Efficient membrane assembly of the KcsA potassium channel in *Escherichia coli* requires the protonmotive force

Annemieke van Dalen⁺, Hildgund Schrempf¹, J. Antoinette Killian and Ben de Kruijff

Department Biochemistry of Membranes, Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands and ¹Department of Applied Genetics of Micro-organisms, Faculty of Biology/Chemistry, University of Osnabrück, Barbarastraße 11, 49069 Osnabrück, Germany

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Very little is known about the biogenesis and assembly of oligomeric membrane proteins. In this study, the biogenesis of KcsA, a prokaryotic homotetrameric potassium channel, is investigated. Using *in vivo* pulse-chase experiments, both the monomeric and tetrameric form could be identified. The conversion of monomers into a tetramer is found to be a highly efficient process that occurs in the *Escherichia coli* inner membrane. KcsA does not require ATP hydrolysis by SecA for insertion or tetramerization. The presence of the protonmotive force (pmf) is not necessary for transmembrane insertion of KcsA; however, the pmf proved to be essential for the efficiency of oligomerization. From *in vivo* and *in vitro* experiments it is concluded that the electrical component, $\Delta\psi$, is the main determinant for this effect. These results demonstrate a new role of the pmf in membrane protein biogenesis.

INTRODUCTION

Translocation of many proteins, across and into the endoplasmic reticulum and the bacterial inner membrane, is catalysed by a complex proteinaceous system, with the core component, the translocon, forming the actual translocation site in the membrane. The eukaryotic and bacterial secretion systems share many homologies and the general working mechanisms are roughly known, although the detailed mode of action is still being studied (for reviews see Economou, 1998; Matlack *et al.*, 1998). Targeting to the translocon in eukaryotic cells is mediated by the signal recognition particle (SRP) pathway. In *Escherichia coli*, this pathway is specifically used for the targeting of inner membrane proteins (de Gier *et al.*, 1997). Most membrane proteins make use of the translocon, although some small single-

spanning membrane peptides, such as M13 and Pf3 coat proteins, can directly enter the lipid membrane (Rohrer and Kuhn, 1990; Kusters *et al.*, 1994). The energy required for the insertion can be derived from ATP hydrolysis at the translocon by SecA, which is especially necessary for translocation of large periplasmic loops (von Heijne, 1989; Koch *et al.*, 1999). In addition, the protonmotive force (pmf) may play an important role. It can increase the efficiency of translocation (Schiebel *et al.*, 1991), or even be required for correct insertion (Andersson and von Heijne, 1994). Membrane insertion in *E. coli* has been studied mainly for proteins containing one or two transmembrane segments and for a few polytopic membrane proteins (see for example, Bassilana and Gwizdek, 1996; de Gier *et al.*, 1998). However, many processes at the membrane are regulated by (larger) protein complexes, composed of homo- or hetero-oligomeric structures. How these oligomeric proteins are integrated into the membrane and correctly assembled is not known. A rather simple, oligomeric membrane protein was identified some years ago: the potassium channel of *Streptomyces lividans* (KcsA; Schrempf *et al.*, 1995). KcsA could be expressed with an N-terminal His tag in *E. coli*, which after purification could be functionally reconstituted in lipid bilayers as determined by electrophysiology. The ease of this expression and purification system resulted in a good characterization of KcsA, including its crystal structure (Doyle *et al.*, 1998). KcsA is a small protein of 160 amino acids that has two transmembrane segments connected by the pore region, which contains the ion selectivity filter and is similar to the pore region of eukaryotic voltage gated potassium channels. The channel protein is a homotetramer that is highly stable in detergent solutions (Cortes

⁺Corresponding author. Tel: +31 30 2535512; Fax: +31 30 2522478; E-mail: a.vandalen@chem.uu.nl

Fig. 1. Expression and identification of His-tagged KcsA in *E. coli*. BL21(λ DE3)/pT7-KcsA cells were labelled for 30 s without (lane 1), or after (lanes 2–4) induction of KcsA synthesis. Samples were analysed by SDS-PAGE without prior heat treatment, except in lane 3. Lane 4, Ni^{2+} affinity precipitation from solubilized cells. As a reference, purified KcsA on a Coomassie-stained gel is shown without (lane 5) or with prior heat treatment (lane 6) and a protein size marker (in kDa) is shown on the right. The monomeric (M) and tetrameric (T) forms of KcsA are indicated.

and Perozo, 1997; Heginbotham *et al.*, 1997). The X-ray structure showed the assembled tetramer with 4-fold symmetry around a central pore. Each subunit contributes its C-terminal transmembrane α -helix to the lining of the pore, while the other (N-terminal) helix faces the lipid membrane.

