The Chloroplast Protein Import Channel Toc75: Pore Properties and Interaction with Transit Peptides

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ABSTRACT The channel properties of Toc75 (the protein import pore of the outer chloroplastic membrane) were further characterized by electrophysiological measurements in planar lipid bilayers. After improvement of the Toc75 reconstitution procedure the voltage dependence of the channel open probability resembled those observed for other β-barrel pores. Studies concerning the pore size of the reconstituted Toc75 indicate the presence of a narrow restriction zone corresponding to the selectivity filter and a wider pore vestibule with diameters of ~14 Å and 26 Å, respectively. Interactions between Toc75 and different peptides (a genuine chloroplastic transit peptide, a synthetic peptide resembling a transit peptide, and a mitochondrial presequence) show that Toc75 itself is able to differentiate between these peptides and the recognition is based on both conformational and electrostatic interactions.

INTRODUCTION

According to the endosymbiont hypothesis chloroplasts originated from gram-negative, cyanobacteria-like ancestors, which were ingested by a primitive eucaryotic cell via endocytosis (Gray, 1992). During the course of the subsequently evolved symbiotic relationship the majority of the genes of the symbiont were transferred to the nucleus of the host (Martin et al., 1998). This necessitated a mechanism for the import of the now nucleus-encoded proteins, which are synthesized at free ribosomes in the cytoplasm. The components of the import machinery in the outer chloroplastic membrane have already been identified to a considerable extent and are now generally referred to as Toc proteins (translocon of the outer chloroplastic membrane) (Schnell et al., 1997). For recent reviews, see Chen and Schnell (1999), Vothknecht and Soll (2000), and Schleiff and Soll (2000). The major components identified so far are Toc160, Toc64, Toc34, and Toc75. Toc160 and Toc34 are GTP-binding proteins (Kessler et al., 1994; Seedorf et al., 1995; Bölter et al., 1998a) and at least Toc160 and perhaps also Toc34 seem to be the first contact sites of the preprotein (Kouranov and Schnell, 1997; Ma et al., 1996). Toc64 functions early in preprotein translocation, presumably as a docking protein for cytosolic cofactors (Sohrt and Soll, 2000).

Toc75 is a protease-insensitive, integral component of the outer envelope, and on this account it was already early on presumed to be the actual pore of the import machinery (Perry and Keegstra, 1994). Furthermore Toc75 was also reported to interact specifically with preproteins (Ma et al., 1996), thus accounting for the finding that the initial binding to Toc159 can be bypassed (Chen et al., 2000). Both notions are further corroborated by our first electrophysiological measurements of the reconstituted Toc75 channel (Hinnah et al., 1997).

The Toc proteins show no homologies to the components of other protein-transport systems, not even to the Tom proteins (translocon of the outer mitochondrial membrane), which have an analogous assignment. Only recently a homologue to Toc75 termed synToc75 has been identified in the outer membrane of the cyanobacterium *Synechocystis* PCC6803 (Reumann et al., 1999; Bölter et al., 1998b). The functional properties of synToc75 as determined by electrophysiological measurements are nearly indistinguishable from the properties of Toc75 as presented here (Bölter et al., 1998b).

We have previously described patch clamp experiments performed with reconstituted Toc75 (Hinnah et al., 1997). However, these experiments were hampered by the fact that only the bath side of a patch is accessible for exchange of the electrolyte solution and addition of peptides. Therefore, we started to perform bilayer measurements, but initially the fusion rates of proteoliposomes with the planar lipid bilayer were very low (Hinnah et al., 1997). As the used fusion method is dependent on the presence of open channels (Woodbury and Hall, 1988), this was to be expected considering the rather unusual voltage dependence of the open probability of Toc75 as revealed by patch clamp experiments. We have since then changed the reconstitution method. Toc75 is now reconstituted by dialysis in the presence of the detergent Mega9 or alternatively with the detergent Triton and subsequent removal of the detergent by adsorption to BioBeads. This resulted in a markedly altered voltage dependence of the open probability and sufficiently high fusion rates (for details see below). The latter now allowed for a detailed characterization of further electrophysiological properties of the Toc75 channel, in particular the effects of different (transit-) peptides on the channel conductance and selectivity. The experiments presented here deal mainly with the following questions. 1) Does the

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Toc75 channel specifically recognize transit peptides, and is it able to distinguish between a genuine transit peptide, a synthetic peptide of similar structure, and a mitochondrial presequence? 2) What kind of interaction between transit peptides and the Toc75 channel takes place (electrostatic interaction or interaction based on conformational information)?

Moreover, experiments have been performed to determine more precisely the pore size of Toc75. The previously proposed diameter of 8 to 9 Å (Hinnah et al., 1997) has repeatedly been subject to debate (Chen and Schnell, 1999; Schatz, 1998) as it seemed rather small in comparison to the diameters of other protein-conducting channels (Hill et al., 1998; Kunkele et al., 1998; Hanein et al., 1996). Indeed, this value was only a first estimation whose calculation was based solely on the conductance of the channel and was moreover dependent on several assumptions (Hille, 1992; Hinnah et al., 1997). Now we present a reconsidered value for the diameter of the constriction zone (and an additional value for the vestibules of the channel), which is in better agreement with the diameters of other protein conducting channels and would allow the translocation of partially folded preproteins.

MATERIALS AND METHODS

Expression and purification of Toc75

Heterologous expression in *Escherichia coli*, and purification of Toc75 was performed as already described in Hinnah et al. (1997). The reconstitution of in this way prepared Toc75 was performed with Mega9 (see below).

Additionally Toc75 has been expressed and purified as described in Rogl et al. (1998) with the following modifications: insoluble parts have been removed by centrifugation at 100,000 × *g* instead of gel filtration and afterwards the preparation was concentrated with centripreps (Amicon, Beverly, MA) to 1 mg/mL in 20 mM Tris/HCl, pH 8.0, 10% glycerol, 1 mM EDTA, and 0.1% Triton X-100. This preparation was reconstituted with Triton X-100 (see below). Both preparations of Toc75 were used and showed no significant differences.

Reconstitution

Reconstitution with Mega9

Small unilamellar liposomes were obtained from purified azolectin (type IV-S, Sigma, St. Louis, MO) as described (Hinnah et al., 1997), and Mega9 was added up to a concentration of 80 mM. Purified Toc75 at a concentration of 0.7 to 1.2 mg/ml in 6 M urea, 10 mM Tris/HCl, pH 6.9 was also supplemented with Mega9 up to a final concentration of 80 mM. Both solutions were mixed adjusting the lipid to protein ratio to 0.5 to 0.8 mg of protein/10 mg of lipid. The mixture was then dialyzed overnight against 5 l of 10 mM MOPS/Tris, pH 7.0 as described previously (Hill et al., 1998).

Reconstitution with Triton X-100

The Toc75 solution with 1% Triton X-100 was mixed with a liposome solution (see above) yielding a final concentration of 0.5 mg of Toc75/10 mg of lipid with 0.7% Triton X-100. The mixture was incubated with ~20:1 (w/w BioBeads/Triton X-100) BioBeads (Holloway, 1973) for 2 h with gentle stirring.

Planar lipid bilayers

Planar lipid bilayers were produced by the painting technique (Mueller et al., 1963). A solution of 80 mg/ml azolectin (type IV-S, Sigma) in *n*-decan (analytical grade, Merck, Rahway, NJ) was applied to a hole (100–500 μM diameter) in a Teflon septum, separating two bath chambers (volume 3 ml each). Both chambers were equipped with magnetic stirrers. Through continuously lowering and then reraising of the solution level, the lipid layer across the hole was gradually thinned out until a bilayer was formed. This formation was monitored optically and by capacitance measurements. The resulting bilayers had a typical capacitance of 0.5 μF/cm^2 and a resistance of >100 GΩ. The noise was 3 pA (r.m.s.) at 5-kHz bandwidth. After the formation of a stable bilayer in 20 mM KCl, 10 mM MOPS/Tris, pH 7.0 (in both chambers = symmetrical conditions), the solutions were changed by perfusion to asymmetrical conditions 250 mM/20 mM KCl, 10 mM MOPS/Tris, pH 7.0, *cis*/*trans*. An osmotic gradient of a channel-permeant solute, is, among the absolute necessity of the channel in the proteoliposome being in the open state (Woodbury and Hall, 1988), a prerequisite for fusion of proteoliposomes with the bilayer (Cohen et al., 1989). To promote attachment of the proteoliposomes to the bilayer, $CaCl_2$ was added to the *cis* chamber to a concentration of 10 to 20 mM (Niles and Cohen, 1987; Zimmerberg et al., 1980). Proteoliposomes were then added to the *cis* chamber directly below the bilayer, causing a slow flow of proteoliposomes along the bilayer surface. After fusion the electrolytes were changed to the final composition. Because we did not observe any asymmetric response of the Toc75 channels incorporated into the planar bilayer so far we did not have any experimental access to verify whether the channels were incorporated with random orientation or whether preferentially oriented Toc75 channels reveal symmetric pore properties. However, the later possibility is likely because also with only a single channel incorporated no asymmetric responses were detected at all.

The Ag/AgCl electrodes were connected to the chambers through 2 M KCl-agar bridges. The electrode of the *trans* compartment was directly connected to the headstage of a current amplifier (Axon Gene Clamp 500, Axon Instruments, Union City, CA). Reported membrane potentials are always referred to the *trans* compartment. The amplified currents were digitized at a sampling interval of 0.2 ms, filtered with a low-pass-filter at 1 kHz (Frequency Devices 902, Haverhill, MA), and fed into a Digidata1200 A/D converter (Axon Instruments) to store on the hard disk of an IBM compatible PC. For analysis, a WINDOWS-based analysis software ("SCIP" single channel investigation program) developed in our laboratory was used in combination with Origin 6.0 (Microcal Software Inc.).

