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Cleavage of the precursor of pea chloroplast cytochrome *f* by leader peptidase from *Escherichia coli*

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Leader peptidase from *Escherichia coli* was able to process the precursor of pea cytochrome *f* synthesised in vitro. N-Terminal sequencing established that cleavage by leader peptidase generated the same mature sequence as in pea chloroplasts. Processing by leader peptidase was much more efficient co-translationally rather than post-translationally, and the extent of post-translational processing declined with time suggesting that the cytochrome *f* precursor folded to an uncleavable conformation. Detergent extracts of pea thylakoid membranes were unable to process the cytochrome *f* precursor co- or post-translationally.

Cytochrome *f*; Leader peptidase; Thylakoidal processing peptidase; Co-translational processing; Post-translational processing

1. INTRODUCTION

Most chloroplast-encoded integral membrane proteins of the thylakoid membrane are synthesised without a cleavable presequence [1]. Only four proteins, cytochrome *f*, CF_0 subunits I and IV of ATP synthase, and the K protein of photosystem II, are currently recognised as being synthesised with cleavable N-terminal presequences [2–5] and, of these, the synthesis of only cytochrome *f* has been studied in detail. Cytochrome *f* is synthesised on thylakoid-bound ribosomes [6,7] and assembled into the cytochrome *bf* complex in isolated pea chloroplasts [8]. Comparison of the sequence of mature cytochrome *f* with that predicted from the cytochrome *f* gene (*pet*A) indicates that the protein is synthesised with an N-terminal presequence of 35 amino acid residues [2,9,10]. The cleavage site for removal of the presequence conformed to the consensus for signal sequence cleavage sites recognised by von Heijne [11,12]. Gene-fusions of the 5′ region of the pea *pet*A gene with *lac*Z indicated that the N-terminal presequence acted as a signal sequence to direct β-galactosidase to the plasma membrane of *Escherichia coli* in a *sec*A-dependent manner [13] and it is assumed that in chloroplasts the presequence similarly directs cytochrome *f* to the thylakoid membrane.

The functional similarity between the cytochrome *f* presequence and bacterial signal sequences suggests that presequence cleavage may also be similar in chloroplasts and bacteria. Kirwin et al. [14] have identified a processing activity in thylakoid membranes which removes the thylakoid targeting domain from plastocyanin, a nuclear-encoded lumenal protein. The purified thylakoid processing peptidase also removes the thylakoid targeting domains of the 33 kDa and 23 kDa polypeptides of the oxygen-evolving complex [15,16]. The reaction specificity of the thylakoid processing peptidase has recently been shown to be identical to that of *E. coli* leader peptidase [17]. This suggests that the thylakoid processing peptidase acting on nuclear-encoded proteins may also be responsible for removing the presequence from the cytochrome *f* precursor. The aim of the present study was to investigate the processing of the cytochrome *f* precursor by bacterial leader peptidase and by thylakoid extracts.

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2. MATERIALS AND METHODS

2.1. *Preparation of radiolabelled precursor protein*

A vector for the expression of the pea preapocytochrome *f* gene (*pet*A) was constructed from pT7CF-35, a plasmid containing a 1.38 kbp *Rsa*I-*Xba*I fragment of pea chloroplast DNA [2] inserted in the *Sma*I and *Xba*I sites of pGEM-3 (Promega). This plasmid was cut with *Asp*718I, treated with S1 nuclease and then cut with *Hin*dIII to give a 1.38 kbp fragment encoding the entire preapocytochrome *f* polypeptide. This fragment was inserted into the prokaryote expression vector pKK223-3 [18] which had been cut with *Eco*RI, end-filled and then cut with *Hin*dIII. This produced a construct with a *tac* promoter and ribosome-binding site positioned just upstream from the coding region of the pea *pet*A gene. Radiolabelled cytochrome *f* precursor was produced from this construct in vitro using a coupled transcription-translation system prepared from *E. coli* strain PR7 [19,20]. Coupled transcription/translation was carried out at 37°C in a total volume of 5–30 μl with 0.3–2 μg plasmid DNA and 3–15 μCi L-[^{35}S]methionine (1200 Ci · mmol^{-1}), as described by Collins [21]. Immunoprecipitation of the products of the coupled transcrip-

tion/translation system with antibodies to charlock cytochrome *f* [6,22] and protein A-Sepharose was carried out as described by Howe et al. [20].

