The triose phosphate-3-phosphoglycerate – phosphate translocator from spinach chloroplasts: nucleotide sequence of a full-length cDNA clone and import of the in vitro synthesized precursor protein into chloroplasts

Ulf Ingo Flügge, Karsten Fischer, Armin Gross, Walter Sebald1, Fritz Lottspeich2 and Christoph Eckerskorn2

Institut für Biochemie der Pflanze, Untere Karspüle 2, 3400 Göttingen, 1Physiologisch-Chemisches Institut, Koellikerstrasse 2, 8700 Würzburg, and 2Max-Planck-Institut für Biochemie, Am Klopfersitz 18, 8033 Martinsried, FRG

Communicated by M. Klingenberg

The nucleotide sequence of several cDNA clones coding for the phosphate translocator from spinach chloroplasts has been determined. The cDNA clones were selected from a λgt10 library prepared from poly(A)+ mRNA of spinach leaves using oligonucleotide probes modeled from amino acid sequences of tryptic peptides prepared from the isolated translocator protein. A 1439 bp insert of one of the clones codes for the entire 404 amino acid residues of the precursor protein corresponding to a mol. wt of 44 234. The full-length clone includes 21 bp at the transcribed non-coding 5' region with the ribosome initiation sequence ACAATGG, a 1212 bp coding region and 199 bp at the non-coding 3' region excluding the poly(A) tail which starts 17 bp downstream from a putative polyadenylation signal, AAATAA. According to secondary structure predictions the mature part of the chloroplast phosphate translocator exhibits high hydrophobicity and consists of at least seven membrane-spanning segments. Using plasmid-programmed wheat germ lysate the precursor protein was synthesized in vitro and could be imported into spinach chloroplasts where it is inserted into the inner envelope membrane.

Key words: chloroplast/cDNA/nucleotide sequence/phosphate translocator/protein import

Introduction

The triose phosphate-3-phosphoglycerate – phosphate translocator, in short the phosphate translocator, can be regarded as one of the main transport systems of the inner envelope membrane of chloroplasts (Flügge and Heldt, 1984). It mediates the export of fixed carbon from the chloroplasts into the cytosol in the form of triose phosphates. Thus, fixed carbon is delivered to the cytosol for the synthesis of, e.g., sucrose and amino acids. The phosphate which is released during biosynthesis is shuttled back via the phosphate translocator into the chloroplasts where it is used for the formation of ATP. The translocator protein has a mol. wt of 29 kd as determined by SDS-PAGE. In its functional state, the phosphate translocator exists as a dimer made up of identical subunits (Flügge, 1985). The translocator protein is coded for by nuclear DNA (Flügge, 1982) and synthesized on soluble cytosolic ribosomes as a precursor with an apparent mol. wt of 40 kd (Flügge and Wessel, 1984). During post-translational import into chloroplasts the precursor is processed to its mature size.

Presumably due to its low abundance (-0.1% of the chloroplast protein) attempts to determine the primary structure of the translocator have so far been unsuccessful. In this report we present the nucleotide sequence of a full-length cDNA clone encoding the spinach chloroplast phosphate translocator and its deduced amino acid sequence. It is the first primary sequence of a chloroplast metabolite translocator and, in addition, the first sequence of a chloroplast envelope membrane protein. The efficient insertion of the translocator precursor protein into the inner envelope membrane is also demonstrated.

Results

cDNA cloning, nucleotide sequence and predicted amino acid sequence of the spinach phosphate translocator protein