Experiments

"Voltage ramp" refers to the continuous increase of voltage with a rate of 10 mV/s. It is important to note that every voltage ramp starts at 0 mV. In the diagrams of the results section two ramps are always depicted together: one from 0 mV to a positive voltage and the other from 0 mV to a negative voltage. The sequence of the ramps was regularly switched but proved to be of no consequence for the experiments. The only exceptions are the experiments with TrOE33 where the sequence is especially mentioned and important for the interpretation of the experiments.

Calculation of pore size

The pore size has been calculated according to Hille (1992, p. 294f):

$$d = \frac{\rho G}{\pi}\left(\frac{\pi}{2} + \sqrt{\left(\frac{\pi}{2}\right)^2 + \frac{4\pi l}{\rho G}}\right)$$

in which d is the diameter of the pore, G is the conductance (410 pS in 250 mM KCl symmetrical solution), l is the length of the constriction zone, assumed to be 5 Å (following the model of Hille (1992) with a very short

TABLE 1 Hydrodynamic radius of PEG

PEG*	Hydrodynamic radius† [Å]
100 mM KCl	–
PEG 300	6 ± 0.02
PEG 400	7 ± 0.03
PEG 1000	9.4 ± 0.3
PEG 1500	10.5 ± 0.1
PEG 2000	12.2 ± 0.1
PEG 3000	14.4 ± 0.4
PEG 4000	19.2 ± 0.3
PEG 6000	25.0 ± 0.3
PEG 8000	31

*The solution contains 100 mM KCl, 10 mM MOPS/Tris, pH 7.0 with 20% (w/w) of the referred PEG.
†As given by Krasilnikov et al. (1992), value for PEG 8000 extrapolated.

constriction zone flanked by wide vestibules), and ρ is resistivity of the solution, 49.5 Ωcm for a 250-mM KCl solution, but taking into account the correction factor of Smart et al. (1997) the value is 247.5 Ωcm.

Polyethylene glycol method

This method allows the estimation of the pore size, according to Sabirov et al. (1993), Krasilnikov et al. (1992), and Bezrukov and Kasianowicz (1997).

The effect of the presence of 20% of PEG (polyethylene glycol) of different molecular weight on the channel conductance was measured. PEG are sphere-like, neutral polymers and their hydrodynamic radii are given in Table 1. The principle of the measurement is as follows. The electric conductivity of the bulk solution is lowered in the presence of PEG. At low hydrodynamic radius the PEG can enter the channel and lower the conductance of the channel by the same factor as the bulk conductivity, but as their radius increases the PEG are progressively excluded from the channel interior and the conductance begins to recover.

The conductance ratio G_{20}/G_0 is plotted against the hydrodynamic radius of the PEG, and the data are fitted according to Bezrukov and Kasianowicz (1997) with the following (modified) equation:

$$G_{20}/G_0 = \beta(1 - (1 - \sigma'/\sigma\beta)\exp(-(x^2/w_0^2))^{P3})$$

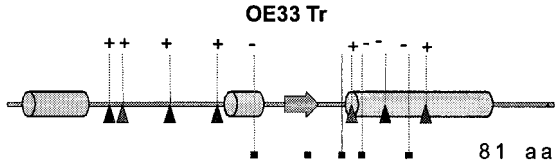
This equation yields the characteristic "cutoff" polymer radius w_0, at this radius the concentration of PEG in the pore is reduced to $1/e$ of the original concentration. Therefore, w_0 is according to Bezrukov and Kasianowicz (1997) the characteristic radius of the channel. G_0 is the conductance in 100mM KCl solution, G_{20} is the conductance in 100 mM KCl solution with 20% PEG, σ'/σ ratio of bulk conductivities in the presence and absence of polymer is 0.553, w_0 is the "cutoff" polymer radius, which is the characteristic radius of the channel, β is the maximal conductance, and P3 is set as 1.

TrOE33

TrOE33 is the transit peptide of the 33-kDa subunit of the oxygen evolving complex, which is associated with photosystem II at the lumenal side of the thylakoid membrane (Hashimoto et al., 1997). The transit peptide is accordingly bipartite, N terminus (amino acids 1–43) is the stroma targeting domain and C terminus follows the thylakoid targeting domain (amino acids 44–81), which accounts for the transport into the lumen of the thylakoid after removal of the stroma targeting domain by the CCPase (Cline et al., 1993). Only the N-terminal domain is relevant for the experiments presented here and shows a composition typical for stroma targeting domains: N terminus an uncharged region, followed by a region rich in positively charged amino acids (bold and underlined), and the whole sequence is rich in hydroxylated amino acids (bold).

MAA**S**LQAAA**T** LMQP**T**<u>**K**</u>L<u>**R**</u>**S**N **T**LQL<u>**K**</u>**S**NQ**S**V
S<u>**K**</u>AFGLEHYG A<u>***K***</u>V

At pH 7.0, TrOE33 (the complete peptide) carries four positive charges, whereas at pH 4.0 this number is increased to six. A secondary structure prediction of the stroma targeting domain resulted in the prediction of two α-helical regions (see below), contrasting the general opinion that transit peptides are perfect random coils (von Heijne and Nishikawa, 1991).



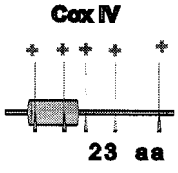
TrOE33 was expressed in *E. coli* and was in a concentration of 0.3 to 0.5 mg/mL in 100 mM NaCl, 50 mM imidazol, 8 M urea, and 20 mM Tris/HCl, pH 8.6.

CoxIV

CoxIV is a synthetic peptide identical to the mitochondrial presequence of the cytochrome oxidase subunit IV. It carries 4.8 positive charges at pH 7.0 (5 at pH 4.0) and is rich in hydroxylated residues. The structure is predicted to be an amphiphilic α-helix, characteristic for mitochondrial presequences (von Heijne et al., 1989). CoxIV fused to cytochrome oxidase subunit IV was transport-competent in import studies with isolated mitochondria (Allison and Schatz, 1986).

CoxIV comprises 23 amino acids and the sequence is as follows:

M L **S** L <u>**R**</u> Q **S** I <u>**R**</u> F F <u>**K**</u> P A T <u>**R**</u> **T** L **S S S** <u>**R**</u> Y

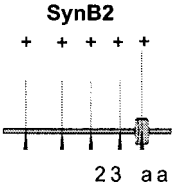


SynB2

SynB2 is an artificial peptide whose sequence is derived from CoxIV (Allison and Schatz, 1986). It possesses an identical charge of 4.8 positive charges at pH 7.0 but is in contrast to CoxIV enriched in polar amino acids (R, Q, S):

M L S <u>**R**</u> Q Q **S** Q <u>**R**</u> Q **S** <u>**R**</u> Q Q **S** Q <u>**R**</u> Q **S** <u>**R**</u> Y L L

According to our secondary structure prediction it forms a random coil, thus displaying the characteristics of a "typical" chloroplastic transit peptide as described by (von Heijne and Nishikawa, 1991). SynB2 is not transport competent in import studies with isolated mitochondria (Allison and Schatz, 1986).



CoxIV and SynB2 were a kind gift of Prof. N. Pfanner (Freiburg). The lyophilisated peptides were dissolved at a concentration of 10 mM in 10 mM MOPS/Tris, pH 7.0.

RESULTS

Heterologous expression of Toc75 in *E. coli* and purification were performed as described before (Hinnah et al., 1997).

The most frequent current transition of the reconstituted Toc75 channel corresponded to the largest conductance (Fig. 1 *A*) and is therefore named main conductance in the following. This trace (Fig. 1 *A*) also best illustrates the slow and occasional closures of Toc75 channels and the absence of openings at a high membrane potential. As derived from the current voltage relationship this main conductance has a value of 1.32 nS ± 38 pS in 1 M KCl symmetrical solution (Fig. 1 *C*) (the conductances cited in the text are the mean values of five independent measurements with 3–12 active Toc75 channels per each bilayer). Furthermore the Toc75 channel displayed two smaller and rarely encountered subconductances (Fig. 1 *B*). Subconductance 1 is represented by transitions from open to closed* (Fig. 1 *B*) (or from closed to open*, not shown) and has a value of 860 ± 41 pS (1 M KCl symmetrical), and subconductance 2 is constituted by the transition from closed to closed*, Fig. 1 *B* (or from open to open*, not shown) with a value of 460 ± 33 pS. The complete transition from open to closed, representing the main conductance, is noticeable in Fig. 1 *B*, thus corroborating the classification of the subconductances (Laver and Gage, 1997).

The results of the preceding patch-clamp measurements lead to the assumption that the conductance of 145 pS at 150 mM KCl symmetrical represented a value close to the saturation value (Hinnah et al., 1997). However, these experiments were hampered by the fact that high salt concentrations reduced the stability of the GΩ-seals and so a detailed study was not feasible. Consequently we now reexamined the single-channel conductance at numerous different KCl concentrations (0.1–3 M; data not shown) and obtained somewhat different results. The single-channel conductance revealed a linear current voltage relationship (Fig. 1 *C*) and increased almost linearly with concentration in the range of 0.1–1 M KCl, and only above 1 M KCl the single-channel conductance gradually began to saturate. The calculation of the saturation value of the conductance using a modified Michaelis-Menten equation (Hille, 1992, p. 362) resulted in $G_{max} = 6.2$ nS and $K_M = 2.49$ molal.

