Mechanism of ΔpH/TAT-dependent protein transport at the thylakoid membrane

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LIST OF ABBREVIATIONS

Alb3	albino 3
amp	ampicillin
APS	ammonium peroxodisulphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Bis-acrylamide	N'N'-methylene-bisacrylamide
BSA	Bovine serum albumin
CAP	$m_7G(5')ppp(5')G$
cDNA	copy (or complementary) DNA
CF_oII	Chloroplast F_o ATP synthase subunit II
C-terminal	Carboxyl-terminal
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DTT	1,4-Dithiothreitol
ECL	Enhanced chemiluminescence
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
ER	Endoplasmic reticulum
Ffh	Fifty-four homologue
FtsY	Filamentous temperature sensitive mutant Y
g	Gram
g	Gravity
GTP	Guanosine triphosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HM	Hepes/magnesium buffer
hsp	Heat shock protein
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-thiogalactopyranoside
IVT	*in vitro* translation
kDa	Kilo-Dalton
l	litre
Leu	Leucine
LHC	Light harvesting complex
LHCP	Light harvesting chlorophyll a/b binding protein
M	molar
Met	Methionine
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimole per litre

mRNA	Messenger RNA
µg	Microgram
µl	Microlitre
nm	Nanometer
N-terminal	Amino-terminal
NTP	Nucleoside triphosphate
OD	optical density
OE16	16 kDa OEC protein
OE23	23 kDa OEC protein
OE33	33 kDa OEC protein
OEC	Oxygen-evloving complex
Oxa-1	Cytochrome oxidase assembly 1
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Plastocyanin
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
Pre-	Precursor
PS I	Photosystem I
PS II	photosystem II
PsbW	Photosystem II subunit W
PsbX	Photosystem II subunit X
PsbY	Photosystem II subunit Y
Rieske	Rieske iron-sulfur protein of the cytochrome complex
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rounds per minute
SDS	Sodium dodecyl sulphate
SPP	Stromal processing peptidase
SRP	Signal recognition particle
TEMED	N,N,N’,N’-tetramethylethylenediamine
TIC	Translocase of the inner chloroplast envelope membrane
TOC	Translocase of the outer chloroplast envelope membrane
TPP	Thylakoidal prcessing peptidase
Tris	Tris(hydroxymethyl)methylamine
Tween20	Polyoxyethylenesorbitan monolaurate
v/v	Volume/volume
w/v	Weight/volume
°C	Degree Celsius
ΔpH	Proton gradient
ΔΨ	Membrane potential

INTRODUCTION

Membranes of a living cell form permeability barriers between the cell and the environment, and in addition, carry out several critical biochemical functions. To allow each membrane-bound compartment to carry out its role, there is considerable protein traffic between compartments. Proteins must be able to pass across these membrane barriers or, in the case of membrane proteins, insert into the bilayer. Protein translocation is taking place both in prokaryotes and eukaryotes. However, in eukaryotic cells this process becomes more complex, because the eukaryotic cell is compartmentalized by the membranes into organelles. A high proportion of cytosolically synthesized proteins has to cross one or more cellular membranes to reach their final destination, either outside the cell or within an intracellular compartment (Agarraberes and Dice, 2001).

Chloroplasts are the photosynthetic organelles residing in plant cells which also in addition carry out many other critical functions. They are organelles of endosymbiotic origin that are believed to have evolved from free-living oxygenic photosynthetic eubacteria (primary endosymbiosis) (Weeden, 1981). During evolution, they transferred most of their genetic information to the host nucleus. To perform their function, they therefore have to import those gene products posttranslationally from the cytosol into the chloroplasts. Chloroplasts have inherited several protein translocation systems from their ancestors that are still similar in function and mechanism to those of free-living bacterial cells like *Escherichia coli*. In this section, the similarities and differences among those protein translocation systems will be discussed.

1. Structure of higher plant chloroplasts

Chloroplasts are endosymbiotic organelles with a prokaryotic origin that still exhibit some structural and functional similarities to prokaryotes (Weeden, 1981). Similar to those of the gram-negative bacteria, two units of membranes, termed the outer envelope membrane and

inner envelope membrane, respectively, surround them. Chloroplasts are the most complex organelles, both structurally and functionally. In addition to the envelope membranes surrounding the chloroplasts, these organelles contain an extra membrane system, the thylakoid membrane, on which the light phase of photosynthesis takes place. Therefore, chloroplasts are divided into at least six distinct regions: outer envelope membrane, intermembrane space, inner envelope membrane, stroma, thylakoid membrane, and thylakoid lumen. The chloroplast thylakoid membrane is unique among biological membranes in its structure and composition. The photosynthetic machinery of thylakoid membranes comprises at least five multisubunit oligomeric complexes, including the photosystems I and II and their light harvesting antenna (LHC, light harvesting complex), the cytochrome complex and the ATP synthase (Andersson and Barber, 1994; Herrmann, 1996). Each complex consists of approximately 15-30 proteins. The thylakoid membrane is utilized for multiple different applications, including protein transport. In addition, the thylakoid lumen contains many proteins that are important for processes like water splitting, electron transport etc.

Chloroplasts still contain residual genomes that encode some of their proteins. They are transcribed translated within the organelles, by using the organellor protein synthesis machinery. However, the protein synthesis capacity of these organelles is strongly reduced, as many genes originally encoded within the organelle genomes have been transferred to the nucleus of the host cells. At present, a typical chloroplast genome encodes only about 100 proteins. Considering that a typical cyanobacteria contains more than 3000 genes (Kaneko et al., 1996), several thousand genes were either transferred to the nucleus or lost. Recently, the genome of the model plant *Arabidopsis thaliana* was sequenced completely and predicted to contain 25498 genes (The Arabidopsis Genome Initiative, 2000). According to predictions by cellular localization programs, up to 14% of the gene products, i.e. about 3,500 proteins, are likely to have a chloroplast localization (The Arabidopsis Genome Initiative, 2000; Emanuelsson et al., 2000). Those proteins are thus synthesized in the cytosol, using the protein synthesis machinery of the host cells. To complete the full function of the organelles, the proteins have to be targeted into the organelles by protein import machineries located at

both the outer and inner envelope membrane of chloroplasts. For the biogenesis of those proteins that are located in the thylakoid membrane or in the thylakoid lumen, import across the envelope membranes is only the first step. They must be subsequently transported into or across the thylakoid membrane.

2. Protein import into chloroplasts

2.1. Transit peptide for chloroplast targeting

Nuclear encoded chloroplast proteins are synthesized as precursor proteins in the cytosol with N-terminal targeting signals termed transit peptides. For the thylakoid located proteins, an additional signal is required for targeting to the thylakoid membrane or to the thylakoidal lumen. The transit peptides of thylakoid proteins can be divided into two types: one type carries only the envelope transit signal; the other type carries the envelope transit signal and a thylakoid transit signal in tandem and is therefore termed bipartite transit peptide. For those thylakoid proteins that carry the first type of transit peptides, an internal, uncleaved thylakoid targeting signal located in the mature part of these proteins is required.

The envelope transit signal, which mediates post-translational import of chloroplast proteins across the envelope membranes, is located at the N-terminus. Although the envelope transit peptides have no common sequence motifs and are highly variable in length (from 20 to more than 120 residues), they share several general features (von Heijne et al., 1989; Claros et al., 1997): (1) an uncharged N-terminal domain of about 10 amino acids that is terminated by glycine or proline; (2) a central domain lacking acidic residues but enriched in hydroxylated amino acids, (3) a C-terminal domain that is enriched in arginines. They do not fold into secondary or tertiary structure in an aqueous environment, but might form amphipathic β-strands or α-helices in a hydrophobic environment (von Heijne and Nishikawa, 1991; May and Soll, 1999; Wienk et al., 1999). This flexibility may allow for significant conformational adaptability to the multiple components involved in protein translocation (Bruce 2001).

Recently, direct interaction of the transit peptides with the protein translocation machinery has been reported (Subramanian et al., 2001). Most of the envelope transit peptides have a carboxyproximal region with a loosely conserved sequence Ile/Val-x-Ala/Cys-Ala (x refers to any amino acid) at the proteolytic processing site of the stromal processing peptidase (SPP) (indicated by the arrow) (Gavel and von Heijne, 1990). For most of the thylakoid proteins, the envelope transit peptides are removed by SPP soon after they reach the stroma. SPP is composed of two antigenically related proteins with molecular masses of 143 and 145 kDa (Oblong and Lamppa, 1992).

The C-terminal part of bipartite transit peptides provides the signal for transport into or across the thylakoid membrane and is termed thylakoid transfer domain or signal peptide. The thylakoid transfer signals are necessary and sufficient for pathway-specific transport of different precursor proteins. Therefore, the structure of the thylakoid targeting domains of the transit peptides are necessarily more complex. The features of the thylakoid transfer domains from each pathway will be discussed in detail later. Signal peptides usually end with an A-X-A motif for cleavage by the thylakoid processing peptidase (TPP) (Kirwin et al., 1987).

2.2. Translocation machinery at the chloroplast envelope membrane

Precursor protein translocation across the outer and inner envelope membrane is mediated by the translocon at the outer membrane of chloroplasts (Toc) and the translocon at the inner membrane of chloroplasts (Tic). Only few components of the import machineries have homologues in prokaryotic cells like *Synchocystis* PCC 6803 (e.g. Reuman et al., 1999), suggesting that these components have a prokaryotic origin, although the precise function of the prokaryotic homologues remains unknown. Translocation via Toc and Tic occurs simultaneously for most proteins, probably at regions where the outer and inner membranes are in close contact (Chen and Schnell, 1999; May and Soll, 1999). Toc contains several transmembrane proteins: Toc159, Toc75, Toc34, Toc36, and a recently identified component Toc64 (Chen and Schnell, 1999; Sohrt and Soll, 2000). Toc34 and Toc159 function as

receptors for precursor proteins (Kessler et al., 1994), while Toc75 forms the aqueous pore through which the precursor proteins are translocated (May and Soll, 1999). The diameter of this pore is only 8-9 Å, suggesting that proteins must be fully unfolded during translocation into the Toc protein pore (Chen and Schnell, 1999). Tic consists of Tic110, Tic55, Tic22 and Tic20 (reviewed by Jarvis and Soll, 2002), but the precise organization and function of these proteins is not clear. Insertion of the precursor proteins into the Tic complex requires ATP hydrolysis within the stroma (Chen and Schnell, 1999). Several kinds of molecular chaperones are required for protein transport across the inner membrane, including ClpC and the chloroplast Hsp70 (cpHsp70) (Marshall et al., 1990; Shanklin et al., 1995). Current models depict ClpC as the motor driving precursor import (Keegstra and Cline, 1999). Another chaperone, Cpn60, binds to the precursor proteins and assists in protein refolding in the stroma (Tsugeki and Nishimura, 1993).

3. Protein export systems – from bacteria to thylakoids

Protein translocation systems are structurally and mechanistically diverse from one membrane system to another, but can nevertheless be divided into roughly two major types: the export system and the import system (Schatz and Dobberstein, 1996). Import system transports proteins into a compartment that is functionally equivalent to, or evolutionarily derived from, the cytosol. Export system transports proteins from the cytosol to an extracytosolic compartment. According to this classification, translocation of nuclear-encoded proteins into mitochondria or chloroplasts is facilitated by import pathways, whereas protein translocation into the bacterial periplasma and the thylakoidal lumen is facilitated by export pathways. All export systems are characterized by many common features and are phylogenetically related to the bacterial secretion systems. Therefore, any knowledge of the bacterial protein transport systems leads to stimulating investigation on the chloroplast systems, and *vice versa*.

3.1. Protein translocation systems at the *E.coli* plasma membrane

Protein translocation across the bacterial cytoplasmic membrane has been studied extensively in gram-negative bacteria such as *Escherichia coli*. Gram-negative bacteria are surrounded by two membranes, the inner membrane and outer membrane, and therefore possess a functional periplasm residing between these two membranes. A wide range of proteins with a function in the periplasmic space or outer membrane has to be transported to their final location. These proteins are synthesized in the cytoplasm as precursors with a cleavable amino-terminal signal peptide. Depending on the nature of the precursors, different translocation/secretion pathways are employed for the transport across the inner membrane (Danese and Silhavy, 1998; Agarraberes and Dice, 2001).

3.1.1. The Sec-dependent pathway

Most periplasmic proteins and outer membrane proteins are transported across the plasma membrane via a general secretion pathway, which is characterized by a peripheral ATPase, SecA, and is therefore designated as the Sec-dependent pathway. Proteins transported by this pathway are synthesized in cytosol as precursors with an N-terminal signal peptide of 18-26 amino acid residues (von Heijne, 1986; Randall and Hardy, 1998; Chou, 2001). These signal peptides have three characteristic regions: a positively charged region with alkaline amino acids at the N-terminus (n-domain), a highly hydrophobic region in the middle (h-domain), and a polar region containing the signal peptidase cleavage site at the C-terminus (c-domain) (Berks, 1996). Signal peptides alter the folding properties of the mature part of the precursors (Park et al., 1988) and are recognized by SecA and often also by a cytosolic chaperone, SecB (Hartl et al., 1990).

Sec-dependent translocation across the plasma membrane is accomplished by the Sec translocon. Sec translocon consists of two cytosolic proteins SecA and SecB, and at least six integral membrane proteins, notably SecY, Sec E, Sec G, Sec D, Sec F and YajC (reviewed by Driessen et al., 1998). These integral membrane proteins form two distinct trimeric complexes: SecYEG and SecDFyajC. The first complex forms the protein-conducting

channel, through which the precursor proteins are inserted into the plasma membrane. SecB binds to the newly synthesized precursor proteins in the cytosol, mediating the interaction between preproteins and the translocon. *In E. coli*, SecA is the most abundant component of the translocase (Driessen, 1994) and has a balanced cellular distribution between soluble and membrane-bound states (Cabelli et al., 1991). SecA exhibits high affinity interaction with SecB/preprotein complexes upon binding to SecYEG (Hartl et al., 1990; Economou and Wickner, 1994). Hydrolysis of ATP bound to SecA leads to the insertion of the precursor protein into the SecYEG channel and the release of SecA from the membrane. Reiteration of the SecA insertion-deinsertion cycle results in translocation of the entire precursor protein (Economou 1998). ATP is absolutely required for this process, and a requirement for proton-motive force is also common but not universal (Schiebel et al., 1991; Nishiyama et al., 1999).

Not all of the components mentioned above are essential for Sec-dependent translocation. SecA, SecY and SecE are the minimum entity to perform Sec-dependent protein translocation in bacteria (Murphy et al., 1995; Prinz et al., 1996), whereas SecG, SecDFyajC are non-essential proteins, required only for maximal rates of protein translocation by regulating insertion-deinsertion cycles (Pogliano and Beckwith, 1994; Duong and Wickner, 1997).

3.1.2. The SRP-dependent pathway

In *E. coli*, targeting of several highly hydrophobic inner membrane proteins, such as FtsQ and leader peptidase, is carried out co-translationally by the signal recognition particle (SRP) system (MacFarlane and Müller, 1995; der Gier et al., 1996). This system is homologous to the mammalian SRP system involved in the co-translational targeting of ribosome-nascent chain complexes (RNCs) to the endoplasmic reticulum (ER) membrane (reviewed by Keenan et al., 2001). The mammalian SRP system is composed of a soluble cytoplasmic signal recognition particle (SRP) and a membrane-embedded Sec61 translocon. The mammalian SRP contains one molecule of RNA (7SL) and a complex of six polypeptides (Walter and Blobel, 1980; 1983), including a 54 kDa subunit. The bacterial SRP was determined as a

cytoplasmic ribonucleoprotein complex that consists of a 4.5S RNA molecule and a 48 kDa protein (Ffh = fifty-four homologue) with homology to the mammalian SRP54 component (Bernstein et al., 1989; Poritz et al., 1990). In addition, the SRP receptor-like protein FtsY was also identified (Bernstein et al., 1989; Romisch et al., 1989). FtsY is localized both to the plasma membrane and to the cytosol. *In vitro* reconstitution experiment demonstrated that the Ffh, FtsY and 4.5SRNA are functionally sufficiently to target proteins to the membrane-embedded translocon (Koch et al., 1999). Bacterial SRP binds to the RNCs through the interaction of the SRP with the newly synthesized polypeptide, and the resulting RNC-ribosome-SRP complex binds subsequently to FtsY in a GTP-dependent manner. The RNCs are delivered by the SRP and FtsY to the SecYEG protein channel, through which the polypeptides are inserted into the plasma membrane. Therefore, the Sec- and the SRP-dependent mechanisms converge at the translocon (Valent et al., 1998; Neumann-Haefelin et al., 2000). Remarkably, SecY and SecE show homology to two components of the Sec61 channel at the mammalian ER membrane, the Sec61α and Sec61γ protein, respectively, indicating that the bacterial Sec/SRP system is phylogenetically related to the mammalian SRP system (Gorlich et al., 1992).

Recently, a novel and evolutionarily conserved component that plays an essential role in the SRP-dependent translocation has been identified from *E. coli*. This plasma membrane protein, YidC, is a homologue to the mitochondrial protein, Oxa1 (for a review, see Luirink et al., 2001). Oxa1 is a nuclear-encoded mitochondrial inner membrane protein that presumably forms a novel translocase in the mitochondrial inner membrane (Herrmann et al., 1997). Oxa1 was shown to be essential for correct integration of a subset of inner membrane proteins encoded by both the mitochondrial and nuclear genomes (Hell et al., 1998; Hell et al., 2001). The bacterial YidC was found to be associated with the SecYEG translocon and to interact with the transmembrane segments of the membrane protein FtsQ during its insertion into the membrane (Scotti et al., 2000). However, depletion of YidC does not impair transport of SRP-dependent proteins such as leader peptidase and ProW (Samuelson et al., 2000). It was suggested that YidC functions in recognizing transmembrane regions and facilitating their

lateral movement into the membrane (Scotti et al., 2000; Berk et al., 2001; Urbanus et al., 2001).

3.1.3. The bacterial TAT pathway

Up to 95% of the periplasmic proteins are transported by the Sec-dependent pathway in an unfolded formation. However, a subset of periplasmic proteins binds complex cofactors in the cytoplasm (e.g. iron-sulfur clusters, nickel and iron cofactors), and are thus obliged to fold prior to translocation (Bogsch et al., 1998). Translocation of these proteins takes place in a Sec-independent manner. Interestingly, almost all of the precursors of these proteins bear a characteristic twin-arginine (RR) motif within their signal sequences (Berks, 1996), which is a unique feature of the ΔpH-dependent protein translocation pathway at the thylakoid membrane (Chaddock et al., 1995). Accordingly, this pathway was termed TAT (twin-arginine translocation) pathway in both the bacterial and the thylakoid system (Sargent et al., 1998; Dalbey and Robinson, 1999; Berks, 2000). The major feature of the TAT-dependent mechanism is that this pathway is capable of transporting tightly folded globular proteins (Rodrigue et al., 1996; Chanal et al., 1998; Santini et al., 1998; and reviewed by Müller and Klösgen, 2005). The bacterial TAT system is even able to export complexes of proteins formed in the cytosol by a “hitch-hacker” mechanism (Wu et al., 2000). At least one experiment has shown that secretion of protein through the TAT system requires the proton-motive force ($\Delta\mu H^+$) (Santini et al., 1998).

TAT pathway signal peptides have a similar tripartite organization as Sec signal peptides: a positively charged n-domain, a middle h-domain and a c-domain. The twin-arginine motif is located at the end of the n-domain, within a conserved (S/T)-R-R-x-F-L-K sequence motif (Berks, 1996). The two arginine residues, especially the second one, are invariant and the other motif residues occur at a frequency of more than 50%. Several experiments have shown that TAT signal peptides target Sec pathway proteins or foreign proteins to the TAT-dependent pathway (Cristobal et al., 1999; Thomas et al., 2001), while Sec signal peptides

direct TAT pathway substrates to the Sec apparatus (Rodrigue et al., 1999), although the folded structure of the TAT pathway substrates do not allow the full translocation. Thus, in *E. coli*, the signal peptide alone mediates mutually exclusive sorting of precursor proteins between the TAT and Sec pathway.

Identification of the bacterial TAT system components have resulted from homology searches to the first component of the plant ΔpH-dependent import pathway called Hcf106 (Settles et al., 1997; Weiner et al., 1998). Three homologous genes to *hcf106* were found in the *E. coli* genome, notably *tatA, tatB* and *tatE*. The *tatA* and *tatB* genes are located in one operon together with *tatC* and *tatD* in tandem, whereas the *tatE* gene forms an independent cistron (Bogsch et al., 1998; Sargent et al., 1998). Mutagenesis has shown that the TatA/B/E proteins are essential TAT pathway components, and that TatA and TatE can replace each other in function (Bogsch et al., 1998; Sargent et al., 1998). Investigation of the *tatABCD* operon has led to the identification of a further critical component of this system, TatC. Disruption of the *tatC* gene leads to a total block of Tat-dependent export (Bogsch et al., 1998), indicating a central role for TatC in this pathway. On the other hand, *tatD* encodes a DNase of cytosolic localization, which is apparently not involved in Tat-dependent protein transport (Wexler et al., 2000). All bacterial Tat components are membrane proteins of the plasma membrane. TatA, TatB and TatE span the plasma membrane once, with an N_{out}-C_{in} topology. TatC sapns the plasma membrane six times, with both the N- and C-terminus on the cytoplasmic side (Gouffi et al., 2002). It has been shown that TatB and TatC are associated together in equivalent amount to form complexes of approximately 600 kDa molecular weight, and variable amount of TatA could be detected in these complexes (Bolhuis et al., 2001; Sargent et al., 2001; de Leeuw et al., 2002). However, electron microscopy of several of these complexes did not yield images sufficiently unique to allow for the assignment of the obtained structures to individual Tat proteins, suggesting a flexible assembly of the TAT translocase from a varying number of subunits. Overexpressed TatA and TatB are tightly associated *in vivo*, and form complexes with a molar ratio from 15+4 to 19+4 when TatC is also present (Sargent et al., 2001, de Leeuw et al., 2001). Electron microscopy of such

complex shows a double-layered ring structure, suggesting that TatA is involved in formation of the protein conducting channel.

3.1.4. YidC-dependent membrane protein insertion

Also several membrane proteins are inserted into the bacterial plasma membrane by a Sec-independent mechanism. These proteins include the procoat protein of phage M13 and phage Pf3. Both proteins are synthesized in a precursor form, with a cleavable N-terminal signal peptide. The signal peptide consists of a positively charged N-terminus, followed by a hydrophobic region. Targeting of the protein to the membrane requires basic residues in both the N- and C-terminal regions, indicating an electrostatic binding of procoat to the acidic phospholipid head groups (Gallusser and Kuhn, 1990). The hydrophobic regions in the signal sequence and the mature protein synergistically contribute to drive proteins insertion into the membrane (Soekarjo et al., 1996). Translocation of the loop between the two hydrophobic regions is stimulated by the proton motive force (pmf) across the membrane (Kuhn et al., 1990). Once inserted, the signal sequence is cleaved by the leader peptidase to generate the mature protein in the membrane (Shen et al., 1991).

M13 procoat is even able to insert into protein-free liposomes (Geller and Wickner, 1985; Soekarjo et al., 1996). Experiments have also shown that Pf3 procoat is able to insert into a trypsin-pretreated membrane (Kiefer and Kuhn, 1999). All of these experiments substantiated the widely held view that the membrane insertion of this kind of proteins is independent of any protein factors. However, later experiments have revealed the involvement of YidC in the membrane insertion process. Membrane insertion of the M13 procoat is strongly inhibited in YidC-deficient E. coli cells, while the secretion of the periplasmic proteins is not affected (Samuelson et al., 2000). Direct interaction of YidC with M13 procoat and Pf3 procoat was furthermore observed during membrane insertion (Samuelson et al., 2001; Chen at al., 2002). This suggests a dual role of YidC in both Sec-dependent and Sec-independent protein insertion into the bacterial membrane.

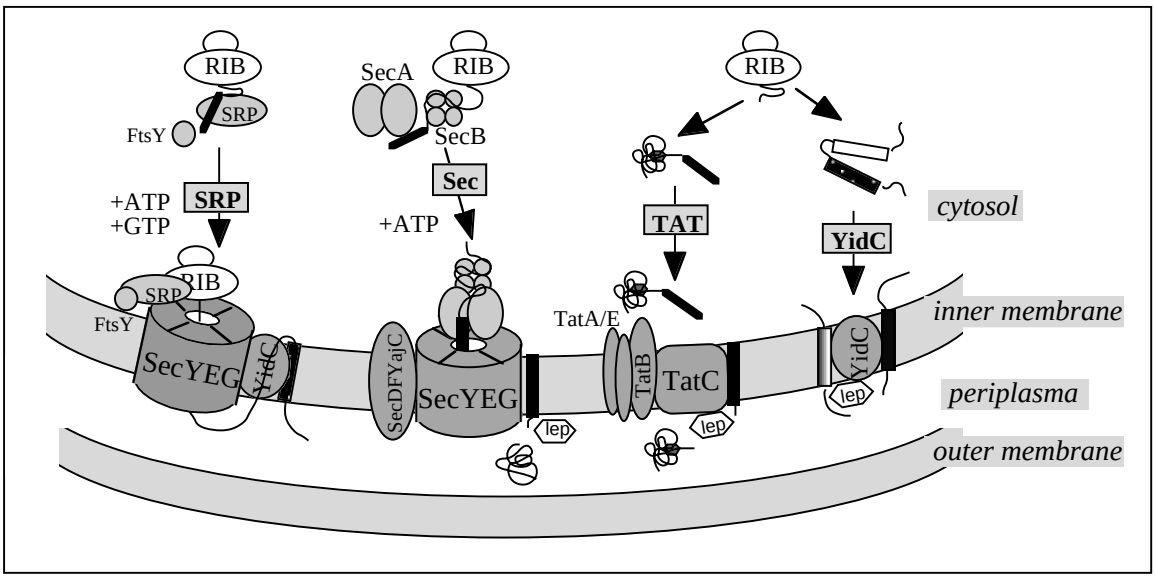

Fig. 1. Protein transport pathways in E. coli. The majority of the periplasmic proteins is synthesized in precursor form with an N-terminal signal peptide and transported across the plasma membrane by either of two pathways, the Sec-dependent or the Tat-dependent pathway. A subset of inner membrane proteins is co-translationally inserted into the membrane by the SRP-dependent pathway through the SecYEG channel. Other inner membrane proteins are inserted by assistance of YidC. Upon translocation, the signal peptides are removed by leader peptidase (Lep), which has its activity exposed to the periplasmic side of the inner membrane. Arrows indicate the protein translocation pathways from the cytosol to the inner membrane and the periplasm. Specific requirements of energy and characteristic factors of each pathway are indicated. RIB = ribosome.

3.2. Protein translocation systems at the thylakoid membrane

Nearly all proteins of the thylakoid are encoded in the nucleus and synthesized in the cytosol as precursor proteins. Most of them undergo a two-stage import process: first, chloroplast envelope translocation and stroma targeting result in a stromal intermediate form generated after removal of the import signal by SPP; second, thylakoid transport and lumen targeting lead to the generation of a mature form, generated by cleavage of the intermediate by TPP in the lumen. Once in the stroma, the precursor proteins enter precursor-specific transport pathways. To date, four distinct pathways for protein transport across the thylakoid membrane have been described. In contrast to the protein import systems at the envelope membrane,

these pathways are directly related to the protein transport pathways operating at the bacterial plasma membrane. Accordingly, they are defined as Sec-dependent pathway, SRP-dependent pathway, ΔpH-dependent pathway, and spontaneous insertion pathway, respectively.

3.2.1. The Sec-dependent pathway of chloroplasts

A subset of thylakoid lumen proteins, including the 33-kDa photosystem II protein (33K), plastocyanin (PC), and PSI-F, are transported by a mechanism that relies on ATP and soluble stromal factors (Hulford et al., 1994). Thylakoid transport of 33K, PC and PSI-F is sensitive to sodium azide (Knott and Robinson, 1994; Karnauchov et al., 1994; Henry et al., 1994), which is known as an inhibitor of the bacterial SecA protein (Oliver et al., 1990), which suggests already that a conserved protein transport mechanism is functional within chloroplasts.

i. Components of the thylakoidal Sec-dependent machinery

Preliminary evidence for a Sec-dependent pathway existing in thylakoids includes the presence of SecA- and SecY-homologous genes in the chloroplast genomes of several algae (Scaramuzzi et al., 1992; Douglas, 1992). Later on, SecA-homologous genes were identified from different plants by a homology-based approach (Berghöfer et al., 1995; Nohara et al., 1995), and were designated cpSecA. CpSecA was also identified independently by a genetic approach, in which the maize mutant *tha1* was found to result from disruption of the maize Sec-dependent protein translocation (Voelker and Barkan, 1995). CpSecA is localized in the chloroplast stroma, and pre-incubation of stroma extract with anti-cpSecA antibodies blocked transport of 33K and PC into thylakoids (Nakai et al., 1994).

A chloroplast SecY homologue was identified in the Arabidopsis EST collection (Laidler et al., 1995) and led to the isolation also of the homologous cDNA from spinach (Berghöfer, 1998). A gene homologous to bacterial *secE* gene is also present in the Arabidopsis genome sequence (Bevan et al., 1998). CpSecE forms a 180 kDa complex together with cpSecY

(Schuenemann et al., 1999). Homologues to the other components of the bacterial Sec pathway, notably SecB, SecG, SecD, SecF or YajC, are apparently lacking from the Arabidopsis proteome. Since in the bacterial Sec-dependent system, the SecYE complex and SecA form a minimal translocase to perform protein secretion, the plant thylakoid Sec-dependent system appears to operate with the minimal number of required components. It is possible though that additional factors, like for example stromal chaperones such as ClpC, cHsp70 or Cpn60, might be involved in the Sec-dependent translocation in the chloroplast system.

ii. Mechanism of thylakoidal Sec-dependent protein transport

The homology observed between the bacterial and chloroplast Sec-dependent machineries suggests that they both operate in a similar manner. Indeed, although the mechanism of the chloroplast Sec-dependent translocation has been studied only in a limited number of experiments, it seems to be largely similar to its bacterial counterpart. Thylakoid precursor proteins bind to the membrane in a cpSecA-dependent manner (Keegstra and Cline, 1999) and form a complex within the membrane that also contains cpSecY (Mori and Cline, 2001). CpSecA partially inserts into the lipid bilayer, carrying a fragment of the precursor protein (Berghöfer, 1998). ATP is absolutely required for thylakoid Sec-dependent translocation, because depletion of ATP by apyrase treatment completely prevents Sec-dependent translocation (Hulford et al., 1994). Inhibition of ATP hydrolysis by sodium azide or by the ATP-analog AMP-PNP results in a permanent insertion of the SecA into the thylakoid membrane (Berghöfer, 1998), suggesting that ATP hydrolysis is required for the release of cpSecA upon translocation of the precursor protein. Tightly folded proteins are not transported by the thylakoid Sec-dependent pathway (Hynds et al., 1998). A trans-membrane potential is not essential for Sec-dependent transport, although translocation of some precursor proteins is stimulated by ΔpH across the thylakoid membrane (Yuan and Cline, 1994; Mant et al., 1995).

3.2.2. The SRP-dependent pathway of chloroplasts

Thylakoid membrane is one of the most condensed membrane containing a large number of membrane proteins. In the light of the completion of the genome sequencing of Arabidopsis, nearly 350 proteins are predicated to locate within the thylakoid membrane (Peltier et al., 2002), most of which are integrated into the thylakoid membrane by the chloroplast SRP pathway, a counterpart of the bacterial or mammalian SRP pathway. The majority of these proteins is encoded by the nucleus and is imported into the stroma after being synthesized in the cytosol. Only a few of them are encoded by the plastid genome and synthesized in the stroma of chloroplasts. Obviously, co-translational insertion is possible in chloroplast only for the plastid-encoded thylakoid proteins. The nuclear-encoded proteins that are synthesized in the cytosol need to be inserted post-translationally into the thylakoid membrane. Chloroplast SRP (cpSRP) is unique in that it is capable to insert membrane proteins both co-translationally and post-translationally. So far, understanding of the mechanism of the post-translational SRP-dependent protein insertion is largely based on studies of the major light-harvesting chlorophyll a/b binding protein (LHCP), which is encoded in the nucleus. Yet, the mechanism of the co-translational SRP-dependent protein insertion is by far less well studied, mainly due to technical constrains.

i. Components of the chloroplast SRP-dependent pathway

Identification of a chloroplast SRP54 homologue (cpSRP54) (Franklin and Hoffman, 1993) and the discovery of the interaction between cpSRP54 and LHCP in the stroma (Hoffman and Franklin 1994) led to the conclusion that SRP is operating in thylakoid membrane protein insertion. Further characterization of cpSRP failed to identify an RNA component, and instead led to the identification of a novel 43 kDa protein subunit (cpSRP43) (Schuenemann et al., 1998). Unlike cpSRP54, the evolutionary origin of cpSRP43 remains uncertain, since a prokaryotic homologue was not identified so far. It might have evolved to cope with the obligatorily post-translational mode of insertion for nuclear-encoded thylakoid membrane

protein. Reconstitution of a functional transit complex consisting of cpSRP54, cpSRP43 and LHCP demonstrated that a RNA component, as well as any further stroma factor, is not required for insertion of LHCP (Schuenemann et al., 1998). A chloroplast homologue of the SRP receptor, cpFtsY, was identified in Arabidopsis proteome (Kogata et al., 1999). Anti-cpFtsY antibodies specifically inhibit the integration of LHCP into isolated thylakoids, indicating that cpFtsY plays an essential role in the insertion process. However, unlike the bacterial SRP system, the involvement of the SecYE translocon in the insertion of LHCP has not been proven. Antibodies to SecY, which block the translocation of lumenal proteins via the Sec translocon, have no effect on the insertion of LHCP (Mori et al., 1999). Instead, Alb3, the chloroplast homologue to mitochondrial Oxa1 and bacterial YidC proteins, was shown to be essential for thylakoid membrane insertion of LHCP (see below). Alb3 is located in the thylakoid membrane. Antibodies raised against Alb3 specifically inhibit the insertion of LHCP, but have no effect on Sec- and ΔpH/TAT-dependent protein translocation (Moore et al., 2000).

ii. Mechanism of post-translational insertion of thylakoid membrane proteins by the SRP-pathway

Studies on post-translational insertion of thylakoid membrane protein were almost exclusively performed with a single model protein, notably LHCP. Like most other nuclear-encoded thylakoid proteins, LHCP carries a cleavable N-terminal import signal, but no cleavable thylakoid-targeting signal. Instead, the thylakoid targeting information is located within the mature part of LHCP (Lamppa, 1988; Viitanen et al., 1988). Once in the stroma, LHCP binds rapidly to cpSRP54 and cpSRP43 to form a transit complex (Payan and Cline, 1991; Schuenemann et al., 1998). Formation of the SRP/LHCP complex prevents hydrophobic LHCP from aggregation and misfolding in stroma (Payan and Cline 1991). CpFtsY binds to the SRP/LHCP transit complex in a strictly GTP-dependent manner to form a super-complex (Tu et al., 1999). Like their cytoplasmic homologues, both cpSRP54 and cpFtsY are GTPases (Hoffman and Franklin, 1994).

It is still unknown how the transit complex is targeted to the thylakoid membrane. It was suggested that cpFtsY pilots the transit complex to the membrane, like its homologue does in the bacterial cytosol (Tu et al., 1999). Once at the membrane, the transit complex is disassociated upon GTP hydrolysis, delivering LHCP to the membrane component (Groves et al., 2001). As mentioned above, Alb3 is essential for insertion of LHCP. It was suggested that Alb3 forms a separate translocon, which is independent of the SecYE complex (Moore et al., 2000; Eichacker and Henry, 2001), indicating that post-translational integration of LHCP takes place by a mechanism that is quite different from that of co-translational protein export.

iii. Mechanism of co-translational insertion of thylakoid membrane protein

In addition to its role in post-translational protein export, cpSRP appears to have retained co-translational targeting activity. A subset of thylakoid proteins, including PsaA, PsaB, PsbA and PsbD, are encoded by plastid genes. *In vitro* transcription/translation of *psbA* gene by extracts from chloroplast stroma has shown that a PsbA-RNC is found, which interacts with cpSRP54 (Nilsson et al., 1999). Analysis of Arabidopsis mutants has shown that lack of cpSRP54 has an effect on the membrane insertion of reaction center proteins such as PsaA and of LHCP, whereas the lack of cpSRP43 affects only the membrane insertion of LHCP but not that of the reaction center proteins (Jonas-Straube et al., 2001). This suggests that cpSRP43 is not involved in the co-translational transport of thylakoid membrane proteins. Surprisingly, co-translational transport of thylakoid membrane proteins was also not affected in an Arabidopsis mutant lacking cpFtsY (Amin et al., 1999), suggesting that cpFtsY is not essential for co-translational insertion. In analogy to the bacterial SRP system, co-translational integration of thylakoid membrane proteins is apparently dependent upon the SecYE translocon. In a maize cpSecY null mutant, translocation of PsbA was also severely affected (Voelker et al., 1997; Roy and Barkan, 1998), indicating that cpSecY is required, for example, for ribosome binding during co-translational transport of plastid-encoded proteins. The role of Alb3 in co-translational transport, one the other hand, is not yet established. The thylakoidal ΔpH stimulates the co-translational SRP-dependent export (Zhang et al., 2000;

Muhlbauer and Eichacker, 1999), as well as the post-translational SRP-dependent membrane insertion of LHCP (Cline et al., 1992).

3.2.3. The ΔpH/TAT-dependent pathway of chloroplasts

In vitro studies of protein import into isolated thylakoids revealed that a subset of precursor proteins is transported by a mechanism that is different from the Sec- or SRP-dependent pathways. These proteins include the 16- and 23-kDa subunits of photosystem II (16K and 23K), photosystem II subunit T (PSII-T), and photosystem I subunit N (PSI-N) (Mould and Robinson 1991; Mould et al., 1991; Cline et al., 1992; Klösgen et al., 1992; Henry et al., 1994). Import of those proteins requires neither nucleoside triphosphates nor soluble stromal factors but is instead totally dependent on the ΔpH across the thylakoid membrane. Therefore, this pathway was designated the ΔpH-dependent pathway.

i. Pathway selection of precursors: distinctive signal peptides

Competition studies with chemical amounts of precursor proteins showed that the Sec- and ΔpH-dependent pathways are precursor-specific and that they operate independently from each other in the translocation of thylakoid proteins (Cline et al., 1993). No genuine precursor targeted by both pathways has so far been found, although recombinant precursors and the cyanobacterial CtpA, when analyzed in the heterologous chloroplast import assays, showed transport by both pathways *in vitro* (Karnauchov et al., 1997). Several studies have shown that the choice of pathway is dictated by the thylakoid-targeting signal peptides of the respective transit peptides (Robinson et al., 1994; Henry et al., 1994; Karnauchov et al., 1994).

Thylakoid transfer signals of the Sec- and ΔpH-dependent proteins share several common structural features: a hydrophilic, positively charged N-terminal region (n-domain), a hydrophobic core region (h-domain), and a polar C-terminal region (c-domain). Domain

swapping experiments and mutagenesis studies showed that pathway specificity is determined by subtle differences between the signal peptides for the Sec- and ΔpH-dependent pathways (Henry et al., 1997; Chaddock et al., 1995; Brink et al., 1998). A twin-arginine motif is found immediately before the h-domain, which is a characteristic of almost all ΔpH-dependent signal peptides and distinguishes them from those of the Sec-dependent pathway. Site-directed mutagenesis has shown that this motif plays an important role in pathway recognition. Even the conservative substitution of a single of these arginines to lysine dramatically impairs the ability of the precursors to be transported (Chaddock et al., 1995). Therefore, the ΔpH-dependent pathway was also termed the twin-arginine translocation pathway (TAT-pathway). Although the RR motif is strictly required for TAT-dependent translocation, a few natural exceptions have been so far identified, as well as in *E.coli* (Molik et al., 2001, Hinsley et al., 2001; Ignatova et al., 2002). At least a KR motif is compelling for a TAT-dependent transport. Typical signal peptides for the ΔpH/TAT-dependent pathway, as well as for the Sec-dependent and the spontaneous pathways, are shown in Fig. 2.

ΔpH/TAT-dependent pathway

```
Sp   23K                AQKQDDNEANVLBSGVS**RR**LALTVLIGAAAVGSKVSPADA
Wh   23K                 AQKNDEAASDAAVVTS**RR**AALSLLAGAAAIAVKVSPAAA
Sp   16K                      AQQVSAEAETS**RR**AMLGFVAAGLASGSFVKAVLA
Ma   16K                     ASAEGDAVAQAG**RR**AVIGLVATGIVGGALSQAARA
Ara  16Ka                        AQQSEETS**RR**SVIGLVAAGLAGGSFVQAVLA
Ara  16Kb                        NVSVPESS**RR**SVIGLVAAGLAGGSFVKAVFA
Bar  PSI-N                   AAAKRVQVAPAKD**RR**SALLGLAAVFAATAASAGSARA
Cot  PSII-T                 VQMSGERKTEGNNG**RR**EMMFAAAAAAICSVAGVATA
Ara  PSII-T                 TPSLEVKEQSSTTM**RR**DLMFTAAAAAVCSLAKVAMA
Ara  p29                  CSKIEPQVSGESLAFH**RR**DVLKLAGTAVGMELIGNGFINNVGDAKA
Ara  Hcf136                 SPSPSSSSSSLSFS**RR**ELLYQSAVSLSLSSIVGPARA
Ara  p16       ...SKKNQIAYSGNSKNQTSSSLLWK**RR**ELSLGFMSSLVAIGLVSNDRRRHDANA
Ara  Rieske           ...ACQASSIPADRVPDME**KR**KTLNLLLLGALSLPTGYMLVPYATFFVPPG
Sp   Rieske           ...TCQATSIPADNVPDMQ**KR**ETLNLLLLGALSLPTGYMLLPYASFFVPPG
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Sec-dependent pathway

```
Sp   33K      ...SSGG**R**LSLSLQSDL**K**ELAN**K**CVDAT**K**LAGLALATSALIASGANA
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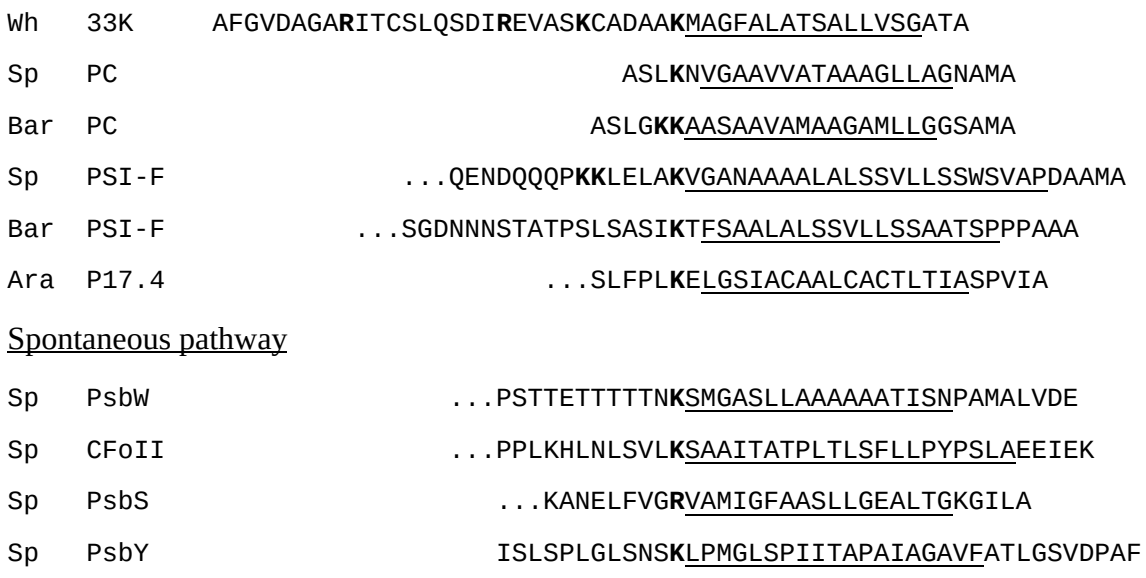


Fig. 2. Signal peptides for ΔpH/TAT-dependent, Sec-dependent and spontaneous thylakoidal protein transport pathways. Signal sequences are shown for representative proteins from spinach (Sp), wheat (Wh), maize (Ma), Arabidopsis (Ara), barley (Ba) and cotton (Cot). The hydrophobic domains (H-domain) are underlined. The conserved twin-R motif of ΔpH pathway signals (see text) is shown in bold, as are the positively charged residues found in the n-domains of the Sec-pathway and spontaneous pathway signal peptides.

Two observations indicate that signal peptides for the ΔpH/TAT-dependent pathway have specificity determinants in addition to the twin-arginine motif. First, substitution of the RR motif to KR, RK, or KK is not sufficient to convert a ΔpH-pathway signal peptide to a Sec type targeting signal peptide (Chaddock et al., 1995). Second, replacing the h/c-domains of a ΔpH-dependent signal peptide by the corresponding domains of a Sec type signal peptide is tolerable for ΔpH/TAT-dependent pathway recognition, but not *vice versa* (Henry et al., 1997; Bogsch et al., 1997), suggesting a Sec-avoidance motif in these regions of ΔpH/TAT-dependent signal sequences.

ii. Components of the ΔpH/TAT-pathway machinery

Identification of the genes for components of the ΔpH/TAT translocation machinery has confirmed that this pathway is in fact highly conserved between bacteria and chloroplasts.