A spinach cDNA library constructed in the vector λgt10 was screened for the spinach chloroplast phosphate translocator by in situ plaque hybridization (Benton and Davis, 1977) with three mixed oligonucleotide probes. These probes had been constructed using information on amino acid sequences of peptides obtained by tryptic digestion of the translocator protein. Only those clones which strongly hybridized with all three oligonucleotides were selected and further analysed. From 3 × 107 phage plaques examined we obtained several independent positive clones (PTBC3, PTBC1, PT8, PT3 and PT19). The inserts of these clones were excised, subcloned into the plasmid vector pT7T3 and sequenced at both 5' and 3' ends using the dideoxy chain termination method (Sanger et al., 1977). One of the clones, PTBC3, represented a full-length clone and the strategy for its sequence analysis is outlined in Figure 1. The nucleotide sequence of the full-length cDNA and its deduced amino acid sequence are shown in Figure 2. The cDNA sequence contains a single open reading frame (ORF) starting at nt 1 and continuing to the TGA stop codon at nt 1234. However, the protein-coding sequence most likely starts at the methionine initiation codon ATG at nt 22 although the clone contains no stop codons further upstream. But the heptanucleotide motif ACAATTG (nt 19–25) represents the consensus eukaryotic ribosome initiation sequence (Kozak, 1984) and the presence of a second initiation site that is 5' to this sequence appears to be most unlikely. Thus, the full-length phosphate translocator cDNA clone consists of 21 bp of a 5' untranslated sequence, a 1212 bp coding region and 199 bp of a 3' untranslated sequence excluding the poly(A) tail. The 3' untranslated region includes the putative polyadenylation signal AAATAAT which is located 17 bases to the 5' side of the poly(A) tail and coincides exactly with the consensus sequence proposed for plants (Dean et al., 1986). The 5' non-coding region is relatively short, but nucleotide sequence analysis showed that inserts from three different clones (PT19, PT3 and PTBC3)
all terminate at exactly the same nucleotide. This suggests that the clones terminate either near the 5'-end of the translocator mRNA or at a strong stop site for the reverse transcriptase. Northern blot analysis allowed estimation of the size of the mRNA in spinach poly(A)^+ mRNA which corresponds to positive phosphate translocator cDNA clones. Figure 3 shows that the nick-translated cDNA insert of clone PT8 (544 bp) strongly hybridized to a ~1450 base long poly(A)^+ mRNA species. Identical results were obtained using cDNA inserts from clones PTBC3, PT3 and PT19 (not shown) indicating that the 1432 bp cDNA insert [excluding the poly(A) tail] shown in Figure 2 does indeed represent a full-length clone.

The ORF of the PTBC3 cDNA insert codes for a polypeptide of 404 amino acid residues corresponding to a mol. wt of 44,234. This value for the phosphate translocator precursor protein is higher than that determined by SDS-PAGE (40 kd) (Flügge and Wessel, 1984).

**Protein characteristics**

The overall polarity index (Capaldi and Vanderkooi, 1972) of the translocator protein is calculated to be only 36%, and it is even lower for the mature part of the protein, thus emphasizing the hydrophobic character of this translocator (Table I). The protein contains a >2-fold excess of basic amino acids compared to the acidic residues resulting in an isoelectric point of ~10.2. The hydropathy profile analysis of the phosphate translocator, calculated with an 11 amino acid residue span, also demonstrates its high hydrophobic nature, which continues through almost the whole sequence (Figure 4). The mature part of the translocator contains at least seven regions of strong hydrophobicity (I–VII) which might be required to traverse the inner envelope membrane. It also possesses a highly polar domain at the C-terminus (Lys-Ala-Lys-Met-Glu-Glu-Glu-Ala-Lys-Arg).

Comparison of the phosphate translocator protein sequence with itself by DIAGON blot analysis (Staden, 1981) revealed only two very short repeats. One of these occurred at amino acids 136–140 and 282–286 [Ile-Ala(His)-Leu-Phe-Val]. This segment is located inside the transmembrane segments II and V, respectively. The other occurred at amino acids 181–186 and 251–256 (Ser-Asn-Val-Ser-Phe). The amino-terminal residue was found to be blocked and could not be determined by Edman degradation. Thus, we do not know yet the exact start of the mature part of the translocator protein. From SDS–PAGE analysis an unusually high mol. wt. of 10–11 kd was calculated for the transit sequence of the spinach chloroplast phosphate translocator (Flügge and Wessel, 1984) suggesting that the signal sequence cleavage site occurs roughly at amino acid position 85–95. Indeed, in this sequence segment a change in the hydrophathy index is observed (Figure 4) and computer analysis revealed an extraordinarily high degree of chain flexibility in the sequence corresponding to amino acid position 83–89 (Ser-Gly-Ser-Gly-Glu-Ala) a sequence which might provide a putative target for the processing peptidase. According to these considerations, the mature part of the translocator protein exhibits a mol. wt of a least 34 kd and this value is clearly higher than that of 29 kd obtained by SDS–PAGE.

