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The chloroplastic protein import machinery contains a Rieske-type iron–sulfur cluster and a mononuclear iron-binding protein

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Transport of precursor proteins across the chloroplastic envelope membranes requires the interaction of protein translocons localized in both the outer and inner envelope membranes. Analysis by blue native gel electrophoresis revealed that the translocon of the inner envelope membranes consisted of at least six proteins with molecular weights of 36, 45, 52, 60, 100 and 110 kDa, respectively. Tic110 and ClpC, identified as components of the protein import apparatus of the inner envelope membrane, were prominent constituents of this complex. The amino acid sequence of the 52 kDa protein, deduced from the cDNA, contains a predicted Rieske-type iron–sulfur cluster and a mononuclear iron-binding site. Diethylpyrocarbonate, a Rieske-type protein-modifying reagent, inhibits the translocation of precursor protein across the inner envelope membrane, whereas binding of the precursor to the outer envelope membrane is still possible. In another independent experimental approach, the 52 kDa protein could be co-purified with a trapped precursor protein in association with the chloroplast protein translocon subunits Toc86, Toc75, Toc34 and Tic110. Together, these results strongly suggest that the 52 kDa protein, named Tic55 due to its calculated molecular weight, is a member of the chloroplastic inner envelope protein translocon.
Keywords: chloroplast/DEPC/import/inner envelope/precursor protein

Introduction

Although chloroplasts have maintained a functional genome, the vast majority of chloroplast proteins are nuclear encoded and are synthesized in the cytosol as precursor proteins (Chua and Schmidt, 1979; de Boer and Weisbeek, 1991). Accordingly, the targeting and translocation of chloroplast-destined precursor proteins plays a central role during biogenesis of the highly structured chloroplasts. The recognition and transport processes of the precursor proteins involve the cooperation of joint translocation sites localized in both the outer and inner envelope membranes (for reviews, see Fuks and Schnell 1997; Lübeck *et al.*, 1997). Very recently, a uniform nomenclature for proteins of the chloroplast protein import machinery has been introduced (Schnell *et al.*, 1997). Components comprising the outer envelope protein import apparatus are designated 'Toc' for translocon at the outer envelope membrane of chloroplasts with the calculated molecular mass as suffix. The components of the translocon of the inner envelope membrane are named 'Tic'.

The protein import machinery of the outer envelope membrane includes four components, Hsc70, Toc34, Toc75 and Toc86, which tightly associate with early translocation intermediates of a precursor protein (Waegemann and Soll, 1991; Hirsch *et al.*, 1994; Schnell *et al.*, 1994; Tranel *et al.*, 1995; Ma *et al.*, 1996). Translocation of the precursor protein probably proceeds simultaneously across the outer and inner envelope membranes (Schnell and Blobel, 1993; Alefsen *et al.*, 1994). The composition of joint translocation sites was examined under conditions which allow partial translocation, but not complete import of precursor proteins (Wu *et al.*, 1994; Kessler and Blobel, 1996; Lübeck *et al.*, 1996). By treatment of chloroplastic envelope membranes with detergents, membranous protein complexes were isolated, which contained the well-established Toc components and in addition the integral inner envelope protein Tic110 (Kessler and Blobel, 1996; Lübeck *et al.*, 1996). Furthermore, Tic110 was co-immunoprecipitated with antibodies against the precursor of the small subunit of ribulose bisphosphate carboxylase (pSSU) or Toc75 (Lübeck *et al.*, 1996; Akita *et al.*, 1997; Nielsen *et al.*, 1997), indicating that Tic110 might be involved in forming contact sites between the import machinery of the outer and inner envelope membranes. Tic110 can thus be considered as a marker for further investigations on potential constituents of the inner envelope protein translocon. Recently, antibodies against Tic110 immunoprecipitated a protein import complex containing a stromal Hsp100 (ClpC) that probably acts as a molecular chaperone for precursor proteins at the stromal site of inner envelope membranes (Akita *et al.*, 1997; Nielsen *et al.*, 1997). Due to the formation of a cross-linked product with different precursor proteins, further inner envelope proteins of 36 kDa (Schnell *et al.*, 1994), 44 kDa (Wu *et al.*, 1994; Ko *et al.*, 1995) and 21 kDa (Ma *et al.*, 1996) have been proposed to be involved in precursor translocation. However, the role of each of these putative components remains unclear. By analogy with mitochondria, the protein import apparatus of both the chloroplastic outer and inner envelope membranes most probably lie in close proximity during translocation of a precursor protein (Schnell and Blobel, 1993; Alefsen *et al.*, 1994; Schatz and Dobberstein, 1996). There is no evidence that the translocons of outer and inner envelope membranes can act independently from each other, although mitoplasts, which are devoid of the outer membrane, still recognize and translocate precursor proteins (Segui-Real *et al.*, 1993). A membrane potential $\Delta\Psi$,

which is necessary for initiation of precursor translocation across the mitochondrial inner membrane (Martin *et al.*, 1991), is not required at the chloroplastic inner envelope membrane (Flügge and Hinz, 1986). From this it was concluded that chloroplasts have developed a different mechanism for routing precursor proteins to the inner envelope membrane. At present, the exact interaction of the chloroplastic protein import complexes localized in the outer and inner envelope membranes remains unclear, particularly due to the lack of knowledge of the constituents and their function in protein translocation.

In this study, we employed independent experimental approaches to verify a 52 kDa protein as a novel component of the chloroplastic inner envelope import machinery. Blue native gel electrophoresis (BN PAGE) of protein complexes from isolated inner envelope membranes revealed that the 52 kDa protein builds up a protein complex with Tic110 and ClpC, both of which are known to be components of the chloroplastic protein import machinery. Furthermore, the 52 kDa protein was specifically co-purified with a poly(his) precursor protein trapped in the protein translocon. Due to its localization and its calculated molecular weight, we propose to name it Tic55. Surprisingly, Tic55 contains a Rieske iron–sulfur centre and a mononuclear iron-binding site. Translocation of precursor proteins into chloroplasts was diminished by diethylpyrocarbonate (DEPC) at the inner envelope membrane, probably via modification of the histidine residues of the Rieske iron–sulfur cluster.

