A chloroplast envelope-transfer sequence functions as an export signal in *Escherichia coli*

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Abstract The small subunit precursor of pea ribulose-1,5-bisphosphate carboxylase/oxygenase engineered with prokaryotic elements was expressed in *Escherichia coli*. This resulted in a dependable level of synthesis of the precursor protein in *E. coli*. The bacterially synthesised plant precursor protein was translocated from the cytoplasm and targeted to the outer membrane of the envelope zone. During the translocation step, a significant proportion of the precursor was processed to a soluble, mature SSU and found localised in the periplasm. The determined amino acid sequence of the isolated precursor showed that it had a deletion of an arginine residue at position −15 in the transit peptide. Expression of this transit peptide-appended mammalian cytochrome b_5 in *E. coli* displayed a targeting profile of the chromogenic chimera that was similar to that observed with the plant precursor protein.

Key words: Chloroplast signal; Protein translocation/targeting/export; *Escherichia coli*; Recombinant protein production

1. Introduction

Chloroplast biogenesis is dependent upon co-ordinated activities of two independent genetic systems localised in the chloroplast and the nucleus [1]. The vast constituent chloroplast proteins are encoded by the nuclear genes and are cytoplasmically synthesised as precursor forms which contain N-terminal extensions known as the transit peptides. The transit peptide is instrumental for specific recognition of the chloroplast surface and in mediating the post-translational translocation of preproteins across the chloroplast envelope. The import of such precursor proteins into the chloroplast is mediated by a complex process involving components which facilitate various steps of the import such as unfolding, binding to surface receptors, translocation across the envelope membrane and maturation [2,3]. In linkage with an envelope-transfer sequence, the transit peptides of proteins destined for thylakoid lumen contain an ensuing prokaryotic-like intra-organellar sorting domain [4]. Indeed, transfer of thylakoid lumen proteins across thylakoid membrane displays azide-sensitivity akin to the *sec*-dependent pathway of bacteria [5]. In vitro synthesised thylakoid-destined precursors are also processed by *Escherichia coli* signal peptidase [6]. The presence of counterpart chaperoning factors such as GroEL, DnaK, SecA and SecY in the plastids reflect endosymbiotic relatedness between bacterial and plastid transport systems [7]. The thylakoidal transfer signal has been demonstrated to be structurally and functionally similar to the signal sequences that can direct protein translocation across the endoplasmic reticulum and the bacterial cytoplasmic membrane [8].

In contrast, the chloroplast envelope transfer signals are distinctly hydrophilic, basic and enriched in hydroxylated amino acid residues and serve as stromal-targeting sequences. Such a transit peptide in the precursor of the small subunit (PrSSU) of ribulose-1,5-bisphosphate carboxylase (RUBISCO) has been extensively used as a model to understand the details of protein transport into the stroma of isolated chloroplasts. In all higher photosynthetic organisms, the SSU is synthesised as a precursor carrying about 55 to 60 amino acid residues-long 'envelope-transfer' transit peptide that is cleaved off either during translocation or immediately after segregation of the polypeptide into the stroma. The PrSSU protein has been expressed in *E. coli* in order to obtain substrate quantities of the precursor product for more definitive transport and structural studies [9–13]. An opportunity to understand the fate and the sub-cellular localisation of the higher plant preprotein in the host bacterium has been hampered by their accumulation in the form of insoluble inclusion bodies.

Here we report on the heterologous synthesis of a pea (*Pisum sativum*) RUBISCO PrSSU that carries a deletion of an arginine residue at position 15. We show that this transit peptide is functional in eventual targeting of the unprocessed native precursor as well as an appended mammalian haemoprotein passenger to the OM of *E. coli*. Moreover, during transit to the OM, a significant amount of the precursor is processed to the mature forms and found localised in the periplasm.

