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Import of a new chloroplast inner envelope protein is greatly stimulated by potassium phosphate

Stephan Hirsch and Jürgen Soll*
Botanisches Institut, Universität Kiel, 24098 Kiel, Germany (author for correspondence)*

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Abstract

A cDNA clone encoding a major chloroplast inner envelope membrane protein of 96 kDa (IEP96) was isolated and characterized. The protein is synthesized as a larger-molecular-weight precursor (pIEP96) which contains a cleavable N-terminal transit sequence of 50 amino acids. The transit peptide exhibits typical stromal targeting information. It is cleaved *in vitro* by the stromal processing peptidase, though the mature protein is clearly localized in the inner envelope membrane. Translocation of pIEP96 into chloroplasts is greatly stimulated in the presence of 80 mM potassium phosphate which results in an import efficiency of about 90%. This effect is specific for potassium and phosphate, but cannot be ascribed to a membrane potential across the inner envelope membrane. Protein sequence analysis reveals five stretches of repeats of 26 amino acids in length. The N-terminal 300 amino acids are 45% identical (76% similarity) to the 35 kDa α-subunit of acetyl-CoA carboxyl-transferase from *Escherichia coli*. The C-terminal 500 amino acids share significant similarity (69%) with USOI, a component of the cytoskeleton in yeast.

Abbreviations: P_i, phosphate; IEP, inner envelope membrane protein; pIEP, precursor form of IEP; SSU, small subunit of ribulose-1,5-bisphosphate carboxylase oxygenase; $\alpha IEP96_{pep}$, peptide specific antiserum to IEP96; $\alpha IEP96_{pol}$, polyspecific antiserum to IEP96

Introduction

Plastid biogenesis and differentiation is governed by a number of environmental and plant derived signals. In spite of varying functions and internal plastid structure the organelles are always surrounded by the outer and inner envelope membranes [15]. These membranes have essential biosynthetic capacity, for example in lipid and prenylquinone biosynthesis [15, 29]. The inner envelope membrane forms in addition a permeability barrier for most low-molecular-weight sub-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide, Sequence Databases under the accession number Z31559.

stances, which are transported consequently by specific carriers [8, 15]. Most of the polypeptide constituents of the plastids are nuclear-coded [4, 6, 15], synthesized in the cytosol and post-translationally imported into the organelle [6, 10, 31, 32]. The outer and the inner envelope membrane cooperate in this process, which involves a number of proteins from each membrane [9, 30, 36].

Though distinct biological and biochemical functions have been ascribed to the outer and inner envelope membranes only a few out of about 150–200 proteins have been purified or identified on a molecular level [8, 13, 20]. In an attempt to characterize further single proteins of the envelope membranes, a λgt11 cDNA library was screened with antisera against envelope proteins. In the course of the work a cDNA clone for an inner envelope membrane protein of 96 kDa (IEP96) was isolated. The IEP96 shows striking homology to the 35 kDa α-subunit of acetyl CoA carboxyl-transferase from *E. coli* [18, 21] over almost the entire length of the carboxyl-transferase. The translocation efficiency of pIEP96 is greatly and specifically enhanced in the presence of 80 mM potassium phosphate which in contrast is already inhibitory to the translocation of other precursor proteins, such as the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (pSSU).

Material and methods

cDNA cloning and in vitro *transcription-translation*

A polyclonal antiserum raised in rabbits against OEP 86 was used to screen a λgt11 library, made from dark-grown pea leaves. About 500000 plaques were screened. Two different isolates were obtained. One was identified as coding for OEP 86 [12], the second, pisa 96, was subcloned into the vector Bluescript (Genofit, Geneva) and sequenced directly [27]. Both strands were sequenced. The 5′-untranslated region of pisa 96 was deleted by PCR to obtain improved transcription-translation and subcloned into the pet17b plasmid vector (Novagen, Madison, WI). Plasmids containing pisa 96 DNA were isolated from large plasmid preparations after alkaline lysis and further purified by CsCl density centrifugation [26]. The purified plasmids were linearized by *Sal* I and transcribed using T7 RNA polymerase followed by translation in a reticulocyte lysate system (New England Nuclear) in the presence of [^{35}S]-methionine [25].