Identification of the components of this pathway was initiated in higher plant by using genetic approaches. Voelker and Barkan succeeded in isolating a maize mutant *hcf106*, in which the translocation of the ΔpH-dependent pathway precursors but not of Sec-pathway precursors was affected (Voelker and Barkan, 1995). The corresponding gene could be isolated and sequenced, which led to the identification of the first component of the ΔpH/TAT-dependent machinery, the Hcf106 protein (Settles et al., 1997). A few years later, a second component of the ΔpH/TAT-machinery, notably Tha4, was genetically and biochemically identified in maize (Mori et al., 1999; Walker et al., 1999). Sequencing showed that Hcf106 and Tha4 are homologous proteins with high similarity in both structure and sequence, especially in the transmembrane domain (~65% identity). Each protein contains a predicted amino proximal transmembrane domain, through which these proteins are anchored to thylakoids. Their C-terminal domains vary in sequence and in length, but are both located on the stromal side of the thylakoid membrane.

As mentioned above, searching for genes homologous gene to *hcf106* in the *E. coli* genome has led to the identification of the *tatABCD* operon that encodes the components of a ΔpH/TAT-like system in *E. coli.* A *tatC* homology gene has been found in the Arabidopsis genome (Motohashi et al., 2001), as well as in pea (Mori et al., 2001). CpTatC is located in the thylakoid membrane, with both N- and C-termini in the stroma. It is predicted to span the membrane six times. The genes encoding essential ΔpH/TAT pathway components, as well as the predicted topology of these proteins, are shown in Fig. 3.

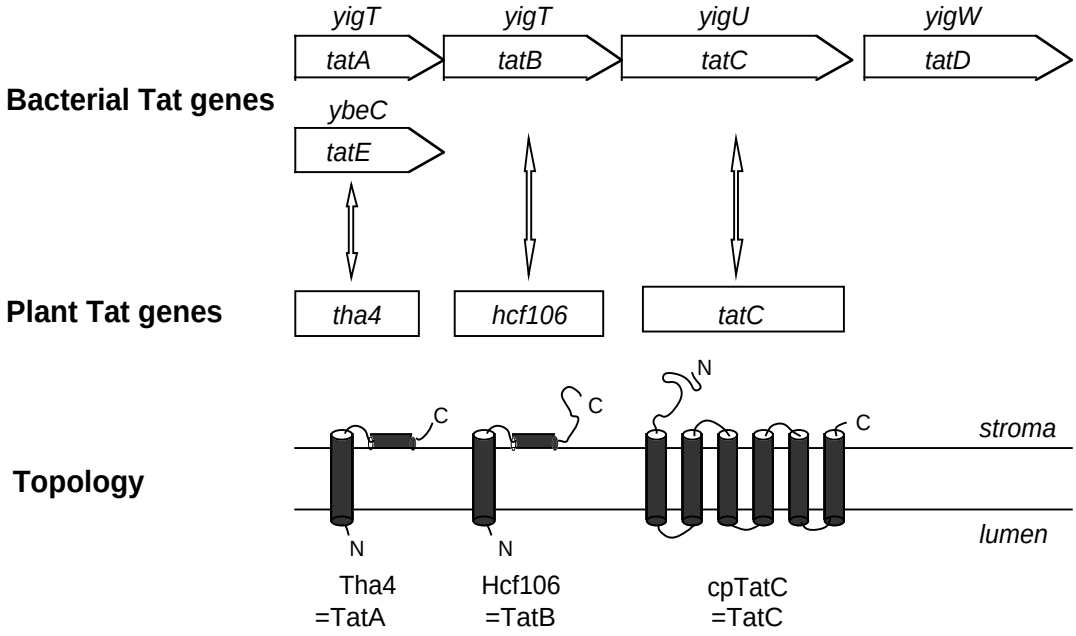


Fig. 3. ΔpH/TAT pathway components of bacterial and plant systems. The bacterial TAT components are encoded by two operons: *yig* and *ybe*. Their corresponding ORFs and the original gene names are indicated. The plant TAT genes are linked to their corresponding bacterial counterparts by arrows. The schematic topology of each protein in the thylakoid membrane is drawn according to Mori and Cline (2001).

iii. The mechanism of ΔpH/TAT-dependent protein translocation

The energy requirements of the ΔpH/TAT-dependent pathway are unique among all protein translocation systems. Transport of precursors by this pathway is independent of nucleoside triphosphates, which are required in most other systems. Instead, the ΔpH/TAT pathway uses the transthylakoidal proton gradient to drive transport. Ionophores, such as nigericin or carbonyl cyanide m-chlorophenylhydrazone (CCCP), inhibit transport. On the other hand, inhibitors for the Sec-dependent pathway, such as azide and apyrase, have no effect on protein transported by this pathway (Mould and Robinson, 1991; Cline et al., 1992; Klösgen et al., 1992). Soluble factors are not important for ΔpH-dependent pathway, suggesting that the initiative step of the transport takes place at the thylakoid membrane, rather than in the stroma (Klösgen et al., 1992; Hulford et al., 1994).

The thylakoidal ΔpH/TAT pathway is able to transport foreign folded proteins that were fused to a ΔpH/TAT signal peptide (Clark and Theg, 1997, Hynds et al., 1998). However, the conformation of authentic substrates during transport has not been established, although indirect evidence suggests that they are folded too. The OE23 (Creighton et al., 1995) and OE16 (Musser and Theg, 2000) proteins have been shown to fold in the stroma prior to transport, but it is unclear if they remain folded also during transport. Further evidence comes from studies on the thylakoidal Rieske protein, a Fe/S containing protein. The Rieske protein gains its Fe/s cluster presumably in the stroma, which is obligatory for ΔpH/TAT-dependent translocation (Molik et al., 2001).

The ΔpH/TAT pathway is able to transport proteins ranging in size from 3.6 kDa to at least 80 kDa (Schubert et al., 2001). The largest known substrate in bacteria is formate dehydrogenase N with a molecular mass of 132 kDa. This protein expands up to 7 nm in diameter when folded, which is larger than the width of the lipid bilayer (Berks et al., 2000). Although the system operates in energy-transducing membranes, large-scale translocation by this pathway appears not to affect the proton permeability (Teter and Theg, 1998). Therefore, the TAT system must have a quite unique translocation mechanism to prevent the leakage of protons. A gated but dynamic flexible channel was supposed to be essential for this system (Robinson et al., 2000). Expansion and contraction of the protein channel could for example be accomplished by adding or removing component monomers. However, the mechanism by which a proton gradient can move the precursor protein are not clear as yet.

iv. Capacity of the ΔpH/TAT-pathway

It appears likely that the bacteria and thylakoids have evolved the ΔpH/TAT-pathway to allow for the translocation of the large folded domains, while preventing ion leakage through the membranes. A proteome analysis of Arabidopsis thylakoid lumen proteins has been performed recently (Schubert et al., 2002), suggesting that more than 50% of the lumenal proteins are synthesized with a typical twin-R motif, indicative of targeting by the ΔpH/TAT

pathway. In contrast, it has been estimated that in bacteria only about 2.5% of the proteins exported are substrates of the TAT-pathway, and almost all of them are cofactor-containing proteins (Berks et al., 2000). Apparently, the number of TAT-substrates has been largely expanded during the endosymbiotic evolution. Unlike the bacterial TAT proteins, many of the passenger proteins of the chloroplast ΔpH/TAT-pathway do not carry cofactors. These proteins include the OE16 and OE23 proteins. For these cofactor-less TAT pathway proteins, they also appear rapid folding kinetics within the chloroplast stroma. In chloroplast, folding of passenger proteins prior to thylakoid translocation likely avoid the impediment of the oscillating acidity of the thylakoid lumen which is directly dependent upon the photosynthetic activity. Obviously, a folded protein is too large for the Sec system to handle (Robinson et al., 2000). Thus, the passenger protein of a ΔpH/TAT pathway protein cannot be transported by the Sec pathway, even when fused to a Sec-type signal peptide (Clausmeyer et al., 1993; Robinson et al., 1994; Henry et al., 1997; Bogsch et al., 1997). A Sec pathway passenger protein, in contrast, can be transported by ΔpH pathway when a ΔpH pathway signal is attached (Clausmeyer et al., 1993; Robinson et al., 1994; Henry et al., 1994).

3.2.4. Spontaneous insertion of thylakoid membrane proteins

Initial studies showed that a range of single-span membrane proteins, including CFoII, PsbW and PsbX, insert into the thylakoid membrane in the absence of SRP, NTPs, ΔpH or a functional Sec machinery (Michl et al., 1994; Lorkovic et al., 1995; Kim et al., 1998). Even pretreatment of the thylakoid membrane with trypsin, which should destroy all stroma-exposed domains of the translocases, has no effect on their insertion (Robinson et al., 1996). A similar mechanism was originally proposed for M13 procoat protein that inserts into the E. coli plasma membrane by a SRP/Sec-independent pathway (Kuhn et al., 1986). Remarkably, the precursors of CFoII, PsbW and PsbX largely resemble the M13 procoat precursor in structure. Like the M13 procoat, they have two hydrophobic domains, one in the signal peptide, the other in the mature protein. Both the N- and C-terminal domains are positively charged, whereas the hydrophilic domain between the two hydrophobic domains is negatively

charged. Like being described for M13 procoat protein (Kuhn, 1987), membrane insertion of the hydrophobic domains of those proteins leads to translocation of the negatively charged hydrophilic domain and formation of a loop-like intermediate in the membrane (Thompson et al., 1998). Therefore, it was previously assumed that a similar spontaneous mechanism is operating in bacteria and chloroplasts.

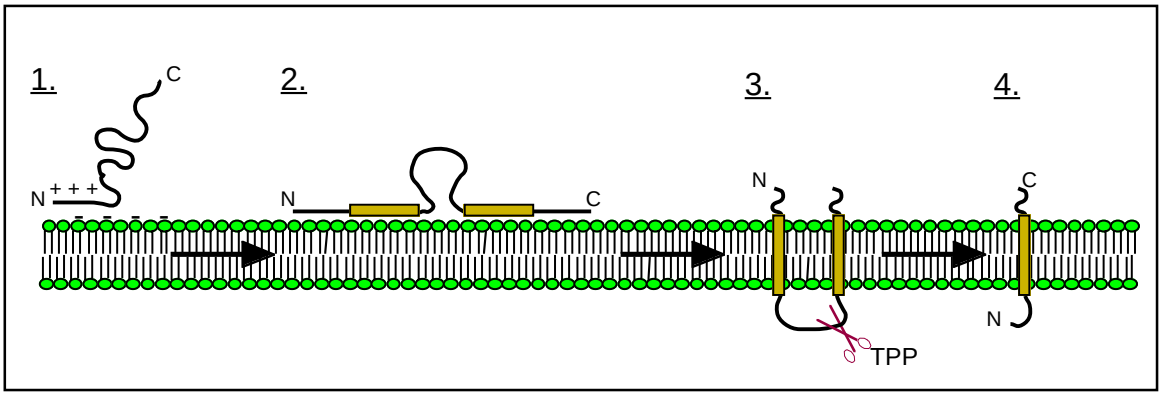


Fig. 4. Model for the "spontaneous" insertion of thylakoid membrane proteins. 1. Targeting of precursor protein to membrane. 2. Formation of hydrophobic α-helixes. 3. Loop insertion and 4. Cleavage of TPP (modified according to Michl et al., 1994). Positively charges are indicated by "+", and hydrophobic α-helixes are drawn as shaded rectangles. TPP is indicated by scissors.

Since insertion of M13 procoat was recently shown to depend on the function of YidC (see above), it was also assumed that Alb3, the chloroplast homologue to YidC, could be involved in insertion of the thylakoid membrane proteins. However, pretreatment of the thylakoid membrane with antibodies against Alb3 strongly inhibits the SRP/Sec-dependent integration of LHCP, but has no effect on the Sec-independent insertion of CFoII, PsbW and PsbX (Woolhead et al., 2001). These data, however, have been obtained in experiments in which the proteins had to be inserted into the isolated thylakoid membrane and therefore may not necessarily correspond to the situation within the chloroplasts. Thus, it still cannot be ruled out that a transport apparatus is involved in the "spontaneous" insertion pathway, but so far there is no positive evidence for this.

Taken together, four distinct pathways are utilized to transport thylakoid proteins across or into the thylakoid membrane. Each pathway transports special protein substrates and, apparently, is subject to differential regulation in biogenesis of the thylakoid membrane. Possibly, these pathways are needed to avoid catastrophic feedback when demands on protein translocation are high. A scheme for the multiple export systems of chloroplasts is shown in Fig. 5.

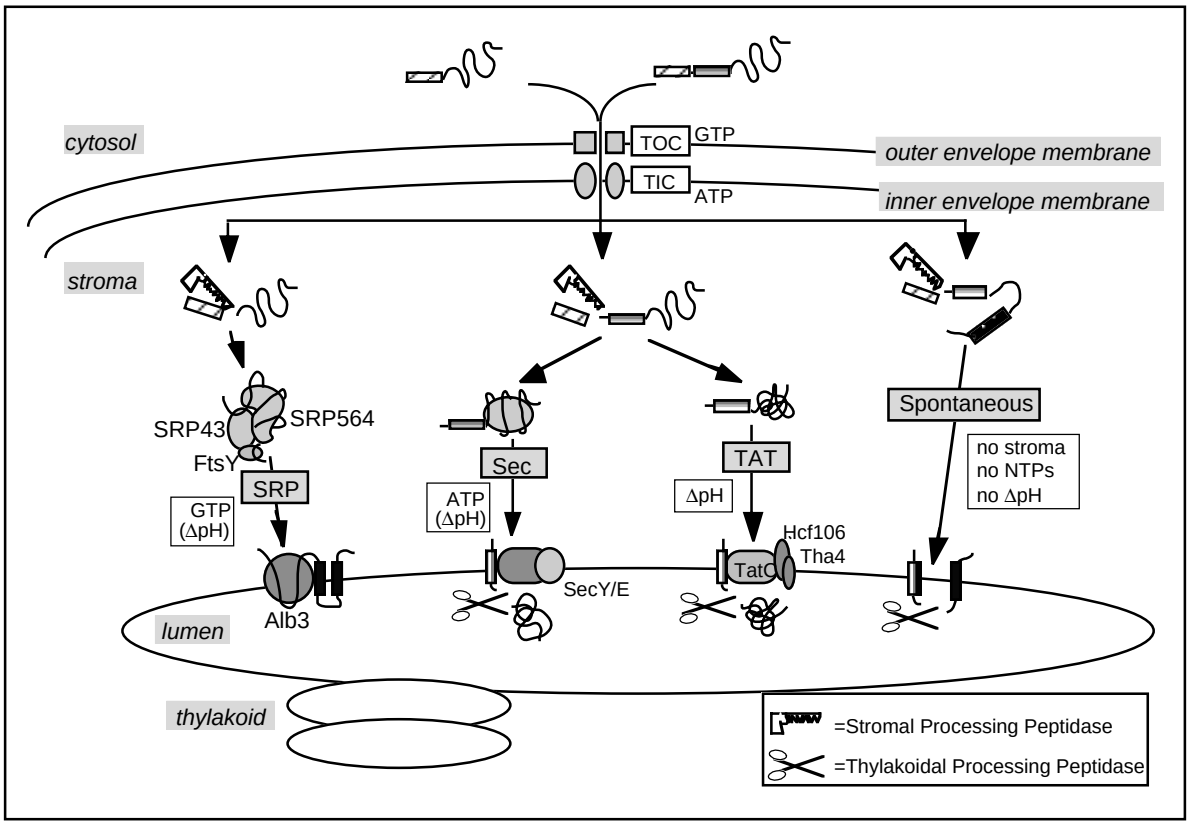


Fig. 5. Import and sorting of nuclear-encoded thylakoid proteins. Nuclear-encoded thylakoid proteins are synthesized in precursor form in the cytosol carrying N-terminal transit peptides. Arrows indicate the path of precursor proteins from the cytosol to the thylakoid membrane. The precursor proteins are imported into the chloroplast stroma through the TOC-TIC complex driven by ATP hydrolysis. The stroma targeting sequences are cleaved off by stromal processing peptidase. The lumenal proteins are translocated by either Sec-dependent or ΔpH/TAT-dependent machineries. Most of the thylakoid membrane proteins are integrated into the membrane by either SRP-dependent pathway or spontaneous insertion. Once in the thylakoid membrane or lumen, the signal peptides are removed by thylakoidal processing peptidase (optionally). Specific requirements of energy and characteristic factors of each pathway are indicated.

4. Goal of the work

It was the goal of this work to characterize the mechanism of ΔpH/TAT dependent protein transport across the thylakoid membrane. For this purpose, in vitro protein transport experiments were performed using isolated intact chloroplasts (*in organello*) or thylakoid vesicles (*in thylakoido*). In the first part of this work, the phylogenetic relationship of the bacterial and thylakoidal TAT-transport systems was analyzed in order to obtain insight into the origin of these systems compare the function of the TAT translocases of both systems. In these experiments, a bacterial protein notably GFOR (glucose-fructose oxidoreductase) was analyzed with the heterologous thylakoid system. In the second part of the work, the translocation steps of ΔpH/TAT-dependent were analyzed by using the chimeric 16/23 protein as a model protein. This allows for studying the mechanism of each translocation step with respect to its energy demands and the requirement of proteinaceous membrane components. In the third part of this work, the involvement of the mature bodies of the chimeric and authentic substrate proteins in the thylakoid targeting by ΔpH/TAT-pathway was analyzed. The final part of this work focuses on the structure of the thylakoidal TAT-translocase. Using a combination of native gel systems and immuno-affinity assays, I have tried to characterize the role of the three TAT-subunits, TatA, TatB and TatC, with respect to their function and organization in each step of the ΔpH/TAT-dependent transport process.

MATERIALS AND METHODS

1. Materials

1.1. Bacterial strains

E.coli strains DH5α (Hanahan, 1983) was used for cloning. Protein overexpression was carried out in *E.coli* strain BL21 (DE3) (Studier and Moffat, 1986).

1.2. Vectors

The following vectors were used for the cloning and transcription: pBluescript II KS- (Stratagene, San Diego), pGEM-T easy (Promega), pBAT (Annweiler et al., 1991). Protein overexpression *in E. coli* was performed in pAR3040 (Rosenberg et al., 1987).

1.3. Plasmids

For cloning and mutagenesis of genes and protein expression, as well as protein import experiments, a collection of plasmids available from the precious work in our laboratory has been used. These plasmids contain cDNA sequences encoding either the authentic precursors of various polypeptides of chloroplast, or the cassettes encoding fusion proteins composed of one transit peptide and one mature portions from different precursor proteins. These plasmids have been described previously () and, if desirable, are specified in detail in the text.

1.4. Plant materials

Pea (*Pisum sativum*) seedings were grown in green house at 25 °C with long day light (10 hours per day), and harvested on the 7th-10th day after sowing.

Arabidopsis thaliana plants were grown on sterile SM medium at 20 °C with short day light (8 hours per day). For preparing DNA or RNA samples, Arabidopsis seedlings were harvested at two weeks after germination. For preparing chloroplasts from Arabidopsis leaves, plants were harvested at four to six weeks after germination.

1.5. Chemicals and other materials

All chemicals were purchased from the following companies: Sigma-Aldrich Chemie (Deisenhofen), Roth GmbH & Co. (Karlsruhe), Serva Feinbiochemica (Heidelberg), Merck AG (Darmstadt), Fluka (Neu-Ulm). If not mentioned, all chemicals used were of analytical grade. Crosslinkers were obtained form Pierce Biotechnology (Rockford, IL).
The Talon metal-chelate affinity matrix was obtained from Clontech (Palo Alto, CA). Protein A-Sepharose was from Amersham Biosciences (Amersham-Pharmacia) (Freiburg).

Nitrocellulose membranes were obtained from Schleicher & Schuell (Dassel), and polyvinylidenfluorid (PVDF) transfermembranes were from Millipore Corporation (Bedford, MA). Secondary antibodies were obtained from Sigma.

DNA molecular weight standard used was 1 kb Ladder from Gibco BRL (Eggenstein). Protein molecular mass standards for SDS protein electrophoresis were obtained from Sigma, which consist of a mixture of 7 proteins: lysozyme (14.3 kDa), soybean trypsin inhibitor (20.1 kDa), trypsiongen (24 kDa), carboanhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa) and bovine serum albumine (66 kDa). Protein Markers used for non-denaturing protein electrophoresis were also from Sigma, and consist of BSA (66-190 kDa), beta-amylase (200 kDa), and thyroglobulin (667 kDa).

Radiochemicals were purchased from Amersham Biosciences and ICN Biomedicals GmbH (Mechenheim).

1.6. Oligonucleotides

Oligonucleotides used for polymerase chain reaction (PCR) and mutagenesis were synthesized by Metabion GmbH (Planegg-Martinsried).

For cloning TAT genes of *Arabidopsis thaliana* or *Pisum sativum*

Arab.TatC forward: TCATATGAGCAGCACAAGCACTAG
Aarb.TatC reverse: GGATCCTCACCGACCTGTGAGCTTG
PeaTatA forward: CATATGGAGATAACACTTTCCATTTC
PeaTatA reverse: GATATCTACAATATCCTTTGTGCTGG
PeaTatB forward: CATATGACACCATCTCTGGCAATTG
PeaTatB reverse: CCCGGGCATTAAATCCGAAGGTAACGACG
PeaTatC forward: CATATGGGTTTGGGAACCACCACTG
PeaTatC reverse: CCCGGGTCCCCGTCCAGCGAGTTTGACCATC

For 16/23 deletion mutagenesis:

23k DC84: TGGATCCTTAACCACCCTCAGAATCAG
23k DC128: CGGATCCTTAATTGCTGGTGGCATCAA
23k C-term151: GGATATCCCAAGCAAAGAGAAAGAG
23k C-term119: GGATATCAAGAAATCCATCACAGAC
23k 84EcoRV: CGATATCACCACCCTCAGAATCAGTC
23k 128EcoRV: CGATATCATTGCTGGTGGCATCAAAG
23k C151EcorV: CGATATCGTTCCATTTTGAAGGTAC

1.7. Enzymes

Molecular biology enzymes were purchased from: Roche Diagnostics GmbH (Mannheim), MBI Fermentas (Vilnius, Lithuania), New England Biolabs (Schwalgach), Stratagene (La Jolla, CA) and USB (Cleveland, OH). Proteases and protease inhibitors were from Sigma. RNase inhibitors was from MBI Fermentas.

2. Methods

2.1. Standard methods

Basic molecular procedures have been carried out as described by Samvrook et al. (1989). Plasmid DNA was isolated by alkaline lysis procedure (Birnboim and Doly, (1979). DNA sequencing was performed either by using the dideoxynucleotide chain termination method (Sanger et al., 1977) or by using ABI method.

2.2. Transcription and translation

To produce analytical amounts of radioactively labelled proteins, *in vitro* transcription and translation of cDNA clones encoding original, chimeric or mutant proteins available from the pervious work and obtained in this study were performed.

2.2.1. *In vitro* transcription

Transcription of cDNAs was performed from gene cassettes cloned in either pBluescript KS or in pBAT vectors. The plasmid DNAs were firstly linerarized downstream of the gene sequence by proper restriction enzymes, then the linerarized plasmid DNAs were subjected for transcription reactions, using either T3 or T7 RNA polymerase in accordance to the recommendations of manufacturers (Stratagene and New England Biolabs, respectively).

Composition of the *in vitro* transcription reaction was as follows:

H_2O	6.5 µl
5 x reaction buffer	5 µl
2.5mM rNTP mixture (GTP: 0.25mM)	5 µl
100mM DTT	2.5 µl
5mM m^7GpppG (capping nucleotide)	2.5 µl
40 U/µl RNase inhibitor	0.5 µl
linerarized plasmid DANN (2 µg)	2.5 µl
RNA polymerase (40U/µl)	0.5 µl
total volume	25 µl

The reaction mixture was pre-incubated at 37°C for 30 min to allow the formation of the cap structure. Incubation was continued for additional 1 h at 37°C after adding 1 µl of 11.25mM rGTP to the reaction, then the reaction was terminated by addition of 100 µl ice-cold DEPC-treated H_2O. The synthesized RNAs were precipitated by addition of 0.1 volume of 4 M NH_4Ac and 3 volumes of EtOH. RNA-ethanol suspension can be stored at -20°C for years. RNAs were collected by centrifugation at 15,000 rpm for 30 min prior to using for *in vitro* translation.

2.2.2. *In vitro* translation

Synthesis of radioactively labelled proteins was performed by *in vitro* translation of mRNA obtained from *in vitro* transcription in the presence of ^{35}[S]-methionine or ^{3}[H]-leucine (Amersham) using an either wheat germ extract- or reticulocyte lysate-based cell-free translation system.

	^{35}[S]-Met	^{3}[H]-Leu
DEPC-H_2O	4.65 µl	3.15 µl
1 M KCl	0.6 µl	0.6 µl
Amino acids mixture (-Met)	0.25 µl	--
Amino acids mixture (-Leu)	--	0.25 µl
100 mM DTT	0.25 µl	0.25 µl
^{35}S-Met	0.5 µl	--
^{3}H-Leu	--	2 µl
reticulocyte lysate	6.25 µl	6.25 µl
total	12.5 µl	12.5 µl

The reaction was carried out for 60-90 min at 30°C, then the resulting *in vitro* translation products were used for import experiments directly or stored at –80 °C for up to one week.

2.3. Isolation of the chloroplast from pea leaves

Green house-grown pea seedlings were harvested at the 7-10 days after germination; the leaves were homogenized in 400 ml of ice-cold SIM using either a Waring Blendor. Homogenate was filtered through two layers of Miracloth and centrifuged in a Serva SLC-250T rotor for 2 min at 4,000 rpm. The crude chloroplast pellet was resuspended with ca. 8 ml of SRM and loaded onto a 35% Percoll cushion. After centrifugation for 7 min at 4,000 rpm in a Serva SL-50T rotor, the pellet of intact chloroplasts was washed twice with 1 x SRM, and the chloroplasts were collected by centrifugation for 2 min at 3,000 rpm in a SL-50T rotor. The chloroplasts were finally resuspended in 2 ml 1x SRM.

The concentration of the chloroplast resuspension was defined by its chlorophyll concentration. Chlorophyll was extracted from 10 µl of the chloroplast resuspension with 1 ml

acetone, and the solution was subjected to a Schimadzu spectrophotometer. The total concentration of chlorophyll a and b was obtained according to the formula (Arnon, 1949): $C_{(a+b)}=A_{663}$ x 8.02 + A_{645} x 20.2

1 x SIM

Hepes/KOH, pH 7,6	25 mM
EDTA	2 mM
Sucrose	350 mM

35% Percoll solution

5 x SRM	2 ml
Percoll	3,5 ml
H_2O	4,5 ml

HM buffer

Hepes/KOH, pH 8.0	10 mM
$MgCl_2$	5 mM

2.4. Import of protein into intact chloroplasts

The standard *in organello* import reaction:

	Volume	Final concentration
chloroplasts	equal to 60 µg chlorophyll	
250 mM methionine	3 µl	5 mM
100 mM Mg-ATP	12 µl	2 mM
1 M $MgCl_2$	1.5 µl	10 mM
in vitro translation reaction	12.5 µl	
1 x SRM	to 150 µl	

Complete assays without *in vitro* translation were briefly preincubated at 25 °C, and the import reactions were initiated by addition of *in vitro* translation and carried out for 30 min at 25 °C in the light. After incubation, samples were transferred into ice and diluted with 350 µl of ice-cold 1x SRM buffer. Chloroplasts were collected from a 50 µl aliquot of the sample by centrifugation at 6,000 rpm for 3 minutes, and denatured with 2 x Laemmli buffer (C^- fraction). Chloroplasts collected from the rest of the sample were resuspended in 1x SRM containing 100 µg/ml thermolysin, and the reaction mixture was chased on ice for 20 min to remove the envelope-bound radioactive protein. Thermolysin treatment was terminated by addition of EDTA to 25 mM. Chloroplasts collected from one tenth aliquot of the assay were denatured with 2 x Laemmli buffer (C^+ fraction), and the rest of the chloroplasts were reisolated by centrifugation through a 35% Percoll cushion at 8,000rpm for 8 min. The intact

plastids were thoroughly washed with 1 ml of 1 x SRM buffer, with EDTA added to 10 mM, and collected by centrifugation for 1 min at 6,000 rpm. Stroma and thylakoid fractions were separated by osmotically lysing chloroplasts in 100 µl of HM buffer containing 10 mM EDTA for 5 min, followed by centrifugation at 10,000 rpm for 5 min. Stromal proteins in the supernatant were precipitated with equal volume of 4 x Laemmli buffer (S fraction). Thylakoid membranes were washed with HM buffer and resuspended in 200 µl of the same buffer. One half of the thylakoids were mock-treated directly (T^- fraction), and the other half was treated with 150 µg/ml thermolysin for 30 min on ice to remove proteins exposed at the surface of the thylakoid membrane. The thermolysin treatment was terminated by addition of EDTA to 10 mM, and the resulted thylakoids were collected by centrifugation at 10,000 rpm for 3 min followed by denaturing with 2 x Laemmli (T^+ fraction). Protein samples were analyzed by SDS-PAGE and autoradiography.

2.5. Import experiments with isolated thylakoids.

In thylakoido import experiments were generally carried out using thylakoids obtained from pea chloroplasts. To isolate thylakoid, chloroplasts were lyzed in HM buffer at a concentration of 0.75 mg/ml chlorophyll for 5 min on ice and then centrifuged for 5 min at 10,000 rpm at 4 °C. The supernatant containing stroma was separated from thylakoid pellets, collected and stored on ice. Thylakoids were washed twice with the HM buffer by centrifugation (5 min, 10,000 rpm) and finally resuspended either in HM buffer or in stroma fraction at a chlorophyll concentration of 0.75 mg/ml.

The standard *in thylakoido* import assay included:

thylakoid suspension	40 µl
in vitro translation product	5 µl
HM buffer	5 µl
Total volume	50 µl

Routinely, thylakoid import reactions were incubated for 30 min at 25 °C in the light. The resulting thylakoid vesicles were re-isolated by centrifugation for 5 min at 10,000 rpm at 4 °C and washed once with HM buffer. Then, half of the thylakoid vesicles were resuspended in 2x Laemmli sample buffer, while the second half were resuspended in HM buffer containing 150 µg/ml thermolysin. After incubation for 30 min on ice, protease treatment was terminated by addition of HME (10 mM Hepes/KOH, pH 8.0, 5 mM $MgCl_2$, 10 mM EDTA) buffer. Thylakoids were collected by centrifugation at 10,000 rpm for 5 min and resuspended in 2x Laemmli sample buffer. After denaturation by heating for 3 min at 100 °C, samples were analyzed by gel electrophoresis followed by radiography.

To examine the influence of inhibitors on the thylakoid translocation of proteins, assays were supplemented with nigericin (to 2 µM) or sodium azide (10 µM). To examine the role of NTPs, apyrase was added (1 U per 50 µl Assay). Competitor proteins were added to the concentration indicated in the respective assays.

Modifications

1, protein import into thylakoids extracted with chaotropic salts and alkaline solutions

In order the function of thylakoid membrane proteins in protein translocation, thylakoid vesicles were pre-treated with solutions of chaotropic salts and alkaline solutions to remove certain thylakoid membrane proteins. In these assays, thylakoids were resuspended at 0.5 mg/ml of chlorophyll in HS buffer (10 mM Hepes, pH 8.0, 5 mM MgCl2, sucrose 100 mM) containing 2 M NaBr, 2M NaSCN, 0.1 M Na_2CO_3, 0.1 M NaOH. After incubation on ice for 30 min, thylakoids were reisolated by centrifugation at 10,000 rpm for 5 min and washed three times with HS buffer. The resulting thylakoids were resuspended at 0.75 mg/ml of chlorophyll in HM buffer and subjected to import assays. One fraction of the thylakoids were mock-treated, and the remained proteins were separated by SDS-PAGE and analyzed by Western blot using antisera against certain thylakoid proteins.

2, protease pre-treatment assays

Protease protection studies with thylakoids were carried out in order to characterize the degree to which different polypeptides are exposed on the stromal surface of thylakoid membrane. Thylakoids were resuspended at 0.5 mg/ml of chlorophyll in HM buffer. This suspension was divided into aliquots which were incubated with the following proteases: thermolysin (100 µg/ml; $CaCl_2$ was added to 5 mM), trypsin (100 µg/ml), and proteinase K (150 µg/ml), or without any additions either for 20 min at 20 °C or for 30 min on ice. Subsequently, thylakoids were diluted with 1 ml of an ice –cold HM buffer, and appropriate protease inhibitors were added: EDTA for thermolysin (to 20 mM), Soybean trypsin inhibitor for trypsin (to 50 µg/ml) and PMSF for proteinase K (to 2 mM). Thylakoids were collected by centrifugation for 10 min at 30,000 *g*, washed once with HM buffer containing corresponding protease inhibitors, and finally resuspended in the same buffer before subjecting to import experiment. One fraction of the thylakoid membranes were mock-treated, and proteins remained in the thylakoid membranes were separated by SDS-PAGE followed by Western hybridization.

3, import with the immune-pretreated pea thylakoids

Pea thylakoids obtained from lysis of the chloroplasts were resuspended in 20mM Hepes/KOH, pH8.0, 10mM $MgCl_2$, 1% BSA at the concentration of 0.5 µg/µl chlorophyll. The corresponding antibody IgG was added to a final concentration of 0.5 µg/µl, or as

described in the text. Binding of the IgG to the thylakoid membrane was carried out at 4°C for 40 mins with agitation, and the resulting thylakoids were reisolated and washed with 10mM Hepes/KOH, pH8.0, 10mM $MgCl_2$ to get rid of the unbound IgG. Finally, thylakoids were resuspended in HM buffer or in stroma at the concentration of 0.75 µg/µl chlorophyll, and subsequently used for *in thylakoido* import assays as described above.

2.6. Electrophoresis of proteins

2.6.1. SDS polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of SDS-denatured proteins was performed according to the description of Laemmli (Laemmli 1970). As a rule, polyacrylamide gels with an acrylamide gradient from 10-17.5% were used except being mentioned. Composition of a 10-17.5 % gel is shown below.

	stacking gel	**10%**	**17,5%**
H_2O	13.9 ml	15.4 ml	0
1 M Tris-HCl (pH 6.8)	2.5 ml	--	--
2 M Tris-HCl (pH 8.8)	--	7.5 ml	7.5 ml
80% sucrose	--	2.4 ml	8.8 ml
30% AA	3.34 ml	14.3 ml	23.3 ml
10% SDS	200 µl	0.4 ml	0.4 ml
10% APS	160 µl	160 µl	160 µl
TEMED	16 µl	16 µl	16 µl
Total volume	20 ml	40 ml	40 ml

Electrophoresis was performed overnight at approx. 25 mA, maximal power 160-180 V.

30% acrylamide stock	acrylamide	292 g
	N,N´-methylenebisacrylamide	8 g
	H_2O	ad to 1000 ml
1 x Laemmli running buffer	Tris	25 mM
	glycine	195 mM
	SDS	0.1%

2.6.2. Non-denaturing electrophoresis of membrane proteins (blue native electrophoresis)

Separation of membrane polypeptides under non-denaturing conditions presents an inherently complex problem as these proteins are prone to aggregation and frequently require different conditions for their optimal recovery from the membrane. For the isolation of photosynthetic

complexes of the membrane, the blue native gel electrophoresis introduced by Schägger and von Jagow(1991) was modified. With these alterations, the method reproducibly allowed high-resolution separation of the principal oligomeric complexes of the thylakoid membrane. A typical separation of thylakoid protein complexes is shown below.
Preparation of samples

Lysis buffer:

Stock solutions	Volume	Final concentration
0.5 M Bistris, pH7,0	500 µl	50 mM
2M ε-aminocaproic acid	2500 µl	1 M
0.5 M EDTA, pH 8.0	50 µl	5 mM
0.1 M $MgCl_2$	25 µl	0.5 mM
0.1 M PMSF (in isopropanol)	50µl	1 mM
0.1 M DTT	50 µl	1 mM
H_2O	ad 5 ml	

Solubilization:

For solubilization of the membrane protein complexes, thylakoid membranes (equivalent to 30 µg of chlorophyll) were resuspended with 15 µl of lysis buffer and 7.5 µl of 5% digitonin. After incubation for 30-60 min at 4°C under agitation, unsolubilized membrane material was spun down at 40,000 *g* for 1 h. The supernatant was supplemented with 1.5 µl of 5% Coomassie Briliant Blue G-250 in lysis buffer, bound on ice for 10 min, and centrifuged at 40,000 g for 3 min. The supernatant of this centrifugation was used for loading onto the gel.

Electrophoresis Gel solutions:

Stock Solutions	**Stacking gel**	**5%**	**13,5%**	**10%**
H_2O	5,1 ml	7,43 ml	1,38 ml	3,08 ml
10x Bistris (0,5M, pH 7,0)	1,0 ml	2,27 ml	1,5 ml	1,5 ml
30% acrylamide/bisacrylamide	1,3 ml	3,85 ml	6,75 ml	5 ml
2M 2-aminocaproic acid	2,5 ml	5,68 ml	3,75 ml	3,75 ml
87,5% glycerol	--	1,23 ml	1,5 ml	1,5 ml
5% digitonin	60 µl	136 µl	90 µl	90 µl
10% APS	90 µl	88 µl	46 µl	50 µl
TEMED	9 µl	8,8 µl	4,6 µl	5 µl
total volume	10 ml	20,8 ml	15 ml	15 ml

10x running buffer: 0.5M tricine, 0.15 M Bistris, pH 7,0

Protein samples were resolved at approx. 10 mA (with power restricted at 280 V) at 4°C. 1x running buffer contained 0,0075% of coomassie G250. The extent of separation of most photosynthetic complexes could be significantly improved by using Bistris-propane buffer instead of Bistris in all solutions.

For the isolation of the photosynthetic complexes of thylakoid membrane, colored bands corresponding to distinct multimeric complexes were cut out of the gel and, after equilibration with and SDS-containing buffer, resolved in the second dimension under denaturing conditions. Detection of proteins in these gels was performed by either silver staining or radio- and fluorography.

Native gel equilibration:

Equilibration buffer:	Tris	25 mM
	Glycine	192 mM
	ß-mercaptoethanol	140 mM
	SDS	1%

Incubate Blue-native gel in the equilibration buffer at 50°C for 5-10 min to denature the proteins.

2.7. Staining of proteins

For detection of protein bands on gels, staining with Coomassie Brilliant Blue R-250 was routinely used (following standard procedures). To increase the sensitivity of detection, the proteins were stained with silver essentially following the method of Heukeshoven and Dernick (1988). SDS-PAGE gels were fixed overnight in fixing solution, and subsequently equilibrated with 50% ethanol 3 time for 20 min with agitation. Gels were swelled in 0.02% $Na_2S_2O_3x5H_2O$, and then washed 3 times for 20 sec with H_2O bidistil. Gels were then stained in silver nitrate solution for 20 min. After washing 2 times with water, stained proteins bands were visualized by development in developing solution until an appropriate pattern was achieved. Development was terminated by transferring the gels into stop solution. Before being dried, gels were washed to remove acetic acid in 50% methanol for at least 30 minutes.

fixing solution	methanol	50 ml
	acetic acid	12 ml
	formaldehyde 37%	50 µl
	H_2O bidist.	ad 100 ml
silver nitrate solution	$AgNO_3$	0.2 g
	formaldehyde 37%	75 µl
	H_2O bidist.	ad 100 ml

developing solution	Na_2CO_3	60 g
	Formaldehyde 37%	50 µl
	$Na_2S_2O_3x5H_2O$ 0.2%	20 µl
	H_2O bidist.	ad 100 ml
stop solution	methanol	50 ml
	Acetic acid	12 ml
	H_2O bidist.	ad 100 ml

2.8. Immunodetection of proteins – Western blot

For detection of proteins using specific antisera, proteins were firstly blotted onto the Immobilon-P membrane (PVDF membrane) purchased from Millipore. Right after electrophoresis, gels were incubated in transfer buffer (150 mM glycine, 20 mM Tris, 10% methanol). Electrophorestic transfer was performed in a semi-dry transfer apparatus (Gibco-BRL) with transfer buffer. Prehybridization, hybridization with primary and secondary antisera were carried out in 1 x PBS containing 1% v/v Tween 20 and 5% dry skimmed milk. Specific antisera were used usually in a 1:1,000 dilution. Secondary antisera conjugated to horseradish peroxidase were used in a 1:30,000 dilution. After the hybridization with the secondary antisera, the PVDF membranes were washed three times with 1 xPBS containing 0.1% v/v Tween 20. For visualization of protein bands, the secondary antibodies were developed with ECL (enhanced chemiluminescence) reaction (Voelker and Barkan 1995). The developing reagent was set up before the reaction freshly from stock solution. After incubation for 1 min in the developing reagent, PVDF membranes were wrapped in plastic foil and exposed to an X-ray film for an appropriate time.

10 x PBS	NaCl	750 mM
	KCl	30 mM
	Na_2HPO_4	45 mM
	KH_2PO_4	5 mM

ECL reagent	stock solution	volume	concentration
	1 M Tris-HCl, pH 8.5	500 µl	50 mM
	250 mM luminol (in DMSO)	50 µl	1.25 mM
	90 mM p-coumaric acid (in DMSO)	22 µl	200 µM
	30% H_2O_2	3 µl	2.7 mM
	H_2O bidist.	Ad to 10 ml	

2.9. Coimmunoprecipitation under non-denaturing conditions

Washed thylakoid membranes (30µg chlorophyll) were solubilized in 100 µl resuspension buffer with digitonin (50 mM Hepes/KOH, pH 7.0, 150 mM KAc, 0.05% BSA, 1 mM PMSF, and 1% digitonin). After incubation for 30 min at 4 °C with agitation, the solubilized

membrane material was gained from the 40,000 *g* supernatant, and was mixed with 1-5 µg IgG. 20 µl of 10% protein A–Sepharose CL4B (Pharmacia) was then added to the mixture, and the suspension was incubated for 1 h at 4°C with agitation. The unbound proteins were recovered by centrifugation for 5 min at 10,000 rpm, and the protein A–Sepharose beads were washed with resuspension buffer described above except containing only 0.1% digitonin. Bound proteins were recovered by incubating the beads in 7 M urea, 2% SDS, 100 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 0.1% bromophenol blue for 10 min at room temperature followed by centrifugation. Protein samples were separated by SDS-PAGE followed by autoradiography.

2.10. Ion-exchange chromatography

Isolated thylakoid membranes (equal to 150 µg chlorophyll) were resuspended in 400 µl solubilization buffer (20 mM Hepes/KOH, pH 7.6, 10 mM $MgCl_2$, 5 mM EDTA, 0.5 mM PMSF). After addition of 100 µl 5% digitonin, the solubilization was performed at 4 °C for 60 min. The non-solubilized material was removed by sediment at 40,000 *g* for 40 min. The supernatant was transferred to a cold room and subjected to a 1 ml DEAE-sepharose column equilibrated with solubilization buffer containing 0.1% digitonin. The column was washed subsequently with 2 ml of solubilization buffer with 0.1 % digitonin and 0 mM $(NH_4)_2SO_4$, 10 mM $(NH_4)_2SO_4$, 40 mM $(NH_4)_2SO_4$, 0.2 M $(NH_4)_2SO_4$, and 1 M $(NH_4)_2SO_4$. The elution was collected in 500 µl fraction, and proteins were precipitated with 10% TCA. After centrifugation at 15,000 rpm for 30 min, the protein pellets were dissolved in 2 x Laemmli buffer and subjected to SDS-PAGE. Proteins of each fraction were analyzed by either Western hybridization or autoradiography.

2.11. Co-purification of thylakoid membrane proteins through metal affinity chromatography

Thylakoid membranes (equal to 50 µg chlorophyll) were re-isolated after performance of import with 10 µg of overexpressed 16/23His protein and washed three times with HM buffer to remove the non-imported 16/23His. Thylakoid membranes were resuspended in 200 µl of phosphate buffer, then digitonin was added to 1%. After centrifugation at 40,000 *g* for 1 hour, the supernatant was applied to an equilibrated 0.5 ml TALON metal affinity resine (Clontech, San Jose, CA, USA) column. The unbound proteins were washed subsequently with 10 ml of phosphate buffer and washing buffer, and the bound proteins were eluted with 2 ml of elution buffer. Proteins in each fraction were precipitated with 10% TCA and collected by centrifugation at 15,000 rpm for 30 min. The protein pellets were dissolved in 2 x Laemmli buffer and subjected to SDS-PAGE followed by Western analysis with different specific antisera.