2. Materials and methods

The molecular biological techniques were followed essentially as described [14]. Dr. S. Smith (Edinburgh University, UK) kindly supplied the pea PrSSU cDNA [15]. For expression of PrSSU, controlled through the tightly-regulated λP_L promoter, *E. coli* N4830-1 cultures, pre-grown in Luria broth (ampicillin 75 μg/ml) at 30°C to an OD = 0.6 units, were thermoinduced at 39 ± 1°C for durations specified elsewhere. *E. coli* subcellular fractionations were performed as described previously [16]. Proteins were analysed by SDS electrophoresis using a 12–18% gradient of polyacrylamide gels [17] with sample loadings

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Abbreviations: IM, inner membrane(s); OM, outer membrane(s); RUBISCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; PrSSU, precursor of small subunit of RUBISCO (Arg^{-15} residue deleted); SSU, mature small subunit of RUBISCO

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ranging from 50 to 100 μg per lane. For Western blots, electrophoresed proteins were transferred from an unstained gel onto nitrocellulose sheets and the rabbit anti-pea SSU reactive components detected by activity staining with horse-radish peroxidase conjugated to goat anti-rabbit IgG [9]. The membrane-associated PrSSU, isolated by electroelution, was microsequenced by Edman degradation (Alta Laboratories, Birmingham University, UK). The periplasmic anti-SSU reactive 14-kDa protein was isolated by electroelution following filtration of a ten-fold concentrated periplasmic fraction through an Amicon 30 filter unit. For immunoelectron microscopy, prefixed ultrathin sections of *E. coli* were treated with 0.5% bovine serum albumin, 0.2% gelatin in phosphate-buffered saline to block non-specific binding. After incubation with affinity-purified anti-SSU antibodies (1:500) the sections were extensively washed and labelled with 10 nm protein A-coupled colloidal gold particles, essentially as recommended by Biocell conjugates (UK). After post fixation in osmium tetroxide, the sections were stained with 2% uranyl acetate and lead citrate [18] and examined using a Jeol JEM-100 CX transmission electron microscope at 100 kV.

3. Results and discussion

The 5′-proximally-modified PrSSU cDNA, containing an optimised ribosomal binding site and a choice of codons ideal for expression in *E. coli* was placed under the control of the thermoinducible λP_L promoter in the derivative plasmid pYPS (Fig. 1). The tandemly co-expressed cytochrome b_5 gene, placed downstream of *prSSU*, aided identification and isolation of the clone that expressed PrSSU through the pink reporter system [19]. Thermoinduction of *E. coli* pYPS directed the synthesis of two proteins of 20 and 12 kDa (Fig. 2). The latter, identified as the co-expressed cytochrome b_5 (see below), constituted approximately 9% of the total cellular protein (Fig. 2A, cf. lanes T+ and T−). Whereas, the former appeared to represent a significantly smaller amount ($\sim$1%) of the total protein; its detection by Coomassie blue staining proved possible only when the total cellular fraction was separated on a (15–18%) gradient polyacrylamide gel that provided a higher resolving capability (Fig. 2C). The total cellular polypeptide profile, probed with anti-pea SSU antibodies, signalled cross-reactivity against the 20-kDa induced band and, to a lesser extent, with another 14-kDa protein (Fig. 2B, cf. lanes T+ and T−). An electroeluted preparation of the 20-kDa recombinant protein (see Section 2) was subjected to 35 rounds of automated Edman degradations. This yielded an N-terminal sequence which was identical to that deduced from the nucleotide sequence of the PrSSU cDNA, except for the absence of the initiator methionine and the −15 arginine residue in the transit peptide. The absence of the formylmethionine initiator suggested that the PrSSU was processed in accordance with the substrate specificity of the cytoplasmic methionine aminopeptidase of *E. coli* [20].