In vitro *import reactions*

Chloroplasts were isolated and purified from pea leaves by standard procedures [36]. Binding and import experiments were carried out in the dark under green safety light to avoid synthesis of ATP by photophosphorylation. Chloroplasts, equivalent to 15 μg chlorophyll, were used in a 100 μl standard import reaction for 15 min at 25 °C [36]. The amount of reticulocyte lysate never exceeded 5% of the total import reaction volume. ATP was supplemented at different concentrations, for example 50–100 μM ATP for binding, 3 mM ATP for translocation [24, 36]. Organelles were repurified through silica sol gradients after completion of the translocation reaction and if neccessary treated with the protease thermolysin [5, 14]. Chloroplasts were recovered by centrifugation, washed once and finally subjected to SDS-PAGE [19]. Radioactive proteins were visualized by fluorography.

Subfractionation of chloroplasts

Chloroplasts according to 200 mg of chlorophyll were lysed in hypertonic buffer solution by 50 strokes in a dounce homogenizer [16]. Intact organelles and most of the thylakoids were removed by low-speed centrifugation (1500 × *g*) for 10 min, washed twice and used as thylakoid preparation. The former supernatant was freed from membranes by a high-speed centrifugation (150000 × *g*) and used as stroma. The membranes were further fractionated on a discontinous sucrose density gradient to separate inner and outer chlo-

roplast envelope membranes. Chloroplasts subfractions were stored at −80 °C until further use.

Immunological procedures

An antiserum was raised against IEP96 in a rabbit from the SDS-PAGE-purified protein (11). A second antiserum was obtained in a rabbit against chemically synthesized peptides coupled to cyanogen-bromide activated sepharose (Pharmacia). The pepetide sequences were deduced from the open reading frame of pisa 96. Western blotting was done as described [33] either after one-dimensional SDS-PAGE [19] or after two-dimensional gel electrophoresis as described [23].

Results and discussion

An antiserum against OEP 86 was used to screen a λgt11 cDNA expression library made from poly(A)$^+$ mRNA isolated from etiolated pea leaves. Two different classes of positive isolates were picked after screening about 500 000 plaques. One coding for OEP 86 [12] the second class exhibited cDNA inserts of about 3300 bp in size (pisa 96) with an open reading frame of 2625 bp coding for a protein of 875 amino acids (Fig. 1). The calculated molecular mass of the protein is 96450 kDa. The N-terminal 50 amino acid segment showed typical characteristics of a chloroplast stromal targeting signal, i.e. high content of hydroxylated amino acids and low proportion of acidic ones, furthermore the first nine amino acids were uncharged and contained neither proline nor glycine [34]. These data suggested that the cDNA clone pisa 96 codes for a plastid-localized polypeptide. To test this notion and to clearly localize the protein we raised a peptide-specific antibody in a rabbit against a mixture of two peptides corresponding to amino acids 204–217 and 225–238 (compare Fig. 1, αIEP96$_{pep}$). Initial immunoblot experiments using the peptide specific antibody indicated that the protein is present in a chloroplast membrane rich fraction (not shown). Chloroplasts were further separated into thylakoids, inner envelope membranes, outer envelope membranes and a soluble protein fraction. Immunoblot analysis of these chloroplast subfractions using the peptide-specific antibody demonstrated that the gene product of pisa 96 is localized in the inner envelope membrane of pea chloroplasts (Fig. 2).

