A mammalian cytochrome fused to a chloroplast transit peptide is a functional haemoprotein and is imported into isolated chloroplasts

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The small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a major chloroplast stromal protein that is cytosolically synthesized as a precursor with an N-terminal extension, known as the transit sequence or transit peptide (Tp). The Tp is essential for the post-translational uptake of the precursor by the chloroplast. The Tp is thought to influence the conformation of the precursor protein and to facilitate polypeptide translocation across the chloroplast envelope barrier via a Tp-selective translocon. To address these issues we have devised a novel strategy to generate substrate amounts of a chloroplast targeting sequence as a fusion with the chromogenic globular domain of cytochrome *b₅* (Cyt). The chimaeric protein is an ideal probe for investigating the conformation of a preprotein and events surrounding protein import into isolated chloroplasts. The Cyt of liver endoplasmic reticulum was fused at its N-terminus with the Tp of the small subunit of Rubisco of *Pisum sativum* (pea). To enhance its production by clearance from the cytoplasm of *Escherichia coli*, the chimaera was

engineered by further N-terminal linkage of a prokaryotic secretory signal. Expression of this tripartite fusion resulted in mg quantities of the signal sequence-processed Tp-Cyt protein, which was eventually targeted to the membranes. The chromogenic nature of the chimaera and its localization to the bacterial membrane facilitated the biochemical isolation of the precursor in a soluble and functional form. The purified preprotein displayed spectral and enzymic properties that were indistinguishable from the native parental Cyt, implying an absence of observable influence of the Tp on the conformation of the haemoprotein. The chimaeric precursor was imported into the stroma of the isolated chloroplasts in a dose-dependent manner. Import was also strongly dependent upon exogenously supplied ATP. The stromally imported chimaeric precursor protein was processed to a size characteristic of Cyt.

Key words: precursor conformation, protein translocation, recombinant protein, signal sequence.

INTRODUCTION

The vast majority of the chloroplast proteins of higher photosynthetic organisms are synthesized in the cytosol as precursors carrying N-terminal transit peptide (Tp) extensions, which mediate their post-translational import into the chloroplast [1,2]. The Tp is necessary to direct the import of the passenger polypeptide into the chloroplast and contains chloroplast-specific targeting information [3,4]. For a protein destined for the stroma, the precursor interacts with the chloroplast surface via Tp-specific receptor(s) [5], which then engage it with a proteinaceous channel to facilitate its translocation across the chloroplast envelope barrier in an ATP-dependent manner; the Tp is cleaved off either during or immediately after transfer of the precursor into the stroma [6]. Significant insight into the import process and the identification of auxiliary transport apparatus has arisen from the use of *in vitro* translated precursors and synthetic Tps, as well as recombinant forms of the precursor proteins [7].

Although Tps are highly variable in length and primary structure, they contain an abundance of hydroxylated residues and lack acidic amino acids. The conformation of the Tps remains unknown and theoretical predictions suggest that they have a propensity to assume unstructured coils [8]. Nevertheless, the fact that Tps from different chloroplast proteins are functionally interchangeable suggest that a more defined secondary structure may be imposed upon interaction with the lipid bilayer of the outer membrane [9]. The actual conformation of a transport-competent precursor protein in solution remains elusive, but there is some experimental evidence to suggest that the

