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Reconstitution of a chloroplast protein import channel

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The chloroplastic outer envelope protein OEP75 with a molecular weight of 75 kDa probably forms the central pore of the protein import machinery of the outer chloroplastic membrane. Patch–clamp analysis shows that heterologously expressed, purified and reconstituted OEP75 constitutes a voltage-gated ion channel with a unit conductance of $\Lambda = 145$pS. Activation of the OEP75 channel *in vitro* is completely dependent on the magnitude and direction of the voltage gradient. Therefore, movements of protein charges of parts of OEP75 in the membrane electric field are required either for pore formation or its opening. In the presence of precursor protein from only one side of the bilayer, strong flickering and partial closing of the channel was observed, indicating a specific interaction of the precursor with OEP75. The comparatively low ionic conductance of OEP75 is compatible with a rather narrow aqueous pore ($d_{pore} \cong$ 8–9 Å). Provided that protein and ion translocation occur through the same pore, this implies that the environment of the polypeptide during the transit is mainly hydrophilic and that protein translocation requires almost complete unfolding of the precursor.

Keywords: bilayer/chloroplast/patch–clamp/precursor proteins/protein import

Introduction

The biogenesis of chloroplasts requires the translocation of polypeptides across the envelope membranes during all stages of chloroplast development and differentiation. Current evidence suggests that the chloroplastic import system is unique in comparison with other protein translocases, i.e. its elements show no relation to other systems, and regulation properties have developed clearly differently from those in other systems such as mitochondria (Soll, 1995; Waegemann and Soll, 1996).

Several components of the chloroplast import machinery have been identified recently in a number of different laboratories (Waegemann and Soll, 1991; Perry and Keegstra, 1994; Schnell *et al.*, 1994; Wu *et al.*, 1994). The chloroplastic outer envelope protein OEP86 serves as a receptor for precursor proteins or is part of a larger multisubunit receptor complex (Hirsch *et al.*, 1994; Perry and Keegstra, 1994). Besides being a major chloroplastic phosphoprotein, OEP 86 has separate GTP and ATP-binding sites (Kessler *et al.*, 1994; Seedorf *et al.*, 1995), thus making it a prime target for a regulatory circuit in precursor recognition. OEP86 shows strong homology to OEP34 in its cytosolically exposed N-terminus and is located in very close physical proximity to OEP75 (Seedorf *et al.*, 1995) to which it can be covalently connected by the *in vitro* formation of a disulfide bridge. OEP75 seems to form the central pore of the outer envelope translocation channel (Schnell *et al.*, 1994; Tranel *et al.*, 1995). Furthermore, OEP75 interacts specifically with the transit sequence of precursor proteins during the import process (Ma *et al.*, 1996). OEP34 represents a new type of GTP-binding protein, showing the classical GTP-binding motifs but no further sequence homology to other G-proteins except to OEP86. Like OEP86, it is located in close physical proximity to OEP75 to which it can be covalently connected by the *in vitro* formation of disulfide bridges (Seedorf and Soll, 1995). Overexpressed OEP34 exhibits GTPase activity (Seedorf *et al.*, 1995). OEP70 is an outer envelope localized hsc70 (heat shock cognate protein of 70 kDa) homologue which could be involved in the translocation process (Marshall *et al.*, 1990; Waegemann and Soll, 1991). The gene for OEP70 has not yet been isolated. Further progress in understanding protein translocation into chloroplasts is limited by the lack of information regarding the molecular functions of components of the transport apparatus and the cooperation of the subunits. We synthesized the chloroplastic import machinery component OEP75 *in vitro* and performed its functional reconstitution into liposomes. Successful reconstitution of protein translocation machineries has been achieved previously either only of total solubilized rough microsome membranes or of *Escherichia coli* plasma membranes (Nicchitta and Blobel, 1990; Nicchitta *et al.*, 1991). Rapoport and co-workers have used biochemically purified components of the cotranslational protein translocation system of the endoplasmatic reticulum of mammals and yeast to study mechanistic details of the transport event (Görlich and Rapoport, 1993; Hanein *et al.*, 1996). Although these elegant experiments give important new insights into functional details, they are hampered by the fact that no modification of the components used is possible, which, however, is a prerequisite to answering mechanistic questions.

In this study we report on the successful reconstitution of *in vitro* synthesized OEP75 which forms the central translocation channel of the chloroplastic outer envelope translocase. The electrophysiological properties of OEP75 show that it is capable of forming a rather narrow translocation pore suggesting that the passage of precursor protein across the chloroplastic outer envelope occurs in a mainly unfolded and extended conformation.

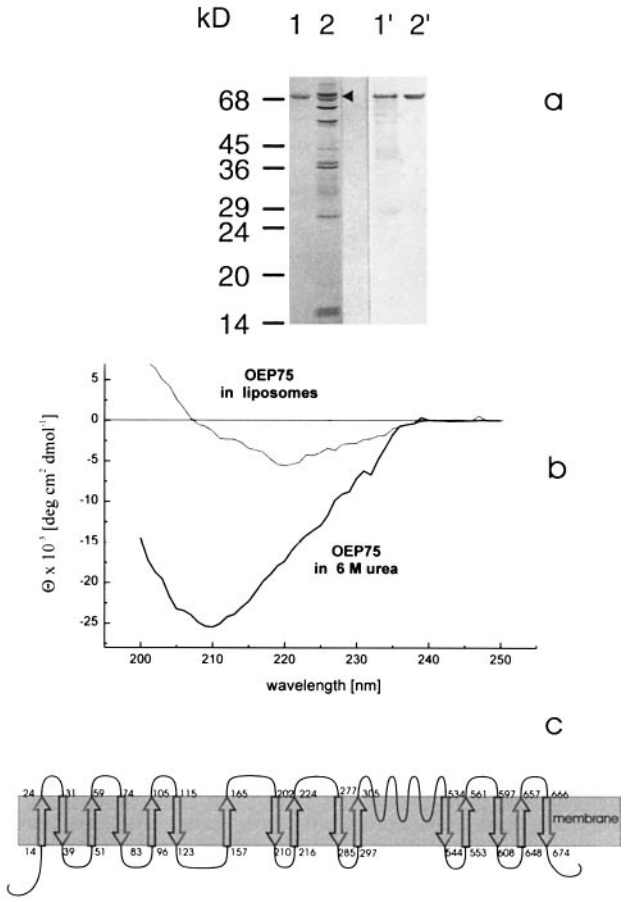

Fig. 1. Expression and structural analysis of 75 kDa pea chloroplastic outer envelope protein (OEP75). (**a**) The purity of *E.coli*-expressed OEP75 and the protein composition of pea chloroplastic outer envelope was analyzed by SDS–PAGE, followed by staining with Coomassie blue (lanes 1 and 2 respectively) and by immunoblot using an OEP75-specific antiserum (lanes 1′ and 2′ respectively). (**b**) CD-spectra of OEP75 in 6 M urea and after reconstitution into liposomes. CD-spectra were recorded between 190 and 260 nm on a Jasko J-500 CD-spectropolarimeter as described in Materials and methods. According to standard determinations (Smith *et al.*, 1985) protein concentrations were 0.05 mg/ml for OEP75 in liposomes (25 mg lipid/ml in 10 mM MOPS–KOH pH 6.9) and 1 mg/ml in 6 M urea. (**c**) Putative secondary structure of OEP75. The hydropathy index was calculated using a pattern database with known 3-D structures based on proteins with mainly β-sheet structures (Gilbert, 1992; Rost and Sander, 1993; Rost *et al.*, 1994). Hydrophobic spans with more than eight residues were assumed to form membrane-spanning β-sheets (Cowan and Rosenbusch, 1994).