The inner envelope membranes from pea and spinach chloroplasts contain a major polypeptide at 96 kDa (Fig. 3a), which has been used as a marker protein for this membrane and termed E 110 [3] or IEP97 [5], respectively. We thus wanted to know whether pisa 96 codes for this major inner envelope polypeptide of 96 kDa from pea chloroplasts. To answer this question a second antibody was raised against the 96 kDa protein isolated from one-dimensional gels (αIEP96$_{pol}$). The different antibodies recognized a protein of identical size after one-dimensional gel electrophoresis and western blotting, indicating that we identified the major IEP96 from pea chloroplast inner envelope (not shown). To further strengthen this indication a two-dimensional gel (Fig. 3b) was overlayed with two nitrocellulose filters and the identical polypeptide pattern transferred to both filters. Each filter was incubated with a different antibody, i.e. either with αIEP96$_{pol}$ (Fig. 3c) or αIEP96$_{pep}$ (Fig. 3d) and the labelled spots compared with a stained polyacrylamide gel (Fig. 3b). The data show that the protein spot recognized by the pepetide-specific antibody co-migrated with the major protein at 96 kDa (Fig. 3b). This protein is also recognized by the αIEP96$_{pol}$ antiserum, which recognized one additional polypeptide of 96 kDa but of lower isoelectric point. We thus conclude that pisa 96 codes for the major 96 kDa protein of the inner chloroplast envelope of pea.

Sequential transcription-translation of pisa 96 resulted in one major radiolabelled translation product of an apparent size of 102 kDa on SDS-PAGE (Fig. 4). The radiolabelled protein binds to intact chloroplasts in the presence of 10–50 μM ATP in a protease accessible form indicating that it is still on the outside of the organelle. Raising the ATP concentration above 250 μM resulted in the increased appearance of a lower molecular

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IEP 96     MASSSATLVG STASDLLRSS TTGFTGVPLR TLGRAGLVLK RRDLTVSVTA▼   50

Acc A                                                 .*.**.*.
IEP 96     KLRKVKRREY PWSSNPDPNM KGGRLRHLST FQPLKQPPKP VILEFEKPLI  100

Acc A      ..* **...  *.** .... ....  * .*   .   .... .*.. * . .
IEP 96     NMEKKINDFR KVAEKTGVDL SDQILALEAK YQKALVELYT NLTPIQRVTV  150

Acc A      ****.**  * *..    ..*  ** ***.   ** **..*.. ..**.. *.*
IEP 96     ARHPNRPTFL DHMYNMTEKF VELHGDREGY DDPAIAAGLG SIDGKTYMFI  200

Acc A      ******.*** .*.***.**. *.******** *. *..   .* *.********
IEP 96     GHQKGRDTKE NIKRNFAMPT PHGYRKALRL MEYADHHGFP IVTFIDTPGA  250

Acc A      .... .*. * *.****.*** .*   * ***. ..******** ****** ..*
IEP 96     FADLKSEQLG QGEAIAHNLR SMFALKVPVI SIVIGEGGSG GALAIGCANK  300

Acc A      . **. *..  *  **.*..* ****.. **  *** . *.*.  * .*.. *.
IEP 96     LLMLENSVFF VAMPEACGAI LWKSNKAAPK AAERLKITAS ALLDLEIADG  350

Acc A      ******.***  .*
IEP 96     IIPEPLAGAH TDPSWMSQQI KIAINEAMDE LTKSLTEDLI KDRMHKFRKL  400
USO 1                                .*..*   *.* ..   **. .  ..

IEP 96     GVDGIQEGIP LVPSKKVNTK KREIGVPPKR QEVPIPDSQI EAEIEKLKKA  450
USO 1      ......*.*.    ..  ... ... ..  .   ....  ...* . *. .  *.

IEP 96     IFEGEDSSAA KKNPGSQIGS AIDKLKGLFL EGKDSSAAKK TPGSQIVAEL  500
USO 1       *...*. .* *   .... *   .. *..  *...    * . .. ..    .*

IEP 96     DKLKGLYLEA KDSSAAKVPG SQIVAEIEKL KNSIFEDEDS SSAVLPEKIP  550
USO 1      ..*...    . ...... ..    . *  .**.* *..* . *.. ........ .

IEP 96     GSEIAVEIAK LKKNILEGKD SSSEPSKLDL DKTIETLKRE VNREFSEAVK  600
USO 1      ...    .  . * *. **. . .... .*..* .** *.*. * ..........