The reversal potential of the Toc75 channel in the presence of a salt gradient of 250 mM/20 mM KCl, 10 mM MOPS/Tris, pH 7.0, *cis*/*trans* and *trans*/*cis* was 48 mV ($n \cong 120$) and −48 mV ($n = 20$), respectively. Using these values the permeability ratio calculated by the Goldman-Hodgkin-Katz-voltage-equation was $P_{K+}/P_{Cl-} = 14.3$, denoting a marked cation selectivity. The permeability of Toc75 for different monovalent cations as calculated from reversal potentials of measurements under bi-ionic conditions ($n = 2$–3 for each ion) reflects the aqueous mobility sequence of these cations: Cs^+ ($p = 1.14$) > Rb^+ ($p = 1$) ≥ K^+ ($p = 1$) > Na^+ ($p = 0.65$) > Li^+ ($p = 0.57$). This reflects an unrestricted passage of these cations through the pore thus indicating a comparably large pore diameter.

When the pH was lowered to pH 5.0 (250/20 mM KCl, 10 mM NAc/HAc, pH 5.0, *cis*/*trans*) the reversal potential was considerably reduced to 35 mV ($n = 4$) (Fig. 2 *A*), resulting in a permeability ratio of $P_{K+}/P_{Cl-} = 6.3$. Further decrease to pH 4.0 (250/20 mM KCl, 10 mM NAc/HAc, pH 4.0, *cis*/*trans* and *trans*/*cis*) resulted in reversal potentials of 13 mV ($n = 5$) (Fig. 2 *B*) and −14 mV ($n = 2$, data not shown), respectively. The resulting permeability ratio is $P_{K+}/P_{Cl-} = 1.9$, so the Toc75 channel is only weakly cation selective at pH 4.0. This dependence of the selectivity on the pH of the aqueous phase demonstrates that the cation selectivity is apparently caused by an excess of negatively charged groups inside the pore or at the pore mouths. At pH 4.0 these groups should be mostly protonated and uncharged, therefore unable to convey a selectivity to the channel.

As already mentioned briefly, the voltage dependence of the open probability differs markedly from the one previously determined in patch clamp experiments.

In the absence of a membrane potential the Toc75 channels were constantly open (Fig. 1 *D*). In response to membrane potentials of opposite polarity, conductance behavior was symmetrical with respect to channel closure. The open probability was reduced symmetrically at higher membrane potentials; the half-maximal effect was reached at −81 mV and at 90 mV, respectively. Beyond ±120 mV the open probability approached zero.

In a first approximation the pore size of Toc75 has been calculated using an Ohmic model of conductance according to Hille (1992). The resulting diameter of 8 to 9 Å for the constriction zone (Hinnah et al., 1997) was based on the assumption that the conductivity of the electrolyte solution within the pore is equivalent to the conductivity of the bulk solution. However, Smart et al. (1997) have demonstrated that the conductivity of the electrolyte solution within the pore has to be considered to be reduced by the factor 5. Taking this correction factor into account the recalculated value for the constriction zone now equals $d = 15.4$ Å (for details see Material and Methods).

To substantiate the calculated result, the permeability of the Toc75 channel for the large organic cations tetraethylammonium (TEA^+) (cross-section ≅ 6 Å) and tetrabutylammonium (TBA^+) (cross-section ≅ 10 Å) was measured. The reversal potentials under bi-ionic conditions were used to calculate the permeability ratios according to the Goldman-Hodgkin-Katz-voltage-equation. For 250 mM TEA-Cl *cis*/250 mM KCl *trans* we obtained a reversal potential of $E_{rev} = -25 \pm 0.4$ mV ($n = 4$) and for 100 mM TBA-CL *cis*/100 mM KCL *trans* we obtained $E_{rev} = -43 \pm 1.0$ mV

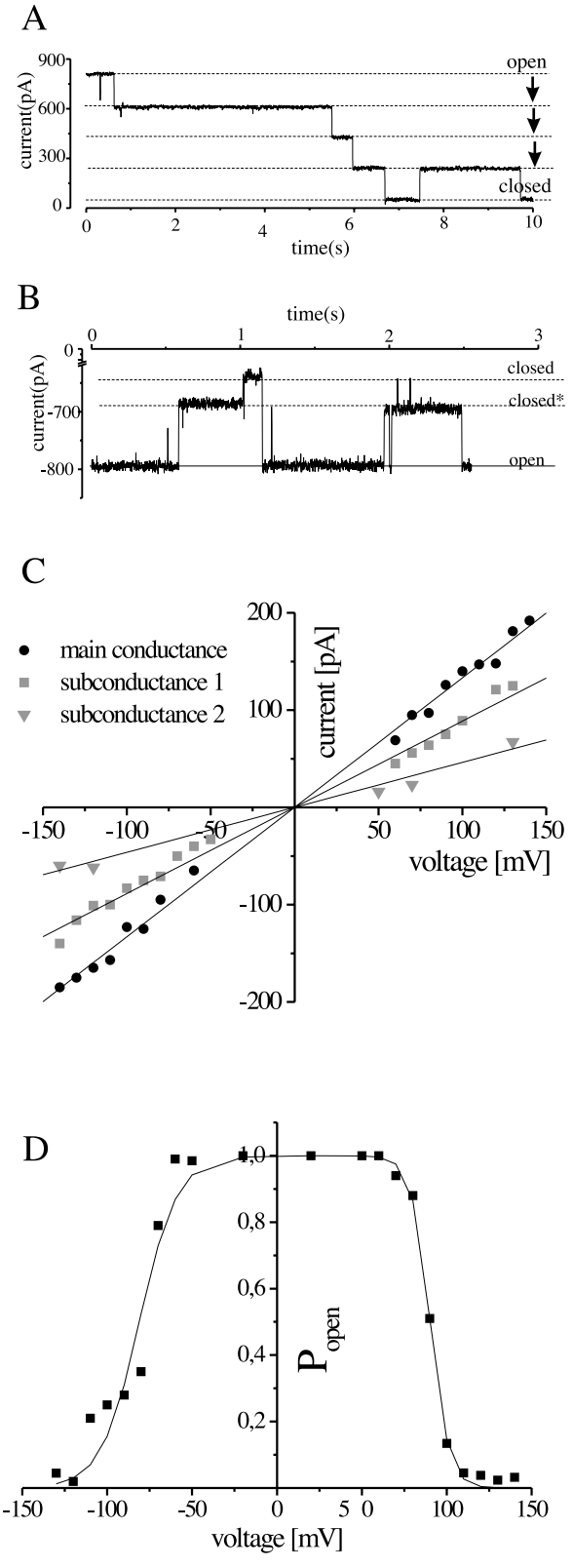


FIGURE 1 (*A–C*) Bilayer in 1 M KCl, 10 mM MOPS/Tris, pH 7.0 symmetrical solution containing nine copies of the Toc75 channel. (*A*) Current trace of this bilayer 10 s after a voltage jump to +140 mV. Only the main conductance is visible, whereas the last four channels are closing. (*B*) Current trace of the same bilayer directly after a voltage jump to −120 mV. The main conductance and the two subconductances are visible, demonstrating that the sum of the two subconductances results in the main conductance. Closed* refers to the partially closed state. (*C*) Current voltage relationship for the single open channel. The slope of the linear regression of the main conductance has a value of 1.33 nS ± 22 pS, subconductance 1 = 886 ± 22 ps and subconductance 2 = 462 ± 32 pS (in 1 M KCl). (*D*) Open probability of the Toc75 channel. Measured with three different bilayers in 1 M KCl, 10 mM MOPS/Tris, pH 7.0 symmetrical solution, each containing multiple copies of the Toc75 channel. Deviation from the mean value was below 6%. Each voltage was applied for 5 min, and the last minute of the recording was used to get the steady-state current for the respective voltage. The open probability is then the quotient of the current immediately after the voltage jump (=all channels open) and this steady-state current. Between voltage jumps the bilayer was held at 0 mV for several minutes to allow closed channels to reopen.

(n = 3). The Toc75 channel is permeable by both cations, P_{K+}/P_{TEA+} = 3.6 (n = 4) and P_{K+}/P_{TBA+} = 4.2 (n = 3). The permeability ratios roughly reflect the mobility of TEA^+/TBA^+ in aqueous solution in comparison with the mobility of K^+. Hence the pore has to be considerably larger than 10 Å to allow for this relatively unrestricted diffusion.

To gain further information on the pore dimensions of the channel, the ability of differently sized soluble nonelectrolytes to partition into the Toc75-channel was measured (PEG method; for details see Material and Methods) (Sabirov et al., 1993; Krasilnikov et al., 1992; Bezrukov and Kasianowicz, 1997). The results are depicted in Fig. 2 *C*, however, the interpretation of the experimental data is not straight forward and yet still controversial. The data are fitted according to Bezrukov and Kasianowicz (1997) and the resulting characteristic mean radius of the channel is r_{mean} = 10.8 ± 1 Å (d = 21.6 ± 2 Å). However, obviously this fit is ill suited to describe our PEG data of Toc75.

As outlined in detail by Smart et al. (1997) it seems rather improbable that a large pore like Toc75 has a single homogenous radius along its entire length (for further details, see Discussion). We therefore tend to interpret the data according to Smart et al. (1997) assuming that Toc75 has a constriction zone flanked by wide vestibules on both sides. The minimal radius of the channel (=radius of the constriction zone) is likely to be in the region where G_{20}/G_0 starts to rise (representing the starting point of PEG exclusion from the pore Fig. 2 *C*). Whereas the region where G_{20}/G_0 approaches the asymptotic end value (denoting the now nearly complete exclusion of PEG from the channel Fig. 2 *C*) is likely to be the end radius. To identify this minimal and end radius, the experimental data were fitted by a mathematically suited logistic equation (see legend to Fig. 2 *C*) and from the second derivative of the best fit (see insert to Fig. 2 *C*) we obtained values of $r_{minimum}$ = 6.8 Å and r_{end} = 12.8 Å, respectively. The minimum diameter of the Toc75 channel would than be d = 13.6 Å and the one of the wide pore mouths d = 25.6 Å.