Results

Isolation of a protein complex from inner envelope membranes containing Tic55 and Tic110

So far, components of the protein import apparatus of the inner envelope membrane have been identified by the use of chemical cross-linkers and/or by immunoprecipitation in the presence or absence of precursor proteins (Wu *et al.*, 1994; Kessler and Blobel, 1996; Lübeck *et al.*, 1996; Akita *et al.*, 1997; Nielsen *et al.*, 1997). These methods do not allow the definition of the composition of an unknown protein complex as a whole, because cross-linking reactions between single constituents may be hampered by chemical or conformational restrictions. On the other hand, the isolation of a native protein complex will shed new light on the assembly of the protein import apparatus of inner envelope membranes. Accordingly, we decided to use BN PAGE, a method which permits a reliable assessment of the number of components related to a protein complex (Schägger *et al.*, 1994; Dekker *et al.*, 1996; Jänsch *et al.*, 1996). Tic110, a component of the protein import apparatus of the inner envelope membrane (Kessler and Blobel, 1996; Lübeck *et al.*, 1996), served as a marker for the detection of proteins related to the protein import machinery of the inner envelope membrane. Different detergents such as digitonin, Triton X-100 or *n*-octyl maltoside have been employed for the preparation of mitochondrial membrane protein complexes by BN PAGE (Schägger *et al.*, 1994). We chose decyl maltoside to solubilize the chloroplastic envelope membranes, because this detergent has been successfully used to isolate the chloroplastic protein translocon (Lübeck *et al.*, 1996; Akita *et al.*, 1997; Nielsen *et al.*, 1997). The purified inner envelope membranes were selectively solubilized by 1–2% decyl maltoside, and the proteins were subsequently subjected to electrophoresis in the presence of Coomassie G-250 dye. The quality of resolution of the inner envelope protein complexes was best after solubilization with 1.3% decyl maltoside. BN PAGE allowed the separation of five dominant and several minor oligomeric complexes in the size range of $<$100 to 530 kDa in the first dimension (Figure 1A). The protein complexes were analysed further under denaturing conditions by Tricine SDS–PAGE. The five major bands represent protein complexes, which differ clearly in the composition of their subunits (Figure 1B, lanes R, 2, 4a, 6 and 7). Several components present in complexes R, 4 and 4a were identified by immunostaining, using antibodies against the large subunit of ribulose bisphosphate carboxylase (LSU), Tic110 and ClpC (Figure 1C). Complex R was characterized as the ribulose bisphosphate carboxylase holoenzyme, which consists of LSU, a 52 kDa protein and a 14 kDa protein, the small subunit (Figure 1B and C). Both subunits form a complex (L_8S_8) of ~530 kDa. Complex 4a has an apparent molecular weight of ~280 kDa and comprises at least six constituents with sizes of 110, 100, 60, 52, 45 and 36 kDa (Figure 1B). The proteins of 110 and 100 kDa were identified as Tic110 and ClpC, respectively (Figure 1C). Both Tic110 and ClpC have recently been described as constituents of the chloroplastic inner envelope protein import machinery (Kessler and Blobel, 1996; Lübeck *et al.*, 1996; Akita *et al.*, 1997; Nielsen *et al.*, 1997). These data indicate that the other components of complex 4a might also be involved in precursor translocation. Therefore we decided to analyse further the prominent protein of ~52 kDa (Figure 1B). Immunostaining with an antiserum raised against this protein (see Materials and methods) revealed that the 52 kDa protein occurs predominantly in fractions 4 and 4a, those which contain the main portion of Tic110 and ClpC (Figure 1C). Although it co-migrates with the LSU of ribulose bisphosphate carboxylase, the 52 kDa protein represents a distinct protein as shown by immunostaining (Figure 1C). After solubilization of the isolated inner envelope vesicles with decyl maltoside, Tic110 was co-immunoprecipitated with the antiserum against the 52 kDa protein (Figure 1D), corroborating the proposal that the 52 kDa protein and Tic110 reside in the same protein complex.

To determine the localization of the 52 kDa protein within the organelle, chloroplasts from pea, and, as a control, mitochondria from potato tubers, were fractionated. Different antisera against marker proteins of the outer and inner envelope membranes, thylakoids and stroma, or the mitochondrial total membrane fraction and matrix, respectively, were used to check the purity of the various fractions. Analysis by immunostaining revealed that the 52 kDa protein is exclusively localized in the enriched inner envelope membrane fraction (Figure 2A). Although the inner envelope fraction showed slight contamination with stromal and outer envelope proteins, the 52 kDa protein was not detected in fractions other than the inner envelope. Alkali treatment of the isolated inner envelope membranes at pH 11.5 showed that the 52 kDa protein behaves like an integral membrane protein (Figure 2B). Some minor cross-reaction with an unknown 23 kDa protein in the inner envelope membranes was observed.

Chloroplastic inner envelope membranes are isolated as outside-out vesicles, as demonstrated by identical proteolytic patterns of Tic110 *in organello* and *in vitro* (Lübeck *et al.*, 1996). Proteolysis of the 52 kDa protein in isolated inner envelope vesicles with various concentrations of trypsin or the less specific protease thermolysin yielded a major degradation product of ~47 kDa (Figure 2C). Although digestion with thermolysin and trypsin resulted in slightly different digestion patterns, both results indicate that only a minor part of the protein is exposed to the intermembrane space. As with the intact protein, the 47 kDa degradation products are not extractable by treatment with 0.1 M Na_2CO_3, pH 11.5 (data not shown). In comparison with Tic110, the amount of trypsin has to be ~10-fold higher to achieve proteolysis of the 52 kDa protein, indicating that most of the protein is either deeply buried within the lipid bilayer or protrudes mainly towards the stroma.

Molecular analysis of Tic55

The co-fractionation of two known components of the Tic complex, i.e. Tic110 and ClpC, with the so far unknown 52 kDa protein (Figure 1) prompted us to proceed with its molecular analysis. The N-terminus of the 52 kDa protein and internal peptides generated by digestion with the endoproteinase glu-C were sequenced. An oligonucleotide mixture derived from the N-terminal amino acid sequence was then used to isolate a cDNA clone of 1863 bp. The cDNA encodes a protein of 553 amino acids, which contains all the peptide sequences from the N-terminus or from the proteolytic fragments, demonstrating that the isolated cDNA encodes the 52 kDa protein of complex 4a (Figure 3A). The N-terminal amino acid sequence data obtained from endogenous 52 kDa protein (AADVKDATLLDGEEDQKVLV) indicate that the protein is made as a larger precursor protein containing a transit peptide of 60 amino acids as deduced from the cDNA. This transit peptide was sufficient to mediate import into chloroplasts and the precursor was processed to its mature form (data not shown). The protein has a calculated molecular weight of 55 kDa and we therefore name it Tic55. Analysis of the deduced amino acid sequence (Claros and von Heijne, 1994) suggests the presence of two membrane-spanning α-helices at the C-terminus, which might anchor Tic55 to the chloroplastic inner envelope membrane (Figure 3A), whereas the N-terminal portion probably consists of amphiphilic β-sheets. By searching sequence databases, a highly conserved Rieske-type iron–sulfur cluster and a mononuclear iron-binding site, characteristic for bacterial aromatic ring-hydroxylating dioxygenases, were discovered at amino acid positions 142–175 and 248–264. The primary structure of Tic55 shows the highest similarity to the Rieske centre- and a mononuclear iron-binding site of LLS1 from maize (Gray *et al.*, 1997), its homologue from arabidopsis and a hypothetical 50 kDa protein from synechocystis. The Rieske centre- and the mononuclear iron-binding site of the choline monooxygenase from spinach