2.2. *Processing of precursor protein*

Processing assays were carried out both co- and post-translationally. Co-translational processing was studied by including 1 μl of *E. coli* leader peptidase (100 μg protein · ml^{-1}) and 1 μl of 0.6% (v/v) Triton X-100 in a 5 μl transcription/translation assay. Incubations were carried out for 1 h at 37°C. Post-translational processing was carried out by incubating 2 μl of the products of transcription/translation in vitro with 1 μl of leader peptidase and 2 μl of 0.3% (v/v) Triton X-100 in 20 mM Tris-Cl, pH 7.0, in a final volume of 5 μl. Processing was allowed to proceed for 1 h at 37°C. Reactions were stopped by addition of 5 μl of electrophoresis sample buffer consisting of 80 mM Tris-Cl, pH 6.8, 10% (v/v) glycerol, 10% (w/v) sodium dodecylsulphate (SDS), 0.002% (w/v) Bromophenol blue, 5% (v/v) 2-mercaptoethanol (added just before use) and boiling for 2 min. Protein products were separated by electrophoresis on 12.5% polyacrylamide gels in the presence of SDS [23] and detected by fluorography using Amplify (Amersham). Thylakoid extracts were prepared from pea chloroplasts as described by Kirwin et al. [14,15]. Purified thylakoid processing peptidase and *E. coli* leader peptidase were gifts from Dr C. Robinson, University of Warwick.

2.3. *Protein sequencing*

Protein sequencing was carried out on the precursor protein produced by coupled transcription/translation in a 30 μl incubation with 60 μCi L-[^{35}S]methionine (1200 Ci · mmol^{-1}), 100 μCi L-[2,3,5,6-^{3}H]tyrosine (100 Ci · mmol^{-1}) and 100 μCi L-[2,3,4,5,6-^{3}H]phenylalanine (130 Ci · mmol^{-1}), and on the processed protein produced in a similar co-translational processing assay with 1 μl leader peptidase (100 μg protein · ml^{-1}) for 90 min at 37°C. Products (30 μl) were separated by electrophoresis on 12.5% polyacrylamide gels in the presence of SDS [23] with 2 mM thioglycollic acid included in the upper reservoir buffer to scavenge N-blocking free radicals. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane [24] by electroblotting in a buffer containing 25 mM Tris, 190 mM glycine and 10% (v/v) methanol for 3 h at 300 mA. After washing in water and air-drying the membrane was autoradiographed to localise the cytochrome *f* polypeptides. Sequencing was carried out on an Applied Biosystems 470A gas-phase sequencer as described previously [25]. Samples from each cycle were assayed for radioactivity in a scintillation counter.

3. RESULTS

Two major products of 41 kDa and 31 kDa were obtained when the expression vector pKK223-3 containing the pea cytochrome *f* gene was transcribed and translated in an *E. coli* S-30 extract (Fig. 1). The 41 kDa polypeptide is the correct size for the precursor of cytochrome *f* and was immunoprecipitated with antibodies to charlock cytochrome *f* (data not shown). The 31 kDa polypeptide is the correct size for the β-lactamase precursor which is encoded within pKK223-3. The addition of *E. coli* leader peptidase and 0.1% Triton X-100 during coupled transcription/translation resulted in the processing of both the cytochrome *f* and the β-lactamase precursors (Fig. 1). The cytochrome *f* precursor was processed to a 37 kDa form which corresponds to the mature protein [2,6], and the β-lactamase precursor was processed to a 29 kDa form. The cytochrome *f* precursor appeared to be a better substrate for leader peptidase than the β-lactamase

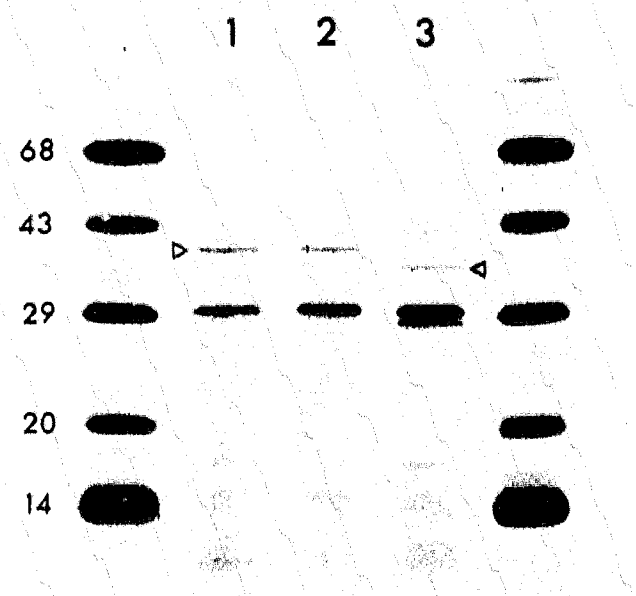

Fig. 1. Co-translational processing of the precursors of cytochrome *f* and β-lactamase by *E. coli* leader peptidase. Lane 1 shows the [^{35}S]methionine-labelled products of coupled transcription/translation of the pea *petA* gene in pKK223-3. Lane 2 contains the products of transcription/translation carried out in the presence of 0.12% Triton X-100. Lane 3 shows the result of including leader peptidase and 0.12% Triton X-100 during transcription/translation. Reactions were carried out for 1 h at 37°C. ^{14}C-labelled molecular mass standards were bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and cytochrome *c* (12.5 kDa). Precursor and processed forms of cytochrome *f* are marked with arrowheads.