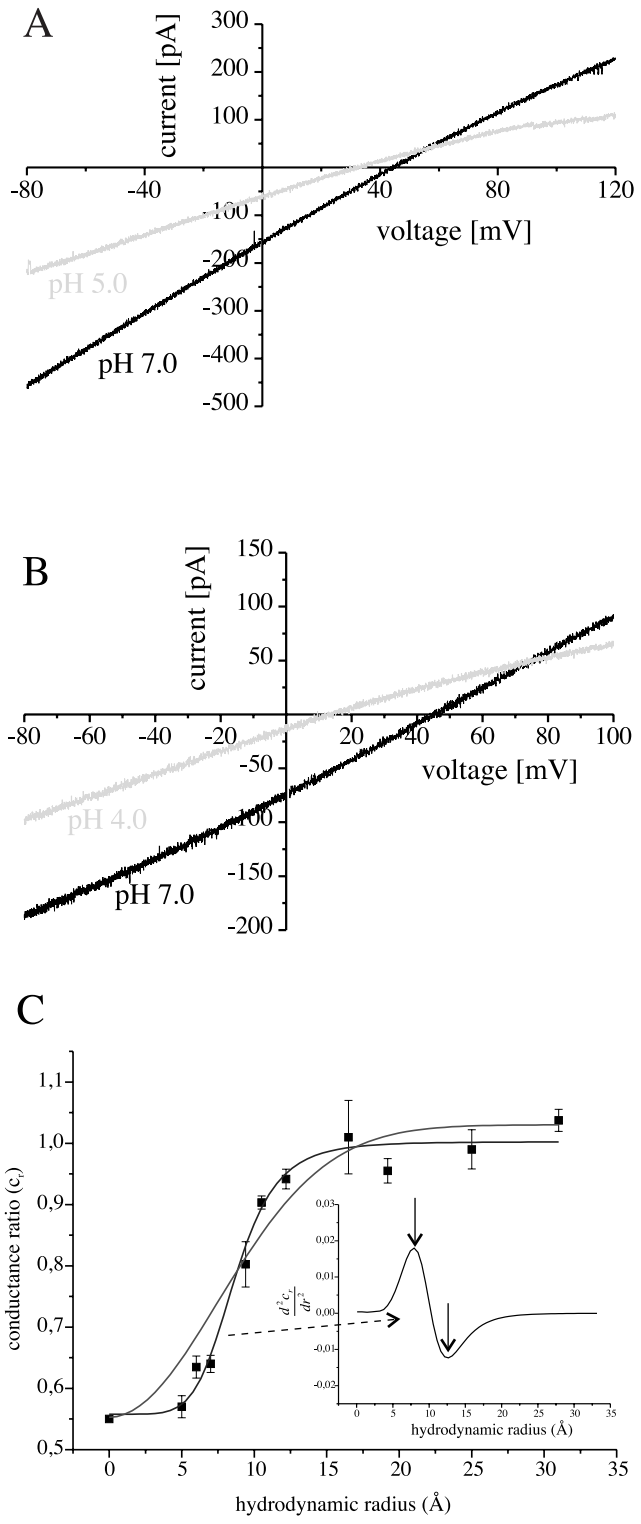


FIGURE 2 Selectivity of the Toc75 channel at different pH values and determination of pore size. Voltage ramps (10 mV/s) were applied to bilayers containing several copies of Toc75 in 250 mM/20 mM KCl, *cis*/*trans* with different pH values. For the sake of comparison, the control and modulated traces are overlaid. (*A*) Current trace of a bilayer containing 15 copies of Toc75. Black, control, 250 mM/20 mM KCl, 10 mM MOPS/Tris, pH 7.0, *cis*/*trans*; gray, the same bilayer after perfusion of both chambers to 250 mM/20 mM KCl, 10 mM NAc/HAc, pH 5.0, *cis*/*trans*. (*B*) Current trace of a bilayer containing six copies of Toc75. Black = control, 250 mM/20 mM KCl, 10 mM MOPS/Tris, pH 7.0, *cis*/*trans*; gray, the same bilayer after perfusion of both chambers to 250 mM/20 mM KCl, 10 mM NAc/HAc, pH 4.0, *cis*/*trans*. (*C*) Conductance ratio is plotted against the hydrodynamic radius of the used PEG. The data are fitted according to Bezrukov and Kasianowicz (1997), yielding a value of r_{mean} = 10.8 Å (d_{mean} = 21.6 Å) for w_0, the characteristic cutoff radius of the channel. Whereas following the interpretation of Smart et al. (1997) the radius of the constriction zone obtained from the 2nd derivative (inset) of the best fit for the PEG conductance profile is r_{min} = 6.8 Å (d = 13.6 Å), and the radius of the wide pore mouths is r_{end} = 12.8 Å (d = 25.6 Å). The experimental data in Fig. 2C were fitted using the following logistic equation: $y = (A_1 - A_2)/(1 + (x/x_0) \times P) + A_2$.

Transit peptides: TrOE33, SynB2, and CoxIV

To address the questions mentioned in the introduction we carried out experiments with three different peptides: the genuine chloroplastic transit peptide TrOE33 (transit peptide of the oxygen evolving complex of 33 kDa); the mitochondrial presequence CoxIV (Cytochromoxidase IV); and the artificial peptide SynB2, which is derived from CoxIV and carries an equal amount of positive charges but adopts a different structure (for details see Materials and Methods).

SynB2

The most obvious effect of the addition of 1 μM SynB2 to the *trans* compartment (250/20 mM KCl *cis*/*trans*) was a complete voltage-dependent block of the channel (Fig. 3 *B*). The block is only noticeable at a positive potential, the interpretation being that at this potential the positively charged peptide is forced into the pore thus plugging it and abolishing the ionic current (for details see Discussion). This explanation is sustained by the fact that the block takes the form of distinct closures (Fig. 3 *B*). Then each closure represents one single Toc75 channel that is "plugged" by SynB2 and the reopening denotes the exit of the SynB2 molecule(s). The block is completely reversible as shown by the unaltered current at a negative potential (Fig. 3, *A* and *B*) or without a potential (data not shown). When the concentrations in the chambers were reversed (20 mM/250 mM KCl, *cis*/*trans*) and SynB2 was added to the *cis* compartment the Toc75 channels were accordingly blocked at a negative potential (data not shown). This interchangeability of the side of the addition was also observed with the other two peptides (data not shown), indicating identical interactions with both sides (in particular with the pore entrances) of the Toc75 channel.

The second major effect of the addition of 1 μM SynB2 to the *trans* compartment was a shift of the reversal potential to a less positive value (control, E_{rev} = 48 mV; 1 μM SynB2, E_{rev} = 41 $\pm$ 1 mV (n = 3)). One possible explanation is the transport of the positively charged SynB2 but to account for the measured reversal potential shift the permeability (calculated according to Goldman-Hodgkin-

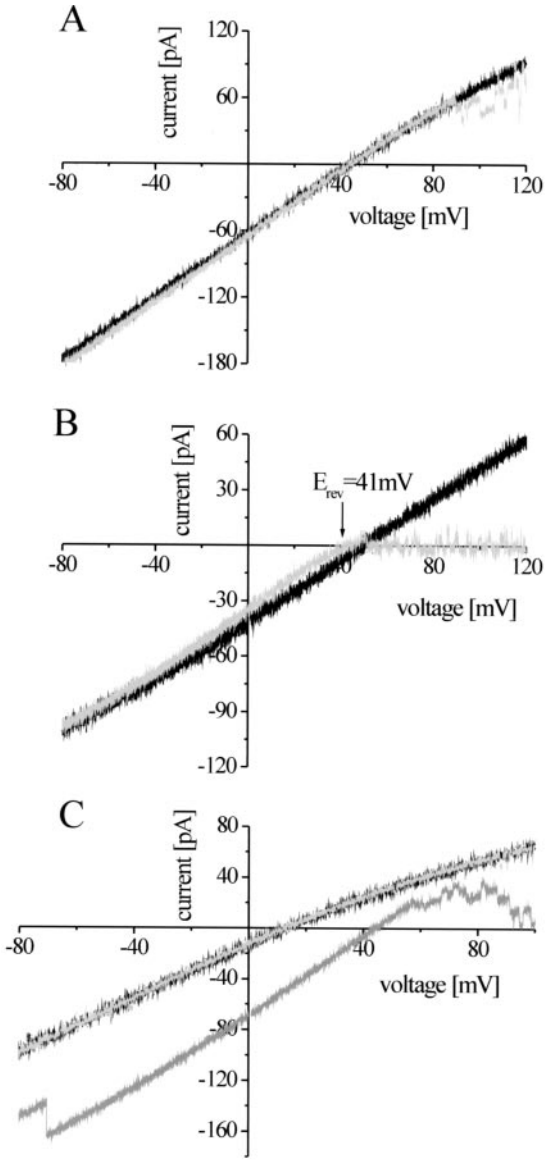


FIGURE 3 Voltage ramps (10 mV/s) were applied to bilayers containing several copies of Toc75 before and after the addition of SynB2. (*A*) Current trace of a bilayer containing five copies of Toc75 in 250 mM/20 mM KCl, 10 mM MOPS/Tris, pH 7.0. Black, control; gray, after the addition of 2 nM SynB2 to the *trans* compartment the current is reduced at higher positive voltages. (*B*) Current trace of a bilayer containing three copies of Toc75 in 250 mM/20 mM KCl, 10 mM MOPS/Tris pH 7.0. Black, Control; gray, after the addition of 1 μM SynB2 to the *trans* compartment the current is nearly completely abolished at a positive potential. (*C*) Current trace of a bilayer containing six copies of Toc75 in 250 mM/20 mM KCl, 10 mM NAc/HAc, pH 4.0. Black, Control; light gray, after the addition of 10 μM SynB2 to the *cis* and *trans* compartment the current is still identical with the control. Gray, After perfusion of both chambers to 250 mM/20 mM KCl, 10 mM MOPS/Tris, pH 7.0 the residue of SynB2 (~0.1 μM) in the *trans* chamber is sufficient to severely reduce the current at a positive potential.