IEP 96     AAGLTKTLTK LRGEISKAKA GNQPLTPLLK VEIKSFNQRL SAAPNSRKLL  650
USO 1      .  *... .  *. *......    .  .  *.  * .. .*.* .. ... . *

IEP 96     KKRGLLREVT KVKLLLDKNK AATRKQELKK KSDEHKEAAR LEQELKKKFD  700
USO 1      .* .*  ... * .  .. ** .    ..*...  .** .....  ....*** *

IEP 96     EVMDTPRIKE KYEALRSEVR RVDASSGSGL DDELKKKIIE FNKEVDLELA  750
USO 1      *. .. . .. . .. .*... . . .. .     .*****     *.. . .*

IEP 96     TAVKSVGLEV ESVKPGHGWN KSSVPEIEEL NKDVQKEIEI VANSSPNVKR  800
USO 1      ...***. *.  ..*  ..    . .  *..** .... *. *   ...  ....

IEP 96     LIEQLKLEVA KSGGKPDSES KSRIDALTQQ IKKSLAEAVD SPSLKEKYEN  850
USO 1        *..* *.        .  ..*  * ... .*.    * .  .. .  ..**.. ..

IEP 96     LTRPAGDTLT DDKLREKVKV NRNFS
USO 1        . *.. *    .**...... ...
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Fig. 1. Protein sequence of pIEP96 from pea chloroplast inner envelope membrane. The sequence was deduced from the cDNA clone pisa 96a. The putative stromal processing protease cleavage site is indicated by an arrow. The repeated protein sequence motifs are overlined by a solid line. Protein sequence similarities of IEP96 to the α-subunit of acetyl-CoA carboxyl-transferase from *E. coli* [18, 21] are indicated above the amino acid lettering, that to USO 1 [22] from yeast below the lettering. Asterisks indicate identities, dots conservative amino acid exchanges.

weight (i.e. processed) form, which is protease protected inside the organelle. Imported and processed IEP96 was largely (90%) recovered in the membrane fraction, while little (10%) off imported mature IEP96 was recovered in the total soluble protein of chloroplasts (not shown), taken together these data indicate, that mature IEP96 had reached the inner envelope membrane. The coding region of pisa 96 thus represents the precursor of IEP96, namely pIEP96 (Figs. 1 and 4).

We further analysed the translocation characteristics of pIEP96 in intact chloroplasts and observed, that protease-sensitive chloroplast surface components are necessary for productive binding and insertion of pIEP96. Furthermore pIEP96 is processed in an organellar free assay [1] to

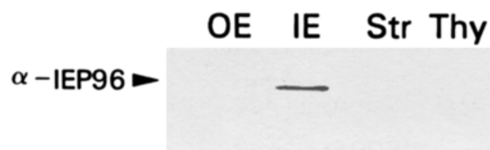

Fig. 2. Localization of pisa 96 gene product in pea chloroplasts. A polyclonal antibody ($\alpha IEP96_{pep}$) was raised in a rabbit against a mixture of two chemically synthesized peptides (amino acids 204–217 and 225–238). Pea chloroplasts were fractionated into outer envelope membranes (OE), inner envelope membranes (IE), stroma (Str) and thylakoids (Thy). Proteins were separated by SDS-PAGE, transferred to nitrocellulose and immunodecorated with $\alpha IEP96_{pep}$. 10 μg envelope protein and 40 μg stroma or thylakoid polypeptides were loaded onto SDS-PAGE.

the mature form indicating that the stromal processing peptidase is involved in the maturation of pIEP96. In addition to these requirements pIEP96 transport was stimulated up to 10-fold in the presence of 80 mM KP_i (Fig. 5B, C). We also observed a concomitant increase of chloroplast-bound precursor at low (Fig. 5a) or high ATP concentration (Fig. 5b) in the presence of KP_i. The inclusion of an ionophore or protonophore (valinomycin or nigericin, respectively) in the presence of KP_i did influence neither binding nor translocation efficiency of pIEP96 (Fig. 5B, lanes 3–8). These results indicate that the stimulatory effect of KP_i is not due to a membrane potential or ΔpH across the chloroplast inner envelope membrane. The yield of pSSU import into chloroplasts dropped 3–4 fold in the presence of 80 mM KP_i.

