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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

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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, AATAAT. 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 3×10^5 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 ACAATGG (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 AATAAT 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)

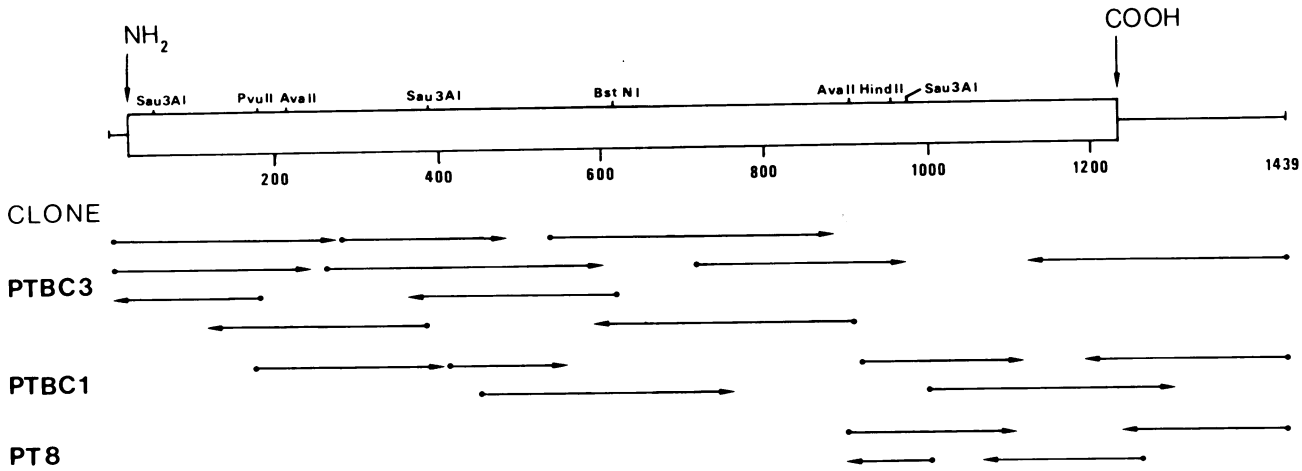

Fig. 1. Strategy for sequencing full-length cDNA coding for the spinach chloroplast phosphate translocator subcloned into the plasmid vector pT7T3. Sequencing was carried out by the dideoxy chain termination method (Sanger *et al.*, 1977) using T7 and T3 primers, dGTP/7-deaza-dGTP and [α-^{35}S]dATP as radioactive label. Some regions of the cDNA were sequenced using exonuclease III/S1 nuclease treated cDNA clones (PTBC3, 1439 bp; PTBC1, 1266 bp; PT8, 544 bp) which had been digested with *Bam*HI and *Pst*I (Henikoff, 1984). In addition, the insert of PTBC3 was digested with different restriction enzymes (*Ava*II, *Pvu*II, *Sau*3AI, *Bst*NI) as indicated and subcloned into the *Sam*I site of pT7T3. The horizontal arrows represent the directions on length of the sequences determined. The positions of the amino- and carboxyl-termini are indicated by arrows.

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-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 hydropathy 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-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-Asn-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 (M_r ~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

1
GTAGTTCTCTCTCTTCCGACA

50 80 110
ATG GAG TCG CGA GTT TTG TCG CGC ACC ACG GCG ATC GCC GCC CTC CCG AAG CTA TTC CGG CCG TCG AGA GAG GCG GCA AGT TTT GGT TTC
Met Glu Ser Arg Val Leu Ser Arg Thr Thr Ala Ile Ala Ala Leu Pro Lys Leu Phe Arg Pro Ser Arg Glu Ala Ala Ser Phe Gly Phe

140 170 200
GCC ACC GGA GTT AAG ACG CCG GTG GGA TTG GTC AAG GAT GGT GGG AGT TTG ACA TGG GGA AGG CAG CTG CGT CCA GTG TTG TTG CTT GAA
Ala Thr Gly Val Lys Thr Pro Val Gly Leu Val Lys Asp Gly Gly Ser Leu Thr Trp Gly Arg Gln Leu Arg Pro Val Leu Leu Leu Glu