Table 1
Marker enzyme activities in subcellular fractions of *E. coli*

Enzyme/protein	Activity/amount (% of total)		
	periplasm	cytosol	membranes
Alkaline phosphatase	94	5	1
Malate dehydrogenase	12	2	86
Succinate dehydrogenase	2	7	91
Fumarase	5	90	5
Isocitrate dehydrogenase	14	82	4
Cytochrome b_5	8	92	0

E. coli N4830-1 harbouring pYPS was thermoinduced at 38.5°C for 4 h. The enzyme activities and the relative content of cytochrome b_5 were determined as described previously [16].

To decipher the sub-cellular location of the PrSSU and the anti-SSU reactive 14-kDa proteins, thermo-induced and non-induced *E. coli* pYPS cells were subfractionated into the periplasmic, cytoplasmic and envelope fractions. The effective separation of the bacterial compartments was confirmed by enrichment of the known marker enzyme activities in the isolated cellular fractions (Table 1) together with almost complete recovery in the cytoplasmic fraction of the co-expressed cytochrome b_5 [21]. The cellular pool of PrSSU appeared enriched in the envelope membranes (Fig. 2, lane E+). The PrSSU protein proved undetectable in the periplasmic and the cytoplasmic fractions (Fig. 2, lanes P+, PD+ and C+). The thermoinduced profile of the envelope membranes revealed another dominant, co-expressed 17-kDa polypeptide that did not cross-react with anti-SSU serum. The anti-SSU reactive 14-kDa protein localised in the periplasmic fraction (Fig. 2B, lanes P+ and PD+) was of a size similar to pea stromal SSU

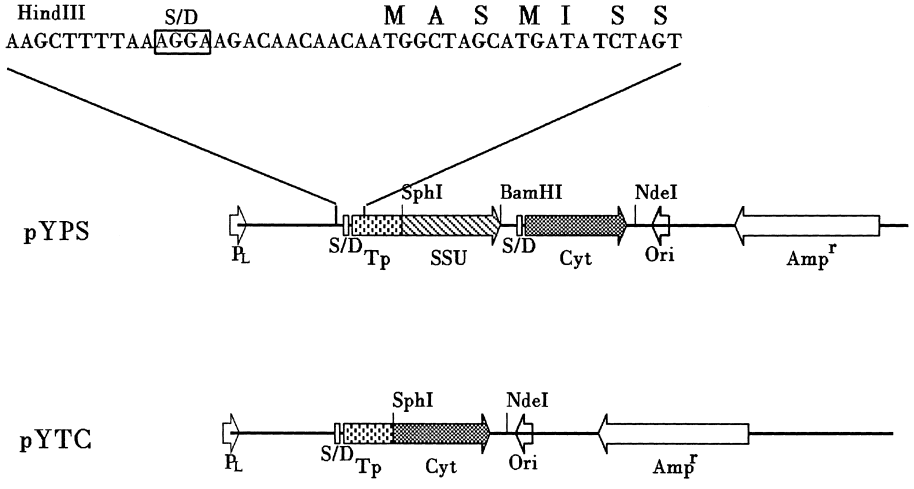

Fig. 1. Gene constructions. pYPS: the upstream *prSSU* segment was modified by replacing the *Hin*dIII–*Pvu*II deletion with a stretch of synthetic DNA duplex incorporating an ribosomal binding site and an intervening sequence designed on *Pseudomonas putida* P450^{cam}. The modified gene was introduced into a region between the thermoregulated λP_L promoter and the downstream mammalian cytochrome b_5 in pλ-1cyt [22]. pYTC: *Sph*I–*Nde*I deletion in pYPS replaced by the cytochrome b_5 gene carrying an engineered *Sph*I site at the N-terminus.