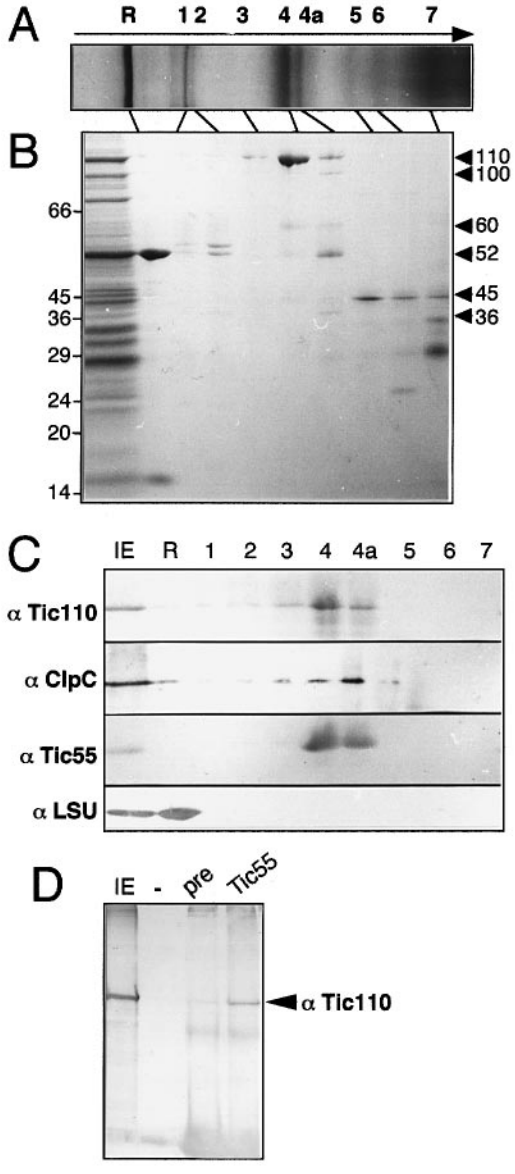

Fig. 1. Two-dimensional resolution of inner envelope membrane protein complexes from pea chloroplasts by BN PAGE and Tricine SDS–PAGE. (**A**) Inner envelope membranes equivalent to 200 μg protein were solubilized with 1.3% decyl maltoside and separated by BN PAGE on a gradient gel of 4% to 14% acrylamide. The designations at the top indicate the numbers of the complexes, starting with complex 1 at top of the gel and continuing to complex 7 at the bottom (R: ribulose bisphosphate carboxylase holoenzyme at ~530 kDa). (**B**) Single complexes were subjected to Tricine SDS–PAGE and stained with Coomassie brilliant blue. A separation of the chloroplastic inner envelope proteins (20 μg) is shown on the left side of the samples. The molecular masses of standard proteins are indicated on the left. On the right, the mobilities of the constituents of complex 4a are indicated by arrowheads and their apparent molecular weights. (**C**) In a parallel experiment protein complexes separated on Tricine SDS–PAGE were used for immunoblotting. The filter was cut into strips and decorated with antibodies against Tic 110, ClpC, Tic55 and the LSU of ribulose bisphosphate carboxylase, respectively. A separation of the inner envelope proteins (10 μg) is shown on the left (IE). (**D**) Co-immunoprecipitation of Tic110 with an antiserum against Tic55. Inner envelope membranes equivalent to 200 μg protein were solubilized with 1.3% decyl maltoside. Insoluble material was removed by centrifugation. Twenty-five μl of an antiserum against Tic55 was used for immunoprecipitation of the solubilized proteins at 4°C. IgGs against Tic55 were recovered by Protein A–Sepharose and the associated proteins were analysed under denaturing conditions on SDS–PAGE followed by immunostaining with an antiserum against Tic110 (lane Tic55). Inner envelope proteins (10 μg) are separated on the left (IE). As a control, Protein A–Sepharose (–) was incubated with solubilized inner envelope proteins. Furthermore, the preimmune serum (pre) does not co-immunoprecipitate Tic110.

