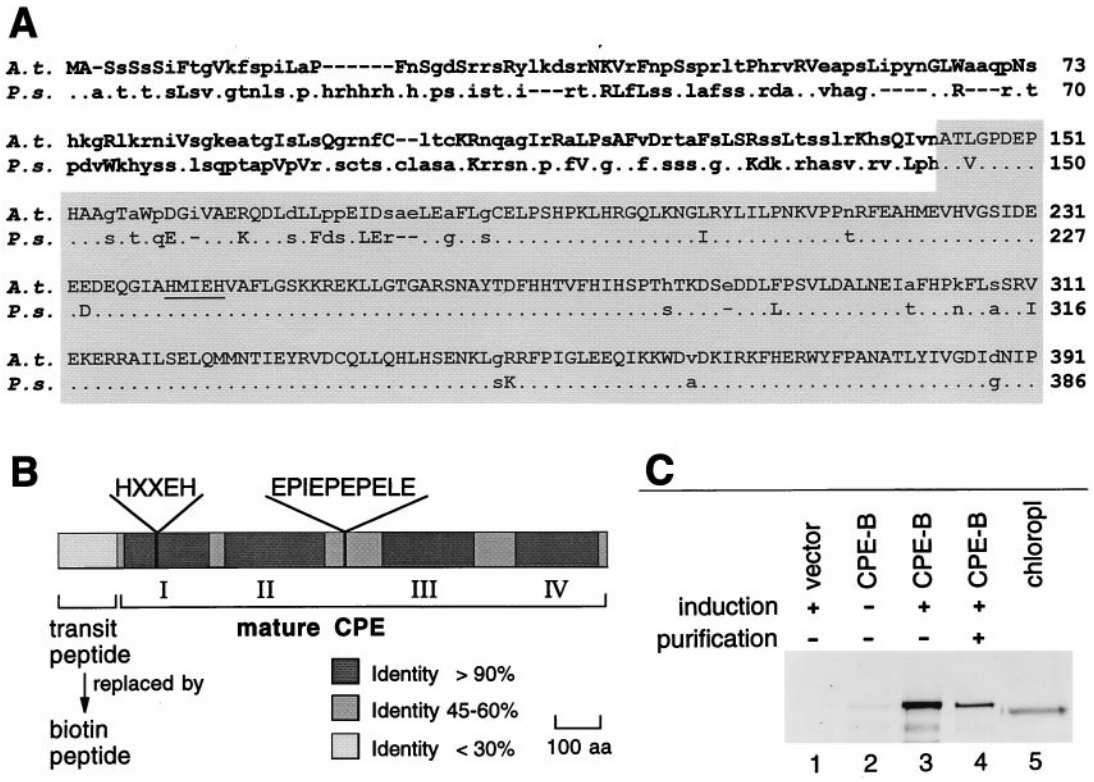

FIG. 1. Structure of CPE and expression of CPE-B fusion protein. (*A*) Alignment of N-terminal amino acid sequences of *A. thaliana (A.t.)* and *P. sativum (P.s.)* preCPE. Lowercase letters indicate different amino acids. Uppercase letters indicate amino acids with a homology index ≥0.8 (28). Dots represent identical amino acids. Sequence of mature CPE predicted from the alignment is indicated by shading. The zinc binding motif is underlined. (*B*) Schematic map defined by amino acid comparison of pea and *Arabidopsis* CPE. The highly conserved regions I to IV, the zinc-binding motif of conserved region I, and a putative flexible linker are indicated. To express active CPE, the predicted transit peptide was replaced by a biotin-containing peptide (CPE-B). (*C*) Western blot analysis of CPE-B expression in *E. coli* using the anti-145/143-kDa antibody was carried out as described (7). Soluble cell extracts of induced cells carrying pBEX5BA (lane 1), and of uninduced and induced cells carrying pCPE-B (lanes 2 and 3, respectively). CPE-B was partially purified with avidin-resin demonstrating biotinylation of the fusion protein (lane 4). Soluble chloroplast extract containing CPE (lane 5) was prepared as described (3).

identity of preCPE-B and CPE-B was demonstrated by using the anti-145/143-kDa antibodies in Western blot analysis of *E. coli* extracts expressing preCPE-B and CPE-B, and compared with a pea chloroplast extract (Fig. 1*C*, not shown for preCPE-B). The chloroplast protein, lacking the biotin-containing peptide tag, migrates ahead of CPE-B.