Katz) would have to be $P_{SynB2} = 6700$ (with unchanged $P_{K+}/P_{Cl-} = 14.3$). But such a high permeability would be in total disagreement with the observed block. Therefore, it seems more probable that SynB2 binds and alters the selectivity of the Toc75 channel for the permeant ions K^+ and Cl^-, a reduction of the cation permeability to $P_{K+}/P_{Cl-} = 9.7$ could easily account for the observed reversal potential shift.

The strength of the block was concentration-dependent, a micromolar concentration inducing a complete block (Fig. 3 *B*) (see above) but even with nanomolar concentrations the current was visibly reduced (Fig. 3 *A*). Moreover the salt concentration exerted a considerable influence. If the peptide was added to the high salt compartment the channel block did not set until a concentration of 5 μM SynB2 was reached (data not shown).

The situation was completely altered after lowering of the pH. At pH 4.0 even the addition of 10 μM SynB2 to each compartment (250 mM/20 mM KCl, 10 mM NAc/HAc, pH 4.0, *cis*/*trans*) did not induce a block (Fig. 3 *C*). However, after perfusion of both compartments with an electrolyte solution of pH 7.0, even the residue of SynB2 in the *trans* chamber (a single perfusion accounts for an ~100-fold dilution $>$ 0.1 μM) caused a nearly complete block (Fig. 3 *C*), in accordance with the previous results (see above). Thus, the conclusion can be reached that the effect of SynB2 on the Toc75 channel at pH 7.0 is completely due to electrostatic interactions (see Discussion).

TrOE33

Essentially the effect of TrOE33 on the Toc75 channel was very similar to the effect of SynB2. The addition of nanomolar concentrations to the low salt compartment induced a voltage-dependent block (Fig. 4 *A*). This block was also concentration and salt dependent (data not shown). However, there are some significant differences between the results obtained with TrOE33 and those obtained with SynB2.

First of all, the voltage-dependent block caused by TrOE33 was even at a concentration as low as 20 nM nearly complete (Fig. 4 *A*), indicating a stronger affinity of TrOE33 to the binding site(s) within the pore as compared with SynB2. This notion is sustained by the fact that the block was not immediately reversible. This is shown in Fig. 4 *A*. First, a voltage ramp from 0 mV to +120 mV was applied and thereafter a voltage ramp from 0 mV to −80 mV. At higher positive potentials the channels are increasingly blocked and apparently some of them stay blocked hence causing the discontinuity of the two current traces at 0 mV. During the second voltage ramp the current increased in a stepwise fashion at approximately −40 to −50 mV, indicating that some of the channels are apparently "cleared" of the obstructing transit peptide(s). Hence, the affinity of TrOE33 to the binding site(s) within the pore is obviously higher than that of SynB2 as the current reduction is not completely reversible and a potential of the opposite polarity is necessary to expel the TrOE33 molecules from the channel.

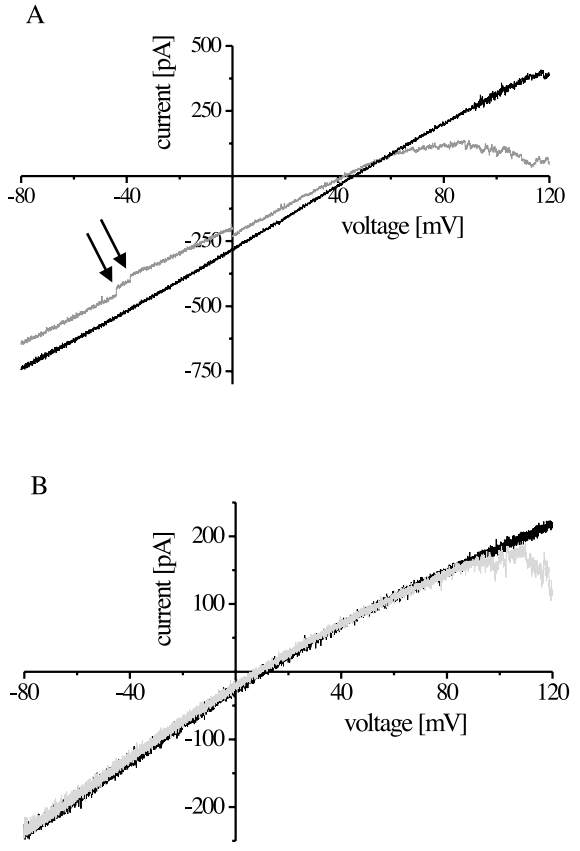


FIGURE 4 Voltage ramps (10 mV/s) were applied to bilayers containing several copies of Toc75 before and after the addition of TrOE33. (*A*) Current trace of a bilayer containing 18 copies of Toc75 in 250 mM/20 mM KCl, 10 mM MOPS/Tris, pH 7.0. Black, Control; gray: after the addition of 20 nM TrOE33 to the *trans* compartment ($\Delta E_{rev} = 5.1 \pm 1.5$ mV ($n = 5$)) the current is reduced at a positive voltage. At first some channels (three) stay closed during the consequently applied voltage ramp from 0 mV to −80 mV and only reopen later (marked by *arrows*). (*B*) Current trace of a bilayer containing 12 copies of Toc75 in 250 mM/20 mM KCl, 10 mM NAc/HAc, pH 4.0. Black, control; light gray, after the addition of 5 nM TrOE33 to the *trans* compartment the current is reduced at a higher positive potential, the effect being nearly identical to that at pH 7.0.

Second, even nanomolar concentrations of TrOE33 are sufficient to shift the reversal potential to a less positive value (Fig. 4 *A*; control, $E_{rev} = 47$ mV; +20 nM TrOE33 *trans*, $E_{rev} = 42$ mV), whereas higher concentrations of SynB2 (~1 μM) were necessary to cause a comparable effect (see above, Fig. 3 *B*, Hinnah, 1999). Following the argumentation for SynB2, the genuine transit peptide TrOE33 thus exhibits not only a higher affinity for the binding site(s) within the pore but also for the binding sites at the channel mouths. As TrOE33 carries only four positive charges at pH 7.0 it is unlikely that the higher affinity is based merely on electrostatic interactions. This notion is corroborated by the results of experiments performed at pH 4.0. Under these conditions the effect of TrOE33 on the Toc75 channel is unaltered (Fig. 4 *B*), 5 nM TrOE33 added to the low salt compartment caused the onset of current reduction and even the reversal potential is slightly shifted (250 mM/20 mM KCl, 10 mM NAc/HAc, pH 4.0, control, $E_{rev} = 11$ mV; +5 nM TrOE33 *trans*, $E_{rev} = 9$ mV ($n = 2$)). At pH 4.0, TrOE33 carries six positive charges as opposed to five for SynB2, so the effect might at least partially be due to enhanced electrostatic interactions. However, TrOE33 already exhibited a higher affinity than SynB2 to Toc75 at pH 7.0, where it only carries four positive charges as opposed to 4.8 for SynB2. Besides, the Toc75 channel is only weakly cation selective at pH 4.0 ($P_{K+}/P_{Cl-} = 1.9$) due to the presumed protonation of otherwise negatively charged amino acids. These considerations render it rather improbable that the effect is solely due to intensified electrostatic interactions. The implications of these findings will be discussed in detail later.

CoxIV

The genuine mitochondrial presequence CoxIV also induces a voltage-dependent block of the Toc75 channel. But as visible in Fig. 5, *A* and *B* higher concentrations than with TrOE33 and also with SynB2 were necessary. CoxIV (17 μM) (Fig. 5 *A*) was added to the *trans* compartment under asymmetric conditions (250 mM/20 mM KCl, 10 mM MOPS/Tris, pH 7.0, *cis*/*trans*) only induced an incomplete block. However, in Fig. 5 *A* the bilayer contains only three Toc75 channels, this experiment can therefore not serve for quantification of the effect, see below (Fig. 5 *B* for depiction of a more representative result). But in return a low number of channels in the bilayer allows a higher degree of resolution and it becomes obvious that the block is caused by an increased frequency of closures (corresponding to the main conductance value) as already perceived with SynB2 and TrOE33 (Figs. 3, *A* and *C*, and 4 *B*). Thus, the same underlying mechanism can be assumed, that is plugging of the pore by the peptide. The block was salt dependent, addition of the same amount of CoxIV (17 μM) to the high salt compartment (*cis*) caused only a slight flickering of the channels toward the closed state (Fig. 5 *A*).

The strength of the block is better represented in Fig. 5 *B*, the dark gray current trace is markedly reduced after addition of 3.33 μM CoxIV to the *trans* side of a bilayer containing ~26 Toc75 channels. But the block is still incomplete, whereas 20 nM TrOE33 (Fig. 4 *A*) was able to induce a block comparable with this one caused by 3.33 μM CoxIV, and 1 μM SynB2 have been sufficient to bring about a complete block (Fig. 3 *B*).

As visible in Fig. 5 *A* the reversal potential was also slightly shifted to a less positive value (from 47–45mV), but the effect was even less pronounced than with 1 μM SynB2. As the amount of available CoxIV was restricted the effect was not quantified by assay of higher concentrations.