Phosphate buffer (pH 7.5)	Na_2HPO_4	58 mM
	NaH_2PO_4	17 mM
	NaCl	68 mM

	PMSF	1 mM
Equilibration buffer (pH 8.0)	sodium phosphate	50 mM
	NaCl	300 mM
	PMSF	1 mM
Washing buffer (pH 7.0)	sodium phosphate	50 mM
	NaCl	300 mM
	Imidazole	10 mM
	PMSF	1 mM
Elution buffer (pH 7.0)	sodium phosphate	50 mM
	NaCl	300 mM
	Imidazole	150 mM
	PMSF	1 mM

2.12. *In vitro* integration of protein into phospholipid membrane

The solid supported lipid membrane TRANSIL (NIMBUS Biotechnologie GmbH, Leipzig) was used to examine the membrane-insertion potential of proteins. TRANSIL consists of porous silica beads each completely coated with a single phospholipid bilayer containing POPG (1-Palmitoyl-2-Olenyl-sn-Glycero-3-Phosphoglycerol)/egg PC (phosphatidylcholine). POPG was present with a concentration of 0%, 5% and 20%, respectively. Before subjected to integration assays, TRANSIL beads were washed with HM buffer and resuspended at 5% in HM buffer. *In vitro* translated radioactive proteins were incubated with TRANSIL beads with agitation for 30 min at room temperature to allow the insertion of the proteins into phospholipid membrane. The resulting beads were collected at 10,000 rpm for 5 min and subsequently washed for three times with HM buffer. One half of the beads were mock-treated, while the other half were resuspended in HM buffer containing 100 µg/ml thermolysin or given proteases. After protease treatment, the beads were collected again and mock-treated. Protein samples were analyzed by SDS-PAGE and autoradiography.

2.13. Screening of peptide libraries on continuous cellulose membrane supports

The solid phase-bound chemical peptide libraries represented the TAT proteins were synthesized by the group of Prof. G. Fischer (Max-Plank Research Unit for Enzymology of Protein Folding, Halle). These libraries have been used for the detection of epitopes which act as binding sites for the substrate proteins or the TAT components themselves. Screening of the peptide libraries was performed according to the description of Kramer and Schneider-Mergener (1995) with minor modifications. Cellulose membranes were rinse with a little volume of methanol for 1 min to avoid the precipitation of hydrophobic peptides, then the membranes were washed three times with an appropriate volume of TBS for 10 min. Membranes were incubated in blocking buffer overnight (about 14 hours) at room temperature with shaking, then the blocking buffer was removed by washing the membrane three times with T-TBS buffer. Membranes were incubated in ligand solutions containing

either *in vitro* translated proteins or overexpressed proteins for three hours, followed by three times washing with T-TBS buffer. Proteins binding on the libraries were transferred to a new nitrocellulose membrane. Radio-labelled proteins were visualized by autoradiography, whereas the overexpressed protein were detected by Western blot using specific antibodies.

TBS	Tris-(hydroxymethyl)-aminomethan Adjust pH to 8.0 with HCl	50 mM
	NaCl	137 mM
	KCl	2.7 mM
blocking buffer	BSA	5% (w/v)
	sucrose	5% (w/v)
	TBS	1 x
T-TBS	Tween 20	0.5% (v/v)
	TBS	1 x
Ligand solutiong	BSA	5% (w/v)
	sucrose	5% (w/v)
	Tween 20	0.5% (v/v)
	TBS	1 x
	in vitro translation	50 µl
	overexpressed protein	0.1 µg/ml
	total volume	50 ml

Regeneration of cellulose bound peptide libraries

After probing, the cellulose peptide libraries can be regenerated using the following procedure. Membranes were washed three times with water for 10 min, followed by three times washing with DMF for 10 min. After removal of DMF by rinsing the membranes with water, the membranes were subsequently incubated three times in regeneration buffer A and three times in regeneration buffer B. After washing twice with methanol for 10 min, the membranes could be reprobed with different ligands.

regeneration buffer A	urea	480.5 g
	SDS	10 g
	β-mercaptoethanol	1 ml
	H_2O	ad to 1 l
regeneration buffer B	ethanol	500 ml
	acetic acid	100 ml
	H_2O	ad to 1 l

RESULTS

1. Thylakoid translocation of a bacterial TAT-substrate – GFOR as a model to compare the bacterial and thylakoidal ΔpH/Tat-pathways

Protein export into the periplasmic space of Gram-negative bacteria has for a long time been considered to rely almost completely on the Sec system (e.g. Pugsley, 1993). In recent years however, compelling evidence for the existence of an additional, Sec-independent transport machinery for the transport of proteins across the plasma membrane has accumulated (Berks, 1996; Santini et al., 1998; Weiner et al., 1998). Many of these studies have been initiated by the finding that most bacterial proteins carrying complex redox cofactors have signal peptides with a twin pair of arginine residues (twin-R motif) preceding the hydrophobic core region and it has been suggested that this motif is a specific targeting signal for a Sec-independent export route (Berks 1996).

The structural similarity of the targeting signals suggests that the two TAT pathways in the prokaryote and the prokaryote-derived organelle are of common phylogenetic origin. However, it is difficult to demonstrate whether the two twin-R pathways also operate with similar mechanisms, due to the lack of established *in vitro* transport systems in bacteria. Swapping experiments analyzing bacterial proteins in chloroplast derived transport systems might provide valuable information about the degree of functional similarity between the two transport pathways, and it has been shown that twin-R signal peptides derived from E. coli proteins are capable of targeting a thylakoidal passenger protein by the ΔpH pathway into the thylakoid lumen (Wexler et al., 1998). On the other hand, an authentic cyanobacterial precursor protein carrying a twin-R signal peptide, CtpA, did not specifically interact with the ΔpH-dependent transport machinery of chloroplasts in such assays (Karnauchov et al., 1997), suggesting a possible influence also of the passenger protein on pathway specificity.

In order to study the homology between the two TAT pathways, the glucose-fructose oxidoreductase (GFOR, gene: *gfo*), a periplasmic protein from *Zymomonas mobilis*, was analyzed by using *in vitro* transport assays based on isolated thylakoid vesicles from higher plant chloroplasts. GFOR is a relatively small protein, which can easily be distinguished from its larger precursor and which carries a complex cofactor (NADP) that is ubiquitous in both prokaryotic and eukaryotic cells. Export of GFOR has been extensively studied in both *Z. mobilis* and *E. coli*, and various alleles of the *gfo* gene are available encoding GFOR-proteins with altered signal peptides or cofactor binding sites (Wiegert et al., 1996; 1997a; 1997b; Halbig et al., 1999).

1.1. GFOR from *Z. mobilis* is efficiently transported across thylakoid membranes from higher plant chloroplasts

In order to determine the compatibility of a bacterial protein carrying a twin-R signal peptide with the thylakoidal protein transport machinery, radiolabelled precursor protein of GFOR from *Z. mobilis* was synthesized by *in vitro* translation and incubated with thylakoids which had been isolated from pea chloroplasts. Although isolated thylakoid vesicles are known to be restrictive with respect to the proteins accepted, refusing even a subset of authentic thylakoid proteins as substrates (e.g. Cline et al., 1993; Karnauchov et al., 1994), the precursor of bacterial GFOR turned out to be an excellent substrate in such experiments. After the import reaction, almost 50% of the protein present in the assay is resistant to externally added protease (Fig. 6) which is indicative of transport into the thylakoid vesicles. The efficiency of the transport process is comparable to that of authentic thylakoid lumen proteins, such as plastocyanin or the 23 kDa subunit of the oxygen-evolving system which were analyzed in parallel (Fig. 6). GFOR accumulating in the thylakoid lumen is quantitatively processed to its mature size indicating that also the thylakoidal processing peptidase (Kirwin et al., 1988) accepts the bacterial precursor polypeptide as substrate.

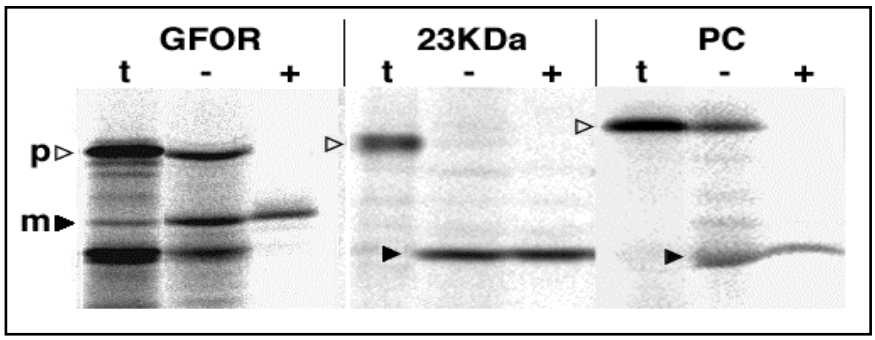


Fig. 6. Transport of GFOR across thylakoid membranes from pea chloroplasts. Precursor proteins of GFOR, the 23 kDa subunit of the oxygen-evolving system, or plastocyanin (PC) were incubated for 20 min at 25°C with isolated pea thylakoids that were resuspended in stromal extract at 0.75 µg chlorophyll/µl. After the import reaction, samples of the assays were analyzed on SDS-polyacrylamide gels either directly (lanes -) or after treatment of the thylakoids with thermolysin (lanes +). The positions of the precursor (p) and mature proteins (m) are indicated by open and closed arrowheads, respectively. *In vitro* translation products are indicated by "t".

1.2. Thylakoid transport of GFOR takes place by the ΔpH-dependent pathway

In order to characterize the mechanism of GFOR transport across the thylakoid membrane, the experiments were repeated under various physiological conditions that are indicative of the different thylakoidal translocation routes. In the first set of experiments, the influence of stromal factors and translocation inhibitors was studied (Fig. 7). It turned out that the transport of GFOR does not depend on the addition of stromal extracts to the thylakoid vesicles and that it is also not prevented by sodium azide, a potent inhibitor of SecA function in prokaryotes and chloroplasts (Oliver et al., 1990; Knott and Robinson, 1994; Henry et al., 1994). In contrast, the transthylakoidal proton gradient apparently plays an important role in this process since GFOR translocation is abolished if the import assays are supplemented with nigericin, a protonophore which dissipates the proton gradient across the thylakoid membrane (Fig. 7).

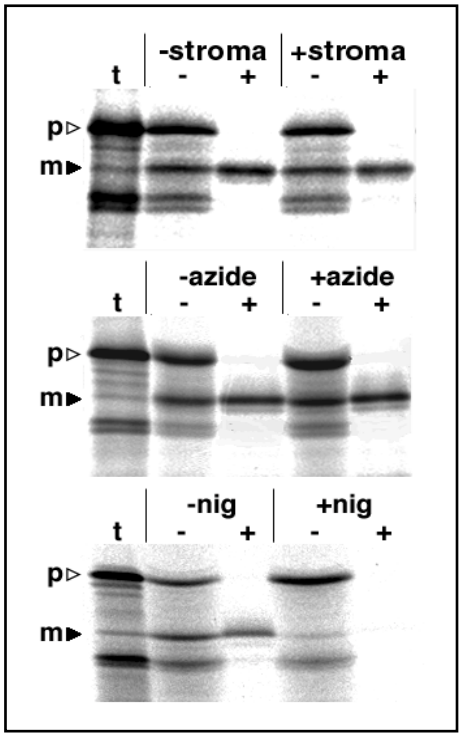


Fig. 7. Thylakoid transport of GFOR requires a transthylakoidal proton gradient but is independent of stromal factors and azide-sensitive components. *In vitro* synthesized GFOR precursor was incubated with isolated thylakoids in the presence and absence of 10 mM sodium azide (azide) or 2 µM nigericin (nig). In the upper panel incubation was additionally performed also in the absence of stromal extracts (-stroma), i.e. with thylakoids that were washed twice and then resuspended in 10 mM HEPES-KOH, pH 8.0, 5 mM $MgCl_2$ rather than in stromal extract (+stroma). For further details see the legend to Fig. 6

In line with these findings, enzymatic destruction of the nucleoside triphosphates in the assays by apyrase does not affect thylakoid transport of GFOR to a significant extent (Fig. 8A):

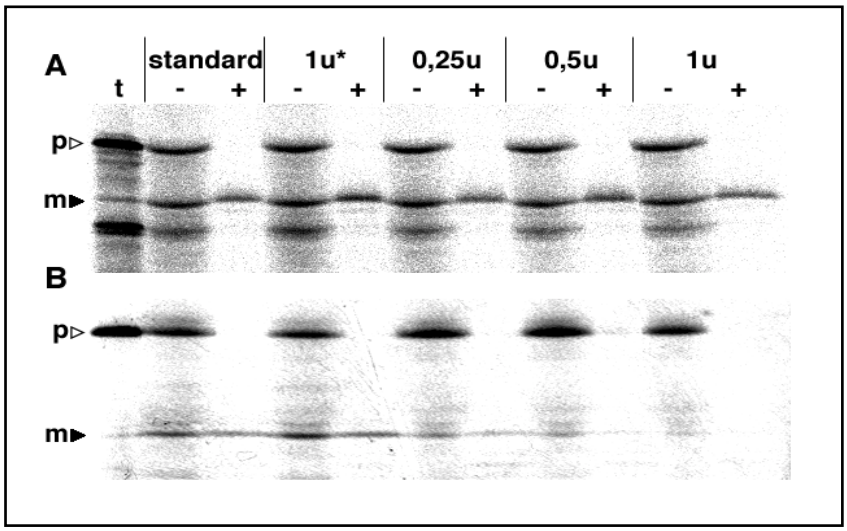


Fig. 8. Thylakoid transport of GFOR is independent of nucleoside triphosphates. Isolated pea thylakoids were incubated with the precursors of GFOR (**A**) or plastocyanin (**B**) in the presence of stromal extract. Before starting the incubation, the mixtures were preincubated with apyrase for 10 min on ice. The activity of apyrase added to each incubation mixture is indicated above the lanes. Into the standard assay, no apyrase was added. The control denoted 1u* contained apyrase that was inactivated by boiling for 5 min. For further details see the legend to Fig. 6.

Plastocyanin, on the other hand, which is targeted by the Sec pathway and which was included as a control, is no longer translocated into the thylakoid lumen under these conditions (Fig. 8B). Together with the azide insensitivity of the translocation process and the lack of requirement for stromal factors (Fig. 7), an involvement of either the Sec or the SRP machineries of the chloroplast can be ruled out. Instead, the requirement for the transthylakoidal proton gradient (Fig. 7) suggests transport by the ΔpH-dependent pathway.

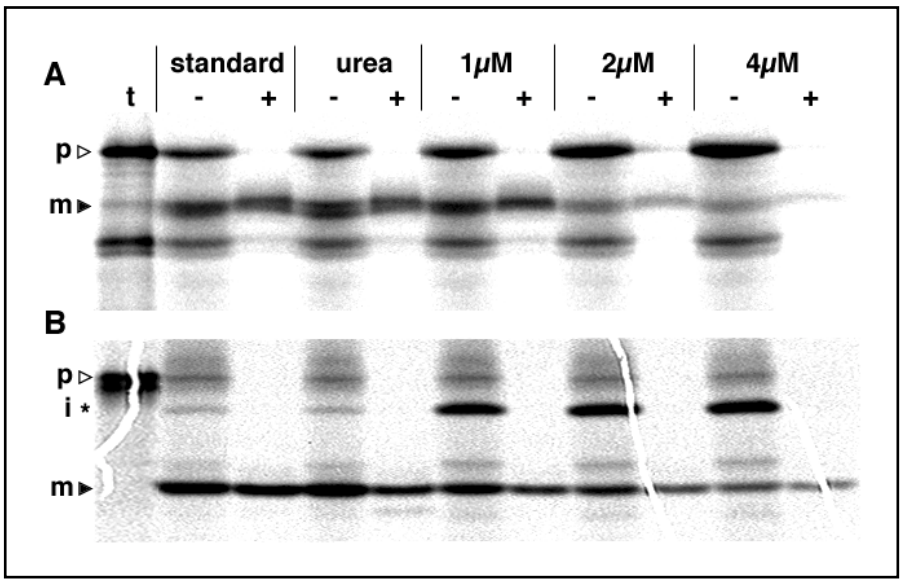


Fig. 9. Thylakoid transport of GFOR is inhibited if the ΔpH-dependent translocation machinery is saturated. *In vitro* synthesized, radiolabelled precursors of GFOR (**A**) or the 23 kDa subunit of the oxygen-evolving system (**B**) were incubated with isolated thylakoids in the presence of increasing amounts of unlabelled competitor protein at concentrations indicated above the lanes. As competitor, the precursor of the 23 kDa protein was utilized which was obtained from overexpression in *E. coli* and prepared by solubilization of inclusion bodies in urea buffers as detailed in Michl et al (1994). All assays except for the standard control contained equivalent amounts of urea. In the urea assay, no competitor protein but only solubilization buffer was added. For further details see the legend to Fig. 6.

Therefore, competition experiments were performed in which the ΔpH-dependent translocation machinery was saturated by high amounts of unlabelled precursor of the 23 kDa subunit from the oxygen-evolving system (Michl et al., 1994), a specific substrate for this

pathway (Cline et al., 1993). With increasing amounts of competitor, GFOR is significantly inhibited in its thylakoid transport and accumulates in its unprocessed precursor form in the incubation mixture (Fig. 9). The degree of competition is similar to that observed for the authentic 23 kDa precursor protein which was analyzed in parallel (Fig. 9). These results unambiguously prove that GFOR from *Z. mobilis* utilizes specifically and exclusively the ΔpH-dependent pathway for its transport across the thylakoid membrane.

1.3. Mutagenesis of the twin-R motif in the signal peptide abolishes thylakoid transport of GFOR

The most significant feature of all thylakoidal signal peptides triggering ΔpH-dependent transport is a twin pair of arginine residues upstream of the hydrophobic core segment. As was shown for the precursor of the 23 kDa protein, conservative replacement of either of these arginines by lysine prevents almost completely the membrane transport of this protein (Chaddock et al., 1995). In order to determine whether the corresponding twin-R motif in the signal peptide of GFOR is of comparable importance for membrane transport, mutant derivatives were analyzed in which these arginine residues were replaced by lysines, either together or each one separately. It turned out that neither of the resulting twin-R mutants (RK-GFOR, KR-GFOR, or KK-GFOR) could be imported into isolated thylakoids, irrespective of whether stromal extract was included in the assays or not (Fig. 10 and data not shown). Thus, also in the heterologous substrate the twin-R motif is an essential element for the interaction with the ΔpH-dependent transport machinery. Neither of the mutants was instead targeted by the Sec- or SRP-dependent pathways, in line with the results described for the twin-R mutants of the 23 kDa protein which likewise could not utilize any of the other pathways for thylakoid translocation (Chaddock et al., 1995). Remarkably though, the GFOR signal peptide lacks charged residues in its C-terminal segment (Wiegert et al., 1996; Kanagasundaram and Scopes, 1992), which are discussed as "Sec avoidance" signals in thylakoidal twin-R signal peptides (Bogsch et al., 1997).

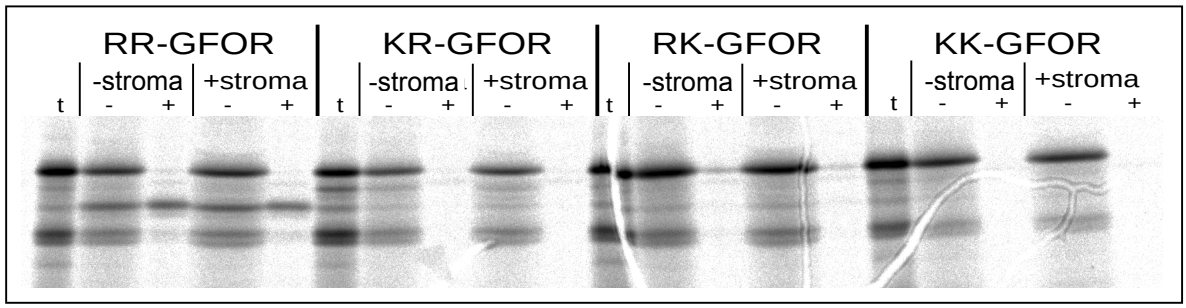


Fig. 10. Mutagenesis of the twin-R motif in the GFOR signal peptide prevents transport of the protein across the thylakoid membrane. *In vitro* synthesized precursors of wild-type GFOR (RR-GFOR) or of mutant proteins, in which one or two of the arginine residues of the twin-R motif was replaced by lysine (RK-GFOR), were incubated with thylakoids in the presence or absence of stromal extracts. For further details see the legends to Fig. 6 and Fig. 7.

2. Mechanism of the ΔpH/Tat-dependent protein translocation across the thylakoid membrane

Current studies on the mechanism of thylakoidal ΔpH/TAT-dependent translocation rely exclusively on the *in vitro* import experiments. Limited number of authentic proteins has been identified to be transported by the ΔpH/Tat-pathway. Investigation of the thylakoid import of artificial chimeric precursor proteins indicated that the transit peptide of the ΔpH/Tat-pathway is able to direct also foreign proteins with high efficiency to this pathway (Robinson et al., 1994; Henry et al., 1994). Analysis of the thylakoid translocation of such chimeric proteins might help us to understand the mechanism of protein transport by this pathway.

2.1. Identification of the translocation steps of the ΔpH/Tat-dependent pathway

2.1.1. Thylakoid translocation of 16/23 is retarded in the thylakoid membrane

Among the numerous authentic and chimeric thylakoid proteins that have been analyzed in thylakoid import experiments, the chimera 16/23 is particularly interesting. The 16/23 protein

was constructed by combining the transit peptide of the 16 kDa subunit (OE16) and the mature part of the 23 kDa subunit (OE23) of the oxygen evolving complex of photosystem II. The authentic precursors of OE16 and OE23 are translocated by the ΔpH/Tat-dependent pathway across the thylakoid membrane (Mould et al., 1991; Mould and Robinson, 1991; Klösgen et al., 1992). As expected, the chimeric 16/23 protein is targeted by the thylakoidal ΔpH/Tat-pathway as well, but it shows a unique behavior during membrane transport (Berghöfer and Klösgen, 1999). In a typical *in thylakoido* import experiment, authentic precursor proteins, such as the OE23 protein, accumulate almost quantitatively as the mature polypeptide in the thylakoids; only a minor fraction of precursor protein is found associated with the thylakoids (Fig. 11). The mature polypeptide was fully translocated and internalized in the thylakoid lumen and is therefore resistant to thermolysin treatment of the thylakoid vesicles. In contrast, the precursor protein is removed by thermolysin treatment and was thus only associated with the thylakoid membrane. When compared to such authentic ΔpH/Tat-dependent proteins, thylakoid import efficiency of the 16/23 polypeptide is relative low. The 16/23 protein is found predominantly as precursor in the thylakoid fraction (Fig. 11). Only a

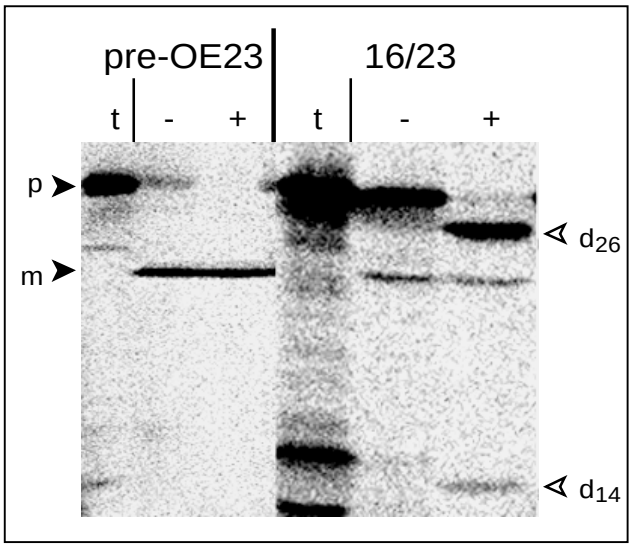


Fig. 11. Thylakoid import of OE23 and 16/23. Radiolabelled pre-OE23 and 16/23 protein were synthesized by *in vitro* translation, and incubated with the isolated pea thylakoid vesicles at 25 °C in the light for 10 min. For more details see legend for Fig. 1. Precursor proteins (p) and mature polypeptides (m) is indicated on the left, and position of the degradation products (d_{26} and d_{14}) is indicated on the right.

small amount of 16/23 is translocated into the thylakoid lumen and processed to the mature 23 kDa polypeptide. The thylakoid-associated precursor protein is degraded by thermolysin, resulting in two degradation products of approximately 26 kDa and 14 kDa, respectively (Fig.

11). This indicates that besides being translocated into the thylakoid lumen, 16/23 is inserted into the thylakoid membrane in two alternative topologies, which are both accessible to externally added protease, leading to two different degradation products. These products might represent different translocation steps.

2.1.2. Topology of the 16/23 translocation intermediates

In order to determine the topology of the two putative translocation intermediates within the thylakoid membrane, a derivative of the 16/23 protein carrying six additional histidine residues at its carboxy terminus was generated. The presence of the His-tag allows determining the topology of the C-terminus during translocation across the thylakoid membrane. Import of the resulting polypeptide (16/23His) into either intact chloroplasts or isolated thylakoids confirmed that the His-tag does not influence the translocation process to any notable extent. Like the untagged protein, the 16/23His polypeptide was retarded at two distinct steps that are characterized by specific proteolytic fragments after protease treatment of the reisolated thylakoid vesicles (Fig. 12 and data not shown). Comparison of the mobilities of the resulting fragments on SDS-PAA gels revealed that the degradation products indicative for the smaller product (d_{14}) are identical in size for the 16/23 and the 16/23His protein. Thus, in this case the C-termini of the proteins are still located on the stromal side of the thylakoid membrane and are accessible to protease. In contrast, the larger products are different in size, indicating that the C-terminal part of the protein was transported across the thylakoid membrane in this instance.

The mobility shift seen with the larger degradation products on SDS-PAA gels corresponds well with the presence or absence of the hexahistidinyl-tag, respectively (Fig. 12). The same is true also for the terminal processing products accumulating within the thylakoid lumen, which show a comparable difference in size (Fig. 12C, m). These results strongly support a scenario in which the smaller degradation product corresponds to an early intermediate (called

Ti-1) that assumes a "horse-shoe" or loop conformation in the thylakoid membrane, with the hydrophobic domain of the transit peptide being integrated into the thylakoid membrane. Part

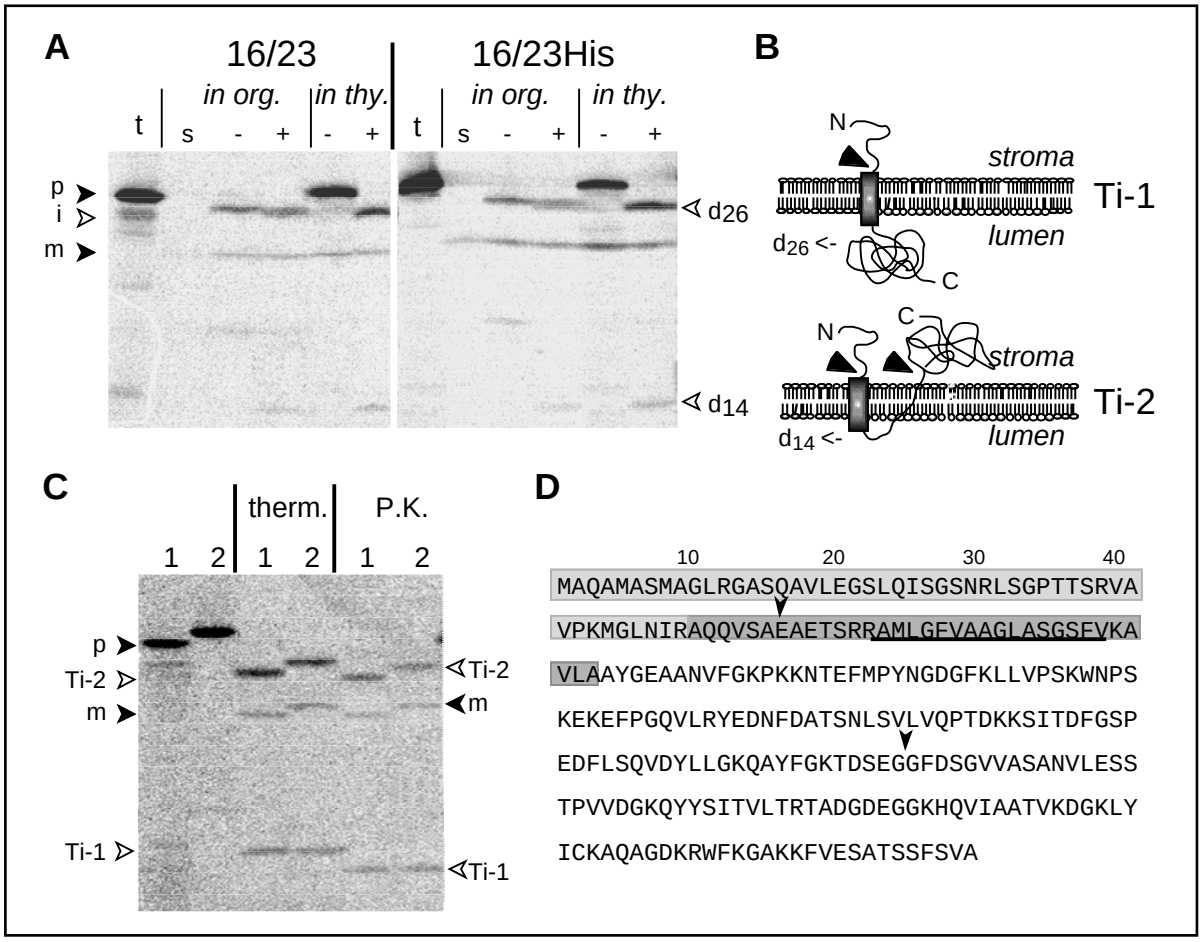


Fig. 7. Identification of the topology of the translocation intermediates. A. *In vitro* translated 16/23 and 16/23His protein were mixed with intact pea chloroplasts (in org.), and incubated for 15 min at 25 °C. After import, the chloroplasts were treated with thermolysin (100 µg/ml) for 20 min on ice, reisolated by centrifugation through a Percoll cushion and fractionated into stroma (s) and thylakoids as described in Materials and Methods. Half of the thylakoid fraction was treated with thermolysin (150 µg/ml) for 30 min on ice (+), and the other half was mock-treated (-). 16/23 and 16/23His were respectively imported into pea thylakoids at 25 °C for 15 min as described the legend to Fig. 1. (in thy.). Samples from both *in organello* and *in thylakoido* import assays were processed by 10-17.5% SDS-PAGE. **B**. Schematic drawing of the topology of the translocation intermediates of 16/23 in the thylakoid membrane, showing the location of the N- and C-termini of both translocation intermediates. **C**. Thylakoids reisolated from in thylakoido import assays were treated with either thermolysin (150 µg/ml) or protease K (150µg/ml) for 30 min on ice. Reactions were terminated by addition of corresponding protease inhibitors (10 mM EDTA for thermolysin, 1 mM PMSF for protease K), and proteins were separated by 10-17.5% SDS-PAGE. **D**. Amino acid

sequence of the 16/23 protein. The stroma targeting domain of the transit peptide is shaded by light gray, and the thylakoid targeting domain by dark gray. The h-domain of the transit peptide is underlined. The deduced thermolysin cleavage sites of Ti-1 are indicated by arrows.

of the mature protein is also inserted into the thylakoid membrane in this scenario. At this stage, both termini of the protein are exposed to the chloroplast stroma. In the next step, the C-terminal domain of the protein is translocated into the thylakoid lumen, giving rise to the translocation intermediate 2 (Ti-2), which is represented by the larger degradation product and which exposes only the N-terminal segment of its transit peptide on the stromal surface of the membrane (Fig. 12B). In the last step of thylakoid translocation, the transit peptide is removed by the activity of the thylakoidal processing peptidase and the "mature" polypeptide is liberated into the thylakoid lumen.

The two putative translocation intermediates were additionally compared also after treatment with an alternative protease, notably proteinase K. Like thermolysin, proteinase K cannot reach the fully translocated mature protein, but has access to the membrane-inserted translocation intermediates. It turned out that the degradation products obtained by proteinase K treatment are smaller than those resulting from thermolysin treatment. Ti-1 is degraded into a 12 kDa fragment and Ti-2 is degraded into a 25 kDa fragment by proteinase K (Fig. 12C). Based on these results, the putative cleavage sites of thermolysin in Ti-1 were rather within the amino acid sequence of 16/23 (Fig. 12D).

Taken together, these experiments have shown that during thylakoid transport of the chimeric 16/23 protein, two translocation intermediates with different topologies within the thylakoid membrane can be identified. Ti-1, which is represented by a 14 kDa thermolysin degradation product, assumes a loop conformation in the thylakoid membrane, exposing both N- and C-termini to the stroma. Ti-2, represented by a 26 kDa thermolysin degradation product, spans the thylakoid membrane only once, with a N_{out}-C_{in} topology.

2.1.3. Kinetics of the translocation intermediates of 16/23

In order to investigate the formation of the two translocation intermediates in more detail, a kinetic experiment was performed. In this experiment, 16/23 was first added to pea thylakoids on ice, while the actual import reactions were carried out at 25 °C in the light for different time periods. Association between 16/23 and the thylakoids took place very efficiently. Already at 0 °C, a large amount of 16/23 was found associated with the thylakoids.

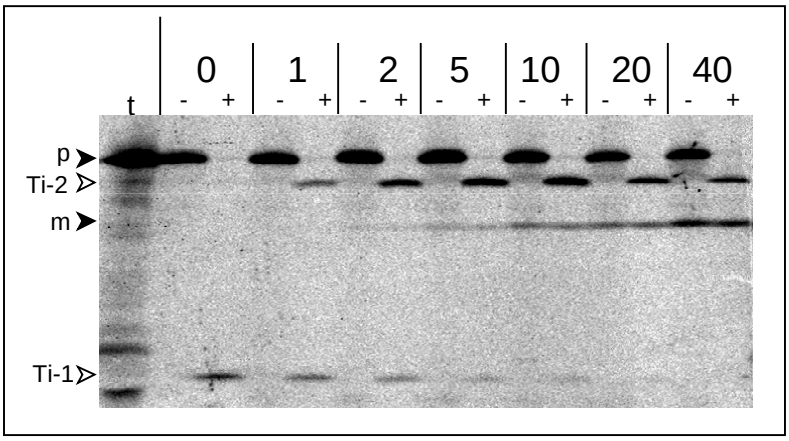


Fig. 13. Thylakoid translocation of 16/23 is retarded in the thylakoid membrane, resulting in two distinct translocation intermediates. *In vitro* synthesized radiolabelled 16/23 protein was mixed with isolated pea thylakoids on ice. Import reactions were carried out at 25 °C in the light for the time periods indicated above the lanes. After incubation, samples were treated as described in Fig. 6 and analyzed by SDS-PAGE.

After this treatment, only the 14 kDa degradation product can be found after thermolysin treatment of the thylakoids (Fig 13, lane 0). The 26 kDa degradation product is obtained only after incubation at 25 °C for some time. This result confirms that the two translocation intermediates are indeed formed consecutively in the thylakoid membrane. The earlier intermediate (Ti-1), which is represented by d_{14}, is formed already at 0 °C, whereas Ti-2,represented by d_{26} needs higher temperature for being made. The time-course shows that the amount of Ti-1 continuously decreases with increasing incubation time and completely disappears when the import was performed for 40 minutes, whereas Ti-2 begins to accumulate

after 1 minute and reaches a peak after approximately 10 minutes. At later time points, Ti-2 is processed to the mature 23 kDa protein.

Since excessive precursor protein was present during the entire incubation time, it could not be concluded whether the formation of Ti-1 is necessarily the initial step of the translocation process or whether the formation of Ti-1 is instead a dead end leading to degradation of the protein within the thylakoid membrane. Therefore, a modified time-course experiment was carried out. Pea thylakoids were incubated with the 16/23 protein at 0 °C to allow for the formation of Ti-1, but not of Ti-2 (Fig. 14). The resulting thylakoids were then reisolated and washed with import buffer to get rid of unbound precursor protein, and were then further incubated at 25 °C in the light. Similar to the result shown in Fig. 13, Ti-1 disappeared from the thylakoid membrane during incubation at 25 °C, whereas Ti-2 accumulated temporarily,

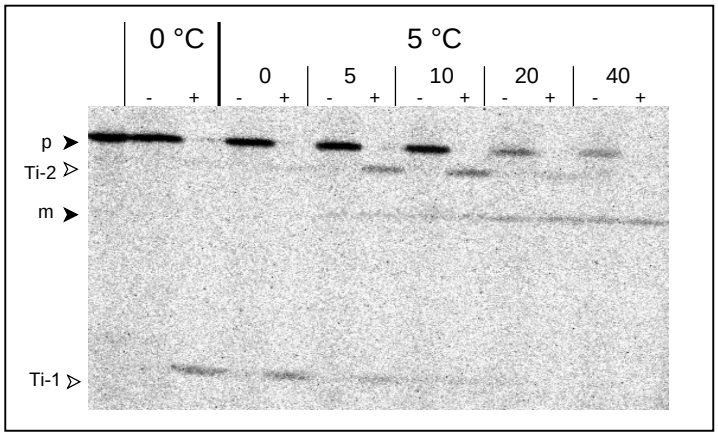


Fig. 14. Membrane inserted Ti-1 is a true translocation intermediate. *In vitro* synthesized 16/23 was incubated with isolated pea thylakoids at 0 °C in the dark. After 5 minutes incubation, the unbound 16/23 protein was removed by washing the thylakoids with HM buffer. Import assays were then carried out at 25 °C in the light for the time periods indicated above the lanes. After incubation, samples were processed as described in Fig. 6 and analyzed by SDS-PAGE.

reaching a peak after 10 minutes. This result proves that Ti-2 is obtained by altering the topology of the 16/23 protein within the thylakoid membrane. Thus, Ti-1 is the initial translocation intermediate that is in a conformation competent for further membrane transport. Translocation of the 16/23 protein can be therefore divided into three consecutive steps: (1)

formation of Ti-1, (2) formation of Ti-2, and (3) processing of Ti-2 into the mature protein. The first two steps, notably insertion of the protein into the thylakoid membrane and the translocation of the C-terminal domain, take place very efficiently, and most precursor proteins are converted into Ti-2 within 10 minutes. However, the last step, i.e., the processing of Ti-2 into the mature protein, takes place only slowly and lasts almost 40 min in the case of the chimeric 16/23 protein, suggesting that the TPP cleavage of the chimeric protein is not as efficient as that of authentic proteins.

2.1.4. Working model for the ΔpH/Tat-dependent translocation of 16/23

The data obtained so far demonstrate that the ΔpH/Tat-dependent translocation of the 16/23 protein takes place in multiple steps. The precursor protein is first inserted into the thylakoid membrane in a loop conformation, leaving both N- and C-termini on the stromal side. Ti-1 is anchored within the thylakoid membrane both with its transit peptide and a segment located in

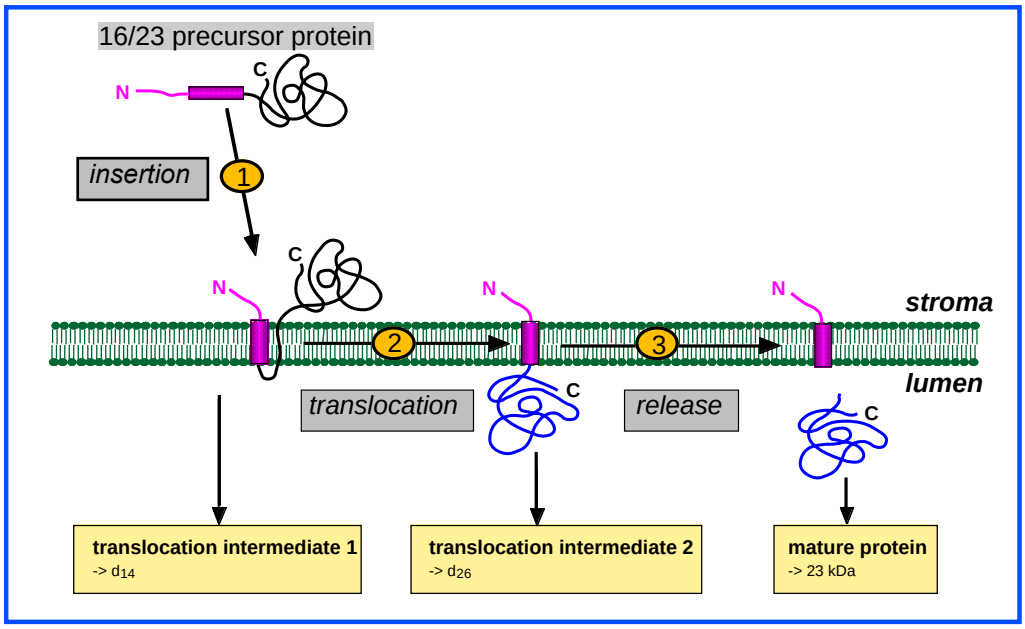


Fig. 15. Working model on the thylakoid translocation of the 16/23 protein. See text for details.

the mature part. In the next step, the C-terminal part of the mature protein is translocated across the thylakoid membrane as an entirety, resulting in the formation of Ti-2 that has a

N_{out}-C_{in} structure. Ti-2 is anchored within the thylakoid membrane only with its transit peptide. Finally, the 23 kDa mature protein is released into the thylakoid lumen after cleavage of the TPP processing site. The transit peptide is not in the thylakoid membrane, suggesting that it is rapidly degraded. A scheme of proposed model is shown in Fig. 15.

2.2. Involvement of the TAT translocase in the transport process of the ΔpH/Tat-pathway

It has been shown that the ΔpH/Tat-dependent translocase consists of at least three thylakoid membrane proteins, TatA (Tha4), TatB (Hcf106) and TatC (cpTatC) (Voelker and Barkan, 1995; Settles et al., 1997; Walker et al., 1999; Motohashi et al., 2001). However, the composition of the ΔpH/Tat-dependent translocase, as well as its functions, has not yet been characterized in detailTaking advantage of the retarded transport process of 16/23, I could investigate the different steps of the ΔpH/Tat-dependent translocation independent from each other.

2.2.1. Both Ti-1 and Ti-2 are associated with the translocase

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a useful method to separate membrane complexes under conditions that keep them mostly intact and can be visualized by Coomassie staining (Fig 16A). Thylakoids from an import reaction with the 16/23 protein were solubilized in non-denatured buffer supplemented with 1.67% digitonin, and separated by electrophoresis on Blue native-PAA gels. Autoradiography of the blue native gel revealed that the radiolabelled 16/23 protein co-migrates with two large membrane complexes of approximately 560 kDa and 620 kDa (Fig. 16A, see also Berghöfer and Klösgen, 1999). Since no major thylakoid membrane complex is detected within this region, it is reasonable to assume that these complexes represent the transient substrate-binding translocase complexes during ΔpH/Tat-dependent translocation of 16/23. In order to examine which forms of 16/23

are present in these two complexes, one lane of this gel was excised, and subsequently supplied, after 90 ° rotation, to a second electrophoresis under denaturing conditions (SDS-PAGE). Autoradiography of this 2D-gel showed that exclusively the precursor protein of 16/23 accumulates in the two complexes (Fig. 16B), whereas the processed, mature form is

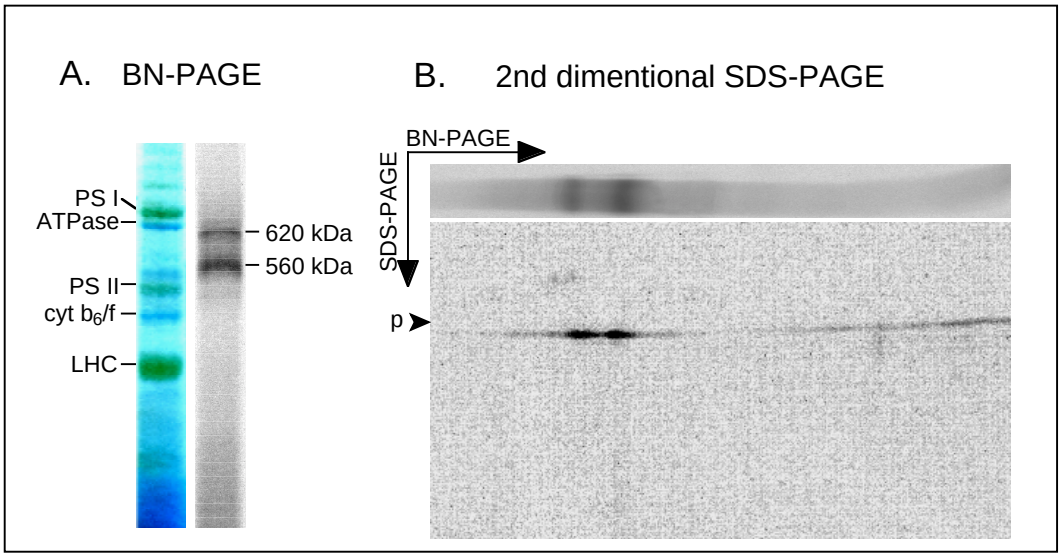


Fig.16. 16/23 is associated with two high molecular weight complexes in the thylakoid membrane. A. After import of radiolabelled 16/23, pea thylakoids were reisolated and solubilized in 1.67% digitonin-containing buffer and processed by blue native-PAGE (BN-PAGE) as described in Materials and Methods. The left panel shows the Coomassie-stained BN-PAGE gel, and the right panel shows the autoradiography of the BN-PAGE gel. The photosynthetic complexes, as well as the radiolabelled complexes, are indicated. **B**. Second dimension SDS-electrophoresis of the BN-PAGE gel. One lane of the BN-PAGE gel containing the imported 16/23 protein was excised and separated in the second dimension SDS-PAGE gel (10-17.5%), after 90 ° rotation. Proteins were visualized by autoradiography. p indicates the 16/23 precursor protein.

absent from both complexes. This result supports the idea that the translocation intermediates of 16/23 are bound to these two membrane complexes, which thus possibly represent the active translocase of the ΔpH/TAT pathway. On the other hand, the processed, mature protein is apparently rapidly released from the translocase, since it is not found in either of these complexes.