presence of a mitochondrial Tp imparts a loosely folded conformation that enhances import competency [10]. However, some authentic precursors such as the chloroplast 5-enolpyruvylshikimate-3-phosphate synthase [11] and several mitochondrial proteins [12,13] are enzymically active implying that at least their active-site conformations must be similar to the mature enzymes. The ability of some precursor proteins to assemble as oligomeric structures [14,15] and to bind prosthetic groups or ligands [16,17] prior to translocation is indicative of retention of the native fold in the mature regions capable of withstanding any structural hindrance from the exposed signals. The precursor of pea ferredoxin-NADP⁺ reductase, synthesized in *Escherichia coli* as a soluble holo protein form containing bound FAD [18], displayed a low level of activity and was import competent. Several groups have reported on the production of recombinant chloroplast precursor proteins as insoluble aggregates and their use, after treatment with chaotropic agents, in the characterization of the protein import process and the associated apparatus [5,19–23]. However, the physiological significance of the structures derived from precursor proteins solubilized from inclusion bodies by use of chaotropic agents remains uncertain, and in any case yielded conflicting results. For example, the precursor of ferredoxin had a low content of defined secondary structure and resembled an unfolded protein [24], and pre-light harvesting chlorophyll *a/b*-binding protein [25] had a partially-folded conformation composed of both α -helix and β -sheet.

In the present study we have devised a novel strategy for substrate-level production of a functional, chromogenic, fusion

Abbreviations used: BPTI, bovine pancreatic trypsin inhibitor; Cyt, globular domain of cytochrome *b₅*; LB, Luria–Bertani; prOE17, photosystem II; Tp, transit peptide; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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protein comprising a higher plant chloroplast Tp and the globular domain of cytochrome b_5 (Cyt) from rat liver. We report on its production, isolation, physicochemical nature, import and processing by isolated chloroplasts, and the influence of the Tp on the conformation of the adjoining passenger protein.

MATERIALS AND METHODS

DNA manipulation, strain and culturing

Standard procedures involving DNA purification, digestion, agarose electrophoresis and bacterial transformation were followed as previously described [26]. Sequencing was performed as recommended by the DNA sequencing version 2 kit [27]. The *E. coli* TB-1 [F^- *ara* Δ (*lac-proAB*) *rps* (Str^r) (ϕ 80d Δ (*lacZ*)M 15) *hsd* R17 (r_{k-} m_{k+})] strain was used in the present study. The host harbouring the relevant plasmid was propagated in either liquid or solid Luria–Bertani (LB) medium [1 % (w/v) Tryptone, 0.5 % (w/v) yeast extract and 1 % (w/v) NaCl] containing 75 μ g of ampicillin/ml. For induction of the recombinant protein, a 2 % (v/v) inoculum of an LB-grown, saturated culture was applied to a phosphate-limited (0.1 mM) Mops medium [28] containing 75 μ g of ampicillin/ml, and batch-cultivated at 35 °C for 6 h with shaking at 125 rev./min.

Isolation of the Tp–Cyt fusion protein and Cyt

Cells harvested (centrifuged at 5000 *g*) from one litre of *E. coli* TB1/pY-STC were suspended in 20 ml of prechilled, resuspension buffer [10 mM Tris/HCl (pH 8), 5 μ M haem and 1 mM Na₂EDTA] containing 1 mg of lysozyme/ml. Cell lysis was promoted by subjecting the suspension to 10×15 s periods of sonication (MSE Soniprep 150), with a 30 s cooling period on ice between each pulse. The membranes were recovered from a post-cellular fraction (1000 *g* for 20 min) by centrifugation at 105 000 *g* for 1 h, and were resuspended in 5 ml of TE buffer [10 mM Tris/HCl (pH 8.0)/1 mM EDTA]. The suspension was mixed with 49 vol. of ice-cold 40 % (v/v) acetonitrile with gentle stirring in a beaker for 5 min. The recombinant haemoprotein recovered in the extract was directly applied on to a DEAE-Sepharose CL-6B column (2.5 cm $\times$ 10 cm) using a Pharmacia LKB FPLC system. The bound Tp–Cyt protein was eluted by applying a linear NaCl gradient, with Tp–Cyt protein eluting at approx. 200 mM NaCl. The fractions enriched in Tp–Cyt protein were pooled and were concentrated by ultrafiltration through an Amicon 10 kDa cut-off filter. The protein was further resolved by preparative non-denaturing gel electrophoresis and the chromogenic band was electroeluted using the Pharmacia-LKB electroeluter system (Milton Keynes, Bucks., U.K.). The Tp–Cyt protein was dialysed for 18 h against three changes of 10 mM Tris/acetate (pH 8) and then freeze-dried. The globular Cyt was isolated from *E. coli* p λ -lcyt as described previously [29].