230 260 290
CCG GTT CAA ACC GGT CCG GTT TGT AGC AGG AGA GAG AAA ACA GCG GTT CAG CCG TGT CGT GCC GCT AGT GGC TCT TCC GGG GAA GCA AAG
Pro Val Gln Thr Gly Pro Val Cys Ser Arg Arg Glu Lys Thr Ala Val Gln Pro Cys Arg Ala Ala Ser Gly Ser Ser Gly Glu Ala Lys

320 350 380
ACT GGG TTT TTG GAG AAG TAT CCG GCT CTT GTC ACT GGC TCC TTC TTC TTC ATG TGG TAC TTC TTG AAC GTG ATA TTC AAC ATT CTT AAC
Thr Gly Phe Leu Glu Lys Tyr Pro Ala Leu Val Thr Gly Ser Phe Phe Phe Met Trp Tyr Phe Leu Asn Val Ile Phe Asn Ile Leu Asn

410 440 470
AAG AAG ATC TAC AAT TAC TTC CCA TAT CCT TAC TTC GTG TCA GTC ATC CAT TTG TTT GTT GGA GTG GTG TAC TGT TTG GCT AGC TGG AGC
Lys Lys Ile Tyr Asn Tyr Phe Pro Tyr Pro Tyr Phe Val Ser Val Ile His Leu Phe Val Gly Val Val Tyr Cys Leu Ala Ser Trp Ser

500 530 560
GTG GGC CTT CCT AAA CGT GCT CCT ATG GAC TCC AAG CTG CTG AAG CTG CTG ATT CCA GTT GCA GTA TGT CAT GCG ATA GGC CAT GTT ACC
Val Gly Leu Pro Lys Arg Ala Pro Met Asp Ser Lys Leu Leu Lys Leu Leu Ile Pro Val Ala Val Cys His Ala Ile Gly His Val Thr

590 620 650
AGC AAT GTC TCA TTT GCT GCT GTT GCG GTC TCT TTC ACC CAC ACC ATT AAA GCC CTG GAG CCC TTC TTC AAT GCT GCT GCT TCT CAG TTC
Ser Asn Val Ser Phe Ala Ala Val Ala Val Ser Phe Thr His Thr Ile Lys Ala Leu Glu Pro Phe Phe Asn Ala Ala Ala Ser Gln Phe

680 710 740
GTT CTC GGA CAG TCT ATT CCC ATT ACT CTC TGG CTC TCC TTG GCT CCT GTT GTG ATT GGT GTT TCT ATG GCT TCC CTG ACC GAA TTA TCA
Val Leu Gly Gln Ser Ile Pro Ile Thr Leu Trp Leu Ser Leu Ala Pro Val Val Ile Gly Val Ser Met Ala Ser Leu Thr Glu Leu Ser

770 800 830
TTC AAC TGG CTT GGT TTC ATC AGC GCT ATG ATT TCA AAT GTT TCC TTC ACT TAC AGG AGT CTC TAC TCA AAG AAA GCC ATG ACT GAC ATG
Phe Asn Trp Leu Gly Phe Ile Ser Ala Met Ile Ser Asn Val Ser Phe Thr Tyr Arg Ser Leu Tyr Ser Lys Lys Ala Met Thr Asp Met

860 890 920
GAC AGT ACC AAT ATC TAT GCC TAC ATT TCC ATA ATT GCT CTG TTT GTT TGC CTT CCC CCT GCA ATC ATT GTC GAG GGT CCT CAA CTT ATG
Asp Ser Thr Asn Ile Tyr Ala Tyr Ile Ser Ile Ile Ala Leu Phe Val Cys Leu Pro Pro Ala Ile Ile Val Glu Gly Pro Gln Leu Met

