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The reaction specificities of the thylakoidal processing peptidase and *Escherichia coli* leader peptidase are identical

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Proteins which are transported across the bacterial plasma membrane, endoplasmic reticulum and thylakoid membrane are usually synthesized as larger precursors containing amino-terminal targeting signals. Removal of the signals is carried out by specific, membrane-bound processing peptidases. In this report we show that the reaction specificities of these three peptidases are essentially identical. Precursors of two higher plant thylakoid lumen proteins are efficiently processed by purified *Escherichia coli* leader peptidase. Processing of one precursor, that of the 23 kd photosystem II protein, by both the thylakoidal and *E.coli* enzymes generates the correct mature amino terminus. Similarly, leader (signal) peptides of both eukaryotic and prokaryotic origin are cleaved by partially purified thylakoidal processing peptidase. No evidence of incorrect processing was obtained. Both leader peptidase and thylakoidal peptidase are inhibited by a synthetic leader peptide.

Key words: chloroplast protein transport/precursor proteins/processing/thylakoid lumen proteins

Introduction

Many cytoplasmically-synthesized proteins are synthesized with amino-terminal pre-sequences which direct transport of the pre-protein across a particular membrane system. Protein translocation across the endoplasmic reticulum, bacterial plasma membrane, and the double-membrane envelopes bounding the chloroplast and mitochondrion involves the interaction of the pre-sequences with membrane-bound elements of the translocation machinery. In some cases, cytoplasmic factors are also required for transport (Ellis and Robinson, 1987; Crooke *et al.*, 1988; Verner and Schatz, 1988; Hartl *et al.*, 1989).

Removal of pre-sequences is carried out by essential, specific processing peptidases during or shortly after translocation across one of the above membranes. Precursors entering the chloroplast stroma or mitochondrial matrix are processed by soluble, metal-dependent peptidases (Robinson and Ellis, 1984; Hawlitschek *et al.*, 1988; Musgrove *et al.*, in press) whereas the corresponding enzymes of the bacterial and endoplasmic reticulum systems are membrane-bound. The latter enzymes, termed leader peptidase (LEP) and signal peptidase, have different structures but identical reaction specificities (Muller *et al.*, 1982; Kronenberg *et al.*, 1983; Watts *et al.*, 1983; Cobet *et al.*, 1989).

Chloroplast biogenesis involves the import of numerous proteins across the envelope membranes, but a subset of these proteins is also transported across the thylakoid membrane into the lumenal space. Cytoplasmically-synthesized thylakoid lumen proteins, such as plastocyanin, thus undergo a particularly complex import pathway which involves traversing three membrane systems. This import pathway can be divided into two phases. Initially, pre-plastocyanin is transported into the stroma and processed to an intermediate form by the stromal processing peptidase. The intermediate is then transferred across the thylakoid membrane and processed to the mature size by a second, thylakoidal peptidase (Hageman *et al.*, 1986; Smeekens *et al.*, 1986). Recent evidence indicates that two other lumenal proteins, the 33 kd and 23 kd proteins of the photosynthetic oxygen-evolving complex (OEC), are imported by a similar mechanism (Kirwin *et al.*, 1989; C.Robinson and R.G. Herrmann, in preparation). The pre-sequences of lumenal proteins thus appear to consist of two domains, specifying 'envelope transfer' and then 'thylakoid transfer'.

As with other processing peptidases, the thylakoidal processing peptidase (TPP) is highly specific in that lumenal protein precursors are cleaved, but not 'foreign' proteins. Unlike the stromal enzyme, however, TPP is an integral membrane protein, with the active site on the lumenal face of the thylakoid membrane (Kirwin *et al.*, 1987, 1988). This property is reminiscent of the leader peptidases in the bacterial plasma membrane and the endoplasmic reticulum. In this article we show that the similarities between TPP and leader peptidases go much further, and that all three enzymes have identical reaction specificities.