Protein sequencing

Purified Tp–Cyt protein (50 μ g) was subjected to N-terminal microsequencing with phenylthiohydantoin, using an Applied Biosystem 477A pulse liquid sequencer with on-line detection using an Applied Biosystems 120A analyser.

Protein and haemoprotein estimations

The Tp–Cyt protein was spectrally quantified using the Soret absorbance peak at either 413 nm (oxidized) or 423 nm (reduced) using the absorption coefficients given previously [30]. Absolute absorption spectra of the bacterial lysates or subcellular fractions

were monitored by scanning samples in TE buffer from 350–450 nm in a cell with a light path of 1 cm. Where haem was used as a supplement to convert apo protein into holo haemoprotein, it was added as a stock 1 mM solution [in 0.1 M Tris/HCl (pH 8.2)/80 % (v/v) ethylene glycol] in equal amounts to the contents of both the test and the reference cuvettes so as to give final concentrations ranging from 2.5–10 μ M. Protein content was determined [31] using BSA as a standard.

¹²⁵I-labelling

Isolated Tp–Cyt or Cyt (1–5 μ g) in 0.2 ml of 0.1 M Tris/HCl (pH 7) was ¹²⁵I-labelled by reaction with carrier-free Na¹²⁵I (37 mBq; Amersham International) using Iodobeads [32] for 15 min at 22 °C. Unbound radioactivity was sequestered by addition of 20 μ l of saturated tyrosine solution. The radiolabelled protein (specific activity of 4 mBq/ μ g of protein) was re-isolated by gel-filtration on a Biogel P5 column, using an elution buffer composed of 50 mM Hepes/KOH (pH 8)/0.33 M sorbitol.

Chloroplast isolation and import reactions

Chloroplasts were isolated from the leaves of 10-day-old pea plants (*Pisum sativum*, Feltham First) grown for 12 h under fluorescent light, and for 10 h in darkness at 22 °C, essentially as described previously [33]. Protein import into isolated chloroplasts was carried out as described by Pain and Blobel [34]. Unless stated otherwise, the import reactions were performed in a final volume of 0.3 ml containing 50 mM Hepes/KOH (pH 8), chloroplasts (equivalent to approx. 50 μ g of chlorophyll), 0.33 M sorbitol, 5 mM magnesium acetate, 40 mM potassium acetate, 2 mM dithiothreitol, 1 mM ATP and 100 μ g of BSA/ml. After preincubation with ¹²⁵I-labelled Tp–Cyt, as specified elsewhere, on ice for 5 min in the dark, import was initiated in ambient white light at 22 °C for 30 min. In some experiments, post-import, the chloroplasts were treated with 50 μ g of thermolysin/ml in the presence of 0.1 mM CaCl₂ for 30 min on ice, re-isolated by Percoll discontinuous gradient centrifugation, and fractionated into stroma and envelope fractions following their lysis by a freeze–thaw cycle.

Microsomes

Rough microsomal vesicles were isolated from sheep pancreas as described previously [19].

Electrophoretic analysis

Proteins were separated by SDS/PAGE [35] and the bands were detected by staining with Coomassie Brilliant Blue R250. The dried gels were subjected to standard autoradiography in order to detect the radiolabelled bands. In some experiments the extent of protein import was quantified from the radioactivity monitored in the excised bands.