950 980 1010
AAG CAT GGG TTC AAC GAT GCC ATT GCG AAA GTG GGG TTG ACC AAA TTC ATT TCT GAT CTC TTC TGG GTG GGA ATG TTT TAC CAT CTC TAC
Lys His Gly Phe Asn Asp Ala Ile Ala Lys Val Gly Leu Thr Lys Phe Ile Ser Asp Leu Phe Trp Val Gly Met Phe Tyr His Leu Tyr

1040 1070 1100
AAC CAG CTA GCT ACC AAC ACC TTA GAG AGG GTA GCA CCA CTT ACC CAT GCT GTT GGC AAC GTT TTG AAA CGA GTG TTT GTC ATC GGA TTT
Asn Gln Leu Ala Thr Asn Thr Leu Glu Arg Val Ala Pro Leu Thr His Ala Val Gly Asn Val Leu Lys Arg Val Phe Val Ile Gly Phe

1130 1160 1190
TCT ATC ATC GCC TTT GGC AAC AAG ATT TCA ACA CAA ACT GCC ATT GGA ACA AGC ATT GCT ATT GCT GGT GTA GCC CTC TAT TCT CTC ATT
Ser Ile Ile Ala Phe Gly Asn Lys Ile Ser Thr Gln Thr Ala Ile Gly Thr Ser Ile Ala Ile Ala Gly Val Ala Leu Tyr Ser Leu Ile

1220 1254 1294
AAG GCC AAA ATG GAA GAA GAG AAA CGA CAA ATG AAG TCT ACT TGA GTATCAAGACTCGGCCCGAAGCTGCATTCGCTTGGAAGGAACTTTGCTGTAGATTTGGA
Lys Ala Lys Met Glu Glu Glu Lys Arg Gln Met Lys Ser Thr End

1333 1373 1413
TAATTTTCCTTCCTGAAAAGAAAAACAACAAAAATGTTGTAAAATTAATTTTTTTTTCGTGTCAATTTATTTTGGAGAATGAACTACTGCGAATCTGTAATCTTCTTGTATTTCAATAAT

AAGCATAATATTGTACG$(A)_n$

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 [^{35}S]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

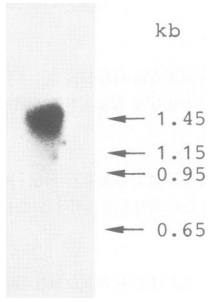

Fig. 3. Northern blot analysis. Poly(A)$^+$ RNA isolated from spinach leaves was resolved on a denaturing 1% agarose gel, blotted onto a Nylon filter and probed with a nick-translated ^{32}P-labelled insert of clone PT8 (544 bp). Markers on the right denote the approximate number of kilobases derived from RNA mol. wt standards.

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

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

precursor protein, both determined by SDS–PAGE. The identity of the labelled polypeptide was verified by competition studies using immunoprecipitation in the presence of an excess of non-radioactive translocator protein. Under these conditions the labelled 40 kd precursor is completely displaced from the immunocomplex suggesting that the 40 kd protein indeed represent the phosphate translocator precursor protein (Figure 5B, lane 4).