Results

OEP75 was expressed in *E.coli* with a C-terminal His_6-tag to facilitate purification. The affinity-purified OEP75 exhibited electrophoretic mobility similar to the endogenous protein present in the chloroplastic outer envelope. The immunoblot analysis further demonstrated the identity between overexpressed OEP75 and the polypeptide *in situ* (Figure 1a). Purified OEP75 was reconstituted into azolectin liposomes using the dialysis technique. We used azolectin, a phosphatidylcholine (PC)-rich lipid mixture from plant membranes, because it has been shown previously to be most suitable for the functional reconstitution of plant membrane proteins (Enz *et al.*, 1993; Schwarz *et al.*, 1994; Heiber *et al.*, 1995). OEP75 proteoliposomes typically had a lipid to protein ratio of ⩾200:1 (wt/wt). Assuming an average liposome diameter of $\bar{d} = 100$ nm, $\bar{M}_{r(lipid)} = 1000$ Da and an average area per lipid molecule of $\bar{A} = 70$ Å^2, the number of OEP75 molecules per 'average small liposome' may be calculated (see Materials and methods for details). With the estimated protein concentration of 10 μg/ml at a lipid concentration of 20 mg/ml we obtain a value of $n \cong 1.1$. The corresponding area density is n ⊤ 19 OEP75 molecules/μm^2 which also applies to the giant liposomes used in patch–clamp experiments (assuming the same lipid/protein ratio as in the smaller liposomes).

CD-spectroscopy of the reconstituted OEP75 was used as a qualitative assay to follow changes in the secondary structure motifs of the protein upon incorporation into the liposome membrane (Figure 1b). Since we used small liposomes in diluted samples (~25 mg lipid/ml, 50 μg protein/ml), scattering artifacts were minimized. As expected, circular dichroism of the purified protein in 6 M urea buffer reveals, by comparison with spectra of proteins with known secondary structures (Venyaminov and Yang, 1996), that the protein adopts mainly a disordered conformation (Figure 1b). However, after reconstitution into small unilamellar liposomes the shape of the spectra changes drastically, indicating refolding of secondary structure motifs as observed previously with other membrane proteins (Wei and Fasman, 1995). Comparison of the OEP75 spectrum with representative spectra of model proteins indicates that dominant secondary structure elements of the reconstituted protein are β-sheet topologies (Venyaminov and Yang, 1996). Moreover, when spectral deconvolution of the spectra was performed using reference spectra of mainly β-sheet proteins (Chang *et al.*, 1978; Venyaminov and Yang, 1996), the data indicate that ~70% of the protein adopts β-sheet topology after reconstitution, while the calculated helix content was less than 12%. Although this method of calculation is not entirely reliable in itself (Chang *et al.*, 1978; Fasman, 1995; Venyaminov and Yang, 1996), the results are in line with those of other secondary structure predictions, which did not indicate the presence of any transmembrane or membrane-associated α-helix (D'Andrea *et al.*, 1989; Gilbert, 1992; Rost and Sander, 1993; Rost *et al.*, 1994). We used the information from the hydropathy plot (not shown) and the CD-spectra, indicating a mainly β-sheet topology for OEP75, to calculate its putative secondary structure. These calculations of secondary structure and solvent accessibility are based on PAM matrices derived from pattern databases obtained from known 3-D structures of proteins with mainly β-sheet topology (Gilbert, 1992; Rost and Sander, 1993). The results of these calculations are depicted in Figure 1c. According to this model, OEP75 contains 16 loop interconnected and membrane-spanning β-strands, clustered in three domains, with a stretch of ~229 amino acids composed of shorter (less than seven amino acids) hydrophobic β-strands from Leu305 to Thr534. This putative structure of OEP75 is distinct from the typical porin-like channels, which are formed by amphiphilic β-strands (Cowan and Rosenbusch, 1994).

OEP75 reconstitution was further evaluated by measuring binding of OEP75 IgGs derived from OEP75 antiserum. Using an inverted phase-contrast microscope we observed immediate precipitation of OEP75 liposomes after addition of OEP75 IgGs (data not shown). Therefore

a significant fraction of the OEP75 liposomes contained the protein in a conformation with the antigenic sites being exposed to the extra-liposomal side. In line with this we estimated by standard protein assay (Smith *et al.*, 1985) that IgGs derived from OEP75 antiserum precipitated ~80% of the OEP75 proteoliposomes. These results indicate that we obtained a high degree of OEP75 reconstitution into the liposomes.

To investigate the electrophysiological properties of the putative channel of the reconstituted OEP75, ion currents across the proteoliposome membrane were measured by the patch–clamp technique and in planar bilayers. In excised patches of giant OEP75 proteoliposomes, single channel currents were observed at positive holding potentials, provided an activating voltage gradient starting from negative holding potentials ($V_h > -100$ mV, duration ≥ 15s) has been applied before (Figure 2). After formation of a high resistance seal ~50% of the attempts ($n > 90$) to detect ion channel activity by the procedure above described were successful. In most cases the activity of at most three channels was observed. Assuming a reasonable area of ~0.2–0.3 μm^2 for the tip of the patch pipette [hard glass pipette with $R_{pip} \top 100$ MΩ, in 100 mM KCl (Sackman and Neher, 1995)], one could expect about nine active channels per patch (see Materials and methods), supposing that the active unit of OEP75 is a monomer and all molecules have been reconstituted into a transport-competent form. Therefore, our observations indicate that either only ~30% of the reconstituted OEP75 proteins were refolded into a transport-competent form or that the voltage activation protocol was only successful to the same degree.