Results

Structures of leader peptides and thylakoid transfer domains

Leader peptides of pre-proteins which are transported through the endoplasmic reticulum or bacterial plasma membrane exhibit little sequence homology but have certain common features: an amino-terminal basic region, a hydrophobic central core, and a short, more hydrophilic carboxy-terminal region. Residues −1 and −3 (relative to the processing site) have small, uncharged side chains (Von Heijne, 1983, 1985). The thylakoid transfer domains of imported lumenal proteins have similar characteristics. In all cases, short-chain residues are found at positions −1 and −3 (almost always Ala-X-Ala). Hydrophobic central core regions are also apparent, and it is likely that the domains are basic at their amino termini, although the precise amino termini are not yet known (the sites of cleavage by the stromal processing peptidase have not been determined). A detailed comparison of lumenal protein pre-sequences is given in Von Heijne *et al.* (1989). In Figure 1 we have

Spinach 33 kDa protein (Tyagi et al., 1987)

-------KCVDATKLAGLALATSALIASGANA▼

Silene plastocyanin (Smeekens et al., 1985)

---IKASLKDVGVVVAATAAAGILAGNAMA▼

Spinach 23kDa protein (Jansen et al., 1987)

-----GVSRRLALTVLIGAAAVGSKVSPADA▼

Erwinia pre-pectate lyase (Lei et al., 1987)

MKYLLPTAAAGLLLLAANPAMA▼

yeast prepro-alpha factor (Kurjan and Herskowitz, 1982)

MRFPSIFTAVLFAASSALA▼

Fig. 1. Comparison of leader peptides and thylakoid transfer peptides. Final cleavage sites are indicated by ▼. Only the second section of thylakoid lumen protein pre-sequences are shown. Apolar regions are underlined.

compared the thylakoid transfer domains of three higher plant lumenal proteins with the leader peptides of two proteins used in this study: *Erwinia* pectase lyase and yeast pro-alpha factor. These peptides are transported across the bacterial plasma membrane and endoplasmic reticulum, respectively.

Processing of thylakoid lumen protein precursors by TPP and LEP

The similarities between leader and thylakoid transfer peptides prompted us to compare the cleavage specificities of LEP and TPP. TPP was extensively purified from pea thylakoids as described (Kirwin *et al.*, 1987) and LEP was purified to homogeneity from *Escherichia coli* (Zwizinski and Wickner, 1980; Wolfe *et al.*, 1982). In both cases, the isolated enzymes are highly specific in that the natural substrates are processed to the mature sizes but no further, and a variety of 'foreign' proteins are not cleaved.

Figure 2 shows assays for the processing of thylakoid lumen protein precursors, using precursors of the 33 and 23 kd proteins (from wheat) as substrates: each precursor is efficiently processed by both TPP and LEP to the mature size. An intermediate size polypeptide generated in the pre-23 kd translation, probably by stromal processing activity in the wheat-germ lysate, is also processed to the mature size. Pre-23 kd is a particularly good substrate for both enzymes; scintillation counting of the labelled bands in Figure 2 indicates that ~90% of pre-23 kd is converted to the mature size by either enzyme. It is, however, difficult to determine whether LEP cleaves lumenal protein precursors precisely as rapidly as TPP, for two reasons. Firstly, TPP has not yet been purified to homogeneity, and we are therefore unable to add equal molar quantities of TPP and LEP to the processing incubations. Secondly, the two enzymes may well have different requirements for optimal activity *in vitro* (e.g. preferred detergent type and concentration) and the two enzymes may not, therefore, be operating with equal efficiency in these processing incubations. Time course analyses of pre-23 kd processing show that maturation is accomplished apparently in a single step, with no evidence for the generation of incorrect cleavage products by either enzyme (Figure 3).