Next, we wanted to know whether the stimulatory effect of KP_i was due to an increase of the salt concentration in the import reaction or whether it was specific for certain ions. To test this, different salts were used in concentrations up to 80 mM, viz. NaP_i, NaOAc, NaCl, KP_i,

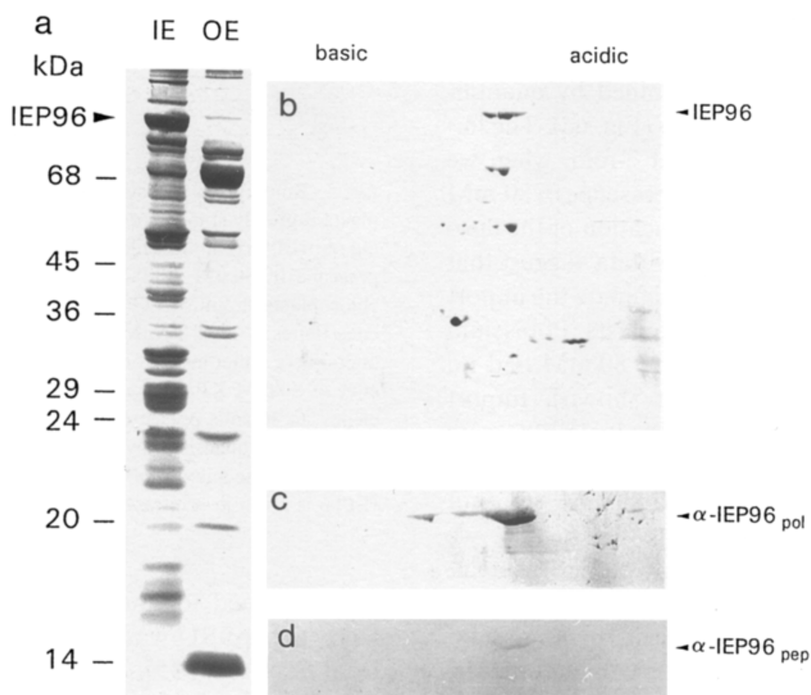

Fig. 3. Pisa 96 gene product represents a major inner envelope membrane protein from pea chloroplasts of 96 kDa. Outer and inner envelope membrane proteins (35 μg each) was separated by one-dimensional SDS-PAGE (a) or inner envelope membrane proteins (150 μg) by two-dimension gel electrophoresis (b). a. A Coomassie Brilliant Blue stained gel is shown. Numbers on the left indicate molecular weight markers in kDa. The position of IEP96 is indicated by an ►. b. A silver-stained two-dimensional polyacrylamide gel is shown. c, d. Immunostaining of the 70–100 kDa region as in b decorated with $\alpha IEP96_{pol}$ (c) and $\alpha IEP96_{pep}$ (d).

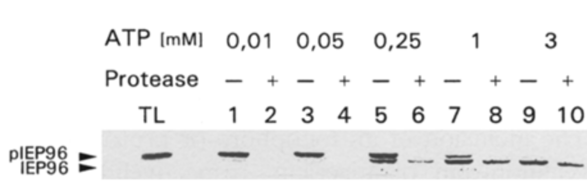

Fig. 4. Binding and import of pIEP96 into intact pea chloroplasts. [^{35}S]-labelled pIEP96 was synthesized by *in vitro* transcription-translation and used in a standard translocation reaction in the presence of various amounts of ATP. Chloroplasts were either not treated or treated with the protease thermolysin (750 μg per mg chlorophyll, 30 min, 4 °C) after import. Translocation products were analysed by SDS-PAGE. A fluorogram is shown. TL, pIEP96 translation product 20% of the amount added to a translocation experiment.