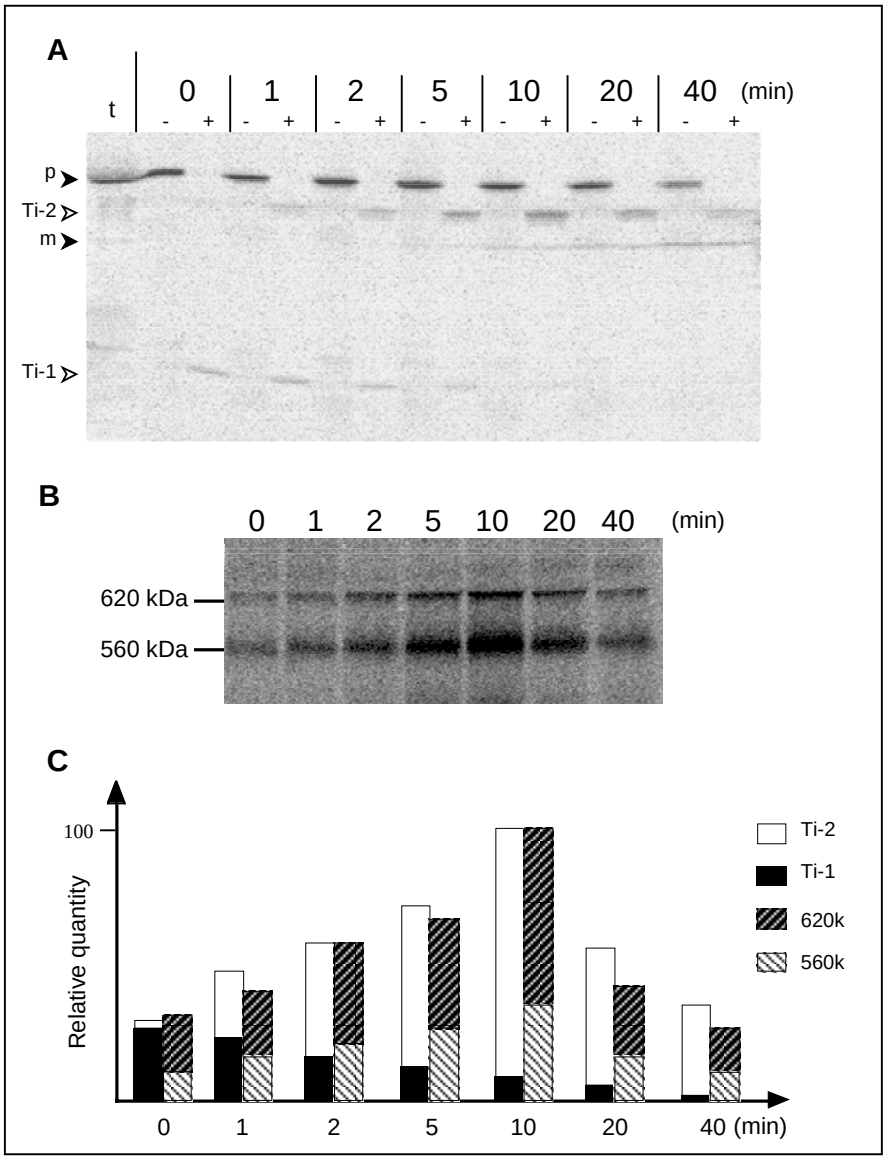


Fig. 17. Translocation intermediates of the 16/23 are associated with the translocase complexes in the thylakoid membrane. *In vitro* translated 16/23 was incubated with pea thylakoids on ice, followed by incubation at 25 °C for the indicated time periods. After import, samples were processed either by SDS-PAGE (**A**) or BN-PAGE (**B**). **C**, Quantification of the radioactive bands of the SDS- and BN-PAGE gels, respectively. The radiation intensity of each band was quantified by using the IQMac V1.2 software (Molecular Dynamics), and shown by the size of the corresponding bars.

In order to determine which of the two translocation intermediates, Ti-1 and Ti-2, are present in the two membrane complexes, BN-PAGE was performed also after kinetic experiments with 16/23. As control, a fraction of the assays was applied also to SDS-PAGE. Autoradiography of the blue native gel showed that already at 0 °C 16/23 was associated with

both membrane complexes (Fig. 17B, lane 0), although at this time point only Ti-1 is formed in the thylakoid membrane (Fig. 17A, lane 0). Remarkably, both complexes are found at each time point, and only the amount of 16/23 protein associated with these complexes varies during incubation time, reaching its maximum at about 10 minutes (Fig. 17B, lane 10). The ratio between the two complexes does not change significantly, in the course of the experiment (Fig. 17C). Even after 40 minutes, still some of the 16/23 protein is associated with the two translocase complexes, although almost all of Ti-1 was processed already into Ti-2, demonstrating that both translocation intermediates are able to form complexes with the translocase. Taken together, these results suggest that before being processed to the mature protein, the translocation intermediates of the 16/23 proteins are associated with the same translocation machinery, despite of their change in topology within the thylakoid membrane. The reason for the finding of the two complexes remains unclear. They might correspond to different compositions of the translocases in the thylakoid membrane, both with the capacity to bind Ti-1 and Ti-2, but this remains speculative so far.

2.2.2. Insertion of the 16/23 chimera into thylakoid membranes is an unassistent process

The data obtained so far showed that 16/23 inserts into the thylakoid membrane and forms Ti-1 with high efficiency even on ice, i.e. under conditions when most of the enzymatic reactions are inhibited. Therefore, it was of interest to examine the mechanism of membrane insertion of 16/23 and the formation of Ti-1 in more detail. In order to find out, whether thylakoid components are involved in the membrane insertion of 16/23, thylakoid vesicles were treated in different ways prior to the import experiment. 16/23 was first imported into thylakoids pretreated with chaotropic salts and alkaline solutions. It was reported that many thylakoid membrane proteins can be removed by extraction with chaotropic salts and alkaline solutions, and all known protein translocation pathways, except for the spontaneous protein insertion, are inhibited by this kind of extraction. In order to check the effect of extraction on the subunits of the TAT-machinery, Western blot analysis was performed (Fig. 18C). It turned out that from the three TAT components, TatA and TatB were resistant to all extraction

procedures, i.e. even after treatment with chaotropic salt or alkaline solutions, significant amounts of these proteins were still present in the thylakoid membrane. TatC, on the other hand, was also resistant to the extraction with chaotropic salts, but was almost quantitatively released from the thylakoid membrane by 0.1 M NaOH.

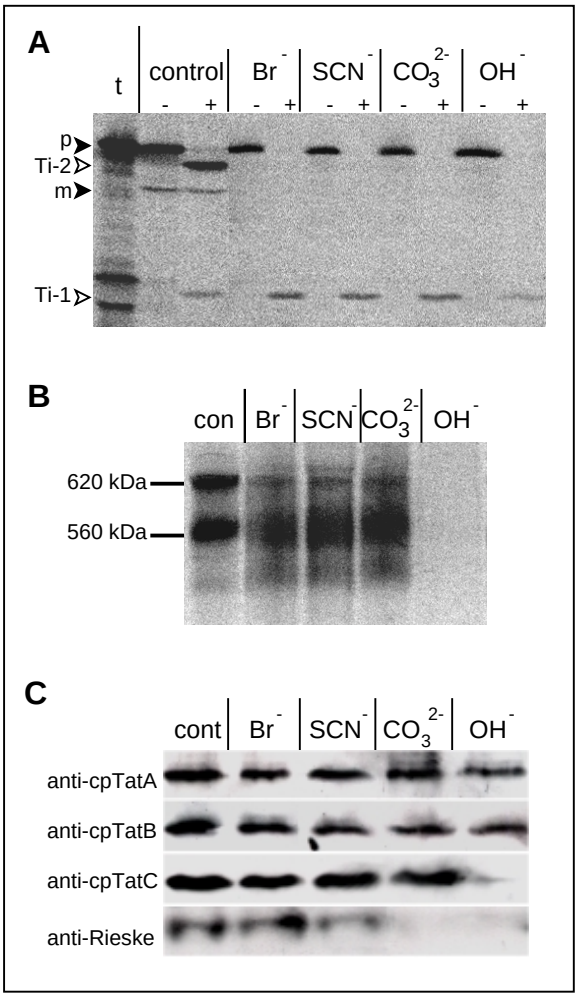


Fig. 18. Import of 16/23 into pretreated thylakoids. Isolated pea thylakoids were first extracted with either 2M NaBr, 2M NaSCN, 0.1 M Na_2CO_3, 0.1 M NaOH, or import buffer (control). After resuspension in import buffer, a fraction of the pretreated thylakoids was subjected to import experiments. Import samples were processed by either SDS-PAGE (**A**) or BN-PAGE (**B**). A second fraction of the pretreated thylakoids was subjected to SDS-PAGE, and subsequent Western analysis using antisera against TAT proteins and, as a control, the Rieske Fe/S protein (**C**). The Western blots were developed by ECL.

Using such pretreated thylakoid membranes in import assays with the 16/23 protein, it turned out that they were still able to bind the protein but had lost their translocation ability (Fig. 18). No mature protein is seen in any of the assays, even not with thylakoids that had apparently kept all three TAT proteins (Fig. 18). On the other hand, 16/23 could still bind to all thylakoids with high efficiency (Fig. 18A, lane-). Thermolysin treatment showed that only Ti-1 accumulated in the thylakoid membrane, i.e., insertion into the membrane in the loop conformation can take place but translocation of the C-terminal domain of the precursor protein is prevented in each case. BN-PAGE showed that the membrane-bound Ti-1 was in

most cases associated with the translocase complexes, too, though predominantly with the smaller (560 kDa) complex (Fig. 18B). In one case, however, no such complex can be detected after BN-PAGE. Pretreatment of thylakoid membranes with 0.1 M NaOH leads to the release of TatC, and prevents all complex formation completely (Fig. 13B, OH^-). This result indicates that the formation of the 16/23-translocase complexes is strictly dependent on the presence of TatC. Unexpectedly though, even without binding to the translocase complex, some Ti-1 was still found in these assays, too.

Since the TatA and TatB are surprisingly stable against extraction with chaotropic salts, involvement of these two proteins in the membrane insertion of 16/23 and the formation of the Ti-1 could be not excluded. Therefore, thylakoid vesicles were pretreated with proteases prior to the import experiment. Pretreatment of the thylakoids with protease should degrade the stroma exposed receptor domains of all translocases, while keeping the thylakoid membrane itself undestroyed. This treatment has been shown to inhibit all known protein translocation pathways at the thylakoid membrane, except for the spontaneous protein insertion pathway that apparently relies solely on the membrane itself (Robinson et al., 1996). In the experiment performed here, thylakoids were pre-incubated with proteinase K, ranging in concentration from 0.1 µg/ml to 20 µg/ml. The efficiency of the protease treatment was investigated in Western blots using antibodies raised to againset the three TAT proteins. It turned out that all three TAT proteins are resistant to low concentration of protease K, but are completely degraded by 20 µg/ml proteinase K (Fig. 19C). The residual amount of the lumenal 33 kDa protein, which was analyzed as control, indicated that at least some of the thylakoids remain intact by this treatment. Translocation of 16/23 into such thylakoid showed that the formation of both Ti-2 and the mature protein is inhibited, already with thylakoids treated with low concentrations of protease, when most of the TAT proteins seemed to be largely unaffected (Fig. 19A). Thus, the translocation step is very sensitive to protease-pretreatment. On the other hand, pretreatment of the thylakoids with low concentration of proteinase K even stimulated the formation of the Ti-1, presumably due to the failure of transforming Ti-1 into Ti-2. In thylakoids lacking all three TAT proteins, Ti-1 is still formed

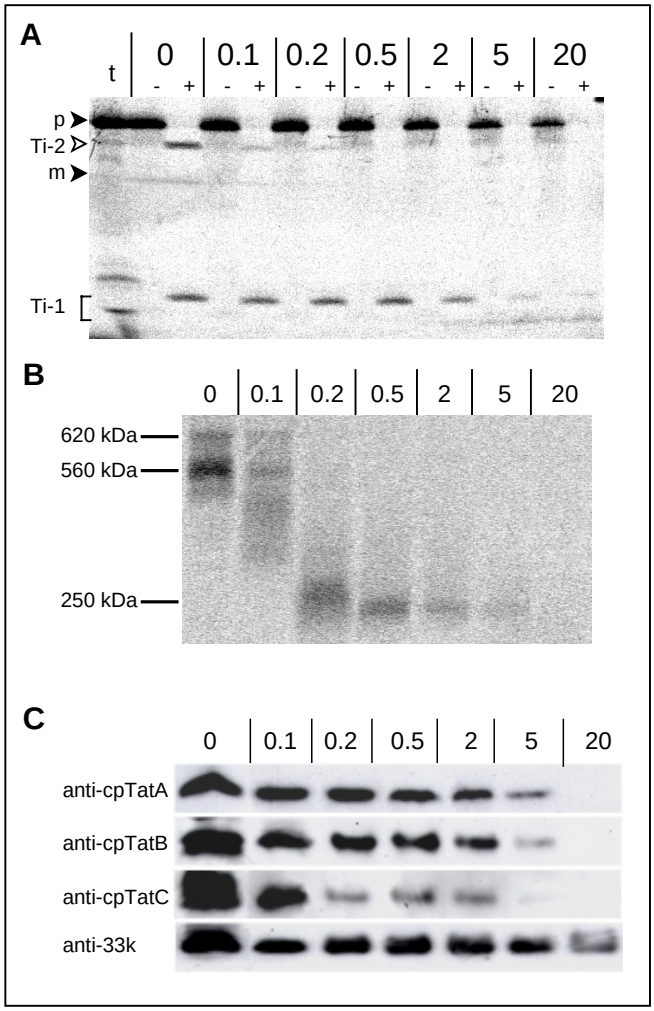


Fig. 19. Import of 16/23 into protease K-pretreated thylakoids. Isolated pea thylakoids were firstly treated with protease K with indicated concentrations. *In vitro* translated 16/23 protein was incubated with the protease K-pretreated thylakoids at 25 °C for 10 minutes. Following incubation, one half of each sample was processed by SDS-PAGE (**A**), and the other half of each sample was processed by BN-PAGE (**B**). **C**. The pretreated thylakoids were subjected to SDS-PAGE followed by Western blot using specific antisera to indicated proteins.

rather efficiently (Fig. 19). In particular, if it is taken into account, one fraction of the 14 kDa degradation product was processed further by residual proteinase K activity into the 12 kDa degradation product (see also Fig. 12). These results therefore strongly suggest that the membrane insertion step of 16/23 can take place independent of the presence of any of the TAT components, possibly in a kind of “spontaneous” insertion event. BN-PAGE confirmed that protease-pretreatment interfered with the interaction of the precursor protein and the translocase. After pretreatment with 0.2 - 2 µg/ml protease K, Ti-1 was not anymore detected in the 560 and 620 kDa complexes but instead in a complex of only about 250 kDa (Fig. 19B). Under these conditions, most of TatC is already degraded, but TatA and TatB are fairly stable. At higher proteinase K concentration, when also TatA and TatB are degraded, even this 250 kDa complex disappears (Fig. 19B), suggesting that this complex represents a TAT-subcomplex of limited properties. It should be noted that essentially identical results have

been observed when trypsin was used for pretreatment of the thylakoids (data not shown). Taken together, these data strongly suggest that the thylakoid membrane insertion of the 16/23 precursor protein can take place independent of any thylakoid membrane protein, including the TAT components, and that the formation of the loop-structure of a precursor protein might be obtained even without interaction with the translocase.

2.2.3. Unassisted thylakoid membrane insertion takes place also with authentic precursor protein

One could argue that the Tat-independent membrane insertion of 16/23 is an artifact of the chimeric protein, because so far no translocation intermediate has been reported for any of the authentic ΔpH/Tat-pathway proteins. In order to exclude that the membrane insertion is specific only for 16/23, the experiments were repeated with an authentic ΔpH/Tat-pathway protein, the pre-OE23 protein. Import of pre-OE23 into untreated thylakoids takes place very efficiently, and already after 5 minutes more than 90% of the precursor protein is found processed to the mature protein (Fig. 20, lane 0). Like with 16/23, trypsin pretreatment of thylakoids strongly inhibits the formation of the mature protein, but in the meanwhile, a 14 kDa fragment was detected in the thermolysin-treated fraction, reminiscent of the 14 kDa degradation product indicative of Ti-1 for the 16/23 chimera. On the other hand, a degradation product corresponding to Ti-2 did not appear. With increasing amount of protease used for pretreatment, the amount of 14 kDa degradation products increased as well (Fig. 20). This behavior is very similar to that of Ti-1 of 16/23, suggesting that it should represent the corresponding translocation intermediate for the authentic pre-OE23 protein. This assumption was confirmed when thylakoids were used that were pretreated with chaotropic salts or alkaline solutions. Again, the translocation of the OE23 protein was inhibited, but the formation of the putative translocation intermediate 1 was stimulated (Fig. 20). These data demonstrate for the first time that also authentic precursor proteins are capable of inserting into the thylakoid membrane as a translocation intermediate independent of the presence of TAT-components.

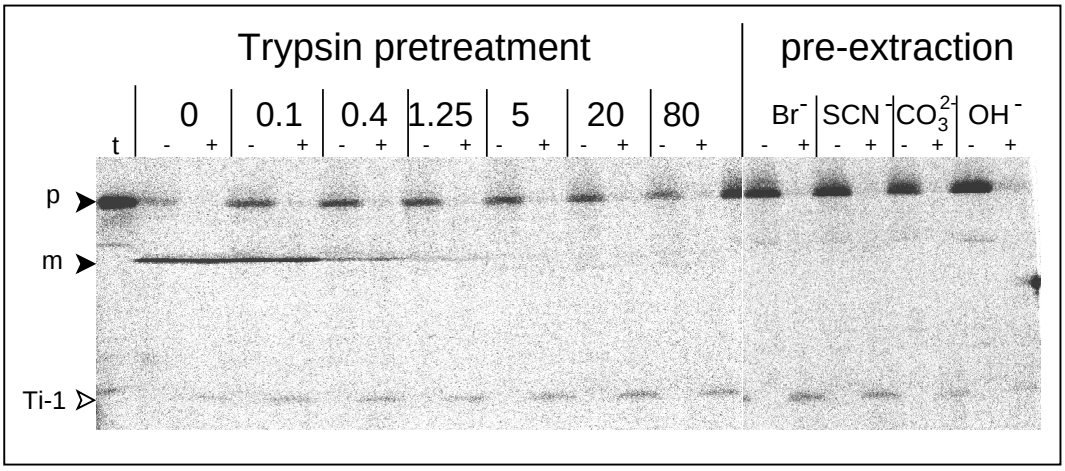


Fig. 20. Import of the authentic pre-OE23 protein into pretreated thylakoids. Thylakoids were treated with the indicated concentration of trypsin for 30 min on ice. After washing with HM buffer containing 50 µg/ml trypsin inhibitor, the thylakoids were resuspended in HM. For pre-extraction assays, thylakoids were pre-incubated with 2 M NaBr, 2 M NaSCN, 0.1 M Na_2CO_3 and 0.1 M NaOH, respectively. After washing with HM buffer, the thylakoids were resuspended in HM buffer. Import reactions were carried out by incubation of pre-OE23 with the pretreated thylakoids for 10 min at 25 °C in the light and then further processed as described in Fig. 6. All samples were analyzed by SDS-PAGE and autoradiography.

2.2.4. Insertion of the 16/23 protein into artificial lipid bilayers

The data shown so far imply that the thylakoid membrane insertion of 16/23 and pre-OE23 protein takes place in a kind of spontaneous mechanism. Membrane insertion in these cases does apparently not need any thylakoid components except for the membrane itself. To examine the potential spontaneous membrane insertion of 16/23 in an independent approach, silica beads coated with phospholipid bilayers (TRANSIL beads) were used, which allow to measure the rate of protein binding to lipid membranes independent of any proteinaceous component (Loidl-Stahlhofen et al., 2001; Schmitt et al., 2001). Since the lipid molecules are not covalently linked to the support and are separated by an ultrathin layer of water molecules, this material has many characteristics reminiscent of biological lipid bilayers. Approximately 93% of the lipid content of pea thylakoids are galactolipids, such as MGDG (Monogalactosyldiacylglycerol), DGDG (Monogalactosyldiacylglycerol) and SQDG

(Sulfoquinovosyl-diacylglycerol), whereas phospholipids contribute to only 7% of the total thylakoid lipid content (Andersson et al., 2001). Unfortunately, it was not possible for technical reasons to obtain TRANSIL beads coated with galactolipids. Therefore, beads coated with phospholipids had to be used instead in all experiments described here. Radiolabelled 16/23 protein was incubated with the TRANSIL beads in HM buffer, and the membrane-bound protein was digested with thermolysin in order to examine whether membrane-protected fragments similar to the translocation intermediates obtained during thylakoid translocation can be found. As a control, 16/23 *in vitro* translation products were diluted into HM buffer also in the absence of TRANSIL beads and digested with thermolysin. When no TRANSIL beads are present, thermolysin treatment of *in vitro* translated 16/23 protein leads to a single degradation fragment of about 10 kDa (Fig. 21A, -transil). In the presence of TRANSIL beads, 16/23 was found to be almost quantitatively bound to the phospholipid-coated beads. Thermolysin treatment of the membrane-bound 16/23 protein led to four degradation products of about 10 kDa, 12 kDa, 15 kDa and 21 kDa, respectively (Fig. 21A, +transil), indicating that membrane binding of 16/23 increases its resistance to proteolysis. However, none of these fragments has the same mobility as the 14 kDa and 26 kDa degradation products obtained in the import assays. In order to distinguish whether the membrane-protected fragments result only from protective folded structures of the 16/23 protein or are true membrane insertion forms, the 16/23 protein was exposed to various concentrations of thermolysin, ranging from 5 µg/ml to 100 µg/ml. It turned out that the 16/23 precursor protein indeed forms some folded structure already in HM buffer that makes the 16/23 protein partially resistant to thermolysin digestion. These structures are responsible for the protease resistant bands of 10 kDa and 21 kDa that are obtained with low concentrations of thermolysin (Fig. 21A, right panel). However, the 12 kDa and the 15 kDa fragment obtained in these assays are obviously not due to folding of the 16/23 protein alone, but presumably represent membrane-protected fractions of the 16/23 protein. To examine the membrane-inserted state of these 16/23 proteins in an independent approach, the TRANSIL beads were also extracted with some chaotropic salts prior to protease treatment (Fig. 21B).

Extraction with two mild chaotropic salts, Na_2CO_3 and NaBr, showed no significant effect on the membrane-inserted 16/23, because both the 15 kDa and the 12 kDa thermolysin fragments

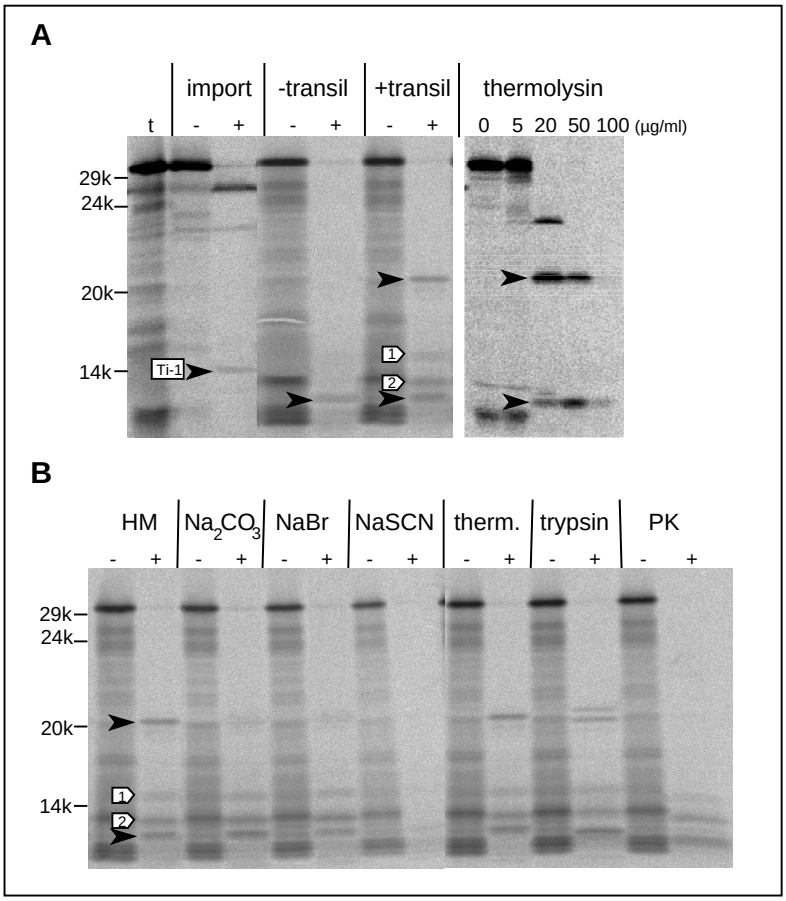


Fig. 21. Membrane insertion of 16/23 into artificial lipid bilayers. A. *In vitro* translated 16/23 was imported into isolated pea thylakoid for 10 min (import), or incubated with HM buffer (-transil) or TRANSIL beads resuspended in HM buffer (+transil) at room temperature for 30 min. After treatment with thermolysin (100 µg/ml) or mock-treatment, proteins were separated by SDS-PAGE. In the right panel, 2.5 µl of *in vitro* translocation product was diluted into 50 µl HM buffer and treated with indicated concentration of thermolysin. **B**. After incubation with 16/23, TRANSIL beads were either washed with different chaotropic salt solutions, or treated with three different proteases. Samples were processed by SDS-PAGE and visualized by autoradiography. Unspecific degradation products are indicated by solid arrowheads, and specific degradation products by empty arrows carrying numbers.

remained detectable, which indicates that the protein has indeed inserted into the artificial lipid bilayers. Extraction removed most of the 21 kDa thermolytic fragment, confirming that this fraction of protease-resistant 16/23 was only associated to the membrane surface. On the

other hand, the strong chaotropic salt NaSCN extracted almost all of the membrane-bound 16/23 (Fig. 21B), proving that insertion of 16/23 into the lipid bilayers is not strong enough to prevent extraction with this chaotropic salt. This might be possibly due to the lack of proteinaceous interaction partners in the lipid membrane. These data were confirmed also by using additional proteases. Treatment of the TRANSIL beads with trypsin and proteinase K rather than thermolysin showed that practically the 12 kDa fragment presumably represents Ti-1 also within artificial bilayers (Fig. 21B).

2.3. The role of the thylakoidal ΔpH in translocation of 16/23

The ΔpH/Tat-dependent translocation pathway was originally defined according to its unique requirement for the ΔpH across the thylakoid membrane (Mould and Robinson, 1991; Cline et al., 1992; Klösgen et al., 1992). However, it is still unknown how the ΔpH facilitates protein translocation while preventing ion leakage across the thylakoid membrane remains unknown. Import assays with 16/23 offer the advantage to investigate the separate steps of protein translocation process independently from each other, and therefore provide the opportunity to study the role of the ΔpH in this process.

2.3.1. Thylakoid membrane insertion of 16/23 is independent of the thylakoidal ΔpH

Ionophores such as nigericin and carbonyl cyanide m-chlorophe-nylhydrazone (CCCP), can dissipate the ΔpH across the thylakoid membrane and by this mean completely inhibit the thylakoid translocation of ΔpH/TAT-dependent proteins. Previous experiments have shown that thylakoid translocation of the 16/23 protein is completely inhibited by nigericin (Berghöfer and Klösgen, 1999). In line with these data, upon addition of nigericin, neither Ti-2 nor the mature protein is detectable in the import assays (Fig. 22). CCCP exhibits a similar but somewhat weaker effect. In contrast to that, the formation of Ti-1 was even stimulated under these conditions (Fig. 22A), indicating that in the absence of the thylakoidal ΔpH, 16/23 is still inserted into the thylakoid membrane and forms Ti-1. BN-PAGE revealed that in

the presence of either of the inhibitors, Ti-1 is still present in the same translocation machineries in the thylakoid membrane, since the resulting complexes have the same mobility on the BN-PAA gel as those of the control assay (Fig. 22B). It can therefore be concluded

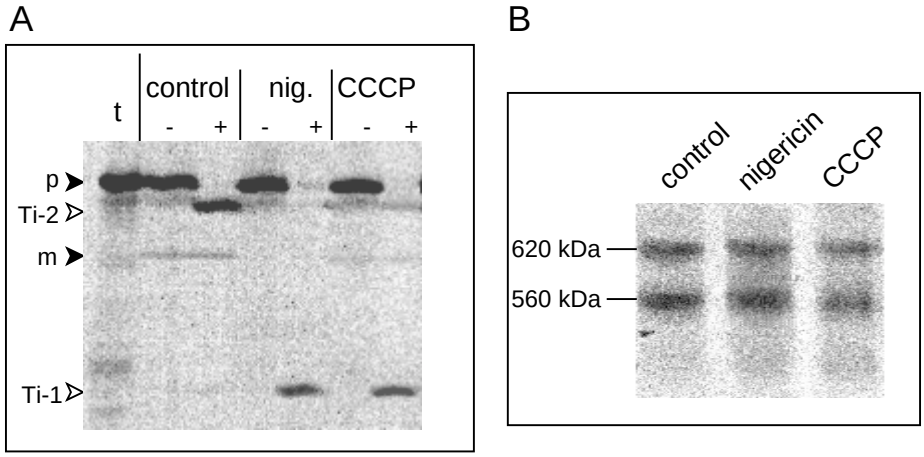


Fig. 22. Thylakoid membrane ΔpH is required for the formation of Ti-2 but not for the formation of Ti-1. Radiolabelled 16/23 was mixed with pea thylakoids in the presence of either nigericin or CCCP, and incubated for 10 min at 25 °C in the dark. Following incubation, one half of each samples was analyzed by SDS-PAGE and autoradiography (**A**). The other half of each samples was analyzed by BN-PAGE and autoradiography (**B**).

from this experiment that the translocation step leading to formation of Ti-1 is independent of the thylakoidal ΔpH. In the absence of ΔpH, 16/23 is still able to form Ti-1 in the thylakoid membrane, and it is captured also by the TAT-translocase. However, the following step of the translocation process, i.e., the translocation of the C-terminal part of 16/23 that results in the formation of Ti-2, is strictly dependent on the thylakoidal ΔpH.

2.3.2. Identification of the ΔpH-dependent step

The next question was whether the last step of the ΔpH/TAT-dependent translocation, i.e. the release of the mature protein, is also dependent on the ΔpH. In order to analyze that, a time-course assay upon addition of nigericin was carried out. In this experiment, the 16/23 was first incubated with the thylakoids for only 5 minutes in the absence of nigericin, to allow for the

formation of Ti-1, Ti-2 and some mature protein (Fig. 23, -nig). Then the reaction was steooped by addition of nigericin and the thylakoids were washed with nigericin-containing buffer to get rid of any free 16/23 protein. Import was then continued in the presence of nigericin in the assays (Fig. 23, +nig). Under these conditions, Ti-1 remains blocked and cannot be transformed into Ti-2, confirming again that this step requires the ΔpH across the thylakoid membrane.

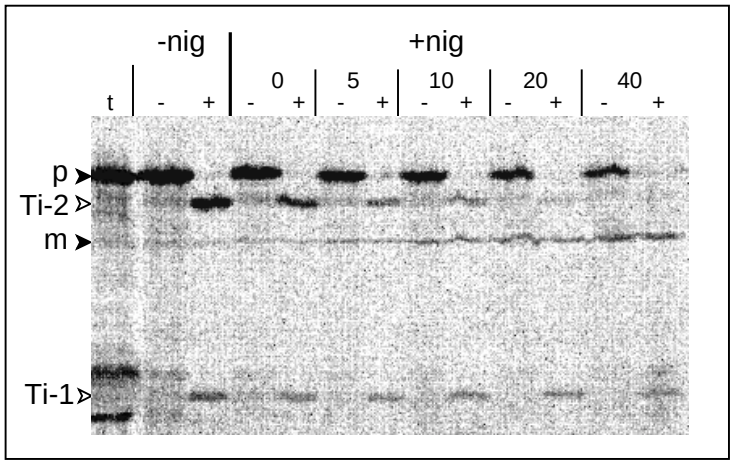


Fig. 23. Identification of the ΔpH-dependent step of thylakoidal translocation of 16/23. *In vitro* translated 16/23 was incubated with thylakoids at 25 °C in the light for 5 minutes, then the thylakoids were re-isolated and washed with import buffer containing 2 µg/ml nigericin. Thylakoids were further incubated in import buffer containing 2 µg/ml nigericin at 25 °C for indicated times. The resulting thylakoids were divided into two halves for either mock treatment (-) or thermolysin digestion (+). All protein samples were separated by SDS-PAGE and visualized by autoradiography.

However, the amount of the Ti-2 decreased with time under these conditions and, in parallel, a simultaneous increase in the amount of the mature protein could be observed (Fig. 23). This demonstrates that the processing of Ti-2 and thus the formation of the mature protein is independent of the thylakoidal ΔpH. After 40 minutes of incubation, Ti-2 was almost exhausted and almost quantitatively processed to the mature protein. This experiment proves that the thylakoidal ΔpH is required only for the actual translocation of the C-terminal part of the passenger protein. All the other steps, i.e., membrane insertion and translocase-binding of precursor protein, as well as the release of the mature protein, are independent of the thylakoidal ΔpH.

2.3.3. The electron transport chain is not involved in the ΔpH/TAT-dependent translocation

The photosynthetic machinery in the thylakoid membrane is very efficient. Upon exposure of isolated thylakoids to light, the electron transport chain along the thylakoid membrane is functional even in the presence of ionophores. It is difficult therefore to exclude that the electron flow locally is still transferred into a membrane potential under these conditions that might be used to generate transiently a local ΔpH across the thylakoid membrane. Since import assays with ionophores were carried out in the light, it could not strictly be excluded that this local ΔpH might facilitate the insertion of the 16/23 into the thylakoid membrane. In order to avoid such possible interference of the electron flow at the thylakoid membrane, the 16/23 protein was imported into isolated thylakoids in the dark. In order to satisfy the energy demands of the import process, a series of pH differences across the thylakoid membrane were generated by resuspending the thylakoids in import buffers with pH ranging from 5.0 - 10.0. It was reported that under dark condition, the lumenal pH of pea thylakoids remains at approximately 6.0 (Pfundel et al., 1994). Thus, the resulting ΔpH in the assays should range from approximately -1 to 4 units. Remarkably, Ti-1 is found in similar amount in each of the assays (Fig. 24), even in the import buffer with pH 5.0, which finally proves that a proton gradient is not required for loop insertion of the precursor protein. Membrane insertion is also independent of the flexibility of the membrane, as it is observed at both 0 °C and 25 °C incubation temperature. In contrast, the formation of Ti-2 and the mature protein is abolished at 0 °C. Only at 25 °C, formation of Ti-2 and the mature protein is found (Fig. 24A), and the rate of the formation is directly dependent on the pH difference, reaching a maximum in buffers of pH 8.0. At pH 9.0, still some Ti-2 is found, whereas higher pH values inhibit the formation of Ti-2. Interestingly, this inhibition is reversible by switching the buffer pH back to 8.0 (data not shown). It has been shown that the physiological pH of the pea chloroplast stroma remains always at about 8.0 (Pfundel et al., 1994), which is in agreement with our observation. Higher pH values affect the translocation efficiency, possibly through interfering

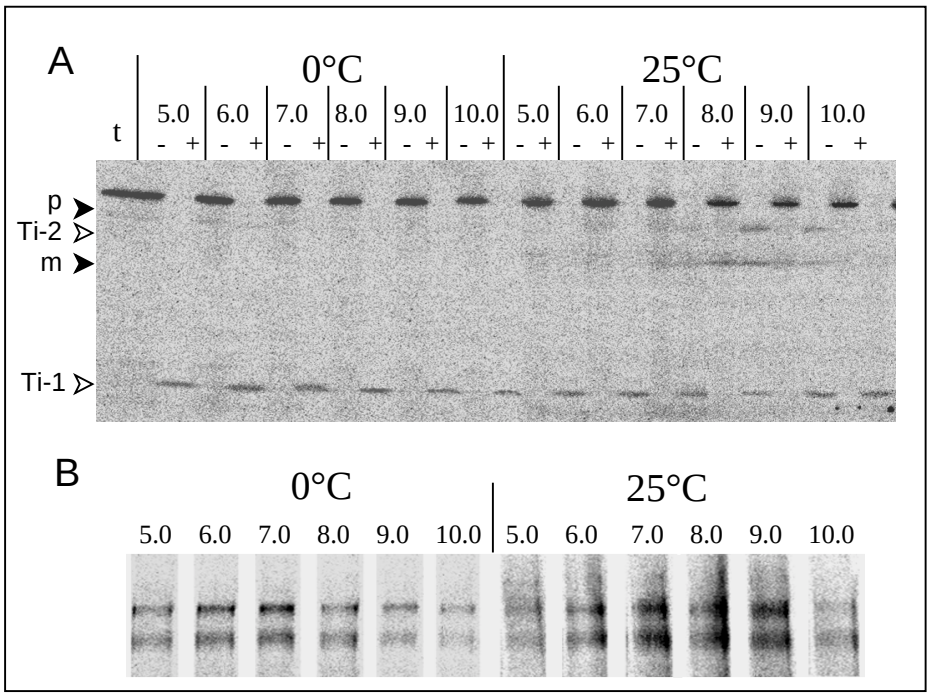


Fig. 24. Translocation of the 16/23 is driven by ΔpH but not by electron transport chain. Isolated pea thylakoids were stored at room temperature for 60 min, then rapidly resuspended into HM buffer of different pH indicated above the lanes a. *In vitro* translated 16/23 was mixed with the thylakoids in green light, and then incubated for 20 min in the dark either on ice or at 25 °C. After import, samples were processed by either SDS-PAGE (A) or BN-PAGE (**B**), and visualized by autoradiography.

the conformation of both the precursor protein and the translocase. This alteration is obviously reversible, since the translocation efficiency is restored when the external pH is switched back to 8.0. BN-PAGE analysis of the membrane complexes indicated that the formation of the precursor-translocase complexes is independent of the ΔpH (Fig. 24B). This experiment proves that the membrane insertion of 16/23 is independent of either ΔpH or electron flow at the thylakoid membrane, whereas the actual translocation step is coupled only to the transmembrane ΔpH, but is not a function of photosynthetic electron transport.

3. Is there targeting information also within the mature polypeptides of proteins targeted by ΔpH/TAT-dependent pathway?

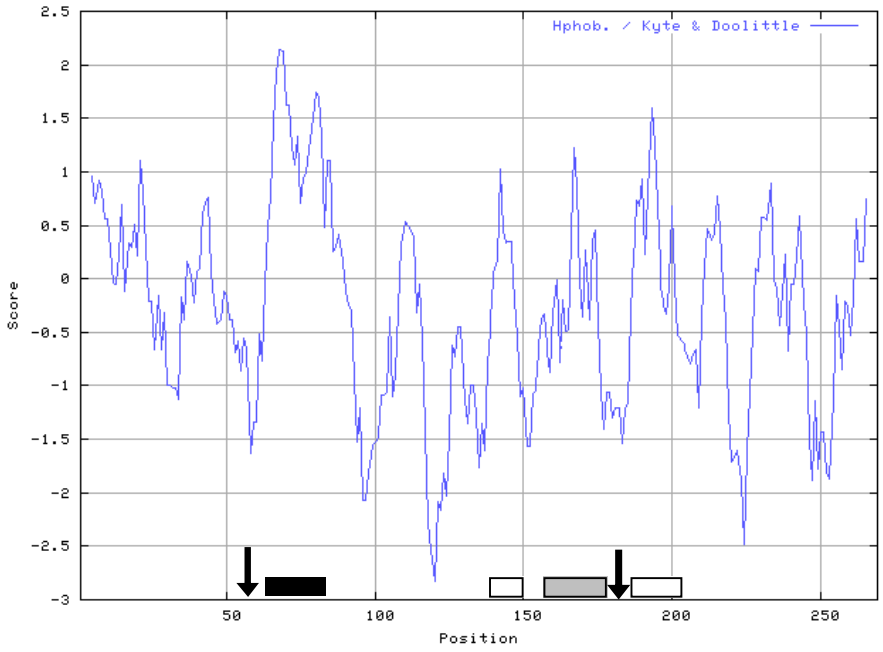
Protein translocation via the ΔpH/TAT pathway occurs in the absence of any soluble stromal component. Therefore, it was assumed that a receptor-like translocase component might be engaged to target the precursor proteins to the membrane-embedded translocon. However, the data shown here clearly demonstrate that a ΔpH/TAT substrate protein is able to insert into the thylakoid membrane without assistance from any proteinaceous components of the thylakoid membrane. This was surprising but it should be mentioned that 16/23 is not the first protein found to insert into the thylakoid membrane in an unassisted manner. A wide range of thylakoid membrane proteins, such as CFoII, PsbW and PsbX, as well as the polytopic membrane proteins Elip2 and PsbS, are capable of integrating into the thylakoid membrane in an unassisted, or "spontaneous" manner (Michl et al., 1994, Lorkovic et al., 1995; Kim et al., 1998, 1999). Thylakoid membrane insertion of these proteins requires neither energy in form of NTPs or a membrane potential nor soluble stromal factors, and protease treatment or chaotropic extraction of the thylakoid membrane does not inhibit their insertion (Michl et al., 1994, Kim et al., 1998). These proteins show no obvious homology in amino acid sequence, but bear some structural similarities. First, they all have a positively charged N-terminal domain that targets them to the negatively charged membrane surface. Second, they have one hydrophobic region in the signal peptide plus one or more hydrophobic regions in the mature protein, which allow them to form a loop structure in the membranes. Third, the central sequence between two hydrophobic regions, which in the loop structure is located on the *trans* side of the membrane, is always negatively charged. Thylakoid membrane insertion of 16/23 shares many similarities to these proteins: First, insertion of 16/23 requires no NTPs, ΔpH or stroma components, and it is not inhibited by protease treatment or chaotropic extraction of the thylakoid membrane. Second, insertion of the 16/23 protein leads to the formation of a transient loop structure in the thylakoid membrane. However, the difference between the insertion of the 16/23 and the spontaneous pathway proteins is also significant: the mature OE23 protein is a soluble lumen protein, whereas all the spontaneous pathway proteins are membrane-integral proteins. Nevertheless, the data obtained so far seem to suggest that insertion of 16/23 into the thylakoid membrane might possibly employ a mechanism similar to that used by the spontaneous pathway proteins.

3.1. Identification of the structural information for the thylakoid insertion of the 16/23 protein

3.1.1. A possible transmembrane helix in the mature part of the 16/23 protein

The 16/23 protein first inserts into the thylakoid membrane in a loop structure, exposing both termini to the stromal side and with the loop on the *trans* side of the thylakoid membrane. To assume this structure, the polypeptide must span the membrane twice. One membrane span is provided by the H-domain of the signal peptide, but the second membrane spanning region must be located within the mature part of the OE23 protein, which is a soluble lumen protein. Except for the hydrophobic region of the signal peptide, no significant hydrophobic transmembrane region is predicted for the 16/23 protein (Fig. 25A). However, three regions of moderate hydrophobicity are found in the mature protein of 16/23 in such prediction analysis, which span the residues from (i) ^{129}V to ^{146}V, (ii) ^{163}F to ^{178}G, and (iii) ^{186}F to ^{204}V, respectively. The putative thermolysin cleavage site recognized to produce the 14 kDa fragment indicative of Ti-1 is located between ^{185}G-^{186}F, which is 8 residues downstream of the C-terminus of the second amphipathic region (from ^{163}F to ^{178}G), suggesting that the other two regions are probably not transmembrane regions. In line with that, the second amphipathic region is long enough to span the thylakoid membrane (16 residues). When using the "Consensus Secondary Structure Prediction" program to predict secondary structure, only this region shows a significant tendency to form an α-helix (Fig. 25B). These data strongly suggest that the amphipathic region from ^{163}F to ^{178}G provides the second transmembrane span for thylakoid membrane insertion of the 16/23 protein, forming the loop structure in cooperation with the H-domain of the signal peptide.