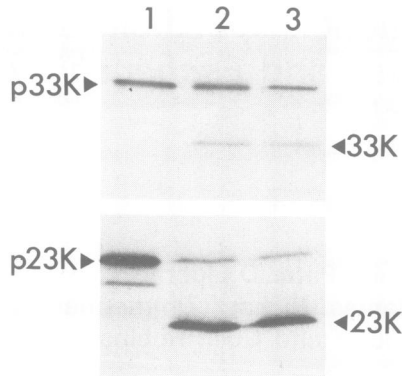

Fig. 2. Processing of thylakoid protein precursors by TPP and LEP. Wheat-germ lysate translation mixtures containing wheat pre-33 kd and pre-23 kd (2 μl) were incubated with 20 μl 20 mM Tris–HCl, pH 7.0, 0.15% Triton X-100 (**lanes 1**) or 20 μl TPP (**lanes 2**) or LEP (**lanes 3**) in the same buffer. Incubation was for 60 min at 27°C. 33 kd, 23 kd, mobility of purified OEC protein markers.

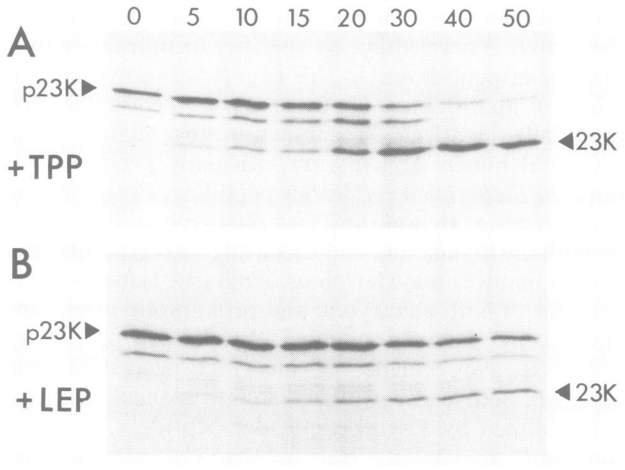

Fig. 3. Time course analyses of wheat pre-23 kd processing by TPP and LEP. Pre-23 kd (10 μl) was incubated with 200 μl TPP (**A**) or LEP (**B**) at 27°C as in Figure 2, except that the peptidase preparations had been diluted 3-fold with 20 mM Tris–HCl, pH 7.0, 0.15% Triton X-100. 20 μl aliquots were removed at times (in min) indicated above the lanes. Symbols as in Figure 2.

TPP and LEP process pre-23 kd at the correct site

The cleavage fidelities of TPP and LEP were analysed more rigorously by determining the site at which each enzyme cleaves pre-23 kd. The precursor was synthesized in the presence of [^{3}H]lysine, incubated with each peptidase, and the processed products were subjected to sequential Edman degradation. Figure 4 shows that the peaks of [^{3}H]lysine appear in cycles 11, 13, and 14, coinciding precisely with the positions of lysine residues in the authentic, purified mature protein. We conclude that both peptidases process pre-23 kd at the correct site. This level of cleavage accuracy suggests that the reaction mechanisms of TPP and LEP are very similar, but it would be premature to suggest that they are identical. Subtle differences in the structures of the peptidase active sites, or in the structure of leader/thylakoid transfer signals, may cause the enzymes to cleave certain heterologous substrates at incorrect sites. We have no evidence that this occurs, but it will be of interest to carry out further tests on the cleavage fidelities of TPP and LEP.