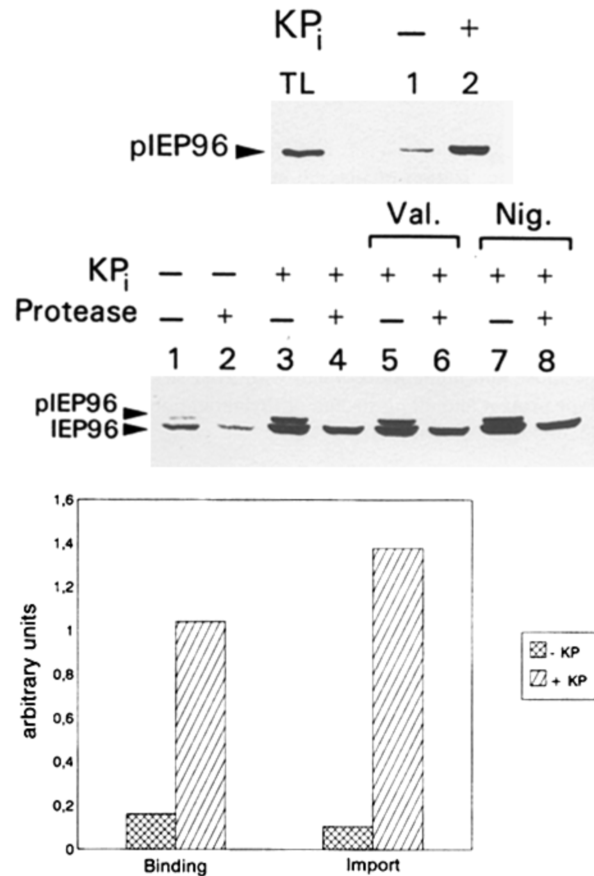

Fig. 5. Binding and translocation of pIEP96 into pea chloroplasts is greatly stimulated by potassium phosphate. A. Binding of pIEP96 to intact chloroplasts is increased by KP_i in the presence of 50 μM ATP. B. Translocation of pIEP96 into chloroplasts at 3 mM ATP in the absence (lanes 1, 2) or presence (lanes 3, 4) of 80 mM KP_i. The addition (1 μM) of the uncouplers valinomycin or nigericin (lanes 5–8) in the presence of 80 mM KP_i does not influence the translocation efficiency. C. Results presented in A lanes 1 and 2 and B lanes 2 and 4 were quantified by laser densitometry of the exposed X-ray film. The amount of bound pIEP96 or imported mature IEP96 is given in arbitrary units.

KOAc and KCl (Fig. 6a). Potassium salts had a more stimulatory effect than sodium salts in every case. On the other hand the positive effect of P_i was clearly more pronounced than that of chloride and acetate, respectively (Fig. 6). In the presence of 80 mM KCl a further addition of NaP_i still resulted in a 6-fold increase in translocation efficiency of pIEP96 as determined by quantification of the fluorogram shown (Fig. 6c). The import yield increased only about 2-fold, when we added up to 80 mM KCl in the presence of 80 mM NaP_i, as determined by quantification of the fluorogram shown (Fig. 6C). These data suggest that P_i and potassium specifically stimulate the import efficiency of pIEP96 into chloroplasts. Potassium phosphate concentrations above 80 mM had no further stimulatory effect (not shown). Import yields of up to 90% of the added pIEP96 were obtained into intact chloroplasts under optimal conditions, i.e. 3 mM ATP and 80 mM KP_i (not shown). The import yields are the highest we observed for any plastidic precursor protein. Whether the increase of pIEP96 binding and translocation into pea chloroplasts by potassium phosphate is due to a stimulation of a factor in the reticulocyte lysate or the chloroplast protein translocation machinery is not known and needs further clarification.