precursor as indicated by the almost complete processing of the former and about 50% processing of the latter. The addition of Triton X-100 alone had no effect on the sizes of the products of the transcription/translation system. Small amounts of the mature-sized products were present even in the absence of added leader peptidase, most probably due to the presence of endogenous leader peptidase in the *E. coli* S-30 extract. Centrifugation of the S-30 extract at 135 000 × *g* for 5 min, to deplete the extract of membranes but not ribosomes, resulted in decreased amounts of the mature-sized products in incubations without added leader peptidase (data not shown).

The site of cleavage of the cytochrome *f* precursor by leader peptidase was determined by N-terminal sequencing of radioactively labelled precursor and product. The precursor protein was synthesised in the coupled transcription/translation system in the presence of [^{35}S]methionine, [^{3}H]tyrosine and [^{3}H]phenylalanine to provide suitably positioned amino acids for sequencing, and was then incubated with leader peptidase. The precursor and product were separated by electrophoresis, blotted on to PVDF membrane and se-

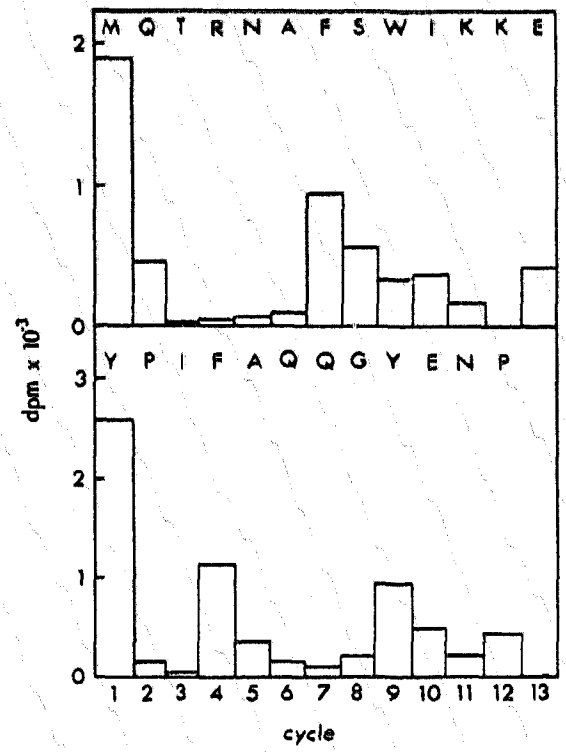

Fig. 2. N-Terminal sequencing of the precursor and processed forms of cytochrome *f* synthesised in the presence of [^{35}S]methionine, [^{3}H]tyrosine and [^{3}H]phenylalanine in the coupled transcription/translation system. The radioactivity in the fractions generated by Edman degradation was determined by scintillation counting. The upper panel shows the results obtained with the precursor, the lower panel shows the results obtained with the processed product. The expected sequence of the polypeptides is shown in the single-letter code.

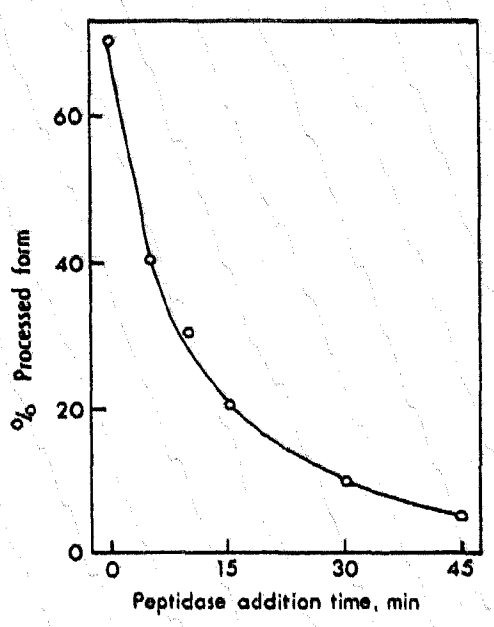

Fig. 3. Post-translational processing of the cytochrome *f* precursor by *E. coli* leader peptidase. Transcription/translation was allowed to proceed for 15 min at 37°C and stopped by transferring the sample to ice. At the various times indicated after the termination of the reaction, samples (2 μl) were added to tubes containing 1 μl of leader peptidase and 2 μl of 0.3% Trixon X-100 and incubated at 37°C for 1 h. Reactions were stopped by boiling the samples in 5 μl of SDS-sample buffer for 2 min, the products were separated by electrophoresis and the labelled polypeptides detected by fluorography. The extent of processing was determined by scanning densitometry with a Molecular Dynamics 300A scanning densitometer.