A. Hydrophobic moment



B. Secondary structure prediction

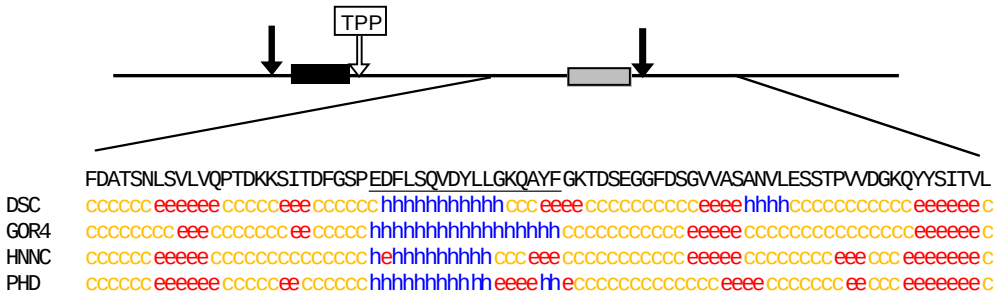


Fig. 25. Identification of a putative amphipathic region within the mature OE 23 protein. A. Hydropathicity of 16/23 was calculated by using the program ProtScale (http://www.expasy.org/cgi-bin/protscale. pl). The h-domain of the signal sequence is indicated by a black rectangle, whereas the three putative amphipathic regions are marked by either empty or shaded rectangles. The two putative thermolysin cleavage sites that are possibly used for d_{14} formation are indicated by arrows. **B**. Secondary structure of the putative amphipathic region and its flanking sequences were calculated by a Consensus Secondary Structure Prediction program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat. pl?page=/NPSA/npsa_seccons.html). Algorithms used are indicated on the left side. DSC is an algotithm introduced by King and Stengerg, (1996), GOR4 by Garnier et al. (1996), HNNC by Guermeur et al. (1997), and PHD by Rost et al. (1994). c = random coil; e = extended strand; h = α-helix. Two putative thermolysin cleavage sites and the TPP processing site are indicated by arrows.

The structure of the 16/23 protein was compared to that of PsbW, a typical protein of the spontaneous insertion pathway. PsbW is a small thylakoid membrane protein of only 54 residues that contains a transmembrane span of 24 residues, which anchors this protein within the thylakoid membrane after translocation. The precursor of PsbW is synthesized in the cytosol with a bipartite transit peptide. Within the thylakoid targeting domain of the transit peptide, there is an H-domain of 20 residues. PsbW shows no sequence conservation to 16/23, but the overall structure of these two proteins bares some similarities: First, both proteins have an H-domain in the transit peptide and a hydrophobic or amphipathic region in the mature part, which might be used as transmembrane span. Second, the N-terminal sequences flanking the H-domain are positively charged. Third, the regions between the two hydrophobic or amphipathic regions are predominantly negatively charged. Taken together, the amphipathic region within the mature part of 16/23 makes the overall structure of it similar to that of PsbW, as indicated in Fig. 26.

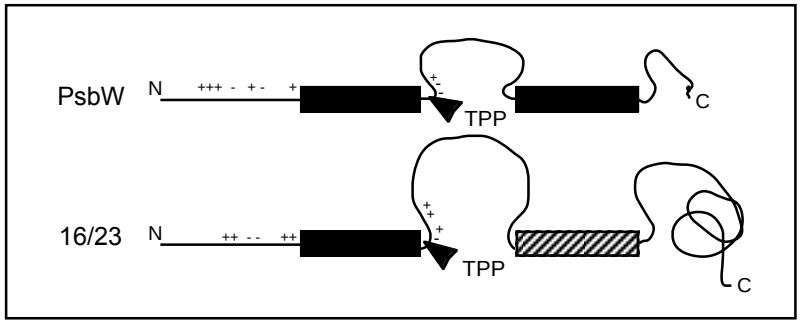


Fig. 26. Schematic structures of PsbW and 16/23. The predicted transmembrane spans are indicated by solid rectangles, whereas the putative amphipathic α-helix is indicated by twilled rectangle. The positively and negatively charged residues flanking the presumed membrane spans are indicated by "+" and "-", respectively.

3.1.2. Importance of the amphipathic region within the mature part of the 16/23

In order to examine whether the putative amphipathic region within the mature part of the 16/23 protein indeed plays a role in the transport process, three deletion derivatives of 16/23 were generated. These mutant proteins either lack the complete hydrophilic C-terminal part

(16/23Δ186-269) or the amphipathic region predicted to operate as membrane span (16/23Δ142-177) or both (16/23ΔC142-269) (Fig. 27A). Each mutant protein exhibits distinct import properties into isolated thylakoids, which are different from that of the “wild type” 16/23 (Fig. 27B). The 16/23Δ186-269 protein, which has retained the second transmembrane span, was translocated into the thylakoids almost as efficiently as the original 16/23, yielding a smaller “mature protein” of the expected size (12 kDa). Interestingly, thermolysin treatment of the membrane-bound 16/23ΔC84 protein yields only a single 14 kDa degradation product. Since this mutant protein was truncated exactly at the thermolysin cleavage site predicted to be recognized also in Ti-1 of the original 16/23 protein, it lacks the C-terminal part of Ti-1 that is exposed to the stromal side. Thus, the 14 kDa degradation product in this case represents both Ti-1 and Ti-2 of the 16/23Δ186–269 mutant protein, despite of their different topology. Translocation of the 16/23Δ186–269 is ΔpH-dependent, because in the presence of nigericin, no final processing product of about 12 kDa is found. As expected, the 14 kDa thermolysin degradation product is still detected. In this case, it should represent only Ti-1, since otherwise some release of the “mature” protein that is independent of the ΔpH should be found. This suggests that the proton gradient is essential even for translocation of the amphipathic region alone, and not only for membrane transfer of the folded C-terminal part of the mature protein. The amphipathic region is essential for the transport process, because translocation of the other two mutants, notably 16/23Δ142–269 and 16/23Δ142-177, was completely abolished. These polypeptides cannot even insert into the thylakoid membrane, because no Ti-1 could be detected, neither in the presence nor in the absence of nigericin. These experiments strongly suggest that an amphipathic helix in the mature protein is essential for membrane insertion of the ΔpH/TAT-dependent precursor proteins, and that the loop insertion takes place by a mechanism that is apparently similar to that of the spontaneous insertion pathway. Finally, these data suggest that the loop insertion step might be the essential first step in ΔpH/TAT-dependent protein transport.

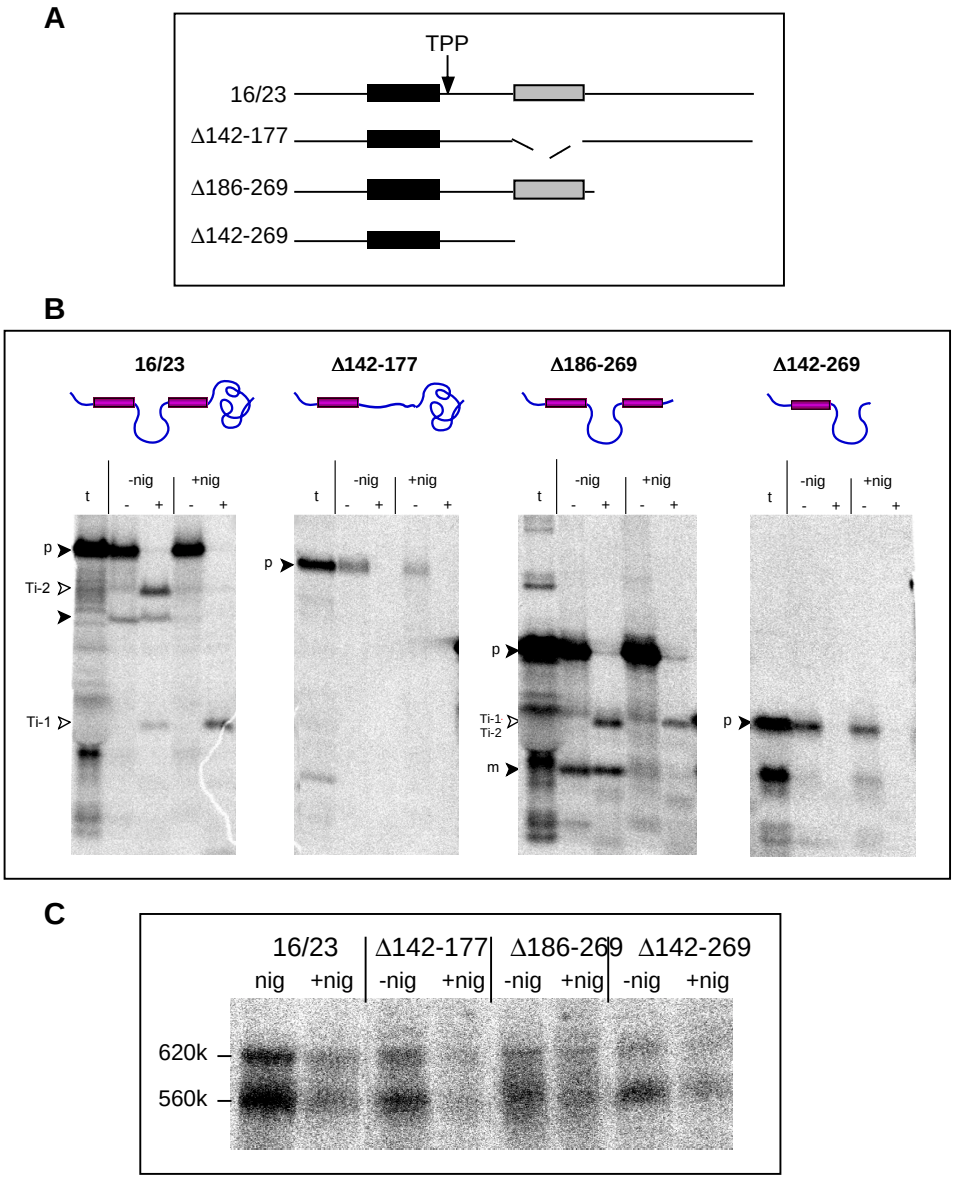


Fig. 27. Thylakoid import of 16/23 deletion mutants. Three 16/23 mutant proteins were generated by deleting parts of the mature polypeptide. The schematic structures of the original 16/23 and the mutant derivatives are shown in **A**. The original16/23 and mutant proteins were imported into isolated pea thylakoids in the absence or presence of nigericin, as described in Fig. 26. Samples were analyzed by SDS-PAGE (**B**) or BN-PAGE (**C**). Fur further details see the legend to Fig. 17.

The derivative proteins were also analyzed with respect to their interaction with the TAT-machinery. BN-PAGE shows that the 16/23Δ186-269 protein is associated with theΔpH/TAT-translocase represented by the two complexes of 560/620 kDa that are observed also during translocation of 16/23. Surprisingly, however, also the 16/23Δ142-269 and 16/23Δ142-177

mutant proteins are associated with these two complexes, although they are not able to insert into the thylakoid membrane (Fig. 27B). Binding of these two mutant proteins to the putative translocase complexes is, however, only weak, because both polypeptides are removed when the thylakoid membranes are washed with 0.1 M Na_2CO_3 solutions. In contrast, both the original 16/23 and the 16/23Δ186-269 polypeptides remain associated with the thylakoids under these conditions (Fig. 28A). This was confirmed by BN-PAGE, showing that neither 16/23Δ142-269 nor 16/23Δ142-177 is associated with translocase complexes during solubilization, whereas the complexes formed with the original 16/23 and the 16/23Δ186-269 proteins are almost not affected (Fig.28B). These results suggest that the mutant polypeptides

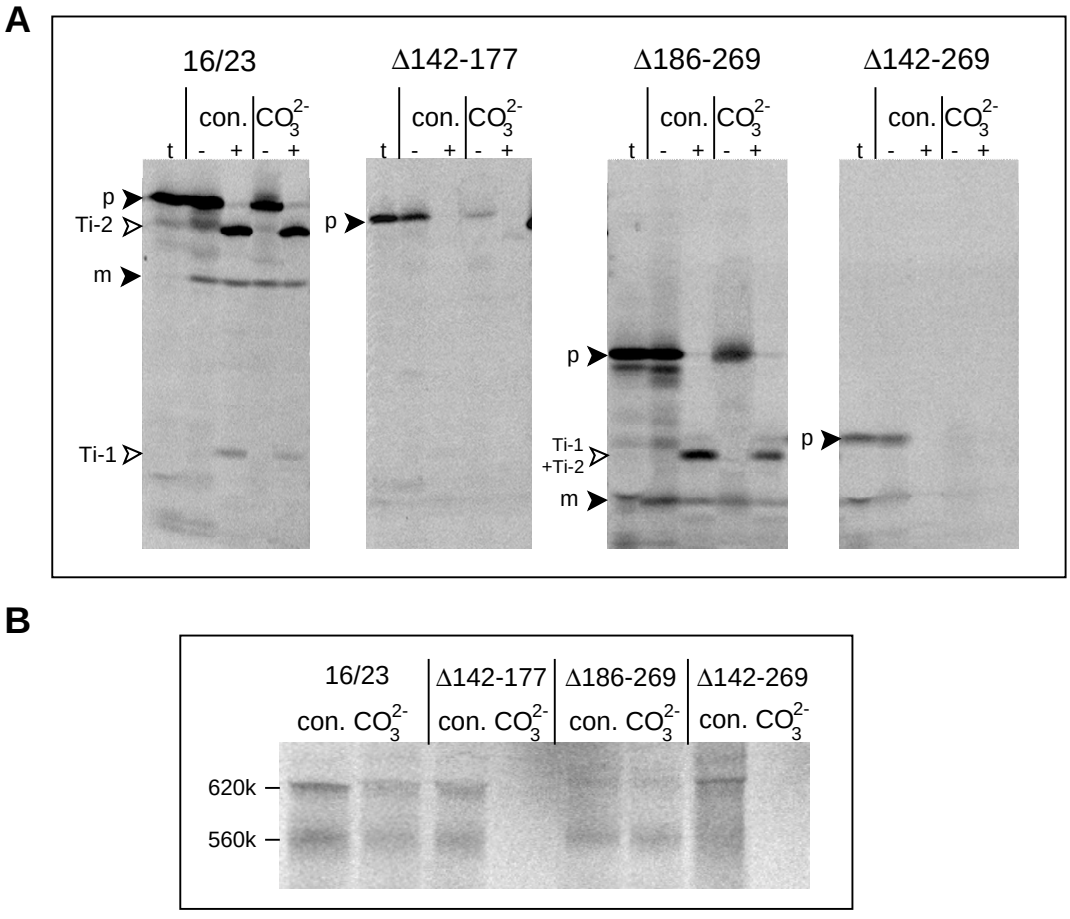


Fig. 28. Thylakoid membrane-associated deletion mutants are recognized by ΔpH/TAT pathway translocase. 16/23 wild type and deletion mutant proteins were imported into pea thylakoids. After import, thylakoids were reisolated and washed with either HM buffer (con) or 0.1 M Na_2CO_3 (CO_3^{2-}). The washed thylakoids were either analyzed by SDS-PAGE after treated with thermolysin or mock-treated (**A**), or analyzed by BN-PAGE (**B**). Protein bands were visualized by autoradiography.

lacking the amphipathic helix are still able to interact weakly with the TAT-translocase under solubilization conditions, most likely via the signal peptide. But the amphipathic helix within the mature protein is essential to stabilize this interaction presumably by inserting into the lipid bilayers and thus fixing the polypeptide in close contact to the ΔpH/TAT-dependent translocase.

3.1.3. Influence of the hydrophilic loop on the thylakoid insertion of the 16/23 protein

The data so far indicate that the membrane-integrated Ti-1 forms a loop structure in the thylakoid membrane, being anchored into the membrane by two membrane spans. The sequence between these two transmembrane spans forms a loop that is translocated across the thylakoid membrane during formation of Ti-1. In the case of 16/23, the central loop contains approximately 79 residues and has a size of about 8.5 kDa. Its sequence is overall hydrophilic and highly charged (in total: 22 charged-residues). A notable feature of the central loop is that both of its termini are negatively charged, thus resembling the translocated loops found in spontaneously inserted thylakoid proteins (Kuhn et al., 1990; Cao et al., 1995). However, the translocated loops of spontaneously inserting thylakoid proteins are smaller and usually contain less than 20 residues (Thompson et al., 1998).

In order to study the relevance of the“16/23-loop” in more detail, three mutants with different loop-lengths were constructed, which are schematically shown in Fig. 29A. In two of these mutants, notably Δ142-151 and Δ119-151, parts of the hydrophilic loop are deleted, while in the third one the loop was extended by 32 residues by repeating the sequence from ^{119}P to ^{151}D (R119-151). Thylakoid import of these mutant derivatives as well as the original 16/23 protein showed that for all the mutants were successfully translocated into the thylakoids, because terminal processing products of the expected size could be detected (Fig. 29). However, in the case of Δ142-151 and Δ119-151, these final processing products were mostly sensitive to thermolysin treatment, indicating that they might be not properly located into the

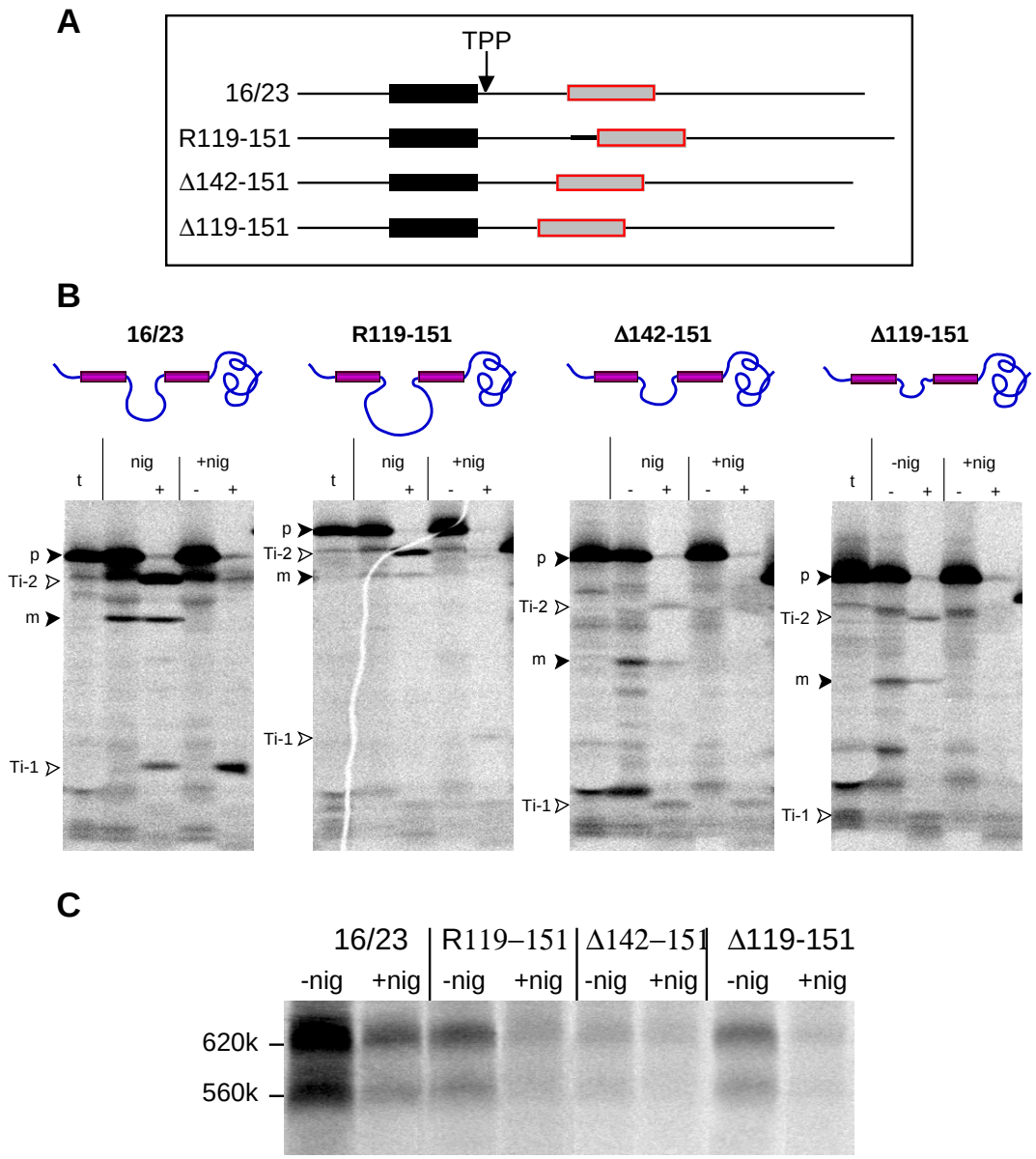


Fig. 29. Thylakoid import of 16/23 mutants. Three 16/23 mutant proteins were generated by either duplication or deletion of one part of the central loop sequence. The schematic structures of the original 16/23 and mutant proteins are shown in **A**. These proteins were imported into isolated pea thylakoids in the absence or presence of nigericin, as described in Fig. 27. Samples were analyzed by SDS-PAGE (**B**) or BN-PAGE (**C**).

thylakoid lumen. One speculative reason for this is that the change of the central loop might interfere in the topology of Ti-1 within the thylakoid membrane, causing earlier TPP processing already at the Ti-1 stage. Of course this needs to be further proven. Like the original 16/23 protein, the three mutant proteins are also integrated into the thylakoid

membrane to form Ti-1 and Ti-2, as indicated by the degradation products obtained after thermolysin treatment. As expected, nigericin inhibits the full translocation of these mutant proteins, and leads to the accumulation of the responsible Ti-1 products in the thylakoid membrane. This suggests that the mutant proteins were transported by the same mechanism as the original 16/23 protein. BN-PAGE analysis indicated that the translocation intermediates of these mutant proteins are able to interact with the ΔpH/TAT-dependent translocases (Fig. 29B). Binding of the Δ142-151 translocation intermediates to the translocase is much weaker than that of the other two mutant proteins and the original 16/23. This might be due to less amount of Ti-2 in the thylakoid membrane, but this needs to be confirmed.

The membrane insertion potential of all 16/23 derivatives described so far, except for the mutant Δ142-151, was examined also using the artificial lipid bilayers of TRANSIL beads (Fig. 30). Membrane insertion of these proteins was monitored by protease protection bands. Three of the five mutant proteins, notably R119-151, Δ119-151 and Δ186-269, showed a strong potential to insert into the phospholipid bilayers, as indicated by protease protected fragments (Fig. 30). This is in line also with their import properties. R119-151, which contains an extended central loop, shows two protease protected fragments, one being significantly larger than that of the original 16/23, as expected. The Δ119-151 derivative shows also two protease protection fragments, but in this case one is smaller than that of the original 16/23, which again in agreement with the shorter central loop. The ΔC84 derivative shows only one protease protected fragment, which is similar in size (12 kDa) to the smaller fragment of the original 16/23 after thermolysin treatment. Again, this was expected, as also the Ti-1 products of 16/23 and Δ186-269 were identical (Fig. 27). The other two mutant proteins, notably Δ142-177 and ΔC128, which both lack the amphipathic helix, do not show any specific protease protected fragment, indicating that they have lost their potential to insert into the artificial phospholipid bilayers. This might explain also, why these two proteins do not show any thylakoid import properties (Fig. 27).

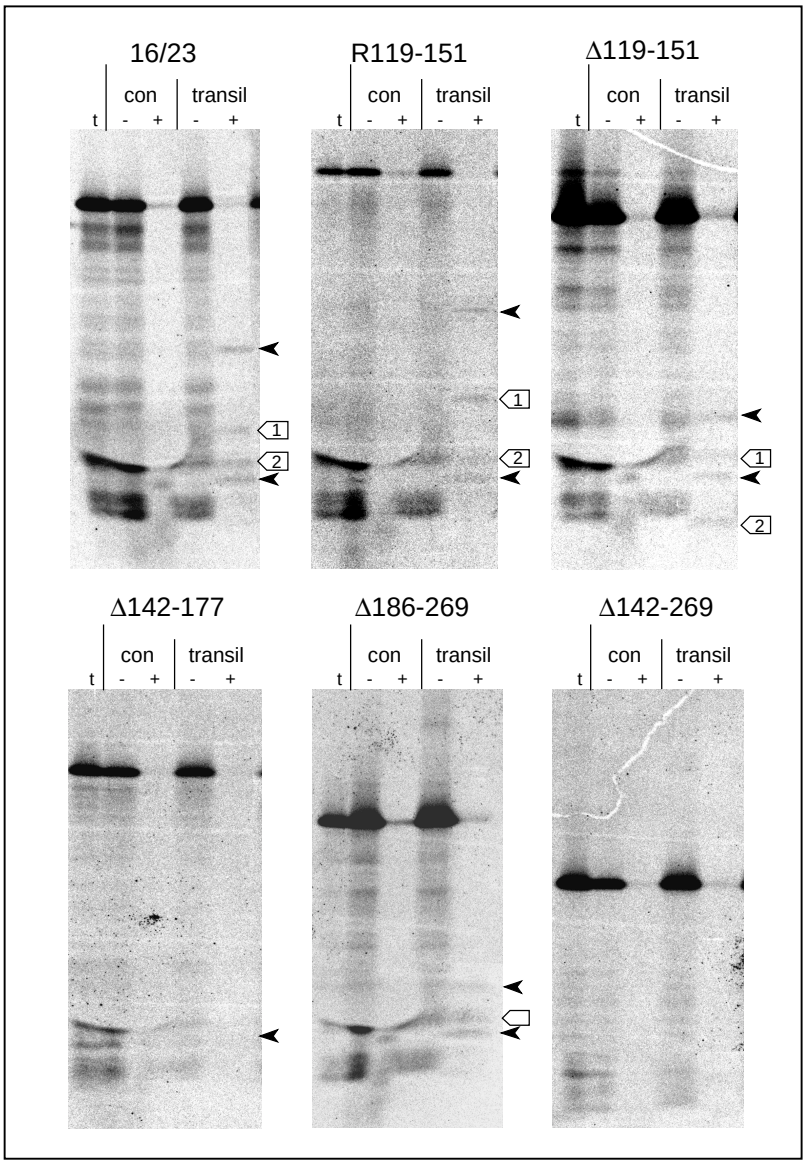


Fig. 30. Membrane insertion of the 16/23 wild type and mutant proteins. Radiolabelled 16/23 wild type and mutant proteins were incubated with either HM buffer (con) or the TRANSIL beads (transil) at room temperature for 30 min. After treatment with thermolysin (100 µg/ml) or mock-treatment, proteins were analyzed by SDS-PAGE. Protein bands were visualized by autoradiography. For more details see the legend to Fig. 21.

Taken together, these results demonstrate that in addition to the signal peptide the mature protein contributes to the membrane insertion of the ΔpH/TAT-dependent precursor proteins. The amphipathic helix within the mature protein presumably provides a second membrane segment required for membrane insertion of the 16/23 protein into the membrane. But also the size of the central loop is critical for this process, since all deletion mutants and the extension

mutant showed lower translocation efficiency. This suggests that a ΔpH/TAT-pathway signal peptide may not be able to direct all passenger proteins to the ΔpH/TAT-pathway. Instead, features within the passenger proteins, especially the presence of an amphipathic α-helix, may determine if they are competent for transport via ΔpH/TAT pathway or not.

3.1.4. The central loop is embedded within the translocase

Since Ti-1 forms a loop structure in the thylakoid membrane, it was of interest to find out whether the central loop is already fully translocated and thus located within the thylakoid lumen. In this case, it should be accessible to protease digestion from the lumenal side of the membrane. To achieve this, inverted vesicles of the thylakoid membrane obtained after import of the 16/23 protein were subjected to protease treatment. Import of 16/23 was performed for only 5 minutes in the presence and absence of nigericin to allow the formation of Ti-1. The samples were divided and inverted thylakoid vesicles were obtained by sonification of one fraction. The loss of a lumenal protein, the 33k OEC-protein, which was monitored by Western blot, indicated that indeed most of the thylakoids formed inverted vesicles under these conditions (Fig. 31). In the absence of nigericin, most of the 16/23 protein had been converted into Ti-2 in the thylakoid membrane (Fig 31, -nigericin, control). The C-terminal hydrophilic part of Ti-2 was degraded in the inverted vesicles by thermolysin, resulting in a 9 kDa degradation product that corresponds to the transit peptide located in the thylakoid membrane and the stroma side (Fig. 31, -nigericin, inverted). On the other hand, Ti-1 was not accessible to thermolysin treatment of the inverted vesicles, because it remained quantitatively stable as the precursor polypeptide in the inverted vesicles (Fig. 31, + nigericin, inverted). This indicates that the central loop of Ti-1 is not exposed in the lumenal space and thus not accessible to protease. Since the central loop is rather hydrophilic it is probably not integrated into the lipid bilayer. Instead, it appears more likely that it remains embedded within the translocases. Thus, it can be concluded from this experiment that the ΔpH/TAT-

dependent translocation machinery forms an aqueous protein-conducting channel in the thylakoid membrane, which houses the hydrophilic loop until the transport process is finished.

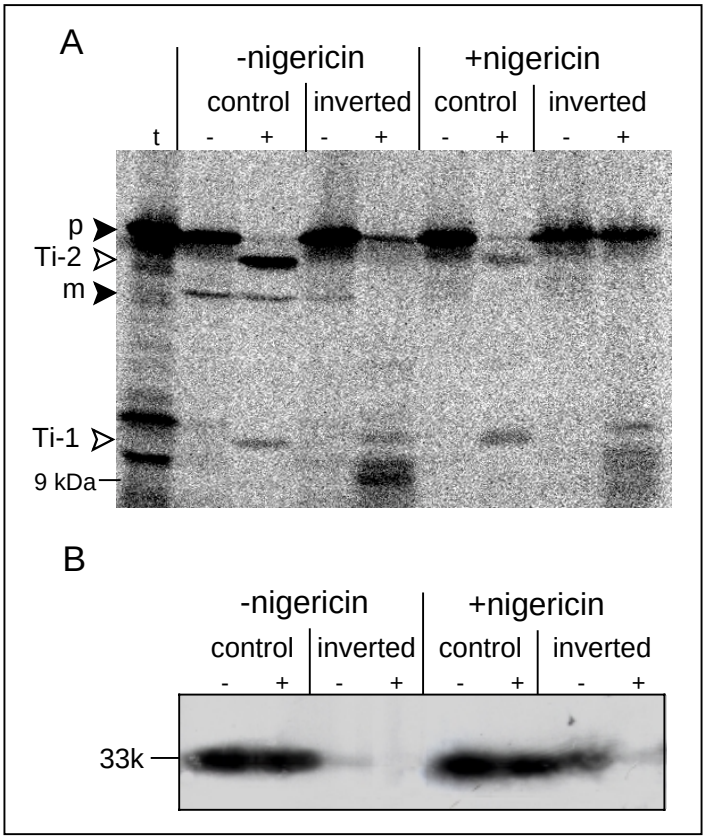


Fig. 31. The central loop of 16/23 is not accessible from lumenal side. Radiolabelled 16/23 protein was incubated with thylakoids either in absence or presence of nigericin at 25 °C for 10 min. One fraction of the thylakoids was sonified at 100 watts for 10 s with a sonifier (Branson Sonic Power Company), leading to the formation of inverted thylakoid vesicles. Both of the sonified and the untreated thylakoid samples were divided and either treated with 100 µg/ml thermolysin (+) or mock-treated (-). Proteins were separated by SDS-PAGE, and subsequently analyzed by autoradiography (**A**) or Western hybridization with antiserum against the 33k protein of the oxygen evolving complex (**B**).

This probably leads to the protection of the cleavage site of Ti-1 from the processing activity of TPP, which otherwise could remove the signal peptide already before the transport of the passenger protein has taken place.

3.2. Unassisted insertion is a general step of the thylakoid translocation also of the authentic ΔpH/TAT-pathway proteins

Unassisted membrane insertion of ΔpH/TAT-dependent proteins into the thylakoid membrane described so far has been observed only with the chimeric 16/23 protein. If unassisted membrane insertion is a general step in ΔpH/TAT-dependent protein transport, one could predict that also authentic TAT-substrates should have an amphipathic region in the mature bodies that could serve as transmembrane segment. Furthermore, these proteins should be able to form translocation intermediates with a loop structure in the thylakoid membrane, if translocation of the C-terminal domain is abolished. Finally, the size of membrane-protected fragments representing the loop structures should be predictable considering the length of the two hydrophobic regions and that of the central loop.

3.2.1. Thylakoid membrane insertion of the authentic pre-OE23 protein

The first authentic TAT-substrate tested was the pre-OE23 protein. In this case, the putative transmembrane segment present in the mature protein was already shown (see Fig. 20). The distance between the hydrophobic region in the signal peptide and the amphipathic region in the mature protein is almost identical to that of the 16/23 (Fig. 25A). Therefore, the predicated thermolysin degradation product of the presumed translocation intermediate-1 should be 14 kDa, too. To test this, pre-OE23 was incubated with thylakoids for 5 minutes, either in the absence or in the presence of nigericin (Fig. 32). It turned out that in the absence of nigericin, most of the pre-OE23 protein is processed into the mature protein, and only a minor fraction of the protein remaining as precursor was associated with the thylakoids. Thermolysin treatment degrades this precursor protein into a sole degradation product of about 14 kDa. In the presence of nigericin, formation of the mature 23k protein is inhibited. Instead, the 14 kDa degradation product accumulates in the protease treated samples, suggesting that it is indeed an early translocation intermediate similar to Ti-1 of the 16/23 chimera. Remarkably, under

no condition a larger degradation product corresponding to Ti-2 is detectable. This strongly suggests that in this case, cleavage by TPP occurs immediately after translocation of the C-terminal hydrophilic domain, in contrast to the 16/23 protein that shows only retarded process

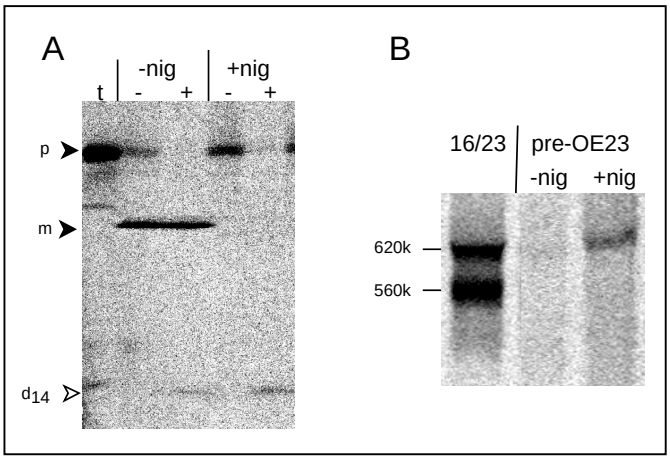


Fig. 32. Thylakoid import of the pre-OE23 protein. Pre-OE23 was *in vitro* translated in the presence of 35S-Met, and was subsequently subjected to thylakoid import assay either in the absence (-nig) or presence (+nig) of nigericin. The imported protein was analyzed by either SDS-PAGE (**A**) or BN-PAGE (**B**), followed autoradiography.

of the precursor protein (Fig. 17). Membrane-associated pre-OE23 protein co-migrates upon BN-PAGE with a single complex of approximately 620 kDa (Fig. 32B). This complex corresponds in size to the larger of the two complexes found after binding of the 16/23 protein to the ΔpH/TAT-dependent translocase. It accumulates in parallel with the accumulation of the 14 kDa degradation product, indicating that the translocation intermediate is present in this complex. This suggests that the ΔpH/TAT-translocase should be also involved in the transport of the OE23 protein.

3.2.2. Thylakoid membrane insertion of the authentic pre-OE16 protein

Sequence analysis of the authentic pre-OE16 protein indicated a relatively hydrophobic sequence within the mature polypeptide (TEAAQRAKVSASEILNVKQFI), which has a high tendency to form an amphipathic α-helix (Fig. 33). This candidate for a transmembrane

region would lead, after loop-insertion, to a predicted degradation product after thermolysin treatment of approximately 10 kDa, considering the distance between the hydrophobic regions

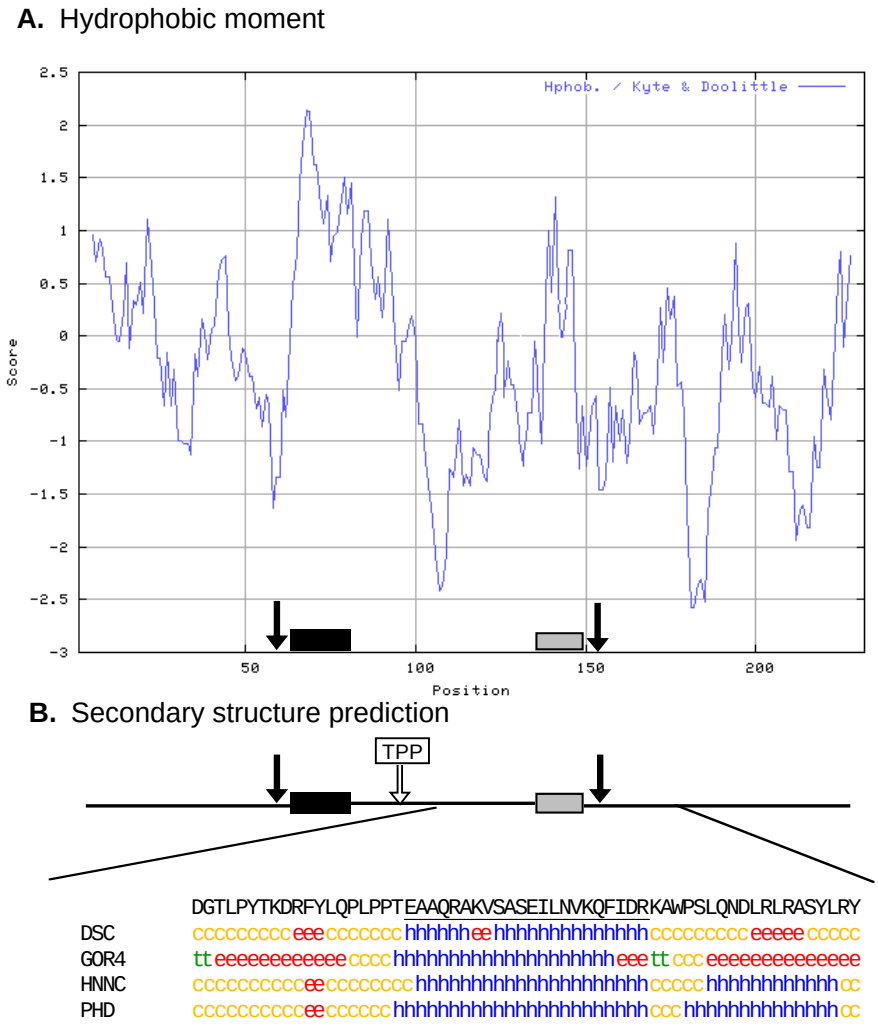


Fig. 33. Identification of a putative amphipathic region within the mature OE16 protein. A. Hydropathicity plot of pre-OE16. **B**. Secondary structure of the amphipathic region and its flanking sequences. For more details see the legend to Fig. 25.

in the signal peptide and the amphipathic region within the mature. This prediction was examined with thylakoid import experiments in the absence and presence of nigericin, using ^{3}H-labelled pre-OE16. It turned out that in the absence of nigericin, pre-OE16 is quantitatively processed to the mature protein within 5 minutes, so that no thermolysin degradation product indicative of a translocation intermediate could be detected. In contrast, in the presence of nigericin, the protein is predominantly associated with the thylakoid

membrane as the precursor polypeptide, which is processed into a 10 kDa product by thermolysin treatment (Fig. 34A), in line with the predicted size for the inserted sequence. This confirms that pre-OE16 is indeed inserted into the thylakoid membrane in a loop structure early in the transport process. BN-PAGE analysis of the inserted pre-OE16 revealed that the protein is present in two complexes that have the essentially the same mobilities as those observed with the 16/23 protein, indicating that the membrane-inserted pre-OE16 is associated with the ΔpH/TAT pathway machinery (Fig. 34B).

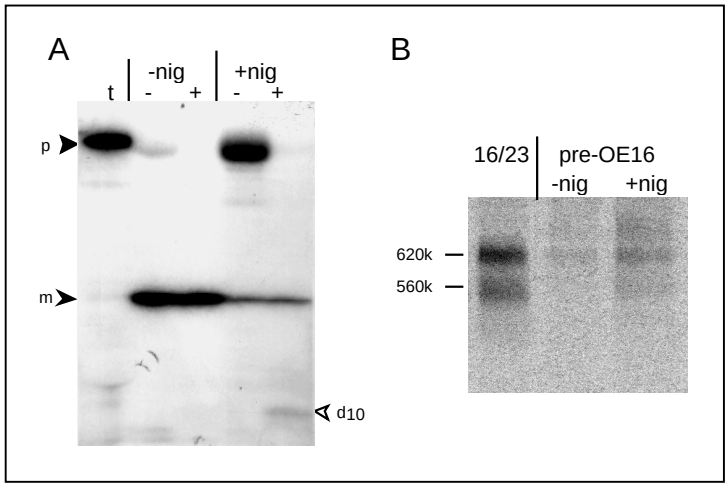


Fig. 34. Thylakoid import of pre-OE16. A. Pre-OE16 was *in vitro* translated in the presence of ^{3}H-Leu and subsequently subjected to thylakoid import assays in the absence (-nig) or presence (+nig) of nigericin. The imported protein was analyzed by SDS-PAGE and exposed to x-ray film for 2 weeks. **B**. Pre-OE16 was *in vitro* translated in the presence of ^{35}S-Met and subsequently subjected to thylakoid import assay in the absence (-nig) or presence (+nig) of nigericin. The imported protein was analyzed by BN-PAGE, followed by autoradiography.

3.2.3. Thylakoid membrane insertion of the Rieske Fe-S protein

The third authentic protein analyzed in this section was the chloroplast Rieske Fe-S protein. The Rieske protein is an integral thylakoid membrane protein that is transported in a ΔpH/TAT-dependent manner (Molik et al., 2001). It carries an uncleavable signal peptide at the N-terminus of the mature protein, which possesses a hydrophobic domain serving as membrane anchor after transport into the thylakoid membrane. The Rieske protein is probably

translocated in a fully folded conformation, because its Fe-S cluster is presumably assembled already in the stroma. Analysis of the amino acid sequence of the Rieske protein reveals that in addition to its membrane anchor, it has also a putative amphipathic region in its lumenal domain, which is located close to the N-terminal end of the cofactor-binding region (Fig. 35). Considering the distance between this amphipathic region and the hydrophobic region at the N-terminal part of the mature Rieske protein, the predicted thermolysin degradation product should be about 11 kDa.

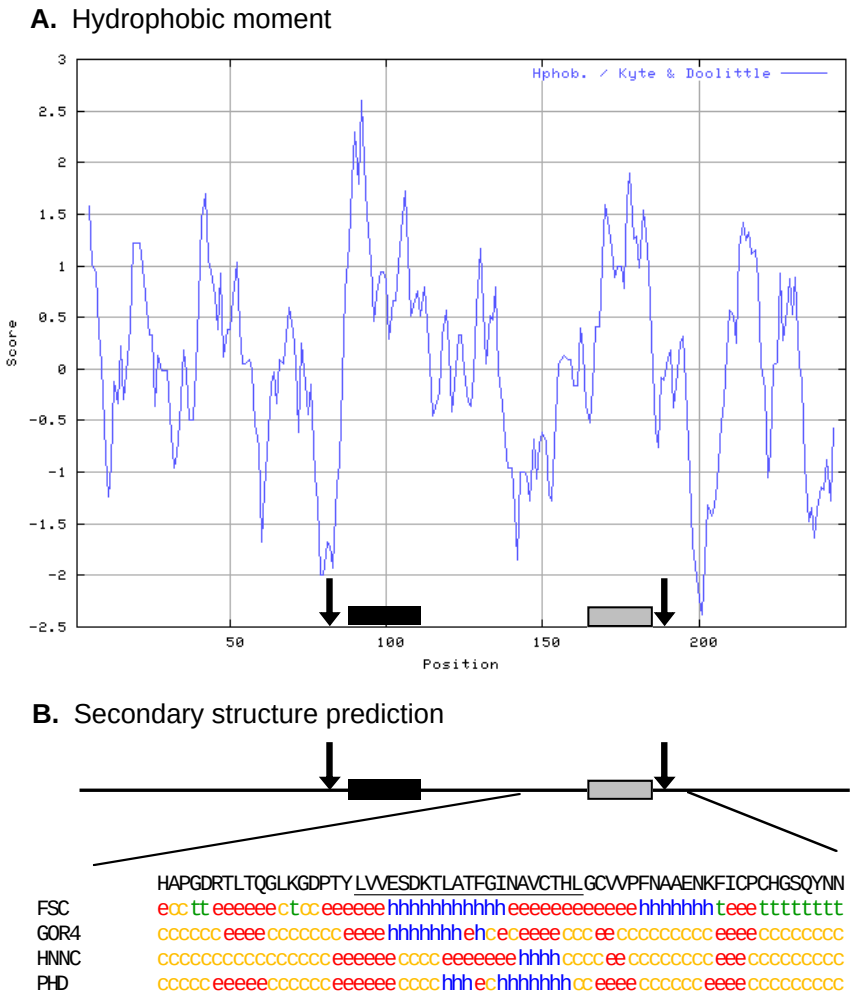


Fig. 35. Identification of a putative amphipathic region within the mature Rieske protein. A. Hydropathicity of pre-Rieske. **B**. Secondary structure of the amphipathic region and its flanking sequences. For more details see legend to Fig. 25.