RESULTS & DISCUSSION

Hybrid gene construction

We have reported that a mutant form of a Tp–Cyt fusion protein, carrying an arginine deletion at position 15 in the Tp portion, was targeted to and accumulated in the outer membrane of *E. coli* [1]. However, similar trials to express the wild-type Tp fusion protein did not prove successful in achieving significant production of the chimaeric protein. To overcome this problem we devised a novel strategy for production of the wild-type Tp fused to Cyt. This involved construction of a tripartite fusion protein in which the Tp–Cyt fusion protein was N-terminally

Figure 1 Gene construction encoding the secretory Tp-Cyt protein

NcoI–*SphI* cleaved *tp* sequence was introduced between the gene encoding the signal sequence and *cyt* in a pA-Cyt progenitor plasmid [28]. $\downarrow$, processing sites of the signal sequence and Tp [30]; *PhoA*, *phoA* promoter; S/D, Shine/Dalgarno sequence; Ss, signal sequence of *E. coli* alkaline phosphatase.

linked to the secretory signal of *E. coli* alkaline phosphatase. Prima facie this scheme would facilitate more efficient clearance from the cytoplasm into the less hostile periplasm, and culminate in the Tp-mediated targeting of the signal sequence-processed chimaera to the outer membrane [1]. The construction scheme of the tripartite protein is depicted in Figure 1. The full-length DNA sequence encoding the Tp of the small subunit of pea ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) [3], cloned within the polylinker site of pSP64 to give pSMS58, was modified by site-directed mutagenesis involving replacement of deleted portions with synthetic oligonucleotide duplex. The smaller *HindIII*–*EcoRV* segment at the 5'-end of the *tp* in pSMS58 was deleted and replaced with a duplex oligonucleotide carrying a unique *NcoI* site at the initiation codon. The *SphI*–*PstI* *tp* DNA fragment was then ligated in-frame between the signal sequence of *E. coli* alkaline phosphatase and *cyt* into identically cleaved pAF-Cyt [28] to give the expression vector pY-STC that encoded the tripartite fusion protein, signal sequence–Tp–Cyt, under the transcriptional control of the *phoA* promoter. The chimaera is comprised of the 21-residue signal sequence of alkaline phosphatase directly fused to the Tp of the small subunit of Rubisco, which in turn is linked to the 99-amino-acid-residue Cyt [29].

Expression, isolation and characterization of Tp-Cyt

Electrophoretic analysis of the lysates of the *E. coli* pY-STC cells cultured in a phosphate-limited medium demonstrated expression of a novel, major, 17.5 kDa protein (Figure 2). The induced protein was purified and identified as a signal-processed Tp-Cyt protein by a variety of biochemical criteria (see below). Subcellularly, the Tp-Cyt protein was dominantly concentrated in the membrane fraction as an integral component (Figure 3) and targeted to the outer membrane (results not shown). The bacterially expressed Tp-Cyt protein was isolated from the envelope fraction to near homogeneity with a specific content of greater than 95% (Table 1 and Figure 3). Although a significant amount of Tp-Cyt was found localized in the periplasm, its isolation from this cellular fraction was difficult. N-terminal sequencing of the isolated protein, by Edman degradation of the first 15-amino-acid residues, correctly matched the DNA-encoded sequence, and confirmed that the signal sequence was correctly processed.

Figure 2 Expression of the Tp-Cyt fusion protein

Total cellular fraction from *E. coli*, harbouring pY-STC, cultured in Mops medium containing 50 mM phosphate (lane 1) or 0.1 mM phosphate (lane 2). Lane 3 contains molecular-mass marker proteins.