Voltage-dependent currents across an excised membrane patch containing multiple copies of the OEP75 channel preactivated by a voltage gradient are shown in Figure 2. Gating of single channel currents is observed at holding potentials above $V_h > +100$ mV. These results indicate that OEP75 can form a hydrophilic pore *in vitro*. At a given holding potential (V_h) the averaged mean current of multiple open channels increased exponentially with increasing positive holding potentials (Figure 3a), while the single channel conductance of the main conductance state was linearly related to V_h (Figure 3b). This shows that the open probability of the channels (P_{open}) increased exponentially with positive values of V_h. In order to prove whether this exponential increase was due to the magnitude of membrane potential (V_m) or its gradient, a voltage step protocol was started from different negative holding potentials (V_h = –50, –100, –150 mV, Figure 3c). Obviously the exponential steepness of the current–voltage relationship for the averaged mean currents of multiple open channels was more pronounced for the voltage gradient with larger absolute values (starting from $V_h = -150$ mV). When a voltage gradient with the same absolute magnitude but opposite direction was applied, no activation was achieved. This clearly demonstrates that the activation of the channel is dependent on both, the absolute value and the direction of the applied voltage gradient. The slope conductance of the most frequently detected open channel amplitude was $\Lambda = 76 \pm 7.3$ pS, ($n = 5$ different preparations) (see Figure 3b). The unvarying dependence of channel activation and activity on the direction of the voltage gradient and on the direction

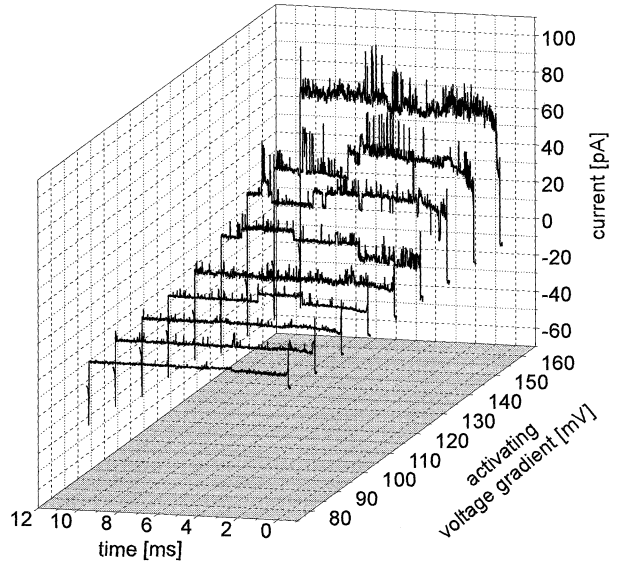

Fig. 2. OEP75 reconstituted into liposomes forms a cation-selective ion channel. Purified OEP75 was reconstituted into liposomes and its electrophysiological characteristics determined by patch–clamping. During the measurements the following solutions were used: bath 50 mM KCl (4 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM MOPS–Tris, pH 7.0), pipette 200 mM KCl (10 mM MOPS–Tris, pH 7.0). Single channel current recordings in excised patches from giant OEP75 proteoliposomes in response to a voltage gradient starting from negative holding potentials (V_h = –150 mV, duration 10s) with increasing amplitudes (10 mV increments starting from +80 mV, duration 10 s).

of the transmembrane electric field demonstrates that the OEP75 channels have been unidirectionally incorporated into the liposome membrane. This notion is further corroborated by the IgG binding and the observations presented below.

In general OEP75 channels exhibited complex gating behavior with multilevel channel openings. Figure 4a and b show current recordings obtained from an excised patch containing multiple copies of active OEP75 channels in response to a voltage sweep from $V_h = 0$ mV to $V_h = +200$ mV and from $V_h = 0$ mV to $V_h = -200$ mV. Before sweeping the command voltage, OEP75 channels were activated by keeping V_h for 15 s at –150 mV. During the voltage sweep from $V_h = 0$ to –200 mV almost no channel activity was observed and the channels mainly remained closed. [At a constant potential of V_h = –150 mV the channels also remained closed (not shown).] However, sweeping V_h from 0 mV to +200 mV frequent channel opening was observed immediately and channels remained mainly open.

Zero current crossing in Figure 4a was at $V_h = -15$ mV ($E_{K+} = -42$ mV), indicating that permeation through the OEP75 channel is slightly selective for cations. The frequently observed open channel amplitudes in different sets of experiments as shown in Figures 2 and 4 ($n > 80$ different experiments from $n = 5$ different preparations) were $\Lambda = 18 \pm 6$ pS, $\Lambda = 70 \pm 15$ pS and $\Lambda = 145 \pm 30$ pS. These values represent slope conductances (see Figure 3b) obtained at holding potentials between $V_h = +80$ mV to +180 mV (see Figure 3 b) with $n > 10$ measurements at a particular holding potential. Closer inspection of the current traces (Figure 5a and b) reveals

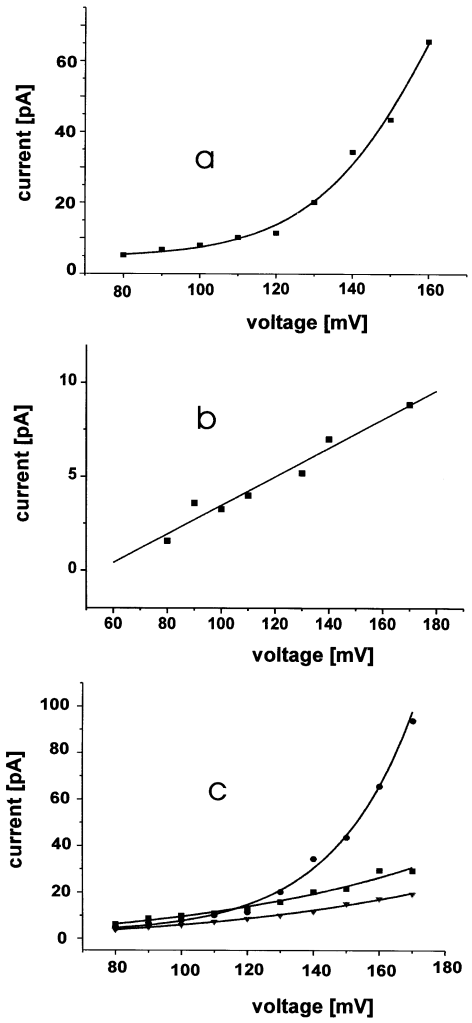

Fig. 3. Mean currents after activation of OEP75 channels depending on different voltage gradients. (**a**) Averaged currents of multiple open channels at positive voltages. Mean values of currents during the time interval when constant positive voltages were applied. Data has been obtained using a step protocol as in Figure 2 starting from a holding potential of V_h = –150 mV. (**b**) Current–voltage relationship for the single open channel (slope conductance $\Lambda = 76 \pm 7.3$ pS). Data was obtained from separate measurements conducted as described in Figure 2. Each data point represents the average from at least 10 independent measurements. (**c**) Averaged mean currents of multiple open channels at a given potential. Three different holding potentials were applied for 1 min before beginning the measurement (see below). A step protocol with 10 mV increments from + 80 mV to +170 mV was used, starting from the corresponding holding potential (see below). Averaged currents (n = 5 different measurements) of multiple open channels at positive voltages are given. Calculated from data as shown in Figure 2. (holding potential ●, –150 mV; ■, –100 mV; ▼, –50 mV).