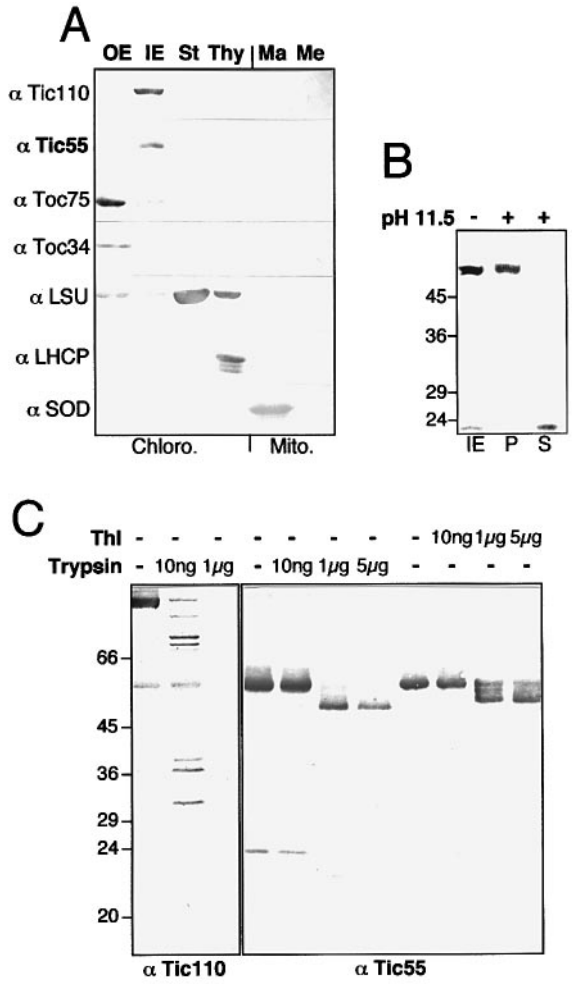

Fig. 2. Tic55 is an integral protein in the inner envelope membrane of pea chloroplasts. (**A**) Immunoblot analysis of the distribution of Tic55 in pea chloroplast subcompartments. Each lane contained outer envelope membranes (OE), inner envelope membranes (IE), stroma (St) and thylakoid membranes (Thy), respectively, equivalent to 10 μg of protein or 20 μg protein of a matrix (Ma) and a membrane (Me) fraction of mitochondria prepared from potato tuber. The purity of the fractions was checked by immunostaining with antisera against marker proteins of the isolated fractions: Tic110, inner envelope; Toc75, Toc34, outer envelope; LSU, stroma; light-harvesting chlorophyll a/b binding protein (LHCP), thylakoid membrane; superoxide dismutase, matrix. (**B**) Inner envelope membranes (20 μg protein) were extracted with 0.1 M Na_2CO_3, pH 11.5. The insoluble protein (P) and the soluble fraction (S) were subjected to SDS–PAGE. An immunodecoration with Tic55 is shown. (**C**) Isolated inner envelope membrane vesicles (20 μg protein) were treated with different amounts of thermolysin (Thl) or trypsin (μg per mg envelope proteins). The proteolytic pattern of Tic55 was compared with that of Tic110 after immunostaining, using antisera against Tic55 and Tic110.

(Rathinasabapathi *et al.*, 1997) share fewer identical amino acids with the amino acid sequence of Tic55 (Figure 3B). The absorption spectrum of the recombinant N-terminal part (24 kDa, see Materials and methods) of Tic55 showed a broad peak at 470 nm with a shoulder around 566 nm and a larger peak at 326 nm (Figure 3C). In contrast to its oxidized form, the intensities of the absorption at 326 nm decreased clearly upon reduction with a 2-fold molar excess of DTT in the presence of 50 μM methyl viologen. The absorption maximum at 470 nm shifted to 460 nm and decreased also. Similar results have been observed for bacterial Rieske-type oxygenases (Mason and Cammack, 1992), indicating that the recombinant 24 kDa peptide of Tic55 contained the iron–sulfur cluster. Furthermore, isolated inclusion bodies are coloured brown.

Inhibition of in vitro import of pSSU by DEPC

DEPC inhibits the electron transfer activity of mitochondrial complex III by ethoxyformylation of the histidine residues involved in forming the Rieske iron–sulfur cluster (Ohnishi *et al.*, 1994). We assayed the effect of DEPC on import of pSSU into chloroplasts, to investigate the question whether a functional histidine residue, e.g. a Rieske iron–sulfur protein, could be involved in the translocation of precursor proteins. Intact chloroplasts were treated with various concentrations of DEPC after binding of the precursor protein to the chloroplastic outer envelope membrane (Figure 4A, lanes 3–8). pSSU binding was permitted at low temperature in the presence of 3 mM ATP. Under these conditions pSSU partially enters the translocation machinery, as demonstrated by the appearance of translocation intermediates Tim3 and Tim4 upon thermolysin treatment (Figure 4B, lanes 1 and 2) (Waegemann and Soll, 1991, 1996). After increasing the temperature, pSSU is completely translocated into the organelle. There, it is processed to the mature form and becomes resistant to proteolytic treatment (Figure 4B, lanes 3 and 4). Much less pSSU could be chased into the processed mature form if the chloroplasts were treated with DEPC after binding of the precursor protein (Figure 4A, compare lanes 4, 6 and 8). The fact that the translocation intermediates Tim3 and Tim4 were still observed, indicates that DEPC had inhibited further translocation of the precursor. Both Tim3 and Tim4 have been shown to co-fractionate with the inner envelope membranes (Waegemann and Soll, 1993), thus indicating that pSSU interacts with the translocons of both the outer and inner envelope membranes, at this stage of precursor translocation. These results suggest that DEPC exerts its effect at the inner envelope membrane. To obtain further evidence for this notion, chloroplasts were pretreated with DEPC and recovered by centrifugation. Translocation of pSSU was then assayed at 25°C in the presence of 3 mM ATP. Again, the yield of translocation was drastically diminished and the translocation intermediates Tim3 and Tim4 accumulated, whereas no effect on binding efficiency was observed (Figure 4B, lanes 5 and 6). Pretreatment of precursor protein with DEPC had no influence on the translocation efficiency of pSSU (Figure 4C). From these data we conclude that DEPC operates at the level of the Tic complex.

Tic55 is a component of a precursor containing protein import complex

Precursor translocation occurs simultaneously through the outer and inner envelope membranes, most probably mediated by joint translocation sites. To elucidate the composition of the translocation apparatus during import, recombinant pSSU containing a histidine tag at its C-terminus was used as precursor protein. Components interacting with the precursor protein at different stages of import should be co-isolated during affinity purification of pSSU-His_6. For this purpose, chloroplasts were incubated in the presence or absence of ^{35}S-labelled pSSU-