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

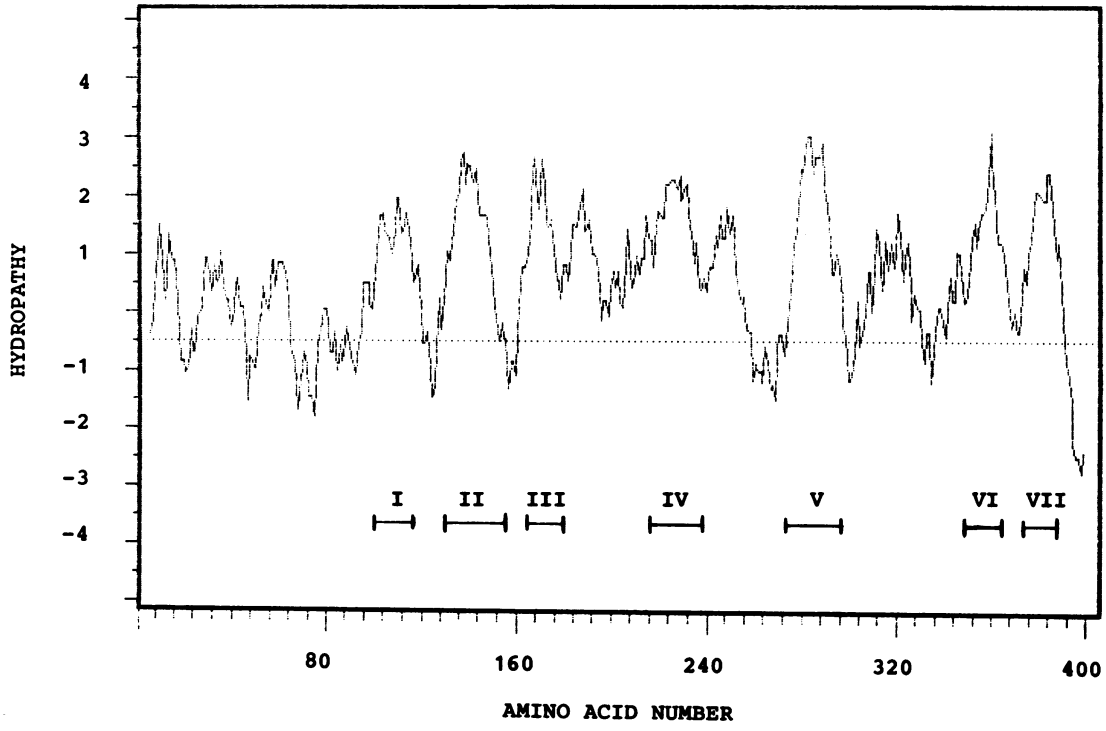

Fig. 4. Hydropathy profile of the amino acid sequence of the spinach chloroplast phosphate translocator. Hydropathy was evaluated according to Kyte and Doolittle (1982) with a span setting of 11 residues (hydrophilic: negative values; hydrophobic: positive values). Tentative prediction of possible membrane spanning segments is indicated by bars (I–VII).

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,

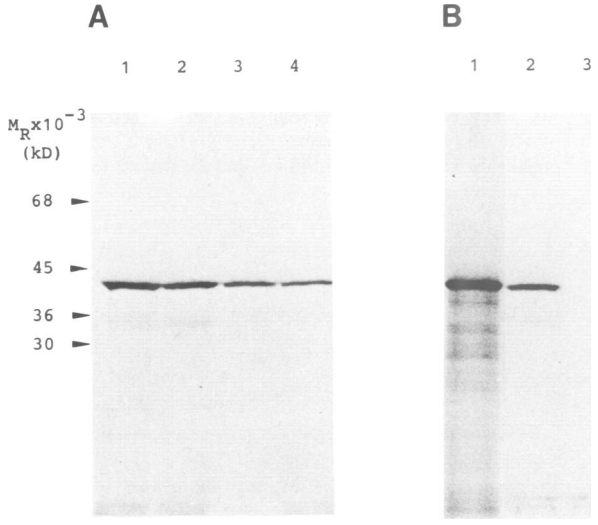

Fig. 5. Hybridization-selected translation and *in vitro* transcription–translation. (**A**) Identification of cDNA clones by hybridization selection and cell-free translation. Plasmids DNAs (50 μg) were immobilized on cellulose filters (Whatman 540) and hybridized with spinach poly(A)$^+$ mRNA (40 μg) as described by Viebrock *et al.* (1982). After elution, mRNA was translated in a wheat germ cell-free system. Immunoprecipitation and analysis of the ^{35}S-labelled antigens by SDS–PAGE were performed as described (Flügge and Wessel, 1984). **Lanes 1–3,** immunoprecipitation of hybridization-selected translation products. Poly(A)$^+$ mRNA had been hybridized with plasmid DNA from clones PT8, PT19 and PTBC3, respectively. **Lane 4,** phosphate translocator precursor protein immunoprecipitated from total cell-free translation products. A fluorogram of the dried gel is shown. (**B**) *In vitro* transcription–translation of the phosphate translocator cDNA cloned into pT7T3. Plasmid PTBC3 was transcribed with T3 RNA polymerase in the presence of m^{7}GpppG. The resulting mRNA was translated in a wheat germ cell-free system in the presence of [^{35}S]methionine. The labelled proteins were analysed by SDS–PAGE and visualized by fluorography. **Lane 1,** total translation products of the mRNA resulting from the *in vitro* transcription. **Lane 2,** immunoprecipitate from the translation products shown in lane 1 using a specific antiserum. **Lane 3,** immunoprecipitation performed as for lane 2 except that 10 μg unlabelled phosphate translocator protein was present during incubation with antibody.