**Further characterization of the cDNA clones and in vitro expression of the translocator protein**

The three translocator peptides that we have sequenced are coded for in the 1212-bp ORF of the cDNA clone: peptide 1, amino acid position 301–310 (Lys-His-Gly-Phe-Asp-Ala-Ile-Ala-Lys); peptide 2, amino acid positions 311–317 (Val-Gly-Leu-Thr-Lys-Phe-Ile) and peptide 3, amino acid positions 394–398 (Met-Glu-Glu-Glu-Lys), respectively. These results strongly indicate that the cDNA obtained indeed codes for the amino acid sequence of the phosphate translocator.

To further confirm the identity of the clones, hybridization-selected translation was performed using plasmids PT8, PT19 and PTBC3 (Viebrock et al., 1982). All three plasmids selected mRNA which directed the synthesis of a translation product with a size identical to that of the phosphate translocator precursor protein (Mr ~40 kd). It was recognized by the antiserum against the phosphate translocator (Figure 5A). Furthermore, the T3 promoter element of the vector pT7T3 was utilized to synthesize
cDNA cloning and import of the chloroplast phosphate translocator

Fig. 2. Nucleotide sequence (coding strand) of the phosphate translocator cDNA, its flanking regions and the deduced amino acid sequence of the phosphate translocator protein. Nucleotide residues are numbered in the 5' to 3' direction. The coding region begins at nt 22. The ribosome initiation sequence and the polyadenylation site are underlined.

mRNA complementary to the cloned PTBC3 cDNA. After translation of the in vitro synthesized mRNA using a cell-free wheat germ system in the presence of [35S]methionine, SDS-PAGE and fluorography revealed only one single major labelled polypeptide, which could be precipitated by antiserum against the translocator protein (Figure 5B). Although the phosphate translocator cDNA insert codes for a 44.2 kd polypeptide (Figure 2), the translated polypeptide displays an apparent mol. wt of only 40 kd, a value which is, however, identical to that of the phosphate translocator
Fig. 3. Northern blot analysis. Poly(A)+ RNA isolated from spinach leaves was resolved on a denaturing 1% agarose gel, blotted onto a Nylon filter and probed with a nick-translated 32P-labelled insert of clone PT8 (544 bp). Markers on the right denote the approximate number of kilobases derived from RNA mol. wt standards.

Table I. Amino acid composition of the phosphate translocator precursor protein

<table>
<thead>
<tr>
<th>Amino acid residues</th>
<th>Polar No. (%)</th>
<th>Non-polar No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg 14 (3.5)</td>
<td>Phe 28 (6.9)</td>
<td></td>
</tr>
<tr>
<td>Lys 23 (5.7)</td>
<td>Leu 41 (10.1)</td>
<td></td>
</tr>
<tr>
<td>Asp 6 (1.5)</td>
<td>Ile 29 (7.2)</td>
<td></td>
</tr>
<tr>
<td>Asn 14 (3.5)</td>
<td>Val 37 (9.2)</td>
<td></td>
</tr>
<tr>
<td>Glu 13 (3.2)</td>
<td>Trp 6 (1.5)</td>
<td></td>
</tr>
<tr>
<td>Gin 9 (2.2)</td>
<td>Ala 40 (9.9)</td>
<td></td>
</tr>
<tr>
<td>His 7 (1.7)</td>
<td>Tyr 14 (3.5)</td>
<td></td>
</tr>
<tr>
<td>Thr 25 (6.2)</td>
<td>Gly 26 (6.4)</td>
<td></td>
</tr>
<tr>
<td>Ser 36 (8.9)</td>
<td>Met 11 (2.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cys 5 (1.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pro 20 (5.0)</td>
<td></td>
</tr>
<tr>
<td>Subtotal 147 (36.3)</td>
<td>Subtotal 257 (63.6)</td>
<td></td>
</tr>
</tbody>
</table>

Import of in vitro synthesized phosphate translocator protein into spinach chloroplasts

To study the energy requirements for the import of the phosphate translocator precursor protein into spinach chloroplasts the precursor protein was synthesized in a transcription—translation system and added to intact spinach chloroplasts.

