Chloroplast import and sequential maturation of pea carbonic anhydrase: the roles of various parts of the transit peptide

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Abstract Chloroplast pea carbonic anhydrase is synthesised in the cytosol with an unusually long bipartite N-terminal extension of the mature sequence previously proposed to serve as a transit peptide. Studies of import into pea chloroplasts show that the N-terminal 69 amino acids of the previously proposed transit peptide is sufficient for translocation and localisation to the stroma, while the acidic C-terminal part does not seem to have any function in these processes. Processing of the in vitro imported precursors is shown to be at a new cleavage site located in the middle of the actual transit peptide. The results indicate that maturation occurs in more than one step. The time–course does not seem to be dependent on the age of the chloroplast but on the age of the translocated precursor.

Key words: Carbonic anhydrase; Chloroplast; Transit peptide; Processing

1. Introduction

Carbonic anhydrase (CA; carbonate hydrolase, EC 4.2.1.1) is a zinc-containing enzyme catalysing the reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. It is a ubiquitous enzyme found in vertebrates, invertebrates, higher plants, algae and in some bacteria. In C_3 dicotyledonous plants CA is mainly found as a soluble chloroplastic enzyme. It forms oligomers composed of six or eight identical subunits of M_r around 25,000 and with each subunit binding one zinc ion [1,2]. The enzyme is a highly efficient catalyst, approaching diffusion control for the CO_2 hydration reaction [2]. The physiological role of CA in C_3 photosynthesis is still not clearly understood, but an attractive hypothesis is that it provides CO_2 for Rubisco by facilitated diffusion [3]. However, recent work using antisense RNA in transgenic tobacco plants resulting in the inhibition of up to 99% of the CA activity showed no significant effect on CO_2 assimilation [4,5].

CA is synthesised as a precursor in the cytoplasm and transported across the envelope membranes. The majority of the chloroplastic proteins are nuclear encoded and are synthesised with an N-terminal chloroplast transit peptide (cTP) that is removed by a stromal peptidase during or after translocation [6]. According to the cDNA sequence encoding the peaCA precursor and the N-terminal sequence of the mature protein, the N-terminal extension of the protein contains 104 amino acids [7,8] and can be seen as composed of two parts. The first, N-terminal part can be recognised as a cTP according to the characteristics given by von Heijne et al. [9]. However, the C-terminal part, comprising 35 residues, is unusual in being rich in acidic residues and does not seem to fit into a cTP pattern. This region is highly conserved among plant CAs as other known chloroplastic CA precursors contain a similar acidic part (spinach [10], tobacco [11], *Arabidopsis thaliana* [12]). We have previously found peaCA subunits of two sizes in pea leaves [13]. From N-terminal sequencing the small subunit was found to correspond to the mature protein while the large subunit started with the C-terminal part of the proposed cTP. When the entire peaCA precursor was expressed in *Escherichia coli*, partial processing occured. This is seen as several bands on an SDS-polyacrylamide gel with sizes ranging from unprocessed precursor to fully processed subunits. Expression of either the small or the large peaCA subunit in *E. coli* gave homogenous, unprocessed products. Nevertheless, oligomerization and catalytic activity was found to be the same in all three products, regardless of the presence or absence of any part of the N-terminal extension [13].

These results prompted us to investigate the role of the different parts of the N-terminal extension of the peaCA precursor in biogenesis or suborganellar localisation. Using in vitro import we found that translocation into the chloroplast and localisation to stroma is equally efficient whether the acidic C-terminal part of the previously proposed cTP is present or not. Interestingly, an unexpected processing of the cTP is observed in the translocated products. Cleavage is obtained at a novel site within the actual cTP. The results indicate that processing of the peaCA precursor is a time-dependent stepwise event, with the first cleavage occurring at this new site.