It is not known yet [7, 8, 13, 20] whether precursor proteins destined for the inner envelope compete for translocation sites with those proteins destined for the stroma or thylakoids. To study this, pSSU was overexpressed and purified from *E. coli* [17, 35]. The insoluble precursor was solubilized in 8 M urea and diluted directly into an import assay which contained either pIEP96 or pSSU as radiolabelled precursors, synthesized in a reticulocyte lysate. *E. coli* synthesized pSSU could successfully compete with [^{35}S] pSSU synthesized in the reticulocyte lysate and mature

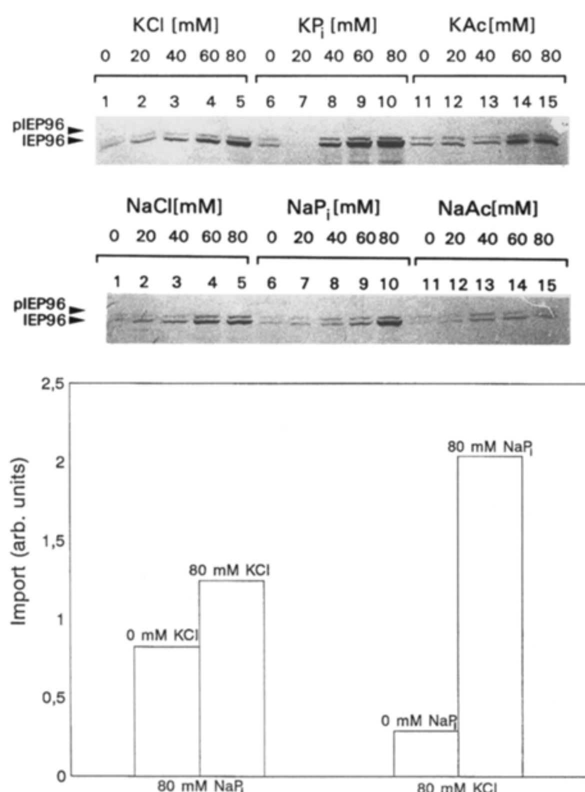

Fig. 6. Stimulation of pIEP96 translocation into pea chloroplasts is more dependent on P_i than on potassium. Different potassium salts (A) and sodium salts (B) were tested for their effect on pIEP96 translocation. The salts were present at concentrations from 0–80 mM as indicated. C. The import stimulation of potassium ions was assayed in the presence of 80 mM NaP_i in comparison to the import stimulation of phosphate ions in the presence of 80 mM potassium chloride. All translocations were in the presence of 3 mM ATP.

[^{35}S]-labelled SSU inside the chloroplasts decreased by 90% (Fig. 7B). When overexpressed pSSU was used to compete with [^{35}S] pIEP96, under conditions which are optimal for pIEP96 import but not for pSSU translocation (see above), i.e. 80 mM KP_i, an import inhibition of about 60% was detected (Fig. 7A, C). These results therefore seem to indicate that pIEP96 and pSSU share components of the protein translocation machinery of chloroplasts. This is corroborated by our observation that the transit peptide of pIEP96 has the typical features of a chloroplast targeting sequence [34].