Subsequently, the chloroplasts were lysed by osmotic shock and the envelope membranes were separated from the thylakoids and the stroma fraction by differential centrifugation. The three compartments were analysed by SDS—PAGE. Figure 6 shows their protein pattern as visualized by Coomassie staining: lane 1, stroma fraction; lane 2, thylakoid membranes and lane 3, partially purified envelope membranes. Compared to the protein pattern of envelope membranes which were purified by sucrose density gradient centrifugation (Figure 6, lane 4) the envelope fractions prepared from the import assays are virtually devoid of thylakoid membrane proteins but contain some stromal contamination, particularly the large subunit of ribulose-bisphosphate carboxylase/oxygenase which strongly binds to the envelope membrane (Joyard et al., 1982). However, fluorographic analyses of the different chloroplast compartments revealed that the processed phosphate translocator protein was completely absent from the stroma (Figure 6, lane 9) and was indeed mainly associated with the enriched
envelope membrane fraction (Figure 6, lane 6). A small amount of the imported phosphate translocator protein, however, was also found in the thylakoid fraction (Figure 6, lane 10). But this observation is most probably due to a contamination of the thylakoids with envelope membranes, which co-sediment with the thylakoids even during low speed centrifugation (Murakami and Strotmann, 1978).

The translocator could only be removed from the envelope membranes by the use of detergents (Figure 6, lane 7), as shown earlier (Flügge and Heldt, 1981). It could be further demonstrated that the solubilized form of the translocator protein was completely digested by treatment with proteases, e.g. thermolysin (Figure 6, lane 8). One can conclude from these experiments that the mature form of the phosphate translocator protein is actually embedded in its target membrane, i.e. the envelope membrane, as an integral component.

Figure 7 describes the energy requirements of the insertion of the phosphate translocator protein into the envelope membrane. In the dark and in the absence of ATP which had been removed by treatment of the chloroplasts with apyrase, only the translocator precursor protein was bound (Figure 7, lane 3). However, if ATP was added externally, the precursor form was efficiently processed to its mature form with an apparent mol. wt of 29 kd (Figure 7, lane 4).

In order to determine the accessibility of the processed translocator protein to proteases, the chloroplasts were subsequently treated with thermolysin. This protease was shown to be unable to penetrate the outer envelope membrane (Cline et al., 1984). In contrast to the bound precursor, the processed mature translocator form was completely resistant to thermolysin treatment (Figure 7, lanes 5 and 9). The isolated translocator protein itself, however, has been found to be digested by this protease (see above). These results indicate that the translocator has been inserted into the inner envelope membrane in a protease-resistant manner.

Import into darkened chloroplasts was not prevented by the presence of the non-hydrolysable ATP-analogue adenosine 5'-[β,γ-methylene]triphosphate, indicating the absolute requirement of ATP hydrolysis for protein translocation. In the light, protein uptake was observed in the absence of externally added ATP (Figure 7, lane 8). Obviously, protein import can also be driven by ATP generated by photophosphorylation. But if the light-induced ATP formation inside the chloroplasts was blocked by the presence of the protonophore carbonylcyanide m-chlorophenylhydrazone in combination with valinomycin/K⁺ (Flügge and Hinz, 1986) translocation of the phosphate translocator precursor protein was strongly inhibited (Figure 7, lane 6). This inhibition, however, could be relieved by the addition of ATP (Figure 7, lane 7). Since the proton motive force across the envelope membrane cannot be restored by externally added ATP in the presence of the ionophores (Flügge and Hinz, 1986), these results clearly demonstrate that ATP itself and not a component of the proton motive force is required for the uptake of the phosphate translocator protein into the chloroplasts.

In contrast to dark conditions where the presence of the ATP-hydrolysing enzyme apyrase completely abolished import activity, the efficiency of protein import in the light was not influenced by the presence of apyrase (Figure 7, lane 11), which removes both external ATP and ADP without, however, affecting the stromal ATP level (not shown). This observation indicates that the import of the phosphate translocator as an inner envelope membrane protein into illuminated is apparently independent of cytosolic ATP, as has been shown for stromal and thylakoid proteins (Flügge and Hinz, 1986; Pain and Blobel, 1987; Hinz and Flügge, 1988).

Chloroplasts which had been pretreated with the protease thermolysin showed an almost complete inhibition of binding and integration of the phosphate translocator into the inner envelope membrane (Figure 6, lane 10). This observation is in accord with the view that both the binding of an envelope membrane protein to chloroplasts and its membrane insertion require a protease-sensitive component of the outer envelope membrane, as has been demonstrated for the import of stromal and thylakoid proteins, respectively (Cline et al., 1985; Bitsch and Kloppsteich, 1986; Hinz and Flügge, 1988).

Discussion

The inserts of several clones coding for the precursor of the phosphate translocator from spinach chloroplasts have been sequenced. The cDNA from clone PTBC3 (1439 bp) was found to contain the entire amino acid-coding region...
U. I. Flügge et al.