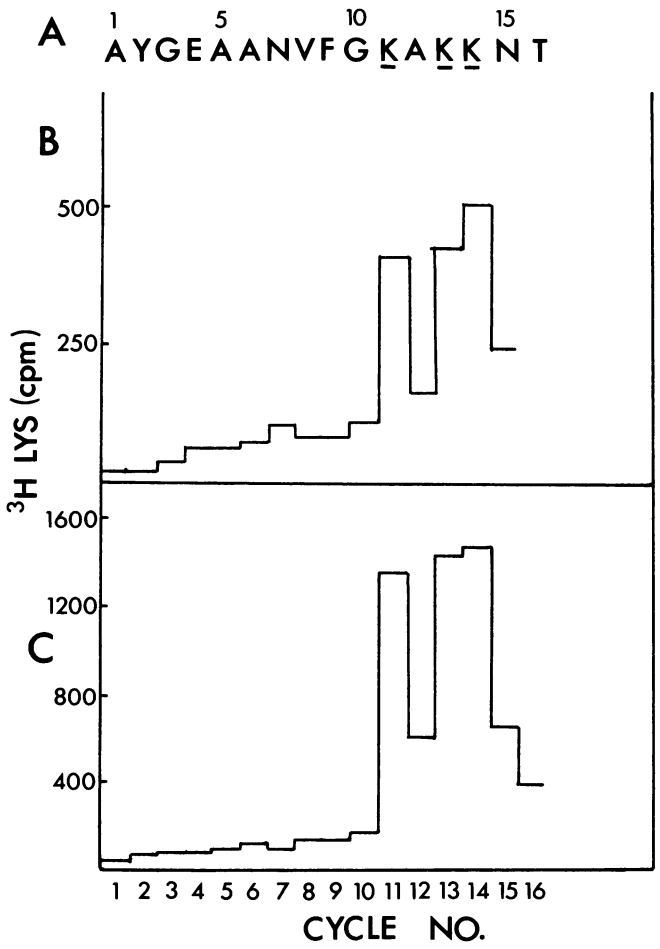

Fig. 4. TPP and LEP process pre-23 kd at the correct site. (**A**) Amino-terminal sequence of purified wheat 23 kd protein, determined as detailed in Materials and methods. (**B**, **C**) Radiosequencing analysis of processed 23 kd after incubation of [^{3}H]lysine labelled pre-23 kd with TPP and LEP, respectively. Fractions generated by each cycle of Edman degradation were counted for ^{3}H radioactivity. The samples subjected to Edman analysis contained 14 600 c.p.m. (**B**) or 38 000 c.p.m. (**C**) [^{3}H]lysine.

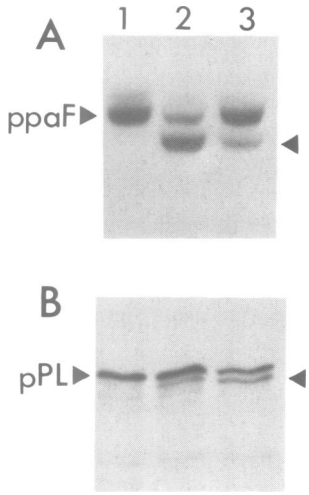

Fig. 5. Processing of leader (signal) peptides by TPP and LEP. (**A**) Wheat germ translations of prepro-alpha factor mRNA were carried out in the presence of 0.15% Triton X-100 (**lane 1**) or TPP (**lane 2**) or LEP (**lane 3**) in Triton X-100. 8 μl translation mixtures contained 2 μl processing peptidase. (**B**) *Erwinia* pre-pectate lyase construct mRNA (see text) was translated in a wheat germ lysate. 2 μl translation mix was incubated for 60 min at 27°C with 20 μl 20 mM Tris−HCl, pH 7.0, 0.15% Triton X-100 (**lane 1**) or TPP (**lane 2**) or LEP (**lane 3**) in the same buffer. PpaF, pPL, prepro-alpha factor, pre-pectate lyase construct translation products. Arrows denote processed polypeptides.

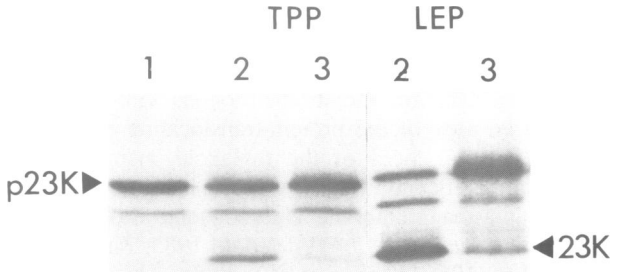

Fig. 6. Inhibition of TPP and LEP by a synthetic signal peptide. Pre-23 kd (**lane 1**) was incubated with TPP or LEP (**lanes 2**) as in Figure 2. A 19-residue synthetic signal peptide (see text) was included at 0.5 mM (**lanes 3**). Symbols as in Figure 2.