Tp-Cyt is a functional haemoprotein

The available three-dimensional crystal structure of Cyt at 2 Å resolution [36] shows that the predominant feature of the molecule is the creation of a spatially defined crevice to accommodate the haem *b* (iron protoporphyrin IX) moiety. The stability of the holo Cyt protein comes from a variety of interactions between the porphyrin ring and the nature of the residues lining the pocket. Such haem group environments display prominent Soret absorption peaks (α , β and γ) in the visible region and are hallmarks in the identification of cytochromes with variations in haem types and interactivity. The haem prosthetic group can be readily incorporated to form the holo Cyt, and although held by non-covalent forces in the crevice, haem removal requires unfolding of the core polypeptide. Thus the influence of the adjoining Tp, if any, on the conformation of the haem-binding globular domain portion could be gauged

Figure 3 Subcellular localization in *E. coli* and purification of Tp-Cyt

Proteins detected after separation by SDS/PAGE and staining with Coomassie Blue in the total (lane 1), cytoplasmic (lane 2), periplasmic (lane 3) and membrane (lane 4) fractions derived from induced *E. coli*/pY-STC. The purity of the Tp-Cyt protein following acetonitrile extraction of the membranes (lane 5), DEAE chromatography (lane 6) and after preparative PAGE (lane 7). Lanes 8 and 9 show the residual membrane after acetonitrile extraction. Lane 10 contains purified Cyt, which was isolated as previously described [29]. Lane 11 contains molecular-mass marker proteins. The intense ~12 kDa band in the membrane fraction (lane 4) is that of the lysozyme used for cell lysis.

Table 1 Purification of the Tp-Cyt fusion protein

Purification step	Total protein (mg)	Tp-Cyt protein		
		Amount (mg)	Specific content (%)	Recovery (%)
Cell lysate*	48	4.9	10.3	100
Envelope membranes	21	3.8	18	76
Acetonitrile extraction	4.5	3	67	60
DEAE chromatography	1.9	1.5	80	30
Preparative PAGE	1.1	1	95	19

* Starting with 1 litre of culture.

spectrally and enzymically. Firstly as shown in Figure 4, Tp-Cyt is a *b*-type haemoprotein with a Soret band at 413 nm in the oxidized protein; the reduced protein showed an increased Soret peak at 423 nm and distinct peaks at 555 nm and 527 nm. The absorption spectrum of the oxidized versus reduced forms of the Tp-Cyt protein was indistinguishable from Cyt alone in having a characteristic maximum at 423 nm and a minimum at 409 nm (Figure 4). The determined extinction coefficients of both the oxidized and reduced forms of the recombinant haemoprotein at various absorption peaks were similar to the values previously reported for the mammalian liver [37] and erythrocyte [38] Cyts. Secondly, Tp-Cyt was enzymically functional in accepting electrons from cytochrome *b*₅ reductase at a rate comparable to Cyt (Table 2). The CD spectra of Cyt and Tp-Cyt (Figure 4),

Figure 4 Spectral characteristics of the isolated Cyt and Tp-Cyt proteins

Purified Tp-Cyt (2.5 mg/ml) or Cyt (1.85 mg/ml) protein (150 μ l) was made up to 3 ml with TE buffer (top panel). Spectra were monitored using a Hewlett-Packard Diode Array spectrophotometer. The reduced spectra were recorded after dissolving a few crystals of sodium dithionite in the same samples in their cuvettes. Ox, oxidised; Red, reduced.

Table 2 Microsomal NADH-cytochrome *b*₅ reductase catalysed reduction of Cyt and Tp-Cyt proteins

The assays were performed by incubation at 30 °C in a buffer containing 50 mM Tris/HCl (pH 7.8), 25 mM KCl and 5 mM MgCl₂ in a final volume of 1 ml containing 0.5 mM NADH, sheep pancreatic microsomes (510 μ g of protein) and purified haemoprotein. Activity is expressed as the increase in absorbance at 555 nm/min per mg of microsomal protein. ND, not detected.