The simplicity of the protein and the ease of expression in *E. coli* make KcsA a good model protein to study how subunits integrate in the membrane and assemble into an oligomeric organization. In the present study, we use pulse-chase experiments to demonstrate the insertion and subsequent tetrameriza-

tion of KcsA in the *E. coli* inner membrane. Our results reveal a novel effect of the pmf in membrane protein biogenesis. We show that it is required for the efficient formation of the tetrameric structure.

RESULTS AND DISCUSSION

KcsA expression and assembly in *E. coli*

To study the assembly of KcsA, *E. coli* strain BL21(λ DE3) was transformed with the plasmid pT7-KcsA containing the *kcsA* gene and used for *in vivo* labelling. The proteins synthesized during a 30 s period were analysed on SDS-PAGE, shown in Figure 1. In the protein pattern several additional bands appeared after induction with isopropyl- β -D-thiogalactopyranoside (IPTG) (compare lanes 1 and 2). One of these bands runs at a molecular weight of ~67 kDa and another at a much lower molecular weight of ~18 kDa. They run at the same position as the tetrameric and monomeric form of the purified KcsA (lanes 5 and 6) and were absent when the empty vector was used (not shown). The identity of the other bands is not clear, but they are not related to KcsA (see forthcoming results). Boiling the sample before electrophoresis resulted in the disappearance of the 67 kDa band and in a more intense 18 kDa band (lane 3), similar to the behaviour of purified tetrameric KcsA for which it is known that boiling results in a tetramer to monomer transition (Cortes and Perozo, 1997), and as verified for purified His-tag KcsA in lanes 5 and 6. In addition, making use of the His tag fused to the N-terminus, both bands could be specifically precipitated by Ni^{2+} -NTA beads (lane 4), further proving that upon induction the monomeric (M) and the tetrameric state (T) of KcsA were formed in *E. coli*.

The observation of both the monomer and tetramer of KcsA allows the analysis of the kinetics of tetramerization in a pulse-chase experiment (Figure 2A). Immediately after the pulse, a considerable amount of tetramer is already present, which remains so during the chase. No intermediate dimers or trimers

Fig. 2. Kinetics of KcsA insertion and assembly. Cells were pulse labelled for 30 s, followed by a chase for the indicated periods. The monomeric (M) and tetrameric (T) KcsA are indicated by arrows. (A) Pulse-chase experiment. (B) Quantification of the ratio of radioactive label in monomer and tetramer during the chase. (C) Na_2CO_3 extraction of labelled cells after the pulse (0') or 10' chase. P: pellet, containing integrated membrane proteins. S: supernatant, containing soluble proteins.

Fig. 3. Energy requirements for KcsA assembly. **(A)** Pulse–chase experiment with 2 mM sodium azide added 1 min before labelling to block ATP hydrolysis. **(B)** Pulse–chase experiment after dissipation of the pmf by 100 μM CCCP, 45 s before labelling. **(C)** Quantification of the ratio of radioactive label in monomer and tetramer during the chase in the presence of azide (filled squares) or CCCP (filled circles). For comparison the data under normal conditions are included (open circles).

are observed. The amount of monomer formed during the pulse period decreases significantly in the subsequent chase, such that after 10 min almost no monomeric protein can be detected. Quantification of the labelled KcsA reveals that the monomer/tetramer ratio decreases with time, suggesting a conversion of the monomer to tetramer in the chase period (Figure 2B). However, from this experiment it cannot be excluded that the decrease of the monomer/tetramer ratio is in part due to degradation of possibly mis-inserted monomers. In any case, it is clear that tetramerization is a highly efficient process since within the 30 s pulse a large amount of tetramer is already formed. Lowering the temperature to 25°C did not have a significant effect on the efficiency of tetramerization. Expression of the *kcsA* gene under an *E. coli* promoter, which is weaker than the T7 promoter, led to similar behaviour of the monomer and tetramer in the experiments (data not shown). In conclusion, the pulse–chase experiments suggest a rapid assembly of KcsA monomers directly into tetramers, without the detectable formation of dimers or trimers.