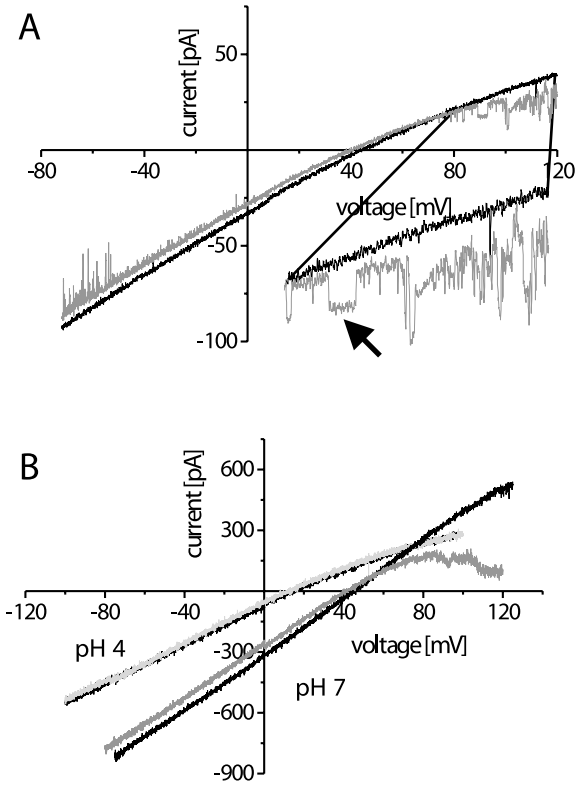


FIGURE 5 Voltage ramps (10 mV/s) were applied to bilayers containing several copies of Toc75 before and after the addition of CoxIV. (*A*) Current trace of a bilayer containing three copies of Toc75 in 250 mM/20 mM KCl, 10 mM MOPS/Tris, pH 7.0. Black, Control; gray: after the addition of 17 μM CoxIV to the *cis* and the *trans* compartment ($\Delta E_{rev} = 7.3 \pm 1.9$ mV ($n = 3$)). The current reduction at a positive voltage results mainly from an increased frequency of closures (see *arrow* in enlarged section), whereas the addition of the same amount of CoxIV to the high salt compartment (*cis*) only induces a slight increase in flickering of the channel toward closed states. (*B*) Current trace of a bilayer containing ~26 copies of Toc75 in 250 mM/20 mM KCl. Black (steeper rising trace to the right), control at pH 7.0; black (lower trace), control at pH 4.0; light gray (identical to control at pH 4.0), after the addition of 33.3 μM CoxIV to the *trans* compartment at pH 4.0 the current remains unaltered; darker gray (partly identical to control at pH 7.0), after the addition of 3.33 μM CoxIV to the *trans* compartment the current is markedly reduced at a higher positive potential.

DISCUSSION

Conductance

The main conductance of a channel is primarily a valuable identifying feature. The main conductance of Toc75 of 1.32 nS in 1 M KCl is identical to the main conductance of synToc75 (1.3 nS in 1 M KCl (Bölter et al., 1998b)), stressing the homology of the two proteins on a functional level. Toc75 shows two additional subconductance levels at 66% and 33% of the main conductance. As indicated by the dissimilarity of the subconductance levels and the direct transitions between the fully closed and fully open state (Laver and Gage, 1997), these are most likely subconductances of a single-pore channel. They may either be caused by long-lived conformational states as, e.g., described for the "allosteric" cyclic-nucleotide gated channels (Miller, 1997) or they may be due to slight alterations in the electrostatic properties in the channel's entrance vestibules (Dani and Fox, 1991). The two subconductances of Toc75 are in good accordance with the two previously determined values (Hinnah et al., 1997) and furthermore with the two subconductances of 965pS and 334pS observed with synToc75 (Bölter et al., 1998b).

The main conductance of Toc75 corresponds well to that of porins (-monomers) e.g., OmpF, OmpE, ScrY show a value of 1.4 nS in 1M KCl (Benz et al., 1985; Schülein et al., 1991), and that of Tom40, which shows under identical conditions (bilayer measurements, 1 M KCl symmetrical solution) a main conductance of 900 pS with one subconductance of 375 pS (Hill et al., 1998).

Open probability

When previously assayed by patch clamp experiments Toc75 displayed a rather unusual voltage dependence of the open probability (Hinnah et al., 1997). Only after preactivation at voltages above −100 mV and subsequent voltage jumps to potentials above +100 mV channel activity could be induced. Because the protein import of chloroplasts is not dependent on a voltage gradient (Douwe de Boer and Weisbeek, 1991), we already suspected at that time that the reason for this in vitro requirement might be incomplete reconstitution. Indeed, circular dichroism-spectra indicated a higher degree of refolding after preincubation with Mega9 (Hinnah et al., 1997). The voltage-gradient may have been necessary to effect a conformational change by interaction with charged protein parts of the incompletely reconstituted Toc75 thereby inducing a transport-competent form. A similar kind of pore-formation has been described for Colicin (Lazdunski, 1995). This concept is now substantiated by the fact that after reconstitution with Mega9 or Triton X-100 Toc75 reveals the typical, porin-like voltage dependence that has meanwhile been proposed to be an intrinsic feature of all β-barrel pores (Bainbridge et al., 1998). Secondary structure prediction, circular dichroism-spectroscopy measurements and topology studies indicate a putative structure for Toc75 consisting of 16 transmembrane β-sheets (Hinnah et al., 1997; Sveshnikova et al., 2000). These would allow folding into a β-barrel, the typical structure of a multitude of pores, e.g., bacterial porins (Jap and Walian, 1996; Kreusch et al., 1994), the mitochondrial protein import pore Tom40 (Mannella et al., 1996), and some pore-forming toxins like aerolysin (Bainbridge et al., 1998). These pores show no significant sequence homology but apart from their structure they share a strikingly similar voltage-dependence of their open probability. Thus, the observed voltage-dependence corroborates the hypothesis that after reconstitution with detergents Toc75 refolds into a β-barrel structure. In

addition, the voltage-dependence significantly resembles that of synToc75 when measured under identical conditions (Bölter et al., 1998b) and of Tom40. However, as already mentioned above this voltage dependence is considered to be an in vitro effect without significance for regulation in vivo (Klebba and Newton, 1998). According to the new concept of regulated solute exchange across the outer chloroplast envelope (Neuhaus and Wagner, 2000), the opening of the Toc75 channel should be regulated to avoid an uncontrolled flux of ions across the outer envelope.

The block of ionic current by preproteins as presented in this study suggests a way by which Toc75 could be indirectly controlled. Possibly the negatively charged receptor Toc160 (155 negative charges at pH 7.0) attracts the positively charged preproteins and augments their concentration in direct proximity of the pore entrance. Together with the high affinity of Toc75 for preproteins this would ensure the constant occupation of the pore, which prevents any uncontrolled ionic current. Thus, it is plausible that the preproteins close the channel as they are transported whereas other Toc components keep the pore closed when no transport occurs.

Selectivity and pH effect

The pronounced cation-selectivity of Toc75 at pH 7.0 ($P_{K+}/P_{Cl-} = 14.3$) is identical to that of synToc75 ($P_{K+}/P_{Cl-} = 13.3$), once more confirming their similarity. Such a high selectivity is rarely found with large pores, e.g., most porins exhibit only a weak selectivity (Jap and Walian, 1996; Cowan et al., 1992). However, the mitochondrial protein import pore Tom40 is also fairly cation-selective ($P_{K+}/P_{Cl-} = 7.7$) (Hill et al., 1998). Generally the ability to differentiate between cations and anions can be ascribed primarily to electrostatic interactions between ions and charged and/or polar groups of the channel (Laio and Torre, 1999). These groups may be localized within the pore and/or at the pore mouth(s) (the latter performing a preliminary screening of the potential solutes) as, e.g., demonstrated for porins (Karshikoff et al., 1994; Jap and Walian, 1996; Cowan et al., 1992). In the case of Toc75 the cation-selectivity can be ascribed to the presence of negatively charged groups, because at low pH (5.0 and 4.0) protonation of these groups rendered the channel progressively unselective. The localization of these groups remains to be elucidated, however, our data (namely the measurements with peptides) strongly suggest that they are not only situated in the constriction zone but also at both channel entrances (see below). The cation-selectivity can be reconciled with the natural substrates of the channel, the transit peptides, which carry an overall positive charge (von Heijne et al., 1989). But as demonstrated in the present study the recognition is not solely based on the positive charge (see results with TrOE33), so the electrostatic interactions between the transit peptide and the negative binding site(s) of the channel could have different and/or additional functions. According to the acid chain hypothesis of protein import into mitochondria (Komiya et al., 1998; Schatz, 1997), the transport of the positively charged presequences is actually driven by their interaction with negatively charged binding sites (among others binding sites at the channel Tom40) of increasing affinity. It is tempting to imagine a similar scenario between transit peptides and binding sites of Toc160, Toc75, and perhaps additional components of the inner envelope.