2. Materials and methods

cDNA encoding the peaCA precursor was previously isolated and cloned into a mutagenesis/expression vector giving the plasmid pPCAt [13]. The T7 RNA polymerase promoter was placed in front of the peaCA insert. An *Nco*I site was introduced at the initial ATG codon and a unique *Hin*dIII site was placed downstream from the stop codon. This plasmid was used to make deletion constructs by introducing additional *Nco*I sites using site-directed mutagenesis [14] followed by digestion with *Nco*I and re-ligation. Purification of peaCA from the *Escherichia coli* strain BL21(DE3) over-expressing peaCA has been described [13]. For in vitro transcription the plasmids were linearised with *Hin*dIII and then transcribed using T7 RNA polymerase (Epicentre Technologies) in the presence of the cap analogue diguanosine triphosphate (Pharmacia) according to the manufacturers instructions. In vitro translations were performed in a wheat germ extract (Promega)

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Abbreviations: CA, carbonic anhydrase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; cTP, chloroplast transit peptide.

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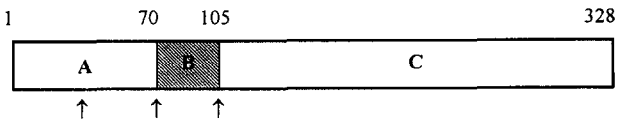
containing 2 μg mRNA and 25 μCi [^{3}H]leucine (Amersham; specific activity 147 Ci/mmol) in a total volume of 100 μl. Reaction mixtures were incubated for 60 min at 27°C. Post-ribosomal supernatants were prepared by centrifugation for 30 min at 100 000 × *g* (4°C) and stored at −70°C. Intact chloroplasts were isolated from 10- to 11-day-old pea seedlings c.v. Feltham First [15,16]. Chlorophyll was assayed according to Bruinsma [16]. Eppendorf cups used for the import experiments were precoated with bovine serum albumin. The import buffer was composed of 50 mM HEPES/KOH, pH 8.0, 330 mM sorbitol, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 200 μg/ml antipain and 2 mM MgATP. Precursors were added last before the chloroplasts (30 μg chlorophyll per 150 μl import reaction). Samples were incubated for 20 min at 26°C in the light. For the time–course study, separate incubations were used for each time point (20, 45, 90 and 180 min). The import was stopped by putting the incubations on ice, and 5 vols. of ice-cold import buffer was added. The chloroplasts were washed, treated with thermolysin and re-isolated as described [16]. Stromal and membrane fractions were obtained by osmotic lysis of protease-treated and re-isolated chloroplasts. The supernatant obtained after centrifugation for 5 min in an Eppendorf centrifuge at full speed was used as the stromal fraction. The pellet was washed in 50 mM HEPES/KOH, pH 8.0, 330 mM sorbitol, pelleted again and used as the membrane fraction containing thylakoid membrane and most of the envelope membranes. Samples were analysed by SDS-PAGE according to Laemmli [18] followed by fluorography. Radioactive bands, corresponding to added precursor and imported processed protein, were excised, rehydrated and prepared for liquid scintillation counting in a 11:9 (v/v) mixture of Lumasolve and Lipoluma (Lumac, Belgium). The import efficiencies were calculated as described by Pilon et al. [19].

For the analysis of pea leaf extracts, plants were grown with a 17 h day/7 h night cycle at 26°C/15°C and harvested after 6–24 days. The tissue was ground with an ice-cold mortar and pestle in 50 mM Tris-SO_4, pH 8.0, 10 mM DTT using 2 ml of medium/g fresh tissue, and then centrifuged at 20,000 × *g* for 10 min at 4°C. The supernatant was analysed by SDS-PAGE and immunoblotting using anti-peaCA antiserum from rabbits and peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad). N-Terminal amino acid sequences were determined by Edman degradation of proteins excised from blotting membrane (PVDF Trans-Blot Transfer Medium, Bio-Rad) in an Applied Biosystems model 477A sequencing system.

3. Results and discussion

To analyse the role of different N-terminal domains of the peaCA precursor in import, processing and localisation, we constructed deletion mutants (Fig. 1). From the plasmid pPCAt, carrying the coding region for the entire peaCA precursor, the deletion constructs pPCAs, l and x were obtained. PeaCA expressed from these plasmids carry different parts of the previously proposed cTP as presented in Fig. 1. PCAs and PCAl correspond to the small and the large subunits, respectively. In PCAx the acidic part B of the precursor, i.e. the first part of PCAl, has been removed.