This predication was examined in thylakoid import experiments similar to those described for the OE23 and OE16 proteins, except that the thylakoid vesicles were resuspended in stroma solution. Almost half of the Rieske protein is processed to the mature form by stroma processing peptidase, but no mature protein is found translocated into the thylakoid membrane, neither in the absence nor presence of nigericin. This might be caused by the failure to assemble the iron-sulfate cluster into the Rieske protein when using diluted stroma solutions (Molik et al., 2001). Despite of that, the membrane associated Rieske protein is processed by thermolysin to a 11 kDa degradation product, which corresponds well with the prediction, suggesting that the mature Rieske protein is likewise inserted in a loop structure into the thylakoid membrane (Fig. 36B).

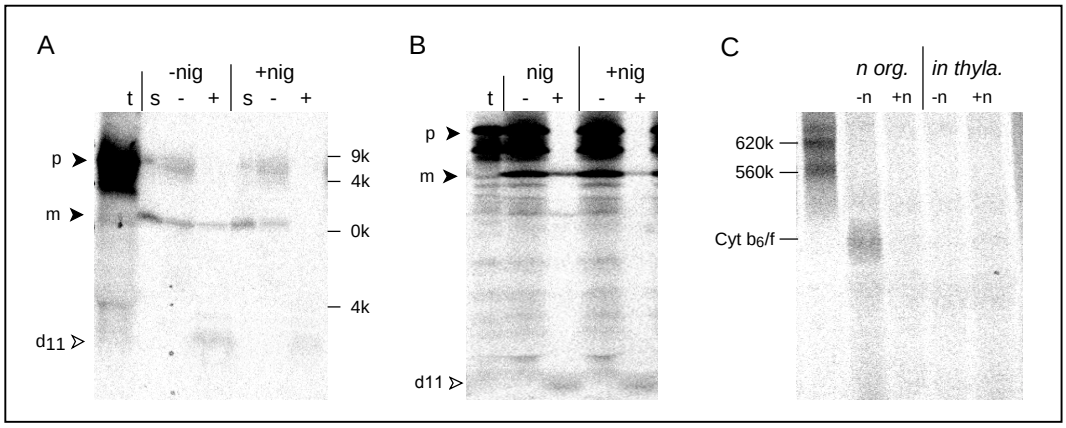


Fig. 36. Import of the pre-Rieske protein into chloroplasts and thylakoids. A. *In vitro* translated pre-Rieske protein was mixed with isolated intact pea chloroplasts in the absence or presence of nigericin, and incubated for 30 min at 25 °C. After import, the chloroplasts were treated with thermolysin (100 µg/ml) for 20 min on ice, reisolated by centrifugation through a Percoll cushion and fractionated into stroma (s) and thylakoids as described in Materials and Methods. Half of the thylakoid fraction was treated with thermolysin (150 µg/ml) for 30 min on ice (-), and the other half mock-treated (+) (**B**). Import of pre-Rieske into pea thylakoids was carried out in the absence of or presence of nigericin. One half of each sample from both *in organello* and *in thylakoido* import assays was processed by 10-17.5% SDS-PAGE, while the other half (**C**) was processed by BN-PAGE. All proteins were visualized by autoradiography.

In order to exclude that this result is an artifact caused by the thylakoid import system that is not capable of translocating the Rieske protein to its final destination (Molik et al., 2001), the

experiment was repeated with intact chloroplasts in an *in organello* approach (Fig. 36A). In this experiment, the Rieske protein was incubated with intact chloroplasts in the absence or presence of nigericin, followed by protease treatment to remove the non-imported protein. The recovered chloroplasts were fractionated into stroma and thylakoids. Most of the Rieske protein was found processed into the mature form in the stroma, but in the absence of nigericin ,only some of it was further translocated into the thylakoids. Thermolysin treatment of the recovered thylakoids revealed that only a part of the membrane-associated protein was fully translocated and therefore resistant to the thermolysin. The rest was only inserted and degraded by thermolysin to a 11 kDa product, i.e., to the same size observed with *in thylakoido* import experiment and thus to the size predicted for a loop intermediate of the Rieske protein. Nigericin totally blocks the translocation of the Rieske protein, but leads again to an 11 kDa degradation product, indicating that the loop insertion of the Rieske protein is independent of the membrane ΔpH. This experiment confirms that also cofactor-containing Rieske protein is able to insert into the thylakoid membrane in a loop conformation and that membrane insertion probably involves an amphipathic region close to the Fe/S-binding domain. This implies that the Rieske protein is partially unfolded at its N-terminus prior to translocation.

BN-PAGE shows that the fully translocated Rieske protein is assembled into the cytochrom b6/f complex of the thylakoid membrane. However, no binding to the TAT-translocase complex can be detected (Fig. 36C). The reason remains unknown so far but it could be speculated that the interaction between the Rieske protein and the ΔpH/TAT-translocase is not so strong, because the targeting signal of the Rieske protein carries a KR-sequence rather than the typical RR-motif.

In summary, it can be concluded that membrane insertion of precursor proteins prior to translocation is an inherent feature of ΔpH/TAT-dependent translocation. Membrane insertion of the ΔpH/TAT-dependent proteins takes place in a spontaneous, unassisted manner. It requires a hydrophobic region in the signal peptide and an amphipathic region in the mature

protein. The membrane-inserted proteins form a loop structure in the thylakoid membrane, and the size of the membrane-embedded fragments can be predicated. After membrane insertion, the proteins bind to the ΔpH/TAT-translocation machinery, which apparently has a size of approximately 620 kDa and/or 560 kDa. However, depending on the substrates, the translocation machinery might have different composition, suggesting that the ΔpH/TAT translocation machinery might have the ability to adapt its composition to various passenger proteins.

4. The role of the TAT proteins in the ΔpH/TAT-dependent translocation

To date, three components of the ΔpH/TAT-translocase in plants are known. Originally, they were denominated Tha4, Hcf106 and cpTatC (Mori et al., 1999; Settles et al., 1997; Motohashi et al., 2001; Mori et al., 2001). But according to their bacterial counterparts, they are meanwhile mostly called TatA, TatB and TatC, respectively. All three proteins are integral components of the thylakoid membrane, which carry either one (TatA, B) or 6 (TatC) (Gouffi et al., 2002) transmembrane spans. They are all essential to build the active translocase of the ΔpH/TAT pathway, since knock-out of either of the three corresponding genes leads to serious defects on ΔpH/TAT-dependent translocation (Voelker and Barkan, 1995; Walker et al., 1999; Budziszewski et al., 2001). However, the precise function of these proteins is yet unknown. Using the chimeric 16/23 protein that accumulates during transport in the ΔpH/TAT-translocation channel, I have tried to characterize the composition and the function of the ΔpH/TAT-dependent translocation machinery.

4.1. Cloning of the TAT genes of Arabidopsis and pea

Soon after identification of the maize *hcf106*, two homologous *Arabidopsis thaliana* cDNA-clones were identified in the MSU EST-databank (clone 200I10T7 and 182I20T7; Berghöfer, 1998). Alignment of the deduced polypeptides from these two cDNA-clones and the known

TAT proteins indicated that the clone 200I10T7 encodes the Arabidopsis homologue to Hcf106 (atTatB), whereas the 182I20T7 encodes the Arabidopsis Tha4 homologue (atTatA).

The Arabidopsis *tatC* gene was found by screening the EST-databank for homology to the bacterial *tatC* gene. Based on the genomic sequence of Arabidopsis *tatC* gene, PCR-primers were generated, which allowed amplifying the complete coding region of *tatC* gene from an Arabidopsis cDNA library. Sequencing of the PCR fragment verified that it contains the coding region# for a protein homologous to bacterial TatC. By using the online prediction program TargetP (http://www.cbs.dtu.dk/services/TargetP/), this protein was predicted to be a thylakoid membrane protein, and was therefore termed atTatC.

Homologous TAT genes were also identified in pea (Summer et al., 2000; Mori et al., 2001). Three TAT genes were cloned by RT-PCR of mRNA isolated from pea seedlings, using primers specific for each TAT gene (the sequences of the primers are given in Material and Methods). Sequencing of the amplified fragments confirmed that the DNA fragments cloned carry the open-reading frames that encode the pea homologues to Hcf106, Tha4 or TatC gene, respectively, and they were termed therefore psTatB, psTatA and psTatC, respectively.

TatA of Arabidopsis and pea share high identity to each other (54%), and both are homologous to TatA from *E.coli* (72%, Fig. 37). Both of them are predicted to be thylakoid membrane protein, with an N-terminal transit peptide of 59 residues for Arabidopsis and 54 residues for pea. The transit peptides of the plant TatA proteins have apparently only stroma-targeting properties, indicating that the thylakoid targeting information is probably found in the mature polypeptides. The Arabidopsis TatA possesses 88 aminoacid residues and has a calculated size of 9.3 kDa. The pea TatA contains 83 residues with a calculated molecular weight of 8.9 kDa. All TatA proteins possess a putative transmembrane span located in the N-terminal segment of the mature protein, and a relatively large C-terminal hydrophilic domain that is characterized by an amphipathic α-helix.

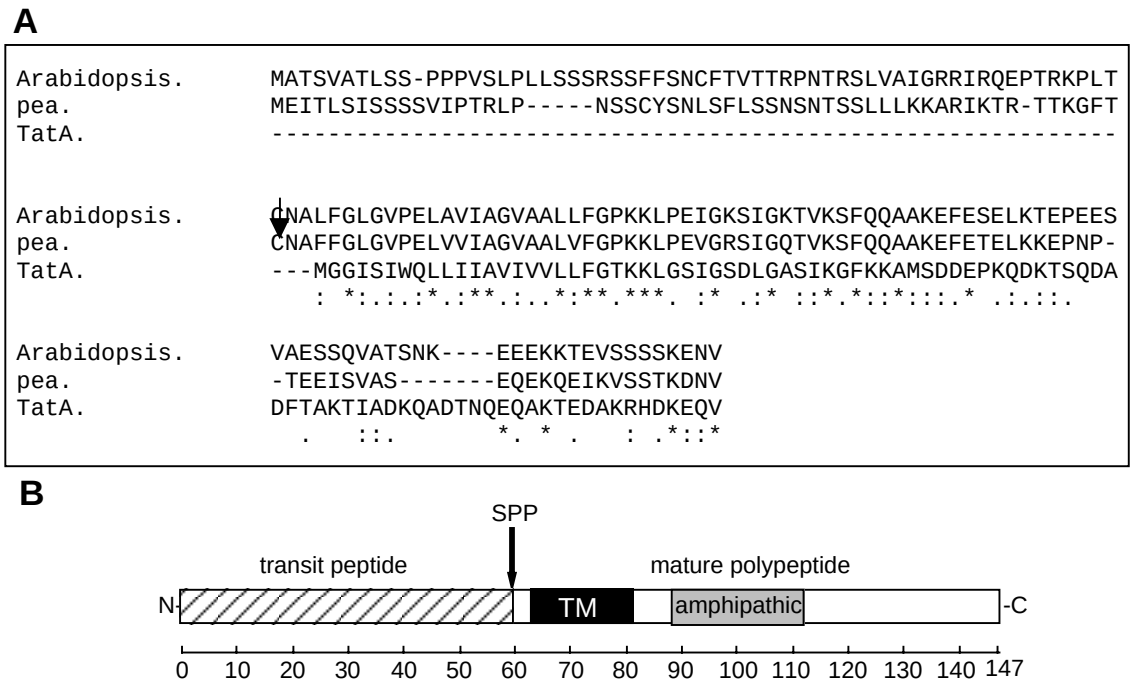


Fig. 37. Analysis of TatA protein sequences. A. Alignment of the TatA protein sequences from Arabidopsis, pea, and *E.coli*. Identical residues are indicated by asterisks, and homologous residues by one or two points. **B**. Schematic structure of the plant TatA proteins. The transit peptide is marked by twilled rectangle, and the transmembrane domain (TM) and putative amphipathic region are indicated in black or gray.

The TatB proteins of Arabidopsis and pea are also highly conserved to each other (52% identity), and both show homology to TatB of *E.coli* (54%, Fig. 38). All TatB proteins identified from different organisms are predicated to be membrane proteins. TatB has a similar structure as TatA, i.e. an N-terminal membrane span followed by a hydrophilic C-domain carrying an amphipathic α-helix. TatB in plants are synthesized with a transit peptide of 83 residues for Arabidopsis and 86 residues for pea, carrying information for stroma targeting of these proteins. The Arabidopsis TatB possesses 177 aminoacid residues with a calculated size of 19.0 kDa. The pea TatB contains 175 residues of 18.9 kDa calculated molecular weight.

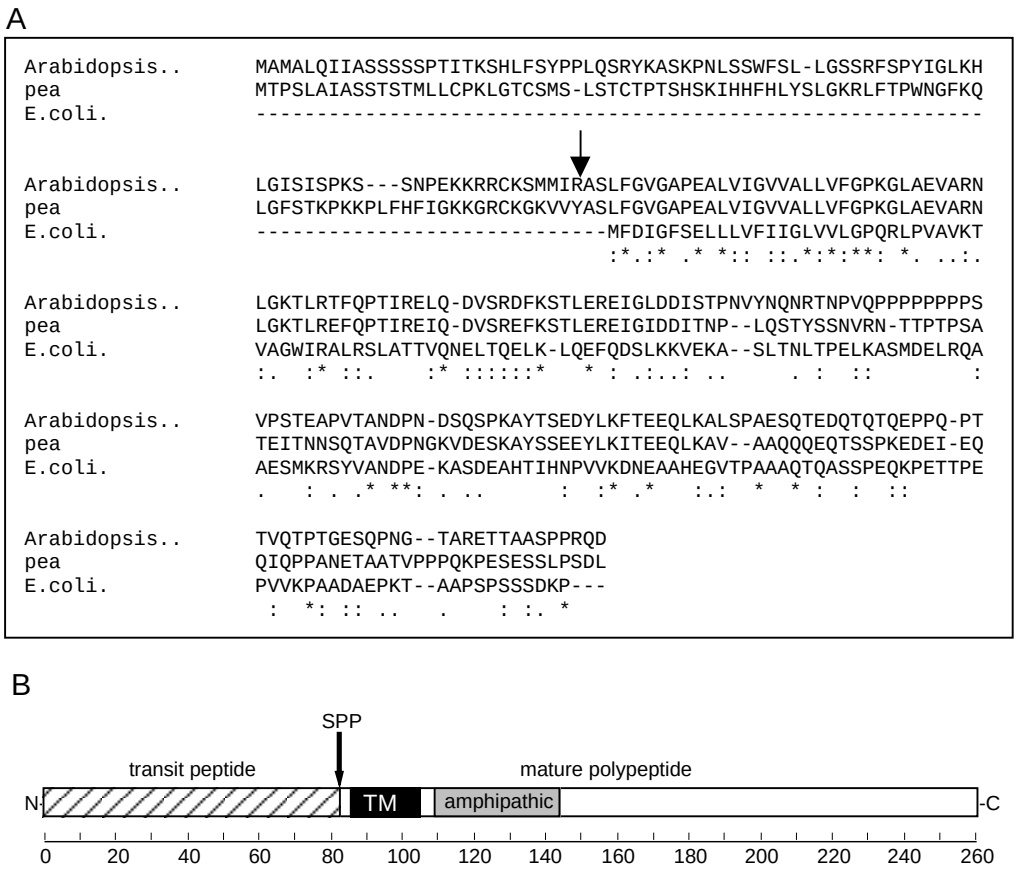


Fig. 38. Analysis of TatB protein sequences. A. Alignment of the TatB protein sequences from Arabidopsis, pea, and *E.coli.* Identical residues are indicated by asterisks, and homologous residues by one or two points. **B**. Schematic structure of the plant TatB proteins. For more details see the legend to Fig. 37.

The TatC proteins of Arabidopsis and pea are also conserved (76% identity) and are both phylogenetically related to the TatC protein of *E.coli* (66%, Fig. 39). They possess an N-terminal transit peptide of 28 residues for Arabidopsis and 27 residues for pea, carrying information only for stroma-targeting. Both TatC proteins are predicated to be integral membrane proteins that span the membrane six times. They have a relatively large hydrophilic N-terminal domain that is exposed on the stromal face of the thylakoid membrane. The Arabidopsis TatC contains 312 aminoacid residues with a calculated size of 34.3 kDa, and the pea TatC possesses 326 residues with 35.9 kDa calculated molecular weight.

A

```
Arabidopsis.      --MSSTSTSSALIHHFRLTTRNLGSPTKQRCPYAVTFCNSWREAGLRYSVTQRRSKGFGP
pea.              MGLGTTTVPTNILPQFGLHRTHLN-------PIRVN-----NSTGFSYPLSLRKNKSFDR
E.coli.           ------------------------------------------------------------


Arabidopsis.      VSALNDDDSP--------TETTPGVGSAVEDRPPDS--------------SED--RSSSVY
pea.              LVCFAVDDEIREKQQQQLSTSSTRLGSAVEERPENKDMIDGISEEALENFKEDGERS-AIY
E.coli.           ------------------------------------------------------------


Arabidopsis.      EFLYPRKEELPDDKEMTIFDHLEELRERIFVSVLAVGAAILGCFAFSKDLIVFLEAP-VK
pea.              DFLYPSKELLPDDKEMSIFDHLEELRERIFISVLGVGGSILGCFAFSKDLVKILEAP-VK
E.coli.           ---------MSVEDTQPLITHLIELRKRLLNCIIAVIVIFLCLVYFANDIYHLVSAPLIK
                            :. :.  .:: ** ***:*:: .::.*   :*  . *::*:  ::.** :*

Arabidopsis.      --TQGVRFLQLAPGEFFFTTLKVSGYCGLLLGSPVILYEIIAFVLPGLTRAERRFLGPIV
pea.              --SEGVRFLQLAPGEFFFTTLKVSGYCGLLLGSPIILYEIIAFIIPGLTKEERKFLGPIV
E.coli.           QLPQGSTMIATDVASPFFTPIKLTFMVSLILSAPVILYQVWAFIAPALYKHERRLVVPLL
                    .:*  ::    .. ***.:*::   .*:*.:*:***:: **: *.* : **::: *::

Arabidopsis.      FGSSLLFYAGLAFSYWVLTPAALNFFVNYAEGVVESLWSIDQYFEFVLVLMFSTGLSFQV
pea.              LGSSVLFYAGITFSYLVLVPAALNFFVNYAEGAVESLWSIDQYPEFVLVLMFSTGLSFQV
E.coli.           VSSSLLFYIGMAFAYFVVFPLAFGFLANTAPEGVQVSTDIASYLSFVMALFMAFGVSFEV
                  ..**:*** *::*:* *: * *:.*:.* *   *:   .* .* .**:.*::: *:**:*

Arabidopsis.      PVIQLLLGQVGVVSGDQMLSIWRYVVVGAVVAAAVVTPSTDPVTQMLLATPLLGLYLGGA
pea.              PIIQLLLGQLGLVSGDKMLSVWRYVVVGAVVAAAVVTPSTDPLTQVLLAAPLLGLYLGGA
E.coli.           PVAIVLLCWMGITSPEDLRKKRPYVLVGAFVVGMLLTP-PDVFSQTLLAIPMYCLFEIGV
                  *:  :**  :*:.* :.: .   **:***.*.. ::** .* .:* *** *:  *:  *.

Arabidopsis.      WMVKL---TGR-----------------
pea.              WMVKL---AGR-----------------
E.coli.           FFSRFYVGKGRNREEENDAEAESEKTEE
                  :: ::    **
```

B

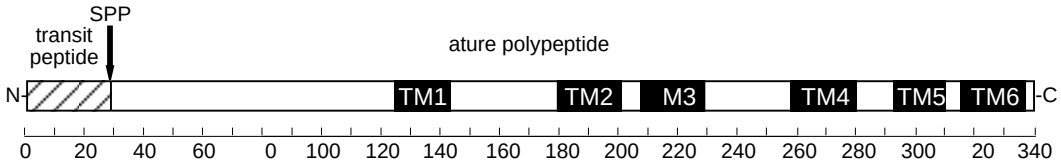


Fig. 39. Analysis of TatC protein sequences. A. Alignment of the TatC protein sequences from Arabidopsis, pea, and *E.coli*. Identical residues are indicated by asterisks, and homologous residues by one or two points. **B**. Schematic structure of the plant TatC proteins. For more details see the legend to Fig. 37.

4.2. *In vitro* import of pea TAT proteins

In order to find out whether the three TAT-proteins reach their destination also after *in vitro* import, *in organello* import experiments were performed. For comparison, pre-OE23 was analyzed in parallel. While most of the 23 kDa protein processed to the mature after 20 minutes and accumulated in the thylakoid lumen, the three TAT proteins were efficiently

imported into the organelles but accumulated to a large extent in the stroma fraction, both as the precursor and being processed to the size of the mature protein (Fig. 40). The major portion of TatA was found in thylakoid fraction, which could be degraded by thermolysion to

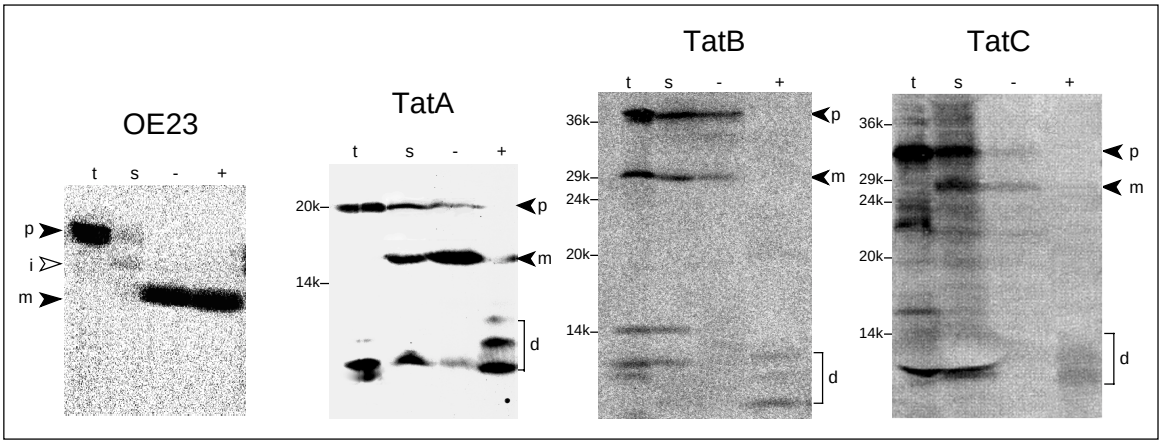


Fig. 40. *In organello* import of the pea TAT proteins. Radiolabelled TatB and TatC, as well as pre-OE23, were obtained by *in vitro* translation in the presence of ^{35}S-Met, whereas TatA was obtained by *in vitro* translation in the presence of ^{3}H-Leu. The resulting proteins were incubated with isolated intact pea chloroplasts for 20 min at 15 °C in the light. After import, the chloroplasts were treated with thermolysin (100 µg/ml) for 20 min on ice, reisolated by centrifugation through a Percoll cushion and fractionated into stroma (s) and thylakoids as described in Materials and Methods. Half of the thylakoid fraction was treated with thermolysin (150 µg/ml) for 30 min on ice (+), and the other half mock-treated (-). Protein samples were separated by 10-17.5% SDS-PAGE and visualized by autoradiography.

3 products smaller than 8 kDa. Imported TatB is poorly processed by SPP in the stroma, and a minor amount of TatB is associated with thylakoids in both precursor and mature form, which also appear 3 small protease protection fragments. Imported TatC accumulates almos quantitatively in the stroma, and only a little amount of TatC mature protein is integrated into the thylakoid membrane. 2 or 3 small protease protection fragments were found also for TatC. These experiments confirmed the predicted thylakoid location of the three TAT proteins, and the protease protection fragments observed from each TAT protein suggest their membrane integral topologies. However, *in vitro* import efficiency of them is lower than that of pre-OE23, which might be du their higher hydrophobicity.

4.3. Generation of the TAT protein antisera

In order to analyze the role of the TAT proteins in chloroplasts, it was required to generate specific antibodies against those proteins. Three antisera were raised in rabbits using precursor of atTatA, the C-terminal 154 residues representing the stromal hydrophilic domain of atTatB and the N-terminal 123 residues corresponding to the stromal hydrophilic domain of atTatC, respectively, as antigen. All three protein or protein domains were obtained by overexpression in *E.coli* and isolated by preparative SDS-PAGE (data not shown). The resulting antisera were tested for specificity in Western blots using thylakoid proteins isolated from Arabidopsis and pea (Fig. 41). It turned out that anti-TatA antiserum recognizes predominantly a 15 kDa protein from Arabidopsis thylakoids, and a 16 kDa protein from pea

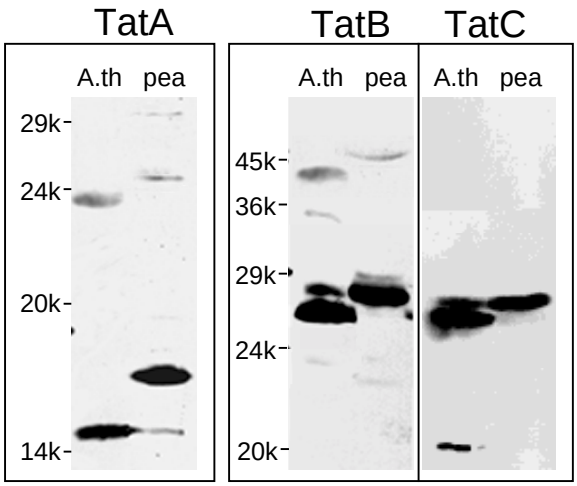


Fig. 41. Western blot with TAT protein antisera. Thylakoid proteins (equal to 7.5 µg of chlorophyll) from Arabidopsis (A.th) and pea (pea) were separated by 10 - 17.5% SDS-PAGE, and subsequently blotted onto a PVDF membrane. Antisera raised against TatA, TatB and TatC were incubated with this membrane, and the protein bands were visualized by ECL reaction after binding of horseradish peroxidase-coupled secondary goat anti-rabbit IgG.

thylakoids. The size of the endogenous thylakoid TatA is in line with the size observed after *in vitro* import psTatA, as well as that of the overexpressed atTatA. However, their sizes are apparently larger than the calculated molecular weight pea TatA (see above). Likewise, the anti-TatB antiserum recognizes predominantly a 28 kDa protein from Arabidopsis thylakoids,

and a 29 kDa protein from pea thylakoids that corresponds well with to the size of the imported psTatB, but are both apparently larger than the calculated size of TatB. It seems that TatA and TatB have strange behavior upon SDS-PAGE, which is also observed by other groups (e.g. Fincher et al., 2003). The reason for that remains unknown. Also the anti-TatC seems to be specific for TatC, because it recognizes predominantly a 27 kDa protein from Arabidopsis thylakoids, and a 28 kDa protein from pea thylakoids, but in this case, the size of the endogenous TatCs observed in immunoblots are both larger than the size calculated for mature TatC from both plants. This might be due to the high hydrophobicity of the mature TatC from both plants. These data indicate already that all three antisera have probably correct specificities. Furthermore, they confirm that the processing products obtained in the *in vitro* import assays represent indeed the mature polypeptides of each TAT protein.

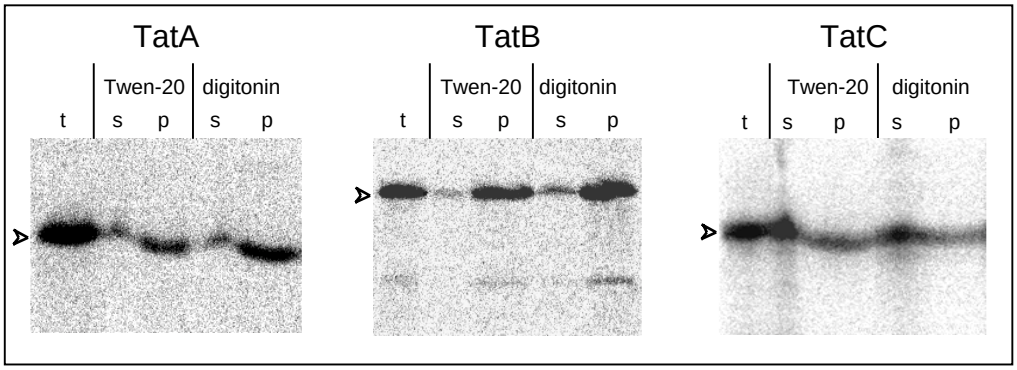


Fig. 42. Immunoprecipitation of the TAT proteins. *In vitro* translated psTatA, psTatB and psTatB were mixed with corresponding antisera IgGs in binding buffer containing 1% (v/v) Twen-20 or 1% (w/v) digitonin, respectively. After incubation for 1 hour at room temperature, protein A-Sepharose was added to a final concentration of 1% (w/v). The unbound proteins in the supernatant were separated from the protein A-Sepharose by centrifugation and applied to SDS-PAGE. The pellets containing the protein A-Sepharose and the antigen-antibody complexes were washed two times with binding buffers and subsequently boiled off in the presence of SDS-loading buffer. Samples were then analyzed by SDS-PAGE and autoradiography. The precursors are indicated by arrow heads.

The specificity of these antisera and their cross-activity to the corresponding pea TAT proteins was further demonstrated by immunoprecipitation experiments, in which the *in vitro* translation products of psTatA, psTatB and psTatC were immunoprecipitated by the

respective antisera. For psTatA and psTatB, immunoprecipitation was almost quantitative, whereas psTatC was immunoprecipitated by the antibodies raised against the Arabidopsis protein only to approximately 40% (Fig. 42). Detergent (Twen-20 or digitonin) present in the immuno-precipitation assays has almost no effect on the reaction efficiency. Pre-immuno sera were not able to precipitate any of the translation products (data not shown), confirming that the reaction between the antibodies and the corresponding TAT proteins is specific.

4.4. Topology of the TAT proteins in the thylakoid membrane

The topology of the TAT proteins in the thylakoid membrane was investigated by protease protection experiments. Protease treatment of thylakoid vesicles from pea followed by Western analysis allows for estimation if a polypeptide is exposed on the stromal surface of the thylakoid membrane. For this purpose, thylakoids were treated with three different proteases (trypsin, thermolysin and Proteinase K) at either 0 °C or 25 °C. After termination of the reaction by trypsin inhibitor, EDTA or PMSF, respectively, the thylakoids were recovered and subjected to Western analysis using specific antisera to the TAT proteins (Fig. 43).

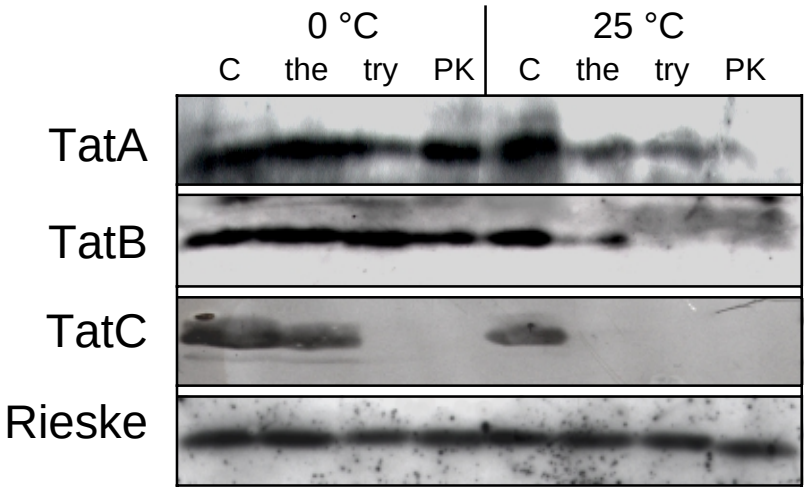


Fig. 43. Protease protection assay of the TAT proteins. Isolated pea thylakoid vesicles were either treated with trypsin (100 µg/ml, try), thermolysin (150 µg/ml, the), proteinase K (150µg/ml, PK), or mock treated (con) for 30 min at either 0 °C or 25 °C. Proteolysis was terminated by addition of soybean trypsin inhibitor (50 µg/ml), EDTA (10 mM), or PMSF (2 mM), respectively. Proteins were

separated by SDS-PAGE followed by Western analysis, using antisera against TatA, TatB, TatC, and the Rieske protein, respectively, as indicated on the left side.

As a control, the Rieske protein, an integral thylakoid membrane protein that has only a short stromal tail and a large lumenal hydrophilic domain was analyze parallelly. As expected for the results in the literature (Karnauchov, et al., 1997), the Rieske protein was not digested by either of the three proteases. However, it was slightly shortened by proteinase K at 25 °C, which is obviously due to the removal of its N-terminal stromal-exposed tail. Protease protection of the Rieske indicated that the protease treatment at both temperatures did not destroy the intact thylakoids, and that proteolysis occurs only at the stromal side of the thylakoid membrane. Under these conditions, TatA and TatB are partially protected from degradation by trypsin and thermolysin, but completely removed by proteinase K at 25 °C. These results demonstrate that the hydrophilic domains of these two proteins are exposed to the stroma, but that they are not easily accessible to protease, suggesting that these domains are folded in a manner preventing their degradation during protease treatment. One possibility is that the amphipathic regions are tightly associated with the thylakoid membrane, which would increase the resistance of these proteins to protease. In contrast, TatC is more sensitive to protease degradation. Already at 0 °C, the N-terminal hydrophilic part of TatC is degraded by both trypsin and proteinase K, and only thermolysin needs 25 °C to efficiently degrade it. This demonstrates that the N-terminal domain of TatC which was used for antibody production, is exposed to the stroma. Considering the position of the transmembrane domains of the three TAT proteins as predicted from computer programs, the following scheme of the topology of the three TAT proteins can be drawn (Fig. 44)

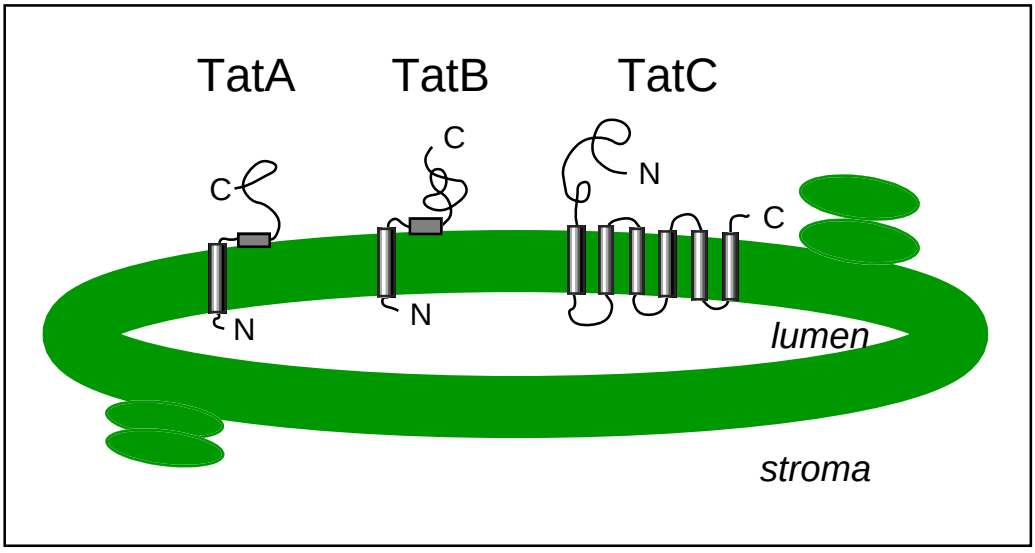


Fig. 44. Presumed topology of the TAT proteins at the thylakoid membrane deduced from computer prediction and protease protection assays. See text for details.

4.5. Function of the TAT proteins during ΔpH/TAT-dependent translocation

Genetic investigation has indicated that all three TAT proteins, i.e. TatA,TatB and TatC, are essential for TAT-dependent translocation in chloroplasts (Voelker and Barkan, 1995; Mori et al., 1999; Budziszewski et al., 2001), yet the precise function of these proteins has not been established so far. In order to examine the function of the TAT proteins during protein translocation by the ΔpH/TAT-pathway, *in vitro* thylakoid import experiments were performed with pea thylakoids that were pre-incubated with purified IgGs against each of the TAT proteins. This procedure should allow the IgGs to bind to the stroma exposed domains of the TAT proteins, and by this means to block their binding site for protein substrates. In order to avoid the effect of unspecific factors present in the rabbit antisera, thylakoid vesicles were pre-incubated in parallel also with IgGs purified from the corresponding pre-immune sera. The results show that pretreatment of the thylakoids with either of the TAT-antibodies does not affect thylakoid translocation by the Sec-pathway, since plastocyanin (PC) is imported into all thylakoids with an efficiency comparable to that of the control assay (Fig. 45B). Likewise, IgGs from either of the pre-immune sera had almost no effect on the translocation

of an authentic substrate of the ΔpH/TAT-pathway, notably the pre-OE23 protein. However, translocation of OE23 was strongly inhibited or even abolished if antibodies to any of the three TAT proteins were added to the thylakoids (Fig. 45), demonstrating that all three TAT proteins are required for ΔpH/TAT-dependent translocation. Remarkably, the concentration of TatA antibodies required to obtain an inhibitory effect were significantly higher than for the other two antibodies (0.5 μg/ml rather than 0.1 μg/ml) (Fig. 45 and data not shown), implying already that the function of TatA is different from that of the other two TAT proteins.

The role of each of the TAT proteins in the three translocation steps identified (see above) was further investigated. When the 16/23 protein was incubated with the antibody pre-treated thylakoids, its translocation across the membrane was completely abolished in each case (Fig. 45C). Neither Ti-2 nor the mature 23 kDa protein could be detected in these assays. However, Ti-1 was still found accumulating in the thylakoid membrane in each case.

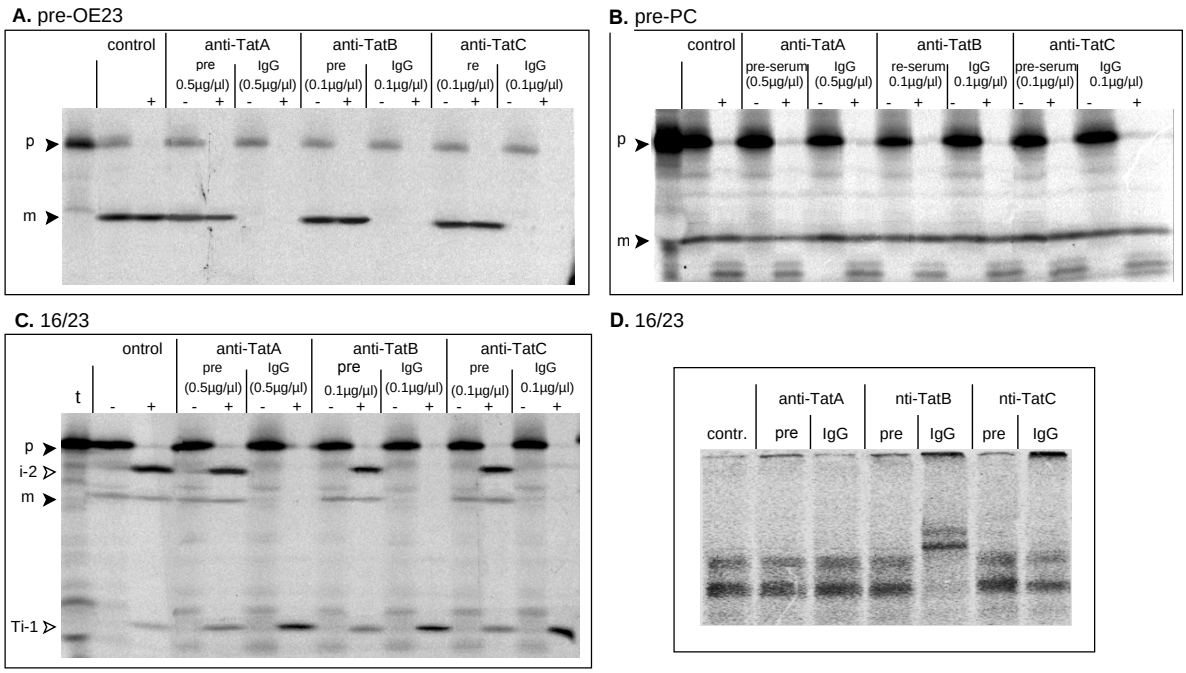


Fig. 45. ΔpH/TAT-dependent translocation was blocked by specific antibodies to TAT proteins. Pea thylakoids were pre-incubated for 1 hour at 4 °C with agitation with purified IgGs of either pre-immuo sera or antisera against TatA, B or C, respectively. The concentration of IgGs used in each assay is indicated above the lanes. After incubation, thylakoids were reisolated, washed with HM buffer, and applied to import assays with radiolabelled pre-OE23 (**A**), pre-PC (**B**) or 16/23 proteins (**C**

and **D**), respectively. Import assays were carried out as described in Fig.6, and the samples were processed to SDS-PAGE (**A** - **C**) or BN-PAGE (**D**) and analyzed after autoradiography.

Actually, it was present in even higher amounts than in the control assays (Fig. 45C). This result demonstrates that the TAT proteins are involved in the translocation of the C-terminal part of the protein, leading to the formation of Ti-2. But neither of the three TAT-components is apparently required for the loop insertion of the 16/23 protein into the thylakoid membrane, which leads to the formation of Ti-1. This confirms the results of the experiments with protease-treated thylakoids (Fig. 19) which show that the membrane insertion step of the 16/23 protein can take place in an unassisted manner independent of the function of the TatA, B, and C protein.

Remarkably though, the loop-inserted Ti-1 of the 16/23 protein is still associated with the TAT translocase, even if the thylakoids were pre-incubated with antisera IgGs against the TAT proteins, proving that the association of the precursor to the TAT complex still took place. Both the 560 kDa and 620 kDa complexes can be detected after BN-PAGE in almost all instances (Fig. 45D). The only exception is the assay with anti-TatB-treated thylakoids. In this case, a shift of approximately 140 kDa, corresponding roughly in size to a single IgG molecule, can be seen, which suggests that in this case the substrate associated with a complex having bound one IgG-molecule. Thus, in this instance, binding was possible even after blocking the stroma-exposed domains of TatB, whereas in the case of TatA and TatC, complexes devoid of a bound IgG molecule were apparently selected for binding.

4.6. Membrane-inserted 16/23 is associated with TatB and TatC, but not with TatA

In order to examine the interaction of the 16/23 protein with the TAT-components in an independent approach, thylakoid membranes recovered after import of the 16/23 protein were fractionated by BN-PAGE and analyzed by Western analysis (Fig. 46). It turned out that most of the TatC protein is found in four high molecular weight complexes of about 620 kDa, 560

kDa, 420 kDa and 380 kDa, respectively. The two larger complexes co-migrate with the 16/23 protein after import. Anti-TatB antisera label the same four complexes of about 620 kDa, 560 kDa, 420 kDa and 380 kDa, but, in addition, numerous other complexes of lower molecular weight are detected (Fig. 46). Furthermore, both TatB and TatC are present also as monomers that migrate together with the running front in such gel systems. In contrast to TatB and TatC, TatA is predominantly found as the monomer upon BN-PAGE. In addition, only a single complex of about 350 kDa is weakly labelled by the anti-TatA antisera, but neither the 620 kDa nor the 560 kDa complex seem to contain TatA protein (Fig. 46).

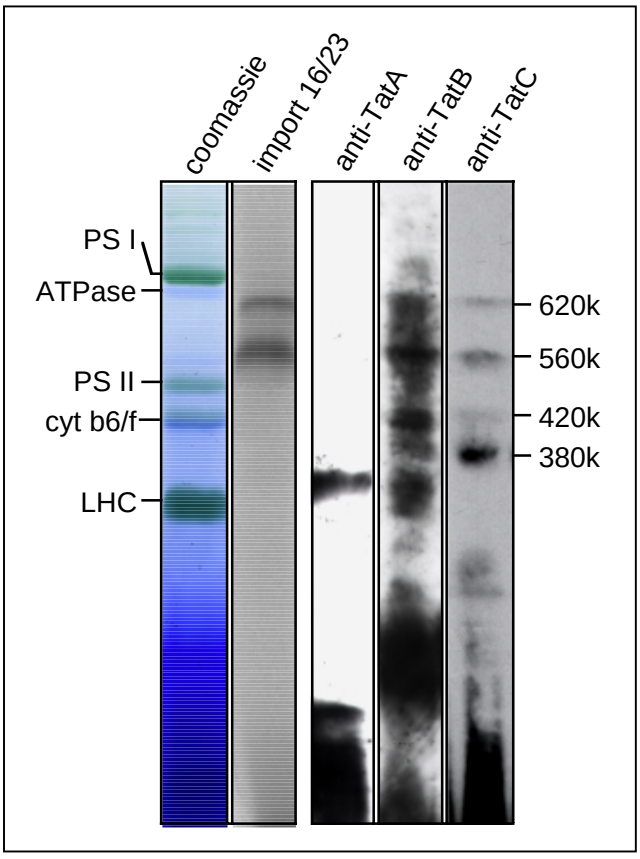


Fig. 46. Western analysis of BN-PAA gels. Pea thylakoids (equally to 60 µg chlorophyll) obtained after import of the 16/23 protein were solubilized in buffer containing 1.67% digitonin, and analyzed by BN-PAGE. Following electrophoresis, the gel was incubated with denaturing buffer (25 mM Tris, 192 mM glycine, 1% SDS, 2.5% β-mercaptoethanol) at 40 °C for 30 min, and subsequently incubated in transfer buffer containing 1% SDS at room temperature for 30 min. The proteins were blotted onto PVDF membranes using a semi-dry blotting device. Western analysis was performed using antisera raised to against TatA, TatB and TatC as described in Materials and Methods.