Pore size

The recalculation of the pore diameter under consideration of the correction factor for the resistivity of the solution within the pore resulted in a value of $d = 15.4$ Å for the constriction zone. However, the calculation is based on another assumption, the unknown length of the constriction zone (here set as 5 Å, according to Hille, 1992), thus still yielding only to an approximate value. Yet, the notion that the pore diameter has to be considerably larger than the previously calculated 8 to 9 Å is substantiated by the nearly unrestricted passage of TBA^+ (see Results). But the method of probing the pore size with charged molecules is disputable because the effective radius of a pore is not only determined by its geometrical dimensions but also by charges and the electric field within the pore (Eisenberg, 1996; Chen and Eisenberg, 1993), which may interact with the charged probes and distort the result. To avoid this problem we used nonelectrolytes (PEG) of different radius to estimate the pore size according to the polymer exclusion method of Krasilnikov et al. (1992) and Bezrukov and Kasianowicz (1997). The fit of the data according to Bezrukov and Kasianowicz (1997) yields a value of $d \cong 22$ Å as the characteristic diameter of the channel. But, as pointed out by Smart et al. (1997), this interpretation is based on the assumption that the pore is a cylindrical conductor having one single characteristic radius. But in reality most channel pores have a more complex internal geometry see, e.g., the hourglass-shaped structure of porins (Cowan et al., 1992). The assumption that Toc75 possesses a comparable geometry, namely that the pore of Toc75 has a constriction zone with $d \cong 14$ Å (thus, confirming the results of the calculation of the pore size) flanked by two wide vestibules with an opening of $d \cong 28$ Å, is corroborated by the data obtained with transit peptides, which are not intelligible without assuming this internal structure of the Toc75 pore (see below). The diameter of ~14 Å of the constriction zone lies well within the range of the values of other protein conducting pores, e.g., Tom40 with ~20 to 26 Å (Hill et al., 1998; Kunkele et al., 1998) and the central pore of the Sec61p complex has a diameter of 20 Å (Hanein et al., 1996). Studies with the preprotein prOE17, which was imported into chloroplasts despite being covalently linked to a tightly folded, protease-resistent BPTI-domain of ~23 Å in diameter (Clark and Theg, 1997) suggests an even larger pore radius for Toc75.

Conversely, this result may also indicate a flexible structure of the pore in vivo, able to expand during import as observed for the Sec61p-complex (Hamman et al., 1997).

Transit peptides

In brief, all of the three peptides caused two major effects: a voltage-dependent block and a shift of the reversal potential.

According to Woodhull (1973) the voltage-dependence of the block demonstrates that the channel is plugged by binding of a peptide within the electrical field (=within the pore). So the distinct closures in the current trace can be attributed to the presence of the peptide within the pore thus blocking the ionic current. Therefore, the voltage-dependent block demonstrates that Toc75 contains a specific binding site within the pore region. The specificity of this binding site is highest for TrOE33 as indicated by the partially irreversible block already caused by nanomolar concentrations. SynB2 is also effectively bound at nanomolar concentrations, but the block was immediately relieved in the absence of a voltage gradient indicating that the attraction was essentially based on the overall positive charge. CoxIV only induced a voltage-dependent block at micromolar concentrations and the block was readily reversible. Thus, the Toc75 channel displays a preference for the genuine transit peptide.

The question of a permeation of the peptides through the Toc75 channel cannot be decided. On account of the electrophysiological data it is impossible to discriminate between a peptide that is stuck in the pore (and consequently retreats) or one that is slowly translocated as both cause a transient block. To demonstrate permeation bi-ionic measurements would be necessary, but this is not feasible because sufficiently high amounts of peptides are not available.

Reversal potential

As already mentioned the observed shift in reversal potential cannot be explained in terms of permeation of the peptides. The required high permeability ratio is incompatible with the observed block. The only possible explanation is an alteration of the channel selectivity resembling that observed at low pH. The peptides bind to the selectivity filter(s) thus screening their negative charge and diminishing the cation selectivity. The selectivity filter(s) in question cannot be situated within the pore, because then binding would also cause block of the ionic current and furthermore be voltage dependent, but both is not the case. Therefore, the selectivity filter(s) have to be localized at the wider pore mouths, so that binding of peptides does not obstruct the passage of ions. Thus, the concept of an hourglass-shaped structure of the Toc75 channel with wide vestibules on either side of a constriction zone, as already indicated by the results of the PEG-method, is further corroborated.

The potency of the peptides to cause a shift of the reversal potential corresponds well with their efficiency in inducing a block. TrOE33 is already effective at a concentration of 20 nM, whereas 1 μM of SynB2 is necessary to induce a comparable result and 17 μM CoxIV is even less effective and causes only a slighter shift. So the binding sites at the pore entrances are likewise capable to distinguish between the chloroplastic transit peptide and the mitochondrial presequence and its derivative. Because TrOE33 is at pH 7.0 even less positively charged than the other two peptides electrostatic interactions seem not to be the only decisive factor in recognition. This observation is further corroborated by the results of experiments at pH 4.0. At this low pH the binding sites in the Toc75 channel are mostly protonated, and it has lost its affinity for SynB2 and CoxIV. TrOE33 on the other hand is now, as before, able to induce a voltage-dependent block and a shift of the reversal potential suggesting that its affinity to all binding sites is nearly unaltered. Furthermore it has already been demonstrated by experiments with deletion mutant Ferredoxin precursors that the uncharged N-terminal region of the transit peptide is essential for import (Rensink et al., 1998; Pilon et al., 1995).

Thus, the specific recognition also seems to depend on the two other possible forms of interaction, namely hydrogen bonds and/or van der Waals interactions. Hydroxylated amino acids are specifically suited to serve as H-donor and -acceptor, and as transit peptides can be distinguished from mitochondrial presequences solely on the basis of their high content in hydroxylated amino acids (von Heijne et al., 1989) it is reasonable to assume an important function in recognition. But hydrogen bonds and van der Waals interactions are relatively weak interactions that can only secure specificity when conformational compatibility exists. This would require a defined secondary structure of transit peptides contrasting the conception that they form perfect random coils (von Heijne and Nishikawa, 1991). Indeed our secondary structure prediction of TrOE33 (see diagram in Material and Methods) indicated α-helical regions in the stromal targeting domain. It has also been demonstrated that conditions that presumably mimic the membrane environment or the contact with lipid surfaces in vitro induce a significant degree of α-helical structure in several transit peptides (Endo et al., 1992; Horniak et al., 1993; Wienk et al., 1999; Krimm et al., 1999). Thus, the necessary secondary structure may in some of the transit peptides not be induced until they reach the direct proximity of the chloroplast envelope. The presumed defined secondary structure seems to consist of α-helical elements, but it has to differ from the amphiphilic α-helix of mitochondrial presequences because CoxIV is not recognized. Interestingly even the synToc75 channel (the presumed predecessor of Toc75) shows a lower affinity to CoxIV than to SynB2, suggesting a highly conserved principle of recognition (Bölter et al., 1998b). In sum our data indicate that the Toc75

channel can recognize preproteins on the basis of charge and conformation.

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REFERENCES

Allison, D. S., and G. Schatz. 1986. Artificial mitochondrial presequences. *Proc. Natl. Acad. Sci. U. S. A.* 83:9011–9015.

Bainbridge, G., I. Gokce, and J. H. Lakey. 1998. Voltage gating is a fundamental feature of porin and toxin β-barrel membrane channels. *FEBS Lett.* 431:305–308.

Benz, R., A. Schmid, and R. E. Hancock. 1985. Ion selectivity of gram-negative bacterial porins. *J. Bacteriol.* 162:722–727.

Bezrukov, S. M., and J. J. Kasianowicz. 1997. The charge state of an ion channel controls neutral polymer entry into its pore. *Eur. Biophys. J.* 26:471–476.

Bölter, B., T. May, and J. Soll. 1998a. A protein import receptor in pea chloropasts, Toc86, is only a proteolytic fragment of a larger polypeptide. *FEBS Lett.* 441:59–62.

Bölter, B., J. Soll, A. Schulz, S. C. Hinnah, and R. Wagner. 1998b. Origin of a chloroplast protein importer. *Proc. Natl. Acad. Sci. U. S. A.* 95: 15831–15836.

Chen, D. P., and R. S. Eisenberg. 1993. Charges, currents, and potentials in ionic channels of one conformation. *Biophys. J.* 64:1405–1421.

Chen, K., X. Chen, and D. J. Schnell. 2000. Initial binding of preproteins involving the toc159 receptor can be bypassed during protein import into chloroplasts. *Plant Physiol.* 122:813–822.

Chen, X. J., and D. J. Schnell. 1999. Protein import into chloroplasts. *Trends Cell Biol.* 9:222–227.

Clark, S. A., and S. M. Theg. 1997. A folded protein can be transported across the chloroplast envelope and thylakoid membranes. *Mol. Biol. Cell.* 8:923–934.

Cline, K., R. Henry, C. Li, and J. Yuan. 1993. Multiple pathways for protein transport into or across the thylakoid membrane. *EMBO J.* 12:4105–4114.

Cohen, F. S., W. D. Niles, and M. H. Akabas. 1989. Fusion of phospholipid vesicles with a planar membrane depends on the membrane permeability of the solute used to create the osmotic pressure. *J. Gen. Physiol.* 93:201–210.

Cowan, S. W., T. Schirmer, G. Rummel, M. Steiert, R. Ghosh, R. A. Pauptit, J. N. Jansonius, and J. P. Rosenbusch. 1992. Crystal structures explain functional properties of two *E. coli* porins. *Nature.* 358:727–733.

Dani, J. A., and J. A. Fox. 1991. Examination of subconductance levels arising from a single ion channel. *J. Theor. Biol.* 153:401–423.

Douwe de Boer, A., and P. J. Weisbeek. 1991. Chloroplast protein topogenesis: import, sorting and assembly. *Biochim. Biophys. Acta.* 1071:221–253.

Eisenberg, R. S. 1996. Computing the fields in proteins and channels. *J. Membr. Biol.* 150:1–25.

Endo, T., K. Kawamura, and M. Nakai. 1992. The chloroplast-targeting domain of plastocyanin transit peptide can form a helical structure but does not have a high affinity for lipid bilayers. *Eur. J. Biochem.* 207: 671–675.

Gray, M. W. 1992. The endosymbiont hypothesis revisited. *Int. Rev. Cytol.* 141:233–357.

Hamman, B. D., J. C. Chen, E. E. Johnson, and A. E. Johnson. 1997. The aqueous pore through the translocon has a diameter of 40–60 angstrom during cotranslational protein translocation at the membrane. *Cell.* 89: 535–544.