Proteolytic Activity of Recombinant CPE. We first tested proteolytic activity of preCPE-B and CPE-B using [^{35}S]methionine-labeled wheat preLHCPII (15). PreLHCPII was processed to a 25-kDa protein only by CPE-B (Fig. 2*A*), whereas preCPE-B was inactive. Mature CPE fused to the biotin-containing peptide was used for further studies and the expression plasmid was named pCPE-B.

N-terminal sequencing of wheat LHCPII generated in an organelle-free assay revealed previously that cleavage of preLHCPII occurred between Lys-40 and Ala-41 (12). Accordingly, conversion of the cleavage site sequence Ala-39–Lys-40–Ala-41 to Thr-39–Thr-40–Lys-41 resulted in a loss of processing (35). To test whether recombinant CPE-B and CPE activity from chloroplasts are sensitive to the same structural modification, processing of wheat preLHCPII and the mutant with an altered cleavage site was compared. During SDS/PAGE, the cleavage product of preLHCPII produced by CPE-B comigrated with LHCPII generated by organelle-free processing using a chloroplast extract, whereas mutated preLHCPII was not processed by either enzyme (Fig. 2*A*). To exclude processing by other *E. coli* proteases, an *E. coli* extract was made from control cells carrying the expression vector without an insert. Upon incubation with preLHCPII no significant proteolytic activity was observed (Fig. 2*A*).

To test if CPE confers general stromal processing activity, in addition to cleaving preLHCPII, the range of substrates assayed was extended to precursors targeted to different chloroplast compartments and those involved in different biosynthetic pathways (Table 1, Fig. 2*B*). Precursors of the stromal proteins RBCS (16) and ribulose-1,5-bisphosphate carboxylase/oxygenase activase (RBCA) (10), and the γ-subunit of ATP synthase ($CF_1\gamma$) (17) that is associated with stromal side of the thylakoid membranes, were processed. Nuclear-encoded precursors of thylakoid lumen proteins have a bipartite transit peptide consisting of two targeting signals. A stromal processing activity has been shown to remove the stromal-targeting-signal yielding an intermediate-size form (36, 37). We tested whether CPE-B also cleaves the precursors of the luminal proteins oxygen-evolving enhancer 1 (OEE1) (18) and plastocyanin (PC) (19), and found that the cleavage products comigrated with the intermediate-size forms generated by the chloroplast extract. Besides maturation of proteins involved in photosynthesis, we tested CPE cleavage of precursors for ACP1 (20), a key component in *de novo* fatty acid biosynthesis, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DHS1) (21) that catalyzes the first step in aromatic amino acid biosynthesis, and heat shock protein HSP21 (22). All three precursors were processed by CPE-B. Importantly, cleavage products of each precursor generated by both CPE-B and the chloroplast extract showed similar sizes during SDS/PAGE. Cleavage efficiency appeared to vary for different precursors. PreRBCA, prePC, preACP, and preHSP21 were typically cleaved to completion within 1.5 h, whereas preLHCPII and preRBCS were still found even after 3 h incubation. These differences may indicate that factors influencing substrate