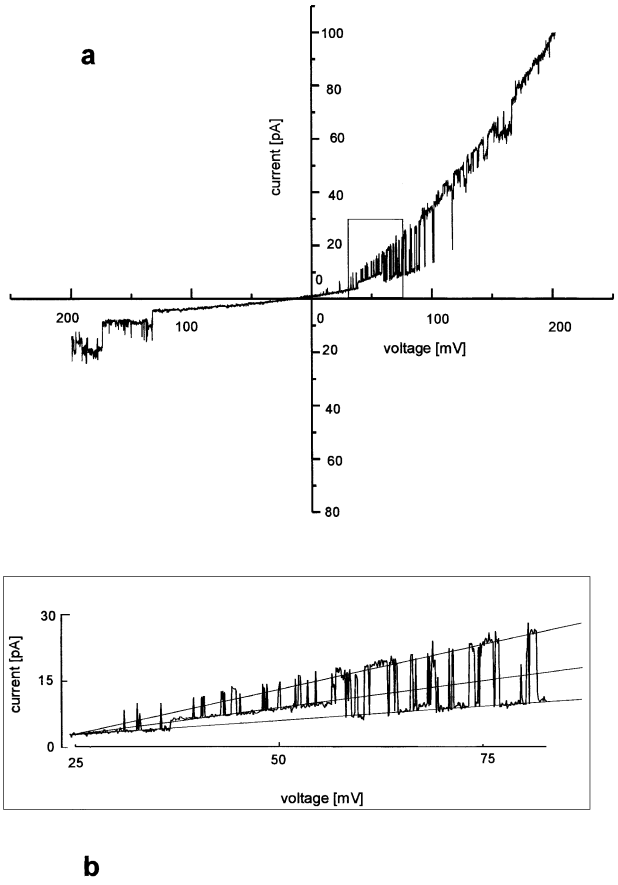

Fig. 4. Current–voltage relationship of the preactivated OEP75 channel. (**a**) Current recordings obtained from an excised patch containing multiple copies of active OEP75 channels in response to voltage sweeps (Δ = 20 mV/s) from V_h = 0 mV to V_h = +200 mV and from V_h = 0 mV to V_h = 200 mV. Channels were activated by keeping V_h for 15 s at –150 mV before sweeping the command voltage. (**b**) Enlarged part of the current recording enclosed by a box in part a.

that in the course of the current traces direct transitions between the closed state and the largest amplitude (fully open channel), as well as direct transitions between the different smaller amplitudes (presumably subconductant states) occur (Figure 5a–c). These gating patterns are extremely unlikely to occur for two or more simultaneously active channels or independent channel entities with different conductances (Dabrowski *et al.*, 1990). We therefore conclude that the distinct channel conductances (Figure 5a–c) originate from a single channel entity, i.e. the OEP75 channel shows multilevel conductance. It is worth noting that the conductance state of the OEP75 channel as well as sublevel conductance values already came close to their maximum in low ionic strength buffer (symmetrical 150 mM KCl, details not shown). Therefore, the above values for the OEP75 conductances obtained at asymmetric 200/40 mM KCl represent saturation values, i.e. the highest conductance of the OEP75 channel is close to $\Lambda = 145 \pm 30$ pS.

Considering the most simple model with the OEP75 channel being a water-filled pore, its diameter would be in the range 8 Å $< d_{pore} <$ 9 Å (see Materials and methods), thus the OEP75 channel should apparently constitute a rather narrow pore. This is a fairly rough estimation, however, it sets the limit for the possible size of the hydrophilic pore at the narrowest point (Hille, 1992). Even based on this rough estimate the above clearly shows that native-like folded protein structures, such as α-helix motifs, having a diameter > 7 Å cannot pass the OEP75 membrane channel in their final folded topology but only in a conformation missing pronounced secondary structure.

In order to reconcile the observed activity of reconstituted OEP75 with channel activities present in the chloroplast outer envelope (Heiber *et al.*, 1995) we investigated

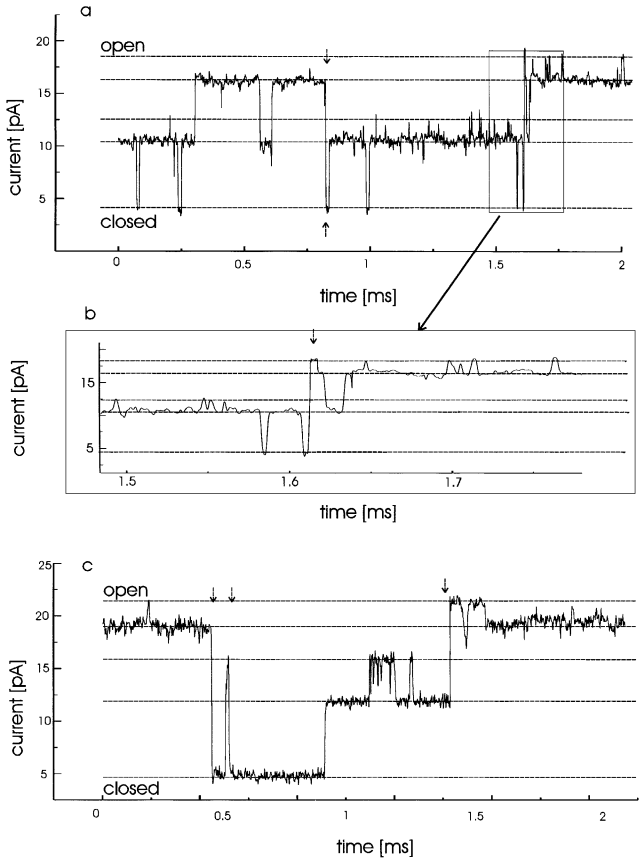

Fig. 5. Subconductance levels of the OEP75 channel. (**a**) Single channel recording obtained from an excised patch containing a single copy of an active OEP75 channel at $V_h = +150$ mV. The channel was preactivated at $V_h = -150$ mV for 15 s. (**b**) Single channel recording obtained from an excised patch showing the shaded part from (a). Single step transitions covering several amplitude levels are indicated by arrows. (**c**) Single channel recording obtained from an excised patch containing a single copy of an active OEP75 channel at $V_h = +180$ mV. The channel was preactivated at $V_h = -150$ mV for 15 s.

channel activities present in purified outer envelope membranes. Purified chloroplastic outer envelopes from pea were reconstituted into liposomes (OM-liposomes) and ion currents across the OM-liposome membranes were measured by the patch–clamp technique. It is worth noting that the gentle reconstitution procedure used only 'dilutes' the outer membranes with lipids without addition of any detergent or other denaturating agent (see legend to Figure 6). Current recordings from other inside out patches ($n > 30$ patches from $n > 5$ different preparations) demonstrated the presence of several ion channels with clearly distinguishable conductances (see Figure 6). The conductances observed may be grouped according to their values into those below and those above $\Lambda \cong 150$ pS. A prominent conductance which could frequently be observed is shown in some detail in Figure 7. Current recordings are from four different patches (originating from two different OM-liposome preparations). This conductance again revealed subconductant states and a non-linear current–voltage relationship for the largest amplitude transition (Figure 6, bottom left). The extrapolated slope conductance (from the linear part at higher positive voltages) was $\Lambda \cong 132 \pm 11$ pS. The corresponding zero current potential from Figure 6 was $V_{rev} = +32$ mV (+59 mV). These values show that the conductance was to a similar extent as selective for cations as the reconstituted OEP75. Moreover, applying a voltage protocol similar to that above for reconstituted OEP75, we obtained at higher positive V_h current recordings with time resolutions sufficient to estimate the open probability at these voltages. With increasing V_h values the open probability of the channel increased exponentially (see Figure 6, bottom right). Thus this channel present as a dominant conductance in OM-liposomes shows characteristics (conductance, selectivity and voltage dependence of the open probability) that are qualitatively identical to that described above for the reconstituted OEP75 channel. We therefore conclude that channel properties of OEP75 in the entire protein import complex present in OM-liposomes may be very similar to the activity of the heterologously expressed and reconstituted protein. The main difference seems to be that the latter requires activation of the channel activity by a voltage gradient (see Discussion).