Sucrose gradient analysis demonstrated that the tetramer of KcsA is found in the fractions containing the inner membrane vesicles of *E. coli* (data not shown). How does the tetramer become integrated into this membrane: by oligomerization of inserted monomers, or is the tetramer already formed in the cytosol or at the membrane interface? Since it is possible to detect the monomer of KcsA during biogenesis of the tetramer, its localization could be investigated by carbonate extraction of pulse-labelled and 10'-chased cells (Figure 2C). Both the monomer and the tetramer were found in the pellet fraction at the different inspected times. This suggests that the monomer is directly, stably inserted into the membrane and remains tightly associated. This was confirmed by treatment of spheroplasts with protease, as both monomer and tetramer are accessible from the periplasmic side of the inner membrane (see Figure 4). Together these results strongly indicate that the assembly into the tetramer occurs in the membrane itself.

Energy requirements for KcsA biogenesis

Energy supply for insertion of proteins into the *E. coli* inner membrane can be expected to involve ATP hydrolysis by SecA at the translocon, and the pmf. To study the energy requirements for KcsA insertion and tetramerization, both possible energy sources were blocked separately, just before pulse labelling. Azide has been shown to block the ATP hydrolysis that is coupled to protein translocation (Oliver *et al.*, 1990). As shown in Figure 3A, the presence of azide in the pulse–chase experiment did not have an effect on the kinetics of KcsA tetramer formation. Large amounts of tetramer were present from the start of the chase and the amount of monomers decreased in time, resembling the situation without azide (compare with Figure 2A). Even increasing the azide concentration (up to 10 mM) did not show any difference (not shown). Quantification of the monomer/tetramer ratio demonstrates clearly that the presence of azide during the experiment had no significant effect on oligomerization (see the two lower lines in Figure 3C). In addition, membrane insertion, as determined by carbonate extraction, was not affected by the presence of azide during the experiment (not shown). This suggests that membrane integration and subsequent tetramerization are not dependent on ATP hydrolysis at the translocon. This result corresponds with the absence of requirement for SecA in membrane insertion of several other (polytopic) membrane proteins, such as the inverted construct of leader peptidase (von Heijne, 1989), LacY (MacFarlane and Müller, 1995), MelB (Basilana and Gwizdek, 1996) and FtsQ (Scotti *et al.*, 1999), which are all proteins with small periplasmic loops. The periplasmic loop of KcsA is also small (~30 amino acids) and in addition contains the pore helix, which is directed partially into the surface of the membrane (Doyle *et al.*, 1998). Therefore, it is likely that the insertion of KcsA does not require the ATP-consuming activity of SecA for translocation of its short periplasmic loop.

To study the effect of the pmf on the insertion and assembly of KcsA, a pulse–chase experiment was performed in the presence

Fig. 4. Transmembrane orientation of KcsA. (A) Schematic presentation of the KcsA monomer orientation, with the possible trypsin cleavage sites in the periplasmic loop indicated by arrows. (B) Trypsin treatment of spheroplasts after pulse labelling in the presence or after dissipation of the pmf by 100 μ M CCCP. As a control for membrane intactness, the spheroplasts were treated with trypsin and proteinase K, with or without solubilization by Triton X-100 and analysed on immunoblot using an antibody against SecB.