In order to confirm that the complexes seen by BN-PAGE/Western analysis were not due to cross-reaction with unspecific background proteins, the fractions obtained above were analyzed in a second-dimension by SDS-PAGE coupled with Western blotting. In this type of analysis, the distribution of the proteins is even more clear. As expected, the radiolabelled 16/23 precursor protein is predominantly found at only two positions corresponding to the 560 kDa and 620 kDa complexes mentioned above (Figs. 46 and 47). The same two complexes are also labelled by TatB and TatC antisera (Fig 46), and SDS-PAGE shows that the labelled proteins have the right sizes corresponding to that of TatB and TatC, respectively

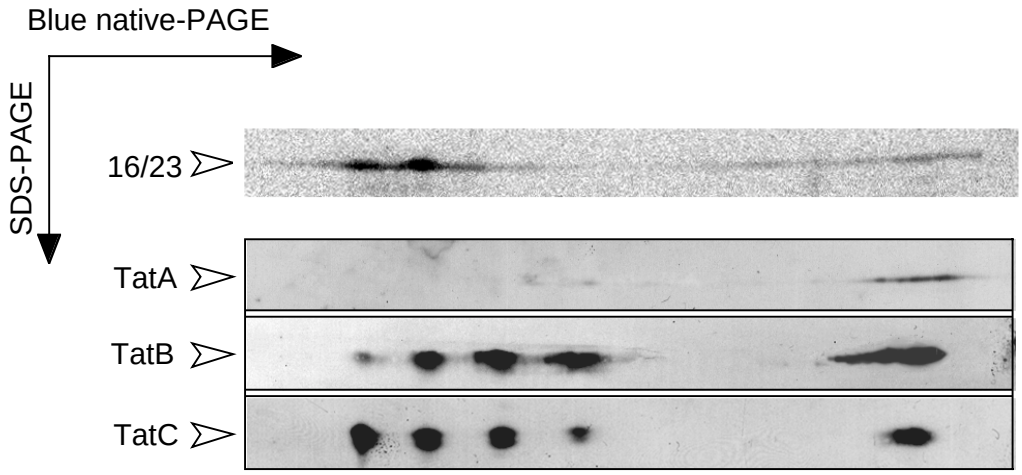


Fig. 47. Two-dimensional electrophoresis of thylakoid complexes. After import of the radiolabelled 16/23 protein, pea thylakoids were reisolated, solubilized in 1.67% digitonin-containing buffer and analyzed first by BN-PAGE, followed by fractionation in the second dimension by SDS-PAGE (10-17.5%). Proteins were blotted onto PVDF membranes, followed by either autoradiography (in case of 16/23) or Western analysis with specific antisera raised against TatA, TatB or TatC.

(Fig. 47). This finally proves that these two proteins are indeed present in the 560/620 kDa complexes together with the 16/23 precursor protein. Notably, the distribution of TatB or TatC in either of the two complexes is completely different, suggesting that these two complexes might have different composition and thus distinct functions, although both complexes show binding of the 16/23 protein. In addition, TatB and TatC are also found together in the two lower complexes, which show no affinity to the 16/23 protein. This

suggests that in addition to being present in the functional ΔpH/TAT-translocase complex, TatB and TatC are able to form subcomplexes which are not able to bind TAT-substrates. In addtion, the main proportion of TatB forms monomers, which might represent the excessive amount of TatB over TatC (see Introduction for details). In contrast, TatA is almost exclusively found as a monomer or dimmer in the thylakoid membrane. A weak signal at approximately 350 kDa is sometimes observed as well (Figs. 46 and 47), but whether this is of any functional meanings remains unclear so far.

In order to examine this result once more independently, a co-immunoprecipitation experiment was carried out. Thylakoids were first incubated with 16/23 either on ice or at 25 °C, to allow for the formation of Ti-1 or Ti-1 and Ti-2 in the thylakoid membrane, respectively (Fig. 48, left panel). The thylakoid membrane was then solubilized by digitonin under conditions that the translocase complexes should remain intact. The solubilized complexes were then pulled down with purified IgGs recognizing either of the TAT proteins. As a contrrol, the corresponding pre-immune IgGs were used. It turned out that inrespective of whether only Ti-1 or also Ti-2 is formed, the 16/23 precursor protein is coimmuno-precipitated by antibodies against TatB and TatC, proving that both TatB and TatC are present in complexes containing membrane-inserted 16/23. In contrast, the antibodies against TatA could not pull down the membrane-inserted 16/23 protein, indicating that TatA is not present in the complex, despite the fact that TatA is definitely required to finally perform ΔpH/TAT-dependent translocation (Fig. 45). As expected, neither of pre-immune sera led to co-immunoprecipitaion of the 16/23 protein. Remarkably, the mature 23 kDa protein was in neither case pulled down by the IgGs, confirming that the terminal processing product is not anymore associated with the translocation machinery, in line with the results shown in Fig. 16 and Fig. 47. Taken together, these results finally prove that TatB and TatC are the main constituents of the ΔpH/TAT-translocase involved in binding the TAT-substrates, whereas TatA may only be transiently present during operation of the translocase complex. Alternatively, the interaction of TatA with the TatB/C translocase complex might be too weak

to remain stable under the experimental conditions applied here. It is obvious though that TatA has apparently a function different from that of TatB, despite of their significant structural similarity.

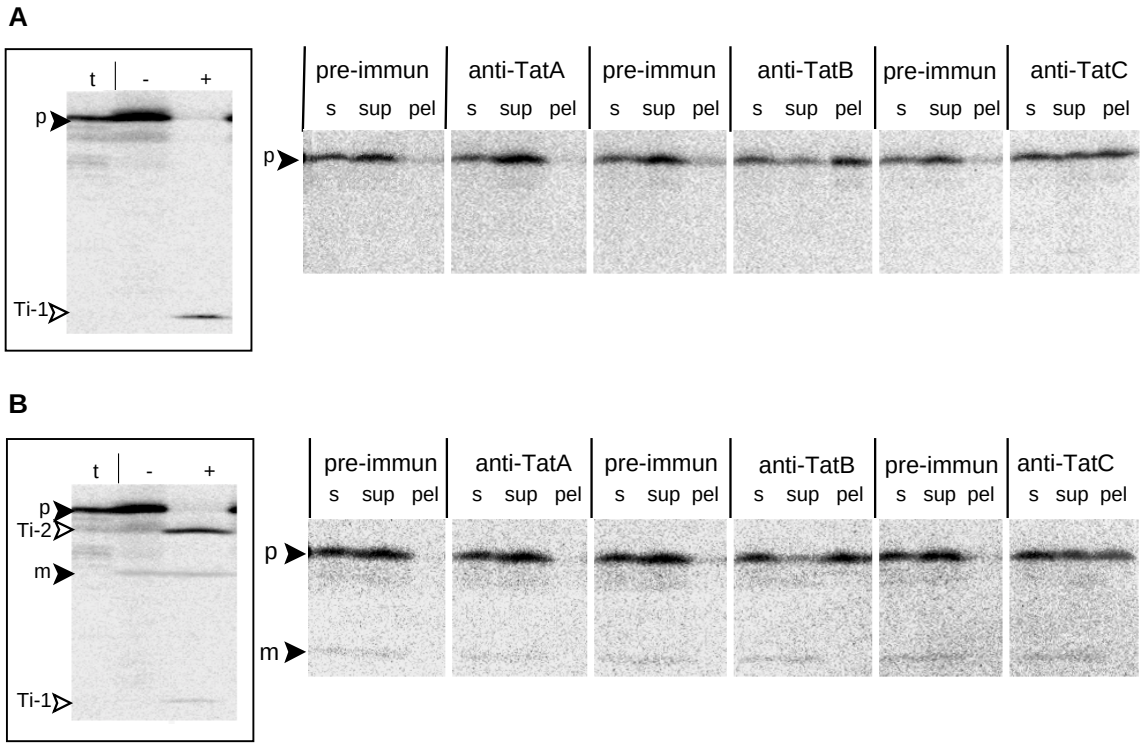


Fig. 48. Co-immunoprecipitation of the 16/23 translocation intermediates with antisera against TatA, TatB or TatC. Radiolabelled 16/23 was incubated with pea thylakoids either on ice (**A**) or at 25 °C for 20 min (**B**), to allow for the formation of Ti-1 and Ti-2, respectively. After import, thylakoids were reisolated and washed to remove unbound 16/23 protein, and finally solubilized in buffer containing 1% digitonin. After sediment of the unsolved material, the solubilized thylakoid complexes (s) were incubated with 20 µg of IgGs purified from either pre-immuno sera or antisera. After incubation for 1 hour at room temperature, protein A-Sepharose was added to a final concentration of 1% (w/v). After centrifugation, the supernatants (sup) and pellets (pel) were recovered, and the pellets were once washed with biding buffers. Finally the samples were analyzed by SDS-PAGE and autoradiography.

4.7. Co-purification of the 16/23 protein with the TAT components

In the next step of the analysis, I have tried to purify the membrane complexes containing TatB and TatC as well as the 16/23 polypeptide. For this purpose, thylakoids after import of

the 16/23 protein were solubilized by digitonin and loaded onto DEAE-sepharose columns. Proteins found in the same complex are usually eluted at the same concentration of the eluent $(NH4)_2SO_4$ (Zielinski and Price, 1980). Autoradiography of SDS-PAA gels performed with the fractions obtained showed that the terminal processing product of 23 kDa protein was eluted with buffers containing 10 mM $(NH_4)_2SO_4$, whereas the 16/23 precursor protein was eluted predominantly in the fractions containing 40-200 mM $(NH_4)_2SO_4$ (Fig. 49). Western analysis of these fractions showed that the TatC protein had an almost identical elution pattern as that of the 16/23 precursor protein, confirming that TatC is present in the same complex together with 16/23. On the other hand, only a minor fraction of TatB was co-eluted with TatC and the 16/23 precursor protein. Instead, the major portion of TatB, as well as all of TatA, was eluted already by buffer containing 10 mM $(NH_4)_2SO_4$. These results re-confirmed that TatC, as well as a minor portion of TatB, are the major constituents of the TAT-complex that is able to bind the TAT-substrates 16/23 protein. Under the experimental conditions described here, it is possible to co-purify the translocase together with the substrate protein, which should allow in future to characterize the composition of the translocase complex in more detail.

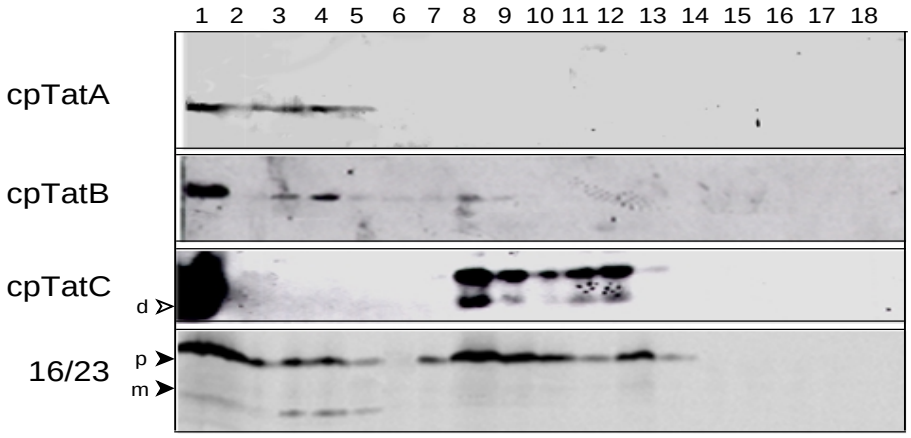


Fig. 49. Co-purification of the TAT proteins together with 16/23 upon ion-exchange chromatography. Thylakoid membranes (equal to 150 µg chlorophyll) were recovered from import assays with the 16/23 protein and solubilized in buffers containing 1 % digitonin. The solubilized proteins (lane 1) were loaded onto a DEAE-sepharose column, which was subsequently washed with buffer containing 0 mM $(NH4)_2SO_4$ (lane 2), 10 mM $(NH4)_2SO_4$ (lane 3-6), 40 mM $(NH4)_2SO_4$ (lane 7-10),

200 mM $(NH4)_2SO_4$ (lane 11-14), and $(NH4)_2SO_4$ (lane 15-18). 500 µl fractions were collected, and proteins were precipitated by 10% TCA. After centrifugation, protein pellets were dissolved in 2 x Laemmli buffer and subjected to SDS-PAGE. Proteins of each fraction were analyzed by either Western hybridization or autoradiography.

4.8. Composition of the 560 kDa and 620 kDa TAT-complexes in pea thylakoids

In order to determine the composition of the 560/620 kDa complexes in more detail, I have tried to isolate them from pea thylakoids. For this purpose, overexpressed 16/23His protien was used to saturate all active TAT-translocase in the thylakoid membrane (Berghöfer, 1998). These thylakoids were then solubilized by digitonin and putative TAT-complexes were separated by BN-PAGE from the photosynthetic complexes which occupy most of the thylakoid membrane mass. Then, I have tried to excise the putative translocase complexes by slicing the gel in the range of 560 kDa to 620 kDa (Fig. 50A). Subsequently, the proteins were eluted from the gel slices, separated by SDS-PAGE and subjected to Western analysis. As expected, The results showed that the 16/23His protein as well as TatB and TatC are found in either of the two gel slices, whereas the TatA is apparently not present (Fig. 50B). This demonstrates that by this means, it is possible to isolate the translocase complex together with its substrate protein 16/23His. Unexpectedly, lots of additional protein bands were found in both complexes upon SDS-PAGE (Fig. 50C). Among these proteins, two bands of about 29 kDa and 28 kDa were recognized by TatB and TatC antisera in Western analysis, respectively (Fig. 50D). These two protein were then purified from the SDS-PAA gel, and further examined by mass spectrometry. The results of mass spectrometry have confirmed that the 29 kDa and 28 kDa protein band indeed represented the pea TatB (86 residues matched) and TatC (50 residues matched) protein (Fig. 51).

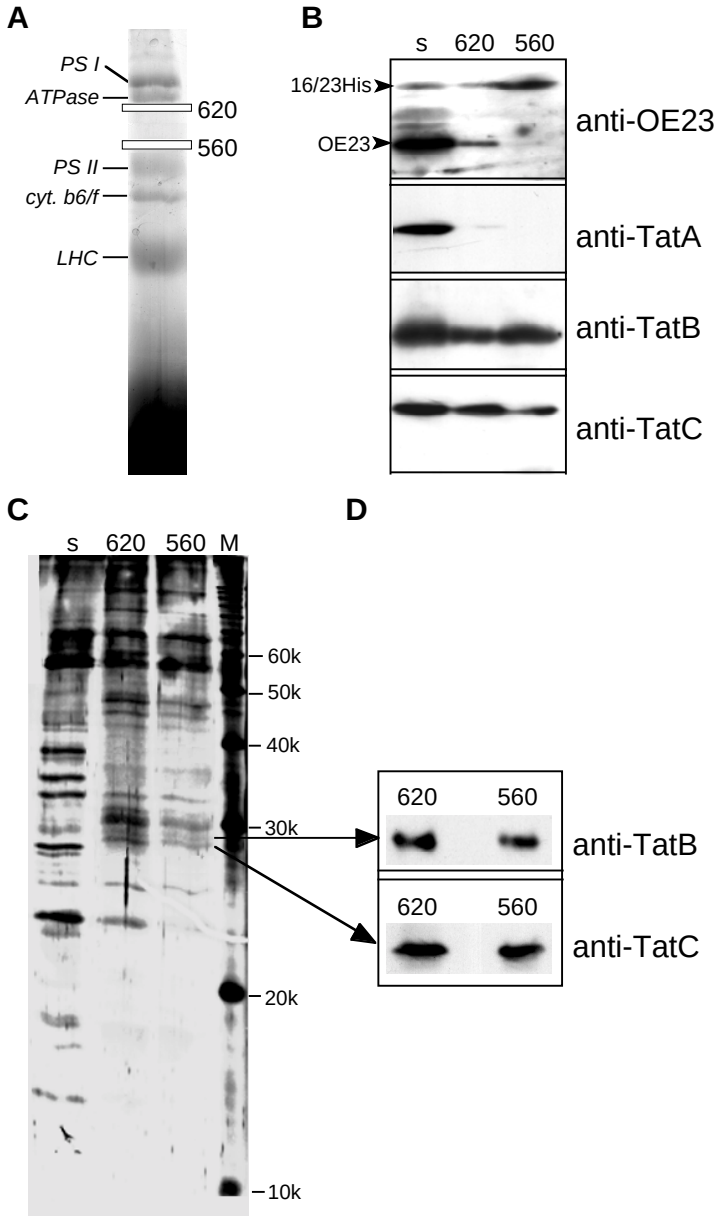


Fig. 50. Purification of the TAT-translocase from pea thylakoids. After incubation of pea thylakoids with 4 mM 16/23His protein obtained by overexpression in *E.coli*, protein complexes of the thylakoid membrane were separated by BN-PAGE. Two gel slices corresponding to protein complexes in the range of 560 kDa and 620 kDa were excised (**A**, lane 620 and 560). Proteins were eluted from these gel slices by electrophoresis, collected by TCA precipitation followed by separation on SDS-PAGE. Protein bands were either analyzed by Western blot using antisera raised against OE23 or the TAT components (**B**), or visualized by silver staining (**C**). Total solubilized thylakoid proteins were also loaded to the SDS-PAGE as a control (lane s). Two protein bands of size of 28 kDa (lower panel) and 29 kDa (upper panel) were excised from the silver stained SDS-PAA gel and analyzed by Western blot using antisera against TatB and TatC, respectively (**D**).

A

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  1 MTPSLAIASS TSTMLLCPKL GTCSMSLSTC TPTSHSKIHH FHLYSLGKRL FTPWNGFKQL
 61 GFSTKPKKPL FHFIGKKGRC KGKVVYASLF GVGAPEALVI GVVALLVFGP KGLAEVARNL
121 GKTLREFQPT IREIQDVSRE FKSTLEREIG IDDITNPLQS TYSSNVRNTT PTPSATEITN
181 NSQTAVDPNG KVDESKAYSS EEYLKITEEQ LKAVAAQQQE QTSSPKEDEI EQQIQPPANE
241 TAATVPPPQK PESESSLPSD L
```

Mascot Search Results

gi:15277532 **Mass:** 28418 **Total score:** 118 **Peptides matched:** 4
(AF284760) HCF106 (*Pisum sativum*)

Query	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Rank	Peptide
2	445.75	889.48	889.47	0.01	0	14	1	EFQPTIR
18	741.38	2221.11	2221.09	0.03	0	51	1	EIGIDDITNPLQSTYSSNVR
20	820.06	2457.16	2457.16	0.00	0	33	1	NTTPTPSATEITNNSQTAVDPNGK
22	1257.94	3770.80	3770.80	0.00	1	19	1	EDEIEQQIQPPANETAATVPPPQKPESESSLPSDL

B