Furthermore, we tested the effect of IgGs derived from OEP75 antiserum on the activity of the reconstituted OEP75 channel in planar bilayers. In different experiments ($n = 8$) addition of OEP75 antibodies neither to the *cis* nor to the *trans* compartment significantly changed the activity of the OEP75 channels. Thus it is evident that although OEP75-IgGs bind specifically to OEP75 liposomes, this binding occurs in regions other than the actual pore region.

In order to see whether the OEP75 channels are affected by import-competent precursor proteins, bilayer measurements were performed in the presence of the precursor of the small subunit of ribulose 1,5-bisphosphate carboxylase (preSSU) and its processed mature form (SSU). The bilayer system is superior when easy access to either side of the membrane is required. Our method for the fusion of proteoliposomes to planar bilayers requires osmotic swelling of the liposomes, which depends on the presence of open channels during the osmotically induced fusion (Woodbury and Hall, 1988; Cohen *et al.*, 1989). As outlined above, the OEP75 channels were closed in the absence of a membrane potential, moreover activation was dependent on a transmembrane voltage gradient with the correct direction. Accordingly, fusion rates of OEP75 liposomes to planar bilayers were poor (<1%).

However, after successful fusion and activation ($V_h = -150$ mV for 15 s) of the OEP75 channel, the addition of preSSU from the *cis* compartment affected the gating of the OEP75 channel. Flickering and significantly more closing events of the channel were observed ($n > 8$). This is shown clearly in Figure 7b depicting current recordings from a bilayer containing multiple copies of active OEP75 channels in the presence of 60 nM preSSU (*cis*-side). When preSSU was added from the *trans* compartment ($n = 8$) no changes in the channel activity were observed (not shown). This was also true when SSU was added from the *cis* compartment ($n = 9$) (Figure 7b) or the *trans* compartment ($n = 8$) (data not shown). Moreover, when SSU (60 nM) was added prior to addition of preSSU (60 nM) from the *cis* compartment ($n = 3$) the effect described above of preSSU on the channel activity was still observed. These results indicate that preSSU interacts specifically from only one side with the OEP75 channel.