Haemoprotein	Microsomes	NADH	Activity
—	+	—	ND
—	+	+	ND
Cyt (50 μ M)	—	—	ND
Cyt (50 μ M)	—	+	ND
Cyt (50 μ M)	+	+	0.11
Cyt (100 μ M)	+	+	0.18
Tp-Cyt (50 μ M)	+	+	0.08
Tp-Cyt (100 μ M)	+	+	0.20
Cyt (50 μ M) and Tp-Cyt (50 μ M)	+	+	0.22

Figure 5 CD spectra of Cyt and Tp-Cyt

The spectra of the isolated Cyt (0.18 mg/ml) and Tp-Cyt (0.16 mg/ml) proteins, dissolved in water, were recorded on a JASCO J40 spectrometer at 22 °C.

monitored from 250–600 nm (Figure 5), showed that both proteins display similar profiles with negative maxima at approx. 280–300 nm and prominent merging negative drops at 420 nm. The spectra are similar to those reported for calf liver full-length cytochrome *b₅* [39]. The absorption bands at wavelengths greater than 300 nm are contributed by haem interactions, but those between 250 and 300 nm are derived from the tryptophan and the four tyrosine residues. The retention of both the intense chromophoric Soret transitions associated with the interactive aromatic residues in the haem pocket together with the UV peaks contributed by the more deeply buried tyrosine residues are additional indicators of significant preservation of the near-native conformation of the core haemoprotein in the Tp-Cyt fusion protein. The slight displacement of the two spectra away from integral major chromophoric depression may suggest some minor deviations in the native conformation in regions away from the haem-binding pocket. The absence in the spectra of any major contribution of the Tp portion, representing 36% of the Tp-Cyt molecule, suggests that the Tp portion is most likely devoid of an ordered structure; findings that are consistent with previous studies by CD [20] and NMR [40] of synthetic Tps from other sources. The fact that the recombinant Tp-Cyt was spectrally, enzymically and structurally similar to the native globular form strongly indicates that the adjoining Tp is probably surface-exposed and unstructured and does not significantly interfere with the fold of the compact, globular haem-binding domain.

Tp-Cyt protein is imported into isolated chloroplasts and processed

In order to assess whether Tp-Cyt was biologically functional, the purified protein was ¹²⁵I-labelled and incubated with the isolated pea chloroplasts for 20 min in the standard import assay. Untreated or thermolysin-treated chloroplasts were re-isolated by centrifugation through Percoll step gradients and the radiolabelled products associated with the chloroplasts were analysed by autoradiography following electrophoresis. Incubation of the recombinant Tp-Cyt with the chloroplasts resulted in a significant proportion of the precursor protein becoming bound to the chloroplasts and the generation of a product smaller in size, by approx. 5 kDa, the expected size following removal of the Tp (Figure 6). That the smaller processed

Figure 6 Chloroplastic import and processing of the radiolabelled Tp-Cyt

Freshly isolated chloroplasts (equivalent to 400 µg of chlorophyll) suspended in a final volume of 1.8 ml of the standard import buffer containing 1 mM ATP, were incubated with 45 nM ¹²⁵I-labelled Tp-Cyt for 2.5 min at 0 °C in the dark. The import reaction was initiated by increasing the temperature to 25 °C and exposing the chloroplasts to ambient light for the indicated durations. Top panel: after allowing the chloroplasts to incubate for 2.5, 10, 20 and 30 min (lanes 2–5), 300 µl aliquots of the reaction mixture were withdrawn, chilled on ice and treated with 75 µg of thermolysin/ml in the presence of 2 mM CaCl₂ for 30 min. The chloroplasts, re-isolated by centrifugation through a 35% Percoll layer, were lysed and the radiolabelled protein in the stromal fraction detected following SDS/PAGE and autoradiography. Lane 1 contains ¹²⁵I-labelled Tp-Cyt protein. Bottom panel: shows the effect of treatment without (lane 1) or with (lane 2) thermolysin following 20 min of the import reaction.

product was within the chloroplasts was confirmed by its insensitivity to thermolysin treatment of the intact chloroplasts. A time-course of the incubation conducted under identical conditions showed that the import rate was linear over the first 20 min, after which uptake was shut down (Figure 6). Figure 7 shows that both the chloroplast–surface interaction and import

Figure 7 Dose-dependency of Tp-Cyt import into isolated chloroplasts

Import assays were performed with increasing amounts of ¹²⁵I-labelled Tp-Cyt ranging from 2.2–88 nM (as indicated). Following incubation at 25 °C in ambient light for 20 min, the chloroplasts were re-isolated and analysed by SDS/PAGE and autoradiography.