```
  1 MGLGTTTVPT NILPQFGLHR THLNPIRVNN STGFSYPLSL RKNKSFDRLV CFAVDDEIRE
 61 KQQQQLSTSS TRLGSAVEER PENKDMIDGI SEEALENFKE DGERSAIYDF LYPSKELLPD
121 DKEMSIFDHL EELRERIFIS VLGVGGSILG CFAFSKDLVK ILEAPVKSEG VRFLQLAPGE
181 FFFTTLKVSG YCGLLLGSPI ILYEIIAFII PGLTKEERKF LGPIVLGSSV LFYAGITFSY
241 LVLVPAALNF FVNYAEGAVE SLWSIDQYFE FVLVLMFSTG LSFQVPIIQL LLGQLGLVSG
301 DKMLSVWRYV VVGAVVAAAV VTPSTDPLTQ VLLAAPLLGL YLGGAWMVKL AGR
```

Mascot Search Results

gi:15277529 **Mass:**38912 **Total score:** 72 **Peptides matched:** 4
(AF284759) TatC (*Pisum sativum*)

Query	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Rank	Peptide
5	429.99	859.91	859.94	-0.03	0	16	1	LGSAVEER
18	639.74	1279.48	1279.47	0.01	0	24	2	LVCFAVDDEIR
19	612.26	1224.51	1224.51	0.00	1	20	1	DLVKILEAPVK
22	1017.74	2015.46	2015.44	0.02	0	12	1	IFISVLGVGGSILGCFAFSK

Fig. 51. Identification of the 29 kDa and the 28 kDa protein bands by MALDI-TOF mass spectrometry. Tryptical digests of the 29 kDa (**A**) and the 28 kDa (**B**) protein bands excised from the SDS-PAA gel were subjected to fingerprint analysis by MALDI-TOF mass spectrometry. MALDI-TOF mass spectrum (Observed) is annotated with the molecular mass (Mr (expt)) and sequence of identified peptides, and is compared with the calculated molecular mass (Mr (calc)). The differences between the experimental and calculated masses are indicated (Delta), as well as potentially missed cleavage sites (Miss). Peptide sequences identified by MALDI fingerprint analysis are highlighted in bold upon the TatB and TatC protein sequence.

4.9. Mapping of protein-protein interaction sites between TAT components and TAT-substrates

As the next step, I have tried to determine the interaction sites between the three components of the TAT-complex and the TAT-substrates. For this purpose, peptide library scans were

performed. Screening an immobilized peptide library is a powerful method to study protein-protein interaction site. A peptide library synthesized on a cellulose membrane may represent the full length of a protein, and the accumulation of many peptides on the surface of the cellulose membrane may to some extent mimic the natural environment, especially for a hydrophobic environment (e.g. Groves et al., 2001; and for a review see Reimer et al., 2002). Therefore, peptide libraries were prepared for all three TAT components (TatA, TatB, and TatC). For this purpose, a series of overlapping 13-mer peptides were synthesized onto a cellulose membrane. 76 peptides, overlapping by 12 residues each, were synthesized to represent the 88 residues of the TatA mature protein. 84 peptides and 151 peptides, with overlaps of 11 residues, were synthesized to represent the 177 residues of TatB mature protein and the 312 residues of TatC mature protein, respectively. For screening, different precursor proteins from both the ΔpH/TAT-pathway and other thylakoid transport pathways were hybridized to these peptide spots. After removal of unbound proteins by washing with detergent-containing buffers, the proteins associated with the peptide spots were transferred to nitrocellulose membranes, and detected by either autoradiography or, if overexpressed precursor protein had been applied, by Western blotting.

4.9.1. Interaction sites between the TAT components and the 16/23 protein

The first protein used to screen the peptide libraries was radiolabelled 16/23 precursor protein obtained by *in vitro* translation. It was synthesized in the presence of ^{35}S-Met and recovered through a Sephadex-G 50 column to get rid of free ^{35}S-Met. Using such 16/23 protein to scan the peptide libraries representing the TAT components, a large number of interaction sites were identified (Fig. 52). The affinity between the 16/23 protein and each peptide was quantified by measuring the radioactivity found in each spot, and projected onto the schematic structure of the corresponding TAT protein. The results obtained show that the 16/23 protein has almost no affinity to TatA, but shows strong interaction with both TatB and TatC. In both instances, the binding domains are predominantly found within the transmembrane spans, but not, as one might have assumed, within the stroma exposed domains of the two TAT proteins

(Fig. 52). Similarly unexpected, among the hydrophilic domains the first two lumenal loops of TatC show highest affinity to the 16/23 protein, whereas almost no interaction with the stromal regions of TatC could be detected.

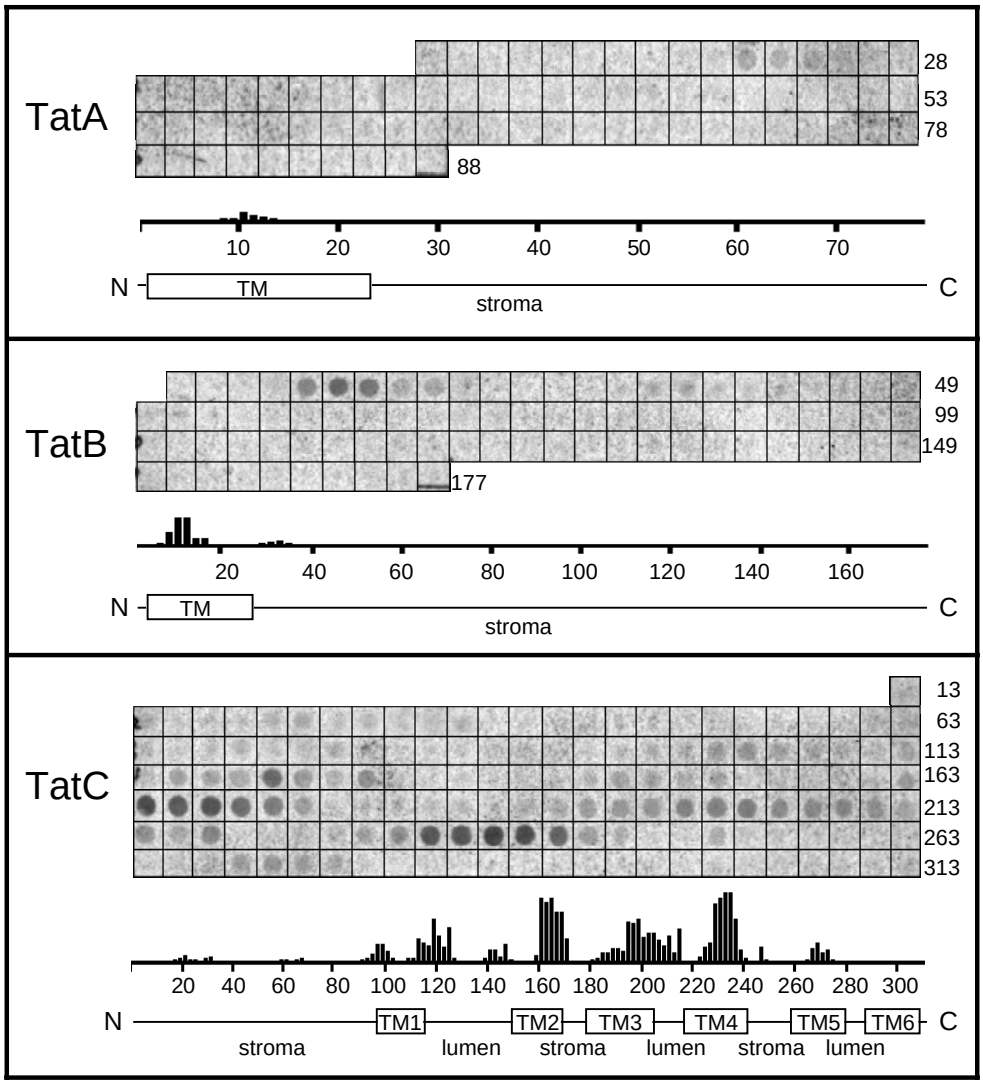

Fig. 52. Screening the TAT components for interaction sites with the 16/23 precursor protein. *In vitro* translated radiolabelled 16/23 was hybridized with the cellulose-based peptide library representing the three TAT proteins. After hybridization, the unbound protein was removed by washing with detergent-containing buffers. Subsequently, the associated 16/23 protein was transferred to a nitro-cellulose membrane and visualized by autoradiography. The amount of radioactivity in each spot was quantified using the IQMac v1.2 software (Molecular Dynamics). The result of this quantification is depicted by the size of the bar shown right above the schematic structure of the respective Tat protein. The length of the bars corresponds of the affinity of the peptide for the 16/23 protein.

Since *in vitro* translation products used for screening the peptide library may contain more than a single radioactive polypeptide, problems with unspecific binding could be envisaged. Therefore, the screen was repeated using overexpressed 16/23His protein as a probe. In this case, Western analysis using antibodies specific to OE23 was used to detect the peptide-associated protein, which should increase the specificity of the signals. With this approach, no

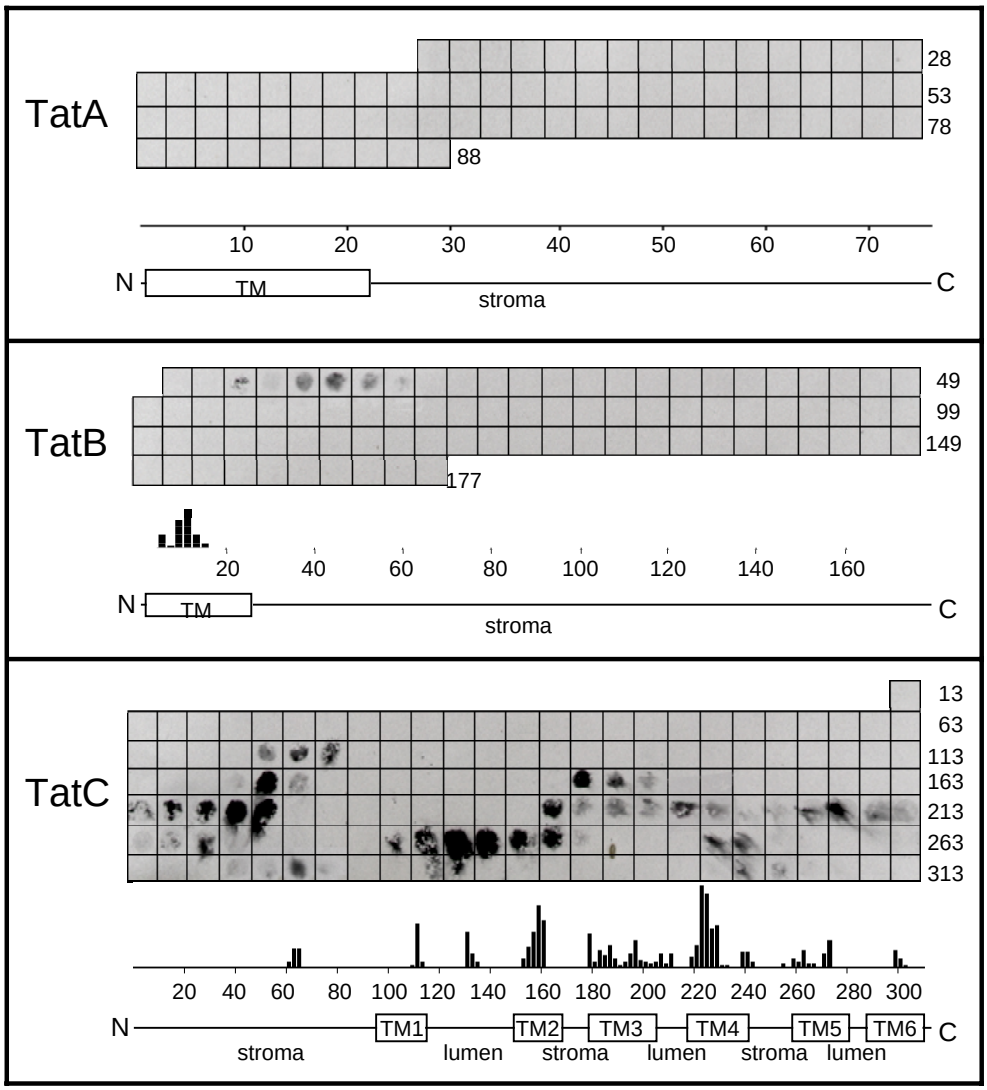

Fig. 53. Screening the TAT components for interaction sites with the 16/23His protein. The 16/23His protein was obtained by overexpression in *E.coli*, followed by purification through a metal affinity column. The purified 16/23His protein was hybridized with the cellulose-based peptide library representing the three TAT proteins. After hybridization, the unbound protein was removed by washing with detergent-containing buffers. Subsequently, the associated 16/23 protein was transferred to a nitro-cellulose membrane, followed by Western analysis using antibodies against OE23. The density of each spot was quantified using the IQMac v1.2 software (Molecular Dynamics). The result

of this quantification is depicted by the size of the bar shown right above the schematic structure of the respective Tat protein. The length of the bars corresponds of the affinity of the peptide for the 16/23His protein.

interaction with TatA could be detected at all, confirming that interaction between TatA and the TAT-substrate is only temporary and is restricted to substrates that have already bound to a TAT-receptor complex. Binding to TatB and TatC is, however, still observed. Again, the binding domains are exclusively found in the transmembrane spans of TatB and TatC as well as in the first two lumenal loops of the TatC protein (Fig. 53). These results confirm the results obtained in the screening assays using the *in vitro* translation product of 16/23, and show that both experimental procedures are suitable for this purpose. Therefore, both procedures were used in subsequent experiments of this work.

In order to examine whether the interaction of the 16/23 protein to the transmembrane spans of TatB and TatC is due only to unspecific affinity of the hydrophobic signal peptide, protein substrates of the Sec-pathway in chloroplasts, notably PC (plastocyanin) and PC/23, were analyzed in the scanning experiment. The signal peptide of PC bears some structural similarity to those of ΔpH/TAT substrates, but it is incompatible with the ΔpH/TAT translocation machinery (Robinson et al., 1994). The overexpressed PC protein shows no binding to either TatA or TatB (Fig. 54). However, it shows strong interaction with TatC. Interaction is particularly strong with the fourth transmembrane span (TM4) of TatC, but also the other TM spans of this protein show some affinity to PC (Fig. 54). This suggests that indeed part of the interaction between the 16/23 protein and the transmembrane spans of TatC is caused by hydrophobic interaction. In contrast, since no binding to the lumenal loops of TatC was found with the PC precursor, this interaction is probably specific, as is that of TatB.

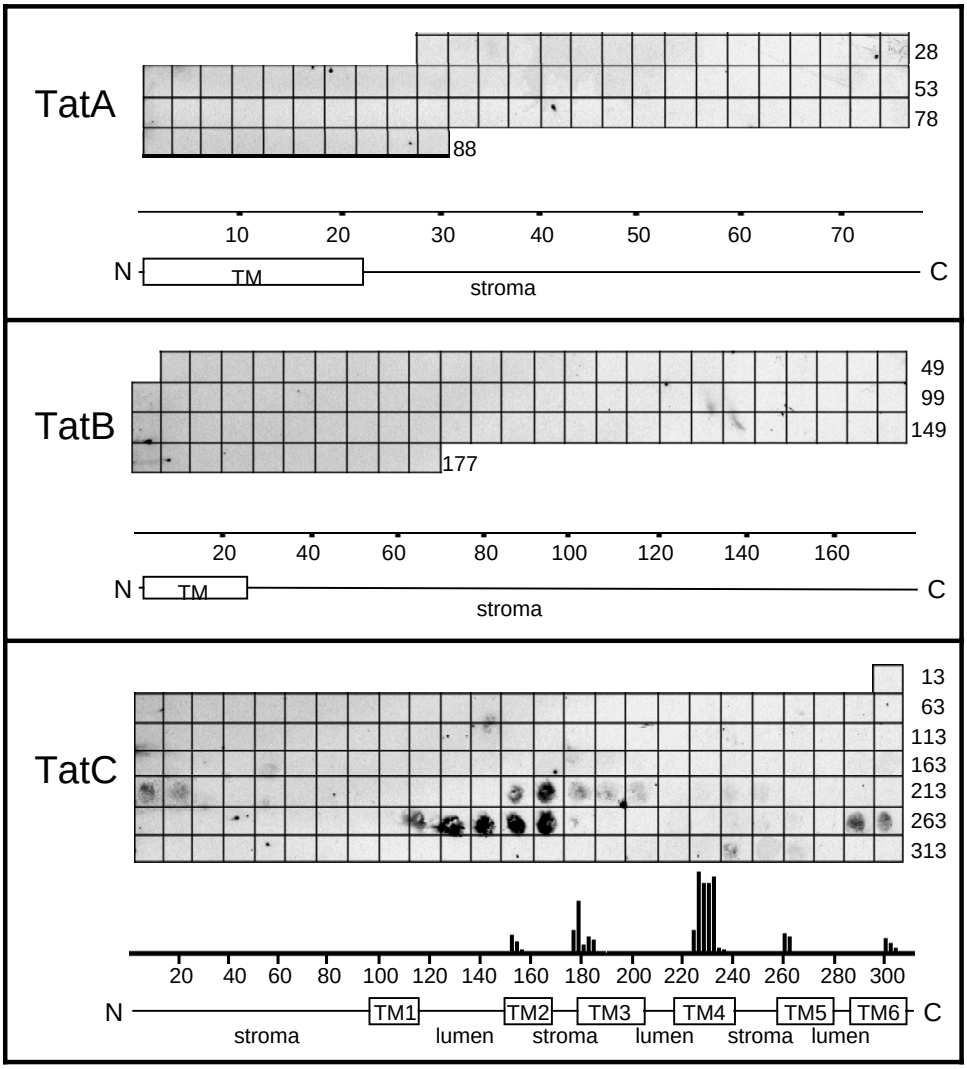

Fig. 54. Screening the TAT components for interaction sites with PC. The PC protein was obtained by overexpression in *E.coli*, followed by purification through a gel filtration column. The purified PC was hybridized with the cellulose-based peptide library representing the three TAT proteins. After hybridization, the unbound protein was removed by washing with detergent-containing buffers. Subsequently, the associated PC was transferred to a nitro-cellulose membrane, followed by Western analysis using antibodies against PC. For more details see the legend to Fig. 53.

PC/23, the second Sec-substrate used as a control, is a chimeric protein consisting of the transit peptide of PC and the mature part of OE23. Although being targeted to the Sec-machinery of the thylakoid membrane, PC/23 is blocked on its transport into the thylakoid lumen (Clausmeyer et al, 1993), presumably because the folded structure of OE23 is not compatible with the Sec machinery. PC/23 was used as a control, because it should help to

identify possible binding sites for the passenger part of Tat substrates, as it carries the same passenger as the 16/23 protein. However, since the binding pattern of PC/23 to the peptide libraries is essentially identical to that obtained with the PC precursor protein (Fig. 55), the OE23 passenger protein does apparently not directly interact with the TAT proteins in a specific manner. In other words, the specific interaction between 16/23 and the TAT proteins is apparently contributed exclusively by the signal peptide.

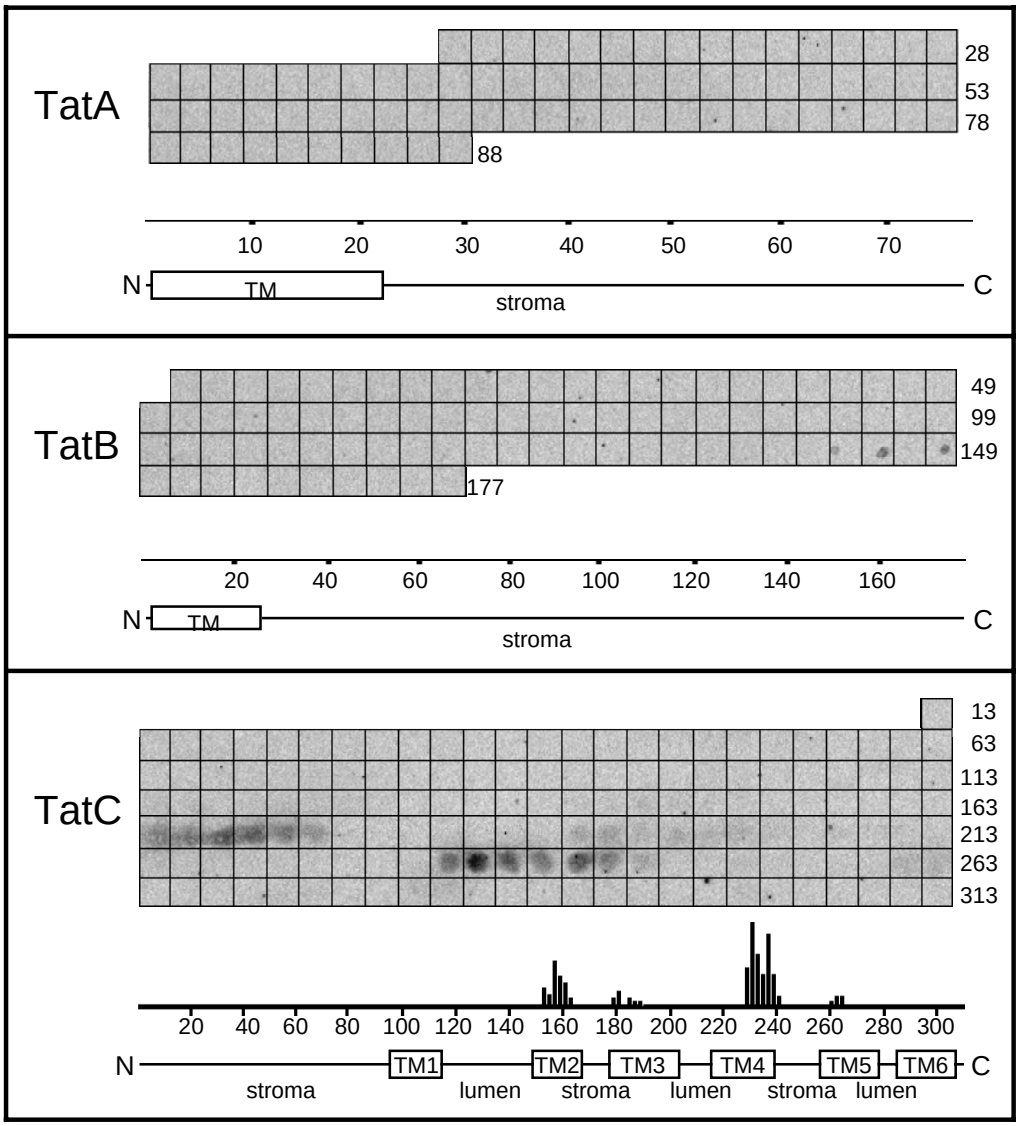

Fig. 55. Screening the TAT components for interaction sites with the PC/23 protein. *In vitro* translated radiolabelled PC/23 was hybridized with the cellulose-based peptide library representing the three TAT proteins. After hybridization, the unbound protein was removed by washing with detergent-containing buffers. Subsequently, the associated PC/23 protein was transferred to a nitro-cellulose membrane and visualized by autoradiography. For more details see the legend to Fig. 52.

As a further hydrophobic control protein, LHCP was subjected to the screening procedure. LHCP is an integral thylakoid membrane protein with three transmembrane spans and thus displays an overall high hydrophobicity. The LHCP precursor contains a hydrophilic transit peptide mediating stroma targeting only. Integration of LHCP into the thylakoid membrane takes place by the SRP pathway, and is mediated by signals present in the mature polypeptide

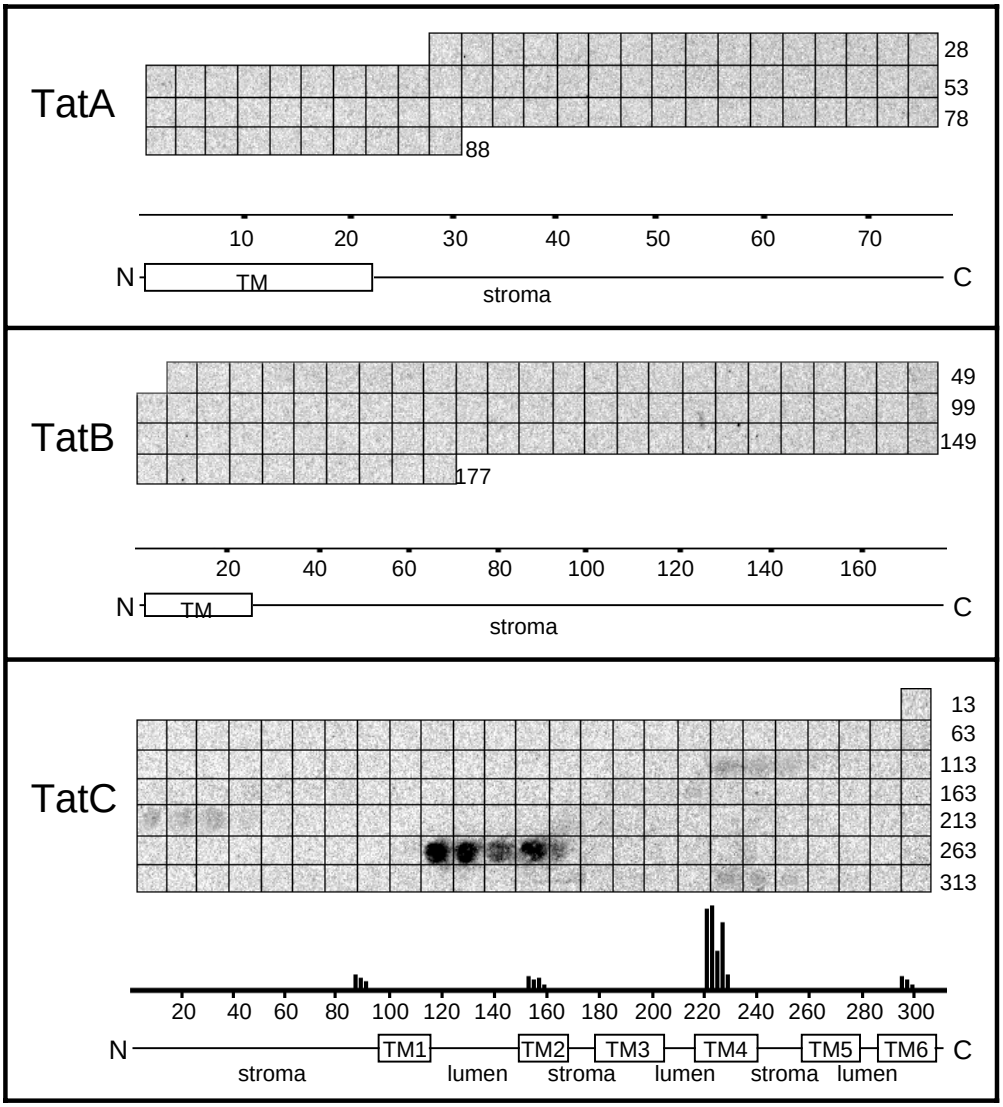

Fig. 56. Screening the TAT components for interaction sites with LHCP. *In vitro* translated radiolabelled LHCP was hybridized with the cellulose-based peptide library representing the three TAT proteins. After hybridization, the unbound protein was removed by washing with detergent-containing buffers. Subsequently, the associated LHCP was transferred to a nitro-cellulose membrane and visualized by autoradiography. For more details see the legend to Fig. 52.

domain (see Introduction). Similar to the results obtained with the two Sec-substrates, interaction of LHCP is observed only with TatC, but not with either TatA or TatB. Again, TM4 of TatC shows the strongest interaction, while TM2 and TM6 exhibit some weaker affinity to LHCP (Fig. 56). This confirms again that the binding sites located within TatB and the hydrophilic regions of TatC are specific for Tat-substrate. Moreover, even the binding sites within the TM2 and TM3 of TatC are apparently specific for Tat-substrates, whereas those of TM4 are able to interact with wide range of hydrophobic protein.

4.9.2. Interaction between the TAT proteins and two authentic Tat-substrates

In the final approach, two authentic substrates of the ΔpH/TAT-pathway, notably the precursor proteins of OE23 and OE16, were analyzed in these assays. Similar to the 16/23 protein, and in contrast to the Sec- and SRP-substrates analyzed above, both pre-OE23 and pre-OE16 interact strongly not only with the transmembrane spans of TatC, but also with the first two lumenal loops of this protein (Fig. 57 and 58). Furthermore, binding to the transmembrane region of TatB is again detected. In contrast, binding to TatA is found only at the background level (Fig. 57 and 58). This confirms that the interaction pattern observed with the 16/23 protein is not an artifact caused by the chimeric substrate, but that it provides a model for specific binding of a Tat-signal peptide to the components of the ΔpH/TAT-dependent translocation machinery.

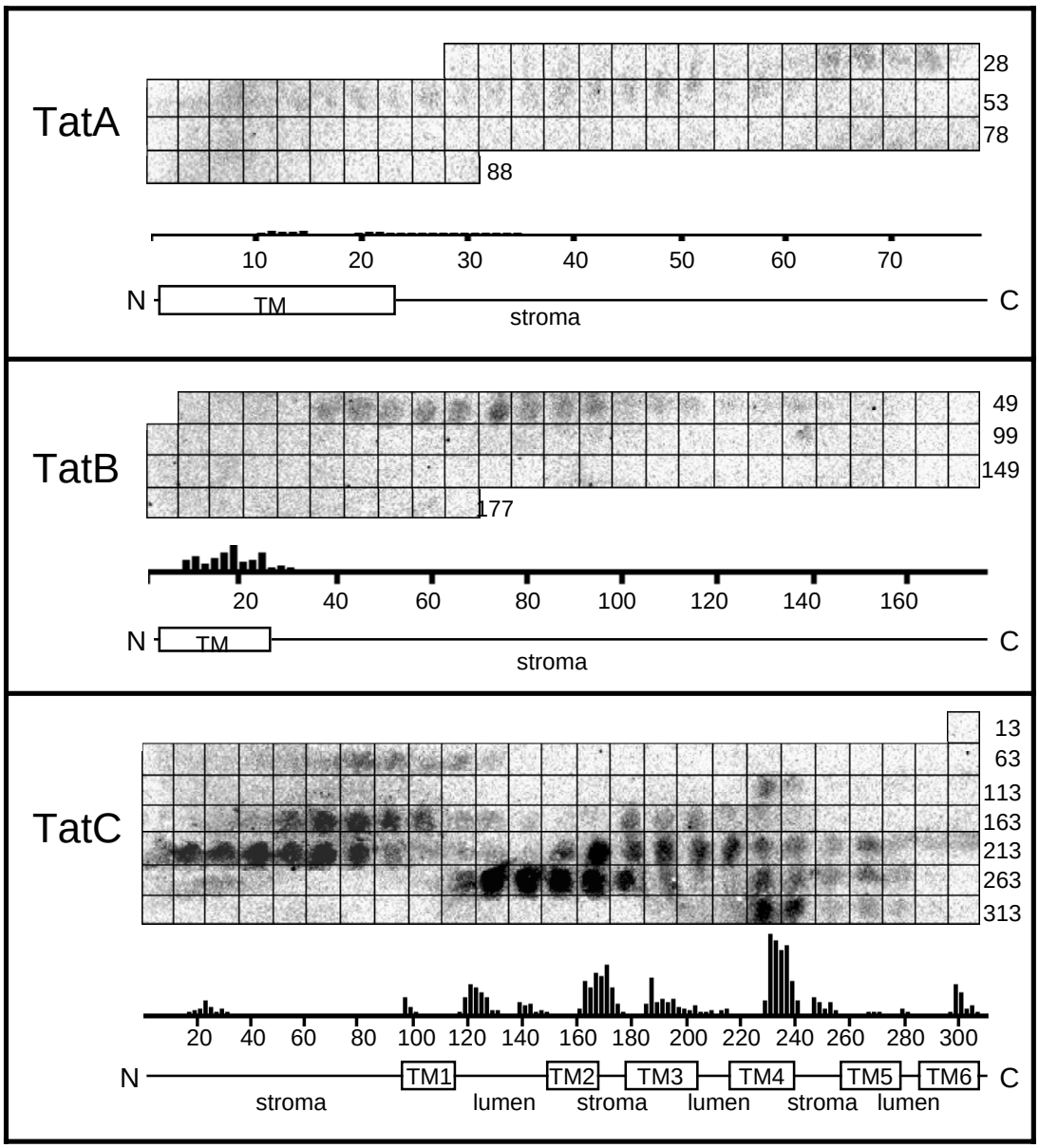

Fig. 57. Screening the TAT components for interaction sites with the pre-OE23 protein. *In vitro* translated radiolabelled pre-OE23 protein was hybridized with the cellulose-based peptide library representing the three TAT proteins. After hybridization, the unbound protein was removed by washing with detergent-containing buffers. Subsequently, the associated pre-OE23 protein was transferred to a nitro-cellulose membrane and visualized by autoradiography. For more details see the legend to Fig. 52.

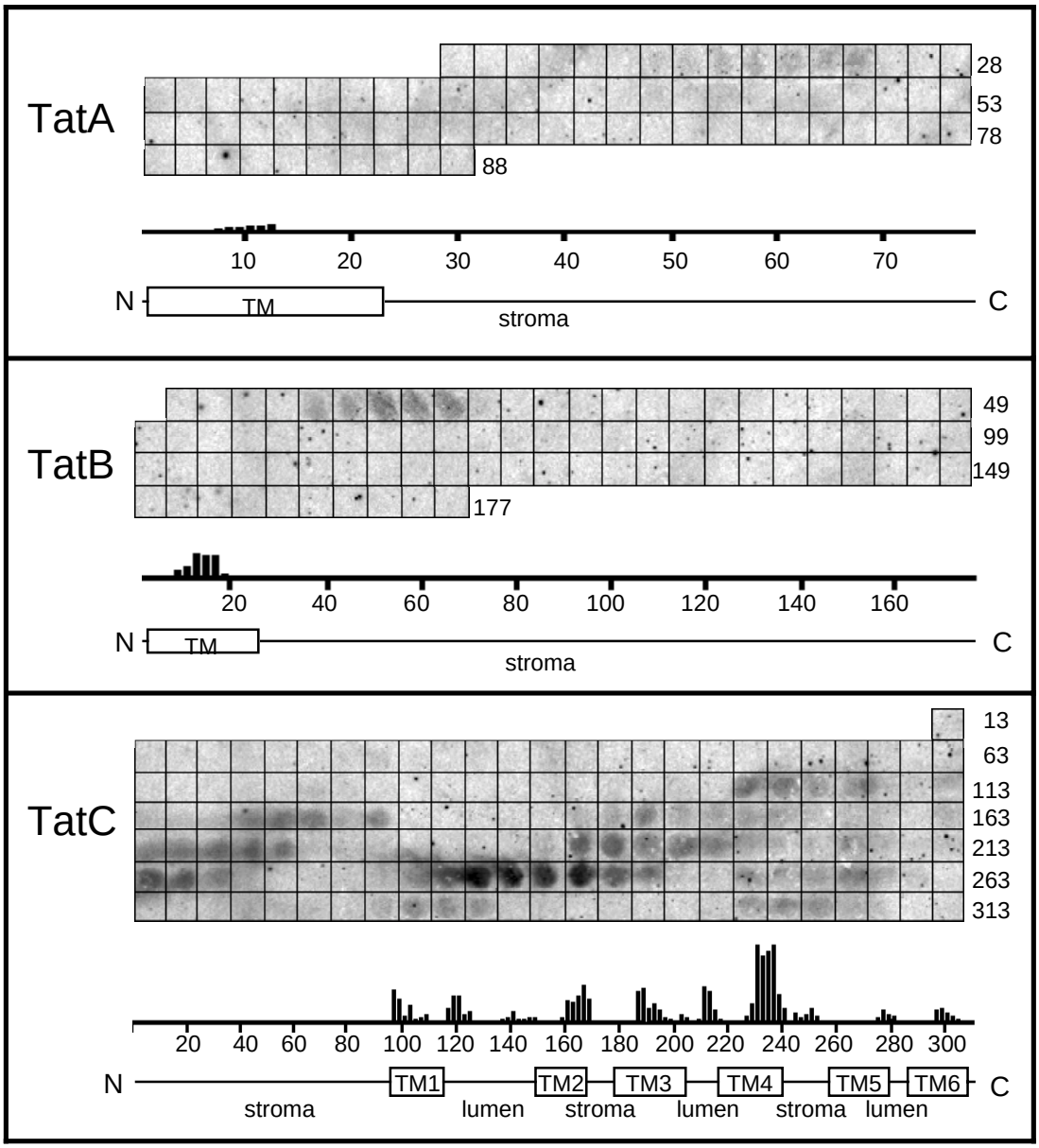

Fig. 58. Screening the TAT components for interaction sites with the pre-OE16 protein. *In vitro* translated radiolabelled pre-OE16 protein was hybridized with the cellulose-based peptide library representing the three TAT proteins. After hybridization, the unbound protein was removed by washing with detergent-containing buffers. Subsequently, the associated pre-OE16 protein was transferred to a nitro-cellulose membrane and visualized by autoradiography. For more details see the legend to Fig. 52.

Taken together, these data provide direct evidence that the precursor proteins of the ΔpH/TAT pathway interact directly and specifically with TatB and TatC. Most of these interactions are

found within the transmembrane spans of these two TAT components, as well as in the lumenal loops of TatC. To access to these interaction sites, the precursor protein obligatorily needs to be firstly inserted into the thylakoid membrane. This corresponds well with the observation that the TAT-substrates insert into the thylakoid membrane prior to association with the TAT translocase (see Fig. 19). No convincing interaction of the TAT-substrates with TatA could be detected so far, which is in line also with the previous observation that the precursor proteins are found in a complex composed of TatB and TatC but lacking TatA (see Figs. 46 and 47). Neither of the stromal domains of the three TAT proteins seem to be involved in the binding of the Tat-substrates, which might explain why the precursor proteins need to insert into the thylakoid membrane prior to binding to the translocation machinery.

DISCUSSION

Proteins are transported across or into the thylakoid membrane via at least four distinct pathways. Among those pathways, the ΔpH/TAT-pathway has received particular attention in the last few years due to its unique features. It is for example capable of transporting presumably folded proteins with a signal peptide carrying twin-arginine motif. This pathway was initially characterized in chloroplasts of higher plants (Mould and Robinson, 1991, Cline et al., 1992), but was recently found to have a prokaryotic origin (Berks, 1996). Meanwhile, it has turned out that this pathway is operating in a wide range of organisms. It was the goal of this thesis to study in detail the mechanism of the ΔpH/TAT-dependent translocation pathway at the thylakoid membrane, and to characterize the function and organization of the TAT components.

1. Thylakoid transport of the bacterial TAT-substrate GFOR

The data on the thylakoid transport of GFOR are important in several aspects. The structural similarity of the targeting signals suggests that the two twin-R transport pathways in the prokaryote and the prokaryote-derived chloroplasts are of common phylogenetic origin. This was confirmed by analysis of the transport of a full bacterial precursor protein carrying a twin-R signal peptide (GFOR) into thylakoid vesicles isolated from higher plant chloroplasts. The principle idea of using this heterologous combination was to characterize the specificities, and possibly homologies, of bacterial and thylakoidal membrane transport processes that are engaged by twin-R signal peptides. The results show that the precursor of GFOR from *Z. mobilis* is efficiently and selectively transported by the ΔpH-dependent translocation system across the thylakoid membrane (Fig.9), which confirms the close relationship of the thylakoidal ΔpH-dependent pathway and the TAT-pathway of bacteria and further emphasizes their putative common evolutionary origin. Furthermore, it shows that not only bacterial twin-R signal peptides but also full precursors are compatible with the

thylakoidal ΔpH-dependent transport machinery. While the former had already been deduced from studies analyzing chimeric polypeptides that are composed of bacterial signal peptides and thylakoidal passenger proteins (Wexler et al., 1998; Mori and Cline, 1998), the latter could not necessarily be expected: the only bacterial twin-R protein analyzed so far in the chloroplast thylakoid system was CtpA from *Synechocystis* PCC6803. This protein could, however, not be imported into isolated thylakoid vesicles (Karnauchov et al., 1997). When analyzed with intact chloroplasts, it did not specifically utilize the ΔpH-dependent pathway but was instead also targeted by the thylakoidal Sec pathway suggesting that it was not capable of distinguishing the two translocation routes in chloroplasts (Karnauchov et al., 1997). It is thus so far unfair to draw any conclusion on the substrate and species specificities of the overall conserved TAT pathways from the limited number of experiments.

The most prominent feature of the ΔpH/TAT-dependent pathway is the transport of fully folded proteins. GFOR carries a complex cofactor (NADP), although the mechanism of incorporation of NADP is not yet known. Interestingly, GFOR from *Z. mobilis* cannot be exported into the periplasmic space of *E. coli* when expressed with its genuine signal peptide (Wiegert et al., 1997a). Only after fusion to Sec-targeting signal peptides like those derived from OmpA or PhoA, export can be observed which notably takes place by the Sec pathway. However, GFOR does not accumulate in the periplasm in these instances but is rapidly degraded in this compartment. It was concluded that proteolysis in the periplasm is due to the lack of cofactor insertion which probably takes place in the bacterial cytosol. Consequently, export of GFOR in *Zymomonas* is assumed to be conducted by a pathway which, in contrast to the Sec pathway, is capable of handling folded proteins (Wiegertet al., 1997a). As yet, it is not clear whether GFOR also binds its cofactor NADP in our import assays and if so, whether binding takes place prior to the membrane transport step.

2. Thylakoid translocation of TAT substrates

Protein translocation across or into the thylakoid membrane via the ΔpH/TAT-dependent pathway bears several aspects that are different from all other systems: translocation is totally dependent on the proton gradient across the thylakoid membrane, as well as on the invariable twin-arginine motif located right in front of the hydrophobic domain of the signal peptide. Proteins translocated by this pathway are folded prior to transport and might carry redox cofactors that are obtained within the stroma of the chloroplasts. However, the knowledge on the mechanism of the recognition event between the precursor protein and the translocation machinery is very limited. It is well known that the transit peptide carries all the information required to allow for interaction between the precursor protein and the translocation machinery. Especially the twin-arginine motif may play a central role in the recognition event, whereas the folded mature polypeptide does probably not contribute to this process. However, how the RR-motif is captured by the ΔpH/TAT machinery is not yet clear. Moreover, translocation of some passenger proteins is apparently slower than that of others, even if they are targeted by the same transit peptide, suggesting that the mature polypeptide may have also some influence on the translocation process (Clausmeyer et al., 1993; Hynds et al., 1998; Marques et al., 2003).

2.1. ΔpH/TAT-dependent translocation takes place in multiple steps

Our current knowledge on the translocation mechanism of the thylakoidal ΔpH/TAT pathway relies to a large extent on *in vitro* studies about the import of authentic or chimeric precursor proteins. However, thylakoid transport of most of those proteins takes place too efficiently to allow for a detailed analysis. Neither translocation steps nor translocation intermediates, which may be helpful in understanding the details of the mechanism, are generally achieved in those experiments. The only example known to date showing retarded ΔpH/TAT-dependent translocation across the thylakoid membrane is the chimeric 16/23 protein, which

is composed of the transit peptide from pre-OE16 and the mature polypeptide from pre-OE23, both of which are typical ΔpH/TAT pathway proteins (Berghöfer and Klösgen, 1999). For this protein, two translocation intermediates were identified in the thylakoid membrane which are represented by two specific protease protected products of 14 kDa and 26 kDa, respectively. The work presented here could demonstrate that these two proteolytic fragments represent two different topologies of the 16/23 protein within the thylakoid membrane. The 14 kDa fragment results from a “horse shoe” or loop insertion structure of the 16/23 protein in which both the N- and the C-termini are exposed to the stromal side (Fig. 12). The 26 kDa fragment corresponds to a single membrane-spanning topology of the 16/23 protein, with the N-terminus located at the stromal side of the thylakoid membrane and the C-terminal part already translocated into the lumen (Fig. 12). Kinetic experiments have revealed that the 14 kDa and 26 kDa degradation products, as well as the terminally processed mature protein, appear consecutively in the translocation process (Fig. 13), demonstrating that they represent true intermediates of the ΔpH/TAT-dependent translocation process. Taking advantage of the slow translocation process of 16/23, it was possible to dissect the ΔpH/TAT-dependent translocation process into three steps: 1. Membrane insertion of the signal peptide and the N-terminal part of the mature protein, resulting in the loop structured Ti-1 in association with the ΔpH/TAT machinery; 2. Translocation of the C-terminal part of the mature protein and formation of Ti-2; 3. Cleavage of the signal peptide by TPP and liberation of the mature protein into the lumen.

2.2. Unassisted or “spontaneous” loop insertion of precursor protein into the thylakoid membrane

The work presented here has focused to some extent on the early steps of the translocation process, e.g. the membrane insertion of the precursor protein leading to the formation of Ti-1. It was surprising to find that membrane insertion of the 16/23 protein occurs efficiently, even at very low temperature, i.e. under conditions that should prevent most enzymatic reactions.

Since ΔpH/TAT-dependent translocation in chloroplasts requires no soluble component of the stroma, it is assumed that there must be a receptor-like component in the thylakoid membrane that guides the precursor protein to a protein-conducting channel. However, the data shown here demonstrate that such a receptor is not necessary for insertion of the 16/23 protein into the thylakoid membrane. Neither extraction of thylakoid vesicles with chaotropic solutions nor digestion with protease, which blocks both the Sec- or SRP-dependent protein translocation processes, affects the formation of Ti-1 of the 16/23 protein (Fig. 18). Likewise, antibodies against the three TAT components block the full translocation of 16/23 and all other ΔpH/TAT pathway proteins, but still allows for the accumulation of Ti-1 (Fig. 45), suggesting that the TAT components are not involved in the first insertion step of the 16/23 protein. Membrane insertion of 16/23 and the formation of the Ti-1 are also independent of the thylakoidal ΔpH, which is, however, essential for the full translocation of the C-terminal part of the mature polypeptide (Fig. 22). These data strongly suggest a model in which the membrane insertion of 16/23 and the formation of Ti-1 is an “unassisted” or even “spontaneous” process.

This assumption was confirmed in thylakoid import experiments using derivatives of the 16/23 protein carrying conservative substitutions of the twin-arginine motif. When the RR-motif is replaced by either KR, RK or KK, the mutant proteins are still able to insert into the thylakoid membrane in a topology identical to that of Ti-1 found with the original 16/23 protein (Frielingsdorf, 2003), This is particularly remarkable for the KK mutant which is not anymore recognized by the ΔpH/TAT dependent translocation machinery. The original 16/23 and all mutant proteins exhibit membrane insertion properties even in experiments with reconstituted phospholipid bilayers, in which no proteinaceous components are present (Fig. 24 and data not shown). These results confirmed that the membrane insertion of the 16/23 protein can indeed take place in an “unassisted” manner. On the other hand, the membrane inserted protein is apparently stable only if it is compatible with the ΔpH/TAT-dependent translocase (Frielingsdorf, 2003), implying that the thylakoid membrane possesses a quality

control or proof-reading mechanism which removes those proteins that are incompatible with the translocation machinery. Therefore, it can be assumed that upon insertion into the thylakoid membrane, the precursor proteins are protected by the translocation machinery. Possibly, the inserted polypeptides are quickly inserted into the protein conducting channel, where they are not anymore accessible to degradation activities. Like the chimeric 16/23 protein, also authentic ΔpH/TAT pathway substrates such as pre-OE16 and pre-OE23 are able to insert into the thylakoid membrane in an unassisted manner (Fig. 20+34). This suggests that loop insertion is the initiation step for all ΔpH/TAT pathway substrates, and not a particular feature of the chimeric 16/23 protein.

2.3. Folding or unfolding of passenger proteins

Another remarkable feature of the ΔpH/TAT-dependent transport is the fact that the passenger protein is apparently translocated in a single step as a complete unit, suggesting that transport of passenger proteins takes place in a folded conformation. In neither of the kinetic experiments shown here, any further intermediate degradation product between Ti-1 and Ti-2 could be detected. This is in clear contrast to the results for the Sec-pathway which show that the passenger polypeptide is pulled through the thylakoid membrane in an extended conformation (Economou et al, 1995). Indeed, in aqueous solutions, the mature part of the 16/23 protein is almost completely folded, because it is degraded by trypsin only a 21 kDa fragment, i.e. to a size that is only 2 kDa smaller than the full-size mature polypeptide of OE23 (Fig. 21), in accord with the results described for the authentic OE23 protein during its passage through the stromal space (Creighton et al., 1995). However, the formation of the loop intermediate of Ti-1 demands for insertion of at least the N-terminal 80 residues of the mature polypeptide into the thylakoid membrane (Fig. 12), which implies that this part of the mature polypeptide needs to at least partially unfold upon insertion. Folding of ΔpH/TAT pathway passenger proteins before their translocation has been demonstrated both *in vitro* and *in vivo*, for example for the Rieske protein (Molik et al., 2002) and the green fluorescent protein

(Marques et al., 2003; Spence et al., 2003). It has been proven also for many bacterial TAT substrates (e.g. Santini et al., 1998; Hynds et al., 1998). In *E. coli*, numerous substrates of the TAT-pathway acquire their cofactors in the cytoplasm, and are thus transported in a folded form. For example, the high potential iron-sulfur protein (HiPIP) from *Allochromatium vinosum* was shown to be translocated into the periplasmic space by the TAT system of *E. coli* (Brüser et al., 2003). This protein contains one [4Fe-4S] cluster that is assembled into the pre-HiPIP already in the cytoplasm, i.e. prior to its TAT-dependent translocation. Similar to the thylakoid translocation of 16/23, pre-HiPIP is apparently inserted into the plasma membrane already before translocation of its C-terminal cofactor-containing domain. Pre-HiPIP is anchored to the plasma membrane through the H-domain of its signal peptide and the N-terminal half of its mature polypeptide, although there is so far no evidence that pre-HiPIP assumes also a loop topology within the membrane. Data have shown that reconstituted pre-HiPIP, which was used in these experiments, is folded into the native conformation. Thus, membrane insertion of this protein requires the unfolding of the N-terminal half of its mature polypeptide without losing its cofactor. This unfolding process presumably needs energy in form of NTPs, but the membrane insertion step seems to be independent of any forms of energy. Membrane insertion of pre-HiPIP is not affected when the TAT components are absent from the plasma membrane, and is also not abolished when the twin-R motif of the signal peptide is changed to KK, suggesting that the TAT machinery is not involved in the membrane insertion step (Brüser et al., 2003). These results strongly suggest that the bacterial TAT-dependent translocation might also be initiated by a spontaneous insertion step involving both the signal peptide and the N-terminal part of the passenger protein. Therefore, not only the signal peptide but also the mature polypeptide of ΔpH/TAT pathway substrates might be critical for the specific targeting of precursor proteins to the translocation machinery. This assumption might lead in future to the identification of targeting information also within the mature parts of ΔpH/TAT-dependent substrates. Indeed, it turned out already that a relatively hydrophobic or amphipathic region within the mature polypeptide is apparently essential for membrane insertion of those proteins (see below).

2.4. The consequences of loop insertion – obligatory for transporting folded proteins?

The unassisted insertion of the 16/23 protein proposed here resembles the mechanism of membrane insertion of the M13 procoat protein into the plasma membrane of *E.coli*. However, recent work has shown that insertion of the M13 procoat is not a spontaneous process but instead depends on an integral membrane protein, YidC. The chloroplast homologue to YidC, Alb3, is probably not required for membrane insertion of the 16/23 protein, although it is found to be involved in protein transport by the chloroplast SRP-pathway, e.g. for LHCP. Antibodies to Alb3, which strongly inhibit thylakoid membrane insertion of LHCP, have no effect on the membrane insertion or any further translocation of the 16/23 protein (data not shown). The ability to insert in an unassisted manner so far appeared to be restricted to some thylakoid membrane proteins. It might be enabled by the unusual composition of the thylakoid membrane, which contains a high proportion of galactolipids. Of course, the data presented here cannot exclude that an as yet uncharacterized proteinaceous component is essential already for the early insertion step and the formation of the loop structure, but so far there is no evidence for this assumption. On the other hand, it is also not known so far whether the spontaneous insertion is an obligatory or just an alternative step during ΔpH/TAT dependent protein translocation.

The biochemical consequences of membrane insertion of the precursor protein need to be further examined in the future. Through formation of a loop structure within the thylakoid membrane, the signal peptide may place the recognition sites such as the RR-motif to the right position to allow their interaction with the translocation machinery. The RR-motif binding site of the ΔpH/TAT machinery must apparently be located within or close to the thylakoid membrane, since the distance between the RR-motif and the hydrophobic domain of the transit peptide is so critical. Insertion of a single hydrophilic residue between the RR-motif and the h-domain of the signal peptide could abolish ΔpH/TAT-dependent translocation (Brink et al., 1998). This is in line also with the fact that almost all of the critical residues of

the three components of the TAT-machinery, notably TatA, TatB and TatC, are located within or close to the membrane-spanning domains and include even loops on the *trans* side, while only few of them are found in the hydrophilic domains on the *cis* side (Lee et al., 2002; Allen et al., 2002). This fits well also with the results of the peptide scans shown here, demonstrating that TatB and TatC but not TatA contain specific interaction sites for Tat substrates. These interaction sites are located almost exclusively within the transmembrane domains of these two proteins as well as in lumenal loops of TatC (Fig. 57, 62+63). Interaction of precursor protein with the transmembrane domains is probably mediated through hydrophobic forces, because also Sec-signal peptides show some interaction to these regions, whereas the interaction to the lumenal loops is strictly TAT-specific (Fig. 59). Unexpectedly, the large stromal domains of the three TAT proteins show no interaction with the precursor proteins, suggesting that they probably do not provide any receptor function. Instead, the data shown here indicate that the substrate proteins are recognized by the TAT machinery only within and on the *trans* side of at the thylakoid membrane, which supports the idea that membrane insertion of the precursor proteins is a prerequisite for TAT-dependent transport.

Almost all other protein transport systems employ a receptor on the *cis* side of a membrane to guide the precursors to the protein conducting channel. Therefore, recognition between the translocation machinery and the substrates normally begins in an aqueous environment. Why does the ΔpH/TAT-pathway employ instead this special mechanism? Upon membrane insertion, the polypeptide of the precursor protein is brought to the protein conducting channel possibly by hydrophobic affinity, instead of penetrating the channel directly from the top, as most other protein translocation pathways do. By this means, the polypeptide can bind to a translocase which is not yet fully assembled, without consuming the thylakoidal ΔpH. Considering that the channel could reach diameters of more than 80 nm, depending on the demands of the folded substrate protein, the channel could remain sealed most of the time, which is essential for maintaining the proton gradient. Therefore, this specialized membrane

insertion step might have been developed to meet the demands of translocating folded proteins while keeping the membrane proton gradient sealed.

2.5. The possible reason for retardation of TPP cleavage

The kinetic experiments have also revealed that the retardation of the translocation process occurs specifically at the last step, i.e. the cleavage of the transit peptide and the release of the mature polypeptide. All earlier steps of the ΔpH/TAT-dependent translocation are very efficient, since most of the 16/23 protein is converted into Ti-2 within 10 minutes incubation. It seems that the combination of the OE16 transit peptide and the OE23 mature polypeptide inhibits TPP cleavage. The reason for the inefficient TPP-cleavage of the 16/23 protein might be the slightly higher hydrophobicity of the c-domain of the OE16 signal peptide in comparison to that of OE23. Since also the N-terminus of the mature OE23 protein is more hydrophobic than the corresponding region of the OE16 protein, in the 16/23 protein the sequences flanking the TPP cleavage site are significantly more hydrophobic. This might hinder the presentation of the TPP cleavage site on the lumenal side of the thylakoid membrane and thus prevent or at least affect the processing by TPP which in turn would retard the liberation of the mature protein into the thylakoid lumen. In line with that, the increased hydrophobicity would stabilize Ti-1 of the 16/23 protein, which would explain also its strong accumulation in the thylakoid membrane. Of course, this scenario is so far still speculative, but it could experimentally be examined in the future.

In summary, ΔpH/TAT-dependent translocation of 16/23 takes place in at least four distinct steps, and is initiated by an unassisted or “spontaneous” membrane insertion, which seems to be a common step also for authentic ΔpH/TAT pathway proteins. Transport of the passenger protein takes place presumably in a folded manner. The TAT-dependent translocase as well as the thylakoidal ΔpH are required for the translocation of the folded C-terminal part of the passenger polypeptide. TPP cleavage of the signal peptide leads to the liberation of the mature

polypeptide into the thylakoid lumen, which is retarded in case of 16/23, possibly due to the change of hydrophobicity around the TPP cleavage site. The working model for the ΔpH/TAT-dependent translocation process is shown in Fig. 64.

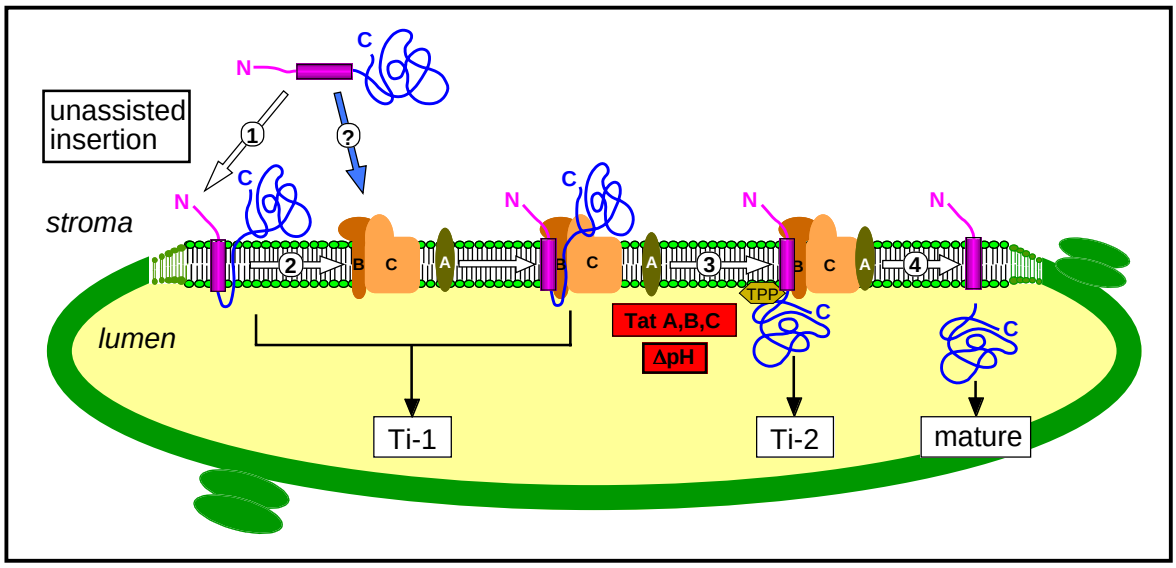

Fig. 64. Working model of the ΔpH/TAT-dependent translocation pathway. The ΔpH/TAT-dependent translocation process can be dissected into at least four steps: 1. Unassisted or "spontaneous" loop insertion of precursor protein into the thylakoid membrane. 2. Binding of Ti-1 to the translocation machinery. 3. Association of TatA to the TatB/C/substrate complex and translocation of the C-terminal part of the passenger protein. 4. TPP cleavage and release of the mature protein. The actual translocation step (3.) requires all three TAT components and the thylakoidal ΔpH. Direct interaction of precursor protein to the translocation machinery, which might take place as well, is indicated by a shaded arrow. For further details see text.

3. Structural basis for the "spontaneous" membrane insertion of the ΔpH/TAT pathway proteins

Selective targeting of the thylakoid proteins to specific translocation pathways was for a long time one of the main topics of this field. It is accepted since long that the lumen targeting domain of the transit peptide carries exclusive targeting information (Henry et al., 1994; Robinson et al., 1994), whereas the nature of the mature polypeptide only dictates the

transport efficiency. In the third section of this work, some targeting information within the mature polypeptide of ΔpH/TAT-pathway proteins has been suggested. It turned out that in addition to the signal peptide, an amphipathic region within the N-terminal part of the mature polypeptide of 16/23 is probably involved in membrane insertion and formation of translocation intermediate of 16/23. This region is characterized by charged residues, and has a strong tendency to form an amphipathic α-helix. Deletion of this region completely abolishes membrane insertion of the protein (Fig. 27). Therefore, it is assumed that this amphipathic α-helix in concert with the h-domain of the signal peptide is responsible for the loop insertion of the 16/23 protein, in analogy to the process of spontaneous insertion of specified thylakoid membrane proteins (Woolhead et al., 2001).

Potential amphipathic regions can be identified in the mature polypeptides of three authentic ΔpH/TAT-substrates (OE23, OE16 and Rieske), and translocation intermediates corresponding in size to the positions of these amphipathic regions were detected during thylakoid transport of each of the respective proteins (Fig. 32, 34, and 36). These data strongly suggest that the loop insertion of a precursor protein is a general step in ΔpH/TAT-dependent translocation. Notably, the Rieske protein is able to insert into the thylakoid membrane in cofactor-loaded state (i.e. during *in organello* import) and in a cofactor-free state (i.e. during *in thylakoido* import) (Fig. 36). This indicates that binding of the cofactor has no influence on loop insertion of this protein, because the amphipathic α-helix is located upstream of the cofactor-binding domain.

A genome-based survey of the thylakoid lumen proteins has been performed. In light of the completion of the *Arabidopsis thaliana* genome sequencing project, the proteome analysis of the Arabidopsis thylakoid lumen was done by Schröder and his coworkers, which showed that the Arabidopsis thylakoid lumen contains roughly 80 proteins, and 55 of them were at least theoretically proved (Schubert et al., 2001). Within these 55 proteins, 25 were predicted to be transported by the Sec pathway, whereas the other 30 proteins by the ΔpH/TAT pathway.

When using the online software ProtScale to calculate the hydropathicity of those proteins, almost all of the predicted ΔpH/TAT pathway proteins except one protein (Q9LM71) appear significant amphipathic helix within the mature part. However, almost one half of the Sec pathway proteins (12 proteins of 25 proteins) show no amphipathic region within the mature part, whereas the other 13 proteins carry amphipathic region within the mature protein. These results indicate that an amphipathic helix with the mature part is the prerequisite for being a ΔpH/TAT-substrate. These results also imply that a Sec-pathway protein is not necessarily transported by the ΔpH/TAT-pathway if an amphipathic helix is absent, which is in agreement with previous observation (Clausmeyer et al., 1993; Chaddock et al., 1995; Brink et al., 1998).

One further remarkable aspect of the membrane insertion of the ΔpH/TAT-pathway proteins is that in this case relatively large hydrophilic loop can be targeted across the thylakoid membrane. While the spontaneous pathway translocates hydrophilic loops containing only 20-40 residues, membrane insertion of ΔpH/TAT-pathway proteins allows translocation of a wide range of hydrophilic fragments covering length from 46 residues (Rieske) to 105 residues (16/23R119-151) (Fig. 36 and 29). These hydrophilic fragments may also play a role in the membrane insertion process, since some changes in length and static charge of these loop regions resulted in failure of insertion (Fig. 29). However, the mechanism of this insertion is still far from being understood, and the present data cannot even exclude the involvement of a protease-resistant factor mediating this insertion.

4. The role of the TAT proteins in ΔpH/TAT-dependent translocation

The involvement of the membrane-embedded translocase in ΔpH/TAT-dependent transport was first analyzed by using native electrophoresis. Membrane-bound 16/23 protein was found to be present in two membrane complexes on blue native-PAA gels, with sizes of 560 kDa and 620 kDa, respectively (Fig. 16; see also Berghöfer and Klösgen, 1999). Further studies

have proven that the two translocation intermediates are associated with both of the two membrane complexes, despite of their divergent topologies in the thylakoid membrane (Fig. 17). The mature protein, on the other hand, is not present in either of these complexes, suggesting that upon TPP cleavage, the mature protein is rapidly release into the thylakoid lumen. Antibodies specific to either of the three TAT components could inhibit thylakoid translocation of ΔpH/TAT-pathway substrates, while having no effect on the transport of the Sec-pathway protein, demonstrating that all three TAT proteins are essential for ΔpH/TAT-dependent translocation. The inhibitionary effect of the TatB and TatC antibodies is much stronger than that of the TatA antibody, suggesting a more central role of these two proteins in the TAT translocase. Indeed, both TatB and TatC were found to form a complex together with the transport substrate 16/23. In contrast, TatA was not found together with either the TatB/C complex or the substrate proteins under the native conditions (Fig. 46 and 47). Instead, TatA is mostly found as a monomer or maximally a dimer, and partially in a complex of about 350 kDa. This was confirmed in several independent experiments, including co-immunoprecipitation and chromatography (Fig. 48 and 49), indicating that interaction of TatA to the other two TAT components and precursor proteins is weak and takes place probably transiently. In the same line, substrate interaction domains could be identified for TatB and TatC by screenings the peptide libraries, while TatA did not show any direct interaction with precursor proteins.

The chloroplast ΔpH/TAT-dependent pathway is capable of transporting a wide range of folded protein from 3.6 kDa to about 80 kDa size (Schubert et al., 2001). Therefore, a dynamic protein channel has also been hypothesized (Mori and Cline, 2001). A complex of roughly 700 kDa which consists of TatB and TatC was also observed by several other groups (Bolhuis et al., 2001; Cline et al., 2001). However, TatA was not detected in this complex. Instead, it was found in smaller complexes ranging in size form 400 kDa to 70 kDa. With respect to the presence of both the substrate protein and thylakoidal ΔpH, TatA was found to complex together with TatBC only in a cross-linking approach (Cline and Mori, 2001; Mori et

al., 2002). These data, along with the fact that an excessive amount of TatA is present in the thylakoid membrane, led to the hypothesis that TatA assemblies might represent the protein conducting channels whose size would be regulated upon the sizes of the transported proteins Müller and Klösgen, 2005). The data obtained in this work support this hypothesis, but in the meanwhile, do not exclude the involvement of other unknown proteins involved in the formation of the TAT translocase, while many further proteins were co-isolated together with TatB and TatC (Fig. 50).

It is remarkable that TatB and TatC of thylakoid are present together in four complexes, though only two of them show affinity to precursor proteins (Fig. 41+42). These data suggest that TatB and TatC might also function in a dynamic manner. It seems that the TAT-dependent translocase might consist of more than one copy of the TatBC dimer, and limited number of the TatBC dimer might form subcomplex. Interaction with the membrane inserted substrate protein promotes assembly of these subcomplexes into a full functional translocase, and this process involves the loading of TatA. Therefore, formation of the full-size TAT-dependent translocase seems to be a complex step-wise process, and many details of this process remain to be determined. Apparently, this reflects the obligatory of transporting vastly differing sizes of folded TAT substrates by the ΔpH/TAT-dependent transport pathway.

SUMMARY

In chloroplasts of higher plants, a subset of thylakoid proteins with signal peptides harbouring a twin-arginine consensus motif is transported by the thylakoidal ΔpH- or TAT (twin-arginine translocation)-dependent pathway. This pathway is phylogenetically related to the bacterial TAT pathway of the cytoplasmic membrane. One of the remarkable features of these pathways is that they are capable of translocating fully folded proteins, which in many cases carry a complex cofactor. Translocation is mediated by a TAT translocase within the thylakoid membrane or bacterial cytoplasmic membrane, which has been suggested so far to comprise the three membrane proteins TatA, TatB and TatC. Translocation of ΔpH/TAT-dependent proteins is demanding, because the membranes must maintain most of their transmembrane potentials while transporting the large folded proteins. The aim of this work was to understand the mechanism of ΔpH/TAT-pathway in more detail. In this work, different substrate proteins have been analyzed by *in vitro* thylakoidal import experiments, and the following results have been obtained:

1. Thylakoid translocation of GFOR. A bacterial TAT protein, the glucose-fructose oxidoreductase (GFOR) from *Zymomonas mobilis*, was imported into thylakoid vesicles isolated from pea chloroplasts. It turned out that thylakoid transport of GFOR is dependent on the thylakoidal ΔpH, and is competed by an authentic thylakoid protein transported by the ΔpH/TAT-dependent transport pathway. These data demonstrate that both TAT pathways from bacteria and chloroplasts are related to each other and probably operate with similar mechanisms.

2. Mechanism of the ΔpH/TAT-dependent translocation at the thylakoid membrane. Studies on the mechanism of the ΔpH/TAT-dependent translocation were predominantly performed with a chimeric protein, called 16/23. Transport of this protein is retarded and two distinct translocation intermediates can be distinguished. The translocation process can be dissected into four steps: *1*. Loop insertion of the precursor protein directly into the thylakoid membrane. *2*. Interaction of the precursor protein with the TAT-translocase located within the

thylakoid membrane. *3*. Translocation of the C-terminal part of the mature polypeptide. *4*. Liberation of the mature protein into the thylakoid lumen. The third step, i.e. translocation of the C-terminal part of the mature polypeptide, involves the function of the TAT translocase and dependent on the thylakoidal ΔpH, and is thus the actual translocation step. In contrast, loop insertion of the precursor protein is independent of any transport machineries and the ΔpH, and is therefore presumably an unassisted or even "spontaneous" process.

3. Is there targeting information also within the mature polypeptide? Loop insertion of 16/23 requires an amphipathic α-helical region in the N-terminal part of the mature polypeptide of 16/23. Deletion of this region inhibits ΔpH/TAT-dependent translocation, although a perfect ΔpH/TAT-signal peptide is still present. Several authentic ΔpH/TAT-dependent passenger proteins show also this feature, indicating that the requirement of an amphipathic α-helical region is a general feature of such processes. These data suggest that in addition to the signal peptide, the passenger protein might also carry decisive information for membrane translocation by the ΔpH/TAT-dependent translocation machinery.

4. Roles of the TAT proteins. Three TAT proteins were cloned from both Arabidopsis and pea. The function and organization of these proteins have been studied. The data presented here show that all three TAT proteins are essential for translocation of ΔpH/TAT-dependent proteins. However, only TatB and TatC form a permanent complex and show affinity to the translocation intermediates of substrate proteins under native conditions. In contrast, TatA appears not to be present in the complex consisted of TatB/C and the translocation intermediates, suggesting a dynamic role of this protein. This has been confirmed by *in vitro* binding experiments in which numerous binding sites for substrate proteins were identified in TatB and TatC, but not in TatA. Purification of a Tat-translocase complex showed that it consisted of TatB and TatC and many additional proteins of unknown origin.

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LIST OF PUBLICATIONS

Hou B., Frielingsdorf S., Klösgen R.B. Characterization of the mechanism for the earlier steps of the thyalkoid ΔpH/TAT-dependent protein transport. In manuscript.

Hou B., Klösgen R.B. The role of the thylakoidal ΔpH in ΔpH/TAT-dependent protein transport of the thylakoid membrane. In manuscript.

Hou B., Klösgen R.B. Identification of a putative amphipathic region required for targeting within the mature bodies of proteins transported by the thylakoid ΔpH/TAT-dependent pathway – Is there targeting information also within the passenger proteins? In manuscript.

Halbig D., **Hou B.**, Freudl R., Sprenger G.A., Klösgen R.B. (1999). Bacterial proteins carrying twin-R signal peptides are specifically targeted by the delta pH-dependent transport machinery of the thylakoid membrane system. *FEBS Lett.* 447(1):95-8.

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Erklärung

Hiermit erkläre ich Eides statt, das ich mich mit der vorliegenden Dissertation erstmals um die Erlangung eines Doktorgrades bewerbe.

Bo Hou

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Erklärung

Hiermit erkläre ich, daß ich diese Arbeit selbständig und ohne Hilfe verfaßt habe und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutze habe. Die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen sind in der Arbeit als solche kenntlich gemacht.

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