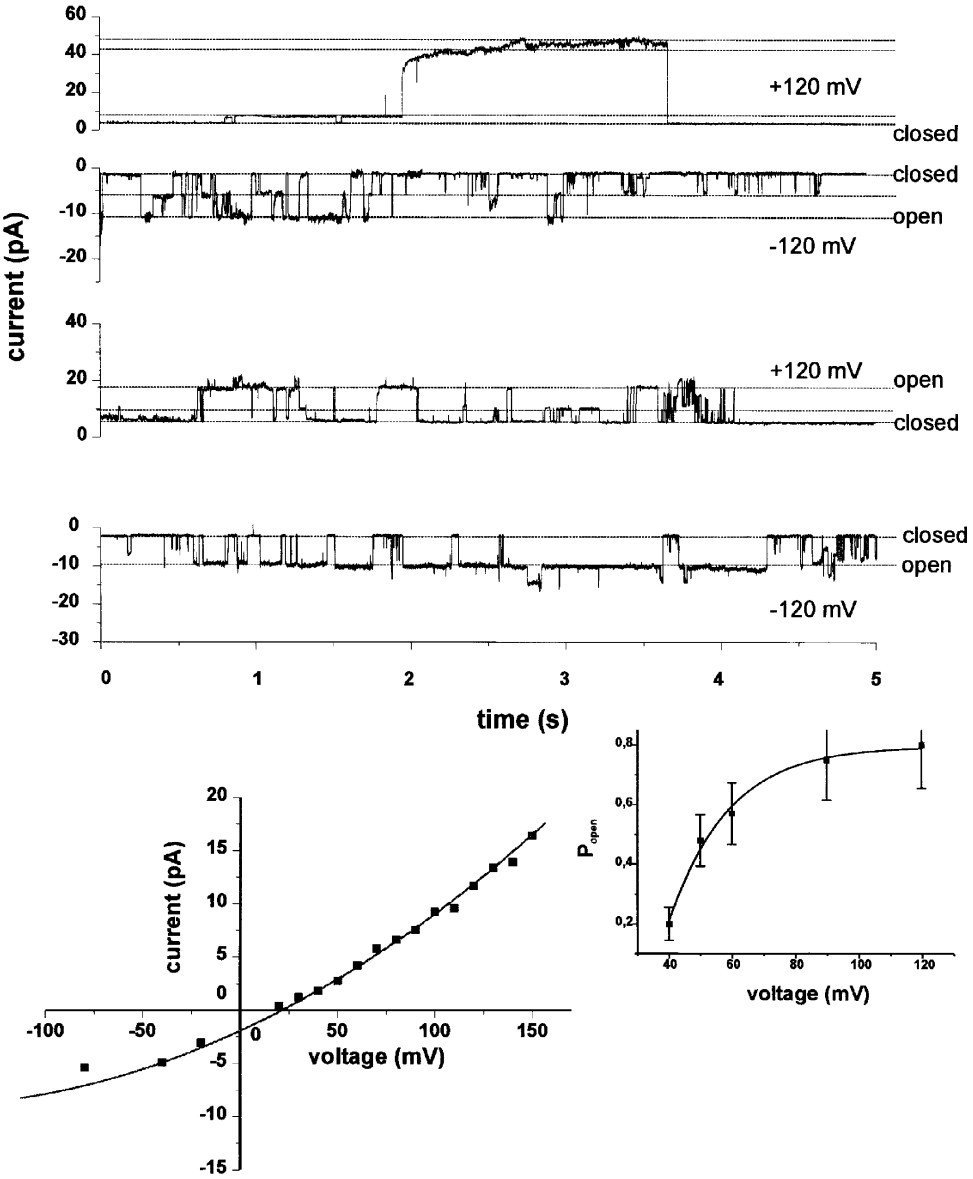

Fig. 6. Ion conductances in purified membranes from pea chloroplasts. Isolated outer envelope membranes from pea chloroplasts (l mg/ml protein) were diluted at 1:50 with artificial liposomes (40 mg/ml azolectin in 10 mM HEPES–KOH pH 7), sonicated and subjected to a freeze–thaw cycle. After reconstitution giant liposomes were formed and single channel activity was followed in excised inside-out patches of OM-liposomes. Solutions: 50 mM KCl bath, 250 mM KCl pipette. All potentials are referred to the pipette. Top: current recordings from excised inside-out patches of OM-liposomes. Recordings from four different patches, from top to bottom: 1. V_h = +120 mV, 2. V_h = –120 mV, 3. V_h = +120 mV, 4. V_h = –120 mV. The large conductance of the upper trace ($\Lambda \cong 400$ pS) belongs to the group of high conductance values ($\Lambda > 150$ pS), while the lower traces ($\Lambda \cong 120$ pS) show channels representative of the low conductance range ($\Lambda < 150$ pS, see text). Bottom left: current voltage relationship of the most frequently observed single channel conductance in OM-liposomes. Recordings from $n = 3$ different OM-liposome preparations. Each data point is the average of $n > 3$ current recordings (duration >200 ms) at the indicated V_h. Slope conductance linearly extrapolated between V_h = +75 to +150 mV was Λ = 132 ±11 pS, V_{rev} = +32 mV (V_K+ = +59 mV). Bottom right: the open probability of the channels was estimated from current recordings of excised patches after application of a voltage gate from V_h = 0 mV to the indicated V_h for 500 ms. P_0 was calculated from all point amplitude histograms by: $P_0 = A\Sigma_{open}/A\Sigma_{closed}$. Each data point represents the average of $n = 3$ independent measurements.

This again supports our conclusion that we obtained a preferential orientation of OEP75 during reconstitution.

In combination the above results demonstrate that the putative protein translocation channel OEP75 forms a voltage-gated aqueous channel *in vitro*.

Discussion

Translocation of proteins across the chloroplast outer envelope proceeds through the oligomeric OEP-complex composed of the three major proteins, OEP86, OEP75 and OEP34 (Soll, 1995; Schnell, 1995). In this paper we provide for the first time direct experimental evidence that OEP75 constitutes a voltage-gated transmembrane channel with a hydrophilic pore. The results presented in this paper add further experimental evidence and support current concepts on the mechanism of protein translocation across the membrane (Isenman *et al.*, 1995; Schnell, 1995; Soll, 1995; Schatz and Dobberstein, 1996).

The heterologously expressed and purified OEP75 could

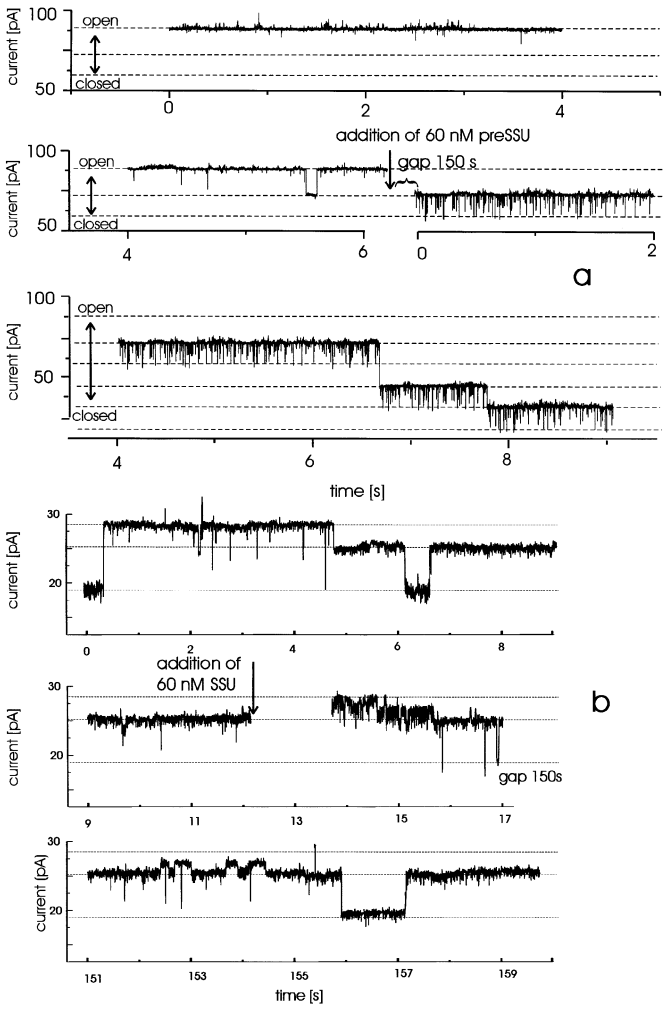

Fig. 7. Effect of preSSU and SSU on the channel activity of OEP75. The labels 'closed' and 'open' give the direction in which closing or opening of channels occurs rather than the open or closed state of a particular channel. In (**a**) the bilayer contained a total of nine active channels which were all open at the beginning, while the bilayer in (**b**) contained only three active channels which were also open at the beginning. Single channel recordings of a planar bilayer containing multiple active OEP75 channels before and after addition of 60 nM preSSU to the *cis* compartment (V_h = 80 mV). Before the addition of preSSU the channels were activated by keeping them at V_h = –150 mV for 15 s. A time gap of 150 s was introduced because flickering and partial closures were observed with a delay of 20–200 s. Mixing artefacts were below 20 s. Single channel recordings of a planar bilayer containing multiple active OEP75 channels before and after addition of 60 nM SSU to the *cis* compartment (V_h = 80 mV). Before the addition of SSU the channels were activated by keeping them at V_h = –150 mV for 15 s. PreSSU and SSU from tobacco (Salvucci and Klein, 1992) were overexpressed in *E.coli* and prepared exactly as described (Waegemann and Soll, 1995). The time gap of 150 s was introduced to demonstrate the long term activity of the OEP75 channel.

be functionally reconstituted into liposomes thereby forming a hydrophilic transmembrane pore highly permeable for small ions. This conclusion is supported by the following results.

The shape of the CD-spectra and the obtained values of the molar ellipticity indicate that the major fraction of the protein is refolded during dialysis into a mainly β-sheet topology. The extent of refolding was significantly increased upon mixture of the unfolded protein (in urea) with a detergent (Mega-9)-containing lipid dispersion prior to dialysis. The electrophysiological experiments confirmed that the refolding resulted in a functional state of the protein, moreover they indicate a preferential insertion into the membrane. Similar results have been obtained for the *E.coli* porins OmpA and OmpF (Surrey and Jähnig, 1992; Surrey *et al.*, 1996). OEP75 antibodies almost quantitatively (~80%) agglutinate OEP75 liposomes. Therefore at least 80% of OEP75 have to be refolded and inserted into the membrane exposing antigenic sites to the extra-liposomal side. The area density of OEP75 channels which could be activated by a specific voltage protocol (~30% of the potentially present channels) also indicate a high degree of functional reconstitution.