of CCCP, an uncoupler of the pmf (Daniels *et al.*, 1981). In the pulse-chase experiment with CCCP pre-treated cells, a large accumulation of the KcsA monomer is observed and only a small amount of tetramer is formed in the 30 s pulse (Figure 3B). During the chase, the amount of monomer is decreasing, and the intensity of the tetrameric band is increasing with time. This demonstrates that tetramerization is possible after dissipation of the pmf, but that this process is largely slowed down (compare with Figure 2A). Furthermore, in this retarded process, the assembly of monomers into the tetramer is unambiguously demonstrated. This also suggests that the previously observed decrease of the monomer in the presence of the pmf is mainly caused by the conversion of monomers into tetramers. The large effect of dissipation of the pmf is further supported by the quantitative data shown in Figure 3C. Directly after the pulse, the monomer/tetramer ratio is increased almost 10-fold due to dissipation of the pmf. At the end of the chase, this ratio reaches approximately the same level as in the presence of an intact pmf. Synthesis of the monomer is still high at the start of the pulse, but tetramer formation is now largely retarded and slowly increases after longer chase periods. The conversion is never as efficient as in the presence of the pmf. These data demonstrate an important effect of the pmf on the membrane assembly of KcsA.

In principle this effect could be caused by differences in the mode of membrane insertion of the monomer in the presence or absence of the pmf. However, carbonate extraction experiments suggested that, even after a 10 min chase, both the monomer and the tetramer were stably integrated in the membrane (not shown). To test whether this carbonate-resistant membrane-associated state represents a transmembrane orientation, the protease accessibility in the periplasm of KcsA was studied both in the presence and absence of the pmf (Figure 4). The expected orientation of a KcsA monomer in the membrane is schematically drawn in Figure 4A and shows that trypsin might cleave the periplasmic loop near the membrane interface. The inverted

orientation is highly unlikely, according to the 'positive inside' rule (von Heijne and Gavel, 1988; Krishtalik and Cramer, 1995). Figure 4B shows pulse-labelled cells converted to spheroplasts and treated with or without trypsin. In the presence of the pmf, the monomer and tetramer are clearly formed during the pulse, and trypsin treatment results in disappearance of the tetramer and most of the monomer. This indicates a transmembrane orientation of KcsA in which cleavage of the tetramer results in disassembly and the resulting fragments of the monomer are too small (6 or 7 kDa) to be detected on the used gel system. After dissipation of the pmf by CCCP, the small amount of formed tetramer and most of the accumulated monomer are also cleaved by trypsin, showing that there is no change in membrane insertion. To check that the cleavage is not a result of a permeabilized membrane, the protection from proteases of the cytosolic chaperone SecB in the spheroplasts containing over-expressed KcsA was verified (Figure 4B). Only after solubilization with detergent could SecB be degraded, confirming the membrane intactness of the spheroplasts. Also in the spheroplasts prepared after CCCP treatment, SecB was protected from proteases (not shown). A protected fraction of KcsA monomer remains both in the presence and the absence of the pmf (20 and 23%, respectively), and could only be degraded after solubilization of the spheroplasts by detergent (not shown). Most likely, the protected fraction represents incorrectly inserted monomers. Importantly, the amount of this protected fraction is not changed significantly by dissipation of the pmf. The formation of an inverted topology in the absence of the pmf can be excluded, since the fragment of 11 kDa that would result is not seen on the gel. This is different from a number of other membrane proteins that are dependent on or influenced by the pmf for their topology or translocation of their periplasmic loops, either short or long (Andersson and von Heijne, 1994; Bassilana and Gwizdek, 1996). From these data on the assembly of KcsA it can

Fig. 5. *In vitro* membrane assembly of KcsA. Protein synthesis from the empty vector (lane 1) and pT7-KcsA (lane 2). Co-translational insertion and assembly in *E. coli* inner membrane vesicles (lane 3), after dissipation of ΔpH by 0.75 $\mu\text{g/ml}$ nigericin (lane 4) and after dissipation of the pmf by 100 μM CCCP (lane 5).

be concluded that the pmf plays an essential role in the high efficiency of tetramerization.