Figure 8 ATP-dependence of Tp-Cyt binding and import

Isolated chloroplasts were placed in the dark for 1 h to deplete their endogenous ATP pool. After addition of ^{125}I -labelled Tp-Cyt (14.5 nM), the suspension was divided and to each aliquot the indicated amount of MgATP was added, and the import reactions were conducted at 15 °C in the dark for 20 min. The re-isolated chloroplasts were analysed by SDS/PAGE and autoradiography.

rates augment with increasing amounts of the precursor substrate, reaching saturation at approx. 90 nM. Thus ^{125}I -labelled Tp-Cyt binds to the chloroplast surface and is translocated in both a time- and dose-dependent manner into the stroma where it is processed into a mature form, comparable in size to Cyt. The fact that the ^{125}I -labelled Cyt was not imported (results not shown) proves that the N-terminal Tp was necessary for transport of the haemoprotein into the isolated chloroplasts.

Exogenous ATP stimulates binding and import of Tp-Cyt

The binding to and import into chloroplasts of *in vitro*-synthesized precursors is an ATP-dependent process [41,42]. Is the energetic requirement for import and binding of the Tp-Cyt in our transport assay (devoid of the cytoplasmic fraction typically employed in translation systems) different from that of native chloroplast proteins, particularly as the passenger protein is a non-chloroplastic protein containing a tightly bound haem prosthetic group? To answer this question, the ATP dependence for the binding and import of the ^{125}I -labelled recombinant protein at a concentration of 14.5 nM was measured, using isolated chloroplasts that had been preincubated in the dark for 60 min to deplete the endogenous pool of ATP. Figure 8 shows that both binding and import events were dependent upon the levels of exogenous ATP supplied. Binding occurred in a saturating manner with increasing amounts of ATP up to approx. 100 μM . Above this concentration the bound precursor enters the translocation step. The ATP-dependent binding and translocation steps were not as clearly distinguishable when import was conducted using a magnitude higher concentration of the precursor and at a higher temperature (results not shown). Under these conditions both binding and import progressed with a much greater efficiency as a function of increasing ATP concentration, although in the absence of ATP a lesser, but significant, extent of import persisted. Like the import of the *in vitro*-synthesized precursor of the small subunit of Rubisco, both the binding and the translocation of ^{125}I -labelled Tp-Cyt was stimulated at a concentration as low as 10 μM ATP. With further increases in ATP levels, both the binding and import increased dramatically, eventually reaching saturating levels when the number of molecules estimated to be bound and imported were 800 and 2400/chloroplast respectively. The number of interactive sites on the chloroplast surface observed with Tp-Cyt was 2-fold lower than the proposed number of high-affinity sites for the

native precursor of the small subunit of Rubisco [43]. Nevertheless, the optimal ATP concentration for ^{125}I -labelled Tp-Cyt import, ranging between 500 μM and 1000 μM , is in close agreement with the previously reported findings [44]. Lesser amounts of ATP appear to be required for binding than import. Using precursor at a saturating concentration, the rate of protein import exceeded the binding by 3-fold implying that the turnover rate of the translocase was energetically determined by ATP.