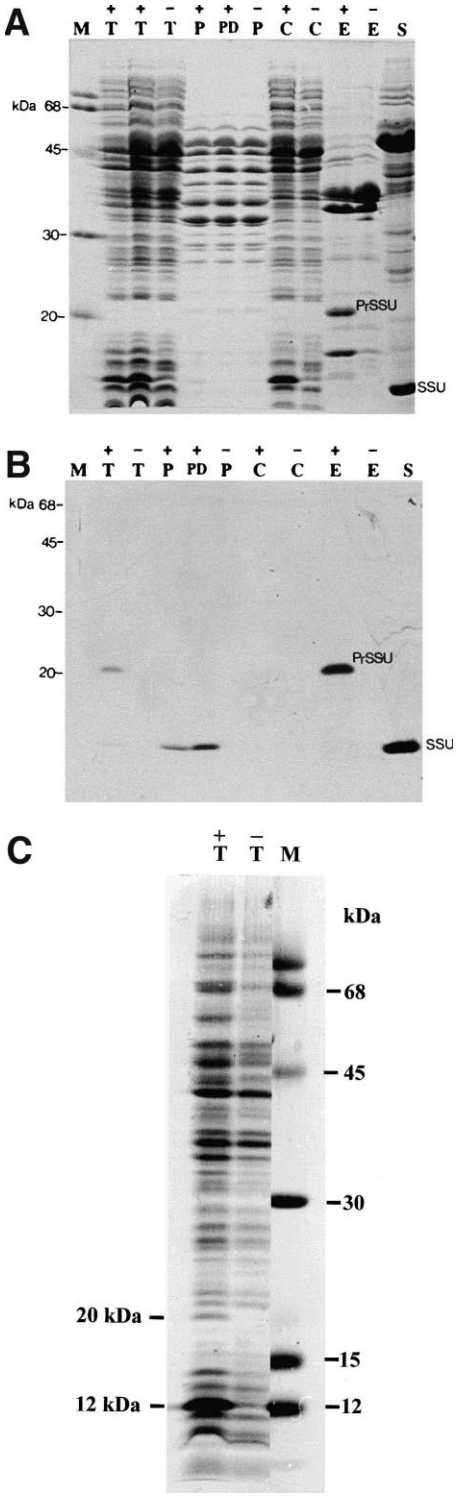

Fig. 2. Subcellular localisation of PrSSU. (A) and (C) Coomassie blue-stained polypeptide profiles, (B) Western blot of (A) probed with anti-pea SSU serum. M, Molecular weight marker proteins; T, total bacterial proteins; P, periplasmic fraction; PD, P after DEAE Sepharose CL-6B chromatography; C, cytosolic fraction; E, envelope membrane fraction. S, pea chloroplast stromal fraction. (+) and (−) denote proteins derived from 4 h thermoinduced or non-induced cells.

(Fig. 2, cf. lanes P+ and S). Moreover, the determined N-terminal sequence of the first five amino acid residues (MQVWP) of the isolated protein matched with the mature SSU sequence.

We considered the possibility that the recombinant PrSSU may have been accumulated in the cytoplasm of the intact bacterium in the form of inclusion bodies which, however, may have co-isolated with the membranes during subcellular fractionation. Therefore, we conducted a detailed time-course analysis by electron microscopy of the thermoinduced *E. coli* pYPS in comparison with the cell-line pλ-1cyt [22] expressing cytochrome b_5 but not PrSSU. This study showed that both strains had normal ultracytomorphology throughout the induction phase of up to 8 h (data not presented). Morphologically, these two recombinant strains were indistinguishable in their cell shape, size and distribution of the nucleoid. The absence of cytoplasmic protein aggregates discounted the likelihood of PrSSU protein being accumulated in the form of inclusion bodies. Unequivocal evidence for the localisation of PrSSU protein in the envelope zone was obtained by immunogold labelling of whole *E. coli* pYPS cell ultrasections (data not shown).