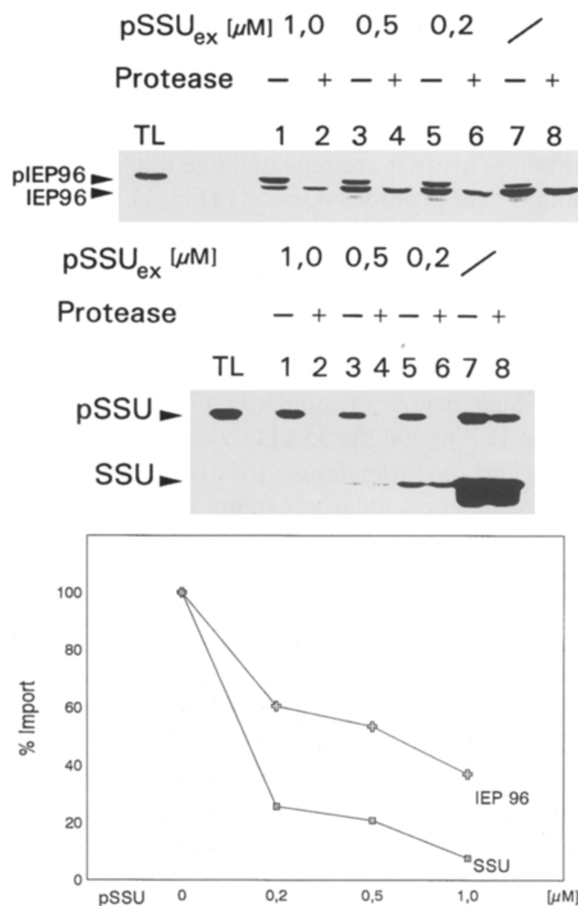

Fig. 7. pIEP96 translocation into pea chloroplasts is competed for by pSSU. pSSU was overexpressed in *E. coli* and solubilized by 8 M urea from inclusion bodies prior to use. The final urea concentration in the import assay was 80 mM. The overexpressed pSSU was present in the concentrations indicated on top of the figure. Import reactions were started by the addition of organelles. After import chloroplasts were either not treated or treated with protease (as in Fig. 3). A. Overexpressed pSSU inhibits pIEP96 translocation. B. Overexpressed pSSU inhibits the translocation of pSSU translation product. C. Quantitation of the results shown in A and B (as in Fig. 5C).

Sequence analysis of IEP96

A putative cleavage site for the stromal processing peptidase is present between amino acids 50 and 51 of pIEP96. The sequence around amino acid positions 48–51 is very similar to the consensus motif for the stromal processing enzyme described in [6, 34]. Furthermore, the shift in

molecular size observed on SDS-PAGE between precursor and mature IEP96 corresponds well with a suggested length of the transit sequence of 50 amino acids. IEP96 contains stretches of 26 amino acids that are repeated five times between amino acid positions 436–581 (Fig. 1). These internal repeats exhibit no homologies to other known proteins as deduced from a computer search using the FASTA program. The function of these repeats of amino acid repeats remains to be determined.

A high degree of similarity (76%) exists between IEP96 and the 35 kDa α-subunit of acetyl-CoA carboxyl-transferase from *E. coli* [18, 21]. The sequence similarity extents almost over the entire length (300 amino acids) of the acetyl-CoA carboxyl-transferase (Fig. 1). When inner envelope membranes were probed with a biotin specific avidin probe [2] or with an antiserum against acetyl-CoA carboxylase from pea chloroplasts [28] positive responses were obtained with inner envelope proteins but none corresponded to IEP96 (not shown). It should be noted that amino acids which are currently thought to be functional in acetyl-CoA carboxyl-transferase from plants and other organisms are not conserved in IEP96 despite of the high sequence similarities between the two proteins. Further analysis is necessary to determine wether IEP96 is involved in a carboxylation reaction.

A second significant stretch of similarity of IEP96 was observed to USO 1, a component of the cytoskeleton in yeast. USO 1 is proposed to function in the intracellular protein traffic [22]. The similarity (69%) spans over 500 amino acids, position 346–873 in pIEP96 (Fig. 1). The regions of similarity between acetyl-CoA carboxyl-transferase and USO1, respectively, and IEP96 do not overlap. USO1 and IEP96 share however only 20% identical amino acids, the final degree of similarity is due to conservative exchanges. We do however think that this similarity is significant, since it spans a region of about 50 kDa, more than half of the entire IEP96. We do not understand yet the purpose and function of a protein such as IEP96 which seems to be made up of 'cassettes'.

Acknowledgement

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Note added in proof

While this manuscript was under review two publications appeared (Schnell DJ, Kessler F, Blobel G, Science 266: 1007–1012 (1994); Wu LB, Siebert FS, Ko K J Biol Chem 269: 32264–32271 (1994)) which described the presence of an inner envelope membrane protein of apparent molecular mass between 97 and 100 kDa in a protein complex together with a plastidic precursor protein. This protein was referred to as IAP100 or CIM97, respectively, and it was suggested that this polypeptide is part of the inner envelope membrane protein translocation machinery. The data described in these publications make us feel that it is very likely that IEP96 presented here is indeed identical to IAP100 and CIM97. Thus, IEP96 would represent the first component of the inner envelope membrane protein translocation machinery identified on a molecular level.

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