The size of the hydrophilic pore can be estimated from the electrophysiological data in a first approximation as being ~8–9 Å in diameter. Although OEP75 antibodies agglutinated OEP75 liposomes already at very low concentrations (20 μg/ml), higher concentrations of antibodies (<100 μg/ml) did not affect gating of and permeation through the pore. This observation is in line with previous observations of Tranel *et al.* (1995). They demonstrated that partial inhibition of *in vitro* pSSU import into chloroplasts occurred only at high Fab antibody fragment concentrations (~5 mg/ml). At these high concentrations no bilayer experiments could be conducted because the bilayer membranes were destroyed. Apparently the antibody epitope of reconstituted OEP75 is not situated close to the pore region.

OEP75 contains a precursor-binding site distinct from that of OEP86, which cooperates in a multi-subunit complex to mediate recognition and translocation (Ma *et al.*, 1996). Therefore, import-competent precursor proteins but not their mature forms were able to interact specifically with OEP75 when added from one side of the membrane, thereby affecting the gating of the pore. Although we cannot exclude that this is an indirect effect of the precursor protein on the OEP75 channel, this seems unlikely due to the observations of Ma *et al.* (1996). It is likely that the transmembrane pathways for small ions and the precursor proteins are identical. This is corroborated by the identification of a channel conductance in purified outer membranes from pea chloroplasts with almost identical properties (unit conductance, selectivity, voltage dependence of channel gating) as the reconstituted OEP75. This channel activity in the outer membrane, which can be clearly distinguished from other porin like higher conductances is probably a manifestation of the OEP75 channel within the entire OEP–protein import complex. By direct patch–clamp measurements in a so-called whole chloroplast configuration with *Peperomia metallica* chloroplasts precursor mediated openings of conductance pathways in the chloroplast envelope have been reported previously (Bulychev *et al.*, 1994). However, at present it is difficult to compare the properties of reconstituted OEP75 with the conductance in the membranes of *Peperomia metallica* chloroplasts described by Bulychev *et al.* (1994).

Our results therefore indicate that the environment of a polypeptide during transport across the membrane is mainly hydrophilic. Similar conclusions have been drawn previously (Crowley *et al.*, 1994) for the movement of secretory proteins across the ER membrane. Fluorescence probes placed at various sites on the nascent protein chain

were used to assay the polarity of the probe environment and its accessibility during the transit for external quenchers (Crowley *et al.*, 1994). Further studies (Hamman *et al.*, 1997) indicate that the aqueous pore in a functional translocon of the ER membrane has a diameter of 40–60 Å. Electron micrographs of the heterotrimeric Sec61p complex, a major component of the protein-conducting channel of the ER, revealed the structure to be a cylindrical oligomer with a diameter of 85 Å and an opening at one side of ~20 Å (Hanein *et al.*, 1996). These electron micrographs present direct structural information on a protein import channel supporting the biochemical data (Hamman *et al.*, 1997). The nascent chain-bound ribosomes might form a tight seal to the SEC61p channel in order to avoid leakage of small ions (Simon and Blobel, 1991), an opportunity not existing in the post-translational protein translocation. Consequently, smaller diameters might have evolved for the pore of the latter process. Assuming that small ions and precursor proteins are translocated across the same pore (OEP75), we propose that the polypeptide is in an almost completely unfolded state during its movement across the chloroplastic outer membrane.

The rather unusual voltage dependence of the OEP75 channel activation and activity may provide some hints to the mechanism of maintaining the permeability barrier across the membrane, and to the energy requirements for channel activation or formation (see below). We observed that channel activation was dependent on the applied voltage gradient and its direction, whereas gating of the activated channel was dependent only on the direction of the applied membrane potential. Although we do not know the relative orientation of the reconstituted OEP75, our data indicate that reconstitution leads to a highly preferential orientation of the protein in the liposome membrane. Similar observations on the functional reconstitution of membrane channels from denatured proteins have been made during reconstitution studies using heterologously expressed *Haemophilus influenzae* porin (Dahan *et al.*, 1996), *E.coli* OmpA and OmpF (Surrey and Jähnig, 1992; Surrey *et al.*, 1996), porin from *Rhodopseudomonas blastica* (Schmidt *et al.*, 1996) or the SecY, SecE protein translocase (Brundage *et al.*, 1990; Akimaru *et al.*, 1991; Driessen, 1992). Unidirectional orientation and membrane insertion has also been observed previously (Surrey and Jähnig, 1992; Dahan *et al.*, 1996; Surrey *et al.*, 1996). It is worth noting that we applied a reconstitution protocol similar to that of Surrey and Jähnig (1992), specifically without using detergents for the protein solubilization. Our results indicate that activation of the OEP75 channel is related to the movement of electrical dipoles, which may be formed by parts of membrane-spanning β-sheets, in the membrane. Accordingly, formation of the OEP75 channel pore to its final transport-competent form *in vitro* requires the interaction of parts of the protein with an external electrical field. Interaction with native lipids or plant sterols which are present in the outer membrane in low but significant amounts (Douce and Joyard, 1990) could also alter the voltage-dependent channel activation, since we recently observed high fusion rates (which requires the presence of open ion channels) with planar bilayers when OEP75 reconstitution was performed into azolectin/ergosterol (9:1 wt/wt) liposomes (S.Hinnah, unpublished). Furthermore, *in vivo* this requirement for an electric field may in part be substituted by the GTP-binding protein OEP34 of the protein import complex. OEP34 is very tightly associated with OEP75 and can indeed be covalently connected to the channel protein by the formation of a disulfide bridge under oxidizing conditions. The precursor receptor OEP86 might actually be a component of the OEP75 channel modulation in a way analogous to the membrane-delimited regulation of the muscarinic K^+(Ach) channel (Hille, 1992). The activated OEP75 channel exhibited significant open probabilities only at positive holding potentials. This shows that transport through the pore is regulated to occur preferentially in one direction. In summary, the voltage dependence of channel activation and gating indicates that formation of the final pore is achieved by movements of parts of the protein in response to dipolar interactions while transport through the pore seems to be regulated to occur mainly in one direction.

Finally, it is worth mentioning that the OEP75 channel shows a slight cation selectivity. This could indicate that permeation and presumably also recognition of the import-competent proteins may also be mediated by charge. This is supported by the fact that chloroplast specific targeting peptides contain an overall positive charge (von Heijne *et al.*, 1989).

Materials and methods

Synthesis of OEP75

A plasmid for the overexpression of the mature OEP75 was constructed into the vector pet24c. An artificial *Nde*I site was introduced by recombinant PCR into the cDNA clone p214C (Innis *et al.*, 1990) coding for the preOEP75 (precursor form of OEP75). Similarly a new *Xho*I site was introduced at the C terminus of the p214. The cloning procedure resulted in replacement of the N-terminal four amino acids by a methionine, and of the C-terminal five amino acids by a leucine and a His_6-tag. The protein was expressed in *E.coli* BL21De3 cells and initially recovered as insoluble inclusion bodies. Inclusion bodies were solubilized in 6 M urea, insoluble material separated by centrifugation, and soluble proteins applied on a Ni^{2+} chelating matrix in 5 mM imidazol, 0.5 M NaCl and 20 mM Tris–HCl pH 7.9. The matrix was washed with six column volumes washing buffer containing 60 mM imidazol, 0.5 M NaCl and 20 mM Tris–HCl, pH 7.9. OEP75 was eluted at 1 M imidazol, 0.5 NaCl and 20 mM Tris–HCl, pH 7.9. All buffers contained 6 M urea. The eluate was dialyzed extensively against 6 M urea in Tris–HCl, pH 7.9.