Whilst the immuno-gold labelling clearly showed that the PrSSU protein was targeted to the envelope zone, the approach did not indicate whether it was enriched in the IM or OM. To gain further insight, the cell envelope fraction of thermoinduced *E. coli* pYPS was further resolved into the IM and OM fractions by discontinuous sucrose gradient centrifugation of the total membrane fraction, obtained by mild lysozyme digestion of EDTA-treated generated spheroplasts [23]. The envelope fraction resolved into two discrete bands with buoyant densities of $\rho = 1.23 \pm 0.02$ for the lower white band (OM) and 1.14 ± 0.03 for the upper brown band (IM), values in close agreement with those previously reported. Greater than 85% of the total succinate dehydrogenase activity in the envelope was recovered with the IM. The distinctive and characteristic polypeptide profiles displayed by the two types of membranes (Fig. 3A) substantiated that effective subfractionation of the two membranes had occurred. In the latter, the major outer membrane proteins OmpA, F and C appeared as the prominent bands but were absent in the IM [24]. Comparison of the immunoelectrophoretogram in Fig. 3B clearly shows that the envelope-localised PrSSU was discretely segregated in the OM and was undetectable in the IM. Detectable build-up of PrSSU in the isolated OM occurs at (6 h) or following a longer induction period (Fig. 3) than that seen at 4 h in the crude envelope fraction (Fig. 2, lane E+), possibly due to loss of some of the precursor protein during the lengthy subfractionation procedures. Localisation of PrSSU protein to the OM is concomitantly coupled with induction of two additional proteins of 15 and 17 kDa. These proteins are not cross-reactive with anti-SSU sera and do not appear in the control thermo-induced cell line pλ-1cyt.

That the PrSSU protein was tightly integrated into the OM was indicated by the inability to extract it from the isolated membranes by washings with either 0.1 M Na_2CO_3 or 1 M NaCl (data not presented), treatments known to release loosely bound and peripheral proteins [25]. To seek whether the PrSSU protein was laterally exposed to the exterior of the cells, 5-h thermoinduced, non-permeabilised, *E. coli* pYPS cells were treated with trypsin. Whilst, this 'shaving' approach specifically depleted a 35-kDa band, the PrSSU band re-

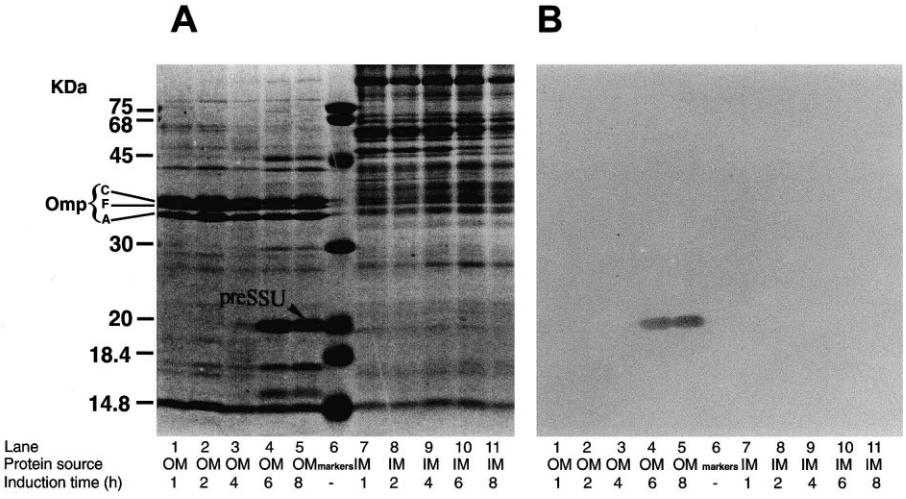

Fig. 3. Immuno-electrophoretic localisation of PrSSU in isolated IM and OM. Envelope fractions of *E. coli* pYPS cells subfractionated into IM and OM. (A) Coomassie blue-stained, (B) Western of a comparable gel, shown in A, probed with anti-pea SSU serum.

mained unaffected, implying that it was most likely not exposed to the exterior to be susceptible to the exogenous protease (data not presented).