Fig. 6. Import of the in vitro synthesized phosphate translocator protein into chloroplasts. Intact spinach chloroplasts (0.6 mg chlorophyll/ml) were preincubated for 15 min in import buffer (Materials and methods) in the dark and in the presence of 2 mM ATP. The final volume was 0.3 ml. Import was initiated by the addition of postribosomal supernatant of the plasmid-programmed wheat germ lysate containing the 35S-labelled phosphate translocator precursor protein (1 × 10^6 d.p.m. protein-bound radioactivity). Incubations were allowed to proceed for 15 min at 25°C and then the samples were cooled to 0°C and treated with thermolysin (50 µg/ml) and in the presence of 1 mM CaCl_2 for 30 min. Afterwards, the chloroplasts were washed twice in medium B and were then osmotically shocked by addition of medium A (Materials and methods). Envelope membranes were separated from the stroma and the thylakoids as described in Materials and methods. The three chloroplast compartments were subsequently analysed by SDS–PAGE and Coomassie-brilliant-blue staining or fluorography. Lanes 1–4 show Coomassie-stained stroma proteins (lane 1), thylakoid membrane proteins (lane 2), partially purified envelope membrane proteins (lane 3) and sucrose gradient-purified envelope membrane proteins (lane 4). Lanes 5–10, fluorographic analyses. Lane 6, partially purified envelope membrane proteins; lane 9, stroma proteins; lane 10, thylakoid membrane proteins. Before SDS–PAGE the envelope membranes of sample 7 were treated with Triton X-100 (final concentration 2%, w/w) and those of sample 8 with Triton X-100 in the presence of thermolysin (50 µg/ml) and 2 mM CaCl_2 for 20 min. The solubilized membranes were centrifuged at 100 000 g for 30 min and the supernatants were then analysed by SDS–PAGE and fluorography (lanes 7 and 8). Lane 5 shows the phosphate translocator precursor protein prepared as in Figure 5B, lane 2. p and m represent the precursor (Mr ~ 40 kd) and the mature form (Mr ~ 29 kd) of the phosphate translocator protein.

including the transit peptide as well as 5′ and 3′ non-coding segments. Several lines of evidence confirm that the cDNA is indeed encoding the phosphate translocator protein.

First, the agreement between the amino acid sequences of three tryptic peptides and the amino acid sequence deduced from the nucleotide sequence of the cDNA clone indicates that the cDNA insert is derived from an mRNA species coding for the phosphate translocator.

Secondly, the mRNA in spinach poly(A)^+ RNA corresponding to the cDNA was found to be 1400–1450 bases long. This is compatible with that of the PTB3 cDNA (1439 bp) indicating that we have isolated virtually a full-length clone. Third, mRNA either selected by hybridization of the cDNA to poly(A)^+ mRNA or synthesized by in vitro transcription of the cloned cDNA using T3 RNA polymerase both directed the synthesis of a 40 kd polypeptide that co-migrated with the authentic phosphate translocator precursor protein. Furthermore, the synthesized polypeptide was recognized by a specific antibody and its immunoprecipitation could be prevented by the presence of an excess of purified phosphate translocator protein.

Finally, the in vitro synthesized precursor protein using plasmid programmed wheat germ lysate was efficiently inserted into the inner envelope membrane of spinach chloroplasts and processed to a polypeptide of the same size as the native phosphate translocator protein.

The sequence of the chloroplast phosphate translocator represents the first sequence of a transport protein of the inner envelope membrane. Functionally related antiport systems have already been isolated from mitochondria. Analysis of the primary sequences of the ATP/ADP translocator, the phosphate/OH^- antiporter and the hamster brown fat uncoupling protein revealed that these translocators are made up of three repeated domains ~ 100 amino acid residues (Aquila et al., 1982, 1985; Saraste and Walker, 1982; Runswick et al., 1987). Since there are extensive sequence homologies between the repeated elements in each of the three proteins it has been suggested that the mito-
The chloroplast phosphate translocator, however, contains no such internal repeated sequences and obviously does not share structural features with the mitochondrial transport proteins. This suggests that chloroplast translocators may represent a class of transport proteins distinct from those of the mitochondria.