Antibody preparation and agglutination of OEP75 liposomes

OEP75 antibodies were raised as described in detail elsewere (Seedorf *et al.*, 1995). The IgG fraction was purified using a protein A–Sepharose column (Pharmacia) according to the manufacturer's recommendations. Precipitation of OEP75 liposomes by the anti-OEP75 IgG fraction was followed by standard protein assay (Smith *et al.*, 1985). For this, OEP75-liposomes, and the anti-OEP75 IgG fraction in the presence and absence of OEP75 liposomes were incubated for 30 min (4°C) and subsequently assayed.

Preparation of OEP75 liposomes and giant liposomes for patch–clamp experiments

Small liposomes were obtained by dissolving 50 mg/ml of azolectin (Sigma, type IV-S) in 10 mM MOPS–Tris (pH 6.9) using a Branson sonifier equipped with a microtip. Liposomes were freeze–thawed once. Fractions containing purified OEP75 (~1 mg/ml) in 6 M urea were mixed with the preformed liposomes (0.1 mg protein/10 mg lipid), sonicated (Branson sonicator, five pulses, 2 s, 40% duty cycle, output control 4), subjected to freeze–thawing and dialyzed overnight against a buffer (2l) containing 200 mM KCl, 4 mM $MgCl_2$, 20 mM $CaCl_2$, 10 mM MOPS–KOH pH 6.9. Giant vesicles suitable for patch–clamp measurements were obtained by a modified dehydration–rehydration procedure according to Keller *et al.* (1988) as described in detail

elsewhere (Schwarz *et al.*, 1994; Heiber *et al.*, 1995). These giant liposome vesicles typically had diameters between 20 and 50 μm. Giant liposomes without OEP75, when used as controls, did not show any single channel activity under the experimental conditions applied. The lipid to protein ratio after reconstitution was determined as described previously (Heiber *et al.*, 1995).

Approximate calculation of the reconstitution efficiency and pore size

We start with the following values:

10 mg/ml lipid (M_r = 1000 *g*)

and

20 μg/ml protein M_r = 75 kDa, OEP75).

We can now calculate: the surface area of an 'average' vesicle is: $\bar{A}_{ves} = 4\pi\bar{r}_{ves}$, ($\bar{r}_{ves}$ = 50 nm) and the number of lipid molecules per average vesicle is: $n_{lipd/ves} = 2(\bar{A}_{ves}/\bar{A}_{lipid})$, with an average surface area for a single lipid molecule of A_{lipid} = 0.7 nm^2 we obtain:

$$n_{lipid/ves} = 8.97\times10^4 \text{ and } m_{ves} = 8.97\times10^7 \text{ g/mol.}$$

This yields a vesicle concentration of: $c_{ves} = (c_{lipid}/m_{ves}) = 3.095\times 10^{-7}$ mol/l, with a protein concentration of: $c_{protein} = 1.33\times10^{-7}$ mol/l it follows that:

$$n_{protein/vesicle} = (c_{protein}/c_{ves}) = 1.1$$

and the area density:

$$n_{area} = (n_{protein/vesicle}/\bar{A}_{ves}) = 38\ \mu m^{-2}$$

The pore size can be calculated according to Hille, 1992:

Briefly:

$$d_{pore} = 2\{1/[4(R\pi)]\} \times \rho\pi + \sqrt{\rho}\ \sqrt{\pi}\ \sqrt{(\rho\pi + 16Rl)}$$

where: resistivity of the solution is ρ ≅ 120 Ωcm, length of the pore l = 5 nm, maximal conductivity of the pore δ_{max} = 145 pS and its resistance $R = (\delta_{max}/l + c_{0.5\ max}/c)^{-1}$, with c = 200 mM and $c_{0.5\ max}$ = 160 mM, we finally obtain d_{pore} = 8.34 Å.

Electrophysiological measurements

Patch–clamp measurements. Giant liposome vesicles were placed in a tissue bath, mounted on an Olympus IMT-2 inverted microscope, and the vesicles were observed using phase-contrast optics. Single channel current recordings using the patch–clamp technique were performed as described (Hamill *et al.*, 1981). Since we do not know the orientation of the reconstituted OEP75 in the liposomes, holding potentials are always referred to the pipette. GΩ-seals ranging from 5 to 50 GΩ could be achieved by slight suction once the pipette tip was brought into contact with the membrane. Sealing was apparently affected by the protein content of the vesicles, the probability of GΩ-seal formation was inversely proportional to the protein concentration. Currents were amplified using a patch–clamp L/M-EPC 7 amplifier (List Medical). Current recordings were digitized at 18 kHz sampling rate using a modified commercial DAT Recorder (Sony DTC 690) and stored on DAT tapes. For analysis, current recordings were filtered with an 8-pole Bessel filter, typically at 1 kHz, digitized at a sampling interval of 0.2 ms with a Digidata 1200 A/D converter (Axon Instr.) and stored on a personal computer.

Planar lipid bilayers

Planar lipid bilayers were produced by the painting technique (Mueller *et al.*, 1962). A solution of 50 mg/ml azolectin (Sigma type IV-S) in *n*-decan (anal. grade, Merck) was applied to a hole (100–500 μm diameter) in a Teflon septum, separating the two bath chambers (volume ⊤ 3 ml, each). Both chambers were equipped with magnetic stirrers. Bilayer 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 1 pA (r.m.s.) at 5 kHz bandwidth. After the formation of a stable bilayer in symmetrical solutions of 20 mM KCl, 10 mM MOPS–Tris pH 7.0, the experimental conditions were changed to asymmetric concentrations. Concentrated solutions of KCl and $CaCl_2$ were added to the *cis* chamber up to a final concentration of 250 mM KCl and 10 mM $CaCl_2$. Liposomes were added to the buffer solution in the *cis* compartment through the tip of a microloader (Eppendorf), effecting a slow flow of liposomes directly across the bilayer (for further details see (Heiber *et al.*, 1995). Data analysis was performed using Windows™ based software developed in our lab (Schwarz *et al.*, 1994) in combination with Origin™ (Microcal Software Inc.).

CD-spectroscopy

CD-spectra were measured using a Jasco J-600 spectropolarimeter, after calibration with (+)-10-camphorsulfonic acid. The spectra were recorded at 20°C in a quartz cell with 0.5 cm optical path length. Scans were performed at a rate of 100 nm/min with a sampling interval of 1 nm and averaged (n = 50) to improve the signal/noise ratio. Relative abundance of secondary structures was calculated as described (Chang *et al.*, 1978; Deleage and Geourjon, 1991; Curtis, 1996; Venyaminov and Yang, 1996).

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