Hanein, D., K. E. S. Matlack, B. Jungnickel, K. Plath, K. U. Kalies, K. R. Miller, T. A. Rapoport, and C. W. Akey. 1996. Oligomeric rings of the sec61p complex induced by ligands required for protein translocation. *Cell.* 87:721–732.

Hashimoto, A., W. F. Ettinger, Y. Yamamoto, and S. M. Theg. 1997. Assembly of newly imported oxygen-evolving complex subunits in isolated chloroplasts - sites of assembly and mechanism of binding. *Plant Cell.* 9:441–452.

Hill, K., K. Model, M. T. Ryan, K. Dietmeier, F. Martin, R. Wagner, and N. Pfanner. 1998. Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins. *Nature.* 395:516–521.

Hille, B. 1992. Ion Channels of Excitable Membranes. Sinauer Associates Inc., Sunderland, Massachusetts.

Hinnah, S. C. 1999. Electrophysiological characterisation of the protein import pore Toc75 and its predecessor SynToc75. Thesis/Dissertation. FB Biologie/Chemie, Universität Osnabrück. 1–155.

Hinnah, S. C., K. Hill, R. Wagner, T. Schlicher, and J. Soll. 1997. Reconstitution of a chloroplast protein import channel. *EMBO J.* 16: 7351–7360.

Holloway, P. W. 1973. A simple procedure for removal of Triton X-100 from protein samples. *Anal. Biochem.* 53:301–308.

Horniak, L., M. Pilon, R. van't Hof, and B. de Kruijff. 1993. The secondary structure of the ferredoxin transit sequence is modulated by its interaction with negatively charged lipids. *FEBS Lett.* 334:241–246.

Jap, B. K., and P. J. Walian. 1996. Structure and functional mechanism of porins. *Physiol. Rev.* 76:1073–1088.

Karshikoff, A., V. Spassov, S. W. Cowan, R. Ladenstein, and T. Schirmer. 1994. Electrostatic properties of two porin channels from *Escherichia coli*. *J. Mol. Biol.* 240:372–384.

Kessler, F., G. Blobel, H. A. Pathel, and D. J. Schnell. 1994. Identification of two GTP-binding proteins in the chloroplast protein import machinery. *Science.* 266:1035–1039.

Klebba, P. E., and S. C. Newton. 1998. Mechanisms of solute transport through outer membrane porins - burning down the house. *Curr. Opin. Microbiol.* 1:238–247.

Komiya, T., S. Rospert, C. Koehler, R. Looser, G. Schatz, and K. Mihara. 1998. Interaction of mitochondrial targeting signals with acidic receptor domains along the protein import pathway: evicence for the acid chain hypothesis. *EMBO J.* 17:3886–3898.

Kouranov, A. and D. J. Schnell. 1997. Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. *J. Cell Biol.* 139:1677–1685.

Krasilnikov, O. V., R. Z. Sabirov, V. I. Ternovsky, P. G. Merzlyak, and J. N. Muratkhodjaev. 1992. A simple method for the determination of the pore radius of ion channels in planar lipid bilayer membranes. *FEMS Microbiol. Immunol.* 105:93–100.

Kreusch, A., A. Neubuser, E. Schiltz, J. Weckesser, and G. E. Schulz. 1994. Structure of the membrane channel porin from *Rhodopseudomonas blastica* at 2.0 Angstrom resolution. *Protein Sci.* 3:58–63.

Krimm, I., P. Gans, J. F. Hernandez, G. J. Arlaud, and J. M. Lancelin. 1999. A coil-helix instead of a helix-coil motif can be induced in a chloroplast transit peptide from *Chlamydomonas reinhardtii*. *Eur. J Biochem.* 265:171–180.

Kunkele, K. P., S. Heins, M. Dembowski, F. E. Nargang, R. Benz, M. Thieffry, J. Walz, R. Lill, S. Nussberger, and W. Neupert. 1998. The preprotein translocation channel of the outer membrane of mitochondria. *Cell.* 93:1009–1019.

Laio, A., and V. Torre. 1999. Physical origin of selectivity in ionic channels of biological membranes. *Biophys. J.* 76:129–148.

Laver, D. R., and P. W. Gage. 1997. Interpretation of substates in ion channels: unipores or multipores. *Progr. Biophys. Mol. Biol.* 67:99–140.

Lazdunski, C. J. 1995. Colicin import and pore formation: a system for studying protein transport across membranes. *Mol. Microbiol.* 16: 1059–1066.

Ma, Y. K., A. Kouranov, S. E. Lasala, and D. J. Schnell. 1996. Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope. *J. Cell Biol.* 134:315–327.

Mannella, C. A., A. F. Neuwald, and C. E. Lawrence. 1996. Detection of likely transmembrane beta-strand regions in sequences of mitochondrial pore proteins using the gibbs sampler. *J. Bioenerg. Biomembr.* 28: 163–169.

Martin, W., B. Stoebe, V. Goremykin, S. Hansmann, M. Hasegawa, and K. V. Kowallik. 1998. Gene transfer to the nucleus and the evolution of chloroplasts. *Nature.* 393:162–165.

Miller, C. 1997. Cuddling up to channel activation. *Nature.* 389:328–329.

Mueller, P., D. Rudin, R. Tien, and W. C. Westcott. 1963. Methods for the formation of single bimolecular lipid membranes in aqueous solution. *J. Phys. Chem.* 67:534–535.

Neuhaus, H. E., and R. Wagner. 2000. Solute pores, ion channels, and metabolite transporters in the outer and inner envelope membrane of higher plant plastids. *Biochim. Biophys. Acta.* 1465:307–323.

Niles, W. D., and F. S. Cohen. 1987. Video fluorescence microscopy studies of phospholipid vesicle fusion with a planar phospholipid membrane. *J. Gen. Physiol.* 90:703–735.

Perry, S. E., and K. Keegstra. 1994. Envelope membrane proteins that interact with chloroplastic precursor proteins. *Plant Cell.* 6:93–105.

Pilon, M., H. Wienk, W. Sips, M. de Swaaf, I. Talboom, R. van't Hof, G. de Korte-Kool, R. Demel, P. J. Weisbeek, and B. de Kruijff. 1995. Functional domains of the ferredoxin transit sequence involved in chloroplast import. *J. Biol. Chem.* 270:3882–3893.

Rensink, W. A., M. Pilon, and P. Weisbeek. 1998. Domains of a transit sequence required for in vivo import in *Arabidopsis* chloroplasts. *Plant Physiol.* 118:691–699.

Reumann, S., J. Davila-Aponte, and K. Keegstra. 1999. The evolutionary origin of the protein-translocating channel of chloroplastic envelope membranes: identification of a cyanobacterial homolog. *Proc. Natl. Acad. Sci. U. S. A.* 96:784–789.

Rogl, H., K. Kosemund, W. Kühlbrandt, and I. Collinson. 1998. Refolding of *Escherichia coli* produced membrane protein inclusion bodies immobilised by nickel chelating chromatography. *FEBS Lett.* 432:21–26.

Sabirov, R. Z., O. V. Krasilnikov, V. I. Ternovsky, and P. G. Merzlyak. 1993. Relation between ionic channel conductance and conductivity of media containing different nonelectrolytes: a novel method of pore size determination. *Gen. Physiol. Biophys.* 12:98–111.

Schatz, G. 1997. Just follow the acid chain. *Nature.* 388:121–122.

Schatz, G. 1998. The doors to organelles. *Nature.* 395:439–440.

Schleiff, E., and J. Soll. 2000. Travelling of proteins through membranes: translocation into chloroplasts. *Planta.* 211:449–456.

Smart, O. S., J. Breed, G. R. Smith, and M. S. P. Sansom. 1997. A novel method for structure-based prediction of ion channel conductance properties. *Biophys. J.* 72:1109–1126.

Schnell, D. J., G. Blobel, K. Keegstra, F. Kessler, K. Ko, and J. Soll. 1997. A consensus nomenclature for the protein-import components of the chloroplast envelope. *Trends Cell Biol.* 7:303–304.

Schülein, K., K. Schmid, and R. Benz. 1991. The sugar-specific outer membrane channel ScrY contains functional characteristics of general diffusion pores and substrate-specific porins. *Mol. Microbiol.* 5:2233–2241.

Seedorf, M., K. Waegemann, and J. Soll. 1995. A constituent of the chloroplast import complex represents a new type of GTP-binding protein. *Plant J.* 7:401–411.

Sohrt, K., and J. Soll. 2000. Toc64, a new component of the protein translocon of chloroplasts. *J. Cell Biol.* 148:1213–1221.

Sveshnikova, N., R. Grimm, J. Soll, and E. Schleiff. 2000. Topology studies of the chloroplast protein import channel Toc75. *Biol. Chem.* 381:687–693.

von Heijne, G., and K. Nishikawa. 1991. Chloroplast transit peptides: the perfect random coil? *FEBS Lett.* 271:1–3.

von Heijne, G., J. Steppuhn, and R. G. Herrman. 1989. Domain structure of mitochondrial and chloroplast targeting peptides. *Eur. J. Biochem.* 180:535–545.

Vothknecht, U. C., and J. Soll. 2000. Protein import: the hitchhikers guide into chloroplasts. *Biol. Chem.* 381:887–897.

Wienk, H. L., M. Czisch, and B. de Kruijff. 1999. The structural flexibility of the preferredoxin transit peptide. *FEBS Lett.* 453:318–326.

Woodbury, D. J., and J. E. Hall. 1988. Role of channels in the fusion of vesicles with a planar bilayer. *Biophys. J.* 54:1053–1063.

Woodhull, A. M. 1973. Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.* 61:687–708.

Zimmerberg, J., F. S. Cohen, and A. Finkelstein. 1980. Fusion of phospholipid vesicles with planar phospholipid bilayer membranes I: discharge of vesicular contents across the planar membrane. *J. Gen. Physiol.* 75:241–250.