Cleavage of eukaryotic and bacterial leader peptides by LEP and TPP

Comparisons of the reaction specificities of TPP and LEP were extended by assaying both enzymes for the cleavage of leader peptides which direct the transport of proteins through the endoplasmic reticulum or the bacterial plasma membrane. Figure 5 shows that both peptidases efficiently process yeast prepro-alpha factor and a construct consisting of the leader peptide of *Erwinia carotovora* pectate lyase fused to an IgG light-chain domain. Removal of the *Erwinia* leader peptide can be carried out post-translationally, whereas processing of prepro-alpha factor is only observed if the peptidases are present during translation. It is possible that prepro-alpha factor misfolds after translation in the wheat-germ system, such that it is no longer efficiently recognized by the peptidases.

It should be pointed out that not all lumenal protein precursors or leader peptides can be processed by the isolated enzymes. For example, the precursor of the 16 kd OEC protein (a thylakoid lumen protein) and preproricin (transported across the endoplasmic reticulum) are not processed by either enzyme, presumably because they assume an unfavourable conformation during translation. However, all of the lumenal protein precursors/leader peptide precursors tested to date are processed either by *both* TPP and LEP, or by neither. The reaction specificities of the two enzymes appear, therefore, to be identical.

A meaningful comparison of the structures of LEP and TPP is presently impossible because TPP has not yet been purified to homogeneity. However, preliminary findings suggest that the structures may be dissimilar. Antisera raised against LEP do not cross-react with TPP, as judged by Western blotting. Furthermore, two of the steps in the LEP purification protocol (Zwizinski and Wickner, 1980), namely ethanol precipitation and non-denaturing gel electrophoresis, completely inactivate the thylakoidal enzyme (not shown).

Neither peptidase is inhibited by any of the standard protease inhibitors tested to date, thereby precluding the assignment of either enzyme to a particular class of protease (Zwizinski and Wickner, 1980; Kirwin *et al.*, 1987). However, LEP has been shown to be inhibited by a synthetic procoat leader peptide (Wickner *et al.*, 1987). In order to

further probe the similarities between TPP and LEP, we tested the effects on both enzymes of another synthetic leader peptide, a 19mer based on the consensus leader peptide sequence described by Austen *et al.* (1984). Figure 6 shows that this peptide effectively inhibits processing of pre-23 kd by both TPP and LEP, reinforcing the similarities in reaction specificity between the peptidases. As a control, we tested the effects of a 10mer of similar overall chemical composition (ILAGNAMAAE). This peptide did not inhibit either TPP or LEP at concentrations up to 1 mM (not shown) suggesting that the consensus signal peptide is a specific competitive inhibitor.

Discussion

In this report we have assessed the similarities between the thylakoidal processing peptidase and the processing peptidases in the endoplasmic reticulum and the bacterial plasma membrane. We have shown that partially purified TPP activity cleaves leader (signal) peptides from both prokaryotic and eukaryotic sources. Similarly, purified *E.coli* LEP processes precursors of two higher plant thylakoid lumen proteins to the mature size. We conclude that the reaction specificities of TPP and LEP are identical. Since LEP and eukaryotic signal peptidase also have identical reaction specificities (Muller *et al.*, 1982; Kronenberg *et al.*, 1983; Watts *et al.*, 1983; Cobet *et al.*, 1989) it is extremely likely that the shared cleavage specificity extends to all three peptidases.

The precise locations of the three peptidases within their respective membranes are also similar. In each case, the peptidases are integral membrane proteins with active sites on the *trans* side of the protein-translocating membrane, i.e. the lumenal faces of the thylakoid membrane and endoplasmic reticulum, and the periplasmic face of the bacterial plasma membrane (Jackson and Blobel, 1977; Zimmermann *et al.*, 1982; Wolfe *et al.*, 1983; Zimmermann and Mollay, 1986; Kirwin *et al.*, 1988).

One of the most interesting aspects to emerge from this, and previous work, is that the peptidases have conserved reaction specificities but markedly different structures. *E. coli* LEP consists of a single polypeptide of M_r 39 000 (Zwizinski and Wickner, 1980) whereas signal peptidase from both higher and lower eukaryotes is a complex of at least two subunits (Evans *et al.*, 1986; Baker and Lively, 1987; Bohni *et al.*, 1988; Shelness *et al.*, 1988). It will be of interest to determine the structure of TPP; preliminary evidence already suggests that some properties of the enzyme differ from those of *E.coli* LEP.