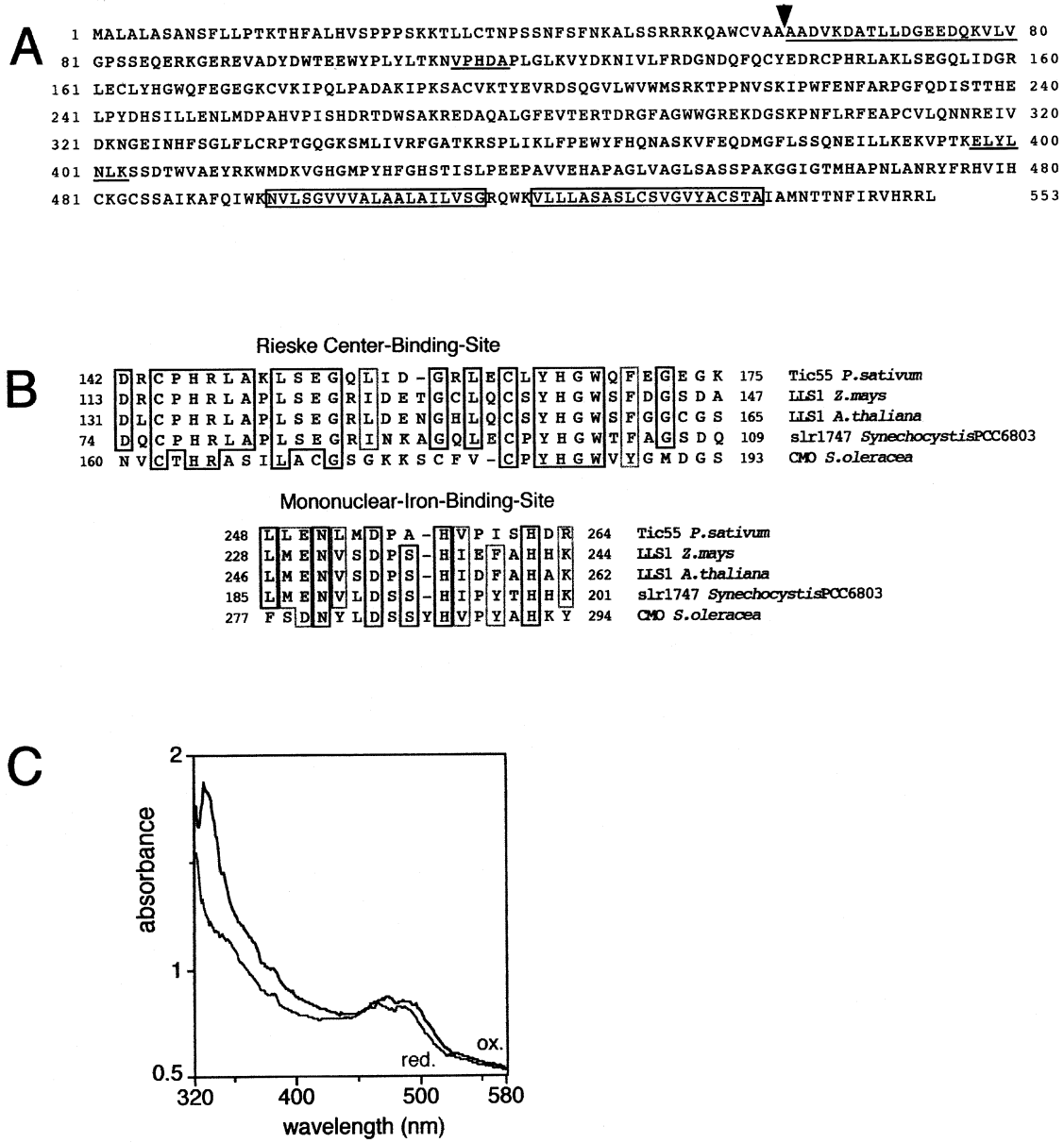

Fig. 3. Amino acid sequence of Tic55 and the alignment of two iron-binding sites with Rieske [2Fe–2S]- and mononuclear iron-binding regions of homologous proteins. (**A**) The amino acid sequence for the precursor Tic55 as deduced from the cDNA is shown. These sequence data have been submitted to the EMBL database under accession number AJ000520. The N-terminus of the mature Tic55 determined by amino acid sequencing of endogenous Tic55 is indicated by an arrowhead. Peptide sequences of the N-terminus or of internal peptides generated by the endoprotease glu-C treatment are underlined. The predicted membrane-spanning domains are boxed. (**B**) The Rieske [2Fe–2S]-centre and the mononuclear iron-binding region of Tic55 are compared with regions of the LLS1 protein of *Zea mays* (acc. no. U77345), its homologue of *Arabidopsis thaliana* (acc. no. H36617), a hypothetical protein (slr1747) of *Synechocystis* PCC6803 (acc. no. D60909), and a choline monooxygenase (CMO) of *Spinacia oleracea* (acc. no. U85780). Residues conserved in at least four sequences are boxed. Numbers on the left and right sides indicate the localization of the oligopeptide within the amino acid sequence. (**C**) Optical spectrum of Tic55 in the oxidized (ox.) and the reduced (red.) state. An optical spectrum (320–580 nm) of recombinant Tic55 (40 μg/ml) was taken in its oxidized state or in the reduced state in the presence of 5 μmol DTT and 50 μM methyl viologen. Samples were degassed with helium before measurement.

His_6, either under binding conditions at 2°C, or followed by a warming-up at 25°C for 2 min. After hypotonic lysis of the chloroplasts, total membranes were solubilized with decyl maltoside and subsequently allowed to bind to Ni-NTA agarose. The agarose was stringently washed, and pSSU-His_6, together with the adhering proteins, were eluted with sample buffer containing EDTA and SDS. The flow-through fraction, the last washing step and the eluate were examined by immunostaining, using antisera against proteins localized in different chloroplastic compartments. Toc86, Toc75 and Toc34, which are well-characterized components of the Toc complex, co-purified almost exclusively in the presence of precursor protein (Figure 5A, compare lanes 3, 6 and 9), whereas OEP24 and OEP16, which are not related to the general import pathway (Pohlmeyer *et al.*, 1997; K.Pohlmeyer and J.Soll, unpublished results), were not observed (Figure 5A, compare lanes 'ft' and 'e'). Tic110, a constituent of the Tic complex, was clearly enriched in the eluate containing precursor protein (Figure 5A and B). As with the components of the import machinery described above, Tic55 was recovered only in the presence of precursor protein

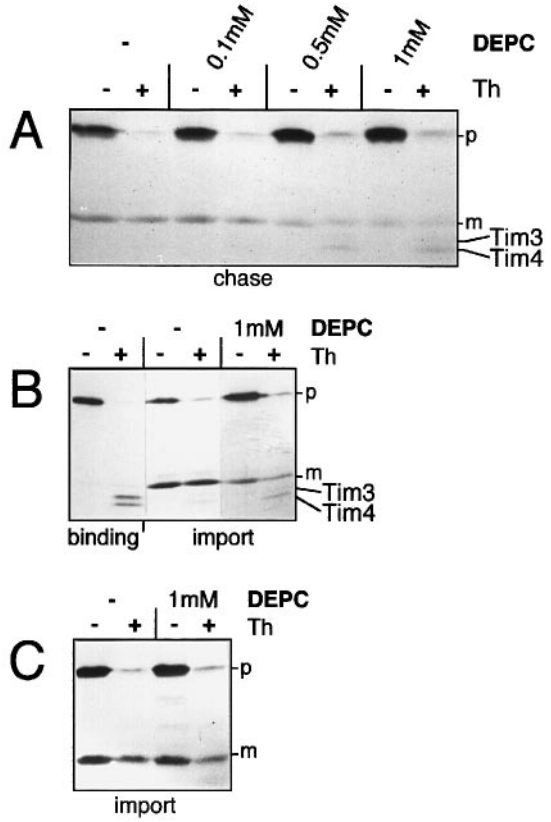

Fig. 4. Import of a chloroplastic precursor protein into isolated chloroplasts is inhibited by DEPC. (**A**) After binding of the precursor protein to chloroplasts (equivalent to 30 μg chlorophyll) in the presence of 3 mM ATP at 2°C, the organelles were treated with 0.1–1 mM DEPC. The chloroplasts were re-isolated and subsequently translocation of precursor protein was allowed to take place for 5 min at 25°C. Thermolysin (Th) treatment (100 μg/mg chlorophyll) was done after recovering the organelles by centrifugation. Translocation intermediates (Tim3 and Tim4) accumulated with increasing concentrations of DEPC. (**B**) Translocation intermediates appear to be temperature- and DEPC-dependent. Partial translocation of pSSU occurs under binding conditions at 2°C in the presence of 3 mM ATP. Raising the temperature to 25°C allows complete translocation of pSSU and processing to its mature form (import). Treatment of chloroplasts before import of the precursor protein resulted in the accumulation of translocation intermediates during translocation in a similar manner to that observed under binding conditions. (**C**) pSSU was treated with 1 mM DEPC before import. The reaction of DEPC was stopped by the addition of 1 mM imidazole. Import of the precursor protein was performed at 25°C for 5 min. 'p', precursor protein; 'm', mature, processed protein.