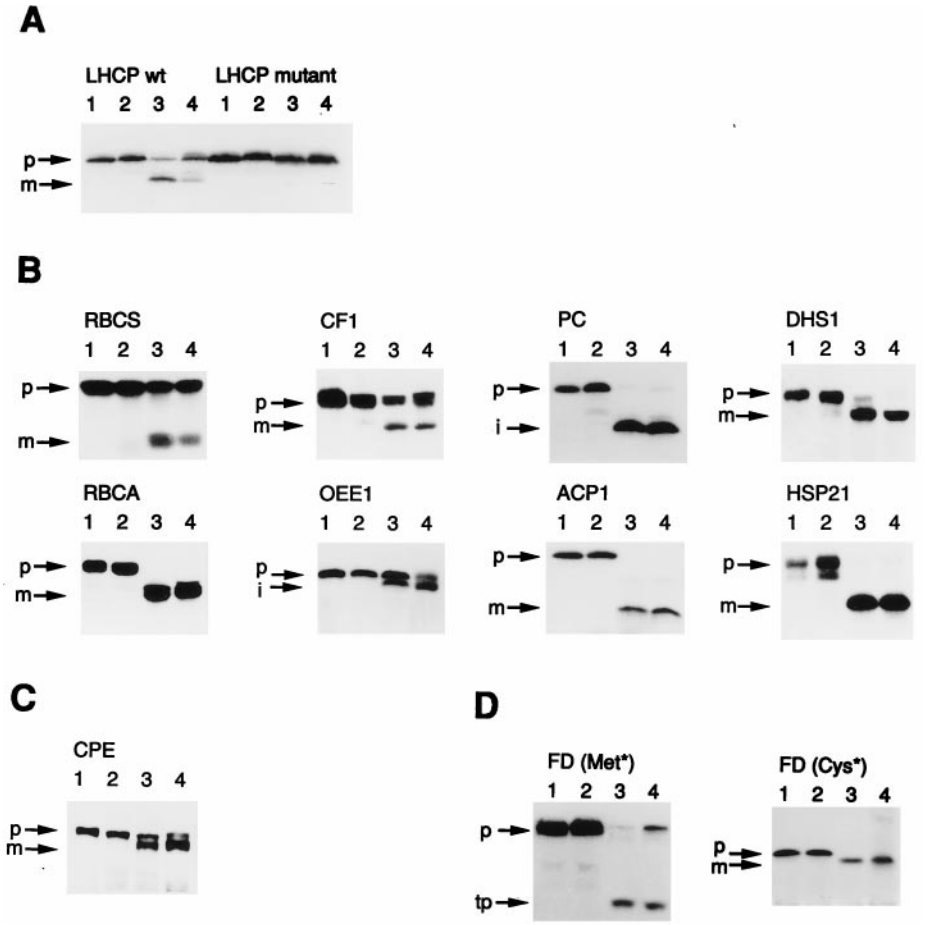

FIG. 2. CPE is the general SPP of chloroplasts. (*A*) Processing of [^{35}S]methionine-labeled preLHCPII wild type (wt) and a processing site mutant. (*B*) Processing of [^{35}S]methionine-labeled preRBCS, preRBCA, pre$CF_1\gamma$, preOEE1, prePC, preACP1, preDHS1, and preHSP21. (*C*) Processing of [^{35}S]methionine-labeled preCPE. (*D*) Processing of preFD labeled with [^{35}S]methionine (Met*) and [^{35}S]cysteine (Cys*), respectively. Lanes 1, radiolabeled precursor. Lanes 2, precursor incubated with control extract of induced *E. coli* cells carrying pBEX5BA lacking the CPE insert, except incubation of preACP was done with magnetic beads that were preincubated with the control extract. Lanes 3, processing with CPE-B extract, except processing of preACP was carried out with immobilized CPE-B due to a proteolytic activity in the *E. coli* extract that degraded preACP. Lanes 4, processing with chloroplast extract. p, Precursor; i, intermediate; m, mature; tp, transit peptide.

conformation, not present in the organelle-free assay, normally determine the accessibility of precursor cleavage sites during translocation into the organelle.

CPE is cytosolically synthesized and imported. To determine if CPE processes its own precursor, [^{35}S]methionine-labeled preCPE was synthesized in a reticulocyte lysate and incubated with CPE-B. PreCPE was processed by CPE-B, demonstrating cleavage in trans. The cleavage product comigrated with that of a chloroplast extract during SDS/PAGE (Fig. 2*C*).

Precursor Processing by CPE Releases the Intact Transit Peptide. Processing of a precursor by CPE releases the mature protein, but what is the fate of the transit peptide? An important question was whether it is removed by a single endoproteolytic step, or if multiple cleavages are required before the production of mature protein. Earlier attempts to detect transit peptides after processing in organelle-free assays failed, indicating multiple cleavages that released small peptides undetectable by SDS/PAGE, or very rapid degradation by another strong stromal proteolytic activity after release of the transit peptide. The availability of recombinant CPE-B, in the absence of other stromal components, allowed us to test whether the processing products included the intact transit peptide. For this purpose we selected the precursor of the stromal protein ferredoxin (FD) (23) because of its distribution of methionine and cysteine residues for radiolabeling. PreFD has four methionines found only in the transit peptide and five cysteines present only in the mature protein. Processing of preFD labeled by [^{35}S]methionine should yield the unlabeled mature protein and the labeled transit peptide. Tricine-SDS/PAGE, which resolves small peptides (14), was employed for analysis. Autoradiography of such a gel revealed a single labeled protein (Fig. 2*D*, lane 3) whose size was estimated to be ≈5 kDa based on comparison with Coomassie-stained protein markers in agreement with the calculated molecular mass of 4,894 Da of the transit peptide. We conclude that this protein is the transit peptide. The absence of labeled mature size product suggests that recombinant CPE-B cleaves preFD at the same site as native CPE because the mature FD sequence starts C-terminal of the fourth methionine of the polypeptide (23). Based on this finding we tried to identify the transit peptide after processing with chloroplast extract. By modification of the standard conditions (see *Materials and Methods*), we were able to detect the transit peptide generated by the organelle-free assay (Fig. 2*D*, lane 4). Production of mature FD was demonstrated by incubation of [^{35}S]cysteine-labeled preFD with CPE-B extract from *E. coli* and a chloroplast extract, followed by standard SDS/PAGE (Fig. 2*D*). These results show that the transit peptide is initially removed from the precursor by a single endoproteolytic step. Importantly, they also suggest that CPE alone may not be sufficient for transit peptide turnover, a problem that remains for future studies.