The similarities in the TPP and LEP reaction mechanisms may be a consequence of the evolution of the chloroplast. It is widely accepted that chloroplasts arose from endosymbiotic cyanobacteria, many of which contain internal thylakoid membranes. Lumenal proteins in several species of cyanobacteria are synthesized with pre-sequences which resemble leader (signal) sequences and thylakoid transfer domains of corresponding imported chloroplast proteins (Kuwabara *et al.*, 1987; Wallace *et al.*, 1989). The enzyme responsible for the maturation of these precursors is presumably related to TPP and LEP, at least in terms of reaction specificity. However, it is also likely that the cyanobacterial plasma membrane contains LEP activity; if so, it remains to be determined whether a single enzyme is involved in the maturation of proteins destined for the plasma membrane, periplasmic space and thylakoid lumen. Very little is currently known about the mechanisms by which these proteins are transported and segregated in cyanobacteria.

Given the highly conserved reaction specificities of TPP and LEP, it is clear that the processing signals within leader and thylakoid transfer peptides are essentially identical. This finding raises the interesting possibility that the translocation signals are also conserved, and that the two types of peptide are functionally interchangeable. Work is in progress to determine whether leader peptides can indeed direct proteins across the thylakoid membrane, and the recent demonstration of protein import by isolated thylakoids (Kirwin *et al.*, 1989) should facilitate a detailed examination of the thylakoidal protein transport system, about which little is currently known. It will be of great interest, for example, to determine whether the stroma contains functional equivalents of trigger factor, or secB, factors which are involved in bacterial protein transport.

Materials and methods

Materials

Radiochemicals were purchased from Amersham International (UK) and the 19mer peptide was synthesized by Ronald Merk, Genzentrum Munchen.

Processing assays

Precursor proteins were synthesized by *in vitro* SP6 RNA polymerase transcription of cloned cDNAs, followed by translation of capped transcripts in a wheat-germ translation system in the presence of [^{35}S]methionine (Anderson *et al.*, 1983; Melton *et al.*, 1984). cDNAs encoding yeast prepro-alpha factor, *Erwinia* pre-pectate lyase were kindly provided by Drs David Meyer (UCLA) and Robert Spooner (Warwick) respectively.

A full-length cDNA encoding the precursor of the wheat 33 kd OEC protein was isolated as described (Kirwin *et al.*, in press), and a 1.1 kbp cDNA insert encoding the entire pre-23 kd protein was isolated from a wheat λgt11 expression library. Nucleotide sequencing data (in preparation) have confirmed that the pre-sequences of both proteins are similar to those of the corresponding spinach proteins (Jansen *et al.*, 1987; Tyagi *et al.*, 1987). *In vitro*-synthesized precursors were incubated with processing peptidases as described in the figure legends. Purified *E.coli* leader peptidase (Zwizinski and Wickner, 1980) was used at a concentration of 100 $\mu g/ml^{-1}$, and pea TPP was used at a concentration corresponding to 1 U of activity per 10 μl enzyme preparation activity having been determined using pre-plastocyanin as a substrate (Kirwin *et al.*, 1987). After incubation, samples were analysed by SDS–polyacrylamide gel electrophoresis followed by fluorography.

Protein sequencing

Amino-terminal sequencing of wheat 23 kd protein was carried out by resolving purified protein on an SDS–polyacrylamide gel according to the conditions described in Applied Biosystems User Bulletin no. 25. The gel was then blotted onto Immobilon membrane (Millipore, UK). The protein band was excised and placed in the cartridge block of an Applied Biosystems model 470 a protein sequencer equipped with a 120a on-line PTH analyser, using the standard O3R PTH program. For radiosequencing, processed translation mix (60 μl) was resolved on the gel, and fractions from each cycle were counted for ^{3}H radioactivity.

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