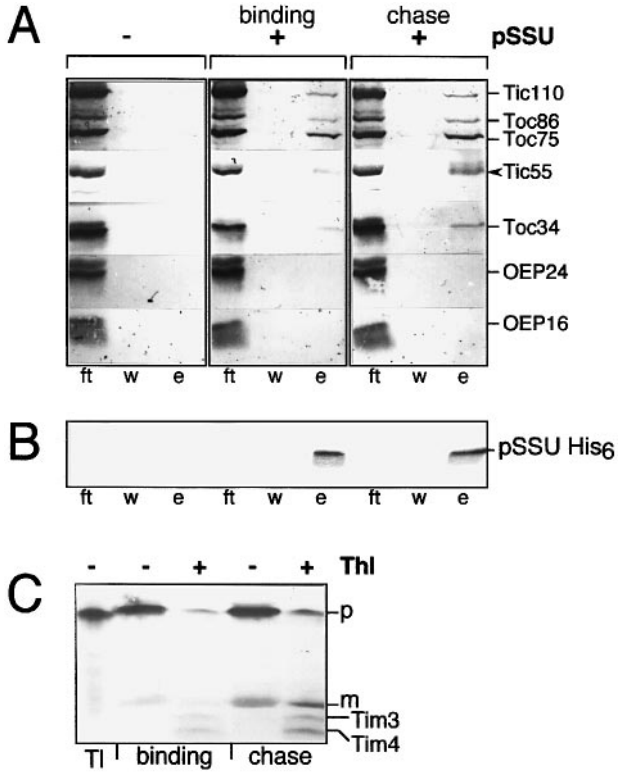

Fig. 5. Tic55 is a constituent of the chloroplastic protein import apparatus. (**A**) Chloroplasts equivalent to 600 μg chlorophyll were incubated with 2 μg recombinant ^{35}S-labelled pSSU-His$_6$ in the presence of 3 mM ATP for 10 min at 2°C (binding) followed by a warm-up for 2 min at 25°C (chase). After lysis of the re-isolated chloroplasts, total membranes were recovered and then treated with 1.3% decyl maltoside. Solubilized proteins were incubated with 60 μl Ni-NTA–agarose for 1 h at 4°C. 10% of the flow-through (ft), the supernatant of the last washing step (w) and the eluate (e) were analysed by SDS–PAGE and immunostaining with antisera raised against Tic110, Tic55, Toc86, Toc75, Toc34 and the outer envelope proteins OEP24 and OEP16, respectively. As a control, chloroplasts equivalent to 600 μg chlorophyll were treated as described above but without the addition of precursor protein. An increasing amount of Tic110, Tic55, Toc86, Toc75, Toc34 was observed in the presence (binding, chase) but not in the absence (–) of precursor protein (compare lanes 'e'). The experiment was repeated ten times with similar results. All the panels shown were taken from the same experiment. (**B**) ^{35}S-labelled pSSU-His$_6$ is recovered quantitatively after eluting the Ni-NTA–agarose with sample buffer containing 10 mM EDTA and SDS. After immunostaining (A) the nitrocellulose membrane was exposed to an X-ray film for detection of the radiolabelled precursor protein. (**C**) The import of recombinant ^{35}S-labelled pSSU-His$_6$ in isolated chloroplasts was carried out under conditions as described in (A). After import of the precursor, chloroplasts were treated with thermolysin (Thl, 100 μg/mg chlorophyll) and subjected to SDS–PAGE. An X-ray film is shown. Under binding conditions the precursor (p) was hardly processed at all to its mature form (m); instead, translocation intermediates (Tim3 and Tim4) appeared after treatment with protease (lane 3). Warming up the import reaction to 25°C (chase) resulted in an increase of translocation intermediates and mature SSU.

(Figure 5A and B, compare lanes 3, 6 and 9). Under binding conditions, processing of pSSU to its mature form hardly occurred at all. Instead, translocation intermediates became visible after protease treatment, indicating that pSSU was involved in interaction with the Toc and Tic complex (Figure 5C, binding). The chase period resulted in increasing amounts of translocation intermediates as well as mature SSU, suggesting that more pSSU had moved from a binding site to a partially translocated state (Figure 5C, chase). The amounts of precursor-associated Toc and Tic components recovered under chase conditions increased (Figure 5A, chase), indicating either that more translocation sites are involved or that the joint translocation sites formed are more stable to the isolation procedure, possibly due to the recruitment of so far unknown components of the import machinery. One critical control is the demonstration that proteins only bind to the Ni-NTA agarose as a result of their specific interaction with pSSU-His$_6$. Poly(his) pSSU was quantitatively recovered in the eluate (Figure 5B), indicating that the Toc and Tic proteins were isolated by Ni-NTA affinity chromatography due to their specific interaction with the poly(his) pSSU. This was supported by our finding that only insignificant traces of the proteins relevant to import, namely Tic110 and

Toc75, were recovered from the Ni-NTA matrix in the absence of pSSU (Figure 5A, –pSSU, lane 'e'). Mature poly(his) SSU was never detected under these conditions (data not shown), although processing occurs under chase conditions (Figure 5C, chase). Probably the C-terminal poly(his) tag is not accessible due to its movement into the protein import machinery. The light-harvesting chlorophyll a/b binding protein, which is a major thylakoid protein, did not bind to Ni-NTA agarose and was not co-purified with the precursor protein. These data support the concept that Tic55 interacts specifically with the precursor protein or acts as a component of the protein import apparatus.