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 promoted 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 carboxylcyanide *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 Kloppstech, 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

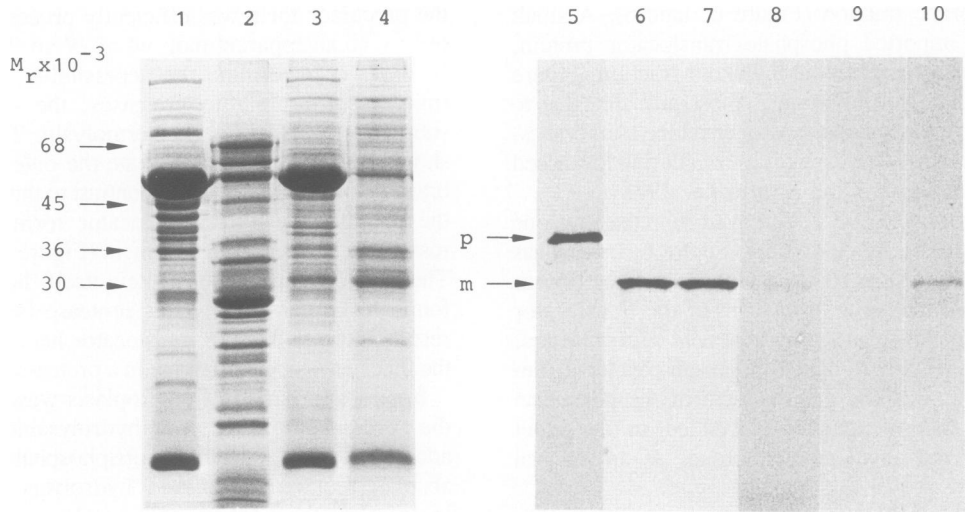

Fig. 6. Import of the *in vitro* synthesized phosphate translocator protein into chloroplasts. Intact spinach chloroplasts (0.6 mg chlorophyll/ml) were preincubated for 15 min in import buffer (Materials and methods) in the dark and in the presence of 2 mM ATP. The final volume was 0.3 ml. Import was initiated by the addition of postribosomal supernatant of the plasmid-programmed wheat germ lysate containing the ^{35}S-labelled phosphate translocator precursor protein ($\sim 1 \times 10^6$ 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 wtih 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 (M_r ~40 kd) and the mature form (M_r ~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 a 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 size is compatible with that of the PTBC3 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