Cleavage Sites Recognized by CPE. The structural requirements for cleavage by the chloroplast processing activity are

still unknown, and only a small number of site-specific mutations have been studied that might help elucidate the determinants for processing (35, 38, 39). Further, comparison of predicted precursor cleavage sites does not suggest a well-conserved consensus sequence (40). Usually the assignment of a cleavage site and the end of the transit peptide is based on N-terminal amino acid sequence of a mature protein isolated from chloroplasts and alignment with the sequence deduced from a full-length cDNA. To date, only a handful of cleavage sites have been determined directly by analysis of the N termini of products of an organelle-free processing reaction. These include preLHCPI (41), preLHCPII (12), preOEE1, preOEE2, and prePC (42) (Fig. 3). To compare with these precursors, here we investigated where recombinant CPE-B cleaves preRBCA. PreRBCA was chosen for this analysis because—in contrast to LHCPI and II of the thylakoid membranes and OEE1, OEE2 and PC of the thylakoid lumen–RBCA is localized to the stroma. In addition, preRBCA is one of the substrates most efficiently processed by CPE-B (Fig. 2*B*). Nonlabeled preRBCA was processed using immobilized CPE-B isolated by streptavidin-coated paramagnetic particles that bound to its biotin-containing peptide tag. The N-terminal sequence of the processing product revealed that cleavage occurred between Lys-57 and Ala-58 of the precursor (Fig. 3). It is striking that four of the five cleavage sites determined previously after *in vitro* processing reactions also contain a basic residue, i.e., lysine or arginine, in position -1, and an Ala at +1, suggesting this may be a preferred site of CPE. However, it is clear that these residues are not sufficient for cleavage, but rather must be found in the right structural context because Lys-Ala and Arg-Ala found elsewhere in the precursor are not recognized by CPE. Previously, Ala-59 was predicted to be the first amino acid of mature RBCA (10). It is possible that another proteolytic step clips an additional alanine from RBCA during its maturation, e.g., via one of the aminopeptidases of unknown function that have been identified in chloroplasts (46), or possibly, limited degradation occurred during protein isolation. Alternatively, it cannot be excluded that in an organelle-free reaction CPE-B cleaves one residue upstream, between Lys-57 and Ala-58, due to the conformation of preRBCA, which could be different from its structure during import. Nevertheless, given that CPE-B cleaves a large diversity of substrates without degradation products, and evidence that the transit peptide is released intact, our results demonstrate the high specificity of the *in vitro* cleavage reaction.

Proteolytic Activity of CPE Depends on Metal Cations. The presence of a zinc-binding motif (7), and earlier *in vitro* processing studies with chloroplast extracts (3), indicated that CPE may be a metallopeptidase. To test this, preRBCA was incubated with CPE-B in presence of specific protease inhibitors (Fig. 4*A*). The strong sensitivity to metal chelators such as 1,10-phenanthroline (only 4% activity at 1 mM) and EDTA

preRBCA	KKHTNVRFPSSSRTTSMTV**K**^{57}	↓**A**AENEEKNTD	(472 aa)
preLHCPI	STKSKFAAAMPVSVGATNS**M**^{40}	↓**S**RFSMSADWM	(246 aa)
preLHCPII	LPSSALIGDARVNMRKTAA**K**^{40}	↓**A**KQVSSSSPW	(266 aa)
preOEE1	MPAKIGGRASSARPSSHVA**R**^{31}	↓**A**FGVDAGARI	(325 aa)
preOEE2	AASARPAPAVGRTQLFVVC**K**^{34}	↓**A**QKNDEAASD	(258 aa)
prePC	STTRAATVKVAVATPRMSI**K**^{41}	↓**A**SLKDVGVVV	(165 aa)
	-1	+1	

FIG. 3. Amino acid sequences of identified CPE cleavage sites. The processing sites of preRBCA (*Spinacia oleracea*)(10), preLHCPI (*Lycopersicon esculentum*)(43), preLHCPII (*Triticum aestivum*)(15), preOEE1 (*T. aestivum*)(44), preOEE2 (*T. aestivum*)(45) and prePC (*Silene pratensis*)(19) are shown. The amino acids found by N-terminal sequencing of the cleavage product of preRBCA generated by immobilized CPE-B are underlined. The length of the precursors is noted in parentheses. Position of the C-terminal amino acids of the transit peptides is shown. The cleavage sites are indicated by arrows.