The findings from this study have shown that the pea PrSSU protein when expressed in *E. coli* was targeted to the OM and to an extent processed en route to generate a counterpart size related to the mature form in the periplasm. Following cytoplasmic synthesis and removal of the N-terminal methionine, PrSSU was rapidly targeted to the OM. Translocation to the OM could occur either directly from the cytosol via the contact zones or in consecutive steps through the IM and periplasm. The former pathway could account for the PrSSU absence in the IM whereas the latter pathway could explain the presence of the processed derivative in the periplasm. In view of the recent findings that auxiliary periplasmic molecular chaperones are involved in the transit of unfolded IM-translocated OM proteins to the OM [26], the role of the chromosomally co-expressed 15- and 17-kDa protein (Fig. 3) in the bacterial export of PrSSU poses intriguing possibilities [27]. Nevertheless, what is the nature and location of the topological sequence(s) in the chloroplastic precursor protein that is functional in delivering the protein to the *E. coli* OM?

To address this question, we substituted the SSU portion in the higher plant precursor gene with the mammalian globular cytochrome b_5 gene in the pYPS vector. The DNA encoding the 99 amino acid residue globular haemoprotein was placed in a direct reading frame with the transit peptide of SSU (Fig. 1B). The results of this expression study were similar to those observed with the PrSSU. A significant proportion of processed globular cytochrome b_5 was localised in the periplasm (data not presented) and the chromogenic chimeric transit peptide-cytochrome b_5 was also targeted to the OM (Figs. 4 and 5) where it was retained as an integral, correctly-folded holoprotein as indicated by its spectral properties (Fig. 6). The OM-targeted transit peptide-cytochrome b_5 displayed indistinguishable spectral characteristics in comparison with the native cytochrome b_5, including the Soret absorption peak at 423 nm and the visible peaks at 555 and 527 nm. Thus, the Arg^{-15}-deleted transit peptide carries targeting information for localisation of a passenger protein to the OM of *E. coli*.

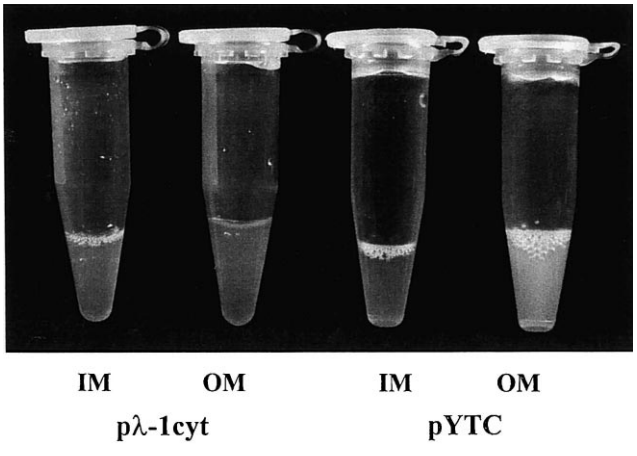

Fig. 4. Expression of transit peptide-fused cytochrome b_5 induces colour transformation of the OM fraction. The isolated membrane suspensions were prepared from thermoinduced *E. coli* harbouring either pλ-1cyt or pYTC expressing the chimeric protein. The (pink) colour of the OM suspension is derived from the presence of the globular form of cytochrome b_5. Note: colour photograph produced in grey tone.

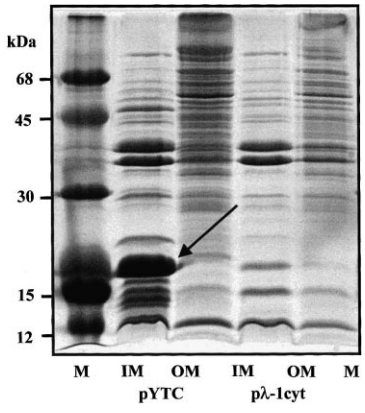

Fig. 5. Transit peptide-cytochrome b_5 is targeted to the OM. The proteins derived from *E. coli* pλ-1cyt (control) and pYTC were analysed by SDS–PAGE as described in Section 2. The arrowhead shows the position of the chimeric transit peptide-cytochrome b_5 protein. M, marker proteins.