Although the pre-sequence of the chloroplast phosphate translocator contains a slight abundance of the hydroxylated amino acids, Ser and Thr, there are apparently no structural similarities to any other known pre-sequences of nuclear coded mitochondrial or plastid proteins (Hurt and van Loon, 1986; Karlin-Neumann and Tobin, 1986; Tyagi et al., 1987; R.Hernnann, personal communication). Since the phosphate translocator is the first inner envelope membrane polypeptide to be sequenced, it might be assumed that the specific targeting information supposed to be contained in the transit sequence is significantly different from that known for proteins directed to other compartments (e.g. stroma or thylakoids).

Import studies into chloroplasts, however, demonstrated that the transit sequence of the phosphate translocator was able to direct the precursor protein to its target compartment, i.e. the inner envelope membrane where the processed form of the translocator is inserted in a protease-resistant manner. The overall process of insertion and processing of the precursor protein can be driven by light or, in the dark, by externally added ATP. It thus resembles the energy requirements for importing stromal and thylakoid proteins, respectively, where ATP was shown to be required as the sole energy source (Grossman et al., 1980; Flügge and Hinz, 1986; Pain and Blobel, 1987; Schindler et al., 1987). This is in contrast to the energy requirements for protein import into mitochondria, where, in addition, a membrane potential was shown to be required (Pfanner and Neupert, 1985).

Since binding and translocating the phosphate translocator precursor protein is completely abolished by pretreatment of the chloroplasts with proteases, components of the outer envelope presumably functioning as import receptors are apparently involved in the import process. Whether phosphorylation of the targeting apparatus is involved during translocation as suggested for the import of other chloroplast proteins directed to other compartments (e.g. stroma or thylakoids)

Import of the phosphate translocator precursor protein requires the hydrolysis of ATP located at the chloroplastic side of the outer envelope membrane, presumably in the intermembrane space (Flügge and Hinz, 1986). But it is apparently independent of cytosolic ATP (i.e. ATP at the cytosolic side of the outer envelope membrane) which could be completely removed by apyrase. This observation appears to exclude the participation of an ATP-dependent cytosolic factor which has been shown to be required to maintain mitochondrial precursor proteins in a transport-competent and unfolded conformation (Pfanner et al., 1987; Vernier and Schatz, 1987). However, the ATP-dependent unfolding activity could be embedded in the outer membrane and may be energized by ATP from the inside, thus rendering protein translocation into chloroplasts independent of a cytosolic factor (Rothman and Kornberg, 1986; Hinz and Flügge, 1988). This also indicates that the energy requirements for importing the phosphate translocator and other proteins into chloroplasts and for importing proteins into mitochondria are significantly different.

The availability of a full-length cDNA for the phosphate translocator provides an excellent system to study the precise mechanism for inserting the translocator into the inner envelope membrane. It should also enable us to elucidate the importance of specific amino acid residues in the active site of the translocator and to gain information on structure-function relationships. Such investigations are currently under way.

Materials and methods

Materials

Radiochemicals were obtained from Amer sham-Buchler (Br auschweig, FRG). Reagents and enzymes for recombinant DNA techniques were obtained from either Pharmacia LKB, Boehringer (Mannheim) or Gibco/BRL if not stated otherwise.

Synthesis and cloning of cDNA

Poly(A)\(^+\) mRNA was isolated from 3–4 week old spinach leaves as described by Apel and Kloppstech (1978). Oligo(GT)\(_{15}\) primer cDNA was synthesized by the method of Gubler and Hoffman (1983). The double-stranded cDNA was protected by methylation with EcoRI methylase, ligated to phosphorylated EcoRI linkers, digested with EcoRI and subsequently size-fractionated by gel filtration on Sepharose 4B. cDNA longer than 400 bp was ligated to EcoRI-cleaved λgt10 DNA (Promega Biotec) and packed into phage particles (Huynh et al., 1985). The phages were grown on E. coli strain C600 hflA at a density of 20 000 plaques/120 mm plate and replicated onto nylon filters (Biodyne) which were screened with \(^32\)P labelled oligonucleotide probes F1 1 – 3.