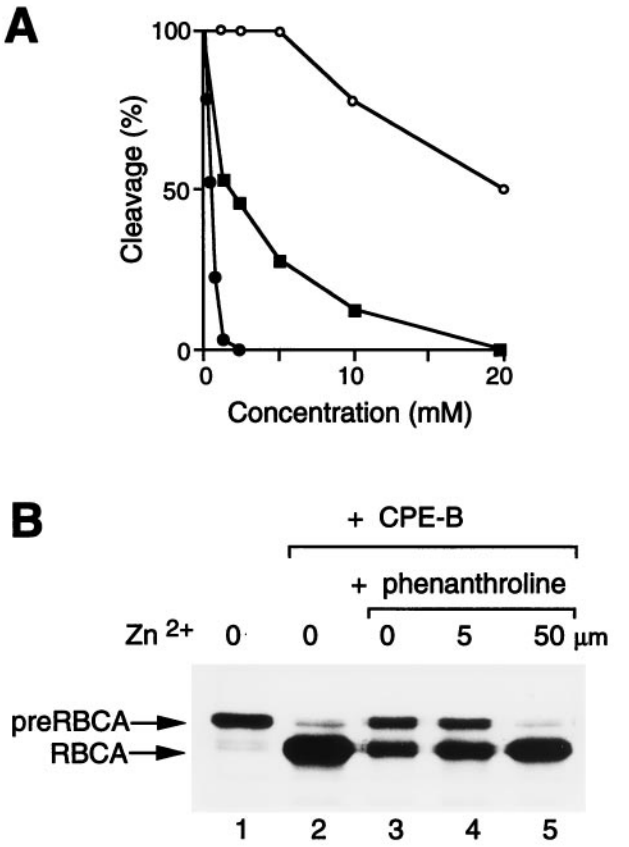

FIG. 4. CPE is a metal-dependent enzyme. (*A*) Percent of cleavage of [^{35}S]methionine-labeled preRBCA in the presence of EDTA (■), EGTA (○), and 1,10-phenanthroline (●). Aliquots of CPE-B extract were preincubated at 28°C for 30 min with each inhibitor and activity was assayed under standard conditions with 1 μl of *in vitro* synthesized preRBCA. Processing products were separated by SDS/PAGE and quantified using PhosphorImager (Molecular Dynamics). Extent of cleavage in absence of inhibitor was taken as 100%. (*B*) Reactivation of immobilized CPE-B by Zn^{2+} in chelating buffer. Untreated [^{35}S]methionine-labeled preRBCA (lane 1), preRBCA incubated with immobilized CPE-B without inhibitor preincubation (lane 2), and after preincubation with 1 mM 1,10-phenanthroline at 0 μM (lane 3), 5 μM (lane 4), and 50 μM (lane 5) $ZnCl_2$.

(29% activity at 5 mM) clearly characterizes CPE as a metallopeptidase. EGTA, another metal chelator with preference for Ca^{2+}, significantly blocked CPE-B only at 20 mM. A serine protease inhibitor, PMSF, had no significant effect (not shown). CPE activity in soluble chloroplast extracts exhibits similar sensitivity to these compounds (3). A metal requirement was directly demonstrated using immobilized CPE-B for processing. The cleavage of preRBCA, which was reduced to 40% in chelating buffer (Fig. 4*B*, lane 3), was restored to 85% at 50 μM Zn2+ (Fig. 4*B*, lane 5).

Our results demonstrate for the first time that one stromal peptidase, CPE, is responsible for the removal of transit peptides from numerous chloroplast precursor proteins synthesized in the cytosol and destined for different compartments and biosynthetic pathways. Both a recombinant form of the enzyme, CPE-B, and the processing activity of chloroplast extracts generated processing products from 11 different precursors that comigrated upon SDS/PAGE, both activities were sensitive to the same mutation of the cleavage site region of preLHCPII, and both were found to depend on metal cations. We show further that CPE indeed acts as an endopeptidase, and significantly, releases the intact transit peptide from preFD by a single proteolytic step. We conclude that CPE is the general SPP, providing a central link between precursor

translocation across the envelope and protein function within the organelle. In our future work, CPE will be referred to as SPP. SPP recognizes an amazing variety of substrates. The recovery of active recombinant SPP will allow us to study the molecular mechanism of the highly specific protein-protein interactions required for precursor recognition and processing, yielding both the mature protein and initially the intact transit peptide.

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