Protein/(plasmid)	Construction	M_r (protein)
PCAs/(pPCAs)	C	24200
PCAl/(pPCAl)	B+C	28100
PCAx/(pPCAx)	A+C	31500
PCAt/(pPCAt)	A+B+C	35400

Fig. 1. PeaCA constructions. (Top) Schematic representation of the primary structure of the peaCA precursor. A is the N-terminal part and B is the acidic C-terminal part of the previously proposed cTP. C represents fully processed peaCA. Putative cleavage sites are marked with arrows. Amino acid sequence numbers are indicated. (Bottom) Table of the peaCA constructs.

In vitro translation using the plasmids pPCAt, pPCAx and pPCAl yielded radiochemically pure proteins of the expected molecular masses, as determined by SDS-PAGE followed by fluorography (Fig. 2). Upon addition to isolated pea chloroplasts only PCAt and PCAx are processed and found in the chloroplast pellet. From Fig. 2 it is seen that these proteins are protected against protease treatment, indicating that the proteins are translocated and not just associated with the outside of the chloroplasts. The chloroplast subfractionation indicates that the translocated and processed precursors accumulate in the stroma. The membrane fractions contain only a very small amount of the imported products, suggesting that any association to the inner envelope membrane or to the thylakoid membrane must be very weak. Of the added precursors 25% were imported in the 20 min reactions. In contrast, with the translation product PCAl we do not get any radioactively labelled protein associated with the chloroplasts. Hence, this form is not imported and it is not associated to the outside of the chloroplasts. From these results we conclude that the peptide corresponding to part A in Fig. 1 is sufficient for both translocation and localisation. Thus, part B is not necessarily part of the actual transit peptide. It also seems as if part B, which, according to theoretical calculations, has a high tendency to form an α-helix with one side of the helix dominated by hydrophobic

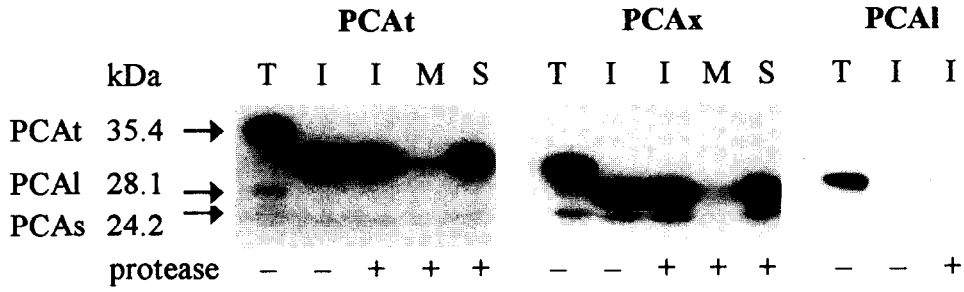


Fig. 2. Import and processing of pre-peaCA deletion constructs. Incubations were for 20 min using in vitro translation products and isolated pea chloroplasts. Samples were analysed by SDS-PAGE and fluorography. T, translation product; I, intact chloroplasts; M, membrane fraction; S, stromal fraction. Protease treatment with thermolysin prior to chloroplast lysis is indicated. Mobilities of purified, unlabelled PCAt, PCAl and PCAs run on the same gel are shown to the left.

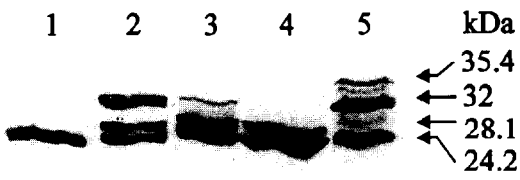


Fig. 3. Western blot analysis of pea leaf extracts. Soluble proteins were separated by SDS-PAGE, transferred to blot filter and probed with anti-peaCA antiserum. Lane 1, purified PCAs; lane 2, 6-day-old plants; lane 3, young leaves from 18-day-old plants; lane 4, mature leaves from 18-day-old plants; lane 5, purified PCAt.

residues and all negative charges on the other side [13], does not mediate stable membrane association.