Tp-Cyt constitutes a non-chloroplastic ^{125}I -labelled passenger protein and yet it is importable into chloroplasts. The incorporation of iodine into one of the four tyrosine residues, occurring only in the Cyt portion of the chimaera, would be expected to result in an increase in size equivalent to a methyl group. Clearly such a chemical modification of the adjoining protein, as reported for the import into mitochondria of artificial precursor proteins containing a branched polypeptide-chain or a C-terminal stilbene disulphonate [45], did not annul the Tp-mediated chloroplast recognition and its subsequent import. Moreover, its import, kinetic behaviour and the requirement of ATP are similar to those of the native chloroplast preproteins [20,46] and cleavage of the Tp by the chloroplast endoprotease [47]. Incubation of the full-length recombinant precursor of the small subunit of pea Rubisco in the import assay inhibited import of the radiolabelled Tp-Cyt in a dose-dependent manner (results not shown), further indicating that precursor segregation into chloroplasts is operative by the normal pathway. Therefore it is reasonable to conclude that Tp-Cyt is imported into chloroplasts via the normal import pathway. The findings that the appended small subunit Tp directs translocation of the foreign Cyt into the chloroplast is in agreement with the previous reports that other non-chloroplastic protein chimaera linked with the same signalling peptide were also imported into chloroplasts and processed therein [44,48,49]. Thus the information for precursor small subunit translocation, in the case of the small subunit of Rubisco, appears to reside in the Tp segment.

The isolated Tp-Cyt when incubated with freshly prepared pea stromal fraction was not processed (results not shown), probably due to inaccessibility of the cleavage site to the processing enzyme. However, the Tp-Cyt was processed during its import into isolated chloroplasts (Figures 5–7). This suggests that the cleavage site in the fusion protein is accessible to the processing apparatus during the translocation step when the precursor is transiently unfolded [50,51]. It is possible that in our organelle-free processing reaction, the absence of membrane/cytosolic components may influence the nature of the Tp-Cyt processing reaction and the sites available for cleavage. The absence of the cytosolic unfoldase factors, such as the heat shock protein HSP70 in the outer-membrane of the chloroplast [52–54], may also be another reason for the inability of the stromal fraction to process Tp-Cyt. However, *in vitro*-synthesized chloroplast precursors have been reported to be successfully processed by the stromal transit peptidase [47,55–57].

Recently Clark and Theg [58] have reported that the chloroplast envelope transmembrane translocon can translocate folded proteins. The 17-kDa subunit of the oxygen-evolving complex of photosystem II (prOE17) was C-terminally cross-linked to the 6.5 kDa bovine pancreatic trypsin inhibitor (BPTI) creating the chimaeric protein prOE17-BPTI. The small, tightly folded BPTI was co-transported across the envelope membrane *en route* to the thylakoid membranes. The small size of Cyt and its tightly folded structure raises the possibility that the haemoprotein may be imported as a holo form. However, our work cannot discriminate between the import pathway(s) employing folded and unfolded structures.

In the present study we have devised a novel procedure that involves the appendage of a bacterial secretory signal to a fusion protein comprising a chloroplast Tp and a mammalian Cyt that results in the successful over-production and isolation of a soluble, processed, chromogenic chimaeric protein. Spectroscopic and enzymic properties of the chimaeric protein suggest that the N-terminally appended Tp appears to be surface-exposed, such that it does not exert any detectable interference on the conformation, folding or the electron-accepting properties of the adjoining Cyt. In the absence of cytosolic factors the Tp-Cyt protein is biologically functional in its ability to be imported into the stroma of isolated chloroplasts where it is correctly processed. Import of the ^{125}I -labelled Tp-Cyt protein occurred in a dose-dependent manner. Transport of the Tp-Cyt protein was also stimulated by exogenous supplementation with ATP in a dose-dependent manner. Modification of the Tp-Cyt protein by ^{125}I -labelling at sites within the passenger portion of the molecule does not impair the import capability of this artificial precursor into the chloroplast. The processing of Tp-Cyt occurs during its import into isolated chloroplasts, but not when the fusion protein is incubated with the stromal fraction, implying that the processing event is coupled with translocation. The recombinant chromogenic Tp-Cyt fusion protein therefore offers, in quantity, a biologically active probe ideal for further investigating the modes of protein targeting to and import into chloroplasts.

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