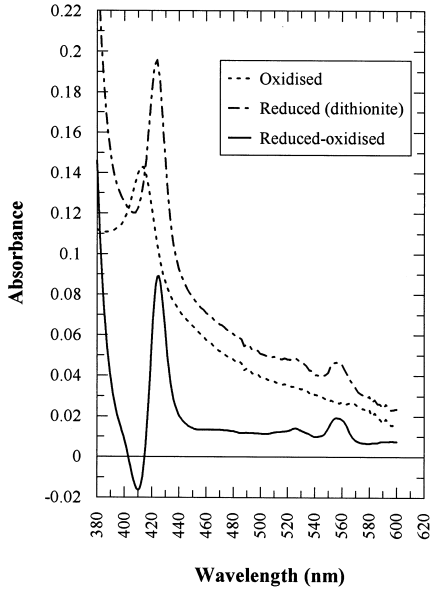

Fig. 6. Spectral characteristics of isolated OM. The membranes, prepared from *E. coli* pYTC expressing transit peptide-cytochrome b_5 fusion protein and suspended at 75 μg protein/ml in a buffer composed of 25 mM Tris–acetate (pH 8), were scanned against the isolated OM from *E. coli* pλ-1cyt (control) at a comparable concentration.

Most chloroplast transit peptides are particularly rich in hydroxylated amino acids and contain at least several evenly distributed, basic residues [28]. Hence, they are considered to be more soluble in an aqueous environment than the corresponding hydrophobic secretory signal sequences [29]. However, hydropathy analysis of the arginine-deleted transit peptide reveals two hydrophobic regions in the transit peptide, a shorter portion at the amino terminus (I) and a longer middle segment (II). Albeit displaying a lower hydrophobicity index, the intragenic region II comprising of 21 residues displays characteristics similar to signal sequences found in the OM proteins of *E. coli* (Fig. 7). These features include an N-terminal region carrying a positively charged residue, a central hydrophobic core and a C-terminal segment which contains a proline located some six residues from a plausible cleavage site according to the −3, −1 rule of von Heijne [30]. Similarly, Neilson et al.'s *Signalp* program [31] predicts a potential signal sequence in the transit peptide portion spanning from 1–21 residues with a probable cleavage site between residues 21 and 22: QSA–AV. Such an N-terminal cleavable or non-cleavable signal sequence segment of the pea transit peptide could act as a membrane insertion loop to initiate the translocation of the passenger polypeptide, possibly by the *sec*-dependent translocation apparatus. Some of the subsequently translocated PrSSU protein may have undergone proteolysis to yield a 'trimmed' form related to the mature SSU in the periplasm. Precisely, how the transit peptide subsequently partitions into the OM remains to be elucidated and the presence of an additional sorting signal cannot be ruled out.

Bacterial expression of another chloroplast precursor, the 33-kDa oxygen-evolving complex protein of wheat, was exported to the periplasm and processed to the mature component [32]. The bacterial translocation and processing of the wheat 33-kDa preprotein has been accounted for by the presence of a second intragenic 'thylakoidal transfer' signal, located downstream of the N-terminal 'envelope transfer' signal, which shares functional similarities with signal peptides [8]. In contrast, the PrSSU transit peptide contains information for translocation of the precursor across the chloroplast envelope only. Although the transit peptide used in this study had a deletion of an arginine, between domains I and II, that is strongly conserved in higher plants, the PrSSU transit peptides of *Silene pratensis* (Swiss-Prot entry Q42516) and *Amaranthus hypochondriacus* (Swiss-Prot entry Q42516) are also devoid of this basic residue. Moreover, the arginine-deleted mutant PrSSU is also competent for importation into isolated pea chloroplasts (data not presented). We have not been successful at expressing the wild type PrSSU in the *E. coli* N4830.