Discussion

In this study, BN PAGE was employed to analyse the composition of the protein import apparatus of the chloroplastic inner envelope membranes. Resolution of the respiratory chain protein complex and the protein translocase of mitochondria from mammals, plants or fungi have revealed that BN PAGE is a powerful tool for defining the composition of membrane protein complexes (Schägger *et al.*, 1994; Dekker *et al.*, 1996; Jänsch *et al.*, 1996). Antibodies against Tic110 and ClpC, two proteins which were recently identified as constituents of the protein import machinery, served as markers for the detection of a protein complex that could be involved in translocation of chloroplastic precursor proteins. In this way we identified Tic55 as a constituent of a protein complex comprising Tic110, ClpC and proteins with sizes of ~60, 45 and 36 kDa. The apparent molecular weight of the single constituents taken together exceeds clearly the apparent molecular weight (280 kDa) of complexes 4 and 4a. The difference between apparent complex molecular weight and the summation of individual components is probably due to a pI effect (pI >5.4: Tic110, Tic55, ClpC) (Schägger *et al.*, 1994).

Other groups have used various cross-linking approaches to examine the chloroplast protein import machinery of the outer and inner envelope membranes. Only those translocation components in close proximity to a trapped precursor protein could be detected either by label-transfer or cross-linking reagents (Perry and Keegstra, 1994; Wu *et al.*, 1994; Ma *et al.*, 1996; Akita *et al.*, 1997; Nielsen *et al.*, 1997). Apart from Tic110 and ClpC, Cim/Com44 and two proteins of 36 and 21 kDa were described as yielding cross-linked products with a precursor protein, although their function in precursor translocation still remains unclear (Schnell *et al.*, 1994; Wu *et al.*, 1994; Ma *et al.*, 1996). However, the generally low yields found in cross-linking experiments hamper the identification and molecular characterization of individual constituents. The assumption that Cim/Com44 and the 36 kDa protein might be identical to those proteins observed in the complex isolated by BN PAGE is tempting, but remains to be established. In addition, complexes recovered after immunoprecipitation frequently contain proteins derived from the antiserum or IgGs (Kessler and Blobel, 1996; Akita *et al.*, 1997), which disturb the analysis of proteins in the 50–60 kDa range. In particular, Tic55 would not be detected as a component of the complex due to its apparent molecular weight of 52 kDa, thereby co-migrating with the heavy chain of IgGs. Thus, BN PAGE represents an additional tool to explore the composition of the protein import machinery.

The simple association of Tic55 with Tic110 and ClpC as described by BN PAGE and co-immunoprecipitation may not reflect an involvement of the protein in precursor protein translocation. In another independent approach, we examined whether Tic55 interacts with a precursor protein or with other known components of the protein import machinery of intact chloroplasts. Accordingly, the translocation of a precursor with a histidine tag at its C-terminus was decelerated by decreasing the temperature, as protein import has been shown to take place ~10-fold more slowly at 5°C than at 25°C in the presence of ATP (Leheny and Theg, 1994). Proteins associated with the precursor were co-purified by Ni-NTA affinity chromatography. In this study, Tic55 was isolated together with Tic110, Toc34, Toc75 and Toc86, which are known constituents of the import complex, whereas proteins of the outer envelope membrane such as OEP16 and OEP24 or the light-harvesting chlorophyll a/b binding protein of the thylakoids were not present on this complex. Other workers have trapped the precursor by complexing a C-terminal histidine-tag with Ni-NTA or by binding a biotin–streptavidin complex, but so far the composition of an associated protein complex has not been described (Froehlich and Keegstra, 1997; Rothen *et al.*, 1997).

In a third approach to establish Tic55 as a bona fide subunit of the inner envelope translocon, we investigated the effect of DEPC on pSSU translocation. DEPC has been described as inhibiting the electron transfer activity of mitochondrial complex III by modification of the Rieske iron–sulfur protein through ethoxyformylation of iron-complexing histidine residues. The efficiency of precursor translocation clearly decreased at concentrations higher than 0.5 mM DEPC, indicating that a functional histidine residue is involved in protein translocation. The outer and inner envelope membranes do not contain any cytochromes, but do contain several iron–sulfur proteins (Jäger-Vottero *et al.*, 1997). It follows that inhibition of precursor translocation could occur due to the modification of a Rieske-type iron–sulfur protein. Furthermore, we could demonstrate that inhibition of precursor translocation by DEPC occurs at a later step of import, most probably at the inner envelope membrane. Although inhibition of precursor translocation by DEPC represents a circumstantial indication, these data support the contention that Tic55 is involved in protein import. Based upon these independent lines of evidence we conclude that Tic55 is not only associated with Tic110 in isolated inner envelope vesicles, but is also part of the protein import apparatus in the presence of precursor protein. In addition, we demonstrated that precursor protein, Toc86, Toc75, Toc34, Tic110 and Tic55 can be isolated as a core protein translocation complex without the use of any cross-linking reagents.

The deduced amino acid sequence of Tic55 exhibits ~30% identity to LLS1, which functions as a suppressor of cell death in plants (Gray *et al.*, 1997). Tic55 also shows this level of identity compared with a hypothetical 50 kDa protein from synechocystis. These proteins have two highly conserved consensus motifs in common, a Rieske-type iron–sulfur cluster and a mononuclear iron-

binding site. These domains have been characterized for the α-subunit of bacterial aromatic ring-hydroxylating dioxygenases (Mason and Cammack, 1992; Jiang *et al.*, 1996). Aromatic ring-hydroxylating dioxygenases are soluble, multicomponent enzymatic systems, whose subunits function as electron transport chains containing flavin and iron–sulfur [2Fe–2S] redox centres and a terminal oxygenase. The latter, the catalytic subunit, hydroxylates an aromatic substrate in the presence of oxygen and Fe^{2+} (Mason and Cammack, 1992). In contrast to the soluble terminal oxygenase, Tic55 behaves like an integral membrane protein. Furthermore, Tic55 shows less amino acid sequence homology to a chloroplastic protein (Rathinasabapathi *et al.*, 1997), which also contains these two non-haem iron-binding motifs and functions as choline monooxygenase in the stroma. Therefore, it seems unlikely that Tic55 still functions as a terminal aromatic ring-hydroxylating oxygenase, but rather as a regulating subunit of the inner envelope translocon. This notion is supported by recent findings that a number of proteins that use iron–sulfur clusters as prosthetic groups act as biosensors (Hidalgo *et al.*, 1997). Allosteric changes in the protein structure of the transcription factor SoxR, which are only mediated by alteration of the oxidation state of the [2Fe–2S] iron–sulfur cluster, regulate the activation of several genes in response to superoxide-generating agents. LLS1, which also contains a Rieske-type iron–sulfur cluster, has been suggested to function as a kind of rheostat for regulation of cell death in maize (Gray *et al.*, 1997). We argue that Tic 55 belongs to a new family of Rieske-type iron–sulfur proteins, which fulfil various new tasks besides electron transfer. A function of Tic55 in signal transduction or redox regulation during protein import is conceivable, as the protein import machineries of the outer and inner envelope membranes have to cooperate during precursor translocation.