Both components of the pmf, the electrical potential, $\Delta\psi$, and the proton gradient, ΔpH , can play a role in the biogenesis of KcsA. Unambiguous determination of the contribution of each component in *in vivo* experiments is difficult and at least requires extensive variations in growth conditions (Koronakis et al., 1991). In contrast, the effect of each component can be studied in an *in vitro* transcription-translation-translocation system under well-defined conditions. Therefore, an *in vitro* system for KcsA was set up (Figure 5). *In vitro* protein synthesis directed from the cloning vector does not show any intense labelled protein bands (lane 1), whereas from the plasmid with the *kcsA* insert an obvious band, corresponding to the monomeric form of KcsA, is detected (lane 2). No tetramers are formed in this membrane-free translation reaction. The tetramer is formed only after the co-translational addition of *E. coli* inner membrane vesicles (IMV), and is localized in the pelleted vesicles (lane 3). The essential presence of IMV for tetramer formation *in vitro* is in agreement with the *in vivo* finding that tetramerization occurs in the membrane. Addition of nigericin to this *in vitro* system has been shown to destroy only ΔpH and leave $\Delta\psi$ intact (Geller et al., 1993; Kiefer and Kuhn, 1999). The presence of nigericin did not affect the tetramerization of KcsA (lane 4). Dissipation of both ΔpH and $\Delta\psi$ by CCCP completely inhibited the tetramer formation (lane 5). Similar results were obtained with nigericin during *in vivo* experiments (data not shown), suggesting the importance of $\Delta\psi$ *in vivo* also. Thus, it can be concluded that $\Delta\psi$ is the essential component of the pmf for efficient tetramerization.

How can the $\Delta\psi$ facilitate the oligomerization of KcsA? It could have an effect on the highly charged cytoplasmic C-terminus that might stabilize the tetrameric structure since its removal resulted in a lower thermostability (Perozo et al., 1999). Possibly, $\Delta\psi$ influences its orientation with respect to the

membrane so that it favours interactions with the C-termini of other subunits. The $\Delta\psi$ might also affect charges located around the pore region, which has been shown to be important for the tetrameric stability of KcsA (Splitt et al., 2000). Besides affecting the protein, $\Delta\psi$, but also ΔpH , may have an effect on other, still unknown factors that play a role in the correct insertion and oligomerization of KcsA. It has been shown for secretion of proteins that the pmf stimulates translocation of proteins through the translocon, possibly by affecting the formation of the translocation channel (Nouwen et al., 1996). For the efficient formation of the KcsA tetramer such indirect influence of the pmf could be present. A direct explanation for the observed effects of the pmf is difficult to obtain.

In conclusion, the experiments presented in this study demonstrate the possibility of following the tetramerization of KcsA in an *in vivo* and *in vitro* situation, and of studying the biogenesis of an oligomeric protein. The requirement of $\Delta\psi$ for efficient oligomerization is the first observation pointing to a novel role of the pmf in membrane protein assembly.

METHODS

Strains, plasmids and growth conditions. *E. coli* M15 (pRep4) carrying the plasmid pQE-837 was used as host to isolate KcsA with an N-terminal His tag (Schrempf et al., 1995). To obtain a more efficient expression, the *kcsA* gene including the His tag coding sequence was cloned under T7 promoter control. Therefore, an *NdeI* restriction site was introduced upstream of the gene by PCR and the *NdeI*–*SalI* fragment was cloned into the *E. coli* expression vector pT7-7, resulting in pT7-KcsA. The construct was checked by sequencing. BL21(λ DE3) (Studier et al., 1990) was used as host strain for pT7-KcsA. Cultures were grown at 37°C in M9 minimal medium supplemented with 0.4% glucose, 5 $\mu\text{g/ml}$ thiamine and all amino acids except methionine. Where appropriate, ampicillin (100 $\mu\text{g/ml}$) and kanamycin (50 $\mu\text{g/ml}$) were added to the medium. *E. coli* strain MRE600 (Cammack and Wade, 1965) was used to isolate S-135 lysate and grown in Giston broth at 37°C until early logarithmic phase. MC4100 (Casadaban, 1976) was grown at 37°C in LB to late logarithmic phase to prepare IMV.