In view of the considerable heterogeneity of chloroplast

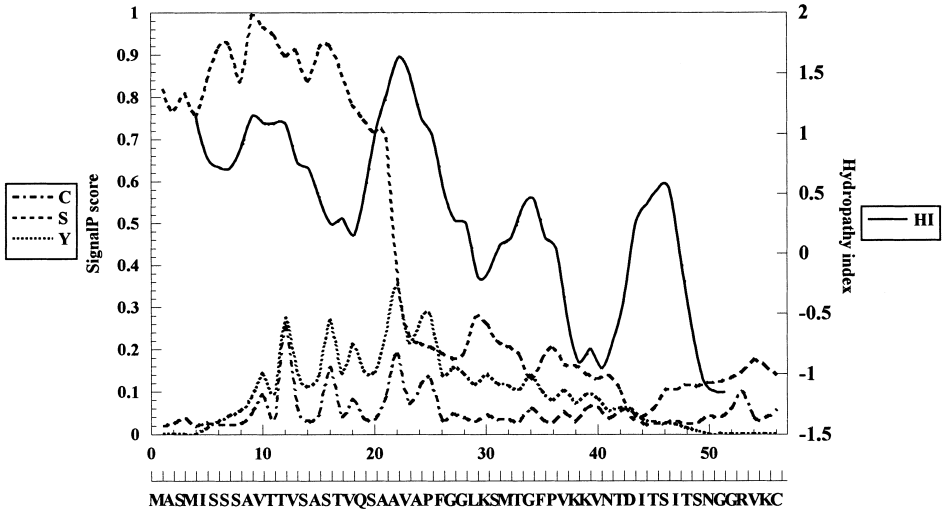

Fig. 7. Predicted hydrophobic character of and signal sequence-like element in pea transit peptide. The hydropathy indices were calculated using Kyte and Doolittle [39] algorithms with a window setting (10% linear weighting with respect to the window centre) of nine residues. The signal peptide score (*S*-score) and the combined cleavage site score (*Y*-score) were obtained using the Signalp prediction program of Nielsen [31].

presequences it is widely assumed that specific recognition by the chloroplast surface is mediated by a secondary structural element, such as amphipathic α-helix or β-sheet structure. Presequences destined for membranes other than chloroplast may also share such substructural elements because of their universal need to establish initial interaction with the lipid phase of membranes prior to gating via the translocase. Per se this could not constitute intracellular mistargeting due to stringent prior screening of the presequence by chaperoning factors and organellar surface receptors. The suggestion that the transit peptide of PrSSU contains an intragenic signal sequence to translocate protein across the IM has serious implications in protein trafficking in the eukaryotic cells since prokaryotic signal sequences are functionally operational in localising proteins into the endoplasmic reticular lumen of the eukaryotes [33]. While the targeting of preproteins to specific organelles occurs with high fidelity in vivo, under certain conditions preproteins can exhibit a degree of promiscuity in the membrane surfaces they will recognise. For example, mitochondrial preproteins can be translocated into mitochondria and microsomes [34], peroxisomal proteins can be imported into the mitochondria [35] or microsomes [36] and chloroplast proteins can be translocated across the membrane of mitochondria [37]. Such infidelity has also been reported with the import of rabbit reticulocyte-synthesised PrSSU into the matrix of yeast mitochondria [38].

It remains to be investigated whether the transit peptide mediated translocation is accomplished via the general secretory pathway or via a signal sequence-independent translocation pathway. In the latter case the origins and parallels of the mode of protein transport across biological membranes of prokaryotes and the energy organelles may be reflecting links with an endosymbiont ancestor. In this context, Cartwright et al. [39] have identified and sequenced an *E. coli* ORFT gene showing a potential ancestral linkage to the genes for the mitochondrial import site proteins ISP42 [40] and MOM38 [41]. In a more recent study, Pang et al. [42] have shown that the introduction into *E. coli* of Toc 36, a 44-kDa component of the chloroplast protein import apparatus, enhanced translocation of endogenous secretory proteins. Moreover, Toc36 also complemented the temperature-sensitive *secA* mutation that interferes with protein translocation.

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