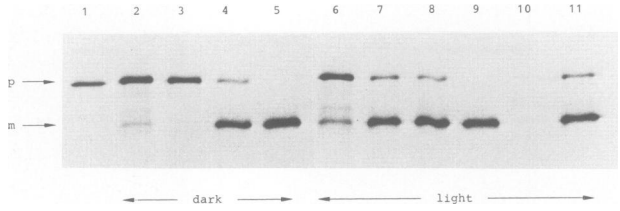

Fig. 7. Energy requirements for the import of the *in vitro* synthesized phosphate translocator protein into the envelope membrane. Intact spinach chloroplasts (0.6 mg chlorophyll/ml) were preincubated for 15 min in import buffer (Materials and methods) either in the dark (**lanes 2–5**) or in the light (**lanes 6–11**) and in the presence of 2 mM adenosine 5-[β,γ-methylene]triphosphate (lane 2), 2 U apyrase (lanes 3 and 11), 2 mM ATP (lanes 3–5 and 9 and 10), 5 mM ATP (lane 7), and 5 μM CCCP and 5 μM valinomycin (lanes 6 and 7). Sample 10 (lane 10) contained chloroplasts which had been pretreated with thermolysin (30 μg/ml) as described (Hinz and Flügge, 1988). The final volume was 0.3 ml. The further processes were as described in the legend to Figure 6. After import, samples 5 and 9 (lanes 5 and 9) were further treated with thermolysin (50 μg/ml) in the presence of 1 mM $CaCl_2$ for 30 min. Partial purification of envelope membrane proteins was performed as described in Materials and methods. Envelope polypeptides were analysed by SDS–PAGE. p and m represent the precursor and the mature form of the phosphate translocator protein. **Lane 1** shows the phosphate translocator precursor protein prepared as in Figure 5B, lane 2. A fluorogram of the dried SDS–polyacrylamide gel is shown.

translocator, the phosphate/OH^- antiporter and the hamster brown fat uncoupling protein revealed that these translocators are made up of three repeated domains of ~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-

chondrial translocators are related to each other and presumably derived from a common ancestor (Runswick *et al.*, 1987).

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.Herrmann, 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 (Hinz and Flügge, 1988) remains to be clarified.

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; Verner 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 Amersham-Buchler (Braunschweig, 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(dT) primer cDNA was synthesized by the method of Gubler and Hoffman (1983). The double-stranded cDNA was protected by methylation with *Eco*RI methylase, ligated to phosphorylated *Eco*RI linkers, digested with *Eco*RI and subsequently size-fractionated by gel filtration on Sepharose 4B. cDNA longer than 400 bp was ligated to *Eco*RI-cleaved λgt10 DNA (Promega Biotec) and packed into phage particles (Huynh *et al.*, 1985). The phages were grown on *Escherichia coli* C600 hfl at a density of 20 000 plaques/120 mm plate and replicated onto nylon filters (Biodyne) which were screened with ^{32}P-labelled oliognucleotide probes F1 1–3.

Preparation of oligonucleotide probes and screening procedures

The phosphate translocator protein was isolated from purified envelope membranes (Douce *et al.*, 1973) by preparative gel electrophoresis, electro-eluted from the gel and precipitated by chloroform/methanol as described earlier (Wessel and Flügge, 1984). Tryptic digestion was performed in 0.2% $(NH_4)HCO_3$, 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 64-fold redundant mixed oligomer 5′ d(AARCAYGGNTTYAAYGAYGCNATNGCNAA) 3′; F1 2, a 24-fold redundant mixed oligomer 5′ d(GTNGGNYTNACNAARTTYATN) 3′; and F1 3, a 16-fold redundant mixed oligomer 5′ d(ATGGARGARGARAAR) 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 × 10^8 c.p.m./μg using [γ-^{32}P]ATP and T_4-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; Albretsen *et al.*, 1988). Phage plaques which strongly hybridized with each of the labelled oligonucleotides (15 out of 3 × 10^5 phage plaques examined) were rescreened, purified to homogeneity and the inserted cDNA was released by digestion with *Eco*RI. Restriction fragments were subcloned into the *Eco*RI site of the polylinker of the plasmid pT7T3 before transformation of the host cell MN522. Sequencing at both the 5′ and 3′ ends was performed according to the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using [α-^{35}S]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 pT7T3 vector with *Bam*HI–*Pst*I. Furthermore the insert of clone PTBC3 was digested with the restriction enzymes *Ava*II, *Pvu*II, *Sau*3AI and *Bst*NI and the fragments obtained were subcloned into the *Sma*I site of pT7T3 by blunt-end ligation.

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

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

PTBC3 cDNA cloned into the *SmaI* site of the linearized vector pT7T3 was carried out in the presence of m^7GppG using 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 $MgSO_4$, 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 Hepes–KOH, pH 7.9, 0.33 M sorbitol, 5 mM EGTA) and were then 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.

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