quenced (Fig. 2). Radioactive amino acids were detected at cycles 1 (^{35}S) and 7 (^{3}H) on sequencing the precursor, indicating that translation had initiated at the first of two in-frame ATG codons to produce the full presequence of 35 amino acid residues. On sequencing the processed form of cytochrome *f*, [^{3}H]amino acids were detected at cycles 1, 4 and 9, corresponding exactly to the positions of the tyrosine and phenylalanine residues. This clearly indicates that leader peptidase cleaved the cytochrome *f* precursor to give the same N-terminal sequence found in the mature isolated from pea chloroplasts [2].

Attempts to carry out post-translational processing with leader peptidase on preparations of the cytochrome *f* precursor which had been stored at −20°C, −80°C or −140°C were unsuccessful. However, post-translational processing was possible provided that the time which elapsed beween the completion of protein synthesis and the addition of leader peptidase did not exceed approximately 45 min (Fig. 3). The most efficient post-translational processing was obtained when leader peptidase was added immediately after the completion of protein synthesis, although the extent of processing was less than that obtained with co-translational addition of leader peptidase. Decreased processing was observed with increased periods of time before leader peptidase addition. After 45 min there was only a very low level of processing observed. This suggests that soon after synthesis the cytochrome *f* precursor adopts a conformation which is not a substrate for leader peptidase. Attempts to detect cleavage of the cytochrome *f* precursor, co- or post-translationally, with extracts of pea thylakoid membranes were unsuccessful. Preparations of the thylakoidal processing peptidase which were active in processing the precursors of nuclear-encoded lumenal proteins [14–17] were unable to process the cytochrome *f* precursor (data not shown).

4. DISCUSSION

Purified leader peptidase from *E. coli* was capable of processing the precursors of cytochrome *f* and β-lactamase co-translationally in vitro. N-Terminal amino acid sequencing of the processed cytochrome *f* product indicated that the bacterial enzyme cleaved the precursor at an alanine-tyrosine bond which is the site at which processing occurs in vivo [2]. Leader peptidase

has also been shown to be capable of processing the precursors of two thylakoid lumen proteins, the 33 kDa and 23 kDa polypeptides of the oxygen-evolving system of photosystem II, to mature size [16,17]. Both of these proteins are nuclear-encoded and are synthesised with presequences consisting of two domains, a chloroplast-import domain which is removed by a peptidase located in the stroma and a thylakoid-transfer domain which is removed by a peptidase located in the thylakoid membrane [16,26]. Correct cleavage of the 23 kDa polypeptide precursor by *E. coli* leader peptidase was confirmed by N-terminal sequencing of the processed protein [17]. It was also demonstrated that in vitro leader peptidase processed several precursor proteins including those for the 33 kDa and 23 kDa polypeptides both co- and post-translationally with equal efficiencies. In contrast, post-translational processing of cytochrome *f* precursor was less efficient than co-translational processing and, furthermore, post-translational processing of the cytochrome *f* precursor was possibile only with freshly prepared protein. Protein which was more than about 45 min old when leader peptidase was added was processed only very poorly, if at all. These results suggest that very soon after synthesis the cytochrome *f* precursor adopts a conformation which is inaccessible to leader peptidase. Halpin et al. [17] obtained similar results with yeast pre-α factor. Leader peptidase was able to process pre-α factor co-translationally, but not post-translationally.

Halpin et al. [17] have recently shown that the reaction specificities of leader peptidase and a partially purified thylakoid protease are identical. However, this thylakoidal processing peptidase was unable to process the cytochrome *f* precursor either co- or post-translationally. This may suggest that a different protease is involved in processing the cytochrome *f* precursor, although such an activity could not be detected in detergent extracts of pea thylakoid membranes. However, it is possible that the thylakoid extracts were not active enough to allow efficient processing of a cytochrome *f* precursor which rapidly folded to a conformation inaccessible to processing. Wallace et al. [27] have recently shown that the reaction specificities of pea and cyanobacterial thylakoid processing peptidases are similar but not identical, and have raised the possibility that two peptidases are required for the processing of the precursor of the 9 kDa lumenal polypeptide. Howe and Wallace [28] constructed a weight matrix which accurately predicted the cleavage sites of several prokaryotic and eukaryotic thylakoid lumen proteins. However, the matrix was much less successful at predicting the processing sites for a number of chloroplast-encoded intrinsic thylakoid proteins including cytochrome *f*, and the existence of more than one thylakoidal processing peptidase was proposed to explain the results. The nature of the peptidase responsible for processing the cytochrome *f* precursor in thylakoid membranes requires further study.

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