Materials and methods

Isolation of chloroplasts and inner envelope membranes

Intact chloroplasts from 12- to 14-day-old pea plants (*Pisum sativum* L. var. Golf) were isolated as described previously (Waegemann and Soll, 1991). After rupturing the organelles by 40 strokes with a Dounce homogenizer (Kontes Instruments, Veneland), the chloroplast inner and outer envelope membranes were purified by sucrose density centrifugation (Keegstra and Yousif, 1986; Waegemann *et al.*, 1992).

Chloroplast protein import assays

Isolation of chloroplasts and the standard import assays were carried out as described. Import was performed at 25°C for 5 min. Organelles were re-isolated by centrifugation, and samples were analysed by SDS–PAGE and fluorography (Waegemann and Soll, 1991). If necessary, chloroplasts were pretreated before import or after binding of the precursor protein with 0.1–1 mM DEPC in wash I (330 mM sorbitol, 3 mM $MgCl_2$, 50 mM HEPES, pH 7.6) for 10 min at 4°C in the dark. After re-isolation of the organelles in wash I, import was performed in 100 µl import-mix [330 mM sorbitol, 3 mM $MgCl_2$, 50 mM HEPES, pH 7.6, 10 mM methionine, 10 mM cysteine, 20 mM potassium gluconate, 10 mM $NaHCO_3$, 2% BSA (w/v), 3 mM ATP].

Purification of a protein import complex by a his-tagged precursor protein

^{35}S-labelled pSSU-His_6 was synthesized in *Escherichia coli* BL21 DE3 in the presence of [^{35}S]methionine/cysteine and isolated from inclusion bodies (Waegemann and Soll, 1995). Chloroplasts equivalent to 600 µg chlorophyll in 900 µl import-mix were incubated with 2 µg ^{35}S-labelled pSSU-His_6 either for 10 min at 2°C (binding conditions) or followed by warming up the reaction for 2 min at 25°C (chase). After re-isolation, chloroplasts were lysed hypotonically in 3 ml 10 mM HEPES, pH 7.6, 0.5 mM PMSF for 30 min at 4°C. A fraction containing outer and inner envelope membranes and thylakoid membranes was recovered by centrifugation for 15 min at 100 000 *g*. The pellet was solubilized in 1 ml buffer I [100 mM NaH_2PO_4, 10 mM Tris, pH 8.0, 1.3% (w/v) decyl maltoside, 8 mM imidazole, 0.5 mM PMSF] for 10 min at 4°C. The insoluble material was removed by centrifugation for 10 min at 100 000 *g*. Solubilized proteins were incubated for 1 h at 4°C with 60 µl Ni-NTA agarose (Diagen, Hilden, Germany), which had been equilibrated with buffer I in the presence of 0.05% (w/v) lactalbumin. The agarose was washed four times with a 5-fold volume of buffer I at pH 6.8, followed by elution of the protein with sample buffer containing 10 mM EDTA and SDS. 10% of the flow-through, the entire final washing fraction and the eluate were subjected to SDS–PAGE and Western-blotting. The filters were cut into strips and incubated with different antibodies raised against proteins localized in the outer and inner envelope membrane, the thylakoids and the stroma. Visualization of immunopositive bands was performed with biotinylated antibodies, avidin and alkaline phosphatase (Vector Laboratories, Burlingame, USA).

As a control, chloroplasts equivalent to 60 µg chlorophyll were incubated with 0.2 µg ^{35}S-labelled pSSU-His_6 under the same conditions. The samples were analysed by SDS–PAGE and exposed to an X-ray film.

BN PAGE

Chloroplastic inner envelope membranes (equivalent to 200 µg protein) were solubilized in 100 µl 1.3% decyl maltoside, 600 mM 6-aminocaproic acid, 40 mM Bis-Tris–Cl, pH 7.0 for 10 min on ice. The insoluble material was removed by centrifugation for 10 min at 100 000 *g*. 5% Coomassie blue G-250 in 500 mM 6-aminocaproic acid was added to give a detergent:Coomassie ratio of 8:1 (w/w). The protein complexes were separated on a gradient gel from 4% to 14% polyacrylamide. BN PAGE was performed as described elsewhere (Schägger *et al.*, 1994). Single bands were cut from the native blue gel and subjected to Tricine SDS–PAGE (Schägger and von Jagow, 1987).

Isolation of a cDNA clone for Tic55

Protein complexes of the inner envelope membranes were separated by BN PAGE and Tricine SDS–PAGE as described above. Proteins blotted onto PVDF membranes were sequenced either from the N-terminus of the protein or after digestion with endoproteinase glu-C (Boehringer-Mannheim, Germany) followed by separation on HPLC. A degenerate oligonucleotide (GAYGGNGARGAYCARAARGT, 256 combinations) was derived from the N-terminal amino acid sequence. The [γ-^{32}P]ATP-labelled oligonucleotide mixture was used for screening a cDNA expression library (Uni Zap XR, Stratagene, USA) made from poly $(A)^+$ RNA of 5-day-old light-grown pea seedlings (*P.sativum* L. var. Golf). One full-length cDNA clone of 1863 bp coding for a protein of 553 amino acids was isolated. DNA cloning and sequencing were performed according to standard procedures (Sambrook *et al.*, 1989). Database searches were performed with TBLASTN and BLAST & BEAUTY (Worley *et al.*, 1995), and sequence alignments with CLUSTALW 1.6 (Thompson *et al.*, 1994) at the BCM Search Launcher (Houston, USA).

Raising of antibodies

For producing an antiserum against a N-terminal portion of Tic55, a nucleotide fragment of G_{226} to T_{765} was subcloned into the pQE60 vector (Diagen) using a *Bam*HI and a *Nco*I restriction site, which was introduced at G_{226} by PCR. The plasmid was transformed to *E.coli* SG13009[pREP4] (Diagen) and the expressed 24 kDa peptide, comprising the first 194 amino acids of the mature protein, was isolated from inclusion bodies.

Miscellaneous

Trypsin or carbonate treatment of isolated inner envelope vesicles, SDS–PAGE and Western blotting and immunoprecipitation were performed according to our published procedures (Waegemann and Soll, 1995; Lübeck *et al.*, 1996).

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