Both PCAt and PCAx are cleaved to homogenous products. The difference in mobility between the precursor and the imported, processed form is the same for both proteins, indicating that parts of cTP of equal size have been cleaved off. An unexpected result is found when we compare the mobilities of the imported and processed products with the mobilities of purified proteins obtained from *E. coli*. In Fig. 2 it can be seen that PCAt is not processed to a size corresponding to either PCAl or PCAs, but to a larger product with a molecular mass around 32 kDa. Similarly, PCAx, which would be processed to the size of PCAs (24.2 kDa) if the complete cTP had been removed, is found to have a mobility corresponding to a molecular mass of about 28 kDa. To investigate whether this processing pattern is influenced by the import reaction time, a time-course study of the import of PCAt with incubations between 20 min and 180 min was performed (data not shown). These incubation times do not affect the processing and no band corresponding to a smaller size is obtained. The 180 min reaction gives a weak signal, probably because the chloroplast intactness is partly lost during during the long incubation time.

Since our in vitro import detected a novel species, we next investigated this in vivo. Western blot analysis of leaf extracts using anti-peaCA antiserum give different results depending on the age of the leaves. When young plants (6 days old) and the youngest, not fully expanded leaves from older plants were examined, three bands were recognised (Fig. 3, lanes 2 and 3). Purified, *E. coli*-produced PCAt and PCAs run on the same gel show that these bands can be identified as PCAs, PCAl and a 32 kDa protein. It seems reasonable to assume that the 32-kDa band corresponds to the processed product from the import experiment of PCAt since both have the same electrophoretic mobility as compared to the bands seen for purified PCAt. Using mature, old leaves from 11- to 24-day-old plants only two bands are detected (Fig. 3, lane 4), corresponding to PCAs and PCAl. To investigate whether this further processing is dependent only on the age of the chloroplast or if it occurs after the protein has been in the plastid for a longer time, we did an in vitro import experiment using chloroplasts isolated from old leaves from 18-day-old plants. The processing of the translocated PCAt and PCAx was still the same as for the import described in Fig. 2 where young leaves from 10 or 11-day-old plants were used for chloroplast isolation (data not shown). The maturation of the peaCA precursor thus seems to be a time-dependent event occuring in more than one step. In addition, the precise processing is not dependent on the age of the chloroplast but presumably on the age of the translocated precursor, with a time scale of at least several hours.

Cleavage at the first processing site of the peaCA precursor results in a 32 kDa product. This is the same size as one of the main bands observed on an SDS-gel of *E. coli*-produced PCAt. We isolated this band from a PVDF-blotting membrane and the N-terminal amino acid sequence was determined. The sequence obtained was FVSASLNTS, corresponding to residues 29–37 of the cTP. The Ser at position 37 is the first in an unusual stretch of seven serines and the first cleavage of the cTP should be close to this region.

Processing of stromal proteins usually occurs in a single step without any detectable intermediates. In a minority of cases there have been reports about processing intermediates (Rubisco small subunit from pea [20], L18 ribosomal protein from *Chlamydomonas*, [21]). However, the lifetimes of these seemed to be very short and inhibition of the second processing event was necessary for detection of the Rubisco small subunit intermediate. In *Chlamydomonas*, Su and Boschetti [22] have shown the existence of at least two different stromal peptidases with different substrate specificites. One of the peptidases cleaved the precursor of the small subunit of Rubisco to an intermediate form while the other peptidase activity resulted in cleavage to the mature size. The maturation of the peaCA precursor could also involve more than one protease activity, resulting in this novel sequential processing giving a long-lived intermediate form. We also note that Fawcett et al. [10] report that immunoblotting of spinach leaves homogenates gives a smear of CA bands from 30 kDa to 24 kDa.

In summary, we have shown that the first part of the previously proposed bipartite transit peptide (i.e. amino acids 1–69, corresponding to part A in Fig. 1) is sufficient for translocation and localisation of the precursor. Moreover, we have found that the processing of the precursor occurs in more than one step with the first cleavage occuring at a site close to a stretch of seven serine residues in the middle of the cTP. The subsequent maturation step (or steps) takes place as an event dependent on the time passed after translocation. It was previously shown that the inhomogeniety in the size of isolated PCAt did not affect oligomerisation or the specific CO_2 hydration activity as compared to PCAs and peaCA purified from pea leaves [13]. Hence, within the chloroplast the partly processed enzyme subunits can probably oligomerise and also posses full catalytic activity.

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