Pulse-chase experiments. Overnight cultures were diluted 1:40 into fresh minimal medium and grown to early logarithmic phase. The expression of KcsA was induced for 30 min with 1 mM IPTG. The cells were labelled with 10 $\mu\text{Ci/ml}$ [^{35}S]methionine (Amersham) for 30 s and subsequently chased with an excess of non-radioactive methionine (final concentration 2.5 mg/ml). To test the influence of ATP hydrolysis by SecA or of the pmf, sodium azide (Merck) or CCCP (Sigma) was added 1 min or 45 s before labelling to a final concentration of 2 mM or 100 μM , respectively. Samples of 300 μl of culture were taken either directly after pulse labelling, or at different time points during the chase, and chilled on ice. Cells were collected by centrifugation and analysed by SDS–PAGE in the presence of 0.1% SDS (Laemmli, 1970). The tetramer of KcsA has been shown to be stable in the presence of high concentrations of SDS, but not at high temperatures (Cortes and Perozo, 1997). Therefore, the samples were never boiled before electrophoresis, unless specifically noted. Gels were scanned in a PhosphorImager (Molecular Dynamics) and quantification was performed with the program Image Quant. For identification of

KcsA, the labelled cells were resuspended in 50 mM Tris pH 7.5, 1% SDS, disrupted by brief sonication and solubilized in 1% Triton in 50 mM Tris pH 8, 150 mM NaCl and 10 mM imidazole. The His-tagged KcsA was precipitated by binding to Ni²⁺-NTA agarose beads (Qiagen), which were spun down and washed in buffer (50 mM Tris pH 8, 150 mM NaCl, 0.5% Triton) containing 20 mM imidazole. His-tagged KcsA was eluted from the beads in 50 mM Tris pH 8, 150 mM NaCl, 2% octyl glucoside and 400 mM imidazole.

Carbonate extraction. Cultures were treated as in the pulse-chase experiment. Cells were resuspended in buffer (50 mM Tris pH 8, 5 mM EDTA) and incubated with 1 mg/ml lysozyme for 15 min on ice. Samples were subjected to three cycles of freezing and thawing to break open the cells, and an equal volume of 0.2 M Na₂CO₃ pH 11.5 was added, followed by 5 min incubation on ice. The stably integrated membrane proteins were pelleted by centrifugation (30 min, TLA100 rotor, 50 000 r.p.m., 4°C). Supernatant and pellet were analysed by SDS-PAGE.

Protease mapping. Pulse-labelled cells were converted into spheroplasts as described (Kiefer *et al.*, 1997). The spheroplasts were incubated on ice in the absence or presence of 500 µg/ml trypsin (Merck) for 1 h. Subsequently, 1 mM phenylmethylsulfonyl fluoride (Sigma) was added and after 5 min incubation on ice the spheroplasts were collected by centrifugation, and analysed by SDS-PAGE. Control of membrane intactness was done by analyses of SecB protection on immunoblot after treatment of the spheroplasts with 500 µg/ml trypsin and 500 µg/ml proteinase K (Sigma), since SecB is resistant to trypsin. This protease mixture was found to degrade purified SecB in spheroplast buffer (not shown).

In vitro transcription, translation and assembly. Plasmid pT7-KcsA was used to direct the transcription of the *kcsA* gene by T7 RNA polymerase (Pharmacia) in a transcription mix containing 20 mM HEPES pH 7.5, 10 mM MgOAc₂, 2 mM spermidine, 200 mM KOAc, 25 mM NaCl, 5 mM dithiothreitol, and 2.5 mM ATP, GTP, CTP and UTP. Isolation of S-135 lysate, preparation of IMV and the *in vitro* translation reaction were performed as described (de Vrije *et al.*, 1987). For membrane assembly, IMV (final concentration 0.4 mg/ml protein) were added co-translationally. To dissipate the ΔpH only or the total pmf, 0.75 µg/ml nigericin or 100 µM CCCP was added, respectively, to the translation mix prior to IMV addition. At the end of translation the IMV were pelleted by centrifugation (30 min, TLA100 rotor, 75 000 r.p.m., 4°C) and analysed by SDS-PAGE.

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