Preparation of oligonucleotide probes and screening procedures

The phosphate translocator protein was isolated from purified envelope membranes (Douc et al., 1973) by preparative gel electrophoresis, electroeluted from the gel and precipitated by chloroform/methanol as described earlier (Wessel and Flügge, 1984). Tryptic digestion was performed in 0.2% (NH\(_4\))\(_2\)HCO\(_3\), 0.1% SDS for 12 h using 50 μg trypsin per mg protein. Subsequently, SDS was removed by filtration on a Sep-Pak C\(_18\) cartridge (Millipore) and tryptic peptides were separated by reverse-phase HPLC (Pro RPC column, Pharmacia LKB) in aqueous 0.1% trifluoroacetic acid with a 0–80% acetonitrile gradient. The amino acid sequences of the purified peptides were determined by Edman degradation using a gas-phase sequencer (470A Applied Biosystems). Three oligonucleotide probes modeled from these sequences were synthesized: F1, 1, a 54-fold redundant mixed oligomer 5′(dAARCYGONTTYYAAXGAGCTNGTACNAATN)3′; F1, 2, a 24-fold redundant mixed oligomer 5′(dGTNGYNTACNAARTTYATN)3′; and F1, 3, a 16-fold redundant mixed oligomer 5′(dATGGARGARGAR­AAR)3′ where R is A or G, Y is C or T and N is A, C, G or T. For plaque hybridization screening (Benton and Davis, 1977) the oligonucleotide probes were labelled at the 5′-end to a sp. act. of 1 – 5 × 10\(^5\) c.p.m./μg using \([\alpha\]32P]ATP and T4-polynucleotide kinase (Maniatis et al., 1982).

Hybridization was performed for 20 h at least 5°C below the minimum melting temperature of the mixed oligonucleotides (LaThe, 1985; Albreten et al., 1988). Phage plaques which strongly hybridized with each of the labelled oligonucleotides (15 out of 3 × 10\(^7\) phage plaques examined) were rescreened, purified to homogeneity and the inserted cDNA was released by digestion with EcoRI. Restriction fragments were subcloned into the EcoRI site of the polylinker of the plasmid pTT73 before transformation of the host cell M15N252. Sequencing at both the 5′ and 3′ ends was performed according to the dideoxynucleotide chain termination method (Sanger et al., 1977) using [\(\alpha\]35S]dATP, dGTP or 7-deaza-dGTP and the DNA polymerase ‘Sequenase’ (Tabor and Richardson, 1987). For sequencing, a deletion series was also generated by digestion of the cDNA clones with exonuclease III/S1 nuclease (Henikoff, 1984). Controlled exonuclease III digestion was carried out using the clones PTBC3, PTBC1 and PT8 after linearization of the pTT73 vector with BamHI – PstI. Furthermore, the insert of clone PTBC3 was digested with the restriction enzymes AvaII, PvuII, Sau3AI and BamHI and the fragments obtained were subcloned into the Smal site of pTT73 by blunt-end ligation.

Hybridization-selected translation, in vitro translation and import of the phosphate translocator precursor protein into spinach chloroplasts

Hybridization-selected translation was essentially performed as described by Vierbrock et al. (1982). In vitro transcription of the phosphate translocator

cDNA cloning and import of the chloroplast phosphate translocator
PTBC3 cDNA cloned into the Smal site of the linearized vector pT7T3 was carried out in the presence of T3 RNA polymerase according to the instructions given by the manufacturer (Pharmacia LKB). Translation in a wheat germ cell-free system was performed according to Flügge and Wessel (1984) and the post-ribosomal supernatant was used for protein uptake studies. Protein import into intact spinach chloroplasts was carried out in a medium containing 0.3 M sorbitol, 15 mM methionine, 25 mM potassium gluconate, 2% bovine serum albumin, 2 mM MgSO₄, 50 mM Hepes–KOH, pH 8.0 (import buffer) and chloroplasts equivalent to 200 μg chlorophyll. Afterwards, the chloroplasts were washed twice in medium B (50 mM tricine–KOH, pH 7.9, 0.33 M sorbitol, 5 mM EGTA) and then were osmotically shocked by addition of medium A (10 mM tricine–KOH, pH 7.9, 5 mM EGTA, 2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride). To remove the thylakoid membranes, the shocked chloroplasts were centrifuged three times for 15 s at 6000 g and, subsequently, the supernatant was centrifuged for 30 min at 100 000 g. The pellet representing the enriched envelope membranes was washed once with medium A and analysed by SDS–PAGE. The supernatant represented the stroma fraction.

Acknowledgements

We thank Professor R.G.Herrmann and his research group for valuable discussions and Professor D.Gallwitz and H.P.Geithe for synthesizing the oligonucleotide probes. This work was supported by the Deutsche Forschungsgemeinschaft (F1 125/2-8) and the 'Fonds der Chemischen Industrie'.

References